Journal of Analytical Chemistry, Vol. 57, No. 8, 2002, pp. 694–700. Translated from Zhurnal Analiticheskoi Khimii, Vol. 57, No. 8, 2002, pp. 827–833.
Original Russian Text Copyright © 2002 by Hong-Wen Gao, Jun Jiang, Lin-Qian Yu.

ARTICLES

Langmuir Aggregation of Coomassie Brill Blue on Protein and Determination of Protein by MPASC[1]

Hong-Wen Gao*, Jun Jiang, and Lin-Qian Yu

Anhui University, School of Chemistry and Chemical Engineering, Hefei-230039, P. R. China

Received December 12, 2000; in final form, April 16, 2001

Abstract—The microphase adsorption–spectral correction technique (MPASC) has been applied to investigate the aggregation of coormassie brill blue G250 (CBBG) on protein in which the microelectrostatic fields exist. The Langmuir adsorption of CBBG in protein was confirmed, and the interaction is sensitive at pH 3.78. Results show that the adsorption ratio of CBBG to five proteins, e.g., BSA, OVA, Hb, Mb and γ-G are 100, 65, 30, 98 and 210, respectively, their adsorption constants are 9.92×10^4, 8.04 x 10^4, 9.38×10^4, 1.66×10^5, and 2.75×10^4, and their absorptivities are 2.33×10^6, 1.56×10^6, 1.98×10^6, 5.61×10^5, and 4.48×10^6 $Lmol^{-1}$ at 670 nm. The study indicates that 1 mg of protein adsorbs about 1.54 mmol of CBBG. For the protein determination of samples, the recovery of protein is between 88.0 and 111% and the relative standard deviation is 2.6%.

INTRODUCTION

Currently, the study of biomacromolecular chemistry is very active, and it attracts many chemists and biologists for research. The interaction of a chromophore with protein is quite helpful to understand its structure and function, and the quantitative analysis of protein is important in clinical tests. The determination of the content of proteins involves commonly used methods, e.g., Biuret [1], Bradford [2], Lowry [3], and Bromcresol Green [4]. Recently, the resonance light scattering technique has been proposed and applied successfully in the investigation of biological macromolecular complex with organic dyes [5–7]. However, the interaction between biological macromolecules and organic dyes has not been elucidated satisfactorily, and earlier observations have not been explained clearly, e.g., Pesavento experiential equation (8) and Scatchard model [9].

Some chromophores and dyes, e.g., neutral red as the adsorption indicators, are usually used in the determination of halogen anions by precipitation titrimetry. The same color change was observed when we used proteins in place of halogen anions. Consequently, the reaction between biological macromolecules and dye molecules has the same interaction mechanism as the precipitation adsorption reaction. The biological macromolecule contains the complex spatial structure. The winding, folds, coils, and others will cause plenty of holes, gullies, or helix grooves. Many secondary bonds are close to each other, such as Van Der Waals bonds, salt bonds, hydrogen bonds, and other polar bonds. The charge density becomes very high around the holes, grooves, and gullies. The like charges aggregate together to form many microelectrostatic fields (as shown Fig. 1). They will selectively attract organic compound with charge until kinetic equilibrium is reached (Fig. 2). The existence of the microelectrostatic field is the basis of the adsorption of dye molecules in macromolecules [10]. In addition, we found that the equilibrium equation of the biochemical reaction [11] is the same as the Langmuir adsorption equation [12]. The microelectrostatic filed is so narrow that organic molecules (with charge) were adsorbed in only the monolayer. The Langmuir equation is used:

$$\gamma = \frac{KNc_L}{1 + Kc_L}. \quad (1)$$

Here, K is the adsorption constant and c_L the concentration of the excess dye (L). N indicates the maximal adsorption ratio of L to protein (M) and γ is the molar ratio of L adsorbed to M. Within the increase in L concentration, γ will approach a maximum, called the adsorption ratio N. If Kc_L approaches to infinity, γ approaches N. Conversely, $\gamma = KNc_L$ if Kc_L approaches zero. Therefore, we calculated the binding constant K = S/N where the symbol S indicated the tangent slope of curve $\gamma(c_L)$ at the grid origin. Of course, we can still calculate K by expression 1 by the determinations of certain a solution where its γ/N is between 0 and 1. Both c_L and γ are calculated by equations given below [13]:

$$\gamma = \eta(c_{L_0}/c_M), \quad (2)$$

$$c_L = (1-\eta)c_{L_0}, \quad (3)$$

where

$$\eta = (A_c - \Delta A)/A_0, \quad (4)$$

where both c_M and c_{L_0} are the concentration of the M and L added initially and η indicates the effective frac-

