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Evidence for a Large and Flexible Region of Human Serum Albumin Possessing High Affinity Binding Sites for Salicylate, Warfarin, and Other Ligands

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SUMMARY

The relations between the single high affinity binding sites for azapropazone, phenylbutazone, chlorpropamide, sulfathiazole, and iophenoxate and the binding regions of human serum albumin represented by the marker ligands diazepam, phenol red, salicylate, and warfarin were examined by a series of competition experiments. Binding was determined by equilibrium dialysis at pH 7.0. In order to ensure an accurate analysis of the competition experiments, the number of moles of ligand bound per mole of protein was usually 0.4 or less to minimize ligand binding to weaker sites. Furthermore, binding of both ligands was determined in all experiments (except for iophenoxate). None of the test ligands competed with diazepam for a common high affinity binding site, but, surprisingly, they were all able to displace two or three of the other marker ligands according to a competitive scheme. These findings show, first, the existence of a particular serum albumin region for high affinity binding of diazepam. Secondly, they imply that it is not necessary to assume the existence of new drug binding regions beyond those existing for phenol red, salicylate, and warfarin. On the contrary, the relatively many examples of competitive binding indicate that the binding regions represented by the last-mentioned three marker ligands are placed quite close to each other in the albumin molecule in a common region, which is suggested to be located at subdomains 1C and 2A-B. The region must be relatively large, because in some cases independent high affinity binding of pairs of ligands is observed. It is probably also rather flexible, inasmuch as no clear relation could be found between the chemical structure of the test ligands and the two or three marker ligands with which they compete. Correlations between primary association constants and partition coefficients for both marker ligands and test ligands, in the unionized forms, between *n*-hexane or 1-octanol and aqueous media showed that hydrophobic forces are important for the binding processes. However, the data also showed that other attractive forces must be operative as well.

Although the unique capability of serum albumin to bind ligands of a very diverse chemical nature has been recognized for a long time and has been the subject of a great number of studies, a detailed explanation of this property at the molecular level cannot be given at present. This is mainly because the three-dimensional structure of the protein has not yet been clarified. However, models, based on the existence of discrete regions at which a varying number of compounds can interact with a high affinity, have been elaborated and tentatively assigned (1–4) to the secondary structure of serum albumin, proposed by Brown (5). It is generally agreed that there exist three separate regions for high affinity binding of (i) long chain fatty acid ions, (ii) Cu^{2+} and Ni^{2+}, and (iii) diazepam, tryptophan, and a few other ligands. However, these only account for a minority of the ligands that can be bound by albumin, which comprise physiological compounds like bilirubin and hemin and a great number of drugs and dyes. To account for the binding of these ligands, additional binding region(s) have been proposed. Sudlow *et al.* (6, 7) proposed the existence of a particular drug binding region for which 5-dimethylaminonaphthalene-1-sulphonamide can be used as a fluorescent marker ligand. Sjöholm *et al.* (8) suggested two regions represented by the high affinity binding sites of digitoxin and warfarin, respectively. Fehske *et al.* (1) operate with three binding regions for which bilirubin, digitoxin, and warfarin plus azapropazone are representative markers.

Kragh-Hansen (9) carried out competitive binding studies with various marker ligands in an attempt to correlate the different models. Instances of competitive binding were obtained compatible with the existence of three or four binding regions in addition to those (i–iii) mentioned above, (iv) bilirubin and phenol red, (v) salicylate and digitoxin, and (vi) warfarin, and perhaps hemin, binds to a further region.

In the present work, these studies have been greatly extended by inclusion of a number of additional drugs in the binding analyses. The purpose was that the present results, by proper combination with previous data, should give fairly definitive evidence for or against the presence of discrete albumin regions for high affinity drug binding. The following ligands were chosen as markers for the putative binding regions: iv, phenol red; v, salicylate; and vi, warfarin. In addition, diazepam was

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included as marker for region iii. The drugs tested were azapropazone, phenylbutazone, chlorpropamide, sulfathiazole, and iophenoxate. These test ligands were chosen because they are strongly bound to albumin and because they have occasionally been suggested as representatives of special drug binding regions of albumin. This is especially true for azapropazone (1) and iophenoxate (10). The results show that the high affinity binding sites for the test ligands differ from those of the marker ligands. However, it is not necessary to invoke new binding regions. Instead, the data suggest that the binding regions iv, v, and vi are not so discrete as originally thought but are situated quite closely in the tertiary structure of human serum albumin.

