Analysis of the Difference in Color Development in the Dye-binding Method Due to the Kind of Buffer Solution

Yuji SUZUKI

Department of Medical Technology, Saitama Prefectural University Junior College,
820, Sannomiya, Koshigaya, Saitama 343-8540, Japan

In a dye-binding method using a pH indicator, color development has reportedly been affected by the kind of buffer solution used in the color reagent. This phenomenon was analyzed by using a calculation based on the assumption that the anion of the buffer solution also reacts with protein. Color development decreases with increases in the anion concentration of the buffer solution and in the equilibrium constant of the reaction between the anion and protein. The differences in color development due to the kind of buffer solution can be attributed to differences in the equilibrium constant of the reaction forming the anion-protein complex and to the concentration of the anion between the buffer solutions.

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Introduction

The dye-binding method based on a protein error of a pH indicator occurring in the presence of a protein has been widely employed for the determination of serum protein during clinical examinations.¹⁻⁶ In this method, the color reagent is comprised of a dye and a buffer solution, which maintain the pH of the reaction mixture to be constant. Methyl orange,¹ bromophenol blue,² bromocresol green,^{3,4} bromocresol purple,^{5,6} etc. have been employed as color reagent dyes. As a buffer solution, citrate buffer,^{1,2} phosphate buffer,³ tartarate buffer,^{4,6} acetate buffer,⁵ etc. have been used. In the dye-binding method, the color development between the dye and protein has reportedly differed according to the kind of buffer solution used^{4,6} in addition to the pH, dye and buffer concentrations.⁷ However, the reason why the color development differs depending on the kind of buffer solution is unclear. Thus, the author investigated the effect on the color development of the type of buffer solution, using a theoretical calculation based on the chemical equilibrium of the protein error previously reported,⁸⁻¹² and then compared these calculated results with experimental results obtained by using bromocresol purple, which is widely employed in clinical examinations, as well as two kinds of buffer solutions.

Experimental

Reagents

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

Buffer solutions: The citric acid/ Na_2HPO_4 buffer solution was prepared by mixing solutions of 0.1 mol l^{-1} citric acid and 0.2 mol l^{-1} Na_2HPO_4 . The acetic acid/sodium acetate buffer solution was prepared by mixing solutions of 0.2 mol l^{-1} acetic acid and 0.2 mol l^{-1} sodium acetate. The pH of the solutions was adjusted, using a Hitachi-Horiba M8 pH meter.

A dye solution, 1 mmol l^{-1} : 540 mg of bromocresol purple (BCP) was dissolved in 10 ml of ethanol, and then diluted to 1000 ml with distilled water.

Color reagent: To 10.0 ml of a dye solution, 10–90 ml of a buffer solution was added, and then diluted to 100 ml with distilled water.

A protein solution, 2 g l^{-1} : 200 mg of human serum albumin, was dissolved in 100 ml of distilled water.

Procedure

A test solution was prepared by adding 2.0 ml of a color reagent to 0.5 ml of a protein solution. A reagent blank was prepared by mixing 2.0 ml of the color reagent with 0.5 ml of distilled water. Ten minutes after the reaction at 25°C, their absorbances were recorded at 590 nm with a Hitachi 7011 Clinical Spectrophotometer against distilled water at 25°C.

Analysis of Dye-binding Method

In this study, the calculation was carried out in the same manner as in previous studies⁸⁻¹² based on the assumption that the anion of the buffer solution (like the dissociated dye anion) also reacts with protein to form a colorless anion-protein complex, so that the following chemical equilibria below are assumed to exist in the test solution:

where HD is the dye, D^- the dissociated dye anion, P^+ the unreacted protein positively charged, M^- the anion of the buffer solution, PM the anion-protein complex, and PD the dye-protein complex. K_D is the dissociation constant of the dye, and K_{PM} and K_{PD} are the equilibrium constants for the reactions forming the anion-protein complex and the dye-protein complex, respectively.

