〈Original Article〉

Reaction principle of turbidity generation of serum albumin by aromatic organic acid salt

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Summary The anion of sulfosalicylic acid (SSA) used for measuring urine protein is said to bind to the positively-charged protein and generate protein turbidity. If this reaction principle is correct, other organic acid salts are also assumed to generate turbidity in the same manner as SSA. Thus, this reaction principle was studied by a calculation based on the chemical equilibrium of the protein error, and by an experiment using seven aromatic organic acid salts. In the experiment the turbidity generation of human serum albumin occurred only in the pH range from about 1.5 to 4.0 in three kinds of salts, but not at all in four kinds. In the calculation the protein/organic anion complex was estimated to form in the wide pH range from 1 to 11. Thus, the calculated result did not accord with the experimental one approximately in the pH range from 4 to 11, but the experimental result can be explained by the supposition that only the no-charged complex generates the turbidity at a lower pH.

Key words: Urine protein, Organic acid salt, Turbidity generation, Reaction principle

1. Introduction

There are the dye-binding method[1-2], the biuret method[3], and the turbidimetric method[4-6] for determining urine protein. Of the turbidimetric methods, a SSA method that is highly sensitive and reliable is used not only to determine the urine protein concentration but also to confirm a false-positive in a test strip and to screen the Bence-Jones proteins from the discrepancy between the test strip and SSA method. The discrimination of the sample with examination data such as a test strip (−) and the SSA method (+) is very important for diagnosis. The reaction principle of the SSA method is explained by the fact that the SSA anion binds to the positively-charged protein electrostatically, and the formed protein/organic anion complex generates turbidity by the coherence of the complexes[7]. The author previously investigated whether its theory is proper or not by an experiment and a calculation based on the chemical equilibrium[8]. This investigation revealed that the experimental result agrees with the calculated one on the assumption that only the no-charged protein/organic anion complex generated from the SSA anion and the positively-charged protein condenses into turbidity. If its reaction principle is valid, it is estimated that the aromatic organic acid except the SSA also generates turbidity. The understanding of the reaction principle is very important to avoid measurement error and the appropriate evalua-

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tion of inspection data. Thus, in this paper the reaction principle of the turbidimetric method was investigated furthermore by an experiment using seven aromatic organic acid salts which are considered to bind to the positively-charged protein, and by a calculation based on the chemical equilibrium of the protein error[9-10].

2. Materials and methods

1. Reagents

All reagents of purest quality available were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Buffer solutions: those with the pH range from 1 to 2 were prepared by a HCl solution. Buffer solutions with the pH range from 2 to 3 were prepared by mixing a 0.1 mol/L HCl solution and a 0.1 mol/L glycine solution containing a 0.1 mol/L NaCl. Buffer solutions with the pH range from 3 to 8 were prepared by mixing a 0.1 mol/L citric acid solution and a 0.2 mol/L Na_2HPO_4 solution. Buffer solutions with the pH range from 8 to 13 were prepared by mixing a 0.1 mol/L glycine solution containing a 0.1 mol/L NaCl and a 0.1 mol/L NaOH solution. pH was measured by a pH meter (TOA Electronics Ltd.).

0.118 mol/L organic acid salt solution: 0.0118 mol of organic acid salt (sodium sulfosalicylate dehydrate, sodium sulfanilate dehydrate, potassium hydrogen phthalate, sodium hippurate, sodium benzoate, sodium salicylate, benzenesulfonic acid sodium salt monohydrate) was dissolved in 100 mL of distilled water.

2 g/L protein solution: 200 mg of human serum albumin (HSA) for biochemistry was dissolved in 100 mL of distilled water.

Bromocresol purple (BCP) color reagent[9]: To 20 mL of 1 mmol/L BCP solution, 40 mL of a buffer solution of pH 5.2 and 8.0 was added, and then diluted to 100 mL with distilled water.

2. Procedure

1) Measurement of turbidity

A test solution was prepared by adding 2.0 mL of a buffer solution with the pH range from 1 to 13 and 2.0 mL of a 0.118 mol/L organic acid salt solution to 1.0 mL of a 2 g/L HSA solution, and reacted for 10 min at 37℃. Its turbidity was measured at 660 nm against distilled water using Hitachi U1500 spectrophotometer.

2) Measurement of pH of reaction mixture

Since the organic acid salt solution is added to a buffer solution containing HSA rather in excess, the pH of the reaction mixture is considered to change from the pH of a buffer solution. Therefore, the pH of the reaction mixture was measured by a pH meter in order to accuratly obtain the relationship between the turbidity generation and the pH. The pH described in this paper does not indicate that of a buffer solution itself but that of the reaction mixture after adding the organic acid salt solution to a buffer solution containing HSA.

