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FORMATION OF A POLYMER COMPLEX BETWEEN BOVINE SERUM ALBUMIN AND SODIUM CHONDROITIN-6-SULFATE IN AN AQUEOUS PHASE AND ON A SOLID/WATER INTERFACE OF KAOLIN

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ABSTRACT

A water-insoluble polyelectrolyte complex (PEC) was formed by mixing bovine serum albumin (BSA) with sodium chondroitin-6-sulfate (Na_2Chs) at a pH lower than an isoelectric point (iep) of BSA. A PEC was also formed on the surface of kaolin via concurrent and/or cooperative adsorption of these two polymers on it. As a result, the aggregation of kaolin particles was induced and accelerated by virtue of an interparticle bridging effect of the PEC formed on the surface. On the other hand, water-soluble PEC was formed in an aqueous phase at a pH higher than the iep of BSA through an electrostatic attractive force between the negative charge of Na_2Chs and the positive charge of patches on BSA. However, a strong effect on the aggregation of kaolin particles was not observed. The binding ratio of BSA to Na_2Chs, R, in an aqueous phase and on the surface of kaolin was obtained by chemical analysis as a function of the pH of its mother solution. The R-value was affected not only by the pH but also by the initial mixing ratio of BSA to Na_2Chs, the concentration and species of an added salt, the net charge of the polymers, the charge patches on BSA, and so on. The effect of binding the phosphate ion to BSA and kaolin on PEC formation was studied in detail. The significance of PEC formation in biological tissue is emphasized.

INTRODUCTION

Polymer molecules in an aqueous solution often associate among like or unlike molecules. Methylcellulose molecules, for example, aggregate at high temperature and at a high concentration of NaCl because of dehydration and hydrophobic interaction, resulting in a high viscosity of the solution [1]. An A-B type of block copolymer, where A is hydrophobic (such as styrene) and B is hydrophilic (such as *N*-vinylpyrrolidone), forms a self-assembly, that is, intramolecular self-organization of a single molecule and intermolecular self-organization of a number of molecules. The block copolymer micelle thus formed showed highly selective solubilization of components from an aromatic–aliphatic mixture. That is, the solubilizate that is more compatible with the polymer block that constitutes the core of the micelle is solubilized to a greater extent [2].

Entanglement also occurs between unlike molecules. When a membrane of poly(ethylene terephthalate) (PET) is treated with poly(ethylene oxide) (PEO) in a good solvent for both polymers, PEO penetrates into the PET membrane by diffusion, and a surface physically interpenetrating network (SPIN) is formed near the surface of the membrane. It was reported that the PET membrane thus modified by PEO becomes extraordinarily resistant to cellular adhesion [3]. Krill protease coprecipitates with water-soluble starch in the presence of an organic solvent such as acetone. It was observed that physically entrapping the enzyme into the starch matrix protects the enzyme from the denaturing environment [4].

Unlike molecules also form complexes through intermolecular hydrogen bonding and intermolecular electrostatic interaction. A membrane composed of poly(vinyl pyrrolidone) (PVP) and poly(methacrylic acid) (PMA) by hydrogen bonding was studied for pervaporation of water–*n*-propanol mixtures [5]. PEO and PMA or poly(acrylic acid) (PAA) in an aqueous phase associate by hydrogen bonding between ether oxygen and a carboxylic acid group, the degree of which decreases with an increase in the degree of neutralization of polyacid [6, 7].

On the other hand, the formation of a water-soluble or insoluble polyelectrolyte complex (PEC) has been widely studied, and its application is important in various industrial and biological fields. A PEC membrane consisting of glycol chitosan and poly(vinyl sulfate) showed ion selective permeability toward Na^+ transport [8], and one of albumin and chitosan was studied for application to hemodialysis in artificial kidney systems [9]. The albumin-blended chitosan membrane exhibited high permeability for low molecular weight compounds and a reduction in platelet attachment through surface modification by albumin. The PEC coacervation of gelatin and acacia [10] or sodium carboxymethylcellulose (NaCMC) [11] has been characterized by chemical analyses of the coacervate itself and the equilibrium fluid phases. Phase separation was accelerated by the addition of PEO [12], owing mainly to its incompatibility with PEC; that is, PEO was not contained in the PEC. These studies are significant as a basis for the design of microencapsulation.

The enzyme activity of papain decreased in the presence of a polycation, poly(L-lysine) [13], for example, due to PEC formation. Park et al. studied complex coacervation between protein [bovine serum albumin (BSA), for example] and cationic or anionic polyelectrolyte by means of turbidimetry and quasi-elastic light scattering. They concluded that the role of the charge patches on the protein is more predominant than that of the net charge on the protein in the formation of the PEC with polyelectrolyte, because the net charge on the protein of the complex was frequently identical in sign to the charge on the polyelectrolyte of the complex [14].

Complex formation of BSA with sodium chondroitin-6-sulfate (Na_2Chs) was studied by Nakagaki et al. by means of static light scattering [15]. The complex was formed even at a pH (5.6–7.2) higher than an isoelectric point (iep) of BSA (around 4.9) by virtue of electrostatic attractive force between the positively charged patch of BSA and negative charge of Na_2Chs. They concluded that the binding of BSA to the polymer chain of Na_2Chs follows the Langmuir-type binding isotherm, where the affinity between them decreases with increases in pH and ionic strength. However, they did not study PEC formation at higher concentrations of polymers and/or lower pHs than the iep.

In the present study, coacervate/precipitate formation in an aqueous phase and complex formation on a solid/water interface are discussed. These are important for understanding the formation of biological assemblies of soft and hard tissues in an animal body. Soft tissues are complexes composed of various proteins and polysaccharides, while hard tissues are complexes of proteins, polysaccharides, and hydroxyapatite (HAP). In this context, we should have used HAP as a solid material, but unfortunately, it is sparingly soluble in water. Because of its solubility, HAP particles become small in size and sometimes disappear from sight during measurement of the zeta potential of a dilute suspension of HAP. Therefore, it was difficult to determine its zeta potential precisely by means of a microscope electrophoresis apparatus after dilution of the HAP suspension. Insoluble kaolin, instead of HAP, was thus chosen as the supporting substrate on which the adsorption of two polymers occurs cooperatively and a complex is formed between them. Concurrent adsorption of unlike species onto a solid surface proceeds competitively or cooperatively. A repulsive force is exhibited between adsorbate molecules in the case of competitive adsorption, while an attractive force is seen in the case of cooperative adsorption. Hence, in this paper we assume that the term "cooperative adsorption" of two unlike adsorbates has almost the same meaning as "complex formation" between them on a solid/water interface.

Complex formation between BSA and Na_2Chs in an aqueous phase and on the surface of kaolin was studied by changing such experimental conditions as mixing ratio, pH, and ionic strength in the mother solution; by determining the PEC composition; and by measuring the zeta potential and mean diameter of kaolin particles.