Materials and Methods

Chemicals. Human serum albumin (97% pure according to the manufacturer) from AB Kabi (Stockholm, Sweden) was defatted by the charcoal method of Chen (11) and then lyophilized for storage in a desiccator at 4° until use. [^{14}C]Warfarin (46 mCi/mmol) and [2-^{14}C] diazepam (54 mCi/mmol) were purchased from Amersham International (Amersham, Buckinghamshire, England) and [7-^{14}C]salicylic acid (53.8 mCi/mmol) was bought from New England Nuclear (Boston, MA). The amount of radiochemical impurities that do not bind to albumin was determined according to the principles of Honoré (12) and was found to be less than 0.1%, about 0.4%, and 1.5–2.0% for the batches used of diazepam, salicylic acid, and warfarin. [^{14}C]Warfarin was purified by thin layer chromatography in toluene/dioxane (90:10) on silica gel plates. After chromatography, the impurities accounted for 0.3–0.5% of the total radioactivity. Warfarin, phenylbutazone, and sulfathiazole were supplied by Sigma Chemical Co. (St. Louis, MO); phenol red, sodium salicylate, and salicylic acid by Merck (Darmstadt, Germany); and digitoxin (puriss.) by Fluka AG (Buchs, Switzerland). The following drugs were received as gifts: nominally pure samples of diazepam from Dumex Ltd. (Copenhagen, Denmark), azapropazone from A/S Ferrosan (Copenhagen, Denmark), and chlorpropamide from Pfizer A/S (Hvidovre, Denmark). Iophenoxic acid was generously donated by Dr. G. Mudge (Dartmouth Medical College, Hanover, NH) and was found to be pure by several different thin layer chromatography solvent systems and plates.

Equilibrium dialysis studies. All binding experiments were performed at 20° with media containing 33 mM sodium phosphate buffer, pH 7.0. For determination of single binding of ligands to albumin, samples containing azapropazone, phenylbutazone, chlorpropamide, or sulfathiazole, with and without 4% (w/v) of albumin, were prepared. Except for sulfathiazole the ligands were added, with magnetic stirring, to the solutions in small volumes (5–120 μl) of a stock solution consisting of the drug dissolved in ethanol (azapropazone and chlorpropamide) or 0.1 N NaOH (phenylbutazone). The final solutions of azapropazone or chlorpropamide contained 0.05–1.20% (v/v) ethanol. Addition of stock solution of phenylbutazone changed the pH of the final solutions less than 0.10 pH unit, and the pH was not readjusted.

For equilibrium dialysis, 4.5 ml of the different drug-albumin solutions were pipetted into cellophane bags (Visking, 18 mm diameter). After closure they were placed in 4.5 ml of corresponding reference solution (solutions with drug but without albumin) in special tubes (diameter, 25 mm). As references, representing 100% free ligand, tubes were prepared with the same albumin-free solution on both sides of the dialysis membrane. The two sets of tubes were carefully closed with Parafilm (American Can Co., Greenwich, CT), placed in a temperature-controlled (20°) waterbath, and shaken gently for 17–18 hr. The concentrations of unbound drug in the outer media were determined, after suitable dilution with phosphate buffer, by spectrophotometry at a light absorption maximum. Linearity of extinction with varying concentrations was verified for all drugs.

