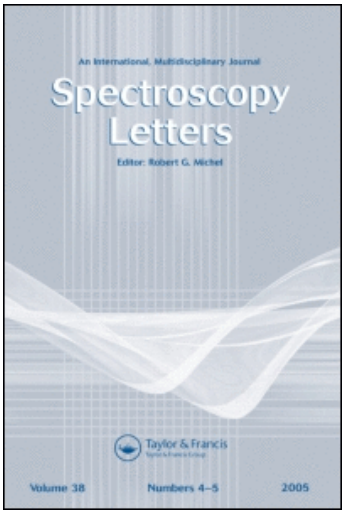

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Mechanism of the Interaction Between Bromophenol Blue and Bovine Serum Albumin

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ABSTRACT

The mechanism of the interaction between Bromophenol Blue (BPB) and Bovine Serum Albumin (BSA) was studied spectrophotometrically. Absorption spectral changes of BPB–BSA and BPB–Chitosan mixtures in different BSA and Chitosan concentrations are discussed. The influence of different experimental conditions on the absorption spectra of BPB–BSA mixtures was investigated. Through these experiments, the conclusion was drawn that the bathochromic shift of the absorption spectra of the BPB–BSA reaction system results mainly from hydrophobic

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interaction between BPB and BSA, while electrostatic force is necessary for the formation of the BPB–BSA complex.

Key Words: Bromophenol blue; Bovine serum albumin; Spectrophotometry; Interaction mechanism.

INTRODUCTION

Protein assays using small dye molecules[1–11] have been widely investigated, but people have different opinions on many aspects of this kind of reaction. So, an investigation on this reaction mechanism should be beneficial not only for protein assays but also for clarification of the chemical nature of the interaction between proteins and small organic molecules of organisms.

Seedher et al.[1] interpreted the reaction of small molecules with albumin as a non-covalent interaction through their investigation of the association constants. Murakami et al.[2] and Anon.[3] thought that dye interacted with proteins to form a ligand and this kind of ligand is displaceable.[3] They investigated the binding sites and binding constants, but when people viewed the molecular structure of BPB and BSA, they found that electrostatic force was the main binding force.[4]

Li Ke-an et al.[4] thought that the color change during the combination was due to the transformation of the free acidic form of the dye species into a bound basic form; Waldmann et al.[5] said that the color change was a displacement toward the quinoid structure of the dye. All these interpretations could be explained by the conjugation theory, which is based on the electrons' translocation.

When investigating the reaction at the molecular level, Trivedi[6] thought that lysine residues of the albumin were very important in the

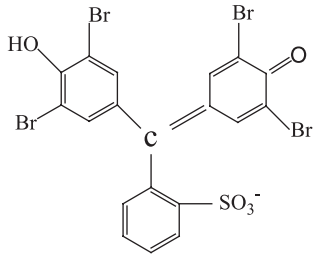

Figure 1. Structure of bromophenol blue.

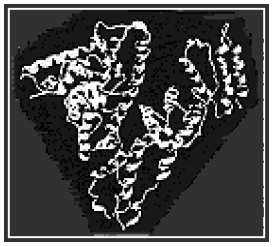

Figure 2. Structure of bovine serum albumin. From Ref. [12].

binding with the dye, while Tayyab et al.[7] held the opposite opinion. Moreover, Polya et al.[8] thought that the reaction between proteins greatly affected the binding reaction. Although there were so many different opinions, the conformational change of protein molecules during the binding was thought to be true.[2,7]

Bromophenol Blue (BPB) is widely used as a protein dying reagent. Figure 1 and Figure 2 show the structure of BPB and bovine serum albumin in its normal form[12] respectively. Under acidic conditions, BPB can interact with bovine serum albumin (BSA) to form a stable blue complex with a bathochromic shift of the BPB absorption spectra.

Our laboratory made a systematic investigation of the interaction mechanism between polysaccharid and spectral probes, and proposed the Spatial Orientation Interaction Model.[13–16] This model established a theoretical basis for an understanding of the interaction between BPB and BSA, especially of their color change.