3) Measurement of precipitated protein concentration

A test solution was prepared by adding 2.0 mL of a buffer solution with the pH range from 1 to 13 and 2.0 mL of a 0.118 mol/L organic acid salt solution to 1.0 mL of a 2 g/L HSA solution, and reacted for 10 min at 37℃. Then, this reaction mixture was centrifuged for 15 min at 2,500 rpm, and the residue was obtained by removing supernate by decantation. The precipitated protein concentration in the residue was measured by the biuret method.

4) Titration of the reaction mixture

As described later, the turbidity generation of HSA occurred only in the limited pH range. Thus, in order to confirm that the turbidity generation depends on the pH, the reaction mixture in which the turbidity does not form even by the addition of the organic acid salt was prepared as follows. To 20 mL of 2 g/L HSA 40 mL of the buffer solution of pH 7.0 and 40 mL of 0.236 mol/L potassium hydrogen phthalate solution were added. The pH of this reaction mixture became around 5.1, and the turbidity did not form. The pH of the mixture was measured while titrating it first by 1 mol/L hydrochloric acid, a part of the solution was taken out appropriately, and the turbidity was measured at 660 nm against distilled water. This titration was continued until the pH of the solution became around 2.5. Then, this solution of pH 2.5 was

inversely titrated with 1 mol/L sodium hydroxide solution, a part of the solution was taken out appropriately, and the turbidity was measured at 660 nm against distilled water. This titration was continued until the pH of the solution became around 5.5.

5) Effect of organic acid salt on color development of HSA by BCP

0.5 mL of the organic acid salt solution was added to 0.5 mL of the HSA solution, and then 4.0 mL of the BCP color reagent was added to this solution. The absorbance was measured against a reagent blank at 590 nm. The reagent blank was prepared by adding 4.0 mL of the BCP color reagent to the solution comprising 0.5ml of distilled water and 0.5 mL of organic acid salt solution.

3. Calculation based on chemical equilibrium

It was assumed that the anion (OA^-) of the organic acid (HOA) binds to the positively-charged protein (P^+) and the formed protein/organic anion complex (POA) becomes turbid by their coherence[8].

$$HOA \rightleftharpoons H^+ + OA^- \qquad \frac{[H^+][OA^-]}{[HOA]} = K_A$$

$$P^+ + OA^- \rightleftharpoons POA \qquad \frac{[POA]}{[P^+][OA^-]} = K_{PS}$$

◎Protein/organic anion complex concentration

The protein/organic anion complex concentration was calculated as follows according to the previous paper[10]:

$$K_A K_{PS}[POA]^2 - (K_A K_{PS} C_A + K_A K_{PS} C_P + [H^+] + K_A)[POA] + K_A K_{PS} C_A C_P = 0 \qquad (1)$$

K_A: dissociation constant of organic acid, K_{PS}: equilibrium constant of the reaction between a positively charged protein and the organic anion, C_P (mol/L): positively-charged protein concentration, C_A: organic acid concentration (mol/L).

Here, the positively-charged protein concentration was calculated by the following equation.

$$C_P = \alpha C_x = \frac{C_x}{1 + \frac{K_w}{K_b[H^+]} + \frac{K_a K_w}{K_b[H^+]^2}}$$

C_X: total protein concentration (mol/L), α: degree of dissociation of positively-charged protein, K_W: ionic product of water, K_a: dissociation constant of acidic side chain of protein, K_b: dissociation constant of basic side chain of protein.

◎Variables in the calculation

pK_A=2～5, $K_{PS}=10^4$, $C_A=2.4\times10^{-2}$ mol/L, $C_X=5.8\times10^{-6}$ mol/L, $K_w=10^{-14}$, $K_a=10^{-8}$, $K_b=10^{-3}$.

3. Results

1. Characteristics obtained by experiment

1) Reaction between HSA and organic acid salt

The reaction between HSA and the seven organic acid salts was investigated, changing the pH of the reaction mixture. HSA became turbid by sodium sulfosalicylate, sodium salicylate and potassium hydrogen phthalate. The turbidity of HSA was observed only in the pH range from about 1.5 to 4.0, and did not occur in the pH range from 1 to 1.5 and above pH 4.0 as indicated in Fig. 1. The pH at which the turbidity generation began to occur was different from the kind of organic acid salt and was 1.5 for sulfosalicylate, 2.5 for salicylate and 3.2 for phthalate. The turbidity became maximum at approximately pH

