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The Characterization of Two Specific Drug Binding Sites on Human Serum Albumin

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SUMMARY

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The binding of a number of fluorescent probe molecules to human serum albumin (HSA) has been studied. Small changes in the amino acid moiety of the dansylamino acids resulted in large changes in the binding of these compounds to HSA. It is suggested that electrostatic and dipolar forces play a role in the specificity and binding affinity of such compounds. Fluorescent probes which had one tight binding site were used for drug displacement studies. Changes in probe fluorescence were shown, by equilibrium dialysis and by fluorescence titrations, to be a result of competitive displacement by drugs. The pattern of displacement of probes by drugs enabled the identification of two specific drug binding sites. The relative affinity of drugs for these binding sites was measured by their ability to displace fluorescent probes specific for the sites. The method provides a rapid and simple means for detecting potential drug interactions based on competition for protein binding sites.

INTRODUCTION

A large number of low molecular weight compounds, including many drugs, bind reversibly to human serum albumin, which functions as the major transport protein in the circulation. This binding can affect the distribution of drugs within the body and their rates of metabolism and excretion. These factors in turn partly determine the time course and intensity of drug effects.

Standard separation techniques, such ultrafiltration and equilibrium dialysis, have provided some information about the number of binding sites for individual drugs on HSA[1] and their strength of binding at these sites (1). However, there has been no systematic study of the total number of strong drug binding sites on HSA or of the specificity of these sites for particular drugs.

Fluorescence spectroscopy is one of the most versatile and sensitive of the optical techniques for studying the interaction between ligands and macromolecules (2). Molecules whose fluorescence characteristics are dependent on the environment have been widely used as probes. Changes in the fluorescence characteristics associated with probe binding to macromolecules are similar to the changes observed when these molecules are transferred from a polar to a nonpolar environment (3, 4). It has been postulated that the probes bind to hydrophobic binding sites on proteins (4, 5), and drugs which displace them are assumed to have a hydrophobic area at the binding site (6–10).

The relative strength of drug binding at

[1] The abbreviations used are: HSA, human serum albumin; DNSA, 5-dimethylaminonaphthalene-1-sulfonamide; ANS, 1-anilino-8-naphthalenesulfonate; CPIB, *p*-chlorophenoxyisobutyrate.

sites on plasma albumins has been studied by the ability of the drugs to decrease the fluorescence of probes bound to the proteins (6–13). In some cases bovine serum albumin has been used, and the binding characteristics may be very different from those measured with HSA. Furthermore, the concentrations of drugs and probes relative to HSA have frequently been so high that nonspecific sites on HSA would have been saturated. Interpretation of fluorescence changes in terms of individual binding sites is very difficult in such circumstances.

A further difficulty is that probe fluorescence may respond in nearly identical ways to a number of unrelated phenomena (14). A decrease in probe fluorescence may result from competitive displacement of the probe. Alternatively, there may be a noncompetitive displacement of the probe or a decrease in the quantum yield of the bound probe as a result of a ligand-induced conformational change in the macromolecule. One approach to the problem is to compare the change in fluorescence with displacement of the probe as measured by equilibrium dialysis. We have recently shown (15) in the case of warfarin that the drug-induced fluorescence changes are due to displacement.

The binding of a number of fluorescent probes to HSA was characterized in the present study. Those probes with a single binding site or two sites with widely separated dissociation constants could be used as specific probes of HSA binding sites. Changes in the fluorescence of these bound probes were used to monitor displacement by various drugs. The nature of the changes in fluorescence was established by equilibrium dialysis and fluorescence titrations with and without drugs.

Two distinct binding sites on HSA for fluorescent probes and anionic drugs (sites I and II) were characterized by this technique. The ability of drugs to displace competitively those probes chosen as specific markers for these sites provides a measure of the specificity and relative strength of binding of drugs and of their ability to compete for binding at these sites.

