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Counter-Ion Dissociation of Chondroitin Sulfate Adsorbed on Hydroxyapatite in Water

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The intrinsic viscosity and the amount of adsorption of sodium and calcium chondroitin-6-sulfate (Na_2Chs and CaChs) on hydroxyapatite (HAP) were measured. The values depended on the degree of counter-ion dissociation of chondroitin-6-sulfate (Chs). Ion activity, a, and apparent degree of dissociation of counter-ions (Na^+ and Ca^{2+}), α, for Chs were determined by means of ion-selective electrodes in the presence or absence of HAP. In the case of salt-added systems (NaCl or $CaCl_2$: 5—20 mM), α, obtained from the slope of the relationship between a and [Chs], was almost independent of the concentration of HAP, [HAP] (0—5 g/dl), although a decreased with increase in [HAP] due to counter-ion adsorption by HAP. In the case of salt-free systems, both a and α apparently decreased with increase in the amount of HAP added, because a portion of the counter-ions dissociated from Chs is consumed by adsorption at the surface of HAP. It was concluded that the apparent degree of ionization (*i.e.* apparent charge density along the polymer chain) of Chs adsorbed by HAP is almost the same as that for Chs free from HAP in the bulk solution, irrespective of the apparent value of α obtained from the relationship between a and [Chs].

Keywords——hydroxyapatite; calcium chondroitin sulfate; sodium chondroitin sulfate; counter-ion dissociation; calcium ion activity; sodium ion activity; intrinsic viscosity; adsorption isotherm; calcification

Chondroitin sulfate (Chs) is found in cartilage, bone, and tooth as a protein complex, which plays an important role in the calcification of biological hard tissues. It may store Ca^{2+} and serve to transport it, making it available to the matrix vesicles at the appropriate time.[1] Chs is also found in human urine. The molecular weight of the urinary Chs is considerably lower than that reported for tissue chondroitin sulfate.[2] This Chs in urine is known to be an inhibitor of renal calculi formation.

Hydroxyapatite (HAP: $Ca_{10}(PO_4)_6(OH)_2$) is the major constituent of biological hard tissues and human renal calculi. Therefore, it is important to understand the interaction of Chs and HAP or Ca^{2+} in connection with biological calcified tissue formation. In the previous paper,[3] it was shown that Chs can bind Ca^{2+} more easily than Na^+ and that the binding isotherms for Ca^{2+} are of the Langmuir type. On the other hand, Chs is adsorbed at the surface of HAP, showing a high-affinity adsorption isotherm. The amount of adsorption of Chs, however, decreases with increase in the concentration of phosphate added due to the competitive adsorption of the anions, phosphate and Chs.[4,5]

In this work, the interaction of HAP with calcium and sodium chondroitin sulfate (CaChs and Na_2Chs) in an aqueous phase is discussed in terms of the counter-ion activity and the amount of adsorption of Chs by HAP.

Experimental

Materials——Na_2Chs was the same sample as that used in the previous papers.[3,4] It is of C-type, *i.e.*

chondroitin-6-sulfate (molecular weight 7×10^4—8×10^4), and was kindly provided by Kaken Yakukako Co., Ltd. According to the analysis by Kaken Yakukako Co., this sample contains a trace amount of chondroitin-4-sulfate (*i.e.* the A-type). CaChs was obtained as follows: Na_2Chs was ion-exchanged to acidic form (H_2Chs) by means of a cation-exchange resin (Amberlite IR-120B), followed by titration to pH 7.5 with a saturated aqueous solution of $Ca(OH)_2$. It was confirmed that the content of Ca^{2+} in the product (CaChs) is equivalent to that of Chs by chemical analyses of both components (Ca^{2+} and Chs). Concentrations of Chs (Na_2Chs and CaChs) are given in terms of the repeating unit as base molarity (*i.e.* mM) in the present paper.

HAP was the same sample as that used in the previous paper.[4] All chemicals used were of analytical grade, purchased from Nakarai Chemicals Ltd. or Wako Pure Chemical Industries Ltd. These were used without further purification. Water was doubly distilled.

