Interaction of Trypan Blue with Protein and Application

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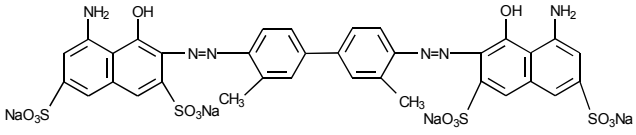
The microphase adsorption - spectral correction (MPASC) technique has been described and applied to the aggregation of trypan blue (TB) in proteins. The formation of the microelectrostatic field in protein causes the Langmuir monolayer aggregation of TB. The adsorption ratio of TB to bovine serum albumin (BSA), ovalbumin (OVA), hemoglobin (Hb) and human γ-globulin (γ-G) was determined to be 14.8, 8.4, 2.8 and 27.6, respectively, and the adsorption constant of the aggregates to be 7.17×10^5, 4.88×10^6, 4.85×10^6 and 2.99×10^6. The adsorption ratio of TB to proteins interestingly indicates almost no relation to the array sequence of amino acid residues. The interaction of TB with proteins is sensitive at pH 3.29, and the reaction was applied to the determination of protein trace with satisfactory results.

Keywords: MPASC technique; Protein; Langmuir aggregation; Trypan blue (TB); Microelectrostatic field.

INTRODUCTION

The interaction of proteins with small ions and molecules has become increasingly important to the chemist and the physician. Combinations of proteins with small anions and cations,[1,2] sulfonamides,[3] dyes,[4,5] alkyl sulfates,[6] fatty acids[7] and aromatic compounds[8] have been described earlier. Today, more and more chemists and biochemists are interested in protein chemistry.[9-13] However, the interaction of a stain with protein has not been elucidated satisfactorily, and earlier observations have not been explained clearly and reasonably, e.g. the Pesavento equation.[5] The present work was undertaken in an attempt to clarify the general principles involved in protein-stain interaction. For this purpose, several proteins, e.g. bovine serum albumin (BSA), human γ-globulin (γ-G), horse myoglobin (Mb) and ovalbumin (OVA) and trypan blue (TB) were selected in this study. In this work, we calculate from the experimental measurements, with the aid of simplifying assumptions, the number of TB bound to each protein molecule and the adsorption constant of the aggregate. In addition, the quantitative determination of proteins is useful[14-18] in clinical tests and accurate study of the interaction of a stain with protein.

The microphase adsorption - spectral correction (MPASC) technique was described and applied to the interaction of trypan blue (TB) with proteins (BSA, OVA, Hb and γ-G). The structure of TB is given below:



Trypan Blue (TB), C. I. 238501

The ionization of the sulfonics forms a quadrivalent anion in one side of TB, and protonation of the aminos forms a bivalent cation in acid solution in the other side of TB. We found that the interaction of TB with proteins (BSA, OVA, Hb, γ-G) is sensitive at pH 3.29 and selective in the presence of EDTA. The experiments indicate the aggregation obeys the Langmuir isothermal adsorption in only a monolayer. The adsorption ratio of TB to BSA, OVA, Hb and γ-G is 14.8, 8.4, 2.8 and 27.6, respectively; the adsorption constant of their aggregates is 7.17×10^5, 4.88×10^6, 4.85×10^6 and 2.99×10^6 and their absorptivity 4.95×10^5, 2.08×10^5, 4.50×10^5 and 3.26×10^6, l $mol^{-1}cm^{-1}$ at 660 nm. In addition, an interesting appearance indicates the aggregation of TB in proteins is in relation to only the number of amino acid residues and no relation to their kinds and sequence.