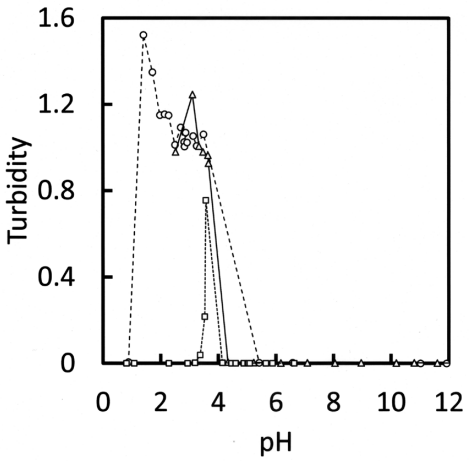

Fig. 1 Relationship between the turbidity and the pH (experimental result)
pH indicates the pH of the reaction mixture after adding the organic acid salt. The turbidity was measured at 660 nm against distilled water.
○: 0.118 mol/L sodium sulfosalicylate
△: 0.118 mol/L sodium salicylate
□: 0.118 mol/L potassium hydrogen phthalate

1.5 for sulfosalicylate, pH 3.1 for salicylate and pH 3.5 for phthalate. Four other organic acid salts did not generate turbidity at all in the pH range from 1 to 11. Fig. 2 indicates the relationship between the precipitated protein concentration and the pH of the reaction mixture. The precipitated protein was detected in the same pH range as the turbidity, and its maximum concentration existed at pH 1.7-3.1 for sulfosalicylate, pH 3.1-3.3 for salicylate and pH 3.5 for phthalate. The pH for sulfosalicylate and salicylate are different from the pH at which the turbidity became maximum.

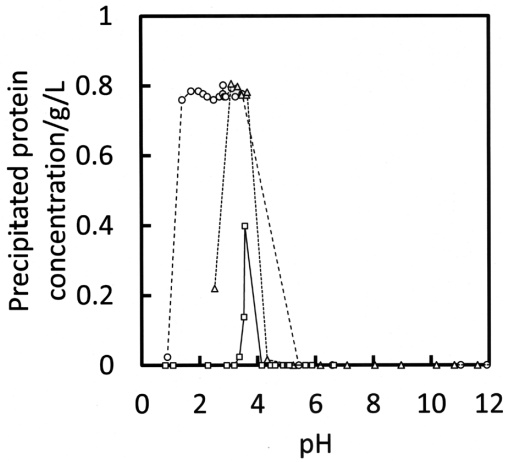

Fig. 2 Relationship between the precipitated protein concentration and the pH (experimental result). The precipitated protein concentration was measured by the biuret method.
○: 0.118 mol/L sodium sulfosalicylate
△: 0.118 mol/L sodium salicylate
□: 0.118 mol/L potassium hydrogen phthalate

2) Effect of organic acid salt concentration on precipitated protein concentration

Fig. 3 indicates the relationship between the precipitated protein concentration and the pH when the SSA concentration varied. As the organic acid salt concentration decreased, the pH at which HSA begins to precipitate shifted to a higher pH.

3) pH dependence of turbidity generation

As mentioned above, the turbidity generation did not occur in the pH range of 1 to 1.5 and above pH 4.0

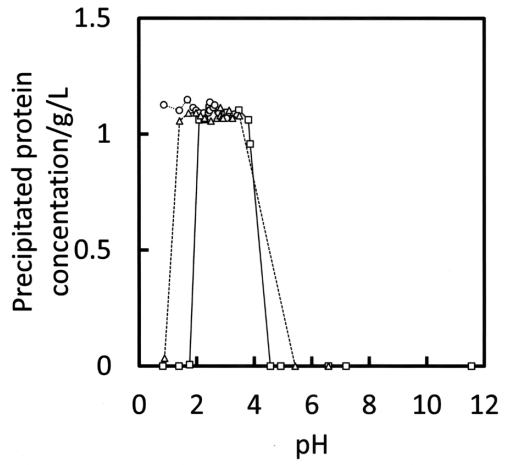

Fig. 3 Relationship between the precipitated protein concentration and the organic acid salt concentration (experimental result).
The measurement was performed using sodium sulfosalicylate as a precipitant.
○: 0.059 mol/L, △: 0.118 mol/L, □: 0.236 mol/L