In double ligand experiments, the effects of azapropazone, phenylbutazone, chlorpropamide, sulfathiazole, and iophenoxate on high affinity binding of the marker ligands diazepam, phenol red, salicylate, and warfarin were examined. For that purpose four different sets of solution were prepared. Samples containing diazepam plus [^{14}C]diazepam, phenol red, sodium salicylate plus [^{14}C]salicylic acid, or warfarin plus [^{14}C]warfarin, with and without 4% albumin, were mixed. In addition, albumin-containing and albumin-free solutions of the same composition as just mentioned were prepared but with a constant total concentration of one of the five test ligands. Azapropazone, phenylbutazone, chlorpropamide, and sulfathiazole were added principally in the same way as mentioned above, and iophenoxate was added in 30 μl of a stock solution prepared in 0.2 N NaOH. Except for phenol red, the marker ligands were added to the solutions in small volumes (15–50 μl) of a stock solution consisting of both nonlabeled and isotopically labeled ligand dissolved in ethanol (diazepam), 0.1 N NaOH (warfarin), or phosphate buffer (sodium salicylate). The final solutions contained at most 0.8% (v/v) ethanol, and the pH was changed less than 0.10 pH unit. The binding was determined by equilibrium dialysis as described above. In the double ligand experiments, both the concentrations of unbound marker ligand and unbound test ligand were determined (except for iophenoxate). The concentrations of diazepam, salicylate, and warfarin were determined by liquid scintillation counting of radioactivity. The concentration of phenol red was determined by spectrophotometry at 559 nm in an alkaline medium. No quenching of the radioactivity of [^{14}C]diazepam, [^{14}C]salicylate, or [^{14}C]warfarin and no influence on the spectrophotometric determination of the phenol red concentration was observed in the presence of any of the five test ligands. On the other hand, the presence of all four marker ligands influenced the spectrophotometric determination of the free concentration of the test drugs. Appropriate corrections for the overlapping light absorbance spectra were carried out on the basis of the known concentration of marker ligand in the samples.

Control experiments with ligand-containing, but albumin-free, solutions showed that the dialysis membrane was fully permeable for all ligands and that equilibrium was established within the period of time used. Protein leakage amounted to 0.05 ± 0.03% (10 determinations, mean ± SD) as measured by the method of Lowry *et al.* (13). In order to correct for this leakage and for any UV-absorbing impurities from the dialysis membrane, the absorbances of media outside bags containing albumin dissolved in buffer were subtracted from the absorbances of media outside bags containing both protein and ligand. Likewise, all sets of experiments included tubes with only phosphate buffer on both sides of the dialysis membrane in order to correct the references for any UV-absorbing impurities from the cellophane membrane. Finally, due to the light sensitivity of azapropazone (14), all solutions with this ligand were made under orange light, and the equilibrium dialysis was carried out in the dark.

Partition coefficient determinations. A total of 50 μmol of drug or dye (phenylbutazone, 1 μmol), 10 ml of polar solvent, and 10 ml of *n*-hexane or 1-octanol were added to test tubes with screw caps. The polar solvent was water (sulfathiazole and digitoxin), 0.1 N HCl (azapropazone, chlorpropamide, and warfarin), 0.5 N HCl (phenylbutazone), or 1 N HCl (salicylic acid, iophenoxic acid, and phenol red). After 20 hr of shaking at 20°, the concentrations of the different compounds in the various phases were determined by spectrophotometry.

Calculations. The binding percentages ($LB\%$) were used to calculate the average number of moles of ligand (L) bound per mole of albumin ($\bar{\nu}_L$):

$$\bar{\nu}_L = 2 \cdot [L_t] \cdot (LB\%)/[P_t] \cdot 100 \qquad (1)$$

where $[L_t]$ and $[P_t]$ are the concentrations of total ligand and total protein, respectively. The molarities of the albumin solutions were calculated on the basis of a molecular weight of 67,000 (4).

Then, the binding parameters n and K were calculated on the basis

of the following equation and fitted to the experimental data by a computer-analyzed nonlinear iteration procedure (15):

$$\bar{\nu}_L = \sum_{i=1}^{j} \frac{n_i \cdot K_i \cdot [L_f]}{1 + K_i \cdot [L_f]} \quad (2)$$

In this equation n_i and K_i represent the number of binding sites and the corresponding association constants in the ith binding class, respectively, and $[L_f]$ is the concentration of free ligand.

