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Heterotropic binding of alclofenac and dansylsarcosine to bovine serum albumin

MEENAKSHI MARUTHAMUTHU* and S KISHORE
Department of Physical Chemistry, University of Madras, Guindy Campus, Madras 600 025, India

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Abstract. The binding data for the interaction of alclofenac (AF) and dansylsarcosine (DS) to bovine serum albumin (BSA) have respectively yielded nonlinear Scatchard plots. The plots have been subjected to Rosenthal's method of analysis and thus the ligands have been found to possess two different kinds of sites in BSA. The binding capacities of these sites have been evaluated. The fluorescence competition studies have revealed that the binding of DS to BSA is noncompetitively inhibited by AF. Therefore, the presence of distinct binding sites for AF and DS in BSA could be inferred. The fluorescence quenching studies have also been able to demonstrate this aforesaid fact. The analysis of the quenching data by the modified Stern–Volmer plot has indicated that both the tryptophan (Trp) residues of BSA are accessible to DS for the quenching in absence of AF, but only one of them is accessible in presence of AF. This has led to suggest that the binding site of DS has been in the vicinity of loop 3–4, involving Trp-134 and Trp-212. The binding of AF at a distinct site from that of DS has exerted heterotropic interactions at the DS binding site and thereby inhibited the binding of DS to BSA.

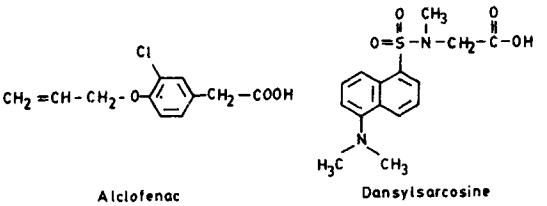
Keywords. Bovine serum albumin; alclofenac; dansylsarcosine; tryptophan residue; quenching; heterotropic interaction.

1. Introduction

Albumin is a plasma protein, which binds various small molecules in the blood (Peters 1976). Some of the earlier studies (Sudlow *et al* 1975; Birkett *et al* 1980) have established the presence of discrete binding sites for the ligands in serum albumin. These studies which have led to reveal the existence of distinct sites for the ligands are based on the fluorescent probe techniques. Dansylsarcosine (DS) is a hydrophobic fluorescent probe and has found application (Wanwimolruk *et al* 1983) for characterizing the binding sites of several arylpropionic antiinflammatory drugs in human serum albumin (HSA). In the fluorescent probe studies, the probe can be either displaced by competitive mechanism (Aarons *et al* 1979) or noncompetitive, allosteric mechanism (Nielson *et al* 1977). These factors led to employ DS as a fluorescent probe for the present investigation.

The drug alclofenac (AF) is a new nonsteroidal antiinflammatory drug. Because of its pharmacological importance, the determination of binding capacity for AF in

* For correspondence



Alclofenac Dansylsarcosine

bovine serum albumin (BSA) acquires significance. Further, the fluorescence probe displacement studies involving DS as the probe and AF as the ligand have been made in order to know the mechanism of the displacement. As BSA exhibits fluorescence, quenching studies have been carried out to find out whether these two ligands have common or dissimilar sites. Consequently, from the present investigation, it would be possible to monitor the location of the sites for the ligands and the resulting DS–AF displacement/interaction occurring during the binding.

2. Experimental

Bovine serum albumin (BSA) (fatty acid-free) and dansylsarcosine (DS) have been purchased from the Sigma Chemical Co., USA. A pure sample of alclofenac (AF) was kindly provided by the Mitsubishi Yuka Pharmaceutical Co. Ltd., Japan. Other reagents used were of analytical grade. The solutions of BSA were prepared based on the molecular weight of 67,000 and the concentrations were checked by measuring the absorbance at 279 nm using $A^{1\%}_{1cm} = 6{\cdot}6$ (Janatova *et al* 1968).

The binding of AF and DS to BSA was investigated respectively by spectrophotometry and spectrophotofluorometry. All the experiments were carried out at pH 7·4 using 0·05 M Na_2HPO_4–KH_2PO_4 buffer.