In this paper, some selected experiments were conducted to better interpret the interaction of Bromophenol Blue (BPB) with proteins. Also, the influence of different experimental conditions was investigated. All our work was designed to explore our postulates that: the bathochromic shift of the BPB–BSA binding reaction results mainly from a hydrophobic interaction between BPB and BSA while electrostatic force is needed for the combination between BPB and BSA.

EXPERIMENTAL

Apparatus

A U-3000 spectrophotometer (Hitachi Co., Japan) was used for recording absorption spectra, and a UV-754 spectrophotometer (Shanghai Fine

Scientific Instrument Co., China) was used for the measurement of absorbance at a given wavelength. A portable pH-HJ90B meter (Beijing Hangtian Computer Co., China) was used for the pH measurements.

Reagents

BSA was obtained from Shanghai Boao Biotechnology Co., China. Calculations reported for BSA are in terms of a molecular weight of 67000. The concentration of the BSA stock solution was 2.000×10^{-4} mol.L^{-1}. The concentration of the BSA operating solution was 2.000×10^{-5} mol.L^{-1}. Bromophenol Blue (BPB) was purchased from The Third Reagent Factory of Shanghai, China. The concentration of the BPB stock solution was 3.000×10^{-3} mol.L^{-1}. The BPB operating solution was 3.000×10^{-4} mol.L^{-1}.

The pH was controlled by a Na_2HPO_4-Citric acid buffer. Hydroxypropyl-β-cyclodextrin (HP-β-CD) was obtained from Jiangsu Taixing Xingxing Medicine Co., China. Chondroitin sulfate was purchased from Shandong Fuda Fine Chemicals limited Co., China. Azur A was purchased from Shanghai Xingzhong Chemistry Factory, China. Chitosan was prepared by our laboratory. Calculations reported for Chitosan are in terms of molecular weight of 10000. All other reagents are of analytical or guaranteed reagent grade.

Methods

The BPB operating solution was transferred into a series of 12×100 mm test tubes, then Na_2HPO_4-Citric acid buffer, BSA and other reagents were added in different amounts to each test tube. The mixture was diluted to a certain volume with deionized water and mixed thoroughly either by inversion or vortexing. After 10 min, spectra from 350 to 700 nm or absorbances at a given wavelength of these solutions were measured with reference to deionized water.

RESULTS AND DISCUSSION

Absorption Spectra of BPB at Different pH

Figure 3 shows the absorption spectrum of BPB. It was obtained by keeping the BPB concentration constant and changing the pH of the solutions. With increase in pH, the absorption peak at 436 nm decreases, while the absorption peak at 590 nm increases stepwise, indicating that the

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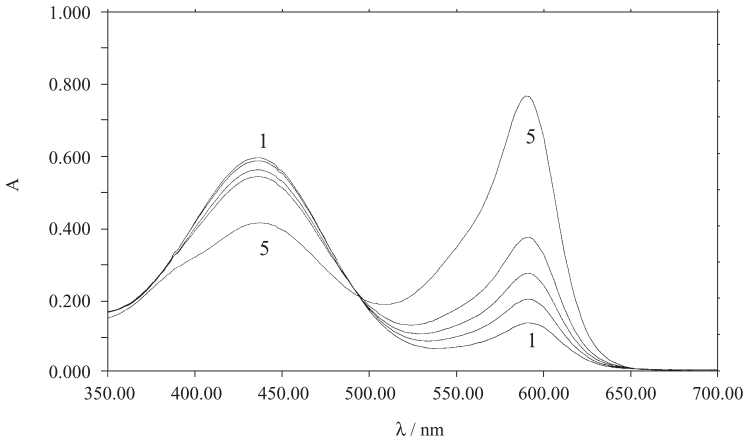

Figure 3. BPB absorption spectra at various pH values BPB concentration constant at 5×10^{-5} mol.L^{-1}. In order of increasing peak absorbances at 590 nm, pH values are 2.6, 2.8, 3.0, 3.2, and 3.6.