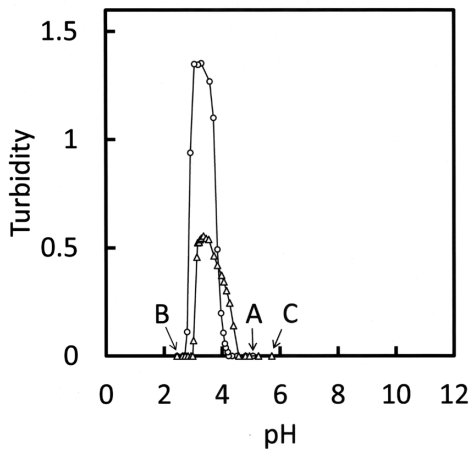

Fig. 4 Reversibility of the reaction (experimental result).
The reaction mixture without the turbidity was titrated first by 1 mol/L HCl (○) from pH 5.1 (A) to pH 2.5 (B). Then, this reaction mixture of pH 2.5 was inversely titrated by 1 mol/L NaOH (△) from pH 2.5 (B) to pH 5.8 (C). The reaction mixture was prepared by adding 40 mL of the buffer solution of pH 7.0 and 40 mL of 0.236 mol/L potassium hydrogen phthalate to 20 mL of 2 g/L HSA. This reaction mixture does not become turbid.

in spite of the addition of the organic acid salt. When the reaction mixture of pH 5.1 without the turbidity was titrated first by 1 mol/L hydrochloric acid, as indicated in Fig. 4 the turbidity began to form at about pH 4.2, reached the maximum at pH 3.1-3.3, and disappeared at about pH 2.8. This titration was continued to pH 2.5. Then, this reaction mixture of pH 2.5 without the turbidity was inversely titrated with 1mol/L sodium hydroxide. The turbidity began to form again at about pH 3.0, reached the maximum at about 3.4, and disappeared at about pH 4.5. In this way, it was confirmed that the turbidity generation depends on the pH of the reaction mixture and is reversible reaction.

4) Effect of organic acid salt on color development of HSA by BCP

The dissociated dye anion (D^-) of bromocresol purple (HD) binds to the positively-charged protein (P^+), forming a dye-protein complex (PD) which is a colored product[10]. In the presence of inorganic salt, inorganic anion binds to a positively-charged protein[9]. As a result, since the positively-charged protein which produces the colored product decreases, the color development decreases. If the organic anion (OA^-) binds to a positively-charged protein like inorganic anion and generates the protein/organic anion complex (POA) as follows, the color development of HSA by BCP is expected to decrease.

$$HD \rightleftarrows H^+ + D^- \qquad HOA \rightleftarrows H^+ + OA^-$$

$$P^+ + D^- \rightleftarrows PD \qquad P^+ + OA^- \rightleftarrows POA$$

Thus, the effect of the organic acid salt on the color development of HSA by BCP at pH of 5.2 and 8.0 was investigated. Fig. 5 indicates the relationship between the color development at pH 5.2 and the organic acid salt concentration. The color development in the absence of the organic acid salt was expressed as 100. The color development decreased in the presence of the organic acid salt; its decrease was far larger than that by sodium chloride. A similar result was provided in the reaction of pH 8. These results indicate that the organic anion binds to the positively-charged protein even at pH 5.2 and pH 8.0 where HSA did not become turbid.

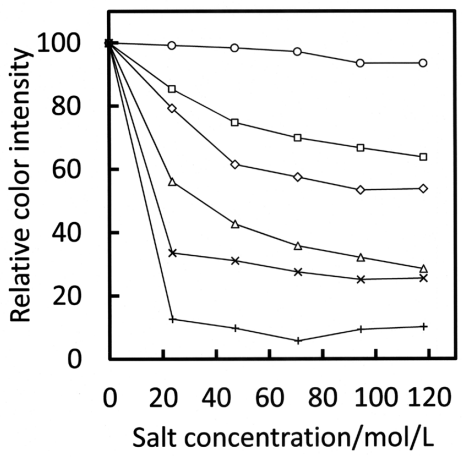

Fig. 5 Effect of the organic acid salt on the color development of the human serum albumin by bromocresol purple (experimental result).
The measurement was performed at pH 5.2.
○: sodium chloride, △: benzenesulfonic acid sodium salt, □: sodium hippurate, ◇: sodium sulfanilate, ×: sodium benzonate, +: sodium salicylate

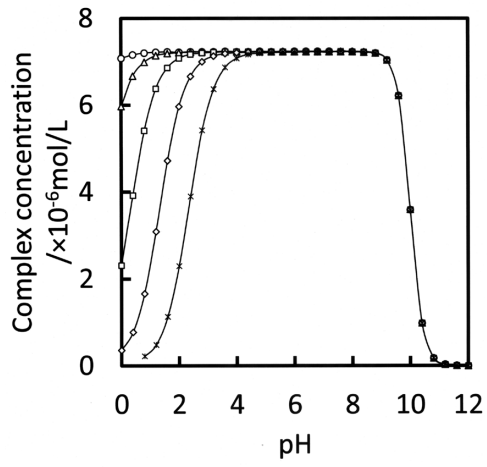

Fig. 6 Relationship between the protein/organic anion complex concentration and the pH (calculated result).
The calculation was performed varying the dissociation constant (K_A) of the organic acid from 10^{-1} to 10^{-5}, assuming that K_{PS} is 10^4. Calculation condition: $K_{PS}=10^4$, $C_A=0.0472$ mol/L, $C_X=7.25\times10^{-6}$ mol/L, $K_A=10^{-1}$ (○); 10^{-2} (△); 10^{-3} (□); 10^{-4} (◇); 10^{-5} (×).

