

Spectral investigations of the interactions of eosine blue and rose bengal with bovine serum albumin

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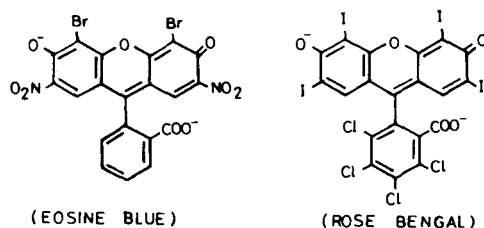
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Received 5 August 1991; revised 25 August 1992; accepted 19 November 1992

The spectrophotometric investigation of the binding of two structurally resembling halogen-substituted fluorescein dyes, eosine blue (EB) and rose bengal (RB), to bovine serum albumin (BSA) at pH 7.4 has resulted in the observance of a single complex for EB but two types of complexes, one absorbing at 540 nm (comp-1) and the other at 555 nm (comp-2), for RB. The analysis of the dependence of binding constant (K) on [BSA] has revealed the binding of EB by ligand-facilitated self-association mechanism. In the case of RB, K has been found to be independent of [BSA] for comp-1 but has showed inverse dependence on [BSA] for comp-2, the mechanisms of binding for both these complexes being different from that of EB-BSA interaction. Moreover, it has been noticed that surface bound comp-1 of RB-BSA is stronger than the interior bound comp-2, i.e., $K_{\text{comp-1}} > K_{\text{comp-2}}$, which could be explained by the bulkiness of EB in preventing its effective interaction with the hydrophobic interior of BSA. Thus contrasting binding mechanisms in the interaction of structurally similar dyes can be monitored.

Serum albumin, a blood protein, is known to bind reversibly a variety of small molecules such as fatty acids, acidic drugs¹ and host of other organic anions²⁻⁵ and thereby transports them in the human circulatory system. 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^{6,7} for the ligands in albumin. Several ligands have found application^{8,9} as templates for probing the binding regions in albumin and the binding regions for the ligands can be same⁹ or different¹⁰ even in the case of ligands possessing similar structural resemblances. That is, factors such as chemical structure and molecular dimensions of the ligand have been found to influence the binding phenomenon as reported in many investigations^{11,12}. Our earlier studies¹³ also revealed that structurally resembling ligand naproxen and 1-anilino-8-naphthalene sulphonate have dissimilar binding characteristics in binding to BSA.

The present study deals with the binding of two structurally resembling dyes namely eosine blue (EB)-dibromodinitrofluorescein and rose bengal (RB)-tetraiodotetrachlorofluorescein to BSA. Both of them have common fluorescein moiety but RB is highly halogenated among the two. Moreover, they are clinically significant dyes and particularly RB has found application in detecting the organic anions in liver plasma¹⁴. Our aim is to look for the binding patterns of the dyes with BSA at the molecular level from the spectrophotometric investiga-



Materials and Methods

BSA (fatty acid free) was obtained from Sigma Chemical Co., USA. Pure samples of dyes eosine blue (Loba Chemie, India) and rose bengal (RB) (BDH, England) were used as received. All other chemical were of analytical grade. The concentration of BSA was checked by using molar extinction coefficient of $\epsilon_{279} = 4.4 \times 10^4 \text{ cm}^{-1} \text{ M}^{-1}$. All the spectrophotometric experiments were carried out at pH 7.4 in 0.05 M of Na_2HPO_4 - KH_2PO_4 buffer at 25°C.

In the spectrophotometric experiments, binding of the dyes has been quantified by absorbance change method¹⁵. A set of typical spectrophotometric experiments of EB binding to BSA were carried out as follows: To a constant $[\text{EB}] = 40.0 \times 10^{-6} \text{ M}$, varying $[\text{BSA}]$ of 2.0 - $40.0 \times 10^{-6} \text{ M}$ were added. The mixtures were maintained at 25°C for 30 min with constant shaking. The absorbances of EB and EB-BSA mixtures were measured at 523 nm using a Carl-Zeiss Specord UV-vis spectrophotometer. Similarly experiments were carried out by keeping constant $[\text{EB}]$ of 16.0, 24.0, 32.0 and $48.0 \times 10^{-6} \text{ M}$

and varying the [BSA] respectively for each of these [EB].

