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Further Characterization of Specific Drug Binding Sites on Human Serum Albumin

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SUMMARY

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The fluorescent probes 5-dimethylaminonaphthalene-1-sulfonamide and dansylsarcosine function as specific markers for two distinct binding sites for anionic drugs on human serum albumin (HSA). The binding of drugs to site I or II was detected by measuring the displacement of these probes. The results give an indication of some of the structural features required for binding at these sites. Stearic acid binding to HSA induced different conformational changes in the albumin at sites I and II, which were detected by changes in the fluorescence and/or strength of binding of probes specific for the two sites. This provides further evidence for qualitative differences between the two sites. The specificity of the sites for particular drugs, even at high drug to albumin ratios, was established, and the specificity and characteristics of the two sites on human adult and neonatal sera were shown to be similar to those on crystalline HSA.

INTRODUCTION

Two distinct binding sites for anionic drugs on human serum albumin have recently been characterized by the use of fluorescent probes (1). The sites were designated I and II, and it was shown that 5-dimethylaminonaphthalene-1-sulfonamide and dansylsarcosine were specific markers for the two sites. The displacement of specific probes from HSA[1] by drugs provided a measure of the specificity and relative strength of binding of drugs to the two sites. Warfarin, phenylbutazone and its analogues, iophenoxic acid, and two sulfonamides were shown to bind specifically to site I, while flufenamic acid, *p*-chlorophenoxyisobutyric acid, and ethacrynic acid bound specifically to site II.

[1] The abbreviations used are: HSA, human serum albumin; DNSA, 5-dimethylaminonaphthalene-1-sulfonamide; CPIB, *p*-chlorophenoxyisobutyric acid.

In this report the structural requirements for binding at each site have been investigated. The specificity of the two sites has been studied at high drug to albumin ratios and in human adult and neonatal sera. Some features of the binding of stearic acid provide further evidence for the discrete nature of the two binding sites.

METHODS

Electrophoretically pure crystalline HSA was obtained from Hoechst Australia, Ltd. The free fatty acid content as quoted by the manufacturer was 0.35 mmole/mmole of HSA. DNSA was obtained from K & K Laboratories or Sigma Chemical Company, and the dansylamino acids, from Sigma Chemical Company. Drugs were obtained as pure substances from the manufacturers. Adult serum was

obtained from normal volunteers who had fasted overnight. Neonatal serum was obtained from cord blood collected at birth.

All experiments were performed using sodium phosphate buffer (0.1 M, pH 7.4) with or without 0.9% NaCl. Experiments were performed at 22° or 37° as stated. The concentration of albumin was measured using the published extinction coefficient $E^{1\%}_{1\,cm}$ of 5.3 at 280 nm (2). 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 7.2–7.6. The sodium salt of stearic acid was evenly dispersed in phosphate buffer by sonication and then incubated with HSA at 37° for 2 hr to obtain a clear solution. Control solutions of HSA were incubated similarly.

Optical density was measured in a Unicam SP 1800 ultraviolet spectrophotometer. Fluorescence measurements were made in a Perkin-Elmer MPF-3 spectrofluorometer.

Drug-Induced Changes in Binding of Fluorescent Probes to Albumin

Fluorescence. The fluorescence of solutions containing probe (2 μM) and HSA (20 μM) was measured at 22° before and after the addition of drugs to concentrations of 20, 40, or 60 μM. The wavelength of the exciting light was generally 350 nm. An excitation wavelength of 370 nm was occasionally used to ensure that the optical density did not exceed 0.05. Fluorescence was measured at 475 nm. Similar experiments were carried out with human adult and neonatal sera diluted to an albumin concentration of approximately 20 μM. Experiments with HSA were repeated at 37° and in buffer without NaCl. The effect of stearic acid on the fluorescence of probes (2 μM) bound to HSA (20 μM) was measured by comparing the fluorescence of probes added to solutions containing either HSA, or stearic acid and HSA, prepared as outlined above.