MATERIALS AND METHODS

Electrophoretically pure crystalline HSA was obtained from Hoechst Australia, Ltd. The free fatty acid content, as quoted by the manufacturer, was 0.5 mEq/100 g. The dansylamino acids were obtained from Sigma Chemical Company. 1-Anilino-8-naphthalenesulfonate and 5-dimethylaminonaphthalene-1-sulfonamide were obtained from K & K Laboratories, and warfarin sodium from Endo Laboratories. All drugs were obtained as pure substances from the manufacturers. All experiments were performed using sodium phosphate buffer (0.1 M, pH 7.4, with 0.9% NaCl). The concentration of albumin was checked using the published extinction coefficient of $E^{1\%}_{1\,cm} = 5.3$ at 280 nm (6). When necessary, the drugs were dissolved initially in a small volume of 0.1 M NaOH or 0.1 M HCl. The final pH of all drug solutions was in the range of 7.2–7.6. Optical density was measured in a Unicam SP 1800 ultraviolet spectrophotometer. Fluorescence measurements were made at 22° in a Perkin-Elmer MPF-3 spectrofluorometer.

Binding of Fluorescent Probes to HSA

The binding of probes to HSA was studied by the fluorescence methods previously described (11). Within the limits of sensitivity, excitation wavelengths were chosen to ensure the least possible absorption of incident light. The optical density change at the excitation wavelengths did not exceed 0.2 during titrations. Emission wavelengths were chosen to give maximum fluorescence of the probe bound to HSA with insignificant fluorescence in buffer. Titration of 2.5 μM probe with HSA was used to determine the limiting fluorescence when this concentration of probe was completely bound. This value was then used to calculate the concentrations of free and bound probe in a reverse titration of HSA with probe. Details of this procedure have been described previously (11). Results were plotted by the method of Scatchard (16) or using the double-reciprocal method described by Hughes and Klotz (17). Results

were calculated as described previously (11). The binding parameters were calculated by linear regression when the Scatchard plot was a straight line.

When the Scatchard plot was curved, the binding parameters were obtained by iteration using the following expression:

$$r = \frac{n_1 K_{a_1} A}{1 + K_{a_1} A} + \frac{n_2 K_{a_2} A}{1 + K_{a_2} A}$$

where r is the number of moles of drug bound per mole of protein; n_1 and n_2, the number of binding sites in each class; K_{a_1} and K_{a_2}, the association constants for these sites; and A, the concentration of free ligand.

Displacement of Fluorescent Probes From HSA by Drugs

Fluorescence. Probes which had either a single binding site or two sites with widely separated dissociation constants were chosen, as these compounds were likely to be bound to specific sites on HSA. Concentrations of both albumin and probe were kept low, and albumin was present in a large molar excess to ensure that the probes with more than one site were bound mostly to their tight binding site. Drugs were added to a 1:1 molar ratio with albumin so that binding to nonspecific sites was kept to a minimum. The percentage displacement of probe is given by

$$\frac{F_1 - F_2}{F_1} \times 100$$

where F_1 is the fluorescence of probe and HSA without drug and F_2 is the fluorescence in the presence of drug. Corrections were made for quenching of probe fluorescence by inner filter effects.

Dialysis. Changes in fluorescence could result from displacement of probes from their binding sites on HSA or from a change in the quantum yield of fluorescence of the bound probe. This was investigated by comparing the fluorescence changes with the displacement of the probes as measured by equilibrium dialysis. Direct determinations of probe displacement were made in 1-ml equilibrium dialysis cells supplied by the Chemical Rubber Company. It was demonstrated that there was no significant binding of the probes to the dialysis membrane at the concentrations used. Solutions were allowed to equilibrate for 16 hr at room temperature (22°). Samples (0.2 ml) were taken from each side of the dialysis membrane and added to 1.8 ml of acetone. Precipitated salts and protein were separated by centrifugation, and the fluorescence of the supernatants was measured at the appropriate excitation and emission maxima for each probe. Total recovery of the probes was obtained after precipitation of the HSA with acetone, and the fluorescence was linear with the probe concentration over the range studied.

In the dialysis experiments the concentrations of probe and drug in the albumin compartment would be different from concentrations in the fluorescence experiments because of distribution of the free compounds into the buffer compartment. The extent of this difference will vary with different drugs and probes, depending on the degree of protein binding. It is therefore almost impossible to duplicate exactly the conditions of the fluorescence experiments.