2.1 *Spectrophotometric method for the binding of AF*

The binding of AF to BSA was carried out at constant [BSA] of 10·0 μM and varying [AF] from 4·0 to 50·0 μM. Spectral measurements were recorded in a Specord UV-Vis spectrophotometer. The spectra were obtained by mixing AF and BSA solutions and keeping them in the sample compartment, the reference being the BSA solution of identical concentration as in the sample cuvette. From the absorbance measurements at 235 nm, the difference absorbance (ΔA_{235}) for the complex were evaluated as proposed elsewhere (Oberfelder and Lee 1985). In order to determine the bound concentration, B, another set of experiments was conducted wherein, a finite [AF] of 10·0 μM is titrated with 4·0 to 16·0 μM of BSA. From ΔA_{235} at different [BSA], the increment in the ligand molar extinction coefficient, $\Delta\varepsilon$[$\Delta\varepsilon = \varepsilon$(bound) $- \varepsilon$(free)] due to binding was evaluated. From ΔA_{235} and $\Delta\varepsilon$, the bound concentration, B, was determined as described earlier (Oberfelder and Lee 1985).

2.2 *Fluorescence method for the binding of DS and the competition studies*

The fluorescence measurements were made using an Aminco Bowman spectrophotofluorometer, which is equipped with a 150 W xenon lamp source. For the DS–BSA

studies, emission was monitored at 480 nm while exciting at the wavelength of 370 nm. In order to monitor the binding characteristics of DS–BSA, [DS] was varied from 2·0 to 30·0 μM and the experiments were carried out at constant [BSA] of 12·0 μM. The absorbances of DS–BSA solutions in the concentration range employed for the experiments do not exceed 0·05 at the excitation wavelength in order to avoid the inner filter effect. The quantification of the binding of DS to BSA was carried out as described elsewhere (Sudlow *et al* 1973).

The competition experiments, involving AF as the inhibitor and DS as the probe were also carried out by fluorometry. In a typical experiment, to a constant [BSA] of 12·0 μm, [DS] were varied from 2·0 to 30·0 μM and the fluorescence was monitored at 480 nm to study the binding of probe DS in absence of AF. Then by employing identical sets of constant [BSA] and varying [DS], the experiments were carried out in presence of AF to study the effect of AF on probe binding. The four different [AF] employed were 3·0, 6·0, 9·0 and 12·0 μM. The results of the competition experiments were analyzed by the Lineweaver–Burk method (Ferhst 1977).

2.3 *Quenching of intrinsic fluorescence of BSA*

The quenching of the tryptophan (Trp) fluorescence of BSA by the addition of DS or AF as quencher was measured at 350 nm with the excitation wavelength at 296 nm. The concentrations of BSA-quencher solutions were chosen such that their absorbances were below 0·1, to minimize the inner filter effect. The experiments were carried out at constant [BSA] of 10·0 μM and the [AF] or [DS] was varied from 2·0 to 30·0 μM to study the effect of quencher on Trp fluorescence.

2.4 *Analysis of the data*

The binding data obtained for the binding of AF and DS were analyzed by the method of Scatchard 1949). For the biphasic, nonlinear type of Scatchard plots, the analytical treatment proposed recently by Zierler (1989) has been followed. Accordingly, for the two different kinds (classes) of binding sites,

$$B = B_1 + B_2 = \frac{n_1 A_T K_1 F}{1 + K_1 F} + \frac{n_2 A_T K_2 F}{1 + K_2 F}, \tag{1}$$

in which, the total concentration of the bound ligand, B, is the sum of the concentrations of two species (kinds) of bound ligand. n_1 and n_2 are the number of binding sites or binding capacity per acceptor of protein in each kind respectively and they are characterized respectively by specific K_1 and K_2 values. A_T and F are respectively total acceptor (BSA) concentration and free concentration of the ligand.

A plot of B/F *vs* B has been made and the nonlinear Scatchard plot (obtained in this study) has been resolved by the method of Rosenthal 1967). By this approach, the binding capacities (n_1 and n_2) of the individual kinds of sites and the respective affinities K_1 and K_2 have been evaluated.

For the fluorescence quenching experiments, the quenching data were analyzed by the modified Stern–Volmer plot (Lehrer 1971).

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a} + \frac{1}{[Q] f_a K_q}, \tag{2}$$

where F_0 and F are the fluorescence intensities at 350 nm in the absence and in presence of the quencher respectively at concentration $[Q]$. K_q is the Stern-Volmer quenching constant and f_a, the fraction of the fluorophore accessible to the quencher. From a plot of $F_0/(F_0 - F)$ *vs* $1/[Q]$, f_a and K_q were determined.