Viscosity——The measurements of solution viscosity were made in a Ubbelohde-type viscometer at 25 °C. The intrinsic viscosity, $[\eta]$, for Na_2Chs in an aqueous solution of NaCl was obtained by extrapolation of the reduced viscosity to infinite dilution with an aqueous solution of a constant concentration of NaCl. The intrinsic viscosity for CaChs was obtained by means of extrapolation of the reduced viscosity of Na_2Chs to infinite dilution with a solution of a constant concentration of $CaCl_2$. The intrinsic viscosity obtained in this way can be approximately regarded as that for CaChs in a given solution of $CaCl_2$, because the concentration of the counter-ion (Na^+) becomes zero but that of Ca^{2+} is kept constant as $[CaCl_2]$ up to infinite dilution, and because the formula weight of the repeating unit of these Chs is almost the same (503 for Na_2Chs and 497 for CaChs). The intrinsic viscosity of Na_2Chs in the salt-free system was obtained by means of the Fuoss–Strauss empirical equation,[6]

$$\left(\frac{\eta_{sp}}{c_p}\right)^{-1} = \frac{1}{[\eta]} + \frac{B}{[\eta]}\sqrt{c_p} \qquad (1)$$

where c_p is the concentration (g/dl) of Na_2Chs, η_{sp}/c_p is the reduced viscosity, and B is a constant. The linear relationship obtained experimentally between $(\eta_{sp}/c_p)^{-1}$ and $\sqrt{c_p}$ is shown in Fig. 1(B), where $[\eta]$ can be determined from the intercept of the ordinate.

Measurement of Amount of Adsorption and Chemical Analysis——HAP (1 g) was suspended in a test tube containing 20 ml of a given adsorbate solution of known concentration, $[CaChs]_t$ or $[Na_2Chs]_t$, at 35 °C and shaken vigorously from time to time. After at least 3 d, the filtrate (Millipore filter, pore size 0.22 μm) was analyzed for Chs. The equilibrium concentration of Chs, $[Chs]_f$, was determined by colorimetry according to the method of Dische *et al.* (at 570 nm).[7] It was confirmed that the Millipore filter does not adsorb Chs, and that the counter-ion (Na^+ or Ca^{2+}) does not interfere with the analysis of Chs. No salt was added to the sample solution of Chs in order to compare the amount of adsorption of Na_2Chs with that of CaChs in the absence of salt effects. The amounts of Chs adsorbed on HAP, 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 Chs, $[Chs]_f$.

Ion Activity——Calcium ion activity ($a_{Ca^{2+}}$) and sodium ion activity (a_{Na^+}) were determined at 35 °C by using Orion calcium- and sodium-sensitive electrodes (type 93-20 and 94-11) connected to an Orion Microprocessor Ionalyzer (model 901). Prior to the measurements on Chs solutions, the calcium and sodium electrodes were calibrated with aqueous solutions of $CaCl_2$ and NaCl, respectively, taking the activity coefficients into consideration.[8] The electrodes exhibited Nernstian response over the range of concentrations of the present work when the other ion was not added.

However, a small amount of calcium ion in the solution interfered with the response of the sodium-specific electrode. Therefore, a_{Na+} could not be obtained in the presence of Ca^{2+}. On the other hand, it was confirmed that the response of the calcium-specific electrode was not disturbed in the presence of low concentrations of added Na^+. Consequently, a_{Na+} was measured only in the system of Na_2Chs with or without NaCl, and a_{Ca2+} was measured in the system of CaChs with $CaCl_2$ or Na_2Chs.

Results

Intrinsic Viscosity

Intrinsic viscosity, $[\eta]$, of Na_2Chs (⊙) in NaCl solution and that of CaChs (⊗) in $CaCl_2$ solution are shown in Fig. 1(A) as a function of concentration of added salt. The value of $[\eta]$ decreases more markedly with increase of the concentration of $CaCl_2$ than that of NaCl.

