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Fluorescence and Circular Dichroism Studies on the Interaction of Bromocresol Purple with Bovine Serum Albumin

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The mechanism of interaction of bromocresol purple (BCP) with bovine serum albumin (BSA) has been investigated by spectrofluorometric and circular dichroism methods. Association constant for the BCP–BSA system showed that the interaction is non-covalent in nature and that there occurs only a partial occupation of a binding site. Binding studies in the presence of hydrophobic probe, 8-anilino-1-naphthalene sulphonic acid, sodium salt (ANS) showed that there is hydrophobic interaction between BCP and ANS and they may share common sites in BSA. Stern-Volmer analysis of fluorescence quenching data showed that the fraction of fluorophore (protein) accessible to the quencher (BCP), was close to unity, indicating thereby that both tryptophan residues of BSA are involved in dye-protein interaction. The rate constant for quenching, greater than 10^{10} $M^{-1}s^{-1}$, indicated that the dye binding site is in close proximity to tryptophan residue of BSA. Thermodynamic parameters, obtained from data at different temperatures, showed that the binding of BCP to BSA involves hydrophobic bonds predominantly. Fluorescence intensity data in the presence of additives showed that hydrophobic interaction plays a prominent role. Significant decrease in concentration of free dye was observed for BCP in presence of paracetamol. Circular dichroism studies revealed the change in helicity of BSA, due to binding of BCP to BSA.

Key words: bovine serum albumin, bromocresol purple, binding sites, interaction studies

Serum albumins are the most abundant proteins in plasma. *In vivo*, albumins act as carrier molecules and bind to a large variety of endogenous and exogenous ligands, both natural and synthetic [1]. Owing to their nearly indiscriminate binding capacity, a plethora of ligand studies have led to the discovery of protein structure-function relationships. The binding to albumin has been characterized by the strength of the binding and the number of binding sites for the ligands as well as by the location of binding regions for the ligands in albumin.

Dyes are being increasingly used for clinical and medicinal purposes [2–4]. The discovery that some dyes would stain certain tissues and not others led to the idea, that dyes might be found, that would selectively stain, combine with and destroy pathogenic organisms without causing appreciable harm to the host. As a result some azo, thiazine, triphenyl methane and acridine dyes came in to use as antiseptic trypanocides

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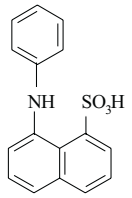
and for other medicinal purposes [5–7]. It is also known, that certain dyes *viz.,* fluorescein and Rose Bengal are preferentially adsorbed by cancerous cells.

Dye-protein interaction governs the duration and intensity of pharmacological effect [8,9]. The use of dyes for protein determination is well established [10–12]. However, other parameters, such as mode of interaction, association constant and number of binding sites are important, when dyes are used as drugs. Critical literature survey reveals, that attempts have not been made so far to investigate the mechanism of interaction of BCP with BSA. This is the first attempt made to investigate the mode of interaction of BCP with BSA.

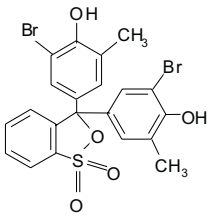
EXPERIMENTAL

Materials: Serum albumin bovine (BSA, Fraction V, approximately 99%; protease free and essentially γ-globulin free) and 8-anilino-1-naphthalene sulphonic acid sodium salt, $C_{16}NSO_3H_{13}Na$ (ANS) were obtained from Sigma Chemical Company, St Louis, USA. AnalaR grade bromocresol purple, $C_{21}Br_2O_5SH_{16}$ (BCP) was used in the study. All other materials were of analytical reagent grade. The solutions of BCP and BSA were prepared in 0.1 M phosphate buffer of pH 7.4 containing 0.15 M NaCl. BSA solution was prepared based on molecular weight of 65,000. Stock solutions of 500 μM of BCP and 250 μM of BSA were prepared.