When the total dye concentration, the total buffer anion concentration, and the protein concentration positively charged, are represented by C_D , C_M , and C_P , respectively, we obtain the following quadratic expression for $[PD]$:¹²

$$(K_D K_{PD} [H^+] + K_D K_{PD} K_{HM}) [PD]^2 - (K_D K_{PD} C_D [H^+] + K_D K_{PD} K_{HM} C_D + K_D K_{PD} C_P [H^+] + K_D K_{PD} K_{HM} C_P + [H^+]^2 + K_{HM} [H^+] + K_{HM} K_{PM} C_M [H^+] + K_D [H^+] + K_D K_{HM} + K_D K_{HM} K_{PM} C_M) [PD] + K_D K_{PD} C_D C_P [H^+] + K_D K_{PD} K_{HM} C_D C_P = 0, \quad (5)$$

where the total protein concentration in the test solution and the mean ionization degree of the amino acid residue in a positively charged protein molecule are represented by C_X and α , respectively, C_P is expressed as follows:

$$C_P = \alpha C_X = \frac{C_X}{1 + \frac{K_W}{K_b [H^+]} + \frac{K_a K_W}{K_b [H^+]^2}},$$

where K_a and K_b indicate the mean dissociation constants of the acidic and basic residues, respectively, and K_W is the ionic product for water.

The absorbance of the test solution measured by a spectrophotometer was calculated as follows:

$$\begin{aligned} E_Z &= E_P - E_B = E_{PD} + E_D - E_B = \varepsilon_{PD} [PD] + \varepsilon_D [D_P^-] - \varepsilon_D [D_B^-] \\ &= \left(\varepsilon_{PD} - \varepsilon_D \frac{K_D}{[H^+] + K_D} \right) [PD] \\ &= \left\{ \varepsilon_D \left(n - \frac{K_D}{[H^+] + K_D} \right) \right\} [PD], \end{aligned} \quad (6)$$

where E_Z (apparent absorbance) is the absorbance of the test solution against the reagent blank, E_P the absorbance of the test solution containing the dye-protein complex and the unreacted dye anion (D_P^-), E_B the absorbance of the dye anion (D_B^-) in the reagent blank, E_{PD} the absorbance of the dye-protein complex formed, E_D the absorbance of the unreacted dye anion (D_P^-) in the test solution, and ε_{PD} and ε_D are the molar absorptivities of the dye-protein complex and the dye anion, respectively. E_P , E_B , E_{PD} and E_D are measured against distilled water. n is the ratio of the molar absorptivities of the dye-protein complex and the dye anion ($= \varepsilon_{PD}/\varepsilon_D$).

E_Z at an arbitrary pH can be obtained by calculating the concentration of PD by Eq. (5), and then substituting this value into Eq. (6), giving arbitrary values to the variables pK_D , K_{PD} , K_{HM} , K_{PM} , K_a , K_b , C_D , C_M , C_X , ε_D and n , as shown below. The pK_D value of the dye was 6.3, and the K_{PD} value of the dye-protein complex was 10^7 . The K_{HM} value was 10^{-3} , and the K_{PM} value of the anion-protein complex varied from 10^2 to 10^3 . The mean dissociation constants of the side chains of the amino acid residue in the protein molecule were $K_a = 10^{-8}$ and $K_b = 10^{-2}$, respectively. The total dye concentration was $8 \times 10^{-5} \text{ mol l}^{-1}$, the anion concentration varied from 0.02 to 0.14 mol l^{-1} ; the total protein concentration ranged from 3.0×10^{-6} to $3.0 \times 10^{-5} \text{ mol l}^{-1}$. Since the n value, semiempirically obtained, was $n = 0.49$ in the reaction of BCP,¹¹ the calculation was carried out using $n = 0.5$. The absorptivity (ε_D) of the dye anion was $7 \times 10^4 \text{ l mol}^{-1} \text{ cm}^{-1}$.

Fig. 1 Relationship between the apparent absorbance and the pH when the K_{PM} value varied (calculated result). Calculation conditions: $pK_D = 6.3$; $K_{PD} = 10^7$; $K_{HM} = 10^{-3}$; $C_D = 8 \times 10^{-5} \text{ mol l}^{-1}$; $C_X = 5.8 \times 10^{-6} \text{ mol l}^{-1}$; $C_M = 0.08 \text{ mol l}^{-1}$; $\varepsilon_D = 7 \times 10^4 \text{ l mol}^{-1} \text{ cm}^{-1}$; $n = 0.5$; $\square$, $K_{PM} = 10^2$; $\triangle$, $K_{PM} = 10^{2.5}$; $\circ$, $K_{PM} = 10^3$.