EXPERIMENTAL SECTION

Apparatus and Materials

Absorption spectra were recorded with a TU1901

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spectrophotometer (PGeneral, Beijing) and an independent absorbance was measured on a Model 722 Spectrophotometer (Shanghai 3rd Instruments). DDS-11A conductivity meter (Tianjin 2ed Anal. Instruments) was used to measure conductivity together with a DJS-1 conductivity immersion electrode (electrode constant 0.98) (Shanghai Tienkuang Devices) in the production of deionized water of less 1 $(\mu\Omega\ cm)^{-1}$. The pH of a solution was measured with a pHS-2C acidity meter (Leici Instruments, Shanghai, China) and Model 620D pH Pen (Shanghai Ren's Electrostatics). The temperature was adjusted and remained constant in a Model 116R electric heated thermostatic bath (Changjiang Test Instruments of Tongjiang, China).

Preparation of Solutions

Stock standard solutions of proteins were prepared by dissolving the commercial bovine serum albumin (BSA) (Beitai Biochemical, Chinese Academy Sciences, Beijing, China), human γ-globulin (γ-G), (Serva, Heidelberg, Germany), hemoglobin (Hb) (Dongfeng Biological Technological, Shanghai) and ovalbumin (OVA) (Shanghai Chemical Reagents Center) in deionized water. The protein content (w, mg/mL) in the above solutions was determined and calculated by the relation: $w = 1.45\ A_{280nm} - 0.74\ A_{260nm}$[19] by measuring their absorbances (A_{260nm}, A_{280nm}) at 260 and 280 nm by UV spectrophotometry. 0.600 mM TB was prepared by dissolving 0.4904 g of trypan blue (TB, content 60%, purchased from Shanghai Reagents Supply Center) in 500 mL of deionized water. The Britton-Robinson buffer solutions (pH 2.21-8.69) were prepared to adjust the acidity of solution. 2.0 M NaCl was used to adjust ionic strength of solution. The Na_2-EDTA solution (1%) was prepared to mask the foreign metal ions possibly co-existing in the samples.

Measurements

(1) *Aggregation of TB with Proteins.* Into a 25 mL calibrated flask were added an appropriate working solution of protein, 2.5 mL of Britton-Robinson buffer solution and 0.40 mL of TB solution. The mixture was then diluted to 25 mL with deionized water and mixed thoroughly. All the absorption measurements were obtained against the blank treated in the same way without proteins. (2) *Preparation of Samples and Determination of Proteins.* Two samples were prepared for evaluating the recommended method. The first (1#) used lake water as background, where 1 mg of acetone, glucose and PO_4^{3-}, 0.1 mg of F^-, Cu(II), Mn(II), Zn(II) and 0.05 mg of Pb(II) and Cd(II) were added. The second (2#) used drinking water as background, where 1 mg of NH_4^+, ethanol, amino acid, sugar, 0.2 mg of F^-, Fe(II), Mg(II), Zn(II) 0.05 mg of Pb(II), Ni(II) and 0.01 mg of Hg(II) were added. In them, an unknown amount of standard BSA, OVA, Hb and γ-G solutions was added, respectively and independently. In the analysis of the samples, the masking reagent, 0.5 mL of Na_2-EDTA solution (1%) was added to complex metal ions. The next successive operation is same as (1).

Principal Equations and Calculation

The biological macromolecule, e.g. protein, often contains a complex spatial structure. The winding, folds, coils and other arrangements leads to many holes, gullies and helix grooves. Because of the hydrophobic interaction, a great deal of the hydrophobic groups are close to each other. The protonation of amino ($-NH_2$) in the polar amino acid residues and the dissociation of the carboxyl (-COOH) in the polar amino acid residues results in the formation of many microelectrostatic fields[20] in a protein molecule. It attracts small cations and anions simultaneously till kinetic equilibrium (Fig. 1). The microelectrostatic field is so narrow that stain molecules are adsorbed in only a monolayer. Therefore, the aggregation is regarded as the monolayer adsorption of the microelectrostatic field. In addition, we found that the equilibrium equation of the conventional biochemical reaction is the same as the Langmuir isothermal adsorption.[21] The adsorption equilibrium occurs: L (aqueous phase, C_L) $\Leftrightarrow$ ML_N (macromolecular phase, C_M) in L-M solution. The Langmuir isothermal adsorption may be expressed as follows:

$$\frac{1}{\gamma} = \frac{1}{N} + \frac{1}{KNC_L} \tag{1}$$

where K is the equilibrium constant and C_L the concentration of the excess L. N indicates the maximal adsorption ratio of L to M and γ the molar ratio of L adsorbed to M. K is calculated from equation 1. Both C_L and γ are calculated by means