EXPERIMENTAL

Materials

BSA (Sigma, St. Louis, Missouri, USA) was Fraction V of an initial fractionation by cold alcohol precipitation. Its iep is almost at pH 4.9, and its molecular weight is $\sim 6.63 \times 10^4$.

The viscosity-average molecular weight of Na_2Chs (Nacalai Tesque Inc., Kyoto, Japan) was 3.02×10^4, which is the same sample used in a previous paper [16]. The apparent degree of dissociation of Na^+ from Na_2Chs in a dilute aqueous solution was determined to be 0.42 by means of a sodium ion selective electrode

(Orion, Ross 86-11) with an ion analyzer (Orion, EA-940) and a vapor pressure osmometer (Corona, type 117). The kaolin used in the present paper was the same as that used elsewhere [17].

Method

A phase diagram (Fig. 1) was obtained by the titration method. That is, an aqueous solution of BSA of known concentration at a given pH lower than its iep as adjusted by HCl was titrated by a standard solution of Na_2Chs of the same pH in the presence of 154 mM NaCl. The concentrations of BSA and Na_2Chs, where a milky turbidity was observed by the naked eye, were determined for various initial concentrations of BSA. The relationship between [BSA] and [Na_2Chs], which shows the boundary for phase separation at a given pH, was thus obtained.

On the other hand, in the absence of NaCl, the pH increased to a certain extent by pouring an aqueous solution of Na_2Chs into that of BSA, although the initial pH adjusted by HCl or NaOH of the former was the same as that of the latter. In a quite similar manner, sodium ion activity (a_{Na}) also increased when an aqueous solution of BSA was added to that of Na_2Chs at a given pH. The titration curves with respect to pH and a_{Na} were obtained by means of a pH meter (TOA, HM-5S) and an ion analyzer (Orion, EA-940) with a sodium ion selective electrode (Orion Ross 86-11), respectively.

Kaolin (0.1 g) was mixed with an aqueous solution (20 mL) of BSA and/or Na_2Chs at various pHs adjusted by sodium phosphate buffer or HCl or NaOH in the presence or absence of NaCl. It was confirmed that 1 day is long enough to attain adsorption equilibrium at 25°C with vigorous shaking from time to time. This suspension at adsorption equilibrium was employed for the determination of the amounts of polymers adsorbed and for measurements of the mean diameter and zeta potential of kaolin particles.

The equilibrium concentrations of Na_2Chs, BSA, and total phosphate ion (Pi) in the supernatant, which was obtained by centrifugation, were determined by colorimetry at 530 nm with carbazole–H_2SO_4 solution [18], at 750 nm with a phenol reagent according to the method of Lowry et al. [19], and at 720 nm through the reduction of phosphate ammonium molybdate by stannous chloride [20], respectively. It was confirmed that the coexistence of Na_2Chs, BSA, and Pi in the sample solution does not cause mutual interference in determinations of Na_2Chs and BSA.

The mean diameter (*d*; median) of the kaolin aggregates in an aqueous phase was obtained by means of a Coulter counter (Coulter Electronics, using a 100-μm aperture tube) after diluting an original suspension (0.5 g/dL) 500 times with 154 mM NaCl. The suspension containing both BSA and Na_2Chs was prepared as follows: a kaolin suspension (0.5 g/dL) containing 10 mg/dL BSA and that containing 10 mg/dL Na_2Chs were prepared separately at a given pH. Immediately before the measurement of *d*, these suspension were mixed at a given ratio in volume and stirred for a while. Therefore, the suspension consists of 0.5 g/dL kaolin and 10 mg/dL of the polymers. The *d* value in the present paper was determined by extrapolation to zero time using the data obtained at 10, 60, and 120 seconds after dilution.

The zeta potential of kaolin was measured by means of a Laser Zee TM (Model 501, Pen Kem) after dilution of an original suspension with a phosphate buffer solution of the same pH and the same concentration (10 or 100 mM). Because of the irreversibility of polymer adsorption, we assumed that the amount of polymer adsorbed after dilution was almost the same as before the dilution.

RESULTS AND DISCUSSION

Complex Formation in the Absence of Kaolin

Figure 1 is a phase diagram, divided into regions of clear and turbid solutions. The precipitate or coacervate appeared in the upper domain due to PEC formation between positively charged BSA and negatively charged Na_2Chs, while a clear solution was retained in the lower region. The boundary curve moves to higher concentrations of both BSA and Na_2Chs, and its slope becomes steeper with an increase in the pH of the sample solution. This is because the positive charge density on the BSA molecule decreases with pH.

The binding ratio R of BSA to Na_2Chs in the PEC was determined by means of chemical analysis of the remaining polymers in the supernatant after centrifugation. That is, R is the ratio of the decrease in concentration of BSA, Δ[BSA], to that of Na_2Chs, $\Delta[Na_2Chs]$, where Δ is the difference in concentration of a polymer in the supernatant before and after precipitation/coacervation. The value of R is shown in Fig. 3 (A) as a function of pH. It increased with pH. This fact is explained as follows. The density of positive charge on BSA decreases when pH increases. The negative charges on Na_2Chs therefore require a greater amount of BSA in weight at high pH than at low pH to form a water-insoluble PEC. This result is compatible with the tendency shown in Fig. 1. It was confirmed that the R-value in the precipitate is almost constant, even though the initial mixing ratio of BSA to Na_2Chs in weight varies from 1:0.25 through 1:4 at a given pH and a given salt concentration (data not shown).

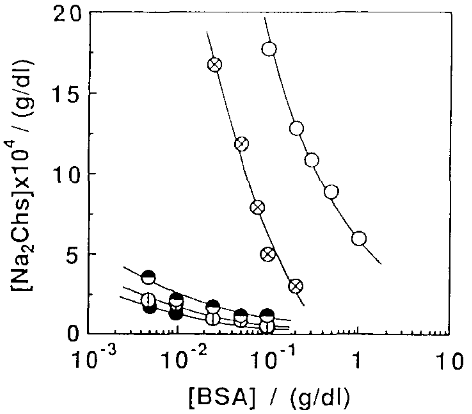

FIG. 1. Phase diagram at a pH lower than the iep of BSA. The solution was clear (one phase) and turbid (second phase) in the region below and above the curve, respectively. 154 mM NaCl was added to a solution of pH 4.0 (●), 4.2 (⦶), 4.3 (◓), 4.4 (⊗), and 4.5 (○).