Fig. 2 Relationship between the apparent absorbance and the buffer anion concentration when the K_{PM} value varied (calculated result). Calculation conditions: $pK_D = 6.3$; $K_{PD} = 10^7$; $K_{HM} = 10^{-3}$; $C_D = 8 \times 10^{-5} \text{ mol l}^{-1}$; $C_X = 5.8 \times 10^{-6} \text{ mol l}^{-1}$; $\varepsilon_D = 7 \times 10^4 \text{ l mol}^{-1} \text{ cm}^{-1}$; $n = 0.5$; pH = 5.4; $\square$, $K_{PM} = 10^2$; $\triangle$, $K_{PM} = 10^{2.5}$; $\circ$, $K_{PM} = 10^3$.

Results

Calculated results

The anion concentration contained in the buffer solution affects the amount of dye-protein complex formed and its apparent absorbance, as described in a previous paper.¹² Namely, as the formation of the dye-protein complex decreases by increasing the anion concentration, the apparent absorbance, *i.e.*, the color development, decreases. When the kind of anion is different from the kind of buffer solution used, the ion radius and the ionic valence differ with the kind of anion. Therefore, the bonding strength between the anion and the protein is thought to differ with the kind of buffer solution. The difference in the bonding strength of the anion to the protein causes a difference in the equilibrium constant of the reaction between anion and the protein. Thus, the apparent absorbance was calculated, changing the equilibrium constant of the reaction. Figure 1 indicates the relationship between the

Fig. 3 Relationship between the apparent absorbance and the pH when two kinds of BCP color reagent containing different buffer solutions were used. The final dye and protein concentrations were $C_D = 8 \times 10^{-5} \text{ mol l}^{-1}$ and $C_X = 5.8 \times 10^{-6} \text{ mol l}^{-1}$, respectively. Color reagents were prepared by mixing 10 ml of a 1 mmol l^{-1} BCP solution with 90 ml of a buffer solution. O, Acetic acid/sodium acetate buffer solution; □, citric acid/ Na_2HPO_4 buffer solution.

apparent absorbance and the pH when the equilibrium constant varies. As the equilibrium constant increases, the apparent absorbance significantly decreases over a wide range of pH values, and the pH at which its maximum absorbance appears shifts to a higher value. Figure 2 shows the relationship between the apparent absorbance and the concentration of the buffer anion when the K_{PM} value varies. When the K_{PM} values of each buffer are different, the apparent absorbances of each buffer also differ, even at the same concentration of the buffer anion. However, if the difference in the K_{PM} value is small, the same apparent absorbance can be obtained by selecting a different buffer concentration for each buffer. For example, the arrows ($\uparrow$) shown in Fig. 2 indicate each buffer concentration at which their apparent absorbances become equal to each other. The degree of the decrease in the apparent absorbance grows greater as the K_{PM} value increases. Furthermore, the K_{PM} value affects the linearity of the calibration curve. The calibration curve approaches a straight line as the K_{PM} value decreases. Thus, the color development is found to be changed by both the anion concentration and the equilibrium constant of the reaction.

Experimental results

The effect on the color development of the kind of buffer solution in the color reagent was examined using citric acid/ Na_2HPO_4 and acetic acid/sodium acetate buffer solutions. The absorption spectra of the colored product formed during the color reaction between human serum albumin and BCP were examined in the presence of the two different buffer solutions. Although the apparent absorbance in the presence of the acetic acid/sodium acetate buffer solution was higher than that in the presence of the citric acid/ Na_2HPO_4 , there was no difference in the absorption maximum wavelength, even when the kind of buffer solution in the color reagent was different. Consequently, the difference in the apparent absorbance in the color reagents containing different buffer solutions was not caused by a shift in the absorption maximum wavelength, but by the difference in the kind of buffer solution used. Figure 3 shows the relationship between the apparent absorbance and the pH when two kinds of different buffer solutions were used. The

Fig. 4 Relationship between the apparent absorbance and the buffer anion concentration in the color reagent. The final dye and protein concentrations were $C_D = 8 \times 10^{-5} \text{ mol l}^{-1}$ and $C_X = 5.8 \times 10^{-6} \text{ mol l}^{-1}$, respectively. Measurements were performed at pH 4.6. O, Acetic acid/sodium acetate buffer solution; □, citric acid/ Na_2HPO_4 buffer solution.