3. Results and discussion

3.1 *Binding of AF and DS to BSA*

The spectral investigation made to study the binding of AF depends on the change in the absorption spectrum of AF on addition of BSA. Figure 1 shows the absorbance spectra for varying [AF] in the presence of constant [BSA]. The spectrum of unbound or free AF shows absorption without showing any characteristic absorption maximum (spectral curve not shown in figure) in the region of 220–260 nm, whereas the absorption spectra of AF–BSA exhibit an absorption maximum at 235 nm (figure 1). The absorbance difference (ΔA_{235}) of AF–BSA has been evaluated as carried out elsewhere (Oberfelder and Lee 1985).

The binding of DS to BSA has been investigated by fluorometry. DS exhibits very low fluorescence in buffer, but its fluorescence is enhanced on binding to BSA. This causes the emission maximum to be blue-shifted and the emission maximum for DS–BSA has been observed at 480 nm. The fluorescence of bound DS as a function of increasing [BSA] gives a plateau at relatively low [BSA] of 48·0 μM, for the range of [DS] employed, so that the fully bound condition for DS can be determined without adopting the extrapolation procedure.

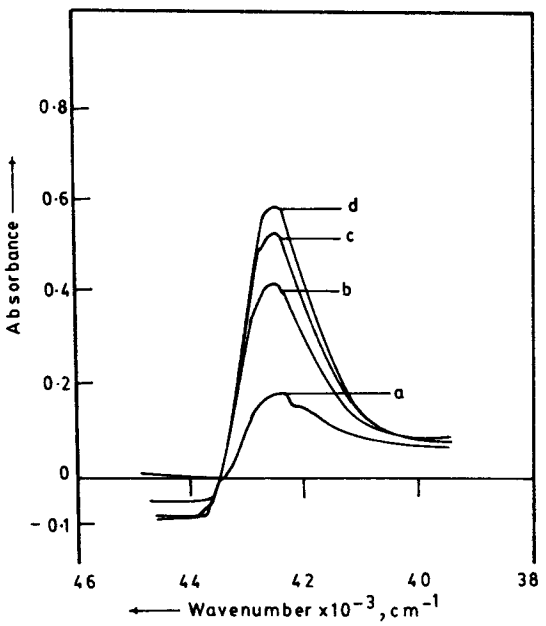


Figure 1. Absorption spectra of AF–BSA at pH 7·4. The spectra were recorded by keeping 5 μM BSA + AF in the sample cuvette and 5 μM of BSA alone in the reference cuvette. The [AF] in μM for various spectra are a:20·0 μM; b:40·0 μM; c:60·0 μM and d:80·0 μM.

The results of AF and DS binding to BSA have been analyzed by the method of Scatchard (1949). The Scatchard plots for the AF–BSA and DS–BSA interactions respectively have been nonlinear in nature (figures 2 and 3). It has been the prevalent misconception in many earlier reports that the nonlinear Scatchard plots have been resolved into two or more linear components. Also, it has been the practice to represent the linearized components as strong and weak classes of sites. But such a misconception has been obviated in the recent report of Zierler (1989). From the Zierler's analysis, the possibilities are that the nonlinear plot can arise, either because there are different classes of sites on a single acceptor (protein) or because there are different kinds of acceptor, each kind containing a specified number of sites. Therefore, both the possibilities can lead to the observance of nonlinear Scatchard plots as obtained in this study (figures 2 and 3). But the latter possibility, namely the presence of more than one kind of acceptor in BSA, does not arise because of the fact that the BSA employed in the present investigation is in the pure form. This is unlike the biological preparations wherein more than one kind of acceptor molecules can be found to exist. Hence, it can be considered that only a single kind of acceptor is present in the BSA system and the nonlinearity of the Scatchard plot is due to the presence of different classes of sites on a single acceptor.