ANS



Bromocresol Purple



Fluorescence studies: Fluorescence measurements were performed on a Hitachi spectrofluorimeter Model F-2000 equipped with a 150W Xenon lamp and slit width of 10 nm. A 1.00 cm quartz cell was used for these studies. On the basis of preliminary experiments, BSA concentration was kept fixed at 10 μM and BCP concentration was varied from 1 to 50 μM. Fluorescence spectra were recorded at room temperature (27°C) in the range 300–500 nm upon excitation at 296 nm. The absorbance of dye-protein mixtures in the concentration range employed for the experiment did not exceed 0.05 at the excitation wavelength in order to avoid inner filter effect.

Binding studies in the presence of hydrophobic probe: Fluorescence studies were also carried out on the interaction of BCP with BSA in presence of an hydrophobic probe, ANS. In the first set of experiments, the interaction of BCP and ANS with BSA was studied under identical conditions. BSA concentration was kept fixed at 10 μM and BCP/ANS concentration was varied (5–30 μM). Fluorescence spectra were recorded in the range of 300–500 nm upon excitation at 296 nm. In the second set of experiments, BSA–BCP interaction was studied in the presence of 5, 10, 15, 20, and 30 μM of ANS. BSA and dye concentrations were kept fixed at 10 μM. Fluorescence spectra were recorded in the range 390–550 nm upon excitation at 370 nm.

Effect of additives: The fluorescence spectra of BCP-BSA were recorded in presence and absence of various additives *viz.*, starch, magnesium-stearate, dextrose, urea and gum-acacia at 344 nm upon excitation at 296 nm. The concentration of BSA and BCP was fixed at 10 μM and 20 μM, respectively and that of each additive was maintained at 20 μM.

Thermodynamics of drug-protein interaction: Thermodynamic parameters for the binding of BCP to BSA were determined by carrying out the binding studies at four different temperatures, 14°, 20°, 26° and 35°C by fluorometric method.

Effect of paracetamol on drug-protein interaction: BSA–BCP binding was also studied in presence and absence of paracetamol using fluorescence spectroscopy. Emission spectra were recorded in the range of 300–500 nm.

Surface tension measurements: Surface tension of BCP solution (0.8%) prepared in phosphate buffer of pH 7.4 containing 0.15 M NaCl at 29°C was determined by drop weight and drop number method using a stalagmometer.

Circular dichroism (CD) studies: CD measurements were made on a JASCO-810 spectropolarimeter using a 1.00 cm cell at 0.2 nm intervals, with 3 scans averaged for each CD spectrum in the range of 200–300 nm. CD measurements of BSA in the presence and absence of BCP were made. A stock solution of 0.1 μM BSA was prepared in 0.01 M phosphate buffer having 0.15 M NaCl. The BSA to BCP concentration was varied (1:1 and 1:5) and the CD spectrum was recorded.

RESULTS AND DISCUSSION

The fluorescence spectra of BSA were recorded in the presence of increasing amounts of BCP. The emission spectra of dye are shown in Fig. 1. It was observed that the interaction of BCP with serum albumin did not result in any noticeable change in λ_{max} of tryptophan fluorescence in albumin. However, the BCP was observed to quench the fluorescence of albumin. The fraction of dye bound, θ, was determined according to Weber and Young [13], and Maruyama *et al.* [14] using the following equation

$$\theta = F_o - F/F_o \quad (1)$$

where, F and F_o denote the fluorescence intensities of protein in a solution with a given concentration of dye and without dye, respectively. The θ represents the fraction of sites on the protein occupied by dye molecule. Fluorescence data was