Experiments involving the binding of RB were also carried out by keeping constant [RB] and varying [BSA]. Constant [RB] of 8.0, 12.0, 16.0, 20.0 and 24.0×10^{-6} M were employed and varying [BSA] were added respectively for each of these [RB]. The absorbance measurements for the mixtures were monitored at 540 nm and 555 nm.

Analysis of the data

The binding of EB and RB can be quantified by the absorbance change method as described elsewhere¹⁵. That is, if A , A_1 and A_2 denote the absorbances of either EB or RB respectively in the absence, partially bound and completely bound condition to BSA, then the fraction of the dye bound to BSA is

$$\frac{C_B}{C_T} = \frac{A - A_1}{A - A_2} \quad \dots (1)$$

where C_B and C_T are respectively bound and total [dye]. From Eq. 1, C_B and hence r , the number of moles of dye bound per mole of BSA can be calculated. The free [dye], $C_F = C_T - C_B$.

Binding data obtained were analyzed by the Scatchard method¹⁶, which is applicable for the binding of ligand to independent, equivalent sites in protein.

$$\frac{r}{C_F} = nK - rK \quad \dots (2)$$

where K is the binding constant of dye-BSA complex and ' n ' is the number of binding sites for the dye in BSA. From the plot of r/C_F versus r , K and n have been obtained.

Results and Discussion

Spectral characteristics and the binding of EB and RB

The change in absorbance of dyes, on addition of BSA has formed the basis for studying the binding (noncovalent reversible complex formation) by spectrophotometric method. Free EB and RB exhibit absorption maximum respectively at 523 nm and 540 nm. BSA does not absorb in this wavelength region and so its interference would not prevail upon the bound forms of the dyes. This allows convenient monitoring of the dye binding in the visible region.

Figure 1A shows the spectral changes for EB with addition of increasing [BSA] to a constant [EB] = 40.0×10^{-6} M. It can be seen that the absorbance of EB has been found to decrease and is

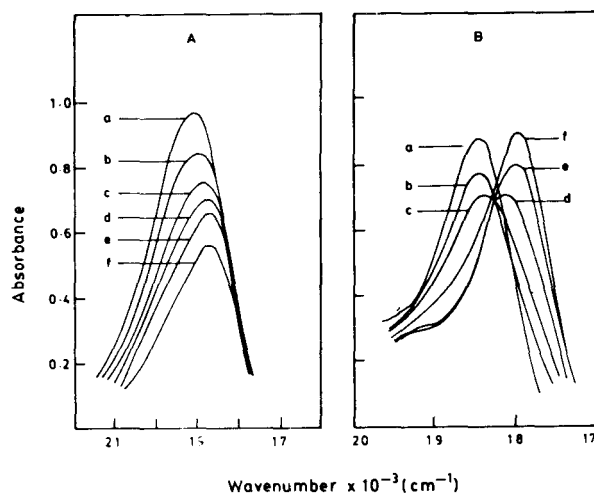


Fig. 1—A ... Visible absorption spectra of EB-BSA complex (a) 40.0×10^{-6} M of EB alone. (b)–(f) in presence of increasing [BSA]. [BSA] $\times 10^6$ (M) are (b) 2.0; (c) 4.0; (d) 6.0; (e) 10.0 and (f) 40.0. B ... Visible absorption spectra of RB-BSA complexes. (a) 20.0×10^{-6} M of RB alone. (b)–(f) in presence of increasing [BSA]. [BSA] $\times 10^6$ (M) are (b) 0.5; (c) 1.0; (d) 2.0; (e) 4.0 and (f) 20.0.