2. Characteristics obtained by calculation

1) Relationship between protein/organic anion complex concentration and pH

Since the electric charge of protein and the dissociation of the organic acid change by pH, the relationship between the protein/organic anion complex concentration and the pH was investigated, varying the dissociation constant (K_A) of an organic acid from 10^{-5} to 10^{-1}. As indicated in Fig. 6, the pH at which the complex begins to form is influenced by the pK_A value of the organic acid, and shifts to a higher pH with increasing the pK_A value. The complex concentration is small at a lower pH, becomes constant with increasing the pH, begins to decrease from around pH 9 and then becomes almost zero above pH 11. Though the complex is expected to form in the pH range of 1 to 11, experimentally the turbidity generation was observed only in the pH range of 1.5 to 4.0. Thus, the experimental result did not fit the theory in the pH range of 1 to 1.5 and 4.0 to 11.

2) Effect of organic acid salt on color development of HSA by BCP

As mentioned above, the color development of HSA by BCP decreased by the addition of the organic acid salt. This phenomenon was investigated by the calculation based on the chemical equilibrium of a protein error, assuming that the organic anion binds to a positively-charged protein. The calculation was carried out, using the next equation derived according to the previous paper[9], while varying the equilibrium constant (K_{PS}) of the reaction between the positively-charged protein and the organic anion;

$(K_DK_{PD}[H^+] + K_DK_{PD}K_A)[PD]^2 + (K_DK_{PD}C_D[H^+] + K_DK_{PD}K_AC_D + K_DK_{PD}C_P[H^+] + K_DK_{PD}K_AC_P + [H^+]^2 + K_A[H^+] + K_AK_{PS}C_A[H^+] + K_D[H^+] + K_DK_A + K_DK_AK_{PS}C_A)[PD] + K_DK_{PD}C_DC_P[H^+] + K_DK_{PD}K_AC_DC_P = 0$

K_D: dissociation constant of BCP, K_{PD}: equilibrium constant of the reaction between the positively-charged protein and the anion of BCP, C_D: concentration of BCP.

Fig. 7 indicates the relationship between the organic acid salt concentration and the dye-protein complex concentration (PD) at pH 5.2. The dye-protein complex concentration decreases by the addition of the organic acid salt, and its decrease

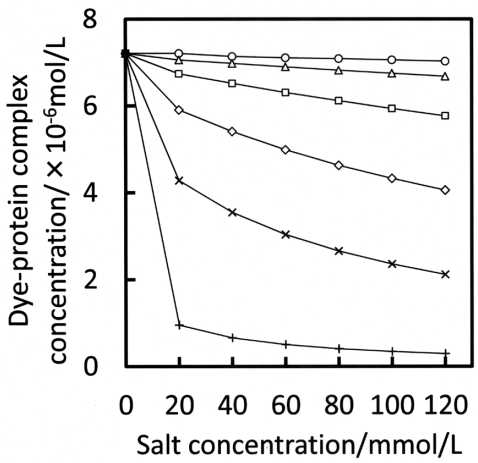

Fig. 7 Relationship between the organicc acid salt concentration and the dye-protein complex concentration when the equilibrium constant (K_{PS}) varies (calculated result).
Calculation condition:pK_D=6.3, K_{PD}=10^7, C_D=8×10^{-5} mol/L, C_X=7.25×10^{-6} mol/L, pH 5.2, ε_D= 69,600 Lmol^{-1}cm^{-1}, n=0.49, K_{PS}=$10^{1.0}$ (○); $10^{1.5}$ (△); $10^{2.0}$ (□); $10^{2.5}$ (◇); $10^{3.0}$ (×); $10^{4.0}$ (+)