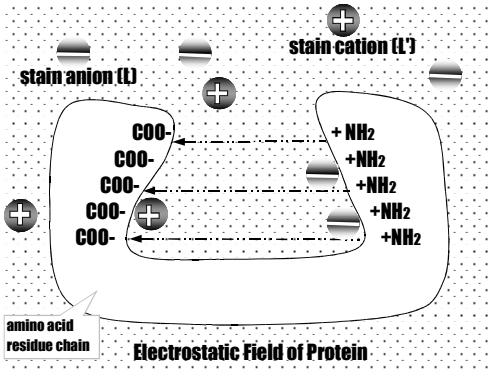

Fig. 1. The formation of microelectrostatic fields in protein and the adsorption process of stains on a microphase surface.

of:[22-28]

$$\gamma = \eta \times \frac{C_{L0}}{C_M} \quad (2)$$

$$C_L = (1-\eta)C_{L0} \quad (3)$$

where

$$\eta = \frac{A_c - \Delta A}{A_o} \quad (4)$$

where both C_M and C_{L0} are the concentration of the M and L added initially and η indicates the effective fraction of L. A_c, A_0 and ΔA are the real absorbances of the M-L product; the measurement absorbance of the reagent blank against water and that of the M-L solution against the reagent blank are directly measured at the peak wavelength λ_2. Within an increase in L concentration, γ will approach a maximum **N**. A_c is calculated by the relation:[24]

$$A_c = \frac{\Delta A - \beta \Delta A'}{1-\alpha\beta} \quad (5)$$

where $\Delta A'$ indicates the absorbance of the M-L solution measured respectively at the valley absorption wavelength λ_1. Usually, α and β are the correction coefficients[25-28] and they are calculated by measuring directly $\mathbf{ML_N}$ and **L** solutions. However, the self-assembly of stain also occurs to form the dimer or polymer.[29,30] Thus, β changes with variation of L concentration (C_{L0}). In addition, the absorptivity (real $\varepsilon_r^{\lambda 2}$ not apparent $\varepsilon_a^{\lambda 2}$) of the adsorption product $\mathbf{ML_N}$ at λ_2 is also directly calculated by the means of:

$$\varepsilon_r^{\lambda_2} = \frac{NA_c}{\delta\gamma C_M} \quad (6)$$

where δ is the cell thickness (cm) and the others have the same meanings as above.

The combination of both the Langmuir adsorption and the spectral correction techniquewill provide a very helpful experimental strategy for study of chromophore or its metallic complex's adsorption in surfactant solution. This method is named Micro Phase Adsorption - Spectral Correction provides a very helpful experimental strategy for study of the aggregation of stain in proteins. The method is called microphase adsorption - spectral correction technique (MPASC). We found that the Pesavento hypothesis is identical with the Langmuir adsorption Equation (1). Consequently, we think that the theoretical foundation of the Pesavento hypothesis comes from the Langmuir adsorption.

RESULTS AND DISCUSSION

Spectral Analysis

The adsorption of TB in proteins (BSA as representative) was carried out, and the spectra of the solution are shown in Fig. 2, which contains 1.00 mg of BSA and 0.240 μmol of TB. From curve 1, the peak absorption of TB is located at 575 nm and that of the BSA-TB aggregate at 620 nm from curve 2. The spectral red shift of the aggregate is only 45 nm. From the relative spectrum 3, the peak and valley absorption of the reacted solution is located at 660 nm and 560 nm, respectively. So these two wavelengths were used in this work. From curve 2, the correction coefficient is calculated to be α = 1.26. From curve 8, we observe that β of TB solution has an obvious change with TB molarity. So the self-aggregation of TB molecules can occur at room temperature.