Satake *et al.*[9] showed that the intrinsic viscosity of sodium dextran sulfate in 50 mM NaCl is three times larger than that of calcium dextran sulfate in 50 mM $CaCl_2$. However, in the case of Chs, the difference of intrinsic viscosities between Na_2Chs and CaChs in the respective chloride solutions does not become as large as that mentioned above, as shown in Fig. 1(A).

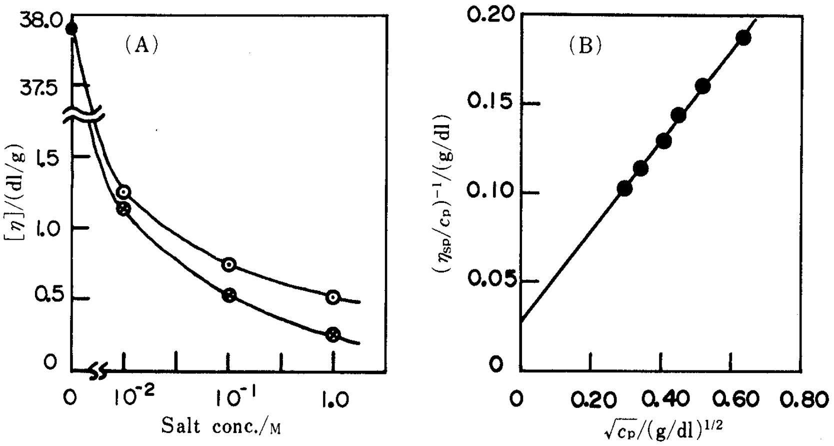

Fig. 1. Relationship between Intrinsic Viscosity of Chs and Concentration of Added Salt (A), or between Reduced Viscosity and Concentration of Na_2Chs in a Salt-Free System (B) at 25 °C

⊙, Na_2Chs with NaCl; ⊗, CaChs with $CaCl_2$; ●, Na_2Chs without any simple salt. The relationship in (B) is expressed by Eq. 1.

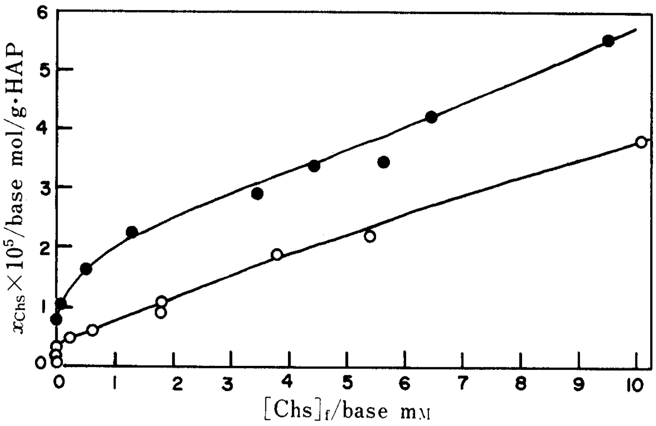

Fig. 2. Adsorption Isotherms for Na_2Chs and CaChs at 35 °C

○, Na_2Chs; ●, CaChs.

Adsorption Isotherm

The amount of adsorption of Chs to HAP, x_{Chs}, are shown as a function of equilibrium concentration of free Chs, $[Chs]_f$, in Fig. 2. The fact that these curves increase steeply along the ordinate at low concentrations of Chs shows a high-affinity adsorption of Chs on HAP. Figure 2 shows that the affinity of CaChs (●) to HAP is higher than that of Na_2Chs (○).