The binding ratio was also determined from changes in pH and in sodium ion activity (a_{Na^+}) in the absence of an added salt. The relationship between an increment in pH (ΔpH, see the Experimental Section) and the concentration of added Na_2Chs is shown in Fig. 2(A). The ΔpH increased and leveled off after attaining a breakpoint. The trace of the breakpoint with respect to an increase in pH of the initial solutions before mixing is shown by a dotted line. The arrows indicate the direction of an increase in pH. The mixing ratio of BSA to Na_2Chs at the breakpoint is regarded as the binding ratio (*R*) as determined by the pH titration method.

On the other hand, a_{Na^+} increased and leveled off when an aqueous solution of BSA was gradually added to that of Na_2Chs of the same pH, as shown in Fig. 2(B). The point where the experimental curve levels off (shown by the arrows) was assumed to be the end point where PEC formation was completed, from which the binding ratio, *R*, was determined. The *R*-values thus obtained are also shown in Fig. 3(A). The data obtained through these various methods were almost in agreement, while those in the presence of 100 mM phosphate buffer (---⊗---) were slightly lower than the others.

Positively charged BSA captures hydroxyl, chloride, and/or phosphate anions (Pi) as its counterions. When it is mixed with Na_2Chs in an aqueous phase, the ions are exchanged and the PEC of $BSA^+ \cdot Chs^-$ is formed as follows;

$$BSA^+ \cdot (OH^-, Cl^-, \text{or Pi}) + Na_2Chs \rightarrow$$
$$BSA^+ \cdot Chs^- + Na^+ + OH^- + Cl^- \text{ or Pi}$$

where the stoichiometric numerical coefficients are omitted. According to the above scheme, free concentrations of OH^- and Na^+, that is, pH and a_{Na^+}, should increase and level off after the binding of BSA^+ with Chs^- is completed. Therefore, the *R*-value can be determined from the changes in pH and a_{Na^+}, as was shown in Fig.

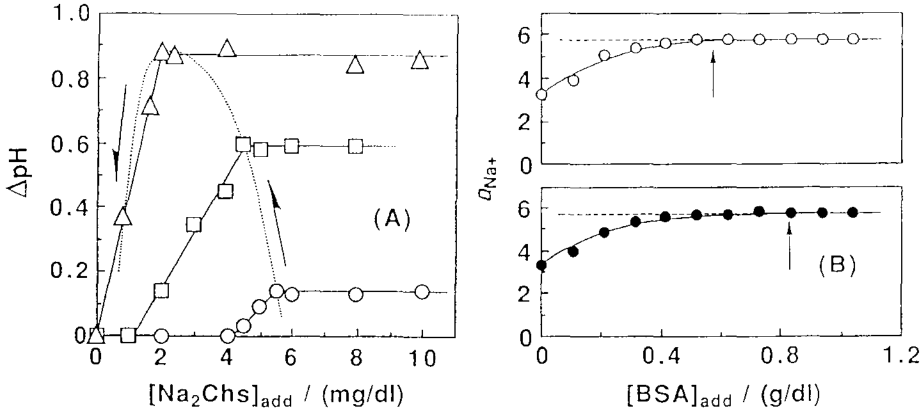

FIG. 2. Determination of the binding ratio, *R*, by means of an ion selective electrode. (A) Change in pH (Δ pH) by the addition of Na_2Chs at 25°C. The initial pH values were 3.5 (○), 4.0 (□), and 5.0 (△), where the BSA concentration was kept constant at 20 mg/dL. (B) Change of sodium ion activity by the addition of BSA at 25°C. The initial pH values were 3.5 (○) and 4.0 (●), where the concentration of Na_2Chs was 0.2 g/dL. The arrows indicate the end points.

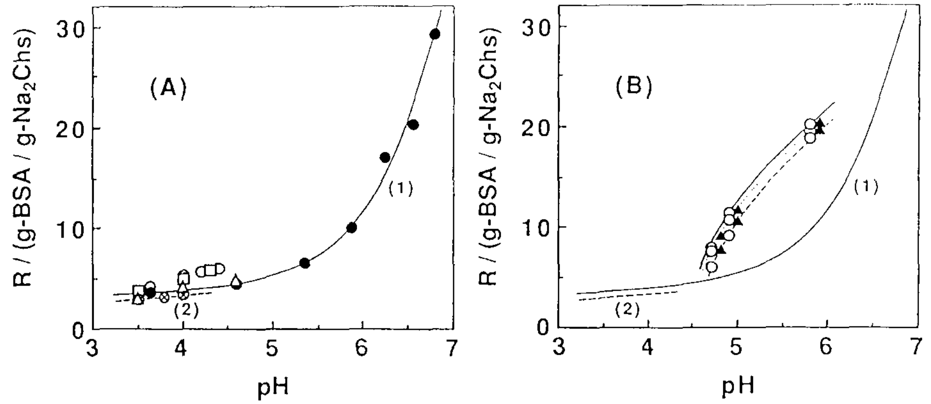

FIG. 3. Binding ratio, *R*, of BSA to Na_2Chs at 25°C. (A) The *R*-value in an aqueous phase was determined from changes in pH (— ● —, see Fig. 2A) and a_{Na^+} (△, see Fig. 2B) from a chemical analysis of a mother solution in the absence of a salt (□) or in the presence of 154 mM NaCl (○) or 100 mM phosphate buffer (---⊗---). *R* is defined here as the ratio of decrease in [BSA] to that in [Na_2Chs], △ [BSA]/△ [Na_2Chs]. (B) The *R*-value on the surface of kaolin. The concentration of phosphate buffer added in order to adjust the pH was 0 (——), 10 (···), or 100 mM (---). The data shown by full lines are those in the presence of 154 mM NaCl and NaOH instead of phosphate buffer. The weight concentration of added BSA was the same as that of Na_2Chs. ○: $[BSA]_{add}$ = $[Na_2Chs]_{add}$ = 20 mg/dL; ▲: $[BSA]_{add}$ = $[Na_2Chs]_{add}$ = 200 mg/dL. *R* is here defined as the ratio of the adsorption amount in weight of BSA to that of Na_2Chs, X_{BSA}/X_{Na_2Chs}. Curves (1) and (2) are quoted from Fig. 3(A) in order to compare the PEC formation in an aqueous phase with that on the kaolin surface.

2(A) and 2(B). Pi exhibits a high affinity for BSA^+ (as mentioned later, see Fig. 6) and competes with Chs^- for the binding sites on BSA^+. Thus, the *R*-value became small in the presence of Pi (see ⊗ in Fig. 3A).

Adsorption Isotherm of BSA and Na_2Chs

The adsorption isotherm of BSA by kaolin at pH 4.7 is shown in Fig. 4(A). The adsorption amount increased stepwise, probably owing to the orientational change of adsorbed BSA from a side-on mode to an end-on one. Furthermore, it increased in the presence of Na_2Chs (compare ○ with □, and ● with ■), where the mixing ratio of BSA to Na_2Chs in a mother solution before adsorption was 1:1 in weight.