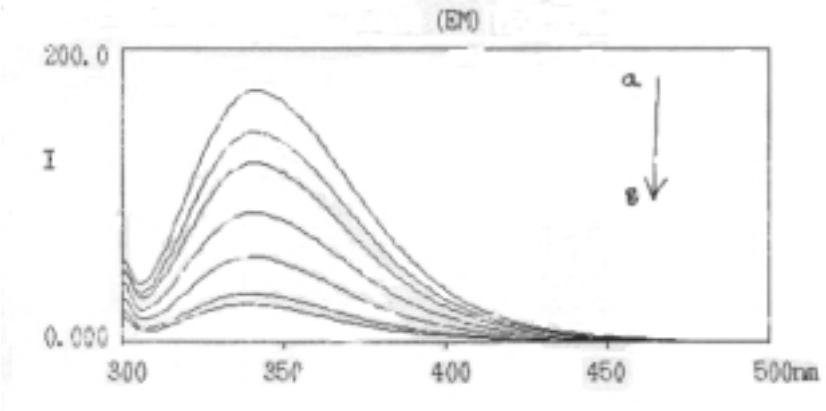


Figure 1. Fluorescence spectra of BSA (10 μM) in the presence of BCP (a – 0, b – 1, c – 5, d – 10, e – 20, f – 30 and g – 40 μM).

analyzed using the method described by Ward [15]. It has been shown that for equivalent and independent binding sites

$$\frac{1}{(1-\theta)K} = \frac{[Dt]}{\theta} - n[P_T] \quad (2)$$

where K is the association constant for dye-protein interaction, n is the number of binding sites, [Dt] is the total dye concentration and $[P_T]$ is the total protein concentration. The $1/1-\theta$ *versus* $[Dt]/\theta$ plot for the dye is shown in Fig. 2. The values of K and n, obtained from the slope and intercept of such plots, are found to be 2.75×10^5 $M^{-1}$and 0.24. Since the data fits equation 2 in all cases, it may be concluded that under the conditions of the experiment, all the binding sites are equivalent and independent. Standard free energy change, ΔG_0 was obtained from the relationship, $\Delta G_0 = -2.303$ RT log K and was found to be –31.19 $KJmol^{-1}$.

Parachor, which is a measure of molar volume of BCP, was calculated from the atomic parachor and other structural features [16]. The value was found to be 1069.5 $(Nm^{-1})^{1/4}m^3$.

Stern-Volmer analysis: Fluorescence intensity data were also analyzed according to Stern-Volmer law,

$$F_o/F = 1 + K_q[Q] \quad (3)$$

by plotting F_o/F *versus* [Q], where F_o and F are the steady state fluorescence intensities at 344 nm in the absence and presence of quencher (BCP) respectively and [Q] is the total dye concentration. The Stern-Volmer plot (Fig. 3) showed positive deviation from straight line, suggesting the presence of a static component in the

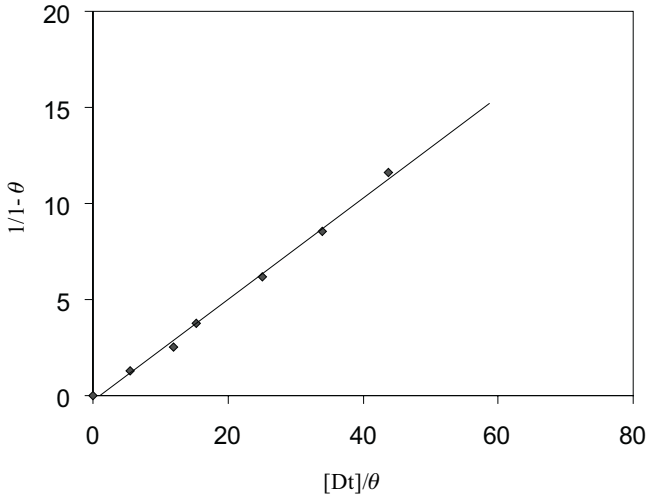


Figure 2. $1/1-\theta$ *versus* $[Dt]/\theta$ plot for the binding of BCP to BSA.