*E-mail: gaohongw@mail.hf.ah.cn; fax: 0086 551 5106110
[1]This article was submitted by the authors in English.

tion of L. A_c, A_0 and A are the real absorbance of the M–L product, the measurement absorbance of the reagent blank against water and that of the M–L solution against reagent blank directly measured at the peak wavelength λ_2. The A_c is calculated by means of [14]

$$A_c = (\Delta A - \beta \Delta A')/(1 - \alpha\beta), \quad (5)$$

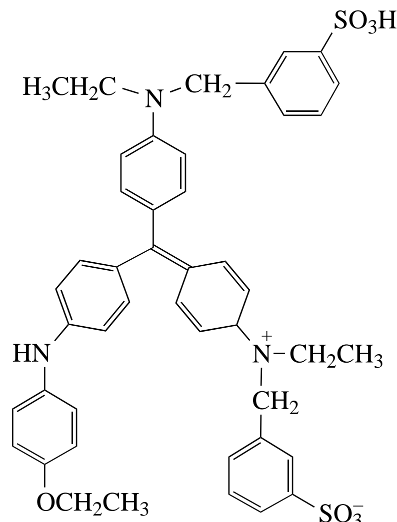
where $\Delta A'$ indicates the absorbance of the M-L solution measured, respectively, at the valley absorption wavelength λ_1. In general, α and β are the correction constants and they are calculated by measuring directly ML_N and L solutions [13]. In addition, the absorptivity (real $\varepsilon_r^{\lambda_2}$ not apparent $\varepsilon_a^{\lambda_2}$) of the adsorption product ML_N at λ_2 is also directly calculated by the following equation:

$$\varepsilon_r^{\lambda_2} = NA_c/\delta\gamma c_M, \quad (6)$$

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

The combination of both the Langmuir adsorption and the spectral correction technique provides a very helpful experimental strategy for the study of aggregation of chromophore on protein. The method is called the microphase adsorption–spectral correction technique (MPASC).

We have studied the reaction between proteins (BSA, OVA, Mb and γ-G) and the organic compound, coormassie brill blue G250 (CBBG) dye, the structure of which is given below:



Coormassie brill blue G250 (CBBG), C.I. No. 42 655

It is about 2 nm in length and 1 nm in width. It can insert into the protein's hole. The reagent was the traditional dye for the determination of protein, but this work is the first to report the aggregation of CBBG molecules on protein macromolecule by being adsorbed. The reaction between CBBG and protein (BSA, OVA, Hb, Mb and γ-G) accords with the Langmuir equation. Results showed that the maximal adsorption ratios of (BSA, OVA, Hb, Mb and γ-G to CBBG at pH 3.78 are 100, 65, 30, 98 and 210, respectively, their adsorption

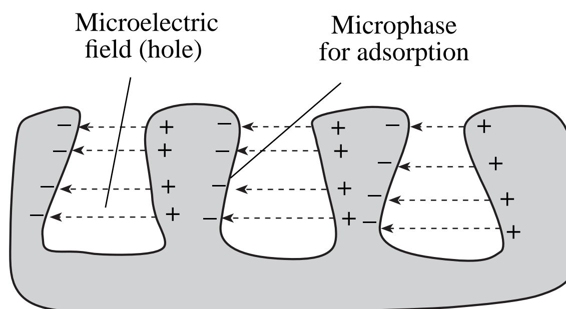


Fig. 1. The microelectrostatic field's hypothesis was proposed in macromolecule.