The adsorption isotherms for Na_2Chs are shown in Fig. 4(B). The adsorption data were obtained from the same sample solution as that used to determine the adsorption amount of BSA in Fig. 4(A). The adsorption amount of Na_2Chs decreased with an increase in the concentration of added Pi (compare ○ with ●) in the absence of BSA, whereas, in the presence of it, the adsorption amount increased with an increase in it (compare □ with ■). The adsorption isotherms in the presence of BSA (□ and ■) increased stepwise again with an increase in a concentration of Na_2Chs (and BSA, [Na_2Chs]/[BSA] = 1/1) in a manner quite similar to those of BSA shown in Fig. 4(A). These facts suggest that Na_2Chs is cooperatively adsorbed

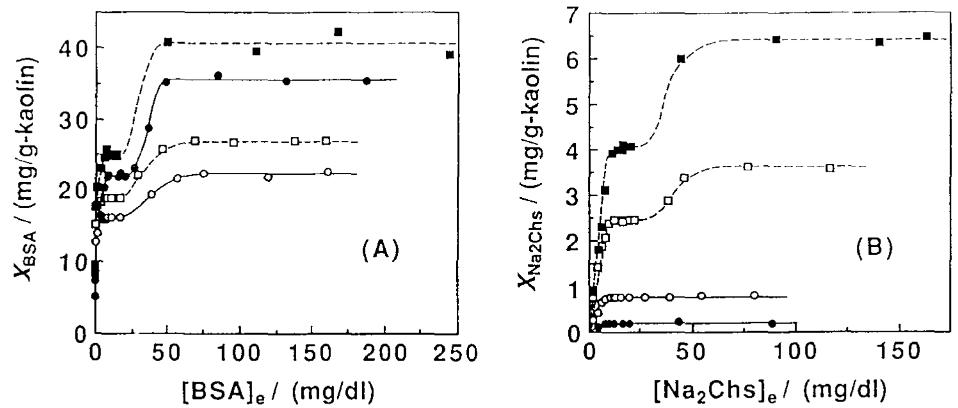

FIG. 4. Adsorption isotherm at pH4.7 and 25°C. (A) The adsorption isotherm of BSA in either the absence or the presence of Na_2Chs. Kaolin + BSA in the absence of Na_2Chs (——, ○, ●) and kaolin + BSA in the presence of Na_2Chs (---, □, ■). The concentration of phosphate buffer was 10 (○, □) or 100 mM (●, ■). (B) The adsorption isotherm of Na_2Chs in either the absence or the presence of BSA. Kaolin + Na_2Chs in the absence of BSA (——, ○, ●) and kaolin + Na_2Chs in the presence of BSA (---, □, ■). The concentration of phosphate buffer was 10 (○, □) or 100 mM (●, ■).

with BSA on kaolin and/or forms a polymer complex on the surface. The properties of the complex on the surface should resemble those in an aqueous solution, although the former might be affected by the physicochemical properties of the adsorbent, such as electric charges, and the affinity for polymers.

The Effect of Phosphate Ion on the Amounts of Polymer Adsorption

Figure 5(A) shows an adsorption amount of BSA (X_{BSA}) from an aqueous solution of BSA of a given concentration (20 mg/dL) and at a given pH, which was adjusted by phosphate buffer or HCl/NaOH. The value of X_{BSA} increased with an increase in the concentration of the phosphate buffer, [Pi]. It was higher in the presence of Na_2Chs (20 mg/dL, - - -) than in its absence (——) except at a low concentration of the phosphate buffer at pH 5.8 (compare □ with ■).

On the other hand, Fig. 5(B) shows an adsorption amount of Na_2Chs (X_{Na_2Chs}) from an aqueous solution of Na_2Chs (20 mg/dL) at a given pH adjusted in a manner similar to that mentioned above. The value of X_{Na_2Chs} decreased with an increase in [Pi] in the absence of BSA (——), while it increased with [Pi] in the presence of BSA (20 mg/dL, - - -). The former suggests a competitive adsorption of Pi with Chs^-. On the other hand, the latter means that a cooperative adsorption and/or complex formation between BSA and Na_2Chs is accelerated in the presence of Pi, especially at low pH.

The data of X_{Na_2Chs} in the presence of BSA (Fig. 5B) and those of X_{BSA} in the presence of Na_2Chs (Fig. 5A) were obtained through the determination of Na_2Chs and BSA in the same sample solution. Therefore, the ratio of X_{BSA} to X_{Na_2Chs} is the binding ratio, R, of the surface complex on kaolin. In a similar manner, the R-value

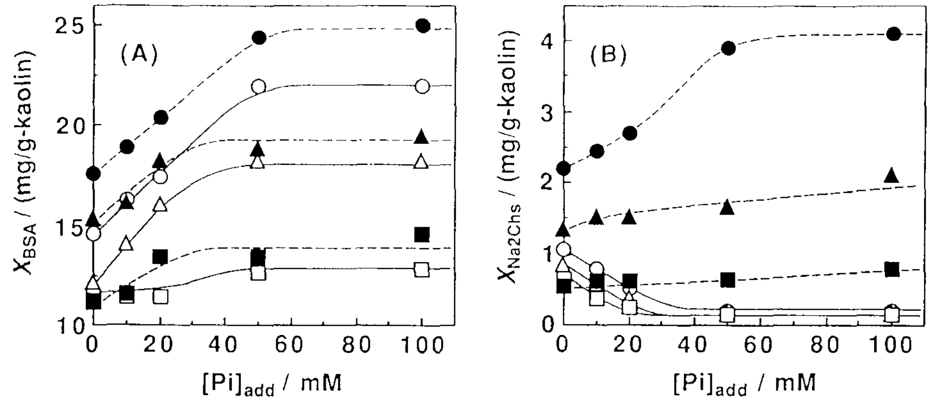

FIG. 5. Effect of phosphate ion concentration ($[Pi]_{add}$) on the adsorption amount of BSA (X_{BSA}) and Na_2Chs (X_{Na_2Chs}). pH = 4.7 (○ and ●), 4.9 (△ and ▲), and 5.8 (□ and ■). $[BSA]_{add} = [Na_2Chs]_{add}$ = 20 mg/dL. T = 25°C. (A) X_{BSA} vs $[Pi]_{add}$ for the system kaolin + BSA in the absence (—— and open symbols) and in the presence of Na_2Chs (--- and closed symbols). (B) X_{Na_2Chs} vs $[Pi]_{add}$ for the system kaolin + Na_2Chs in the absence (—— and open symbols) and in the presence of BSA (--- and closed symbols).

for the surface complex could be evaluated from the data shown in Figs. 4(A) and (B) at given concentrations of Na_2Chs and BSA (20 and 200 mg/dL, for example).