RESULTS

Binding of Fluorescent Probes to HSA

The fluorescence of most of the probes studied was enhanced on binding to HSA, and the emission maximum was blue-shifted. The binding parameters for the probes studied are shown in Table 1, and the Scatchard plot for dansylglycine binding is illustrated in Fig. 1. The curve was fitted assuming $n_1 = 1.0$, $K_{D_1} = 1.67$ μM, $n_2 = 1.0$, and $K_{D_2} = 20$ μM, where K_{D_1} and K_{D_2} are the dissociation constants for the first and second classes of binding sites, respectively. The binding of this probe to HSA was studied similarly by Chignell (6), who found one tight site with a K_D of 2.2 μM using 10 μM HSA and dansylglycine to HSA ratios ranging from 0.25:1 to 5:1. The extra binding site found in this study may be the result of the greater probe to HSA ratio reached in the titration (0.175–18.75).

Over the concentration ranges used, all the probes listed in Table 1 bound to one or

TABLE 1

Characteristics of binding of fluorescent probes to HSA

Fluorescence titrations were performed as stated in the text. n_1 and n_2 are the number of binding sites in each class, and K_{D_1} and K_{D_2} are the dissociation constants for these sites. The excitation and emission wavelengths given are those used in the binding studies and are not necessarily the maxima (see MATERIALS AND METHODS).

Probe	Binding parameters		Excitation and emission wavelengths
Dansyl-L-glutamine	$n_1 = 0.89$,	$K_{D_1} = 50\ \mu M$	360:490
Dansyl-L-glutamic acid	$n_1 = 1.0$,	$K_{D_1} = 37\ \mu M$	370:470
	$n_2 = 1.0$,	$K_{D_2} = 100\ \mu M$	
Dansylglycine	$n_1 = 1.0$,	$K_{D_1} = 1.7\ \mu M$	370:475
	$n_2 = 1.0$,	$K_{D_2} = 20\ \mu M$	
Dansyl-L-proline	$n_1 = 0.98$,	$K_{D_1} = 7.1\ \mu M$	375:460
Dansylhydroxy-L-proline	$n_1 = 1.14$,	$K_{D_1} = 73.6\ \mu M$	370:460
Dansyl-L-α-amino-n-butyric acid	$n_1 = 1.8$,	$K_{D_1} = 13.8\ \mu M$	370:469
Dansyl-γ-amino-n-butyric acid	$n_1 = 1.1$,	$K_{D_1} = 21\ \mu M$	370:460
Dansyl-L-arginine	$n_1 = 0.66$,	$K_{D_1} = 23.6\ \mu M$	370:460
Dansylsarcosine	$n_1 = 1$,	$K_{D_1} = 6\ \mu M$	370:475
Dansyl-L-serine	$n_1 = 1.2$,	$K_{D_1} = 13.8\ \mu M$	370:460
Dansyl-L-β-phenylalanine	$n_1 = 2.4$,	$K_{D_1} = 6\ \mu M$	370:480
Dansyl-L-cysteic acid	$n_1 = 0.65$,	$K_{D_1} = 5\ \mu M$	370:460
	$n_2 = 1.1$,	$K_{D_2} = 50\ \mu M$	
DNSA	$n_1 = 0.5$,	$K_{D_1} = 5.6\ \mu M$	350:460
	$n_2 = 1.7$,	$K_{D_2} = 143\ \mu M$	
Auramine O	$n_1 = 2.1$,	$K_{D_1} = 11.9\ \mu M$	460:500
Warfarin	$n_1 = 0.9$,	$K_{D_1} = 4\ \mu M$	320:380
ANS	$n_1 = 1$,	$K_{D_1} = 1.1\ \mu M$	400:465
	$n_2 = 3$,	$K_{D_2} = 7.7\ \mu M$	

two tight binding sites on HSA. In some cases small differences in the amino acid side chain were associated with marked differences in binding. The Scatchard plot of dansyl-L-α-amino-n-butyric acid and dansyl-γ-amino-n-butyric acid indicated that the α-isomer was bound to two sites on HSA which could not be distinguished whereas the α-isomer was bound to a single site. Dansyl-L-glutamic acid was bound to two sites on HSA with different dissociation constants, whereas danysl-L-glutamine was bound to a single site. Dansyl-L-proline and dansylhydroxy-L-proline were both bound to a single site on HSA, but the hydroxyproline derivative was bound much more weakly.

