RESEARCH PAPER

Accepted 20 May 1998
Received 10 February 1998
Indian J. Pharm. Sci., 1998, 60 (5) pp. 297-301

Spectrophotometric Studies on the Interaction of Bovine Serum Albumin with Triphenylmethane Dyes

NEELAM SEEDHER AND ANJU SAINI
Department of Chemistry
Panjab University, Chandigarh

Spectrophotometric studies on the interaction of three triphenylmethane dyes, bromophenol blue, coomassie brilliant blue R250 and thymol blue with bovine serum albumin have been reported. Data has been analyzed for spectral shifts, extent of combination, composition of complexes, association constants and number of binding sites. Spectral shifts indicated that dyes interact with cationic groups on the protein molecule. Association constants varied between $2.7x10^4$ to $2.3x10^5$, consistent with non-covalent interactions. The number of binding sites varied between 2 and 3 for different dyes. All basic amino acid resdues in protein were not accessible to the dye at pH 2.5.

DYES are being increasingly used for clinical and medicinal purposes[1,2]. 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, triphenylmethane and acridine dyes came into use as antiseptics, tripanocides and for other medicinal purposes[3-5]. Dye-protein interaction is important since most of the administered drugs are extensively and reversibly bound to serum albumin and drug is transported mainly as a complex with protein. Drug-protein interaction governs the duration and intensity of pharmacological effect[6,7].

The use of dyes for protein estimation is well established[8-11] However, other parameters such as mode of interaction, extent of combination, composition of complexes, association constants and number of binding sites are important, when dyes are used as drugs. Present paper reports such studies using three triphenylmethane dyes and bovine serum albumin.

EXPERIMENTAL

Three triphenylmethane dyes bromophenol blue, BPB (Budapest, Hungry), coomassie brilliant blue R250, CBB (Loba Chemie), thymol blue, TB (ACS reagent, Himedia) and protein, bovine serum albumin, BSA (Sigma) were used as such without further purification. All solutions were prepared in 0.05 M glycine buffer of appropriate pH value. Due to limited solubility of thymol blue, its solutions were prepared in buffer containing 20% methanol by volume. Dye and protein stock solutions of following concentrations were prepared : BPB-$1x10^{-4}$M, CBB-2. $5x10^{-4}$M, TB-$4x10^{-4}$M and BSA-$7.246x10^{-5}$M.

Ultraviolet absorption spectra were determined on Hitachi 330 UV/Visible spectrophotometer. Absorption spectra of all the dyes were determined at three different pH values; 2.5, 7 and 9 in the case of BPB and CBB and 7,8 and 9 in the case of TB. For dye-protein complexation, 2 ml of the dye solution was mixed with increasing amounts of BSA and required amount of buffer so as to keep total volume 10 ml in each case. Thus dye concentration was kept fixed at 20 μM for BPB, 50 μM for CBB and 80 μM for TB, whereas, protein concentration was varied from 0 to 60 μM. pH was kept at 2.5 for all dyes except TB. Since TB is not sufficiently soluble at pH 2.5, its solution was prepared in a buffer of pH 7. Ultraviolet absorption spectra (400-800 nm) of dye+protein (sample) against buffer (reference) was recorded in each case.

Table 1 : Wavelength (λmax) of Major Peaks for Various Dyes at Different pH values

Dye	λmax			
	pH 2.5	pH 7	pH 8	pH 9
BPB	430	590	-	590
CBB	630	555	-	555
TB	-	430	430	590

λmax values obtained from ultraviolet absorption spectra of the dyes in 0.05 M glycine buffer.

Table 2 : Molar Absorptivities of Free (t_F) and bound (t_B) dyes

Dye	pH	λ*max	ϵ_F	ϵ_B	$\epsilon_B - \epsilon_F$
BPB	2.5	605	1000	31500	30500
CBB	2.5	605	13400	23400	10000
TB	7.0	435	12125	10000	-2125

*λmax values refer to the protein-bound dye. Subscripts F and B refer to free and bound dye, respectively.

Table 3 : K, n and r_{MAX} values for Different dyes

Dye	pH	K	n	r_{max}
BPB	2.5	2.30×10^5	2.30	9.95
CBB	2.5	7.7×10^4	3.08	10.05
TB	7.0	2.7×10^4	2.50	6.66

Association constant K and number of binding sites n obtained through Scatchard analysis[14]. r_{max} obtained from intercepts of the linear 1/r versus molar ratio of protein to dye plots.