acidic species absorption peak of free-state BPB is at 436 nm and the basic species absorption peak is at 590 nm. This spectral change corresponds to the proton dissociation of BPB. The dissociation constant of BPB in water solution is given by a pk_a=3.85. For simplicity, the above dissociation equilibrium can be written as:

$$HL^- \rightleftharpoons L^{2-} + H^+ \qquad \text{(I)}$$

Absorption Spectra of BPB–BSA Mixtures in Different BSA Concentrations

Figure 4 shows a series of absorption spectra of BPB–BSA mixtures at pH 2.6 under conditions where the BSA concentration is the sole contributor to the spectral features. With an increase in BSA concentration, the BPB absorption peak at 436 nm decreases continuously with a bathochromic shift from 436 to 440 nm, while the absorption peak at 590 nm increases continuously with a bathochromic shift from 590 to 604 nm. Figure 4 indicates that there are interactions between BPB and BSA. In acidic solution, BPB has negative charges and it can be bound with positively charged basic amino acid (Arg, Lys, His) residues of BSA by electrostatic forces,[17] the equilibrium (I) then shifts to the right. On this basis, the hydrophobic benzene rings of BPB can be bound with the

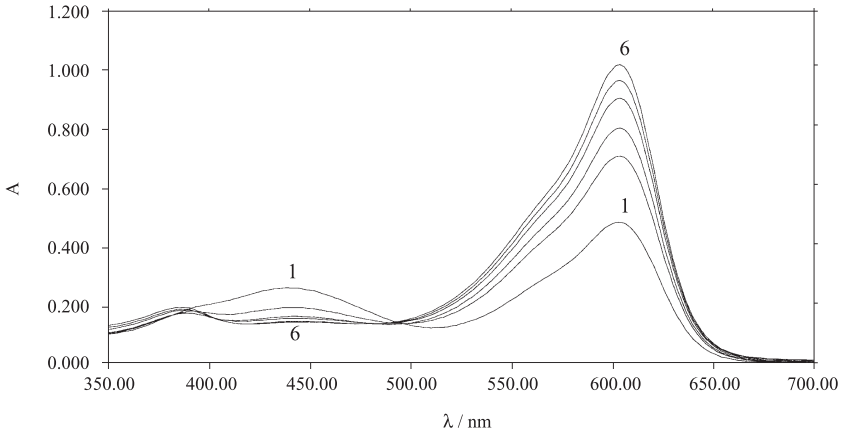

Figure 4. Absorption spectra of BPB–BSA mixtures at different BSA concentrations BPB concentration constant at 5×10^{-5} mol.L^{-1}, pH 2.6. In order of increasing peak absorbances at 604 nm, BSA concentrations are 0.8×10^{-6}, 1.67×10^{-6}, 2.50×10^{-6}, 3.33×10^{-6}, 4.17×10^{-6}, and 5.00×10^{-6} mol.L^{-1}.

hydrophobic cavities of BSA. One BPB molecule can be combined with two BSA molecules at the same time.[18,19] So the BPB–BSA complex is very stable. With an increase in BSA concentration, the yellow reaction system changes to blue and becomes deeper. This color change is mainly caused by the binding reaction, induced by the electrostatic force, between BPB and BSA. Until this experiment was done we supposed that the bathochromic shift of the absorption spectra is caused by an electrostatic interaction between BPB and BSA, but now it appears that the bathochromic shift is caused by a hydrophobic interaction.