Fluorescence as a percentage of the initial fluorescence is given by

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

Titrations to measure the limiting fluorescence of probes completely bound to HSA in the absence and presence of stearic acid at various ratios to HSA were performed as described previously (3). At each point in the titration, the percentage of the probe bound was calculated by

$$\%\ \text{probe bound} = \frac{F_o}{F_b} \times 100$$

where F_o is the observed fluorescence and F_b is the limiting fluorescence when all the probe is bound to HSA in the presence or absence of stearic acid. In all cases the fluorescence of the probes studied was negligible in the absence of HSA.

Dialysis. Direct determinations of drug-induced changes in the binding of probes to HSA were made by equilibrium dialysis as outlined previously (4). The binding of probes as a percentage of control is given by

$$\frac{B_d}{B} \times 100$$

where B is the concentration of probe bound to HSA in the absence of drug and B_d is the concentration of probe bound to HSA in the presence of drug.

RESULTS

Drug and Probe Binding to Sites I and II on HSA

A number of drugs were studied to determine the specificity of binding to sites I and II. DNSA and dansylsarcosine were used as markers for the two sites on HSA, and changes in the fluorescence of the probes provided a measure of drug binding at the sites (Table 1). *RS*-warfarin, *R*(+)-warfarin, *S*(−)-warfarin, phenprocoumon, and acenocoumarin all displaced DNSA (site I) but did not displace dansylsarcosine. There were no differences in the fluorescence changes caused by the two isomers of warfarin. Several drugs were identified which bound specifically to site II. Ibuprofen, flurbiprofen, and Ro 20-5720 (6-chloro-α-methylcarbazole-2-acetic acid) all caused a large decrease in the fluorescence of dansylsarcosine but enhanced the fluorescence of DNSA. Na-

TABLE 1

Comparison of fluorescence and dialysis methods for measurement of displacement by drugs of DNSA and dansylsarcosine

Dialysis. HSA (20 μM) was dialyzed against equal volumes (1 ml) of either DNSA (2 μM) or dansylsarcosine (2 μM) with or without the addition of drugs (20 μM). The concentrations of the probes in the protein and buffer compartments were measured by fluorescence as described previously (4).

Fluorescence. The fluorescence of solutions containing 2 μM probes and 20 μM HSA was measured before and after addition of drugs at a 1:1 ratio to HSA. The excitation wavelength was 370 nm, and fluorescence was measured at 475 nm.

Drug	Binding of Probes to HSA			
	DNSA		Dansylsarcosine	
	Fluorescence	Dialysis	Fluorescence	Dialysis
	% control		*% control*	
RS-Warfarin	62.4	73.3	100	100
R(+)-Warfarin	68			
S(−)-Warfarin	60.5			
Phenprocoumon	38	68.5	100	100
Acenocoumarin	82	91	97	100
Flurbiprofen	127	110	31	70
Ibuprofen	126	113	66	84
Ro 20-5720	126	105	75	82
Naproxen	89	92	38	58

proxen was bound tightly to site II and weakly to site I. Dialysis experiments established that the observed decreases in fluorescence resulted mainly from displacement of the probes from albumin (Table 1).

The specificity of the two sites was also investigated at higher drug to albumin ratios. Figure 1 illustrates the changes in the fluorescence of DNSA or dansylsarcosine bound to HSA on the addition of drugs up to a 3:1 ratio of drug to HSA. At these higher ratios the differentiation of drugs into two separate groups remained, although a small degree of binding of some site I drugs to site II, and vice versa, did occur. Experiments at 37° or with NaCl omitted from the buffer demonstrated that under these conditions the binding patterns were essentially the same.

The range of available dansylamino acids was tested to determine the specificity of their binding to HSA. Drug-induced changes in fluorescence of warfarin and the dansylamino acids were measured (Table 2). Three drugs which bind to site I and three which bind to site II were used, and the probes could be differentiated into three groups on the basis of the