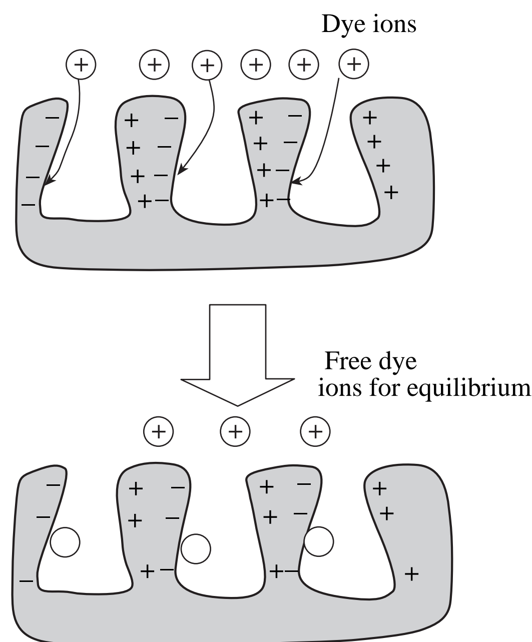


Fig. 2. The adsorption process of dye small molecules on microphase surface.

constants $K_{BSA\text{-}CBBG} = 9.92 \times 10^4$, $E_{OVA\text{-}CBBG} = 8.04 \times 10^4$, $E_{Hb\text{-}CBBG} = 9.38 \times 10^4$, $E_{Mb\text{-}CBBG} = 1.66 \times 10^5$, and $K\gamma\text{-G-CBBG} = 2.75 \times 10^4$, and their absorptivities 2.33×10^6, 1.56×10^6, 1.98×10^6, 5.61×10^5, and 4.48×10^6 $l mol^{-1}$ at 670 nm.

EXPERIMENTAL SECTION

Apparatus and Reagents

Absorption spectra were recorded on a UV/VIS 265 Spectrophotometer (Shimadzu, Japan). The conductivity meter, Model DDS-11A (Tianjin Sec. Anal. Instrument.) was used to measure conductivity together with Model DJS-1 conductivity immersion electrode (electrode constant 0.98, Shanghai Tienkuang Device Works) in the production of nonionic water below

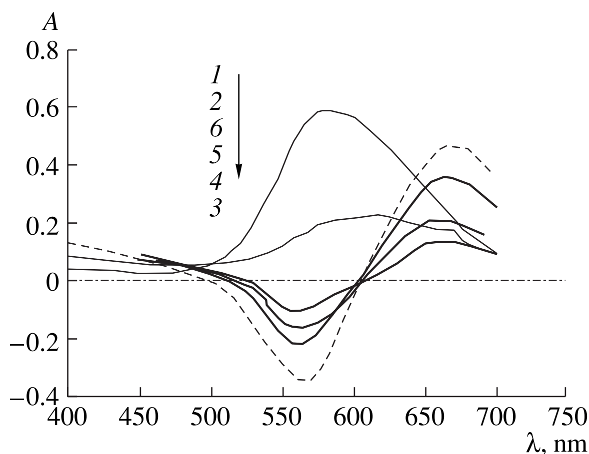


Fig. 3. Absorption spectra of CBBG and its BSA (0.50 mg) solutions: *1*, 0.500 μmol CBBG at pH 3.78, *2*, 0.600 μmol CBBG with 10 mg BSA at pH 3.78, *3*, 0.600 μmol CBBG with 0.250 mg BSA at pH 3.78. *4*, same as *3*, but at pH 4.56, *5*, same as *3*, but at pH 5.02, 6, same as *3*, but at pH 2.21. Both *1* and 2 against water and others against the blank.

0.4 $(\mu\Omega\ cm)^{-1}$. pH of solution was measured on pHS-2C acidity meter (Leici Instrument, Shanghai) and Model 620D pH Pen (Shanghai Ren's Electric). The temperature was adjusted and remained constant in an electrical heated thermostat bath, Model 116R (Changjiang Test Instruments of Tongjiang, China).

Stock standard solutions of proteins were prepared by dissolving the commercial bovine serum albumin (BSA, Beitai Biochem. Co., Chin. Acad. Sci., Beijing, China), human gamma globulin (γ-G, Serva, Heidelberg, Germany), bovine hemoglobin (Hb, Dongfeng Biological Technological Co.), horse myoglobin (Mb, Shanghai Chemical Reagents Centre) and ovalbumin (OVA, Shanghai Chemical Reagents Supply Centre) in non-ionic water. γ-G solution is 1.00 mg/ml and the others are 0.500 mg/ml.