The facts that both X_{BSA} in the presence or absence of Na_2Chs (Fig. 5A) and X_{Na_2Chs} in the presence of BSA (Fig. 5B) increased with [Pi] should be studied experimentally in some detail. The binding data of Pi (X_{Pi}) by BSA and kaolin are shown in Fig. 6 as a function of pH at a given concentration of Pi. These data show that Pi is bound to BSA in an acidic solution (≤pH 6.0) and to kaolin throughout

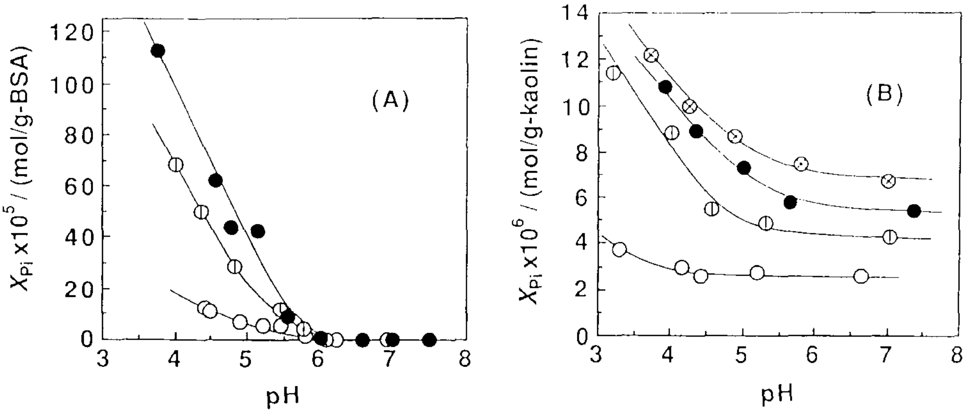

FIG. 6. Affinity of phosphate ion to BSA (A) and kaolin (B) as a function of pH at 4°C. The phosphate ion concentration was kept constant at 1 (○), 5 (⦶), 10 (●), or 20 mM (⊗). (A) Binding ratio of phosphate ion to BSA (20 mg/dL). (B) Adsorption amount of phosphate ion to kaolin (5 g/dL).

the pH's studied. That is, Pi bridges BSA to kaolin, resulting in an increase in X_{BSA} with [Pi] (—— in Fig. 5A). Positively charged BSA, thus adsorbed by kaolin, attracts much of negatively charged Na_2Chs with an increase in [Pi] (- - - in Fig. 5B). The extra Na_2Chs, thus adsorbed, furthermore collects BSA onto the kaolin to form more surface complex (- - - in Fig. 5A) followed by an increase in the amount of surface complex.

Competitive Adsorption and Complex Formation at High pH

The adsorption amount of BSA, X_{BSA}, from 20 mg/dL BSA solution at a given pH (>iep of BSA) is shown in Fig. 7(A) as a function of the mixing ratio of added Na_2Chs to added BSA, $[Na_2Chs]_{add}/[BSA]_{add}$. That of Na_2Chs, X_{Na_2Chs}, is shown in Fig. 7(B) in a similar manner to the above as a function of $[BSA]_{add}/[Na_2Chs]_{add}$. Both of them almost leveled off after decreasing with an increase in the mixing ratio of another polymer. This result shows that the coexistence of a small amount of another polymer causes competitive adsorption, whereas the amount of adsorption never decreases to zero even though the mixing ratio becomes high. This fact supports the formation of the surface complex at a pH higher than the iep, as it was formed in an aqueous phase (see the Introduction and Ref. 15), through an electrostatic interaction between positive charge patches of BSA and negative charges of Na_2Chs [14].

Figures 8(A) and (B) show the effect of pH on the adsorption amounts of BSA (X_{BSA}) and Na_2Chs (X_{Na_2Chs}). They decreased with an increase in pH irrespective of the presence or absence of another polymer owing to the competition with OH^-

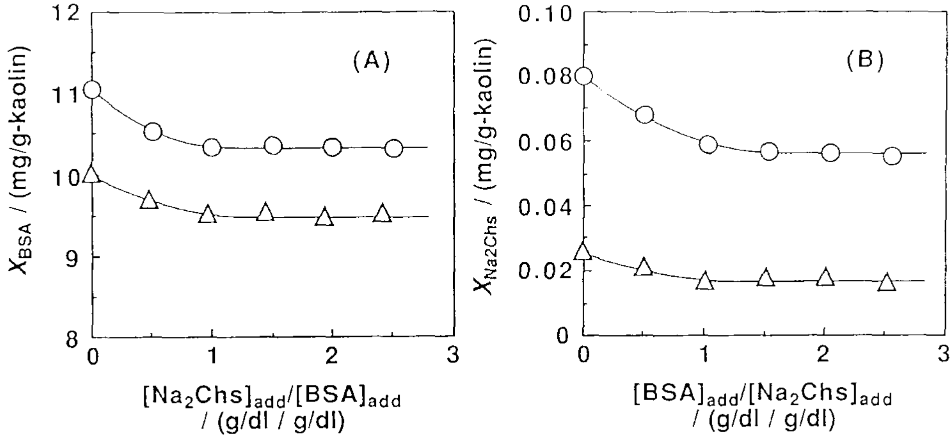

FIG. 7. Adsorption amount of BSA (X_{BSA}) and Na_2Chs (X_{Na_2Chs}) as a function of the mixing ratio (0.5 g/dL kaolin). pH = 6.5 (○) or 7.0 (△). The phosphate buffer concentration was kept constant at 100 mM. T = 25°C. (A) X_{BSA} vs the mixing ratio, $[Na_2Chs]_{add}/[BSA]_{add}$. The BSA concentration was kept constant at 20 mg/dL while the Na_2Chs concentration was changed. (B) X_{Na_2Chs} vs the mixing ratio, $[BSA]_{add}/[Na_2Chs]_{add}$. The Na_2Chs concentration was kept constant at 20 mg/dL while the BSA concentration was changed.