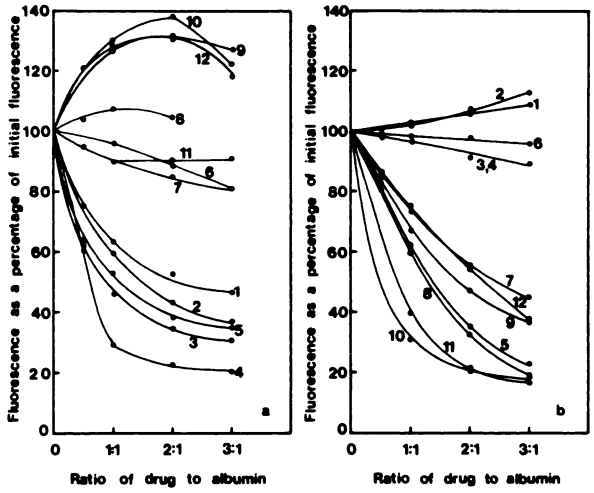


FIG. 1. *Drug-induced changes in fluorescence of DNSA and dansylsarcosine bound to HSA*

The fluorescence of solutions containing 20 μM HSA and 2 μM DNSA (a) or 2 μM dansylsarcosine (b) was measured before and after the addition of drugs. The excitation wavelength was chosen so that the optical density of the final solutions at this wavelength did not exceed 0.05. Fluorescence was read at 475 nm. The drugs used were: *1*, warfarin; *2*, phenprocoumon; *3*, phenylbutazone; *4*, iophenoxic acid; *5*, iopanoic acid; *6*, tolbutamide; *7*, CPIB; *8*, flufenamic acid; *9*, ibuprofen; *10*, flurbiprofen; *11*, naproxen; *12*, Ro 20-5720.

TABLE 2

Characterization of probes binding to specific sites on HSA

The fluorescence of probes (2 μM) in HSA (20 μM) was measured (excitation, 350 nm; emission, 475 nm) before and after the addition of drugs at a 1:1 ratio to albumin. The fluorescence of probes (2 μM) added to solutions of stearic acid and HSA (1:1 and 5:1 ratios of stearic acid to HSA) was compared with the fluorescence of probes added to HSA alone. Values are the fluorescence of the probes bound to HSA in the presence of drug or stearic acid as a percentage of the fluorescence of the probes bound to HSA alone.

Probe	Flurbiprofen	Ibuprofen	Ro 20-5720	Iophenoxic acid	Phenylbutazone	Oxyphenylbutazone	Stearic acid, 20 μM	Stearic acid, 100 μM
	%	%	%	%	%	%	%	%
1. DNSA	127	126	126	30	45	58	169	300
Warfarin	112	115		37	56	72	140	280
Dansyl-L-arginine	152	146	128	25	34	54	184	500
Dansyl-L-glutamine	131	133	128	19	34	54	167	336
Dansyl-L-asparagine	115	115	121	27	47	72	133	169
ε-Dansyl-L-lysine	131	143	123	30	40	61	135	262
Dansyl-L-glutamic acid	111	118	106	20	39	58	139	290
Dansyl-L-aspartic acid	102	112	100	26	49	68	127	187
Dansyl-L-cysteic acid	96	103	97	41	66	76	125	164
Dansyl-L-α-alanine	98	103	102	60	66	80	121	145
Dansylglycine	92	96	93	71	77	85	107	139
Dansyl-L-serine	96	100	98	56	59	76	123	180
2. Dansylsarcosine	31	66	74	95	92	100	93	52
Dansyl-L-proline	29	48	69	96	100	100	89	52
Dansyl-L-tryptophan	75	71	82	93	95	102	89	88
Dansyl-L-phenylalanine	70	68	77	96	92	100	84	70
Dansyl-DL-norleucine	40	56	78	113	104	107	91	89.5
Dansyl-L-norvaline	51	67	74	106	85	95	111	129
Dansyl-L-isoleucine	64	69	79	86	84	94	111	130
Dansyl-L-methionine	57	68	78	100	92	98	100	130
3. Dansylhydroxy-L-proline	52	52	69	64	79	86	93	144
Dansyl-L-valine	81	81	95	74	76	86	116	143
Dansyl-L-leucine	81	77	79	67	75	84	107	127
Dansyl-α-amino-*n*-butyric acid	79	84	80	80	73	85	135	153
Dansylthreonine	73	78	85	68	72	83	186	232
Dansyl-γ-amino-*n*-butyric acid	57	67	82	81	78	88	100	138

changes in fluorescence caused by the drugs.