RESULTS AND DISCUSSION

From the absorption spectra of dyes at different pH values, the wavelengths (λmax) of major peaks are recorded in Table 1. The shift in λmax was seen to be in accordance with the pH range of the indicator. pH 2.5 was selected for dye-protein interaction on the basis of preliminary experiments. Due to limited solubility of thymol blue in the acidic range, its interaction with protein was studied at pH 7. Molar absorptivities of the dyes are recorded in Table 2.

Addition of protein to the dyes resulted in significant spectral shifts, both in the wavelength for maximum absorption and intensity of absorption. Some representative spectra are shown in fig. 1. A shift in wavelength indicates that either a complex is formed between the protein and the dye which absorbs at a different wavelength or the dye

is in different, probably hydrophobic, environment in the presence of protein. It is observed that the major peak shifts towards a lower wavelength; 630 to 605 nm in the case of CBB and towards a significantly higher wavelength, 430 to 605 nm in the case of BPB.

Sulphonephthalein dyes exist in three distinct forms[12] as shown in the below given equilibrium.

$$H_2d \rightleftharpoons HD^- \rightleftharpoons D^{2-}$$

With increase in pH, H^+ ions are used up and the equilibrium shifts to the right. This results in shift in λmax of the dye as shown in Table 1. On comparing the spectra of dye-protein complexes to the spectra of dyes at different pH values, it was found that the direction of shift on the addition of protein is same as that produced by increasing pH. It appears that negatively charged dye anions interact with basic amino and guanidinium groups on the protein molecule resulting in decrease in concentration of dye anions thereby shifting the equilibrium to the right. It has been observed by Klotz[13] that electrostatic interactions must be supplemented by Vander Waal's interaction so that a stable bond may be formed. Further, it was of interest to find that addition of a cationic surfactant produced similar spectral shifts (cf. Fig. 2). This observation indicates thereby that the dye may perhaps be interacting with cationic sites situated in hydrophobic regions of the protein molecule.

For thymol blue no significant shift in λmax has been observed. This may be due to the fact that the dye-protein interaction was studied at pH 7 where the protein carries a net negative charge. Since both the dye and the protein are anionic at this pH, absence of electrostatic interactions appears to be responsible for no shift in λmax. Moreover, the presence of alcohol might have shifted the original peak of the dye to longer wavelength, since this dye was dissolved in 20% alcohol.

The absorbance of the dye also changed significantly with increase in the concentration of BSA added (Fig. 1). The observed shift in molar absorptivity and the dependence of the spectrum on the concentration of protein again shows the existence of inter-molecular dye-protein interactions. Absorbance increased with increase in the concentration of BSA added in the cases of BPB and CBB and decreased in the case of thymol blue. Molar absorptivity of bound dyes were determined under conditions of high protein/dye ratios, where almost all dye molecules are bound to protein. For this purpose absorbance of the dye at infinite concentration of BSA (A_∞) was obtained form the intercepts of the double reciprocal plots; 1/A versus 1/C, where A and C are absorbance of dye at λmax and concentration of BSA, respectively. Molar absorptivities of free and bound dyes are recorded in Table 2.

Extent of Combination

The free concentration of a drug in plasma, on which the pharmacological activity of the drug depends, is determined by the association constant K, the number of binding sites n, and the total concentration of the drug and the binding protein. These parameters for dye-protein interaction have been determined in the following manner[10].

In solutions of low protein concentration which contain both bound and unbound dye, in a cell of 1 cm path length, the absorbance may be expressed as

$$A = \epsilon_F D_F + \epsilon_B D_B \text{ -------(1)}$$

where ϵ_F and ϵ_B are molar absorptivities and D_F and D_B are molar concentrations of free (F) and protein bound (B) dye, respectively. The dye conservation equation, $D_T = D_F + D_B$, allows substitutions of $D_B = D_T - D_F$ into equation (1). Thus

$$D_F = (\epsilon_F D_T - A) / \epsilon_B - \epsilon_F \text{ ------- (2)}$$

knowledge of D_F allows calculation of r, the average number of dye molecules bound per protein molecule.

$$r = D_B/P_T = (D_T - D_F)/P_T$$

where P_T represents total molar protein concentration.