also accompanied by gradual red-shift in $\lambda_{\max}$ of the spectra on addition of increasing [BSA]. The decrement in absorbance represents the change from free EB to bound form of EB. Moreover, the changes in absorbance (here the decrement) throughout the wavelengths of spectral investigation have been observed to occur only in a single direction (Fig. 1A). It has been established in the studies of trypan blue-BSA interaction by Lang and Lasser¹⁷ that there would be only a single complex if the absorbance changes of the bound form of the dye occur in a single direction. Consequently, for the EB-BSA interaction also, the formation of only a single complex can be inferred. However, the gradual red-shift from 523 nm for free EB to 535 nm for the completely bound EB (spectral curve-f of Fig. 1A) indicates that the binding environment around the chromophore is becoming more nonpolar with increase in [BSA]. Further, the red-shift in $\lambda_{\max}$ also invariably suggests the displacement of monomer-polymer equilibria of BSA during EB binding as noticed elsewhere¹⁸.

For the case of RB-BSA interaction, the spectral changes for [RB] of 20.0×10^{-6} M with increasing [BSA] are shown in Fig. 1B. Unlike for the EB-BSA interaction, the spectral changes for RB binding occur in more than one direction. It can be noticed that there is decrease in absorbance of RB at 540 nm, and a simultaneous increase in absorbance resulting in emergence of a new peak with $\lambda_{\max}$ at 555 nm (spectral curve-f of Fig. 1B). Therefore two countable qualitative spectral changes have been moni-

tored. In order to verify whether these two spectral changes correspond to a single complex or more than one complex, the changes in absorbance i.e., ΔA ($\Delta A = A_1 - A$) both at 540 nm and 555 nm have been subjected to the analysis as described by Lang and Lasser¹⁷.

Figure 2 shows the changes in ΔA_{540} and ΔA_{555} values at various $[RB]/[BSA]$ (i.e., D/P) ratios. If there has been only a single complex formation, then the ratio of absorbance changes at these two wavelengths i.e., $\Delta A_{555}/\Delta A_{540}$ should be the same for all the D/P ratios employed. But, it is evident from Fig. 2 that the ratio $\Delta A_{555}/\Delta A_{540}$ varies with D/P ratio. This means that the said ratio depends on $[BSA]$ which implies that the bound species of RB corresponding to 540 nm is found to exhibit distinct spectral characteristics compared to the species corresponding to 555 nm. Therefore, the two countable spectral changes at these two wavelengths suggest the presence of two spectroscopically distinguishable complexes one at 540 nm (comp-1) and the other at 555 nm (comp-2) for RB-BSA interaction. In addition, the absence of isosbestic point in the spectra (Fig. 1B) gives support to the presence of more than one RB-BSA complex in equilibrium with free RB in solution.

Mechanisms for the binding of EB and RB

The binding data obtained for the EB and RB have been analyzed by the method of Scatchard¹⁶. The Scatchard plots for the EB-BSA interactions at four different $[BSA]$ are shown in Fig. 3. The linear

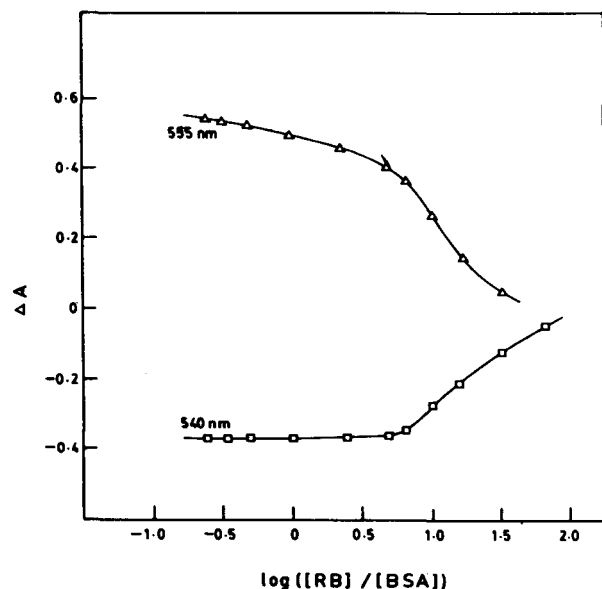


Fig. 2—Variation of absorbance difference ΔA with $\log ([RB]/[BSA])$ at pH 7.4 for RB-BSA complexes at two different wavelengths. $[RB] = 20.0 \times 10^{-6} M$ and $[BSA] \times 10^6 (M) = 0.30$ to 80.0