The CBBG solution, 0.600 mM, was prepared by dissolving 0.2135 g of CBBG (content 60%, provided by Shanghai Chemical Reagents Supply Centre of Chinese Medicine Group) in 250 ml of ethyl alcohol. The Britton-Robinson buffer solutions (between pH 2.21 and 11.58) were used to adjust the acidity of the reaction solution. 2 M NaCl solution was used to adjust the ionic strength of the aqueous solutions. Na_2-EDTA solution, 5% was prepared to mask the foreign metallic ions coexisted possibly in the practical samples. The surfactant solutions, Triton x-100, sodium dodecyl benzene sulfonate (SDBS) and cetyl trimethylammonium bromide (CTMAB) (all 1%), were prepared for studying their effect on sensitivity.

General Procedures

Into a 10 ml calibrated flask were placed an appropriate working solution of proteins, 1.0 ml of Britton-Robinson buffer solution, and an appropriate CBBG solution. The mixture was then diluted with nonionic water to 10 ml and mixed thoroughly. All the absorption measurements were obtained against a blank treated in the same way without proteins.

In the determination of proteins in samples, 0.5 ml of the masking reagent Na_2-EDTA solution (5%) was added to complex metal ions, for example, Pb(II), Cd(II), Hg(II), etc.

RESULTS AND DISCUSSION

Spectra Analysis

The adsorption reaction between CBBG and proteins (BSA representative) was carried out. The absorption spectra of the BSA-CBBG solutions are shown in Figure 3, where 0.50 mg of BSA and 0.60 μmol of CBBG were added. Curves 3–6 give the spectra of CBBG-BSA solutions measured against the CBBG solution. Curves 1 and 2 give that of the CBBG solution and CBBG-BSA product against water at pH 3.78. By comparing curve 1 and 2, we find that the absorption of CBBG locates at 580 nm and that of BSA-CBBG complex at 610 nm. The spectral red shift is only 30 nm. Therefore, the excess of CBBG will certainly affect the measurement of the real absorption of product. From curves 3–6, which represent the absorption difference between CBBG solution and its protein solution, we observe that the two wavelengths, 670 nm (peak) and 570 nm (valley), should be selected as measurement wavelengths so as to obtain the lowest measurement error. A pH of 3.78 gives maximal sensitivity, because curve 3 gives the highest peak and lowest valley. From curves 1 and 2, the correction coefficients $\beta = 0.386$ and $\alpha = 1.00$ are calculated. Because β is high, the spectral correction method should be used instead of ordinary spectrophotometry.

Effect of pH, Ionic Strength, Surfactants, Temperature and Time

By varying the pH of the solution, the absorption of BSA-CBBG solution was measured; the molar ratio of the effective CBBG to BSA is shown in Fig. 4a. We observe that pH 3.5–4.2 gives the highest ratio. Around pH 4, the cation $CBBG^+$ is formed and it is easily attracted into microelectrostatic fields in protein. In this work, pH 3.78 was selected.

The influence of the ionic strength of solution on the binding ratio is shown in Figure 4b. Between the ionic strength 0 and 0.4 M, the binding ratio of CBBG to BSA drops down with an increase in ion strength, especially over 0.2 M. This is because more Na^+ will be attracted into microelectrostatic fields to take up the microphase surface.

The effect of various surfactants on the binding ratio of CBBG to BSA is shown in Fig. 4c. The cationic sur-

factant CTMAB will stop the adsorption of CBBG on microphase surface, because it can be adsorbed strongly instead of CBBG molecules. The surfactants triton x-100 and SDBS also affect the binding ratio, because their micelles will adsorb CBBS molecules to cause a spectral shift.

At various temperatures, the relationship of the binding ratio vs. CBBG addition is shown in Fig. 4d. We find that the binding ratio increases with increase in temperature. At room temperature, the reaction between BSA and CBBG is complete in 5 min (Fig. 4e), because the binding ratio approaches its maximum and then remains almost constant. This indicates that the adsorption reaction between macromolecules and small molecules is often rapid and it is different from the common chemical reaction.

