〈Original Article〉

Reaction principle of the turbidimetric method for urine protein using benzalkonium chloride

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Summary In the previous paper, the reaction principle of the turbidimetric method for urine protein using benzethonium chloride was investigated. When the concentration of benzethonium chloride is low, the turbidity decreased with a rise in pH in the higher pH range. The proposed reaction principle could not explain this phenomenon, So it was examined by an experiment using benzalkonium chloride and by a calculation based on the chemical equilibrium. It was assumed that the reaction of cationic detergent and hydroxyl ion that increases by a rise of pH also participates in this turbidity generation in addition to the reaction of cationic detergent and the negatively-charged protein. By this reaction model the phenomenon by which the concentration of the detergent/protein complex which generates turbidity decreases with increasing the pH in the higher pH range was satisfactorily explained.

Key words: Urine protein, Benzalkonium chloride, Turbidity generation, Reaction principle

1. Introduction

The author previously reported[1] the reaction principle of the turbidimetric method for urine protein using benzethonium chloride (BZ) of cationic detergent[2, 3]. It was clarified that the turbidity generation depends on pH and is reversible. However, when the concentration of BZ in the precipitant is low, a phenomenon was observed by which the turbidity generation decreases with increasing the pH in the higher pH range. The turbidity generation occurred by aromatic organic acid such as sulfosalicylic acid[4-6] was reversible and was observed only in the lower pH range from about 1 to 4, but that occurring by BZ was not observed in such a lower pH range. That the turbidity generation in the turbidimetric method using BZ is caused in the pH range above 5-6 was sufficiently explained by the reaction principle that a positively-charged cationic detergent binds to the negatively-charged protein with a dissociated carboxyl group and the generated complexes cohere by each other's intermolecular forces. However, when the BZ concentration is low, the reaction principle already reported is insufficient to explain the phenomenon that the turbidity generation decreases with a rise in pH in the higher pH range. Thus, the author investigated more the reaction principle which could explain the phenomenon observed when the detergent concentration in the precipitant is low, using benzalkonium chloride of cationic detergent.

2. Materials and methods

1. Reagents

All of the reagents of the purest quality available were obtained from Wako Pure Chemical Industries,

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Received for Publication August 28, 2014
Accepted for Publication October 24, 2014

Ltd., Osaka, Japan.

Buffer solutions: Buffer solutions with a pH range from 0.3 to 2.0 were prepared by a HCl solution. Buffer solutions with the pH range from 2.4 to 8.0 were prepared by mixing a 0.1mol/L citric acid solution and a 0.2 mol/L Na_2HPO_4 solution. Buffer solutions with the pH range from 8.8 to 12.9 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. The pH of the solution was measured by a pH meter (TOA Electronics Ltd.).

BC precipitant: 1.0 g of benzalkonium chloride (BC) was dissolved in 100 mL of purified water.

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

2 g/L γ -globulin solution: 200 mg of γ -globulin was dissolved in 100 mL of purified 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 0.3 to 12.9 and 2.0 mL of a BC precipitant to 1.0 mL of a 2 g/L protein solution, and reacted for 10 min at 37℃. Its absorbance (turbidity) was measured at 660 nm against purified water using a Hitachi U1500 spectrophotometer.

2) Measurement of pH of the reaction mixture

The pH of the reaction mixture was measured by a pH meter in order to grasp the relationship between the turbidity generation and the pH. The pH mentioned in this paper does not indicate that of a buffer solution itself but that of the reaction mixture after adding the BC precipitant to the buffer solution containing protein.