Effect of pH on Aggregation of TB in Proteins

The effect of pH of solution on the adsorption ratio (γ) of TB to protein (BSA as representative) is shown in Fig. 3. The adsorption ratio of TB to BSA approaches maximum between pH 2.8 and 4.0. This is attributed to the fact that the amino ($-NH_2$) on the polar amino acids of protein protonates in acidic solution to form a positive charge, $-NH_2^+$. The positive electrostatic charge density increases in protein and thus it results in enhancement of the attraction of TB on protein.

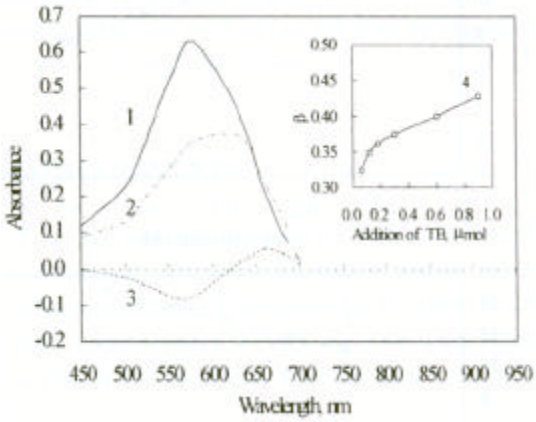

Fig. 2. Absorption spectra of TB and its BSA solutions at pH 3.29: 1- TB (0.180 μmol); 2- TB (0.180 μmol) - BSA (2.0 mg) solution; 3- TB (0.240 μmol) - BSA (0.500 mg) solution and 4- variation of β with TB concentration. 3- against reagent blank and the others against water reference.

Effect of Ionic Strength and Temperature on Aggregation of TB

The influence of ionic strength of solution on the adsorption ratio of TB to BSA is shown in Fig. 4. From curve 1, the adsorption ratio of TB to BSA decreases with an increase in ionic strength. This is attributed to the fact that high concentration NaCl may vary the structure of protein and simultaneously more Cl^- was adsorbed in the electrostatic fields of proteins. The effect of temperature on the adsorption ratio of TB to BSA is shown in Fig. 4, too. The adsorption ratio of TB to BSA increases with an increase in temperature from curve 2. This is different from the other interaction systems.[20,31,32] The self-aggregation of TB is destroyed, and the TB monomer concentration increases at high temperature. The small monomer binds protein easier than the self-aggregate because of the lighter weight and smaller spatial obstacle.

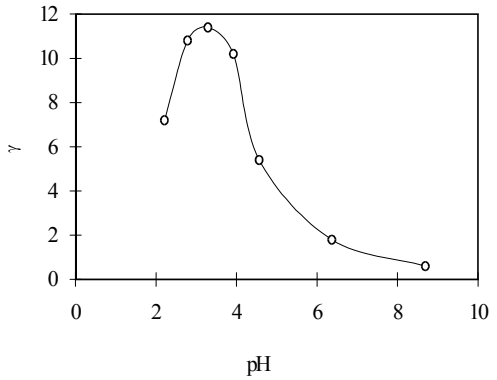

Fig. 3. Effect of pH on the adsorption ratio (γ) of the TB (0.240 μmol) to BSA (0.500 mg) aggregate.