Effect of CBBG Concentration

By varying the addition of CBBG solution, the absorbance of various protein solutions was measured. Calculated γ and c_L of each. Their relationship is shown in Fig. 5, where the dashed lines indicate the Langmuir adsorption, solid lines are the measured points, and dotted lines the tangent of curves. We find that each solid line is close to the dashed curve. Therefore, the biological reaction between macromolecules and dye molecules is just the monolayer adsorption of dye molecules in microelectrostatic field of macromolecule. From curves *1–5*, we can obtain the binding ratio of PCA to BSA, OVA, Hb, Mb, and γ-G are 100,65, 30, 98 and 210, respectively. From the tangent lines T1-T5, we calculated the binding constant of CBBG products with BSA, OVA, Hb, Mb, and γ-G to be 1.00×10^5, 6.57×10^4, 8.16×10^4, 1.26×10^5 and 3.06×10^4, respectively. In addition, we prepared the solutions (Table 1) for the determination of E values and real absorptivity (ε) of protein CBBG product, and the results are listed in Table 1. We find that the mean E calculated from six replicated determinations of each solution is close to the above-calculated data from the tangent slope. From Table 1, the binding constant of the protein CBBG products are arranged as BSA, OVA, Hb, Mb, and γ-G in from high to low order. From ε data, we observe that the higher the absorptivity of protein-CBBG product becomes, the greater the binding ratio N becomes. Their order is γ-G, BSA, Hb, OVA and Mb from great to little. This is in accord with the universal law of chemical reaction. For the determination of the binding ratio and equilibrium constant, the spectral correction method has special advantage in operation and principle by in contrast to classical methods, such as molar ratio [15], continuous variation [16], Scatchard model [9] and equilibrium movement [17].

We tried to calculate the adsorption ratio (R) of CBBG to 1.0 mg of protein by using formula R = N/W, where W indicates the molecular weight of macromolecule. The results are as follows: 1.48×10^{-3} (BSA), 1.59×10^{-3} (OVA), 1.47×10^{-3} (Hb), 1.73×10^{-3}(Mb), and 1.43×10^{-3} (γ-G), the average being 1.54×10^{-3}. The maximal relative standard deviation is only 12%. Therefore, we think that per milligram of protein will bind an almost constant number of CBBG molecules even if their structures are possibly different. Because 1 mg of protein contains approximately an equal number of amino acid residues, a microelectrostatic field consisted of constant amino acid residues.