Twelve of the dansylamino acids (group 1 in Table 2) were classed as site I probes, as they were displaced by iophenoxic acid, phenylbutazone, and oxyphenylbutazone, but were not significantly displaced by the site II drugs. The fluorescence of seven of the site I probes was enhanced by the site II drugs. Eight of the dansylamino acids (group 2 in Table 2) were classed as site II probes, as they were displaced by ibuprofen, flurbiprofen, and Ro 20-5720, but not to any significant extent by site I drugs. The remaining six dansylamino acids were displaced by both site I and II drugs (group 3 in Table 2).

Effects of Stearic Acid on Fluorescence of Probes Bound to HSA

The effects of stearic acid on the fluorescence of probes bound to HSA are also shown in Table 2. The fluorescence of probes classed as site I was in most cases greatly enhanced at both 1:1 and 5:1 ratios of stearic acid to HSA, whereas the fluorescence of probes classified as site II was generally decreased or showed only

minor enhancement. The fluorescence of the remaining probes was generally enhanced.

Albumin titrations of DNSA and dansylsarcosine were performed, maintaining the ratio of stearic acid to HSA at 1:1, 2:1, or 4:1 while increasing the concentrations of both. The fluorescence rose to a plateau when all the probe was bound to HSA. The limiting fluorescence value for DNSA was greater in the presence of stearic acid, whereas for dansylsarcosine the limiting fluorescence was lower in the presence of stearic acid (Table 3). Thus stearic acid binding caused changes in the quantum yield of the fluorescence of both probes bound to HSA. However, when the percentage of DNSA or dansylsarcosine bound to HSA was plotted as a function of HSA concentration (Fig. 2), it was clear that stearic acid enhanced the binding of DNSA to HSA but did not significantly affect the binding of dansylsarcosine. The fluorescence of warfarin was also enhanced by stearic acid, and, as with DNSA, the enhancement was shown to be due partly to tighter binding but mainly to an increased fluorescence quantum yield.

TABLE 3

Limiting fluorescence of DNSA and dansylsarcosine bound to HSA in the presence of varying ratios of stearic acid to HSA

Solutions containing DNSA (2 μM) or dansylsarcosine (2 μM) were titrated with HSA alone or with stearic acid and HSA at 1:1, 2:1, or 4:1 ratios. The limiting fluorescence value is the fluorescence of the probe when completely bound and is normalized with respect to the limiting fluorescence with HSA alone. The wavelength of the exciting light was 350 nm, and fluorescence was measured at 475 nm.

Ratio of stearic acid to HSA	Limiting fluorescence	
	DNSA	Dansylsarcosine
	%	%
0	100	100
1:1	121	95
2:1	129	82
4:1	143	63

Effect of Ibuprofen and Flurbiprofen on Site I

The mechanism of enhancement of the fluorescence of some site I probes by ibuprofen and flurbiprofen was investigated by limiting fluorescence titrations. Albumin titrations of DNSA, dansyl-L-arginine, dansyl-L-glutamine, dansyl-L-glutamic acid, and ε-dansyl-L-lysine were performed in the presence of a 1:1 ratio of ibuprofen or flurbiprofen to HSA. In each case the limiting fluorescence value when all the probe was bound to HSA was greater in the presence of the drugs (Table 4). Therefore the fluorescence enhancement of these probes was due largely to an increase in the fluorescence quantum yield of the bound probe. However, equilibrium dialysis experiments

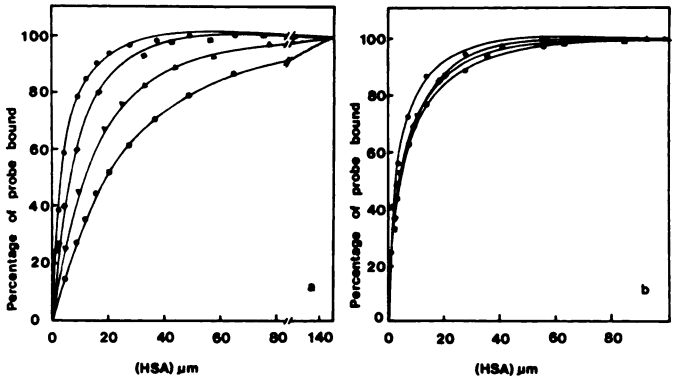


FIG. 2. *Effect of stearic acid on binding of DNSA and dansylsarcosine to HSA*

DNSA (2 μM) (a) or dansylsarcosine (2 μM) (b) was titrated with HSA alone (●——●) or with mixtures of stearic acid and HSA at stearic acid to HSA ratios of 1:1 (▲——▲), 2:1 (■——■), or 4:1 (○——○). Fluorescence was measured at 475 nm, with excitation at 350 nm. The percentage of probe bound was calculated as described in the text.