Effect of Different BSA and Chitosan Concentrations on the Absorbances of BPB–BSA and BPB–Chitosan Mixtures

Figure 5 shows the absorbance changes of BPB–BSA and BPB–Chitosan mixtures at 604 nm, under conditions of changing BSA and Chitosan concentrations, at pH 2.6. Under acidic conditions, as the cationic polysaccharid is positively charged at high charge density, Chitosan can be combined with BPB mainly by electrostatic forces. The positive charge density of Chitosan is higher than that of BSA molecules at the same concentration, but Figure 5 shows that the absorbance spectrum of the BPB–BSA mixtures is significantly different from that of the BPB–Chitosan

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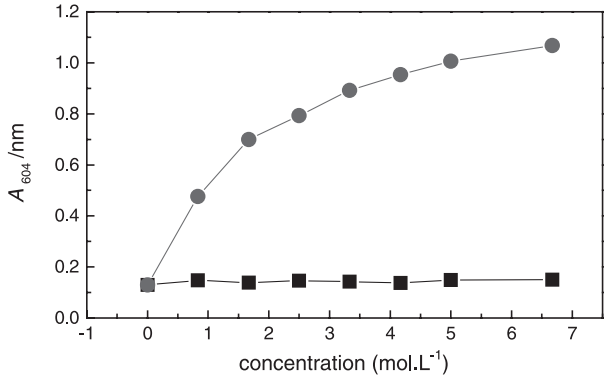

Figure 5. Effect of different BSA and Chitosan concentrations on the absorbance of BPB–BSA and BPB—Chitosan mixtures BPB concentration constant at 5×10^{-5} mol.L^{-1}, pH 2.6. ●—BPB-BSA , ■—BPB-Chitosan. BSA and Chitosan concentrations are 0.00, 0.83×10^{-6}, 1.67×10^{-6}, 2.50×10^{-6}, 3.33×10^{-6}, 4.17×10^{-6}, 5.00×10^{-6}, and 6.67×10^{-6} mol.L^{-1}.

mixtures. This indicates that electrostatic force alone could not lead to the spectral changes seen in Figure 4. Thus, we hold the belief that hydrophobic interaction is the predominant force leading to the spectral changes for the BPB–BSA mixtures.

Effect of NaCl on the Absorbance of BPB–BSA Mixtures

Figure 6 shows that with increased NaCl concentration, the absorbance at 604 nm decreases, while the absorbance at 440 nm increases, resulting in a color change of the reaction system from blue to yellow. This is mainly caused by the shielding effect of co-existing inorganic ions and the competitive combination of inorganic anions with the positively charged amino acid residues of BSA. This phenomenon indicates that the electrostatic force is a prerequisite in the formation of the blue BPB–BSA complex, which shows an absorption maximum at 604nm.

Effect of Ethanol on the Absorbance of BPB–BSA Mixtures

Figure 7 shows that when the ethanol concentration is under 25%, there's almost no change of the absorbance at 440 and 604 nm, but the absorbance at 604 nm increases slowly. When the ethanol concentration is above 25%, the absorbance at 604 nm decreases, while the absorbance at

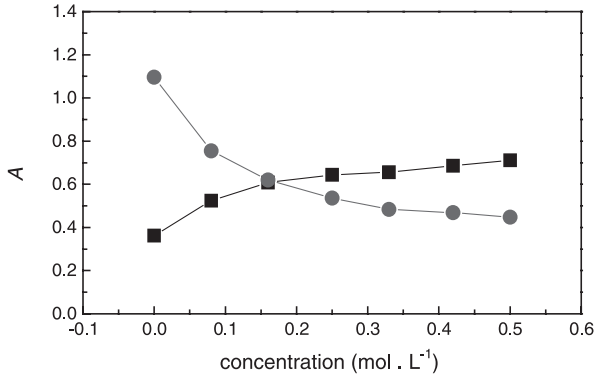

Figure 6. Effect of NaCl on the absorbance of BPB–BSA mixtures BPB concentration constant at 5×10^{-5} mol.L^{-1}, BSA concentration constant at 3.33×10^{-6} mol.L^{-1}. pH 2.6. ●—604 nm, ■—440 nm.