The nonlinear, curved Scatchard plots obtained have been resolved into two straight lines. This makes it possible to fit the curve into the two-term equation (1). The graphical method of Rosenthal (1967) has been followed to evaluate the binding parameters. As illustrated in the report of Zierler (1989), the Rosenthal method involves

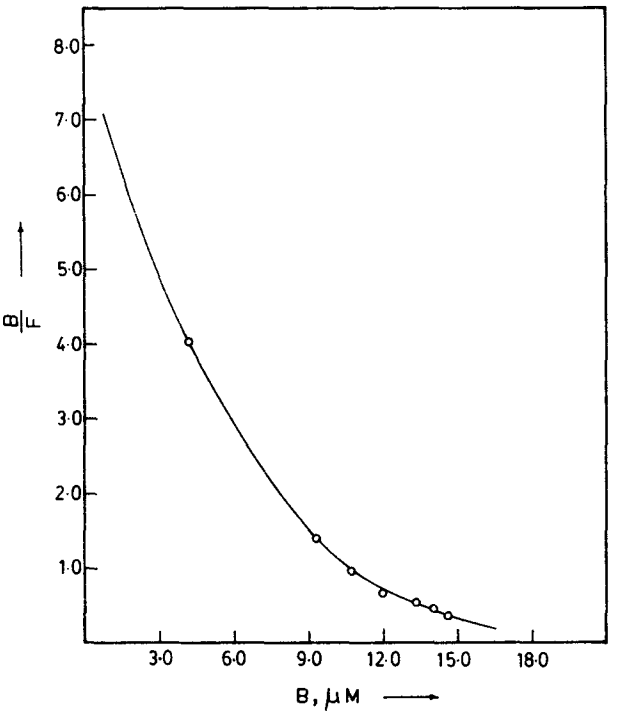


Figure 2. Nonlinear Scatchard plot for the binding of AF to BSA at pH 7·4. [BSA] = 10·0 μM and [AF] = 4·0 to 50·0 μM.

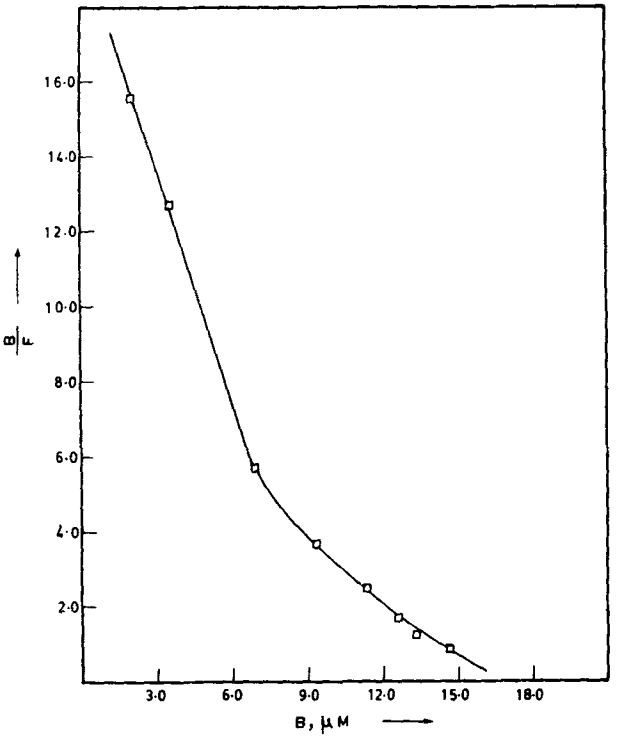


Figure 3. Nonlinear Scatchard plot for the binding of DS to BSA at pH 7·4. [DS] = 2·0 to 30·0 μM. [BSA] = 12·0 μM.

Table 1. Binding parameters for AF–BSA and DS–BSA interactions
(The parameters obtained by resolving the non-linear Scatchard plots by the method of Rosenthal)
Temp. = 25°C; pH = 7·4; [Buffer] = 0·05M.

Ligand	$K_1 \times 10^{-5}$ (M^{-1})	n_1	$K_2 \times 10^{-5}$ (M^{-1})	n_2
AF[a]	5·4	0·73	2·8	0·60
DS[b]	10·2	0·71	6·4	0·52

[a] By the method of spectrophotometry.
[b] By the method of spectrophotofluorometry.
n_1 and n_2 are the binding capacities per acceptor of BSA respectively for the two kinds of sites and they are characterized by the corresponding affinities K_1 and K_2.

the drawing of radial lines from the origin to the point of intersection on the experimental curve. In this way, the individual components of total observed bound concentration, B, have been found to add radially. The binding parameters determined are given in table 1. No attempt has been made in this study to identify the binding capacities (n_1 and n_2) and the corresponding affinities (K_1 and K_2) as belonging to

high or low affinity classes or sites. Thus the binding parameters extracted have been fitted into a binding model involving two classes of sites. However, it is recognised that the curves can be fitted into a model with more than two terms by methods, other than graphical procedure. This may lead to a different binding model and this possibility is not discounted. However, it has been considered here that the model with two kinds (classes) of sites is appropriate within the limits of Rosenthal's method of analysis.