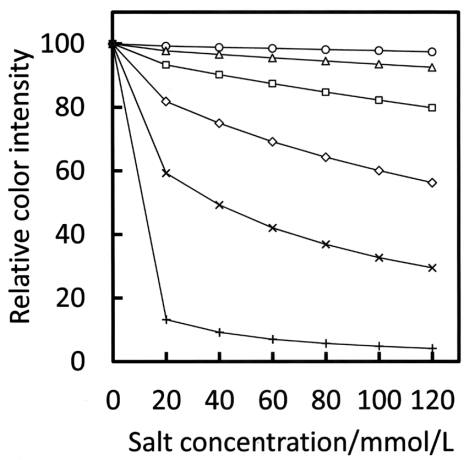

Fig. 8 Relationship between the salt concentration and the color development when the equilibrium constant (K_{PS}) varies (calculated result).
Calculation condition:pK_D=6.3, K_{PD}=10^7, C_D=8×10^{-5} mol/L, C_X=7.25×10^{-6} mol/L, pH 5.2, ε_D =69,600 Lmol^{-1}cm^{-1}, n=0.49, K_{PS}=$10^{1.0}$ (○); $10^{1.5}$ (△); $10^{2.0}$ (□); $10^{2.5}$ (◇); $10^{3.0}$ (×); $10^{4.0}$ (+)

becomes large as the K_{PS} value increases. Fig. 8 indicates the relationship between the organic acid salt concentration and the color development resulting from the dye-protein complex. The color development in the absence of the organic acid salt is expressed as 100. The color development decreases in the presence of the organic acid salt, and its decrease becomes large as the K_{PS} value increases. These calculated results agree well with the experimental results indicated in Fig. 5.

3) Relationship between no-charged protein/organic anion complex concentration and pH

As mentioned above, the protein/organic anion complex is thought to form in the wide pH range of 1 to 11 by the calculation. However, experimentally the turbidity generation occurred only in the pH range of about 1.5 to 4.0. This inconsistency between the calculated and the experimental results is thought to be related to the change of the charge of the complex. Thus, the relationship between the charge of the complex and the pH was investigated by calculation. A carboxyl group (-COOH) exists in a protein molecule, and dissociates with increasing the pH. Accordingly, the negative charge ($-COO^-$) in a protein molecule increases with increasing the pH. From this, at a lower pH the complex formed by the binding of the organic anion to the positively-charged protein is no-charged. However, the carboxyl group in the complex begins to dissociate with increasing pH, by which the charge of the complex becomes negative (POA^-). Since the no-charged complex ($POA^{\pm 0}$) changes to the negatively-charged complex by the rate corresponding to the degree of dissociation of the carboxyl group, the no-charged complex is calculated roughly as follows:

$$[POA^{\pm 0}]=[POA]-[POA^-]=[POA]-[POA]\times \alpha$$

$$[POA^-]=[POA]\times \frac{K_{A\alpha}}{[H^+]+K_{A\alpha}} = [POA]\times \frac{10^{-20}}{[H^+]+10^{-20}}$$

[POA]: total concentration of the complex, $[POA^-]$: concentration of the negatively-charged complex, $[POA^{\pm 0}]$: concentration of the no-charged complex, α : degree of dissociation of the carboxyl group in a protein molecule, $K_{A\alpha}$: mean dissociation constant of α -carboxyl group in a protein molecule.

Fig. 9 indicates the relationship between the non-charged complex concentration and the pH. Assuming that the mean dissociation constant ($pK_{A\alpha}$) of the carboxyl group in a protein molecule is about 2.0, the no-charged complex concentration increases with the rise in pH, reaches maximum, then rapidly decreases, and above pH 4 becomes almost zero. The pH at which the no-charged complex begins to form and its concentration reaches maximum shifts to a higher pH as the pK_A value increases. In addition, the maximum concentration of the no-charged complex decreases as the pK_A value increases.

4) Effect of organic acid salt concentration on no-charged complex concentration

As indicated in Fig. 10, as the organic acid salt concentration decreases, the no-charged complex concentration decreases and the pH at which its concentration reaches maximum shifts to a higher one. At a lower pH the difference in the no-charged protein concentration between the organic acid salt

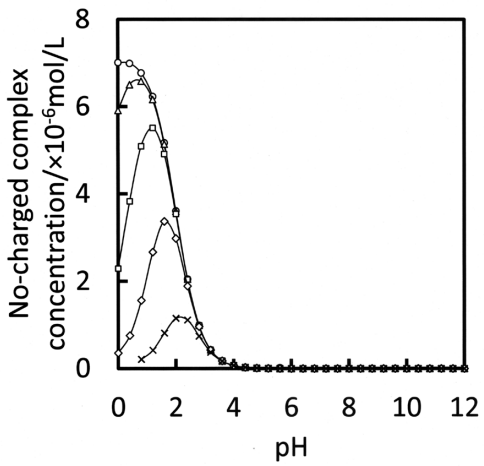

Fig. 9 Relationship between the no-charged protein/organic anion complex concentration and the pH (calculated result).
The calculation was performed, varying the dissociation constant (K_A) of the organic acid from 10^{-1} to 10^{-5}, assuming that the dissociation constant ($K_{A\alpha}$) of the α -carboxyl group in a protein molecule is 10^{-2}. Calculation condition: $K_{PS}=10^4$, $C_A=0.0472$mol/L, $C_X=7.25\times10^{-6}$ mol/L, $K_A=10^{-1}$ (○); 10^{-2} (△); 10^{-3} (□); 10^{-4} (◇), 10^{-5} (×).

levels is large.