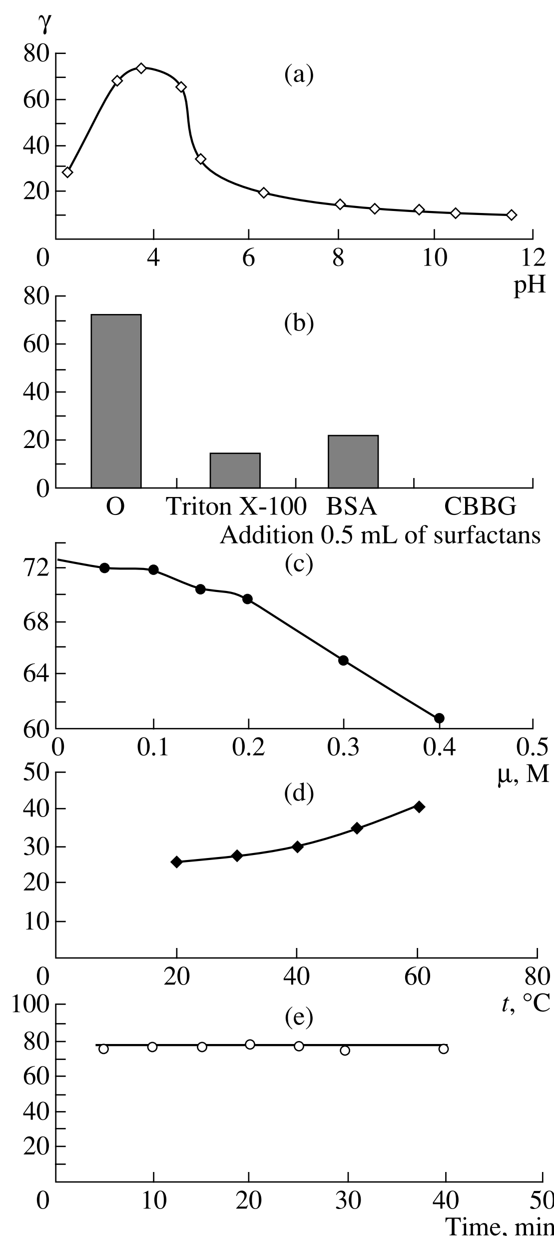


Fig. 4. Effect of pH (a: 0.600 μmol CBBG + 0.500 mg BSA), ion strength (b: 0.600 μmol CBBG + 0.500 mg BSA), surfactants (c: 0.600 μmol CBBG + 0.500 mg BSA), temperature (d: 0.300 μmol CBBG + 0.250 mg BSA), and time (e: 0.600 μmol CBBG + 0.500 mg BSA) on γ.

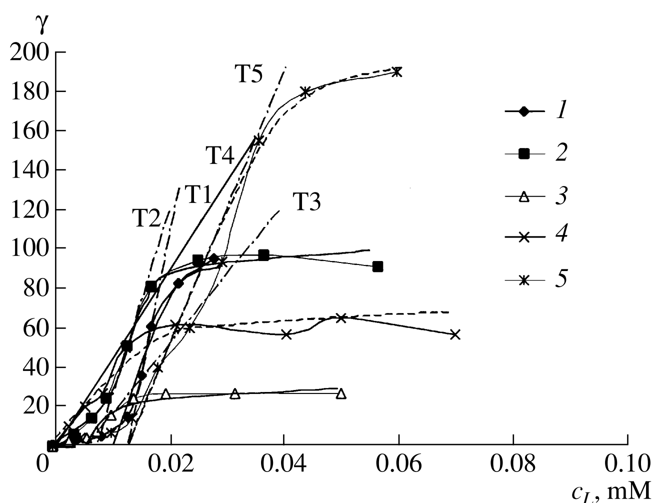


Fig. 5. Relationship between γ and c_L at pH 3.78: *1*, BSA; *2*, Hb; *3*, Mb; *4*, OVA; *5*, γ-G. Solid line, measurement curve; dashed line, Langmuir adsorption; and dash dot line (T1–T5), tangent line of curves.

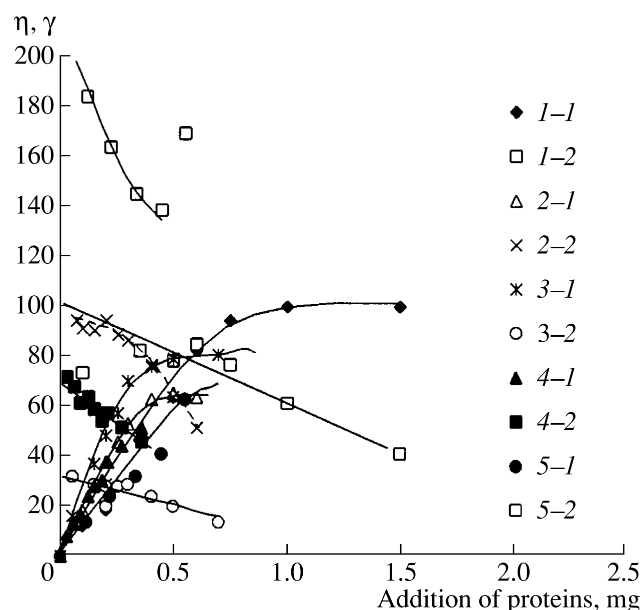


Fig. 6. Effect of addition of proteins solutions on both η(*x* – 1) and η(*x* – 2) where 0.90 μmol CBBG was added: *1*, BSA; *2*, Hb; *3*, Mb; *4*, OVA; *5*, γ-G

Calibration Graph

The standard series of proteins were prepared and measured at pH 3.78, where 1.5 ml of CBBG solution was added. The regression equations between the real absorption (A_c) of the product and proteins (x) are listed in Table 2. They are linear in the recommended concentration scope. From the slopes and linear correlation coefficients of equations, we find that the spectral correction method gives about double sensitivity of ordinary spectrophotometry and higher accuracy. In addition, we calculate the adsorption ratio (*x*) and effective fraction (γ) of CBBG of each solution above, and their curves are shown in Fig. 6. All η curves extend to the vertical axis and the meeting points correspond with the maximal adsorption ratio: 215 (γ-G), 103 (BSA), 97 (Hb), 33 (Mb) and 67 (OVA). They are in accord with the above-calculated data. All η values increase with the increase of protein concentration. It approaches 100% when the protein concentration is much than CBBG concentration.