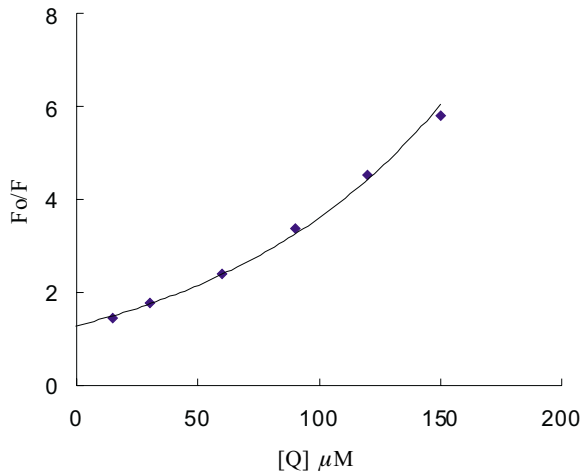


Figure 3. Stern-Volmer plot of F_o/F *versus* [Q] for the binding of BCP.

quenching mechanism [17]. A modified form of Stern-Volmer equation [17] that describes quenching data when both dynamic and static quenching are operative is

$$F_o/F = 1 + K_q [Q] \exp V [Q] \quad (4)$$

where K_q is the collisional quenching constant or Stern-Volmer quenching constant and V is the static quenching constant. The value of V was obtained from (4) by plotting

$$\left(\frac{F_o}{F \exp V [Q]}\right) - 1 \quad \textit{versus} \ [Q]$$

for varying V until a linear plot was obtained. The K_q was then obtained from the slope of above plot through origin (Fig. 4). The values of V and K_q so obtained were found to be 3.15×10^3 and 0.37×10^4 M^{-1} respectively.

According to Eftink and Ghiron [17], upward curvature in the Stern-Volmer plot indicates that both tryptophan residues of BSA are exposed to quencher and the quenching constant of each tryptophan residue is nearly identical, while downward curvature indicates buried tryptophan residues. At a concentration of 90 μM dye about 70.38% of the fluorescence intensity was quenched. The maximum quenching was obtained by extrapolating a plot of $(F_o - F)/F_o$ *versus* 1/[Q] to 1/[Q] = 0 corresponding to infinite concentration of dye. We have observed that at infinite concentration of dye, fluorescence quenching was more than 90% in each case. This again shows that both the tryptophan residues of BSA are accessible to dye molecule. For a bimolecular quenching process, $K_q = k_q \tau_o$, where τ_o is the lifetime in the absence of quencher and k_q is the rate constant for quenching. As τ_o value for tryptophan fluorescence in proteins is known to be equal to 10^{-9} s, [18] the rate constant, k_q,

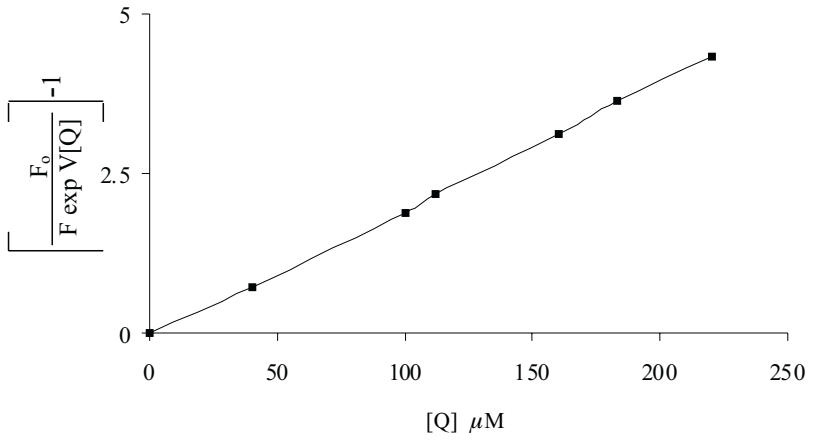


Figure 4. Plot of $\left(\frac{F_o}{FexpV\,[Q]}\right)-1$ *versus* [Q] for BCP.

would be of the order of 10^{13} M^{-1} s^{-1}. The value of k_q depends on the probability of a collision between fluorophore and quencher. This probability depends on their rate of diffusion (D), their size and concentration. It can be shown that

$$k_q = 4\pi \text{ aDNa x}10^{-3} \quad (5)$$

where D is the sum of the diffusion coefficients of quencher and fluorophore, a is the sum of molecular radii and Na is the Avogadro's number. The upper limit of k_q expected for a diffusion-controlled bimolecular process is 10^{10} $M^{-1}s^{-1}$. The high magnitude of k_q in the present study (10^{13} $M^{-1}s^{-1}$) can probably be attributed to a specific long-range interaction between dye molecule and tryptophan residues on protein. Thus the process of energy transfer occurs by intermolecular interaction forces between tryptophan and dye and this is possible only when the dye-binding site is in close proximity to tryptophan residues of BSA.