3) Titration of the reaction mixture

As described later, the turbidity generation of HSA and γ -globulin does not occur in the pH range from 1.0 to 5.5. Thus, in order to clearly indicate that the turbidity generation depends on the pH, the reaction mixture in which the turbidity does not form even by the addition of the BC precipitant was prepared as follows. Forty mL of the buffer of pH 2.61and 40 mL of the BC precipitant were added to 20 mL of 2 g/L HSA or 2 g/L γ -globulin. The pH of this reaction mixture became around 2.8, and the turbidity did not form. The pH of this reaction mixture was measured while titrating it first by 1 mol/L sodium hydroxide solution; a part of it was taken out appropriately, and the absorbance was measured at 660 nm against purified water. The reaction mixture after measuring the absorbance was added to the original reaction mixture, and the titration was continued until the pH of the solution became around 11. Then, this reaction mixture of about pH 11 was inversely titrated with 1 mol/L hydrochloric acid more, a part of which was taken out appropriately, and the absorbance was measured at 660 nm against purified water. The reaction mixture after measuring the absorbance was added to the original reaction mixture, and the titration was continued until the pH of the solution became around 2. Futhermore, this reaction mixture of about pH 2 was again titrated with 1 mol/L sodium hydroxide; then a part of the reaction mixture was taken out appropriately, and the absorbance was measured at 660 nm against purified water. The reaction mixture after measuring the absorbance was added to the original reaction mixture, and the titration was continued until the pH of the solution became around 11. In this titration, the volume of the reaction mixture finally increased from 100 mL to 111.6 mL for albumin and from 100 mL to 112.0 mL for γ -globulin by adding sodium hydroxide and hydrochloric acid solutions. Since the volume change of the reaction mixture by this titration was small, the absorbance correction was not made.

3. Results

1. Relationship between the turbidity generation and the pH

The relationship between the turbidity generation and the pH of the reaction mixture was examined, changing the BC concentration from 0.25 g/L to 1.0 g/L. In the reaction of HSA indicated in Fig. 1, when the BC concentration is 0.25 g/L, no turbidity was generated in the whole pH range from 0.3 to 12.9. When the BC concentration is 0.75-1.0 g/L, the turbidity began to form from about pH 5.5 and reached the peak, but it decreased with increasing the pH, and disappeared at almost about pH 8.0. However, the

turbidity again began to form from about pH 10 and continued increasing to pH 13. When the BC concentration was 0.5 g/L, the turbidity generation from about pH 5.5 almost did not occur. In this case, the turbidity generation began from about pH 9, reached the peak at about pH 10.8 and in the further pH range the turbidity decreased with a rise in pH. In the reaction of γ -globulin indicated in Fig. 2, when the BC concentration is 0.5-1.0 g/L, the turbidity generation began from about pH 6, peaked at about pH 10 and decreased somewhat at about pH 10.5, but in the further pH range the turbidity increased with a rise in pH. The turbidity hardly disappeared at about pH 8.0 as observed in the HSA reaction. When the BC concentration is 0.25 g/L, the turbidity generation began from about pH 6, peaked at about pH 9, and in the further pH range the turbidity decreased with a rise in pH.

2. pH dependence of turbidity generation

As mentioned above, the turbidity generation did not occur within the pH range of less than pH 5.5 for HSA, and in the pH range less than pH 6.0 for γ -globulin. Thus, the turbidity generation by BC also depended on the pH. In order to make this pH dependence more clear, the reaction mixture with no turbidity was prepared as indicated above, and the relationship between the turbidity generation and the pH was examined by titrating the reaction mixture by 1 mol/L sodium hydroxide and 1 mol/L hydrochloric acid. In the case of HSA the turbidity began to form from about pH 10 by the addition of sodium hydroxide to the reaction mixture of pH 2.8, and markedly increased at about pH 10.5 as shown in Fig. 3. When this reaction mixture with turbidity was inversely titrated by 1 mol/L hydrochloric acid, the turbidity decreased with a drop of pH, and neared a constant value in about pH 3. When this reaction mixture with the slight turbidity was again titrated by 1 mol/L sodium hydroxide, the turbidity rose along the titration curve that was provided when it was titrated by hydrochloric acid. In the case of γ -globulin the turbidity began to form from about pH 9 by the