For A_c = 0.010, the detection limit of protein to be 0.003 mg in 10 ml of solution. Seven replicate determinations of 0.100 mg of standard BSA were made, and their mean result was 0.097 ± 0.003 mg.

Effect of Foreign Ions

By adding EDTA-Na_2 (0.5 ml of 5% solution) in the general procedures, the influence of foreign substances, such as cations, anions, sugars, and surfactants on the determination of proteins by using the spectral correction technique was tested at pH 3.78. None of the following substances affected the direct determination of 0.20 mg of BSA (less than 10% error): 2 mg of K^+, Na^+, NH_4^+, Ca(II), SO_4^{2-}, Nl^-, 1 mg of PO_4^{3-}, NO_3^-, SO_3^{2-}, $C_2O_4^{2-}$, I^-, CH_3COO^-, glucose, amino acid, acetone, ethanol, Mg(II), Zn(II), Al(III), Fe(III), 0.5 mg of Cu(II), Mn(II), Ni(U), Co(II), 0.1 mg of Hg(II), Cd(II), Pb(II).

Table 1. Values of γ/*N*, binding constant of complexes CBBG–protein, and corrected molar absorptivity coefficients (averaged by replicate determinations)

Systems	γ/*N*	*K*	ε, at 670 nm
BSA (0.500 mg)–CBBG (0.600 μmol)	(60.6 ± 4.1)/100	$(9.92 \pm 1.31) \times 10^4$	2.33×10^6
OVA (0.150 mg)–CBBG (0.180 μmol)	(27 ± 1.2)/65	$(8.04 \pm 1.22) \times 10^4$	1.56×10^6
Mb (0.250 mg)–CBBG (0.300 μmol)	(16 ± 1.1)/30	$(1.66 \pm 0.51) \times 10^5$	5.61×10^5
γ-G (0.450 mg)–CBBG (0.600 μmol)	(93.4 ± 4.1)/210	$(2.75 \pm 0.61) \times 10^4$	4.48×10^6
Hb (0.250 mg)–CBBG (0.300 μmol)	(51 ± 2.6)/98	$(9.38 \pm 1.55) \times 10^4$	1.98×10^6

Table 2. Parameters of linear regression for calibration dependences for protein determination with CBBG at pH 3.78

Protein	Range of linearity (mg of protein per 10 mL)	Regression equation at 670 nm	Correlation coefficient
BSA	0–0.60	$A_c = 2.81x - 0.022$	0.9939
		$\Delta A = 1.32x + 0.011$	0.9899
OVA	0–0.30	$A_c = 1.91x - 0.011$	0.9821
		$\Delta A = 1.00x - 0.018$	0.9957
Mb	0–0.30	$A_c = 4.03x + 0.004$	0.9996
		$\Delta A = 1.93x - 0.026$	0.9946
γ-G	0–0.30	$A_c = 3.33x - 0.019$	0.9975
		$\Delta A = 1.65x - 0.011$	0.9947
Hb	0–0.30	$A_c = 4.03x + 0.004$	0.9996
		$\Delta A = 1.93x - 0.026$	0.9946

Table 3. Determination of protein in samples with CBBG as reactant at pH 3.78 in the presence of EDTA

Sample	Added	Found, mg protein
Duoqi Drink (contains protein about 2%)	0.040 ml of sample	0.806
		0.781
		0.767
		0.818
		0.807
		0.827
		aver. 0.801 ± 0.021,
		$s_r = 0.026$
	0.300 mg BSA was added in 0.040 ml of sample	0.992
		1.13
		1.08
		aver. 1.07 ± 0.06, recovery 89.7%
Synthetic sample: drinking water; Background (three replicated determinations of 5.0 ml of sample)	BSA 0.100 mg	0.111 ± 0.006 recovery 111%
	OVA 0.100 mg	0.095 ± 0.006 recovery 95.0%
	γ-G 0.100 mg	0.101 ± 0.008 recovery 101%
	Mb 0.100 mg	0.088 ± 0.007 recovery 88%
	Hb 0.100 mg	0.107 ± 0.008 recovery 107%