nature of plots has indicated the existence of independent and equivalent sites for EB in BSA. The total binding constants (nK) and the number of binding sites (n) obtained from the Scatchard plots are shown in Table 1. The strength of EB-BSA interaction is found to be dependent on $[BSA]$ as evident from the enhancement of the binding constant with increase in $[BSA]$ (Table 1). A plot of K versus $[BSA]$ clearly indicates such a said behaviour. So far, only very occasional reports of K increasing with $[protein]$ has been noted in earlier studies and this be-

Table 1—Parameters for the binding of EB and RB to BSA (pH 7.4; temp. 25°C)

Dye	$[BSA] \times 10^6 (M)$	$nK \times 10^{-5} (M^{-1})$	n	$K \times 10^{-5} (M^{-1})$
EB	4.0	9.7	5.8	1.67
	10.0	14.9	4.1	3.63
	16.0	17.6	3.0	5.86
	20.0	17.5	2.6	6.73
RB	0.6	75.2	11.6	6.48
	1.2	75.2	11.6	6.48
	2.0	69.2	10.1	6.85
RB (comp-2)	2.0	22.4	6.0	3.73
	8.0	10.2	4.4	2.31
	20.0	6.2	3.3	1.87

(a) nK —Total binding constant.

(b) K —binding constant.

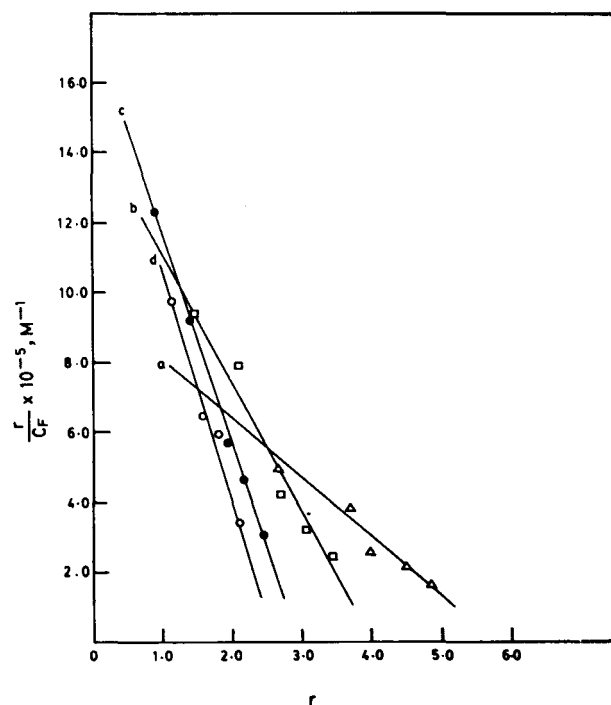
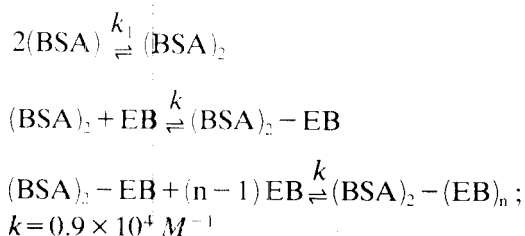


Fig. 3—Scatchard plots for the EB-BSA interactions at various $[BSA]$. $[BSA] \times 10^6 (M)$ are (a) 4.0; (b) 10.0; (c) 16.0 and (d) 20.0.

haviour is exemplified in the case of oxytocin-neurophysin II interaction¹⁹. Taking inferences from the detailed analysis of protein self-association mechanisms by Na and Timasheff²⁰, it has been possible to invoke the operation of ligand-facilitated self-association mechanism for EB-BSA interaction.