The affinity and/or adsorption amount of Chs to HAP may be governed by the following three factors: firstly, the degree of polymer coil expansion owing to intra-molecular repulsion between polymer segments, which also affects the intrinsic viscosity of Chs (Fig. 1(A)); secondly, inter-molecular repulsion between polymer chains; and thirdly, the affinity for dissociated counter-ions (Ca^{2+} and Na^+) at the surface of HAP. It is already known that Ca^{2+} has a higher affinity to HAP than Na^+, and that the adsorption of Ca^{2+} on HAP increases the number of adsorption sites for anions.[10,11] These factors also affect the area occupied by each polymer coil of Chs adsorbed on the surface of HAP. The former two factors are mainly governed by the degree of dissociation of Chs, that is, electric charges on the polymer chain of Chs. Figures 1(A) and 2 suggest that the degree of dissociation (*i.e.* ionization) of Na_2Chs is higher than that of CaChs, in accordance with the result reported elsewhere.[3]

It may be interesting to compare the amounts of adsorption on HAP of chondroitin-6-sulfate (present work) and chondroitin-4-sulfate. Their molecular structures are the same except for the location of the sulfate group, which is assumed to be one of the groups participating in the adsorption on HAP. The difference in location and/or orientation of the sulfate group in the Chs molecules might be responsible for the difference in the amounts of adsorption by HAP.

Degree of Counter-Ion Dissociation from Chs

The activity of the counter-ion (a_{Na^+} or $a_{Ca^{2+}}$) in the presence or absence of HAP is shown in Fig. 3 as a function of the total concentration of Chs added, $[Na_2Chs]_t$ or $[CaChs]_t$. It was found that the relationship between activity and Chs concentration is linear, and may be expressed as follows:

$$a_{Na^+} = a_{0,Na^+} + 2\alpha_{Na_2Chs}[Na_2CHs]_t \qquad (2)$$

for sodium ion activity in the system of Na_2Chs and NaCl, and

$$a_{Ca^{2+}} = a_{0,Ca^{2+}} + \alpha_{CaChs}[CaChs]_t \qquad (3)$$

for calcium ion activity in the system of CaChs and $CaCl_2$.

The intercept of the ordinate, a_{0,Na^+} in Eq. 2 or $a_{0,Ca^{2+}}$ in Eq. 3, gives the cation activity of the added salt, NaCl or $CaCl_2$, in the absence of Chs. The coefficient, α_{Na_2Chs} or α_{CaChs}, is the apparent degree of counter-ion dissociation from Na_2Chs or CaChs, the value of which, strictly speaking, may depend on the inherent degree of counter-ion dissociation from Chs and on the activity coefficient for each counter-ion at the ionic strength in the medium solution. The numerical factor 2 is introduced in Eq. 2, since 1 mol of the repeating unit of Na_2Chs contains 2 mol of Na^+.

In the case of the salt-free systems in the absence of HAP (I (●) and III (●)), α_{Na_2Chs} and α_{CaChs} were obtained as 0.395 and 0.244, respectively. These values are similar to those reported elsewhere (0.40—0.41 for Na_2Chs[3]) and 0.20—0.22 for CaChs[3,12])). However, the slope of the straight line decreases with increase in the concentration of HAP added (I (○) and III (○), for example). The rate of decrease is larger in the CaChs system (III) than in the Na_2Chs system (I). This fact might suggest that the degree of counter-ion dissociation decreases with increase of the concentration of HAP added. Figure 4 shows the relationship between the slope, α_{CaChs}, and the concentration of HAP added, [HAP], for the CaChs–HAP