Sample Analyzed

Two samples were analyzed. One was Duoqi Children's Drink, and the other was mixed with proteins and the following compounds or ions: 1 mg of Ca(II), ethanol, acetate, ctDNA, yRNA, glucose and PO_4^{3-}, 0.5 mg of Ca(II), Mg(II), SO_4^{2-}, NO_3^-, NI^-, 0.1 mg of F^-, Mn(II), Zn(II), 0.02 mg of Pb(II), and Hg(II), where it was drinking water background. The drink sample was first diluted from 1.00 ml of original drink to 100 ml with nonionic water, and then 4.0 ml were taken for analysis. The analysis of samples and recovery of proteins are given in Table 3. According to the data, the calculated concentration 2.0% protein is close to "about 2% protein" marked in the container. Standard BSA was added to the first sample, and its recovery was 89.7% together with the standard deviation 2.6%. In addition, we observe that the recovery of various proteins in the synthetic sample was between 88.0 and 111%.

CONCLUSION

The investigation to the reactions between CBBG and five kinds of proteins powerfully supports the monolayer adsorption principle and the formation hypothesis of microelectrostatic field. Consequently, in fact, both the Pesavento hypothesis and Scatchard model are just microelectrostatic fields' adsorption between macromolecules and dye molecules. However, the MPASC technique has not given a higher sensitivity than other methods, such as RLS [6]. 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 important role in the recognition of biological macromolecules and their property determination.

ACKNOWLEDGMENT

Financial support from the Natural Science Foundation of Anhui Province (No. 99045332) and the Natural Science Foundation of Education Council of Anhui Province (No. 99JL0003) are gratefully acknowledged.

REFERENCES

1. Bradford, M.M., *Anal. Biochem.*, 1976, vol. 72, p. 245.
2. Li, N. and Tong, S.Y., *Anal. Lett.,* 1995, vol. 28, p. 1763.
3. Li, N. and Tong, S.Y., *Talanta*, 1994, vol. 41, p. 1657.
4. Pasternack, R.F., Gibbs, E.J., and Villafranca, J.J., *Biochemistry,* 1983, vol. 22, p. 2406.
5. Arlinghaus, H.F. and Kwoka, M.N., *Anal. Chem.*, 1997, vol. 69, p. 3747.
6. Pasternack, R.F. and Collings, P.J., *Science* (Washington, D. C., 1883), 1995, vol. 269, p. 935.
7. Huang, C.Z., Li, K.A., and Tong, S.Y., *Anal. Chem.*, 1997, vol. 69, p. 514.

8. Pesavento, M. and Profumo, A., *Talanta*, 1991, vol. 38, p. 1099.

9. Scatchard, G., Scheinerg, I.H., and Armstrong, S.H., *J. Am. Chem. Soc.*, 1950, vol. 72, p. 535.

10. Gao, H.W., Jiang, J., and Yu, L.Q., *Analyst* (Cambridge, U.K.), 2001, vol. 126, p. 528.

11. Doumas, B.T., Watson, W.A., and Biggs, H.G., *Clin. Chim. Acta*, 1971, vol. 31, p. 87.

12. Langmuir, I., *J. Am. Chem. Soc.*, 1918, vol. 40, p. 1361.

13. Gao, H.W., *Recl. Trav. Chim. Pays-Bas*, 1995, vol. 114, p. 61.

14. Gao, H.W., Zhang, S.Y., and Ye, S.M., *J. AOAC Int.*, 2000, vol. 83, p. 231.

15. Tikhonov, V.N., *Zh. Anal. Khim.*, 1975, vol. 30, p. 1501.

16. Likussar, W., *Anal. Chem.*, 1973, vol. 45, p. 1926.

17. Lazarev, A.I., *Zavod. Lab.*, 1975, vol. 41, p. 534.