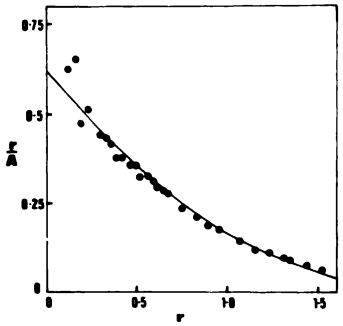

FIG. 1. *Scatchard plot of dansylglycine binding to HSA*

HSA (4 μM) was titrated with dansylglycine, and the fluorescence was measured (excitation, 370 nm; emission, 475 nm). The dansylglycine concentration was in the range of 0.7–75 μM. The concentrations of free and bound dansylglycine were calculated from the fluorescence as described in the text, where r = the number of moles of dansylglycine bound per mole of HSA and A = the micromolar concentration of free dansylglycine. The points represent experimental values, and the solid line was computed using the binding parameters $n_1 = 1.0$, $K_{D_1} = 1.7\ \mu M$, $n_2 = 1.0$, and $K_{D_2} = 20\ \mu M$.

Displacement of Fluorescent Probes from HSA by Drugs

Table 2 shows a comparison between fluorescence and dialysis as methods for measuring the displacement of probes from HSA. Despite differences in the individual

TABLE 2

Correlation between probe displacement as measured by fluorescence and equilibrium dialysis

HSA (16.4 μM) was dialyzed against equal volumes of either [^{14}C]warfarin (2 μM) or 2 μM [^{14}C]warfarin and 30 μM drug in phosphate buffer. Warfarin displacement was determined by changes in radioactivity in the protein and nonprotein compartments of the equilibrium dialysis cells. HSA (20 μM) was dialyzed either against equal volumes of probes (2 μM) or against 2 μM probe and 20 μM drug in phosphate buffer. The concentration of the probes in the protein and nonprotein compartments was measured by fluorescence as described in the text. The values obtained for ethacrynic acid were not included in the calculation of the correlation coefficient (see the text). The correlation coefficient is the coefficient of linear regression for the data obtained by the two methods.

Drug	Displacement of bound probe									
	Warfarin		DNSA		Dansyl-L-cysteic acid		Dansyl-sarcosine		Dansyl-L-proline	
	Fluorescence	Dialysis	Fluorescence	Dialysis	Fluorescence	Dialysis	Fluorescence	Dialysis	Fluorescence	Dialysis
	%	%	%	%	%	%	%	%	%	%
Phenylbutazone	44.2	39.2	54.6	40.7	33.7	40.6	0	2.3	0	2.2
Iophenoxic acid	62.6	73	69.6	44.9	59	53	0	4.8	4	7.5
Oxyphenylbutazone	28.2	23.5	41.9	28.0	24.3	29	0	2.2	0	2.8
Sulfinpyrazone	25	30	29.5	20.6	19	26.1	0	6.3	0	1.5
Sulfadimethoxine	11	11.2	22.5	24	7.4	13.5				
Sulfafurazole			11	13	5.7	3.7	0	4.9	0	0
Tolbutamide	4.1	8	9.4	0	2	0	0	4	0	0
Fursemide	18	10	17.9	18	14.5	20	5.5	5.8	4.2	0
Probenecid	0	0	0	0	0.9	0	1.6	2.9	0	5.7
Debrisoquine	0	0	3.4	0	0	4.8	0	3.7	0	0
Iopanoic acid	28.2	24.9	26.7	24.4	21.5	18.2	36.6	23.5	39	24.4
CPIB	6.9	1.8	5.9	2.3	11	12.4	25	19.7	32.2	20.4
Ethacrynic acid	13.6	4.3				0	42.3	13.9	49	24.4
Flufenamic acid	0		0	0	0	6	30	26.9	42	31.6
Correlation coefficient	0.97		0.96		0.96		0.97		0.96	

figures in Table 2, there is a high degree of correlation between displacement as measured by fluorescence and displacement as measured by dialysis (correlation coefficients, 0.96–0.97). It is concluded, therefore, that with these probes, changes in quantum yield do not contribute significantly to the fluorescence decrease. In the case of ethacrynic acid there were marked differences between the results obtained by the two techniques. Ethacrynic acid is a reactive compound and may not have been chemically stable during the 16 hr of equilibration for the dialysis experiments. Alternatively, changes in the fluorescence of bound probes may have resulted from changes in the quantum yield. As the explanation was uncertain, it was not included in the analysis of these correlation coefficients.