Binding studies in the presence of ANS: Both BCP and ANS quench the fluorescence of BSA, but the magnitude of decrease in fluorescence intensity was much larger for ANS as compared to that for BCP. ANS bound to BSA calculated from the fraction of occupied sites (θ) was found to be 79%, where as the BCP bound to BSA was only 30.17% under identical conditions. It is known that the excitation at 296 nm involves fluorescence due only to tryptophan residues of BSA. Further, under conditions of the experiment tryptophan residue of BSA are partially exposed and their accessibility depends upon the nature of molecules of the interacting species [19]. It thus appears that whereas, tryptophan residues are fully accessible to the hydrophobic probe, ANS, they are only partially accessible to the dye, which has partially hydrophilic character.

In another set of experiments, BSA–BCP interaction was studied in the presence and absence of 5, 10, 15, 20, 25 and 30 μM of ANS. It was found that for a given concentration of BCP, fluorescence intensity slightly decreases when ANS is added to BSA–BCP system. It is known [20] that ANS shows greatly increased fluorescence as a result of hydrophobic interactions with proteins and other macromolecules due to transfer of probe from an aqueous to non-polar environment. Small decrease in fluorescence intensity of BSA–BCP system on the addition of ANS illustrates that ANS competes with dye for the hydrophobic site on the surface of BSA. In other words, ANS inhibits the binding of BCP by displacing the dye from its binding site. This shows that ANS and BCP may share common site in BSA.

Thermodynamics of BCP–BSA interaction: Association constant, K was found to decrease with increase in temperature since,

$$\text{Log } K = -\Delta H_o/2.303RT + \Delta S_o/2.303R \quad (6)$$

Log K *versus* 1/T plot enabled the determination of standard enthalpy change, ΔH_o and standard entropy change, ΔS_o for the binding process. The ΔH_o, ΔS_o and ΔG_o values were found to be +1.91 KJ mol^{-1}, +98.99 JK^{-1} mol^{-1} and –27.78 $KJmol^{-1}$, respectively. The low positive ΔH_o value indicates less-dominant hydrogen bond formation between the substrates while positive ΔS_o value indicates predominant hydrophobic character of binding (Large hydrophobic species such as proteins avoid the water molecules in aqueous solution as far as possible by associating into micelle-like structures with the non-polar portions in contact in the inner regions of the micelles, the polar ends facing the water molecules. This attraction of hydrophobic species, resulting from their unwelcome reception in water, is known as hydrophobic bonding or better, hydrophobic interaction). The positive ΔH_o and ΔS_o values observed in this case indicate less dominant hydrogen bond formation and predominant hydrophobic character of binding between BSA and BCP [21]. These results, together with spectral changes in the fluorescence emission spectra of BSA, induced by BCP, suggest that the interaction may take place in subdomain IA and IIA since these have been proposed to bind drugs and other hydrophobic materials [19].

Surface activity: Surface tension data was expressed as surface activity, which in turn expressed as surface pressure, π, which is the difference between surface tension of the solvent and that of the solution. Thus, surface activity values, expressed as surface pressure, $\pi = \lambda_{solvent} - \lambda_{soln,}$ was found to be 9.9×10^{-3} Nm^{-1}. Reduction in surface tension of solvent or increase in surface pressure is attributed to hydrophobicity of the dye molecule. However, the order of π value suggests that the BCP has hydrophobic character.