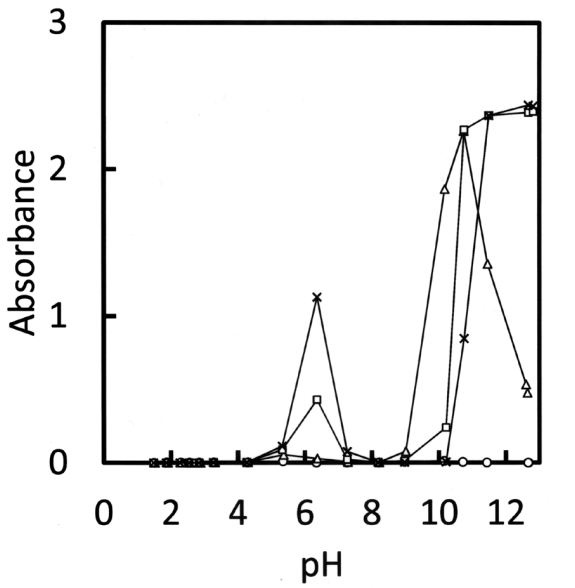

Fig. 1 Relationship between the turbidity generation of human serum albumin and the reaction mixture pH in the reaction of benzalkonium chloride.
The turbidity of human serum albumin was measured at 660 nm against purified water varying the concentration of benzalkonium chloride (BC).
BC concentration: 0.25 g/L (○), 0.50 g/L (△), 0.75 g/L (□), 1.0 g/L (×).

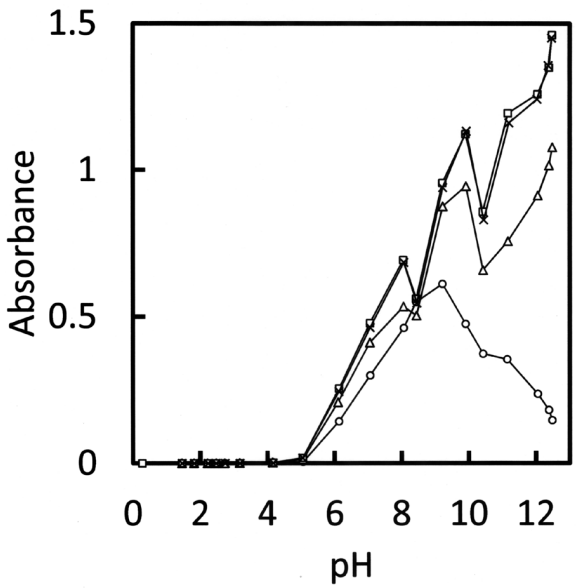

Fig. 2 Relationship between the turbidity generation of γ -globulin and the reaction mixture pH in the reaction of benzalkonium chloride.
The turbidity of γ -globulin was measured at 660 nm against purified water varying the concen tration of benzalkonium chloride (BC).
BC concentration: 0.25 g/L (○), 0.50 g/L (△), 0.75 g/L (□), 1.0 g/L (×).

addition of sodium hydroxide, and markedly increased at about pH 10 as shown in Fig. 4. When this reaction mixture with turbidity was inversely titrated by 1 mol/L hydrochloric acid, the turbidity decreased with a drop of pH, and neared a constant value at about pH 3. When this reaction mixture with the slight turbidity was again titrated by 1 mol/L sodium hydroxide, the turbidity rose approximately along the titration curve that was provided when it was titrated by hydrochloric acid.

3. Titration curve of detergent

In order to examine the reaction between cationic detergent and hydroxyl ion which increases with a rise of pH, 50 mL of purified water, 50 mL of 10 g/L BZ and 50 mL of 10 g/L BC were titrated by 0.01 mol/L sodium hydroxide. In the case of BZ and BC the pH rose little and their increase in pH was smaller than that of purified water as shown in Fig. 5. That is, it was found that the hydroxyl ion becomes trapped by a detergent.