Figure 2 shows the effects of phenylbutazone and iophenoxic acid on the binding of DNSA to HSA as measured by fluorescence. Figure 3 shows similarly the effects of *p*-chlorophenoxyisobutyrate, ethacrynic acid, and iopanoic acid on the binding of dansylsarcosine. The quantum yield of fluorescence of these probes bound to HSA was assumed to be the same in the presence and absence of these drugs, and this is supported by the results shown in Table 2. The limiting fluorescence measured in the absence of drug was therefore used to calculate the amount of probe bound in the presence of drug. The results were plotted by the double-reciprocal method of Hughes

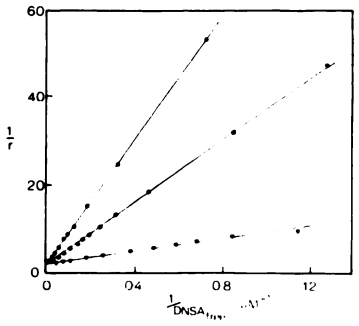

FIG. 2. *Double-reciprocal plot of DNSA binding to HSA in the absence and presence of drugs*

HSA (10.1 μM) was titrated with DNSA (▼——▼), and the fluorescence was measured (excitation, 350 nm; emission, 460 nm). The DNSA concentration was in the range of 1–100 μM. Similar titrations were performed in the presence of phenylbutazone (◆——◆) and iophenoxic acid (●——●) at a 1:1 ratio with HSA. The results were calculated as described in the text, where r = the number of moles of DNSA bound per mole of HSA.

and Klotz (17), and clearly show that displacement of these probes by the drugs studied is competitive in nature. The changes in fluorescence on addition of drugs therefore probably represent a direct displacement of the probes from HSA. Table 3 shows the displacement of 11 probes by a number of highly protein-bound drugs as measured by fluorescence.

DISCUSSION

The binding of the dansylamino acids to HSA was surprisingly variable both in the number of sites and in the tightness of binding to these sites. Small changes in the amino acid moiety resulted, in some cases, in large changes in the binding parameters. These results suggest that electrostatic and dipolar forces and also steric factors play a role in both strength and specificity of binding. This is in accord with results of Chignell (7), who concluded on the basis of circular dichroism measurements that the aromatic portion of flufenamic acid was inserted into a hydrophobic crevice on albumin while the carbonyl group was associated with a cationic group on the protein surface.

Examination of the drug-induced changes in the fluorescence of bound probes (Table 3) indicates that the probes studied could be divided into two clear subgroups, specified by the drugs displacing them. For example, DNSA was strongly displaced by phenylbutazone, oxyphenylbutazone, sulfinpyrazone, iophenoxic acid, warfarin, and sulfadimethoxine, whereas dansylsarcosine was not significantly displaced by these drugs. By contrast, dansylsarcosine was strongly displaced by CPIB, flufenamic acid, and ethacrynic acid whereas DNSA showed only minor displacement by these drugs.

DNSA and dansylsarcosine were chosen as representative of the two groups, and the displacement of other probes by all the drugs was correlated with them (Table 4). The division into two separate groups is quite clear. Warfarin, dansylcysteic acid, dansyl-L-arginine, and dansyl-L-glutamine all showed a high correlation with DNSA, but no correlation with dansylsarcosine. Dansyl-α-amino-*n*-butyric acid, dansylhydroxy-L-proline, ANS, and dansyl-L-proline correlated well with dansyl-

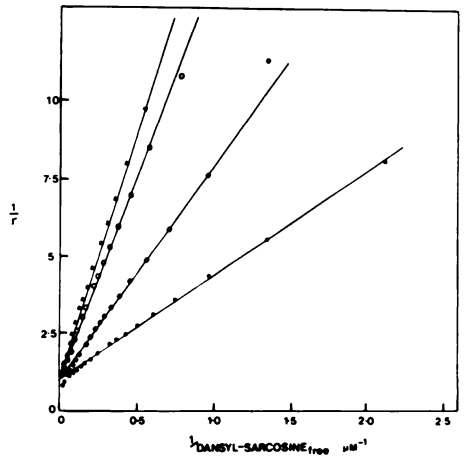

FIG. 3. *Double-reciprocal plot of dansylsarcosine binding to HSA in the absence and presence of drugs*

HSA (5.57 μM) was titrated with dansylsarcosine (●——●), and the fluorescence was measured (excitation, 370 nm; emission, 475 nm). The dansylsarcosine concentration was in the range of 1–100 μM. Similar titrations were performed in the presence of iopanoic acid (▲——▲), ethacrynic acid (○——○), and CPIB (◆——◆) at a 1:1 ratio with HSA. The results were calculated as described in the text, where r = the number of moles of dansylsarcosine bound per mole of HSA.