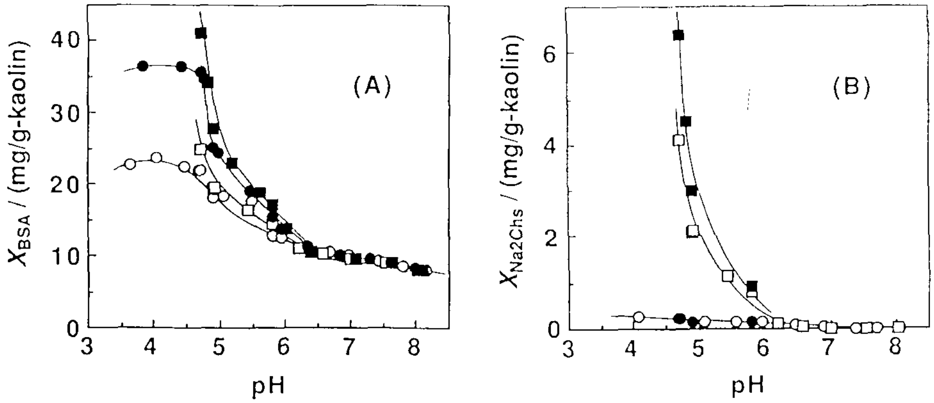

FIG. 8. Effect of pH on the adsorption amounts of BSA (A) and Na_2Chs (B) at 25 °C in the presence of 100 mM phosphate buffer. The concentrations of added BSA and Na_2Chs were kept constant at 20 (○, □) or 200 mg/dL (●, ■), respectively, where $[BSA]_{add} = [Na_2Chs]_{add}$. (A) X_{BSA} vs pH for kaolin + BSA in the absence of Na_2Chs (○, ●), and for kaolin + BSA in the presence of Na_2Chs (□, ■). (B) X_{Na_2Chs} vs pH for kaolin + Na_2Chs in the absence of BSA (○, ●) and for kaolin + Na_2Chs in the presence of BSA (□, ■).

and Pi and/or to the increase in negative charge on the kaolin surface. On the other hand, they were significantly higher in the presence than in the absence of another polymer at a pH lower than 6.5 because of the complex formation on the surface. The adsorption data in the presence of another polymer at a pH lower than 4.5 are not given here due to coacervate/precipitate formation in a mother solution before adsorption. (Incidentally, a suspension containing both BSA and Na_2Chs for a measurement of the mean diameter of kaolin was prepared in a different manner, as mentioned in the Experimental Section.)

When the pH became higher than 6.5, X_{Na_2Chs} decreased to almost zero while X_{BSA} retained a definite value. The slope of X_{Na_2Chs} was remarkably steeper in the presence than in the absence of BSA (Fig. 8B), while the difference in the slopes of X_{BSA} in the presence and absence of Na_2Chs was small (Fig. 8A).

Binding Ratio of the Surface Complex

The binding ratio, R, of BSA to Na_2Chs in the surface complex in weight was shown in Fig. 3(B) as a function of pH, where the concentrations of added Pi, BSA, and Na_2Chs were kept constant. The initial mixing ratio of added BSA to added Na_2Chs in weight, $[BSA]_{add}/[Na_2Chs]_{add}$, was also kept constant at unity for convenience, as mentioned later. These data were extracted from those shown in Figs. 4–8.

The initial concentrations of BSA and of Na_2Chs, where $[BSA]_{add}/[Na_2Chs]_{add} = 1$, were 20 and 200 mg/dL, respectively. These concentrations were chosen as representative for the 1st and 2nd steps of the adsorption isotherms (see Figs. 4A and 4B). Although the mixing ratio was arbitrarily fixed at unity, this ratio is the most convenient for comparison of the binding ratio (R) of the complex between that in an aqueous phase and that in the surface phase. The *R*-value for the complex formed in the water phase was almost constant over a wide range of the initial mixing ratios of BSA to Na_2Chs, as mentioned before (Fig. 3A), where unity is the median for these mixing ratio.

The *R*-value increased with an increase in pH and decreased with an increase in the concentration of the phosphate buffer, $[Pi]_{add}$. This tendency of *R* is quite similar to that found in the complex formation in an acidic aqueous phase (compare curve(1) with curve(2) in Fig. 3(A)). The Curves (1) and (2) in Fig. 3(B) are quoted from those in Fig. 3(A) in order to easily compare the *R*-values obtained by different methods.

Each value of *R* for the surface complex at a given pH is higher than that for the complex formed in an aqueous phase. This is because the adsorption amount of Na_2Chs is relatively smaller than that of BSA, especially at high pH, owing mainly to the repulsion between negatively charged kaolin and Na_2Chs.

The data at a given concentration of phosphate buffer, [Pi], is on a curve, although the initial concentrations of the polymers were different (see ○ and ▲). However, they decreased with an increase in [Pi] (——, · · ·, - - -) probably because Pi bound to BSA (Fig 6A) interrupts the binding of BSA to Na_2Chs on the surface and the adsorption of BSA to kaolin, which also adsorbs Pi (Fig. 6B).

The binding ratio (*R*) increased with an increase in pH at a given [Pi] and at a given initial concentration of the polymers. This fact is explained as follows: Negative charge densities on BSA and kaolin (see Fig. 9) and, therefore, the degree of mutual repulsion among BSA, kaolin, and Na_2Chs, increase with an increase in pH. As a result, the adsorption amounts of each polymer and the complex should

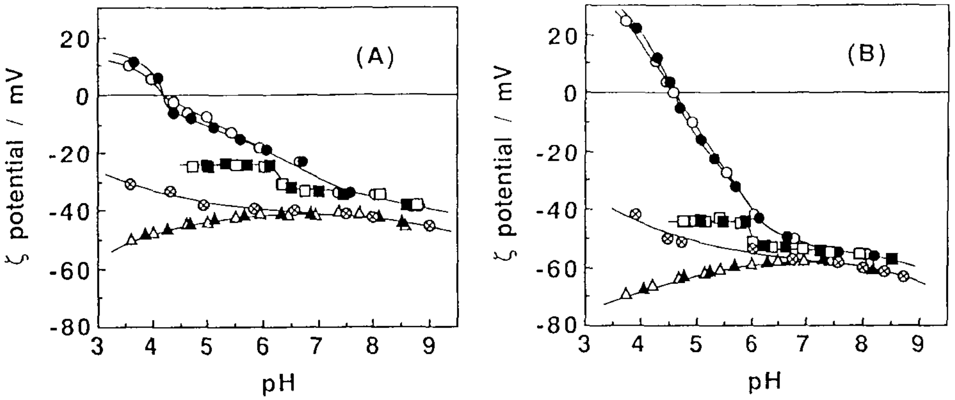

FIG. 9. Zeta potential of kaolin particles in 100 (A) and 10 mM phosphate buffer solution (B) as a function of pH. The concentrations of added BSA and Na_2Chs were kept constant at 0 (⊗), 20 (○, △, □), or 200 mg/dL (●, ▲, ■), respectively. The zeta potential of kaolin particles was measured in the absence of any polymer (⊗), in the presence of BSA but the absence of Na_2Chs (○, ●), in the presence of Na_2Chs but the absence of BSA (△, ▲), and in the presence of both polymers (□, ■), where $[BSA]_{add} = [Na_2Chs]_{add}$.

decrease. However, the rate of decrease in X_{Na_2Chs} with pH is larger than that in X_{BSA} (Figs. 8A and B), resulting in an increase of X_{BSA}/X_{Na_2Chs} with pH.