4. Relationship between the detergent/protein complex concentration and the pH

The turbidity generation is estimated to occur as follows. The positively-charged detergent (S^+) binds to the negatively-charged protein ($PCOO^-$) with the dissociated carboxyl group in a protein molecule (PCOOH), the detergent/protein complex (PCOOS) is produced and a large number of the complexes cohere by each other's intermolecular forces. In addition,

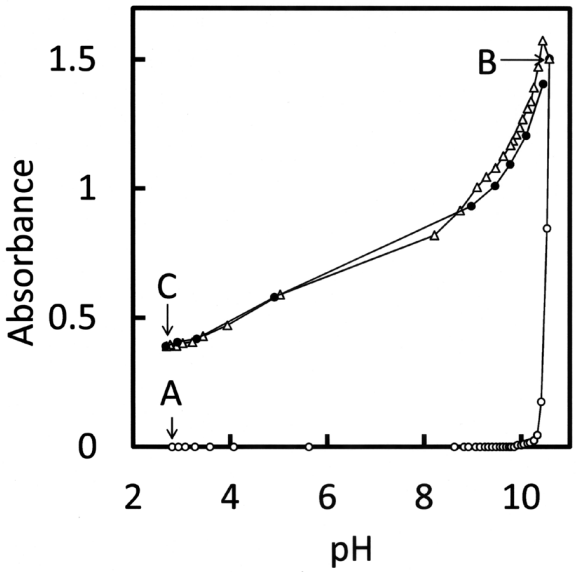

Fig. 3 Reversibility of the turbidity generation of human serum albumin.
The test solution was prepared as follows. 40 mL of the buffer solution (pH 2.61) and 40 mL of 1.0 g/L BC were added to 20 mL of 2 g/L human serum albumin.
○: reaction mixture Ⅰ was obtained by titrating the test solution with no turbidity by 1 mol/L NaOH (start from A).
△: reaction mixture Ⅱ was obtained by titrating the reaction mixture Ⅰ by 1 mol/L HCl (start from B).
●: reaction mixture Ⅲ was obtained by titrating the reaction mixture Ⅱ by 1 mol/L NaOH (start from C).

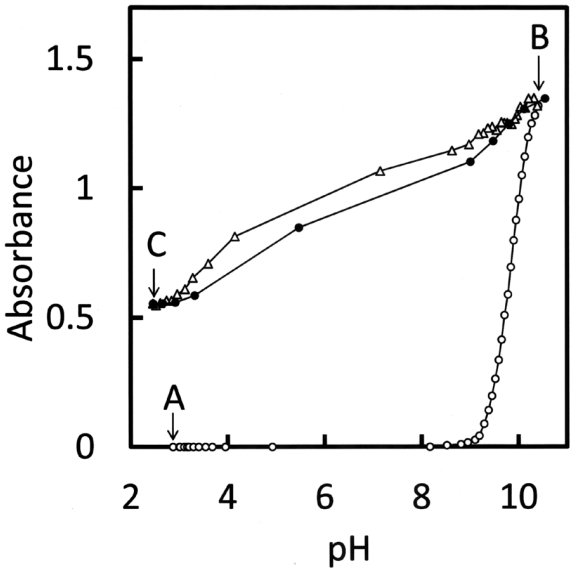

Fig. 4 Reversibility of the turbidity generation of γ-globulin.
The test solution was prepared as follows. 40 mL of the buffer solution (pH2.61) and 40 mL of 1.0 g/L BC were added to 20 mL of 2 g/L γ-globulin.
○: reaction mixture Ⅰ was obtained by titrating the test solution with no turbidity by 1 mol/L NaOH (start from A).
△: reaction mixture Ⅱ was obtained by titrating the reaction mixture Ⅰ by 1 mol/L HCl (start from B).
●: reaction mixture Ⅲ was obtained by titrating the reaction mixture Ⅱ by 1 mol/L NaOH (start from C).

when the pH rises and hydroxyl ion (OH^-) increases, the reaction by which the hydroxyl ion binds to the positively-charged detergent progresses, the concentration of the detergent which can bind to the negatively-charged protein decreases, and therefore the concentration of the detergent/protein complex is thought to decrease.

$$PCOOH \leftrightarrows PCOO^- + H^+ \qquad K_{PH} = \frac{[PCOO^-][H^+]}{[PCOOH]}$$

$$PCOO^- + S^+ \leftrightarrows PCOOS \qquad K_{PS} = \frac{[PCOOS]}{[PCOO^-][S^+]}$$

$$S^+ + OH \leftrightarrows SOH \qquad K_{SO} = \frac{[SOH]}{[S^+][OH]}$$

$$n PCOOS \leftrightarrows (PCOOS)_n \qquad K_{PO} = \frac{[(PCOOS)_n]}{[PCOOS]^n}$$