TABLE 4

Effect of Ibuprofen and flurbiprofen on limiting fluorescence of site I probes bound to HSA

Solutions containing probes (2 μM) were titrated with HSA alone or with HSA and ibuprofen or flurbiprofen maintained at a 1:1 ratio to albumin. The limiting fluorescence is the fluorescence of the probe when completely bound to HSA and is normalized with respect to the limiting fluorescence with HSA alone. Excitation was at 350 nm, and fluorescence was measured at 475 nm.

Probe	Limiting fluorescence		
	HSA alone	HSA + ibuprofen	HSA + flurbiprofen
	%	%	%
DNSA	100	120	120
Dansyl-L-glutamine	100	120	132
Dansyl-L-arginine	100	138	
Dansyl-L-glutamic acid	100	116	121
ε-Dansyl-L-lysine	100	126	

(Table 1) suggested that ibuprofen, flurbiprofen, and Ro 20-5720 also caused a minor increase in the amount of DNSA bound to HSA.

Multiple Drug Experiments

The ibuprofen-induced enhancement of the fluorescence of DNSA was significantly reduced by flufenamic acid, a drug which does not affect the fluorescence of site I probes, confirming that these drugs compete for binding at site II (Table 5).

In contrast, the displacement of DNSA by phenylbutazone was not altered by the presence of flufenamic acid or ibuprofen (site II drugs) at 1:1 ratios to albumin, and the displacement of dansylsarcosine by flufenamic acid was not significantly affected by the presence of phenylbutazone, thus confirming the clear distinction between the two sites.

Comparison of Crystalline Albumin with Diluted Human Sera

The distinction between the two sites was also studied using diluted sera from human adults and neonates. Phenylbutazone and ibuprofen were chosen as drugs specific for sites I and II, respectively, and the drug-induced displacement of the two corresponding probes, DNSA and dansylsarcosine, was measured. The results (Fig. 3) show that the two distinct sites were present with both human and neonatal sera. Furthermore, the patterns of displacement were very similar to those observed with HSA. However, the increased fluorescence of DNSA caused by ibuprofen, which occurred with HSA, was seen with only two of the eight adult sera studied and with none of the neonatal sera.

TABLE 5

Multiple drug binding to HSA

The fluorescence of solutions containing 2 μM DNSA and either 20 μM HSA alone or 20 μM HSA and the same concentration of flufenamic acid was measured before and after addition of ibuprofen. Fluorescence was measured at 475 nm, with excitation at 350 nm. Values are the fluorescence of DNSA in the presence of drugs as a percentage of the fluorescence in their absence.

Ibuprofen	Fluorescence of DNSA	
	HSA alone	HSA + flufenamic acid, 1:1
μM	%	%
10	121	103.6
20	128	104
30	130	107
40	129	109

DISCUSSION

There is now considerable evidence that drugs can bind to a number of separate sites on HSA. Two distinct binding sites (I and II) for anionic drugs on HSA were recently characterized by the use of fluorescent probes (1). Further evidence for the existence of the two spatially distinct drug binding sites on HSA is summarized below.

1. Additional drugs and probes which bind specifically to either site I or II have now been identified. Flurbiprofen, ibuprofen, and Ro 20-5720 bind specifically to site II, whereas phenprocoumon and acenocoumarin bind to site I. A total of 12 dansylamino acids have been shown to bind to site I, whereas eight are specific for site II (Table 2).