The binding of the ligand to the polymeric forms of a macromolecule has been defined as the self-association mechanism²⁰. When [BSA] is increased, self-association of BSA would be occurring so as to cause the observed increase in K for EB-BSA interaction. When [BSA] is decreased, the self-association of BSA would be diminished leading to the observed decrease in K value. That is, EB is found to show preferential affinity towards self-associated BSA. Further, self-association would be nil at [BSA] = 0, and the binding constant at this instance is called intrinsic binding constant (k). The k value of $0.9 \times 10^4 M^{-1}$ has been determined for EB-BSA interaction and it would be unaffected by [BSA]. That is, indefinite self-association occurring at very high [BSA] would lead only to the observance of large K values, with k remaining constant. Therefore, it is suggested that the self-association mechanism alone can explain the observed increase in K with [BSA].

From the aforesaid discussions, it would be possible to depict such ligand-facilitated self-association by Scheme 1.



Scheme 1

In the Scheme 1 only dimerization and not indefinite self-association is considered. Further in the absence of EB, a weak self-association of BSA (i.e., very low k_1 value) has been assumed. That is self-association of BSA is found to be facilitated by the presence of EB. Moreover, as number of sites n for EB in BSA, is found to decrease as [BSA] is increased (Table 1), the possibility of the masking of some of the binding sites due to the self-association of BSA cannot be discounted. The cause of the red-shift in λ_{max} of EB with increasing [BSA] (Fig. 1A) can also now be understood as the self-association of BSA would provide greater nonpolar environment for EB than the monomer of BSA.

Considering the RB-BSA interaction, the binding data for both comp-1 and comp-2 have been ana-

lyzed by Scatchard method¹⁶. It is evident from the linear nature of Scatchard plots at three different [BSA] (Figs. not shown) that the sites are equivalent for both the complexes of RB-BSA. From Table 1, both K and n have been found to be independent of [BSA] for comp-1 while K and n show inverse dependence with [BSA] for comp-2. Further, it can be also noticed (Table 1) that K of $6.5 \times 10^5 M^{-1}$ for comp-1 of RB is almost twice in magnitude to that of $3.7 \times 10^5 M^{-1}$ determined at [BSA] of $2.0 \times 10^{-6} M$ for comp-2. This observation seems to be contrary to expectations, since the presence of comp-2 in more nonpolar environment than comp-1 (evident from spectra in Fig. 1B) would likely to facilitate greater short-range hydrophobic interactions for the formation of the former than the latter. This argument is reasonable and justified because many earlier studies^{21,22} have established hydrophobicity to be a key factor for the strength of ligand-albumin complexes. If the primary consideration is hydrophobicity alone, then, $K_{\text{comp-2}}$ would have been greater than $K_{\text{comp-1}}$. But a reverse trend has been noticed in this study.

To seek explanation for this, molecular dimensions (qualitatively) of RB have to be considered. RB has eight halogen substituents including four iodine atoms with high covalent radius. This makes RB, very bulky in character. As nonpolar ligands have been envisaged to bind in the hydrophobic clefts present in the interior of albumin¹², the difficulty in accommodating the bulky RB suitably inside the clefts should have made RB unable to interact effectively at the binding site. This would be the probable cause for the weakening of comp-2 of RB leading to the observance of low K for comp-2 than comp-1 even though the binding sites of comp-2 happen to be in more nonpolar environment.