When the protein and detergent concentrations in the reaction mixture are represented by C_X and C_S, respectively, the detergent/protein complex concentration can be calculated as follows:

As for the protein concentration, the following equation holds.

$$C_X = [PCOOH] + [PCOO^-] + [PCOOS] + [(PCOOS)_n]$$

$$= \frac{[PCOO^-][H^+]}{K_{PH}} + [PCOO^-] + [PCOOS] + [(PCOOS)_n]$$

$$= [PCOO^-]\left(\frac{[H^+]}{K_{PH}} + 1\right) + [PCOOS] + K_{PO}[PCOOS]^n$$

$$= [PCOO^-]\left(\frac{[H^+] + K_{PH}}{K_{PH}}\right) + [PCOOS] + K_{PO}[PCOOS]^n$$

Hence,

$$[PCOO^-] = (C_X - [PCOOS] - K_{PO}[PCOOS]^n)\left(\frac{K_{PH}}{[H^+] + K_{PH}}\right) \quad (1)$$

As for the detergent concentration, the following equation holds.

$$C_S = [S^+] + [PCOOS] + [(PCOOS)_n] + [SOH]$$

$$= \left(\frac{[PCOOS]}{K_{PS}[PCOO^-]}\right) + [PCOOS] + K_{PO}[PCOOS]^n + K_{SO}[OH^-][S^+]$$

$$= \left(\frac{[PCOOS]}{K_{PS}[PCOO^-]}\right) + [PCOOS] + K_{PO}[PCOOS]^n + K_{SO}[OH^-]\left(\frac{[PCOOS]}{K_{PS}[PCOO^-]}\right)$$

$$= \left(\frac{1}{[PCOO^-]}\right)\left(\frac{[PCOOS] + K_{SO}[OH^-][PCOOS]}{K_{PS}}\right) + [PCOOS] + K_{PO}[PCOOS]^n$$

Hence,

$$[PCOO^-] = \frac{[PCOOS] + K_{SO}[OH^-][PCOOS]}{K_{PS}(C_S - [PCOOS] - K_{PO}[PCOOS]^n)} \quad (2)$$

Here, since $n \gg 1$ and $[PCOOS] \gg [PCOOS]^n$, it is similar to the parentheses of equation (1) and equation (2) as follows:

$$(C_X - [PCOOS] - K_{PO}[PCOOS]^n) \fallingdotseq (C_X - [PCOOS])$$

$$(C_S - [PCOOS] - K_{PO}[PCOOS]^n) \fallingdotseq (C_S - [PCOOS])$$

Therefore, it is similar to equation (1) and equation (2) as follows.

$$[PCOO^-] = (C_X - [PCOOS])\left(\frac{K_{PH}}{[H^+] + K_{PH}}\right)$$

$$[PCOO^-] = \frac{[PCOOS] + K_{SO}[OH^-][PCOOS]}{K_{PS}(C_S - [PCOOS])}$$

Combining both equations, the calculating formula of the detergent/protein complex concentration is provided.

$$K_{PH}K_{PS}[H^+][PCOOS]^2 + (K_{PH}K_{PS}C_X[H^+] + K_{PH}K_{PS}C_S[H^+] + [H^+]^2 + K_{SO}K_W[H^+] + K_{PH}[H^+] + K_{PH}K_{SO}K_W)[PCOOS] + K_{PH}K_{PS}C_SC_X[H^+] = 0 \quad (3)$$

The detergent/protein complex concentration was calculated by this equation (3), giving arbitrary values to these variables as shown below. The equilibrium constant for K_{PS} and K_{SO} were 10^3-10^5 and 10^3, respectively. The mean dissociation constant for carboxyl group in a protein molecule was $K_{PH}=10^{2.5}$. The