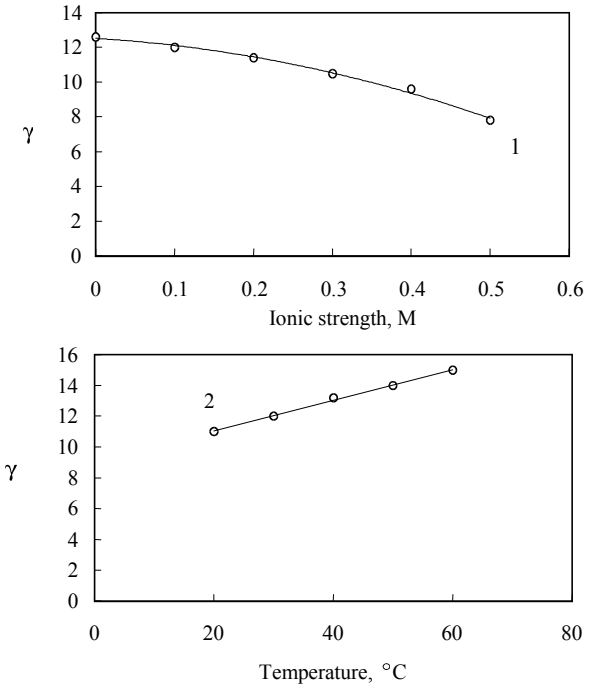

Fig. 4. Effect of ionic strength (1) and temperature (2) on the adsorption ratio (γ) of the TB (0.240 μmol) - BSA (0.50 mg) aggregate at pH 3.29.

Effect of TB Concentration and Determination of Characteristic Constants of Aggregates

By varying the addition of TB solution, the absorbance of a solution was measured, and both the γ and C_L of each were calculated. Their relationship is shown in Fig. 5. All measured points are linear so the aggregation of TB in proteins obeys the Langmuir monolayer adsorption. From the line intercepts, the adsorption ratio of TB to BSA, OVA, Hb and γ-G was calculated to be 14.8, 8.4, 2.8 and 27.6, respectively. By Equation (1), the adsorption constant of the TB aggregates with BSA, OVA, Hb and γ-G were calculated, and their adsorption constants were 7.17×10^5, 4.88×10^6, 4.85×10^6 and 2.99×10^6, respectively. By Equation 6, the real absorptivity (ε) of the aggregates was calculated to be 4.95×10^5, 2.08×10^5, 4.50×10^5 and 3.26×10^6, respectively. Additionally, we tried to calculate the adsorption number (*R*) of TB in per 1 mg of protein. The results are as follows: BSA 0.21, OVA 0.19, Hb 0.18 and γ-G 0.18. Because the *Rs* are near to each other, 1.0 mg of protein binds constant TB molecules in spite of the different array of amino acid residues. It also indicates one electrostatic field consists of constant amino acid residues, and it is in almost no connection with the sequence of amino acid residues. In the determination of the adsorption ratio and equilibrium constant, the spectral correction method has special advantage in operation and principle by comparing the classical methods such as molar ratios,[33] continuous variations[34] and equilibrium movements.[35]

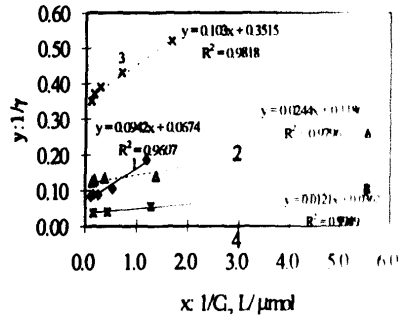

Fig. 5. γ^{-1} vs. C_L^{-1}: 1- BSA (0.500 mg) solution, 2- OVA (0.600 mg) solution, 3- Hb (0.500 mg) solution and 4- γ-G (1.80 mg).