440 nm increases. The reason is that when the ethanol concentration is under 25%, with the increase of the concentration, the helical degree of BSA decreases slightly and the hydrophobic cavity emerges. So the steric hindrance effect of the hydrophobic interaction decreases, the hydrophobic interaction between BPB and BSA increases, and BPB combines with BSA. With the ethanol concentration under 25% in the reaction system, the hydrophobic activity center of BSA is still not destroyed by the hydrophobic ethanol solution. With the increase of ethanol concentration above

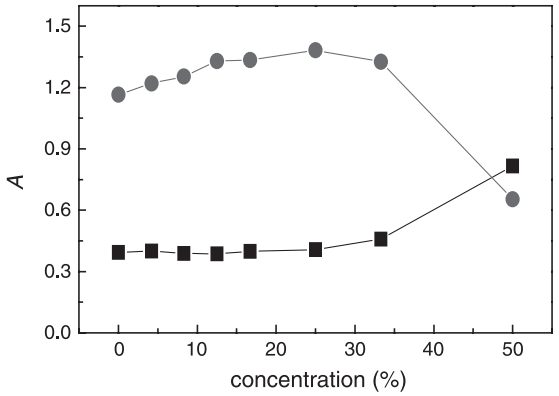

Figure 7. Effect of ethanol on the absorbance of BPB–BSA mixtures BPB concentration constant at 5×10^{-5} mol.L^{-1}, BSA concentration constant at 3.33×10^{-6} mol.L^{-1}. pH 2.6. ●—604 nm. ■—440 nm.

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25% in the reaction system, denaturation of the BSA molecules occurs, the hydrophobic cavities are destroyed and the hydrophobic interaction between BPB and BSA decreases, leading to a decrease of the binding ability of BPB and BSA. At this point, the blue color of the reaction system turns yellow and becomes gradually deeper. This experimental phenomenon conforms to the explanation of Klotz[20] and it also indicates indirectly that there should be very strong hydrophobic interaction during the formation of the BPB–BSA complex.

Effect of Hydroxypropyl-β-cyclodextrin on the Absorbance of BPB–BSA Mixtures

In order to study the strength of the hydrophobic interaction between BPB and BSA, we investigated the effect of hydroxypropyl-β-cyclodextrin (HP-β-CD) on the hydrophobic interaction of BPB–BSA mixtures and Azur A-Chondroitin Sulfate (AA–CS) mixtures. Because of the hydrophobic cavity of HP-β-CD, there is the inclusion reaction between HP-β-CD and aromatic substances, destroying the hydrophobic interaction among molecules. Figure 8 shows that with an increase in HP-β-CD concentration, there is almost no change in the absorbance at 440 nm in the reaction system of BPB and BSA, while the absorbance at 550nm (the peak absorption of AA) increases continuously in the reaction system of AA and CS. This indicates that the hydrophobic strength of the BPB–BSA complex

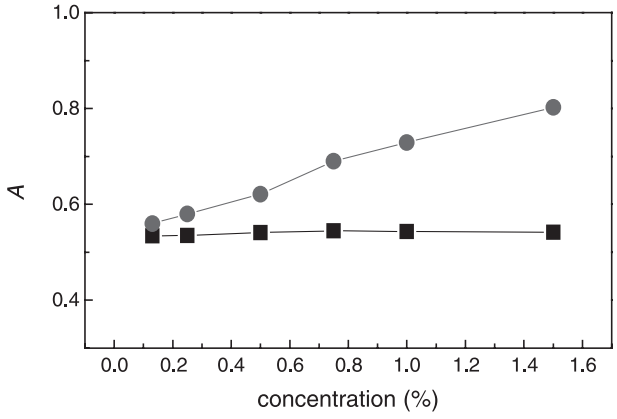

Figure 8. Effect of hydroxypropyl-β-cyclodextrin on the absorbance of BPB–BSA and AA–CS systems BPB concentration constant at 5×10^{-5} mol.L^{-1}, BSA concentration constant at 3.33×10^{-6} mol.L^{-1}. AA concentration constant at 9.12×10^{-5} mol.L^{-1}, CS concentration constant at 8.33×10^{-8} mol.L^{-1}. pH 2.6. ●—550 nm. ■—440 nm.

is greater than that of the AA–CS complex. It is determined by the hydrophobic activity of the BSA macromolecules.