The binding capacity values per acceptor of BSA for the interaction of AF–BSA are $n_1 = 0{\cdot}73$ and $n_2 = 0{\cdot}60$ (table 1). These values are comparable to the corresponding values of $n_1 = 0{\cdot}71$ and $n_2 = 0{\cdot}52$ (table 1) for the interaction of DS with BSA. Hence it can be inferred that both AF and DS possess similar binding capacities for the two kinds of sites respectively in BSA. The capacities have been characterized by specific K values for the ligands which are indicated respectively as K_1 and K_2, indicating affinities in table 1. Moreover, if the affinity of the binding is $< 10^4\,M^{-1}$, the binding of the drugs to albumin is known to affect the pharmacokinetics of the drugs (Jusko and Gretch 1976). In this context, as both K_1 and K_2 of AF–BSA are in the order of $10^5\,M^{-1}$, the binding of AF to albumin would influence the distribution and elimination of AF in blood plasma.

3.2 *Competition studies involving AF and DS*

The binding capacities of BSA respectively for the binding of DS and AF are similar and so it can be expected that they share common sites in BSA. Consequently, the displacement of the fluorescent probe DS from its site in BSA by AF has been monitored by fluorometry. When the studies have been carried out at four different [AF], the fluorescence of DS–BSA has been found to decrease with increase in [AF]. In the displacement studies, the probe fluorescence may respond in nearly indentical ways to both competitive as well as noncompetitive inhibition of the probe. As the method of Lineweaver–Burk (Fersht 1977) is one of the best tools to monitor the mechanism arising out of inhibition, the data obtained from the fluorescence competition studies at four different [AF] have been subjected to such analysis. Figure 4 shows the plots of $1/F$ *vs* 1/[DS] (Lineweaver-Burk plots) for the inhibition of DS by AF. The plots at differnt [AF] give a common intercept on the abscissa (i.e., the same value of $-1/K_M$ irrespective of the [inhibitor]) (figure 4). The observance of the identical value of the Michaelis constant, K_M at varying [inhibitor] is a proven inference for the operation of noncompetitive displacement of the probe. This leads to suggest that AF non-competitively displaces DS from its site in BSA. The noncompetitive inhibition is possible only if the two ligands bind at two separate sites, but displaces each other. Further, the secondary plot (inset of figure 4) gives the inhibition constant, $K_i = 5{\cdot}6\,\mu M$. This K_i cannot be considered as an insignificant one and hence the displacement of DS by AF is effective in spite of their distinct binding sites.

3.3 *Quenching of Trp fluorescence by AF and DS*

When BSA is excited at wavelengths between 295 nm and 305 nm, only Trp side chains of BSA are excited and not tyrosyls, resulting in the emission of only Trp residues (Steinhardt *et al* 1971). Consequently in the present study, the emission of

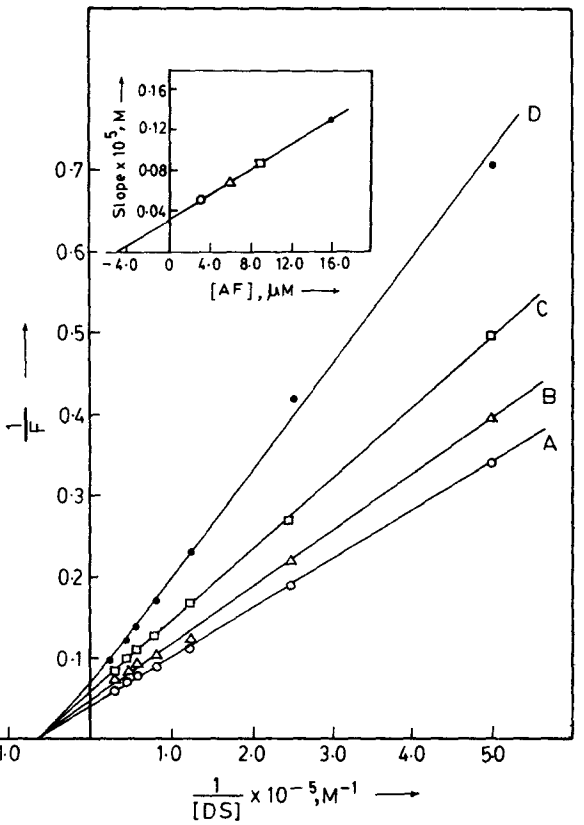


Figure. 4 Lineweaver–Burk plots showing the noncompetitive inhibition of DS binding at various [AF]. [AF] in μM are A:3·0; B:6·0; C:9·0 and D:12·0. Inset: Secondary plot made for the determination of the inhibition constant K_i.