Zeta Potential

Figures 9(A) and 9(B) show the zeta potential of kaolin particles as a function of pH adjusted by a phosphate buffer solution. The zeta potential of a bare kaolin particle (⊗) was negative over the pHs investigated, where its absolute value increased with pH. This fact shows that its original surface has negative charges as a whole although both a positive edge double layer and a negative double layer on the flat surface contribute to its zeta potential [21], and that the density of negative charges on its surface on the average increases with pH due to the adsorption of OH^- and Pi.

On the other hand, the zeta potential in the presence of Na_2Chs (△ and ▲) at pH 3.5–7.0 was lower than that in the absence (⊗), while the former was asymptotic to the latter with pH. This is because the adsorption of Na_2Chs was detectable at low pH but negligible at high pH (see ○ and ● in Fig. 8B). The adsorption of Na_2Chs gives a negative charge to kaolin, resulting in a lower zeta potential at low pH.

When BSA was added (○ and ●), the zeta potential was positive at pHs lower than 4.2 (Fig. 9A) and 4.6(Fig. 9B). The apparent iep was lower than that of BSA itself. This is owing to the fact that kaolin has a negative charge, as mentioned above, and that Pi is adsorbed/bound by kaolin (Fig. 6B) and BSA (see Fig. 6A). When the pH became higher than the iep, the zeta potential was reversed to a negative one and decreased with pH. However, it was not asymptotic to that of the bare particle (⊗), as in the case of Na_2Chs, because BSA was still adsorbed by kaolin even at pH 8–9 (see Fig. 8A).

In the case of the coexistence of BSA with Na_2Chs (☐ and ■; 1:1 in weight), the zeta potential changed stepwise in both 10 and 100 mM phosphate buffer solutions and at both 20 and 200 mg/dL BSA, respectively. These data were between those in the presence of BSA (○ and ●) and those in the presence of Na_2Chs (△ and ▲) by virtue of the complex formation of BSA with Na_2Chs on the surface. They were asymptotic to the zeta potential of kaolin covered with BSA (instead of being asymptotic to the zeta potential of the bare particle) with an increase in pH. This fact is explained as follows: Na_2Chs is easily desorbed with increase in pH, whereas adsorbed BSA remains on the surface of kaolin even at high pH, as shown in Fig. 8. Therefore, the effect of BSA on the zeta potential becomes more dominant than that of Na_2Chs at high pH. However, no simple explanation for the two-step change in zeta potential can be offered at present.

Comparing the data at 100 mM (Fig. 9A) with those of 10 mM phosphate buffer (Fig. 9B), the absolute values of the former were less than those of the latter. This is explained in terms of the effect of ionic strength. That is, with an increase in ionic strength prepared by phosphate buffer, the Debye length and/or the thickness of the electric double layer around the kaolin particle decreases, followed by a decrease in the absolute value of the zeta potential. On the other hand, it might be difficult to explain this effect in terms of the adsorption amounts of the polymers, because the adsorption amount of BSA increases while that of Na_2Chs increases or decreases with an increase in the concentration of the phosphate buffer, as shown in Fig. 5.

Mean Diameter of Kaolin Particles Aggregated

Figure 10 shows the mean diameter of kaolin in the presence of BSA and/or Na_2Chs as a function of pH (A) and of the weight fraction of BSA with respect to the added polymers (B). A mean diameter, *d*, was almost constant over the pH studied (pH 4-6) in the absence of any polymer (⦶), in the presence of 10^{-5} to 10^{-1} g/dL Na_2Chs (●), or 10^{-4} g/dL BSA (⊙). However, the effect of the species of added polymer on *d* was definitely different. The *d* value was lowest in the presence of Na_2Chs because it is a dispersing agent toward kaolin [16, 17]. To the other hand, the *d* value increased with the concentration of BSA and exhibited a maximum around its iep. This result suggests that BSA play a role in the interparticle bridging of kaolin after adsorption. The bridging was the most effective at an iep where the net charge was zero and the mutual electrostatic repulsion was the smallest, resulting in a high *d*-value.

Figure 10(B) shows the relationship between the *d*-value and the weight fraction of the added BSA with respect to the total polymers added ($=10^{-2}$ g/dL). There was a maximum in *d* at pH 4.0 (◓), while the *d*-value monotonously increased with the weight fraction of BSA at pHs of 4.7 (⊗) and 5.8 (△). The values shown on the ordinates are those in the presence of a mere 10^{-2} g/dL Na_2Chs without BSA, or a mere 10^{-2} g/dL BSA without Na_2Chs (see Fig. 10A). The maximum reflects the most suitable weight fraction for aggregation by bridging through

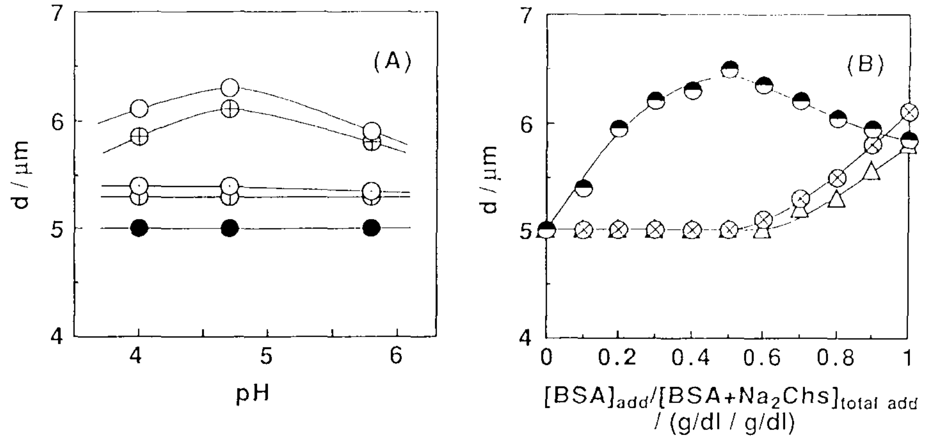

FIG. 10. Mean diameter of an aggregate of kaolin particles in 154 mM NaCl. (A) Diameter *d* vs pH. The added polymer was 10^{-5}-10^{-1} g/dL Na_2Chs (●), 10^{-1} (○), 10^{-2} (⊕), 10^{-4} (⊙) g/dL BSA, or none (⦶). (B) The *d*-value vs the weight fraction of added BSA with respect to the total amount of added polymers at 154 mM NaCl. The total concentration of added BSA and Na_2Chs, $[BSA + Na_2Chs]_{total\ add}$, was kept constant at 10 mg/dL before dilution. The solution pH was 4.0 (◓), 4.7 (⊗), or 5.8 (△).

complex formation between positively charged BSA and negatively charged Na_2Chs adsorbed on separate kaolin particles.