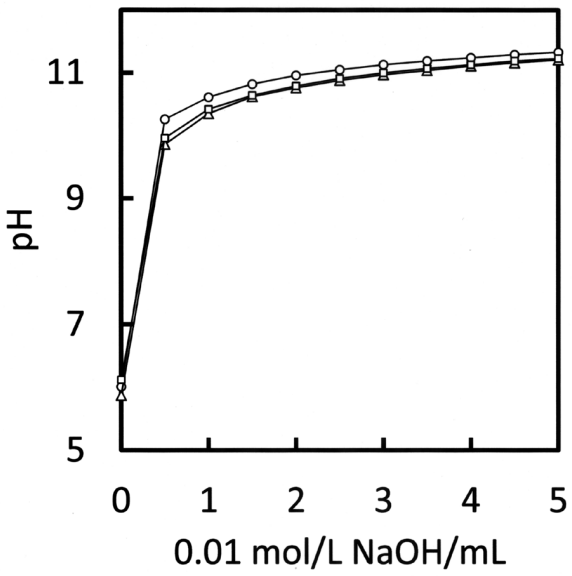

Fig. 5 Comparison of the titration curve.
50 mL of purified water, 50 mL of 10 g/L benzethonium chloride and 50 mL of 10 g/L benzalkonium chloride were titrated by 0.01 mol/L NaOH.
○: purified water, △: benzalkonium chloride, □: benzethonium chloride.

concentrations of protein and detergent were C_X=5.8×10^{-6} mol/L and C_S=1.47×10^{-3}-4.41×10^{-6} mol/L, respectively.

In addition the concentration (C_P) of the protein (P-NH_3^+) with a positively-charged group such as α -NH_3^+ with a pK_a of 7.85-10.60 and ε -NH_3^+ with a pK_a of 9.67-10.53[7] is calculated as follows:

$$P\text{-}NH_3^+ \leftrightarrows P\text{-}NH_2 + H^+ \qquad K_a = \frac{[P\text{-}NH_2][H^+]}{[P\text{-}NH_3^+]}$$

As for the protein concentration, the following equation holds.

$$\begin{aligned} C_X &= [P\text{-}NH_3^+] + [P\text{-}NH_2] \\ &= [P\text{-}NH_3^+] + \frac{K_a[P\text{-}NH_3^+]}{[H^+]} \\ &= [P\text{-}NH_3^+]\left(1 + \frac{K_a}{[H^+]}\right) \\ &= [P\text{-}NH_3^+]\left(\frac{[H^+] + K_a}{[H^+]}\right) \end{aligned}$$

Therefore,

$$C_P = [P\text{-}NH_3^+] = \frac{C_X[H^+]}{[H^+] + K_D}$$

Fig. 6 indicates the relationship between the detergent/protein complex concentration and the positively-charged protein concentration, and the pH when the BC concentration varies. The complex concentration is small in the lower pH range, but increases with a rise in pH and reaches the constant value. However, its concentration decreases when pH rises more. The detergent/protein complex concentration increases as the detergent concentration increases.

When the mean pK_a values of α -NH_3^+ and ε -NH_3^+ are assumed to be 8.5 and 10.5, respectively, the concentrations of the protein with α -NH_3^+ and ε -NH_3^+ begin to decrease from about pH 5.5 for the former and from about 7.5 for the latter due to the dissociation of α -NH_3^+ and ε -NH_3^+. That is, the repulsion between the complexes with similar electric charge weakens from about pH 5.5, and it is considered that the complexes are easy to cohere within this pH range.

4. Discussion

In the case of BC, the turbidity generation occurred in the pH range above approximately 5.5 for HSA, and in the pH range above approximately 6.0 for γ -globulin like BZ. Thus, the pH at which the turbidity generation begins varies with the kind of protein. It is thought that this occurs because of the difference in the equilibrium constant of the reaction and the isoelectric point of the protein. As HSA and γ -globulin are negatively-charged in the pH range where the turbidity generation occurs, the negatively-charged protein is found to participate in the turbidity generation. When the BC concentration in the precipitant is low, the turbidity decreases with a rise of pH in the higher pH range. Since the α -carboxyl group with a pK_a of about 1.59-3.60[7] in a protein molecule almost completely dissociates in the higher pH range, the concentration of the negatively-charged protein should reach a constant value. Therefore, it is thought that the decrease of the turbidity generation in the higher pH