In the present investigation, r/D_F was plotted against r (scatchard plot[14]) for the determination of association constant K, and the number of binding sites (n). K and n values for different dyes are recorded in Table 3. Association constants varied between 2.7×10^4 and 2.3×10^5 for different dyes. It is observed that the K value for BPB is about 3 times higher than that of CBB and about 8.5 times higher than that of TB. This difference in K values can be attributed not only to the difference in affinity of sites but also to the dissimilar specificity and structural requirements for different dyes. The values are consistent with non-covalent

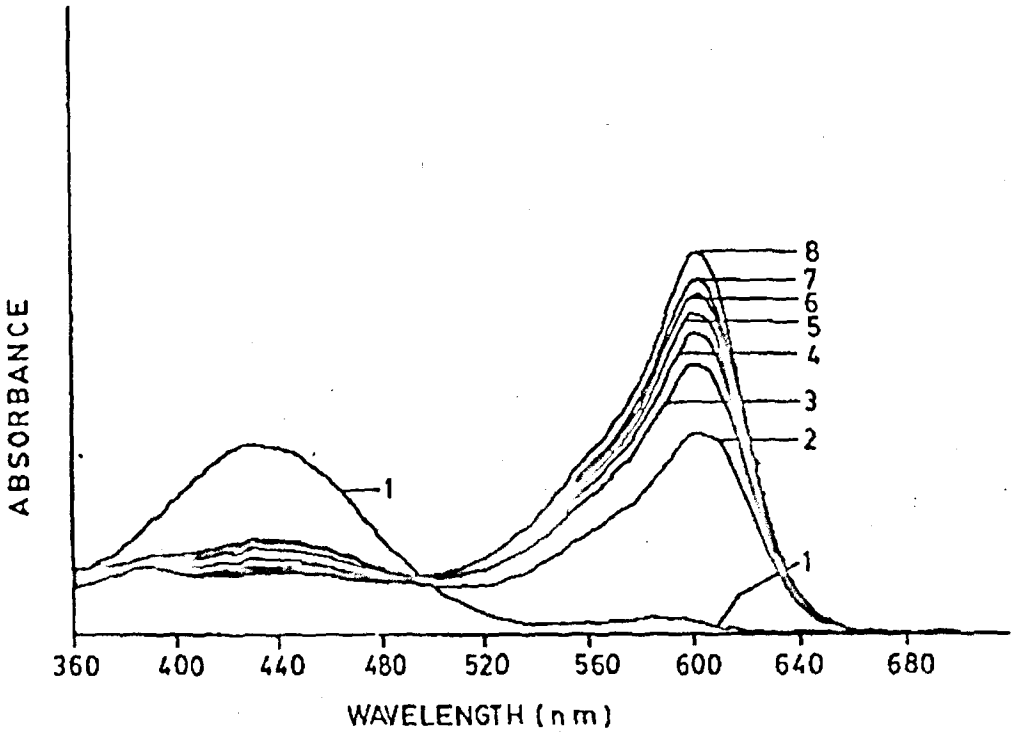

Fig. 1 Absorption spectra of BPB (2 x 10^{-5}M) in the presence of increasing concentrations of BSA (μM) (1) 0.00, (2) 1.89, (3) 3.79, (4) 7.57, (5) 15.15, (6) 22.72, (7) 45.45, (8) 60.60

interactions. Dye-protein interaction appears to involve both electrostatic and hydrophobic interactions as has been observed by other workers[15,16] as well. The number of binding sites varied between 2 and 3 for different dyes. Since binding experiments were carried out at molar protein/dye ratios varying from 0.75 to 3.0, binding of dye is expected to occur only to strongest class of binding sites. The number of strong sites is seen to be much less than the number of arginine (26) and lysine (60) residues in BSA; indicating thereby that a structural feature other than a basic amino acid side chain may be involved. Congdon et al.[10] while studying binding of CBB G250 with proteins at low pH (below 1) have also reported three strong binding sites with BSA.

Composition of Complexes

It is observed that r continues to increase with increase in the moles of dye added per mole of protein. It was possible, however, to determine maximum value of r (r_{max}) at infinite concentration of dye from the intercepts of the linear plots of 1/r versus molar ratio of protein to dye. The r_{max} values for different dyes, given in Table 3, are seen to vary from 6.66 to 10.05. The r_{max} should represent the total number of binding sites, since the extrapolation is done at high molar dye/protein ratio. The values are still much less than the total number of basic amino acid residues in the protein. Some investigators[9,10] while studying dye-protein interaction as a method of protein analysis, have reported a good correlation between the total number of binding sites and number of basic amino acid side chains in the protein at low pH (less than 1). Since the pH at which present investigation was carried out is 2.5, it appears that some of the reactive amino acid residues are buried inside the protein and are accessible to dye molecules only at much lower pH where protein is present in partially unfolded state..Chial and Splittgerber[9] have also observed a significant decrease in the number of binding sites at higher pH.