To elucidate this phenomenon more clearly, the *d*-value was shown as a function of the *R*-value after determining the adsorption amounts of BSA and Na_2Chs. The maximum in *d* was at $R = 4.1$, as shown in Fig. 11. This *R*-value fairly coincides with that obtained by means of the coacervation method at pH 4.0, as shown in Fig. 3(A). This agreement means that PEC formation in an aqueous solution (Fig. 3) is quite similar to that on the surface of kaolin (Figs. 10 and 11). In other words, kaolin particles carrying BSA and those carrying Na_2Chs electrostatically attract each other in an aqueous phase, resulting in the aggregation of kaolin particles and/or the formation of a pseudocoacervate of BSA–Na_2Chs–kaolin.

Needless to say, this coacervate was diluted and redispersed in an aqueous phase immediately before the measurement of *d*. Therefore, the degree of PEC formation on kaolin is reflected in the *d*-value even after dilution. The *d*-value at *R* other than the optimum ($R = 4.1$) was smaller than that at the optimum because an excess amount of adsorbed BSA or Na_2Chs gives an extra electric charge to kaolin, resulting in fair electrostatic repulsion between the particles.

A maximum in *d* was not observed at pHs 4.7 and 5.8, although the complex was formed both in an aqueous phase and on the surface of kaolin, as shown in Fig. 3. This is probably because the attractive force for PEC formation between BSA and Na_2Chs on kaolin might be weak at pH values close to and higher than the iep. Incidentally, the zeta potential and, therefore, the surface charge of the respective particles covered with/without the polymers was negative at pHs higher than 4.6, as mentioned before (see Fig. 9).

In conclusion, water-insoluble PEC or coacervate was formed in an aqueous phase at a pH lower than the iep. It was also formed on the surface of kaolin via concurrent and/or cooperative adsorption of two polymers. As a result, kaolin

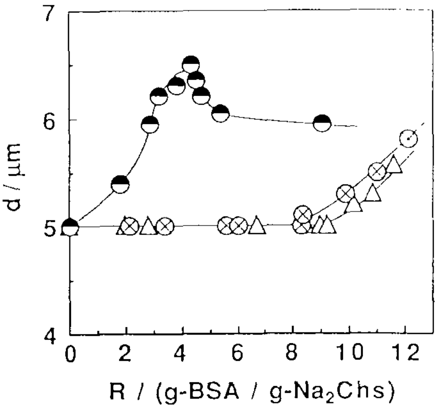

FIG. 11. Mean diameter, *d*, as a function of the binding ratio, *R*, at 154 mM NaCl. The solution pH was 4.0 (◓), 4.7 (⊗), or 5.8 (△). The sum of the concentrations of added BSA and Na_2Chs was kept constant at 10 mg/dL while that of kaolin was 0.5 g/dL before dilution. These data were obtained after rearrangement of those in Fig. 10(B).

aggregation was accelerated by virtue of the effect of interparticle bridging through the PEC formed on kaolin. On the other hand, water-soluble PEC was formed in an aqueous phase at a pH higher than the iep. However, no remarkable effect on aggregation was observed because the attractive force between BSA on kaolin and Na_2Chs on kaolin was weak because these three species have negative charges at high pH. The factors affecting the binding ratio, R, of BSA to Na_2Chs were 1) initial mixing ratio of BSA to Na_2Chs, 2) concentration and species of an added salt, 3) pH, 4) net charge of the polymer, 5) charge patches on BSA, and so on.

According to our preliminary experiment, the PEC was also formed on the surface of HAP through a concomitant adsorption of BSA and Na_2Chs. The R-value on HAP was, however, strikingly higher than that on kaolin. The binding ratio or the composition of the PEC on a solid surface thus depends on the species and chemical composition of an adsorbent. The authors are planning to study the PEC formation on HAP in detail in order to compare it with that on kaolin. The formation of PECs between proteins and mucopolysaccharides on biological HAP and/or modification of the surface properties of HAP by these biopolymers is indeed important for the formation/construction of hard tissues of mammalian animals. Hard tissue is, in fact, a composite material of inorganic and organic compounds.

REFERENCES

[1] W. B. Neely, *J. Polym. Sci., Part A, 1*, 311 (1963).
[2] R. Nagarajan, M. Barry, and E. Ruckenstein, *Langmuir, 2*, 210 (1986).
[3] N. P. Desai and J. A. Hubbell, *Macromolecules, 25*, 226 (1992).
[4] L. Randen, J. Nilson, and P. Edman, *J. Pharm. Pharmacol., 40*, 763 (1988).
[5] J. Nieuwenhuis, Y. Y. Tan, and G. O. R. Alberda van Ekenstein, *Angew. Makromol. Chem., 147*, 83 (1987).
[6] I. Illiopoulos and R. Audebert, *Polym. Bull. 13*, 171 (1985).
[7] Y. Osada and M. Sato, *Nippon Kagaku Kaishi*, p. 175 (1976).
[8] Y. Kikuchi and N. Kubota, *Bull. Chem. Soc. Jpn., 60*, 375 (1987).
[9] T. Chandy and C. P. Sharma, *J. Colloid Interface Sci., 130*, 331 (1989).
[10] A. S. Michaelis, *Ind. Eng. Chem., 57*, 32 (1965).
[11] G. L. Koh and I. G. Tucker, *J. Pharm. Pharmacol., 40*, 309 (1988).
[12] H. Jizomoto, *J. Pharm. Sci., 74*, 469 (1985).
[13] C. I. Mekras, J. B. Lawton, and R. J. Washington, *J. Pharm. Pharmacol., 41*, 22 (1989).
[14] J. M. Park, B. B. Muhoberac, P. L. Dubin, and J. Xia, *Macromolecules, 25*, 290 (1992).
[15] M. Nakagaki and Y. Sano, *Bull. Chem. Soc. Jpn., 45*, 1011 (1972); *Nippon Kagaku Kaishi*, p. 875 (1976).
[16] S. Shimabayashi, K. Nishino, and M. Nakagaki, *Colloids Surf., 63*, 121 (1992).
[17] S. Shimabayashi, M. Okuda, M. Yagiu, and M. Nakagaki, *Colloid Polym. Sci., 265*, 1099 (1987).
[18] T. Bitter and H. M. Muir, *Anal. Biochem., 4*, 330 (1962); Z. Disch, *J. Biol. Chem., 167*, 189 (1947).

[19] O. H. Lowry, N. J. Rowebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, *193*, 265 (1951).
[20] A. Gee, L. P. Domingues, and V. R. Deitz, *Anal. Chem.*, *26*, 1487 (1954).
[21] H. van Olphen, *An Introduction to Clay Colloid Chemistry*, Wiley-Interscience, New York, 1963, pp. 93–94.