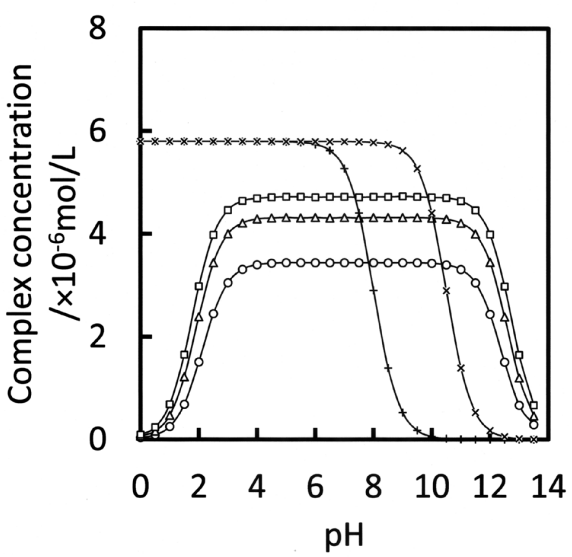

Fig. 6 Relationship between the complex formation and the detergent concentration (calculated result). The calculation was performed by varying the detergent concentration.
Calculation condition: pK_{PH}=2.5, K_{PS}=$10^{3.5}$, C_X=5.8×10^{-6} mol/L, C_S=1.47×10^{-3} mol/L (○); 2.94×10^{-3} mol/L(△); 4.41×10^{-3} mol/L(□).
○, △, □: detergent/protein complex concentration.
＋: concentration of the positively charged protein with α -NH_3^+ group.
×: concentration of the positively charged protein with ε -NH_3^+ group.

range is due to BC molecule itself. It is clear from the result shown in Fig. 5 that BC binds to the hydroxyl ion which increases with a rise of pH. Since the electric charge of the BC molecule connected with hydroxyl ion is zero, this BC molecule cannot be combined with the negatively-charged protein, and increases with a rise of pH. That is, the concentration of BC which can be combined with the negatively-charged protein decreases as the BC concentration in the precipitant lowers. In other words, this means that the detergent/protein complex concentration lowers so that the BC concentration is low as shown in Fig 6. Since the intermolecular force between the complexes is inversely proportional to the 6th power of a distance between molecules[8], when the complex concentration becomes one half, the intermolecular force will fall to a one-64th and the decrease of the complex concentration is thought to greatly influence the coherence of the complexes. It is understood that the phenomenon by which the turbidity decreases with a rise of pH in the higher pH range in the case of the low BC concentration is because the intermolecular force markedly decrease due to the decrease of the complex concentration and an interaction not to reach the threshold of the intermolecular force required for coherence of the complexes increases. On the other hand, even when the BC concentration is low (C_S=1.47×10^{-3} mol/L), the formation of the detergent/protein complex begins from about pH 1, and 60% of the protein change to the detergent/protein complex in the pH range from 3 to 11 as can be seen from Fig. 6. However, the turbidity generation does not occur in the pH range from about 1 to 5.5. The reason why the turbidity generation does not occur in the lower pH range is estimated from Fig. 6 as follows. Since the mean pK_a values of α -NH_3^+ and ε -NH_3^+ in a protein molecule are about 8.5 and 10.5, respectively; in the pH range from 2 to 5, the complex is positively charged, and therefore the complexes do not cohere by repulsion between a similar electric charge. It is estimated that as the positively-charged protein decreases from about pH 6 where α -NH_3^+ begins to dissociate, the complexes trend to readily cohere and turbidity generation occurs.

5. Conclusion

The reaction principle of the turbidimetric method for urine protein using benzalkonium chloride was investigated. The phenomenon that the turbidity generation decreases with a rise of pH in the higher pH range, which is observed when the BC concentration is low, is explained by the reaction principle that BC binds to not only the negatively-charged protein but also to hydroxyl ions which increase with a rise of pH.

Acknowledgments

This work was supported by a Saitama Prefectural University Research Grant.

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