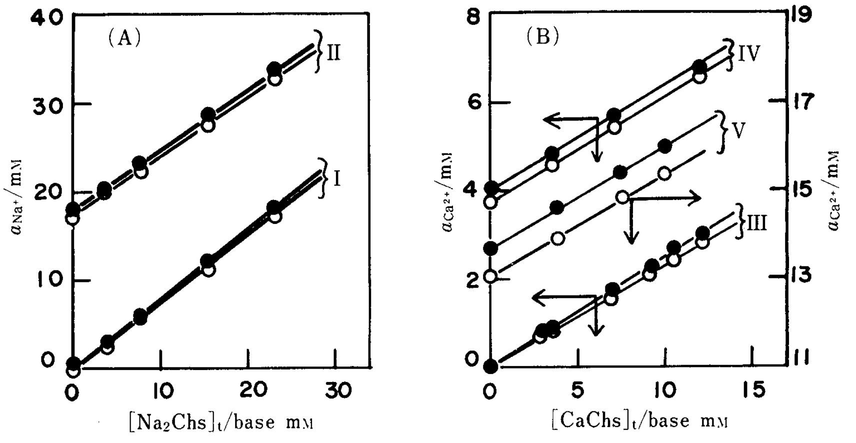

Fig. 3. Counter Ion Activity for Na_2Chs and CaChs

Concentration of added HAP: [HAP]/g/dl = 0 (●), and 5 (○).
Concentration of added salt: $[NaCl]_t$/mM = 0 (I) and 20.0 (II); $[CaCl_2]_t$/mM = 0 (III), 5.17 (IV), and 20.7 (V).

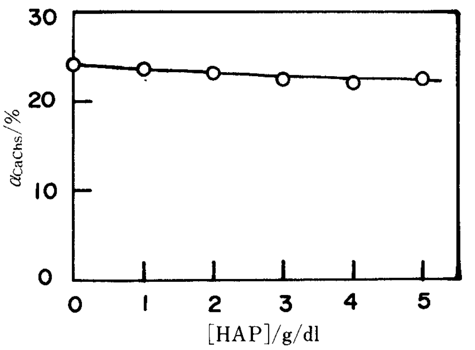

Fig. 4. Apparent Relationship between α_{CaChs} and Concentration of HAP Added

Simple salt was not added to the sample solution.

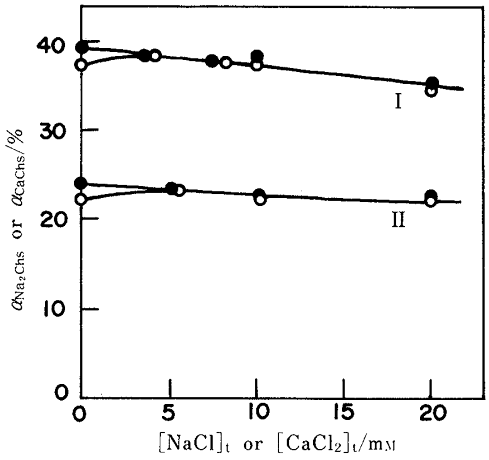

Fig. 5. Relationship between Apparent Degree of Dissociation of Counter-Ion and Concentration of Simple Salt Added

I, α_{Na_2Chs} *vs.* $[NaCl]_t$; II, α_{CaChs} *vs.* $[CaCl_2]_t$. Concentration of added HAP: [HAP]/g/dl = 0 (●) and 5 (○).

system. The value of α_{CaChs} decreases by 7.4% while that of α_{Na_2Chs} decreases by 3—4% when [HAP] is increased up to 5 g/dl (not shown).

In the case of the salt-added systems (II, IV, and V in Fig. 3), a pair of straight lines (● and ○) running almost in parallel were obtained. The vertical distance along the ordinate between the closed and open circles shows the amount of adsorption of counter-ion by HAP (5 g/dl) in activity units.

The apparent degree of counter-ion dissociation, obtained by means of Eqs. 2 and 3, is shown in Fig. 5 as a function of the salt concentration added ($[NaCl]_t$ or $[CaCl_2]_t$). The values of α in the presence (○) and absence (●) of HAP are almost the same, except in the case of the salt-free systems (○ and ● on the ordinate in Fig. 5). The values of α_{CaChs} and α_{Na_2Chs} decrease by *ca.* 5% and *ca.* 10% when $[CaCl_2]_t$ and $[NaCl]_t$ are increased to 20 mM, respectively, owing mainly to the increase of the ionic strength.