Effect of Triton X-100 on the Absorbance of BPB–BSA Mixtures

Figure 9 shows that with an increase of Triton X-100 concentration in the reaction system, the absorbance of BPB–BSA mixtures decreases at 604 nm and increases at 440 nm. As a non-ionic surfactant, Triton X-100 has very strong emulsification ability. With its addition, the hydrophobic activity center and the hydrophobic cavity of BSA are destroyed. At this point, it is more difficult for BPB to be combined with BSA through the hydrophobic interaction and the binding ability of BPB decreases gradually.[20] The blue color of the reaction system turns into yellow and becomes deeper. This also indicates that the bathochromic shift of the absorption spectra of BPB–BSA reaction system results mainly from the hydrophobic interaction between BPB and BSA.

Effect of Temperature on the Absorption Spectra of BPB–BSA Mixtures

The BPB–BSA complex is very stable. When the temperature of the reaction system is under 75°C, the blue color of the solution of BPB–BSA mixtures is not influenced. When the temperature of the reaction system

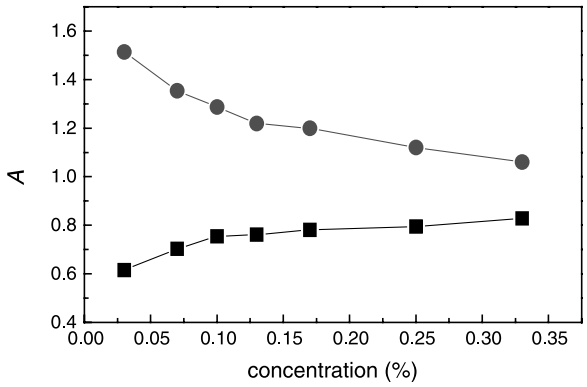

Figure 9. Effect of Triton X-100 on the absorbance of BPB–BSA mixtures BPB concentration constant at 5×10^{-5} mol.L^{-1}, BSA concentration constant at 3.33×10^{-6} mol.L^{-1}. pH 2.6. ●—604 nm. ■—440 nm.

reaches beyond 75°C, the blue fades gradually and turns into yellow entirely at 85°C. When cooling the reaction system slowly to 75°C, the yellow solution begins to turn back to blue. This experimental phenomenon results from the denaturation of the BSA molecules at high temperature and renaturation at low temperature. During the course of denaturation, the steric structure of the BSA molecules is destroyed and could be characterized by a significant loss in helical content[21] and a rise in the hydrodynamic axial ratio[22] and thus, lacks the cavity with a strong hydrophobic microenvironment, leading to a decrease of binding ability between BPB and BSA. Accordingly, the blue color of the reaction system turns yellow. During the course of renaturation, the beta-sheets, formed when albumin was heated,[23] turns into the alpha-helical structure and the cavity with strong hydrophobic microenvironment forms again, resulting in a combination of more BPB and BSA molecules. The yellow color of the reaction system turns back into blue. Because BPB–BSA complex can be destroyed only at very high temperature, the combination of BPB–BSA complex should be very stable. Also, this indicates that there should be a very strong hydrophobic interaction besides the electrostatic interaction between BPB and BSA.

CONCLUSIONS

This paper analyzes absorption spectra of BPB at different pH values and that of BPB–BSA mixtures at different BSA concentrations, discusses the rules of absorption spectra changes of BPB–BSA and BPB–Chitosan mixtures at different BSA and Chitosan concentrations, and investigates the effect of different experimental conditions on the absorption spectra of BPB–BSA mixtures. After the study of the interaction mechanism between BPB and BSA, some reasonable explanations are proposed. We can conclude from the experimental results that the bathochromic shift of the absorption spectra of the BPB–BSA reaction system results mainly from a hydrophobic interaction between BPB and BSA, while an electrostatic interaction is necessary for the formation of the BPB–BSA complex.

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