the two Trp residues (of BSA) have been monitored at 350 nm, the excitation wavelength being 296 nm.

By addition of increasing concentration of either AF or DS, quenching of Trp fluorescence has been monitored (figure 5). It can be noticed from the figure that DS has induced the quenching of Trp fluorescence whereas AF does not cause quenching. It is a fact that the presence of binding site at or in close proximity with the Trp residues alone would facilitate the process of quenching and so the binding site of DS is at or near the Trp residues of BSA. As, AF has failed to cause quenching, it indicates the noninvolvement of Trp residues for the binding. Therefore, quenching studies are also able to demonstrate the presence of distinct binding sites for DS and AF in BSA and are able to support the inferences made from the fluorometric displacement studies.

3.4 *Quenching and the mechanism for heterotropic interaction*

The modified Stern-Volmer plots [cf. (2)] (Lehrer 1971) have been employed to determine the number of Trp residues (out of the two in BSA) accessible to the quencher, DS, both in the absence and in the presence of AF (as AF by itself does

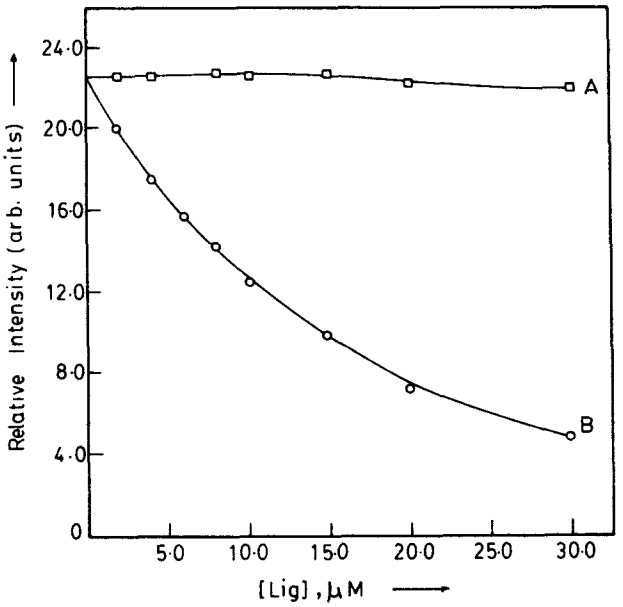


Figure 5. The effect of quenchers AF and DS on the Trp fluorescence of BSA. For monitoring the quenching the excitation and emission wavelengths were respectively 296 and 350 nm. A: AF as quencher; B: DS as quencher.

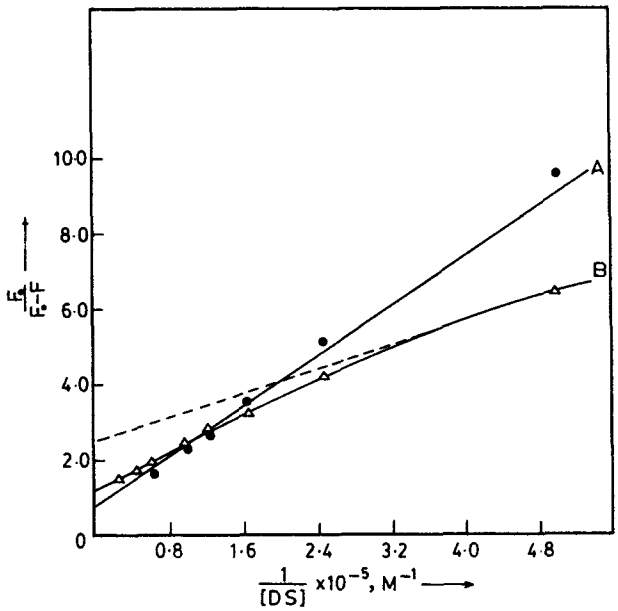


Figure 6. Modified Stern–Volmer plots, plotted according to (2) for the quenching of Trp fluorescence by DS both in the absence and in the presence of AF. A: In absence of AF; B: In presence of 15·0 μM of AF.