Effect of additives and paracetamol on BCP–BSA interaction: To understand further the nature of interaction involved, fluorescence spectra of BSA–BCP were recorded in the presence of each of 10 μM urea, gum-acacia, dextrose, magnesium-stearate and starch solution. It was observed that increase in dye-albumin intensity in presence of urea, dextrose and magnesium-stearate indicate that they inhibit the BCP–BSA binding whereas decrease in intensity of dye-albumin intensity

in presence of starch and gum-acacia shows that they induce the BCP–BSA binding. Thus, urea, gum acacia, dextrose, magnesium stearate and starch alter the micro-environment of the binding sites by affecting the iceberg structure of water [22]. The results of analysis are given in Table 1.

Table 1. Fluorescence intensity of BCP–BSA system in the presence of additives.

Sample	Fluorescence intensity at 344 nm
Only BSA	54.82
BSA + BCP	19.46
BSA + BCP + Urea	24.61
BSA + BCP + Magnesium-stearate	24.91
BSA + BCP + Dextrose	27.30
BSA + BCP + Starch	16.94
BSA + BCP + Gum-acacia	16.76

The simultaneous administration of two or more strongly bound dyes can compete with one another for the binding sites on albumin and so result in displacement interactions [23,24]. Although paracetamol is not strongly bound at therapeutic concentrations it can still affect the protein binding behavior of other dyes either by blocking an active site or by causing conformational changes in the protein molecule. Thus, the presence of paracetamol can significantly alter the pharmacological response of other dyes by altering the concentration of free dye in plasma. It was observed that the association constant (K) increased from 2.75×10^5 to 2.93×10^5 M^{-1}in the presence of paracetamol. This means that the availability of free dye in plasma gets decreased in the presence of paracetamol. Once the interference of the paracetamol in the protein binding of dye is established one can anticipate the need for an adjustment in dosage in the presence of paracetamol. The relative ability of paracetamol to interfere in the binding of other dyes can be quantitatively determined from $K_{ratio,}$ the ratio of association constant in the presence and absence of paracetamol. K_{ratio} can, therefore, be a guide to the modified design of dosage forms in the presence of paracetamol.

Circular dichroism method: The BSA to BCP concentration was varied (1:1 and 1:5) and the CD spectrum was recorded. The binding of BCP was also confirmed by CD spectra (Fig. 5). As expected, the α-helices of protein show a strong double minimum at 220 nm and 209 nm [25]. The intensities of this double minimum reflect the amount of helicity of BSA and indicate that BSA contains more than 50% of α-helical structure. Upon addition of the BCP to BSA (1:1) the extent of α-helicity of the BSA decreases and hence the intensity of double minimum is also reduced. On further addition of BCP to BSA (5:1), the intensity of the double minimum increased, a similar observation made in the case of binding of Schiff base to BSA [25]. This is indicative of increase in helicity when the dye is completely bound to BSA.

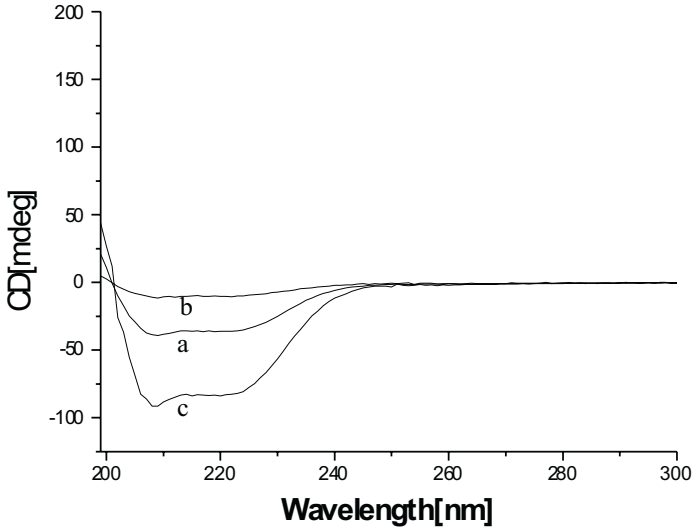


Figure 5. Circular dichroism spectra in the 200–300 nm range; (a) BSA 0.1 μM; [BSA]:BCP = 1:1 (b); and 1:5 (c).

Acknowledgments

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