apparent absorbance in the presence of the acetic acid/sodium acetate buffer solution was higher than that in the presence of the citric acid/ Na_2HPO_4 buffer solution. In this comparison, the anion concentration was higher in the buffer solution of citric acid/ Na_2HPO_4 than in that of acetic acid/sodium acetate. Since the apparent absorbance was thought to be affected by the anion concentration from the calculation result, the color development was measured using the diluted buffer solution. When the diluted citric acid/ Na_2HPO_4 buffer solution was used, the apparent absorbance increased and became equal to that in the acetic acid/sodium acetate buffer solution, as shown in Fig. 4. The calibration curve was more linear in the presence of the buffer solution of acetic acid/sodium acetate than in that of citric acid/ Na_2HPO_4 . Thus, the experimental results largely agreed with those estimated by the calculation.

Discussion

In designing a dye-binding method using a pH indicator, Okamura examined the effect on the color development of the kind of buffer solution added to the BCP color reagent, and reported that the color development by the citrate buffer solution which is tricarboxylic acid was lower than that by succinate and tartarate buffer solutions, which are dicarboxylic acid, and there was no difference between the dicarboxylic acids.⁴ Doumas also examined the relationship between color development and the kind of buffer solution, using malonate, tartarate and succinate buffer solutions, and observed that the color development differed with the kind of buffer solution, reaching a maximum in the presence of the succinate buffer solution.³

In this study, it was estimated by our calculation that the formation of the dye-protein complex and the apparent absorbance decreased when the anion concentration derived from the buffer solution increased. In addition, it was concluded that the larger is the equilibrium constant of the reaction forming the anion-protein complex, the greater is the degree of the decrease in the apparent absorbance. In other words, these calculated results show that in the presence of different buffer solutions, even if the pH of the color reagents is

equal, a difference in the apparent absorbance occurs. In experiments using the acetic acid/sodium acetate and citric acid/ Na_2HPO_4 buffer solutions, when the buffer solution prepared initially was used as is, without dilution, the color development in the presence of the former was higher than that in the presence of the latter. As can be seen from Fig. 4, this result is not explained only by the difference in the anion concentration in both the buffer solutions, indicating that the binding of the acetate ion to human serum albumin is weaker than that of the citrate ion or the hydrogen phosphate ion to human serum albumin. The magnitude of the binding of the anion to the positively charged protein is thought about as follows. Because the anion binds to the protein by the electrostatic interaction, its bonding strength is considered to be proportional to the number of charges of the anion. Therefore, since the number of charges of the acetate ion is less than those of the hydrogen phosphate and the citrate ions, the bonding strength of the former may be smaller than those of the latter. From this, the K_{PM} value of the acetate ion may be smaller than those of the hydrogen phosphate and the citrate ions. If this is correct, the color development and the calibration curve in the presence of the acetic acid/sodium acetate buffer solution are expected to be higher and more linear than those in the presence of the citric acid/ Na_2HPO_4 buffer solution, respectively; these

presumptions coincided with the experimental facts. From these results, it is concluded that the phenomenon of color development differing with the kind of buffer solution used is attributable to differences in the anion concentration of the color reagent and the equilibrium constant of the reaction between the anion and the protein.

References

1. J. S. Bracken and I. M. Klotz, *Am. J. Clin. Pathol.*, **1953**, 23, 1055.
2. P. G. Scheurlen, *Clin. Chim. Acta*, **1959**, 4, 760.
3. F. L. Rodkey, *Clin. Chem.*, **1965**, 11, 478.
4. B. T. Doumas, W. A. Watson, and H. G. Biggs, *Clin. Chim. Acta*, **1971**, 31, 87.
5. P. Carter, *Microchem. J.*, **1970**, 15, 531.
6. K. Okamura, *Rinsho Kensa*, **1974**, 18, 646.
7. Y. Suzuki, *Bunseki Kagaku*, **2003**, 52, 939.
8. Y. Suzuki, *J. Anal. Bio-Sc.*, **2001**, 24, 150.
9. Y. Suzuki, *Anal. Sci.*, **2001**, 17, 1263.
10. Y. Suzuki, *J. Anal. Bio-Sc.*, **2003**, 26, 441.
11. Y. Suzuki, *Anal. Sci.*, **2004**, 20, 1259.
12. Y. Suzuki, *Anal. Sci.*, **2005**, 21, 83.