The presence of binding sites for comp-2 in the interior of BSA gains further validity from the observance of the inverse dependence of K on [BSA] for comp-2 (Table 1). BSA contains large number of aromatic amino acid residues and as proposed by Scholtan²³, the mutual interactions of the side chains of the aromatic amino acid residues would be larger at high [BSA] than at low [BSA]. The greater the mutual interactions of the side chains at high [BSA] the more would be the masking of the binding sites especially when the sites happen to be in the interior of BSA, thereby leading to the weakening of comp-2 formation. This has led to the decrease of K with increase in [BSA]. Further, as n has decreased from 6.0 to 3.3, with increase in [BSA] (Table 1), the masking has also resulted in the loss of some of the sites for comp-2 and indeed suggests the presence

of sites inside the clefts. A similar inference has been made for the observance of inverse dependence of K and n on [BSA] for the binding of benzodiazepine derivatives to BSA²³. On the other hand the invariance of $K_{\text{comp-1}}$ with [BSA], shows that the formation of comp-1 has been neither affected by the bulky nature of RB nor by the masking of the sites at high [BSA]. Hence the binding sites for comp-1 would be distinct from those of comp-2 and also may not involve the hydrophobic clefts.

Thus the present study has been able to distinguish the binding patterns of the dyes EB and RB with BSA. The EB-BSA interaction is found to involve ligand-facilitated self-association mechanism. In contrast, RB forms two complexes, namely comp-1 (surface bound) and comp-2 (interior bound) with BSA. Interestingly, the bulky nature of RB in precluding its effective interaction with the interior of BSA has lessened the affinity of comp-2 than comp-1. Consequently, the presence of the common fluorescein moiety alone in the dyes does not entail the operation of common binding mechanism as other factors have also to be taken into account.

References

- 1 Peters T, *The plasma proteins*, Vol. 1, edited by F W Putnam (Academic Press, New York), 1975, p. 133.
- 2 Rodkey F L, *Arch Biochem Biophys*, 94 (1961) 38.
- 3 Muckle J, *J Chromatogr*, 146 (1978) 77.
- 4 O'Reilly R A, *J Clin Invest*, 48 (1969) 193.
- 5 Muller W E & Wollert U, *Pharmacol*, 12 (1979) 59.
- 6 Kragh-Hansen U, *Biochem J*, 209 (1983) 135.
- 7 Fehske K J, Muller W E & Wollert U, *Biochem Pharmacol*, 30 (1981) 687.
- 8 Sudlow G, Birkett D J & Wade D N, *Mol Pharmacol*, 11 (1975) 824.
- 9 Maruyama K, Nishigori H & Iwatsuru M, *Chem Pharm Bull*, 33 (1985) 5002.
- 10 Birkett D J, Myers S P & Sudlow G, *Mol Pharmacol*, 13 (1977) 987.
- 11 Sjöholm I & Sjödin T, *Biochem Pharmacol*, 21 (1972) 3041.
- 12 Wanwimolruk S, Birkett D J & Brooks P M, *Mol Pharmacol*, 24 (1983) 458.
- 13 Meenakshi M & Kishore S, *colloid Polym Sci*, 268 (1990) 938.
- 14 Yachi K, Sugiyama Y, Sawada Y, Iga T, Ikeda Y, Toda G & Hanano M, *Biochim Biophys Acta*, 978 (1989) 1.
- 15 Peacocke A R & Skerrett J N H, *Trans Faraday Soc*, 52 (1956) 261.
- 16 Scatchard G, *Ann N Y Acad Sci*, 51 (1949) 660.
- 17 Lang J H & Lasser E C, *Biochemistry*, 6 (1967) 2403.
- 18 Sheppard S E & Geddes A L, *J chem Phys*, 13 (1945) 63.
- 19 Nicolas P, Camier M & Cohen P, *J biol Chem*, 251 (1976) 3965.
- 20 Na G C & Timasheff S N, *Methods in enzymology*, Vol. 117, edited by C H W Hirs & S N Timasheff (Academic Press, New York) 1985, p. 496.
- 21 Urien S, D'Athis P & Tillement J P, *Biochem Pharmacol*, 33 (1984) 2283.
- 22 Brown N A, Wilson A G E & Bridges J W, *Biochem Pharmacol*, 31 (1982) 4019.
- 23 Muller W E & Wollert U, *Biochem Pharmacol*, 25 (1976) 147.