4. Discussion

The organic acid salts used in the experiment were classified in two groups; one generates turbidity, the other does not. As can be seen from the experimental result indicated in Fig. 5, the color development of HSA by BCP occurring due to the binding of the dissociated dye anion to the positively-charged protein decreased by the addition of the organic acid salt. This result is the same phenomenon as that indicated by the addition of the inorganic salt, supporting that the organic anion also combines with the positively-charged protein as inorganic anion to produce the protein/organic anion complex even in the pH range where the turbidity generation does not occur. From this, it is considered that not only the binding of the organic anion to the positively-charged protein but also the coherence of the plural complexes is required for the turbidity generation. In the case of benzenesulfonic acid sodium salt, sodium sulfanilate, sodium benzoate and sodium hippurate, the turbidity did not form over all the pH range. The reason why they did not generate turbidity in spite of the formation of the complex is considered as follows. In the case of these organic acid salts except sodium sulfanilate, because only a substituent combined with the positively-charged protein exists on the aromatic ring of these compounds, it is thought that the dipole of the aromatic ring is small and the coherence of the complexes hardly occurs. On the other hand, in the case of sulfosalicylate, phthalate and salicylate, plural substituents exist on the aromatic ring, and a substituent (-OH, -COOH) not related to the combination with the positively-charged protein polarizes due to the difference in electronegativity between hydrogen, carbon and oxygen. The complexes of these organic acid salts are considered to cohere by the dipole-dipole interactions. In the case of sulfanilate, since its complex becomes positive due to the existence of the positively-charged amino group ($-NH_3^+$) bound to the aromatic ring, the coherence between the complexes is assumed not to occur by the repulsive force between the complexes.

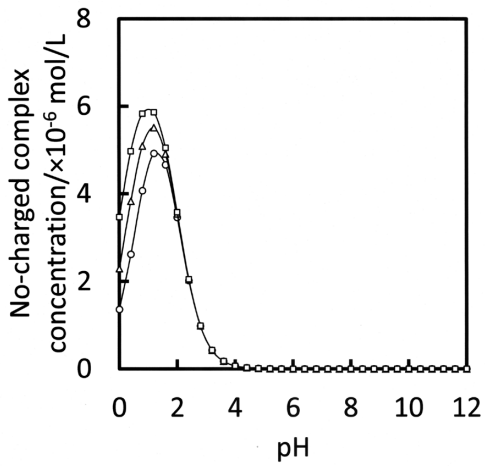

Fig. 10 Relationship between the organic acid salt concentration and the no-charged protein/organic anion complex concentration(calculated result). Calculation condition: $K_A=10^{-3}$, $K_{PS}=10^4$, $K_{A\alpha}=10^{-2}$, $C_X=7.25\times10^{-6}$ mol/L, C_A=0.0236 mol/L (○); 0.0472 mol/L(△); 0.0944 mol/L(□)

It is known that protein is easiest to precipitate at an isoelectric point at which a positive charge and negative charge are equal. Though the isoelectric point of HSA is pH 4.9, the turbidity generation by the organic acid salt used occurred only in the pH range of 1.5 to 4.0. Thus, the fact that the turbidity generation does not occur at pH 4.9 is considered to indicate that at this pH the net charge of HSA does not become zero. In the presence of the organic acid salt, since the positive charge of protein decreases when its anion binds to a positively-charged site of HSA at an isoelectric point of pH 4.9, it is thought that the complex becomes negative and barely coheres. This is the reason why HSA did not precipitate at pH 4.9 by the organic acid salt.

The characteristic described below is presumed according to the calculation based on the chemical equilibrium. From a low of mass action, the concentration of the protein/organic anion complex formed must be proportional to the product of the positively-charged protein concentration and the organic anion concentration. Accordingly, the complex generation is small in a lower pH range where the organic anion concentration is low because the degree of dissociation of the organic acid is small. In addition, assuming