TABLE 3

Drug-induced changes in fluorescence of bound probes

The fluorescence of warfarin (2 μM) in HSA (16.4 μM) and other probes at the concentrations listed in HSA (20 μM) was measured at excitation and emission wavelengths given in Table 1 before and after the addition of drugs. Drugs were added to give a 1:1 ratio of drug to HSA. Concentrations of probes were chosen so as to achieve approximately 6% saturation of probe binding sites as calculated from the dissociation constants in Table 1, assuming binding to the tight site(s) only.

Drug	Decrease in fluorescence of bound probes on addition of drugs										
	Warfarin, 2 μM	DNSA, 2 μM	Dansyl-L-arginine HCl, 1.4 μM	Dansyl-L glutamine, 4.5 μM	Dansyl-L-cysteic acid, 1.5 μM	Dansyl-L-serine, 1.8 μM	ANS, 1 μM	Dansyl-γ-amino-*n*-butyric acid, 2.5 μM	Dansyl-L-sarcosine, 1.8 μM	Dansyl-L-proline, 1.5 μM	Dansyl hydroxy-proline, 6.5 μM
	%	%	%	%	%	%	%	%	%	%	%
Warfarin		28.4	34.6	34	17.3	10.6		0	0	6[a]	7[a]
Phenylbutazone	44.2	54.6	66	54	33.7	29	0	22.4	0	0	18
Iophenoxic acid	63	69.6	75.4	71	59	37.6	15.8[a]	19.5	0	4	20
Oxyphenylbutazone	28	42	46	41	24.3	18	0	12.3	0	0	6
Sulfinpyrazone	25	29.2	38	31	19	13	0	15.7	0	0	6
Sulfadimethoxine	11	22.5	30	21.7	7.4	10	0	3	0	7	10
Sulfafurazole		11	15.2	11.8	5.7	6.7	1.2	3	0	0	1.3
Tolbutamide	4	9.4	16.7	13.9	2	3.8	0	2	0	0	0
Fursemide	18	17.9	14.6	26.7	14.5	8.8	12.2[a]	5.0	5.5	4.2	10
Probenecid	0	0	6	5	0.9	7	2	8	1.6	0	13.4
Debrisoquine	0	3.4	0	0	0	0	0	2.5	0	0	0
Iopanoic acid	28.2	26.7	44	34.2	21.5	36	13	42.2	36.6	39	51.5
CPIB	7	5.9	13	15	11	12.2	7.5	37.5	25	32.2	41.5
Flufenamic acid	0	0	2.5	2	0	12	9	34.2	30	42	13.4
Ethacrynic acid	13.6	2.4	15.2	17.9	10	22.4	17.9	44	42.3	49	56.5
Amitriptyline	0	0	0	0	0	0	0	0	0	0	0

[a] Percentage increase in fluorescence.

TABLE 4

Correlation between displacement of DNSA or dansylsarcosine and displacement of other probes as measured by fluorescence

The fluorescence changes correlated are those listed in Table 3. The correlation coefficient was obtained from a comparison of the displacement by all drugs of either DNSA or dansylsarcosine and the probe indicated.

Probe	Coefficient of linear regression	
	DNSA	Dansyl sarcosine
DNSA		−0.31
Warfarin	0.96	−0.14
Dansyl-L-arginine	0.97	−0.17
Dansyl-L-glutamine	0.97	−0.14
Dansyl-L-cysteic acid	0.94	−0.11
Dansyl-L-serine	0.75	0.37
Dansyl-γ-amino-*n*-butyric acid	0.06	0.89
Dansylhydroxy-L-proline	−0.08	0.86
Dansylsarcosine	−0.31	
Dansyl-L-proline	−0.3	0.99
ANS	−0.28	0.98

sarcosine, but not with DNSA. Dansyl-L-serine showed a better correlation with DNSA than with dansylsarcosine, but neither was as high as for the other probes.