Some structural features of drugs and probes which bind to the two sites can

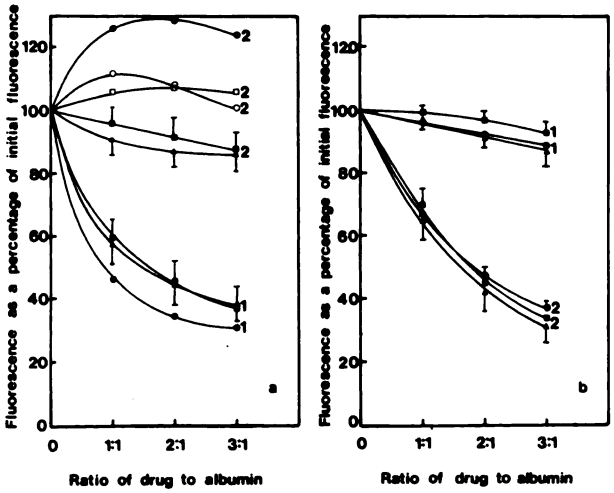


FIG. 3. *Comparison of drug-induced changes in fluorescence of DNSA and dansylsarcosine bound to HSA and diluted human adult and neonatal sera*

Solutions containing HSA (20 μM) and DNSA (a) or dansylsarcosine (b) at concentrations of 2 μM were titrated with phenylbutazone (*1*) or ibuprofen (*2*). The fluorescence was measured at 475 nm, with excitation at 350 nm. Neonatal sera were diluted to an albumin concentration of approximately 21.7 μM, and the values plotted are the means and standard deviations of measurements with sera from five individuals. Adult sera were diluted to an albumin concentration of approximately 20 μM, and the values plotted are the means and standard deviations of measurements with sera taken from eight individuals. Ibuprofen enhanced the fluorescence of DNSA with sera from two individuals (G. G. and D. J. B.). These results are excluded from the analyses and are shown separately. ●——●, Hoechst HSA; ▲——▲, adult sera; ■——■, neonatal sera; ○——○ D. J. B.; □——□, G. G.

now be identified. The structural formulae of the drugs which bind to sites I and II are shown in Table 6. Drugs which bind to site II are all aromatic carboxylic acids, which would be largely ionized at physiological pH. The configuration of these molecules is generally extended, and the negative charge is specifically located at one end of the molecule, away from the nonpolar region. Drugs which bind to site I are also all aromatic acids. However, with the exception of iophenoxic acid, these are more bulky heterocyclic molecules with a negative charge shared between two enol groups, or an enol and a hydroxyl group in the case of the coumarins. The negative charge is therefore more delocalized and is generally at the center of a largely nonpolar molecule. Although iophenoxic acid bears some resemblance to the site II drugs, the negative charge is still centrally located, being surrounded by 3 bulky iodine atoms.

Structural differences between the probes which bind specifically to sites I and II on HSA have also been observed. The dansylamino acids specific for site II all have a hydrophobic amino acid side chain and bear a close structural resemblance to the drugs which bind to this site. Those dansylamino acids which bind to site I in general have hydrophilic substituents on the amino acid side chain.