that the mean dissociation constant of the dissociable group such as the α-NH_3^+ and ε-NH_3^+ in a protein molecule which give a positive charge to protein is about pK_A=9, the degree of dissociation at pH 11 is α=0.99. That is, the complex generation lasts up to about pH 11; above pH 11, the positive charge becomes almost zero and the complex concentration approaches zero. Other than in the lower and higher pH ranges, the complex concentration becomes constant. Thus, the complex is thought to form in the wide pH range. However, the pH where the complex begins to form shifts to a higher pH as the dissociation constant (pK_A) of the organic acid increases as indicated in Fig. 6. The pH at which the turbidity generation begin and the dissociation constant of the substituent which dissociates and binds to the positively-charged protein were pH 1.5 and pK_A<0 for sulfosalicylate, pH 2.5 and pK_A=2.97 for salicylate, and pH 3.2 and pK_A=2.89 for phthalate, respectively. In this way, the pH at which the turbidity generation begin varied according to a dissociation constant level of the organic acid, and the experimental finding agreed with the calculation result. In the experiment, the turbidity generation was observed only in the pH range of about 1.5 to 4.0. The calculated and experimental results agreed approximately in the pH range of 1.5 to 4.0, but above pH 4.0 their results were not consistent at all. As mentioned above, the coherence between the complexes is necessary for the turbidity generation. The reason why the coherence of the complexes does not occur above pH 4.0 is thought as follows. Protein is easy to precipitate at an isoelectric point where its solubility is smallest, and its solubility increases in the pH range except for the isoelectric point. That is, when the net charge is zero, the protein readily coheres. However, when the net charge is positive or negative, the protein hardly coheres due to each other's repulsions. The turbidity by the organic acid salt was highest at around pH 1.5-3.5, and became low at lower and higher pH than this pH range. Therefore, it is clear that the turbidity generation is closely related to the change in the charge of the complex by pH. Since the turbidity generation occurs by the coherence between the complexes, it will not be observed in the case that the coherence between the complexes does not occur even when the complex exists. It is thought that the reaction above pH 4 where the calculated and the experimental results are inconsistent corresponds to the case in which the formed complexes do not cohere. Below pH 4, each of the no-charged complexes are thought to cohere by the attraction between the dipoles of the substituent on the aromatic ring in sulfosalicylate, salicylate, and phthalate. On the other hand, since above pH 4 the complex becomes negative because of the dissociation of the α-carboxyl group, electrostatic repulsion occurs between the coherent complexes. Therefore, it is thought that the complexes break up by repulsion between similar electric charges much larger than a weak intermolecular force between the dipoles; the turbidity thus decreases and disappears. Since the mean dissociation constant of the α-carboxyl group in a protein molecule is around p$K_{A\alpha}$=2, the dissociation of this group begins at about pH 0, and reaches 50% at pH 2, 90% at pH 3, and 99% at pH 4. From this, the percentage of the no-charged complex which readily coheres falls to 50% at pH 2, 10% at pH 3 and 1% at pH 4. That is, above pH 4 the no-charged complex almost does not exist. Comparing the dissociation of the α-carboxyl group and the turbidity generation, the turbidity generation begins near pH 1.5 where the organic anion begins to increase, begins to decrease near pH 2.5 where the α-carboxyl group dissociates more than 75%, and disappears near pH 4 where the α-carboxyl group dissociates almost completely and the no-charged complex becomes almost zero. The relationship between the no-charged complex concentration and the pH resembles closely that between the turbidity and the pH indicated in Fig. 1 and that between the precipitated protein concentration and the pH indicated in Fig. 2.

The inconsistency between the calculated and the experimental results in the pH range of 4 to 11 is considered to be due to the dissociation of the α-carboxyl group. That is, the coherence of the complexes does not occur when the complex becomes negative. By the way, in the lower pH range the turbidity generation did not occur in spite of the existence of the no-charged complex. This indicates that in the lower pH the complex hardly coheres. The

coherence of the complexes is considered to occur by the intermolecular force among the complexes. In the lower pH, since the degree of dissociation of the organic acid is small, the concentrations of the organic anion and the formed complex are low. Therefore, the intermolecular distance between complexes becomes longer, and the intermolecular force becomes small, by which the coherence of the complexes hardly occurs. Since intermolecular force is proportional to the 6^{th} power of a distance between molecules[11], a slight increase in the distance between molecules by decreasing the concentration of the complex is considered to bring about a rapid decrease in intermolecular force. In the case of benzenesulfonic acid sodium salt, sodium sulfanilate and sodium hippurate, the turbidity did not form over all the pH range. This result is thought to indicate that the intermolecular force is very small, or that the repulsion more than the intermolecular force exists even in the lower pH range where the complex is no-charged.

5. Conclusion

The reaction principle of the turbidity generation of albumin by the aromatic organic acid salt was investigated by an experiment and a calculation based on the chemical equilibrium. Not only the binding of the organic anion to the positively-charged protein but also the coherence between the complexes by the intermolecular force is important for the turbidity generation. The turbidity generation is considered to occur only in the lower pH range where the dissociation of the carboxyl group in a protein molecule is incomplete and the no-charged complex without the electrostatic repulsion exists.

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