Table 1. The Linear Regression for Determination of Proteins with TB at pH 3.29

Proteins	Linear scope mg/25 mL	Regression equation at 660 nm	Correlation coefficient
BSA	0-1.0	$A_c = 0.384 \times -0.014$	0.9968
OVA	0-1.0	$A_c = 0.382 \times -0.005$	0.9980
Hb	0-1.0	$A_c = 0.367 \times -0.010$	0.9986
γ-G	0-1.5	$A_c = 0.352 \times -0.013$	0.9949

Application to Determination of Proteins

(1) Calibration Graph for Determination of Proteins. The standard series of proteins were prepared and measured with Tb at pH 3.29. The regression equations are given in Table 1. They are all linear in the recommended scope. Because of the similar slope, the real absorbance of the aggregate has no relation to the proteins' structure. Fig. 6 shows the variation of η of TB with protein and the effective TB takes up 0-100% in various protein solutions at the addition of 0.40 mL of the TB solution. So the absorption of an excess of TB may not be omitted, and its interference is serious to the real absorbance of the aggregates especially in low concentration protein solutions. It is necessary for the spectral correction method to be used in place of ordinary spectrophotometry. For 0.010 of A_c, the detection limit was calculated to be 0.025 mg of protein in 25 mL of solution. Six replicated determinations of 1.00 mg of BSA were made, and the mean is 1.05 ± 0.08 mg of BSA. (2) *Effect of Foreign Ions.* By adding EDTA-Na_2 in solution, the influence of foreign substances and ions on the determination of protein was tested at pH 3.29. None of the following substances affected the direct determination of 0.300 mg of BSA (less than 10% error): 5 mg of K^+, NH_4^+, Ca^{2+}, SO_4^{2-}, 1 mg of Cl^-, F^-, PO_4^{3-}, SO_3^{2-}, $C_2O_4^{2-}$, Triton x-100, glucose, amino acid, Mg(II), Fe(III), 0.5 mg of acetone, ethanol, Cu(II), Mn(II), Zn(II), Pb(II), Ni(II), Co(II), Cd(II), 0.1 mg of Hg(II). (3) *Determination of Protein in Samples.* The analytical results of protein in samples are given in Table 2. The recovery of proteins is between 97.6 and 106%.

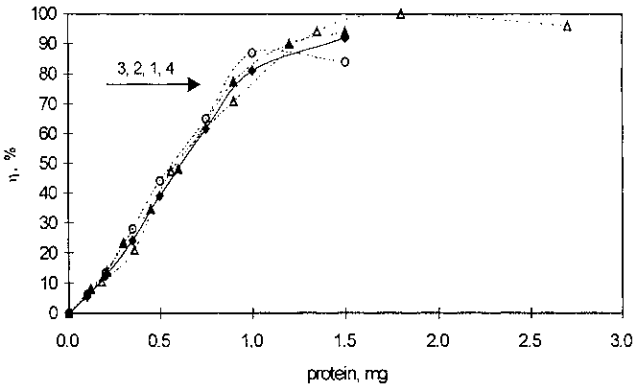

Fig. 6. Effect of the protein concentration on η of TB, where 0.240 μmol of TB was added: 1- BSA, 2- OVA, 3- Hb and 4- γ-G.

Table 2. Determination of Protein in Samples

Sample No.	Added	Found, mg	Average recovery, %
1#	0.500 mg of BSA	0.510 ± 0.033	102
	0.500 mg of OVA	0.502 ± 0.041	100
	0.500 mg of Hb	0.488 ± 0.026	97.6
	1.00 mg of γ-G	1.061 ± 0.062	106
2#	0.500 mg of BSA	0.501 ± 0.024	100
	0.500 mg of OVA	0.505 ± 0.030	101
	0.500 mg of Hb	0.507 ± 0.017	101
	1.00 mg of γ-G	1.02 ± 0.038	102

CONCLUSION

The investigation to the interaction of TB with protein supports the Langmuir aggregation of stain and the microelectrostatic field, though MPASC technique has not given a higher sensitivity than other methods such as RLS.[15] However, it may meet precision and accuracy criteria and offers the additional benefits of simplicity and versatility. We understand the classical method can still play an important role in the study of biomacromolecules.

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