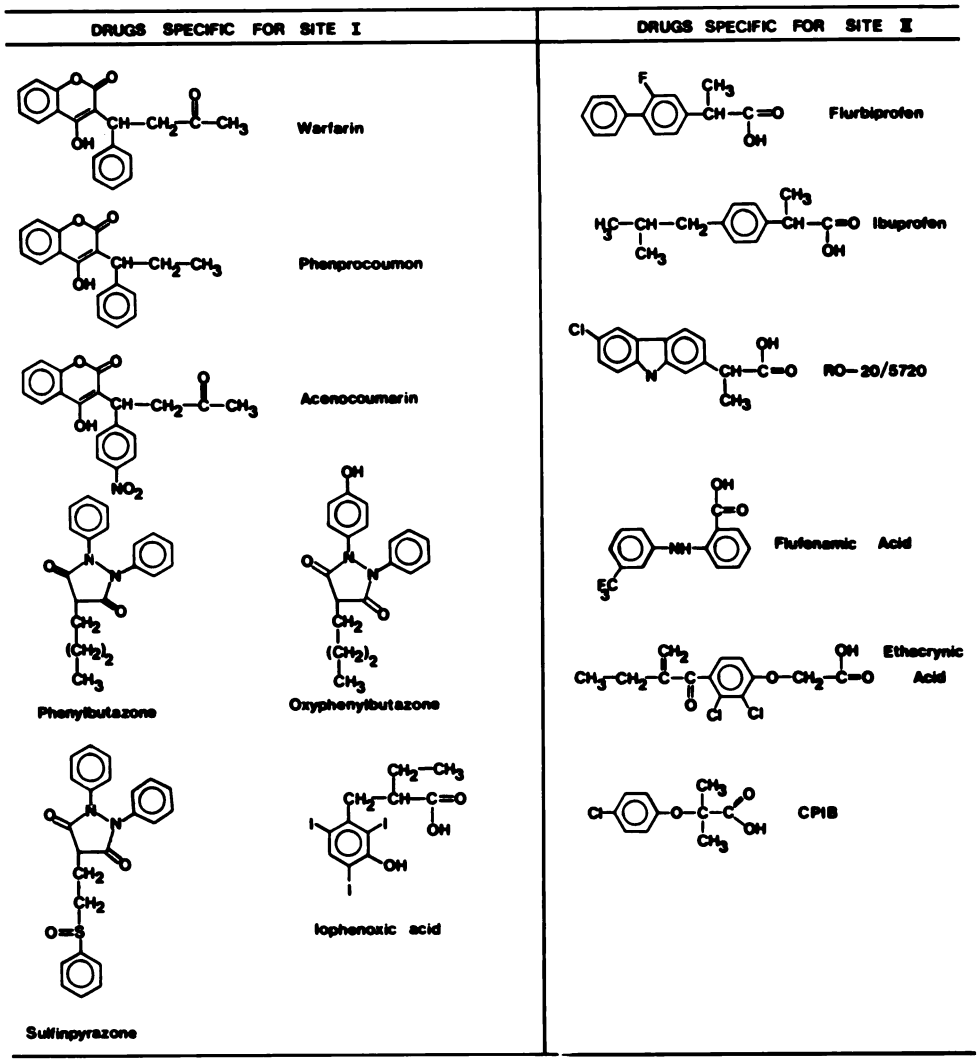
2. The binding of several drugs (flurbiprofen, ibuprofen, and Ro 20-5720) to site II results in enhancement of the fluorescence of a number of probes bound to site I. This effect is likely to be mediated by a drug-induced change in the conformation of the albumin molecule.

3. The displacement of site I probes by site I drugs is not affected by the presence of site II drugs, and vice versa. Furthermore, the ibuprofen-induced enhancement of DNSA fluorescence is reduced in the presence of flufenamic acid, confirming that these drugs share the same site on HSA (Table 5).

4. The addition of stearic acid in general causes different effects on the fluorescence

TABLE 6

Structures of drugs which bind specifically to site I or II on HSA



of site I and site II probes (Table 2). These effects are further discussed below.

The results all support the concept that there are two distinct binding sites for anionic drugs on HSA. A number of studies by other workers also provide evidence in support of the two-site hypothesis. Drugs which have been shown to displace each other from HSA and are therefore assumed to bind to the same site (site I) include phenylbutazone and warfarin (5, 6); warfarin, acenocoumarin, phenylbutazone, and ethyl biscoumacetate (7); ethyl biscoumacetate, phenylbutazone, sulfinpyrazone, tolbutamide, sulfadimethoxine, and phenprocoumon (8); and phenylbutazone and a number of sulfonamides (9, 10).

Fluorescent probe studies have shown that DNSA is displaced by phenylbutazone and warfarin (11) and by a number of phenylbutanzone analogues, other cyclic β-diones, and some sulfonamides (12), and that dansylglycine is competitively displaced from HSA by phenylbutazone (2). Furthermore, it has been demonstrated that ibuprofen, naproxen, and CPIB—all

site II drugs—do not displace DNSA from HSA, except at very high concentrations (12). The acetylation of a specific site on HSA by acetylsalicylic acid increases the binding of flufenamic acid (site II) but does not change the binding of phenylbutazone (site I) (13). These studies are all consistent with results presented in this report.