Table 2. Quenching parameters determined from the modified Stern–Volmer plots for the quenching of Trp fluorescence by DS in the absence and in the presence of AF.

[BSA] = 10·0 μM; [DS] = 2·0 to 30·0 μM.

AF (μM)	f_a	$K_q \times 10^{-5}$ (M^{-1})
0·0	1·0	0·60
15·0	0·4	3·12

not cause quenching). Figure 6 shows such modified Stern–Volmer plots. The plot in absence of AF (plot A of figure 6) is linear in nature. From the intercept, the fraction of the fluorophore accessible to the quencher, f_a, is determined to be unity (table 2), indicating that both the Trp residues of BSA are accessible for the binding of DS. BSA contains two Trp residues at the adjacent loops (i.e., one residue each in loop 3 and 4) (Peters 1976). As both Trp residues are in proximity to the binding site of DS, it leads to the suggestion that the binding site of DS is in the highly nonpolar region involving loop 3 and loop 4 of BSA.

For the quenching by DS in presence of AF, the modified Stern–Volmer plot is curved in nature (plot B of figure 6) and this suggests that both the Trp residues under investigation are not accessible to DS. Since the accessible fluorophore is susceptible to quenching only at low [quencher] (Lehrer and Leavis 1978), the slope at that portion (at low [DS]) of the plot has been extrapolated (cf. figure 6) and the fraction of the Trp residues accessible to DS, i.e., $f_a = 0{\cdot}4$ (table 2) has been determined. That is only one of the Trp residues is available for DS binding in the presence of AF and this can happen if either AF causes direct shielding or conformational changes, resulting in the masking of a Trp residue. But the foregoing discussion has shown that Trp residues are not part of the AF binding site. Hence the masking of a Trp residue by AF arises out of conformational changes occurring at the DS site.

The Stern-Volmer quenching constant, K_q, in the absence and in the presence of AF for the quenching of DS has been found to be respectively $0{\cdot}60 \times 10^5\ M^{-1}$ and $3{\cdot}12 \times 10^5\ M^{-1}$ (table 2). For a bimolecular quenching process, $K_q = \tau_0 k_q$, where τ_0 and k_q are respectively fluorescence life-time in the absence of quencher and rate constant for the bimolecular quenching. As $\tau_0 \simeq 10^{-9}$ s for the Trp fluorophore in proteins (Lehrer 1971), the rate constants k_q, when evaluated would be of the order of $10^{13}\ M^{-1}\,s^{-1}$ and $10^{14}\ M^{-1}\,s^{-1}$ respectively in the absence and in the presence of AF. That is, the rate constant exceeds the upper limit value of $\simeq 10^{10}\ M^{-1}\,s^{-1}$ expected for the diffusion-controlled bimolecular process. Hence, assuming that the probability factor per encounter is unity and that the diffusion coefficient follows the Stokes–Einstein equation (Rohatgi-Mukherjee 1978), the high magnitude of k_q observed in the present study can be attributed to the increase in encounter radii of Trp–DS (i.e., $R_{\text{Trp-DS}}$). This can happen only if the process of energy transfer occurs by dipole–dipole coupling interaction between Trp and DS. Hence the quenching of

Trp fluorescence occurs by dipole–dipole energy tansfer from Trp to DS and this is possible when DS is in close proximity to the Trp residues of BSA.

Therefore, the ligands AF and DS respectively bind to separate sites in BSA. Such simultaneous, independent binding of ligands like warfarin/oleate (Wosilait and Ryan 1980) and bilirubin/diazepam (Brodersen *et al* 1977) onto albumin and the resulting interaction on each other has been reported. In this investigation, the effect of heterotropic interaction exerted on the DS site (at the loop 3–4 of BSA) by the binding of AF has been discussed. This heterotropic interaction has led to the masking of one of the two Trp residues leading to the noncompetitive displacement of DS from its site. As AF is a new antiinflammatory drug, the study relating to structural changes induced by AF on a distinct site in BSA and the mechanism responsible for this are important in albumin binding studies as well as from the pharmacological aspects of drug binding.

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