Apparent Dissociation of Ca^{2+} from CaChs in the Presence of Na_2Chs

The activity ($a_{Ca^{2+}}$) of Ca^{2+} dissociated from CaChs was measured in the presence of Na_2Chs with or without HAP in the salt-free system. The relationship between $a_{Ca^{2+}}$ and $[CaChs]_t$ is linear (Fig. 6(A)). The slope of the straight line for the system containing HAP is smaller than that for the system not containing HAP, as was the case in the salt-free system in Fig. 3. The slope also decreases with a decrease in mole fraction of CaChs, $[CaChs]_t/([CaChs]_t+[Na_2Chs]_t)$. The apparent degree ($\alpha_{CaChs}$) of dissociation of Ca^{2+} from CaChs ($[CaChs]_t$) in the presence of Na_2Chs is obtained from the slope by means of Eq. 3 ($a_{0,Ca^{2+}}=0$). The result is shown in Fig. 6(B) as a function of the mole fraction of CaChs; the curves are S-shaped and the values for the system containing HAP (○) are smaller than those for the system not containing HAP (●) over the whole range. The fact that α_{CaChs} decreases with increase in the mole fraction of Na_2Chs (*i.e.* with decrease in the mole fraction of CaChs) suggests that the presence of Na_2Chs suppresses the dissociation of Ca^{2+} from CaChs. This may occur because the negative charges of Chs capture divalent Ca^{2+} more selectively than monovalent Na^+.

The fraction of Chs which binds Ca^{2+} tightly is given by the term $(1-\alpha_{CaChs})\,[CaChs]_t/([CaChs]_t+[Na_2Chs]_t)$, which is shown as a function of the mole fraction of CaChs in Fig. 7. The experimental points deviate upwards from the dotted line which shows the arithmetic mean value with respect to the mole fraction of CaChs. Figure 7, as well as Figs. 5 and 6, shows a higher affinity of Ca^{2+} than of Na^{+} to Chs.

Discussion

In the case of the salt-free system there are two possible reasons why the apparent degree of dissociation, α (*i.e.* α_{Na^+} and $\alpha_{Ca^{2+}}$), decreases with increase of the amount of HAP added (Figs. 3—6): firstly, the value of α for Chs adsorbed by HAP may be smaller than that for Chs free from HAP; and secondly, the counter-ion (Ca^{2+} and Na^{+}) dissociated from Chs into the bulk solution may be bound to the surface of HAP, resulting in a decrease of the ion activity.

The former might be preferred intuitively on the assumption that dissociation of counter-ions from adsorbed Chs might be depressed by virtue of the restriction of thermal motion due to the adsorption. However, a decrease of α due to the adsorption of Chs by HAP was not found experimentally in the salt-added system (Figs. 3 and 5).

On the other hand, the second possibility is compatible with the fact that α does not decrease upon addition of HAP in the salt-containing system (Figs. 3 and 5). The cations (Na^{+} and/or Ca^{2+}) adsorbed by HAP are supplied from the bulk solution which contains

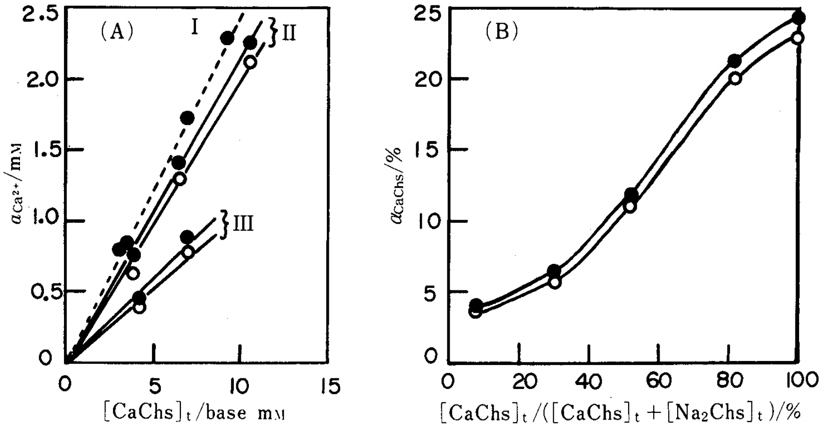

Fig. 6. Apparent Dissociation of Ca^{2+} from CaChs in the Presence of Na_2Chs

No simple salt was added.
Concentration of added HAP: [HAP]/g/dl = 0 (●) and 4 (○).
Mole fraction of CaChs: $[CaChs]_t/([CaChs]_t + [Na_2Chs]_t)$/mol% = 100 (I), 81.3 (II), and 51.8 (III).