The two groups of probes are assumed to bind to two separate sites on the albumin molecule. Site I is occupied by the probes showing a high correlation with DNSA and the drugs which displace these probes. Site II is occupied by the probes showing a high correlation with dansylsarcosine and the drugs which displace these probes.

Inspection of the data in Table 3 shows that phenylbutazone displaces site I probes, but not the probes specific for site II, while flufenamic acid displaces site II probes most strongly. These two drugs are therefore considered to bind selectively to sites I and II, respectively. The displacement of probes by these specific drugs was correlated with the displacement by the other drugs studied (Table 5). Iophenoxic acid, oxyphenylbutazone, sulfinpyrazone, warfarin, sulfadimethoxine, tolbutamide, sulfafurazole, and fursemide showed a high degree of correlation with phenylbutazone, whereas ethacrynic acid and CPIB showed a high degree of correlation with flufenamic acid.

Strong positive and negative correlations between drug and probe displacement, together with clear evidence that the displacement is competitive, is difficult to explain other than by the presence of at least two discrete binding sites with quite different specificity. Convincing evidence in support of this hypothesis is provided by the observation that warfarin, when used as a probe, correlated well with all the site I probes but not the site II probes and, when used as a displacing agent displaced only the site I probes (Table 3).

The specificity and relative strength of binding to HSA of a number of drugs were determined by their ability to displace the probes specific for sites I and II (see Table 3). Iopanoic acid binds strongly to both sites. Probenecid, amitriptyline, and debrisoquine do not bind significantly to either site. As these drugs are strongly bound to

TABLE 5

Correlation between displacement of probes by phenylbutazone or flufenamic acid and by other drugs

The fluorescence changes correlated are those listed in Table 3.

Drug	Coefficient of linear regression	
	Phenyl butazone	Flufenamic acid
Phenylbutazone		−0.89
Iophenoxic acid	0.97	−0.95
Oxyphenylbutazone	0.99	−0.98
Sulfinpyrazone	0.99	−0.89
Sulfadimethoxine	0.89	−0.6
Tolbutamide	0.88	−0.61
Sulfafurazole	0.99	−0.84
Fursemide	0.82	−0.83
Probenecid	0.52	−0.28
Debrisoquine	0.31	−0.28
Iopanoic acid	−0.099	0.49
Ethacrynic acid	−0.86	0.97
CPIB	−0.82	0.96
Flufenamic acid	−0.89	
Warfarin	0.94	−0.82

HSA, there must be at least one other, unidentified, strong drug binding site. There are also, almost certainly, a number of weaker binding sites which were not detected by the present techniques. Because of the high albumin concentrations in serum, a drug could still be highly bound in serum if it were bound weakly to a number of sites on the albumin molecule. As the fluorescence experiments were conducted at low total concentrations of both drugs and albumin, this situation would not be detected by the technique.

The fluorescence of the probes dansyl-L-serine, dansyl-γ-amino-*n*-butyric acid, and dansylhydroxy-L-proline was decreased by site II drugs and most site I drugs. One explanation is that these probes bind to both sites I and II and are therefore displaced by drugs which bind to either of these sites. However, the binding parameters given in Table 1 suggest that these compounds bind tightly to only one site on HSA. It is possible that these compounds bind to a further site (site III) on HSA and that drugs which bind to sites I and II can also bind at site III. The lack of displacement of these probes by warfarin, which has a single site (site I), gives support to this possibility. The available data do not allow confirmation of the existence of a third binding site, but extension of the study to more diverse probe molecules and drugs may resolve the problem.

If each of the drug binding sites on HSA were characterized by a probe, the specificity and relative strength of binding of any drug to particular sites on HSA could be determined readily. As yet, binding sites for cationic drugs have not been characterized, but extension of the work to include a number of diverse cationic probes may provide useful information. Furthermore, studies using this technique together with other spectroscopic techniques, such as circular dichroism and electron spin resonance, may provide useful data about the forces involved in directing drugs to specific sites on HSA and in determining their tightness of binding.

It may eventually be possible specifically to monitor each important drug binding site on HSA by the techniques used in this work. This would allow the rapid screening of drugs to identify their individual binding sites on HSA and their potential ability to cause interactions based on displacement and redistribution.

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