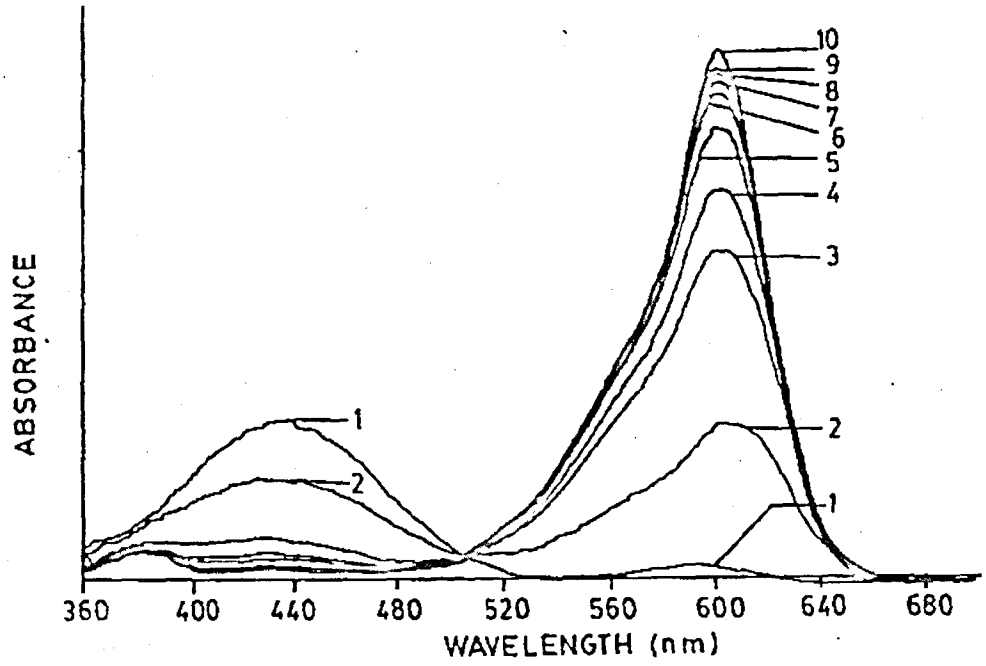

Fig. 2 Absorption spectra of BPB (2 x 10^{-5}M) in the presence of increasing concentrations of CPB (x10^{-4}M) (1) 0.00, (2) 0.50, (3) 1.00 (4) 1.50, (5) 2.00, (6) 3.00, (7) 4.00, (8) 5.00, (9) 6.00, (10) 7.00

REFERENCES

1. Pitschke, M., Fels, A., Schmidt, B., Heiliger, L., Kuckert, E. and Riesner, D., **Colloid Polymer Sci.,** 1995, 273, 740.
2. Przybojewska, B., **Mutat. Res.** 1996, 367, 93.
3. Konielzny, L., Piekarska, B., Roterman, I. and Rybarska, J., **J. Physiol. Pharmacol.,** 1993, 44, 233.
4. Prithipal, S., Michael, A., Prophul, J.P., Pawanjit, S. and Wayne, S., **Indian J. Chem.,** 1993, 32, 44.
5. Weder, H.J. and Bickel, M.H., **J. Pharm. Sci.,** 1970. 59, 1563.
6. Maruthamuthu, M. and Kishore, S., **Proc. Indian Acad. Sci.,** 1987, 99, 273.
7. Lin, J.H., Cocchetto, D.M. and Duggan, D.E., **Clinical Pharmacokinetics,** 1987, 12, 402.
8. Bradford, M.M., **Analytical Biochemistry,** 1976, 72, 248.
9. Chial, H.J. and Spittgerber, A.G., **Analytical Biochemistry,** 1993, 213, 362.
10. Congdon, R.W., Muth, G.W. and Spittgerber, A.G., **Analytical Biochemistry,** 1993, 213, 407.
11. Bonate, P.L. **Analytical Biochemistry,** 1988, 175, 300.
12. Byrne, R.H. **Anal. Chem.,** 1987, 59, 1479.
13. Klotz, I.M., **J. Am. Chem. Soc.,** 1946. 68, 2299.
14. Scatchard, G., **Ann. N.Y. Acad. Sci.,** 1949, 51, 660.
15. Waheed, A.A. and Gupta, P.D., **Analytical Biochemistry,** 1994, 233, 249.
16. Tal, M., Silberstein, A. and Nusser, E., **J. Biol. Chem.,** 1985, 260, 9976.