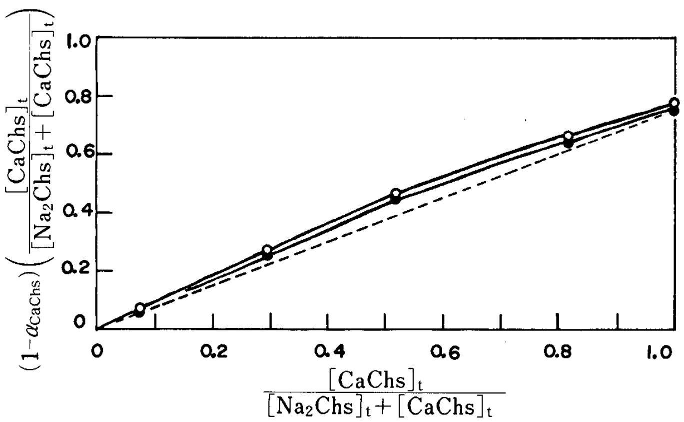

Fig. 7. Apparent Binding of Ca^{2+} to Chs in the Presence of CaChs and Na_2Chs

Concentration of added HAP: [HAP]/g/dl = 0 (●) and 4 (○).

those dissociated from the added salt as well as those dissociated from added Chs. Equilibria of the counter-ion dissociation from Chs (*i.e.* ionization of Chs) and of the counter-ion adsorption by HAP seem to be attained concurrently without any perturbation of the apparent value of α through the aid of counter-ions in the bulk solution. However, cation activity decreases owing to the consumption of cations through adsorption by HAP (Fig. 3, II, IV, and V). Counter anions for the adsorbed cations may be Cl^- and OH^- (from water)$^{10)}$ as well as Chs anion. As for the salt-free system, both a and α, defined by Eq. 2 or 3, decrease, because a portion of the dissociated cations in the bulk solution is consumed through adsorption at the surface of HAP, even though the degree of ionization of Chs (*i.e.* the charge density of the polymer chain) might be constant irrespective of the amount of HAP added.

Polymers are considered to be adsorbed at the surface of the adsorbent as coils of dimensions similar to those of coils which are free in the bulk solution.$^{13,14)}$ It has also been pointed out that a number of ionized groups of the adsorbed polyelectrolyte, although close to the surface, are not actually attached directly to the surface of an adsorbent.$^{15)}$ Therefore, the properties of polyelectrolyte adsorbed on the adsorbent surface may resemble those of polyelectrolyte liberated in the bulk solution with respect to charge density, conformation, and the size of the polymer coil, except as regards the degree of restriction of thermal motion due to the adsorption. Moreover, according to Manning's theory,$^{16)}$ the counter-ion activity of polyelectrolyte, which is intimately related to the degree of dissociation of the polyelectrolyte, is determined only by the distance between charged groups along the polymer chain. Therefore, it is reasonable to conclude that the degree of dissociation of Chs free from HAP is identical with that of Chs adsorbed by HAP.

The apparent degree of counter-ion dissociation (α) can be correctly determined from the slope of the relationship between ion activity (a) and [Chs] when a increases properly with increase of [Chs] (in such cases as the salt-added system and the HAP-free system in Figs. 3 and 5). However, the value of α obtained in this manner is invalid as a measure of the degree of dissociation of Chs if the increment of a does not reflect that of [Chs] properly due to the consumption of counter-ions by concurrent adsorption on HAP (in such cases as the salt-free system in Figs. 3—5).

In conclusion, the degree of counter-ion dissociation (*i.e.* ionization) of Chs (Na_2Chs or CaChs) adsorbed by HAP is almost the same as that for Chs free from HAP in the bulk solution, although a portion of the counter-ions dissociated may be adsorbed at the HAP surface, leading to a decrease of the ion activity.

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