Several studies, however, have presented conflicting data. Whitehouse *et al.* (12) demonstrated a 50% decrease in the fluorescence of DNSA by a 2.4:1 ratio of flufenamic acid to HSA. However, direct measurements of displacements were not made, and changes in optical density at the excitation wavelength for DNSA were not measured. In the present study a 2:1 ratio of flufenamic acid to albumin caused no change in the fluorescence of DNSA, and dialysis studies have shown previously that flufenamic acid, at a 1:1 ratio to albumin, does not displace DNSA (1). Solomon *et al.* (5) have shown that both warfarin and phenylbutazone were displaced by CPIB and stearic acid. However, the ratios of CPIB to albumin were 9.4:1 for phenylbutazone displacement and 4.6:1 for warfarin displacement. Similarly, stearic acid was present at a 35:1 ratio to albumin. Under these circumstances displacement is likely to be due to competition at secondary sites on albumin.

Changes in the conformation of albumin as a result of the binding of small molecules have been observed previously (14–23), and these observations have been extended in the present work. The binding of ibuprofen, flurbiprofen, Ro 20-5720, and stearic acid caused changes in the conformation of the albumin molecule which were detected by changes in the fluorescence of bound probes (Table 3). The fluorescent probe technique monitors relatively small areas of the protein molecule, and the conformational changes observed may well be relatively localized and need not involve changes in the gross structure of the protein.

In the case of stearic acid, the results suggest that there are changes in the structure of the protein at both sites I and II. Up to a 4:1 ratio to albumin, stearic acid does not bind to site I or II on HSA but may, by changes in the albumin structure, affect the binding of other ligands at these sites. Other studies have generally shown that, at less than a 4:1 ratio to HSA, fatty acids do not displace drugs or bilirubin (24–27). These data support the hypothesis that fatty acids bind to sites on HSA which display considerable structural specificity and which are not available for the binding of other organic anions (28).

An inspection of the data in Table 2 allows some subgroups to be distinguished among the probes and drugs which bind to site I or II. Of the site I probes, only some show enhancement of fluorescence on the binding of site II drugs. These are DNSA, dansyl-L-glutamine, dansly-L-asparagine, ε-dansyl-L-lysine, warfarin, and, to a lesser extent, dansyl-L-glutamic acid. These probes also respond to the binding of stearic acid with a greater fluorescence enhancement than do the other site I probes (Table 2). With the exception of dansly-L-glutamic acid, these dansylamino acids all have a second $-NH_2$ substituent on the amino acid side chain. These results suggest that the presence of the second amino group can influence the orientation of the dansyl moiety at site I, or cause an alteration in the structure of the binding site, so that the dansyl group responds to changes in its immediate environment induced by the binding of ligands at distant sites. A division of the site II probes into two groups can also be made on the basis of the effect of stearic acid (Table 2). In this case, however, there are no obvious structural differences between the probes in the two groups.

In a similar fashion, the site II drugs flurbiprofen, ibuprofen, and Ro 20-5720 enhance the fluorescence of some site I probes (Table 2), whereas flufenamic acid, naproxen, ethacrynic acid, and CPIB do not (1). Furthermore, flufenamic acid prevents the ibuprofen-induced enhancement of DNSA fluorescence. This provides convincing evidence that the binding of different drugs to site II can stabilize different conformations of the albumin molecule, and the results are in keeping with the "induced fit" hypothesis proposed by Karush (29) and by Koshland (30).

The differentiation of sites I and II was examined at higher drug to albumin ratios, and it was found that the distinction between the two sites remained clear at drug to albumin ratios of up to 3:1 (Fig. 1). Furthermore, the specificity and characteristics of the two sites with diluted human adult and neonatal sera were almost identical with those observed with the crystalline albumin preparation (Fig. 2). This provides strong evidence that the two sites exist *in vivo*; in agreement with this, the pharmacokinetics of warfarin is not changed by the concurrent administration of ibuprofen (31) or CPIB (32), both of which are site II drugs. In contrast, there is convincing evidence that the interaction between warfarin and phenylbutazone, both of which bind to site I, is due at least in part to displacement and redistribution of warfarin (33, 34). It can therefore be predicted that drugs which bind only to site II will not be involved in displacement interactions with the coumarin anticoagulants or with other drugs which bind only to site I.

The specificity of sites I and II on different commercial preparations of HSA is currently being investigated. There appear to be marked differences between albumin preparations, but the preliminary results suggest that preparations low in fatty acid content most closely resemble normal human sera.

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