In the present study of high affinity binding of pairs of ligands, the possibility that a mutual decrease in binding could be explained by a competitive scheme was analyzed by using the following equations:

$$\bar{\nu}_A = \frac{K_A \cdot [A_f]}{1 + K_A \cdot [A_f] + K_B \cdot [B_f]} \quad (3a)$$

$$\bar{\nu}_B = \frac{K_B \cdot [B_f]}{1 + K_B \cdot [B_f] + K_A \cdot [A_f]} \quad (3b)$$

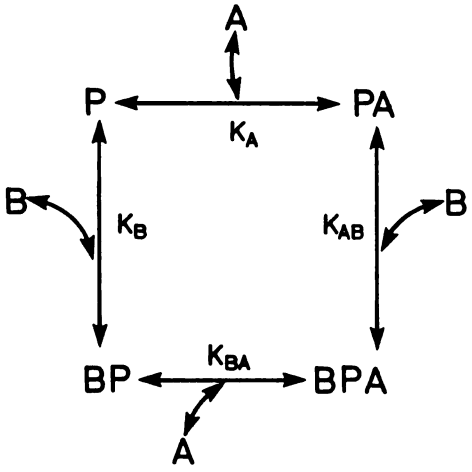
$$[B_t] = [B_f] + \bar{\nu}_B \cdot [P_t]/2 \quad (4)$$

In these general formulations for a protein possessing one binding site in common for ligands A and B, $\bar{\nu}_A$ and $\bar{\nu}_B$ are the average numbers of moles of A and B bound per mole of protein, respectively, and K_A and K_B are the association constants for binding of ligand A and B, respectively. Moreover, $[A_f]$ and $[B_f]$ are the concentrations of the free forms of the ligands. Because the concentrations of total test ligand ($[B_t]$) and protein as well as the association constants for single binding of both marker ligand (A) and test ligand (B) are known, it is possible to calculate a theoretical binding curve for $\bar{\nu}_A$, in the presence of test ligand B, as a function of the concentration of free marker ligand. If the curve constructed by this method is in agreement with the experimental data, the binding mechanism follows a competitive scheme.

However, binding of ligands to serum albumin is usually not characterized by binding to only one site but rather by binding at a high affinity site and a varying number of sites with lower affinity. Therefore the following strategy was used. First, binding of marker ligand to its high affinity site was calculated, as described above, assuming competition between marker ligand and test ligand for that site. Afterwards, the contribution of marker ligand binding to weaker sites, assumed to be unaffected by the presence of test ligand, was calculated by the use of Eq. 2 and added, to obtain total binding. Finally, a binding curve showing total marker ligand binding was made and compared with the experimentally determined data.

In order to minimize ligand binding to weaker sites, the experiments were carried out with ligand-serum albumin molar ratios of 0.4 or less. However, if preliminary studies indicated independent high affinity binding of two ligands, the final experiments were performed with 0.6 mol of test ligand/mol of protein.

Mutual decreases in binding of two ligands to serum albumin cannot always be accounted for by a competitive scheme. In these cases we used a more general formulation:



In this scheme P represents a protein possessing one binding site for ligand A and one binding site for ligand B with the indicated association constants. If A and B bind independently to P, then $K_{BA} = K_A$ and $K_{AB} = K_B$. In all other situations $K_{BA} = x \cdot K_A$ and $K_{AB} = x \cdot K_B$, where the factor x defines a coupling constant. Cooperative binding and anticooperative binding are characterized by $x > 1$ and $x < 1$, respectively. In the present work, no examples of $x > 1$ was found. In case of competitive binding $x = 0$, indicating that simultaneous binding of A and B is impossible.

The values of x can be calculated as follows. The concentration of total protein is equal to the sum of the concentrations of P, PA, BP, and BPA:

$$[P_t] = [P] + [PA] + [BP] + [BPA] \quad (5a)$$

This relationship is readily transformed into:

$$[P_t] = [P] + K_A \cdot [P] \cdot [A_f] + K_B \cdot [B_f] \cdot [P] + x \cdot K_A \cdot K_B \cdot [A_f] \cdot [B_f] \cdot [P] \quad (5b)$$

Furthermore, the concentration of bound A ($[A_b]$) is given by:

$$[A_b] = [A_t] - [A_f] = K_A \cdot [P] \cdot [A_f] + x \cdot K_A \cdot K_B \cdot [A_f] \cdot [B_f] \cdot [P] \quad (6)$$

where $[A_t]$ represents the concentration of total A. Subtracting Eq. 6 from Eq. 5b gives:

$$[P_t] - [A_b] = [P] + K_B \cdot [B_f] \cdot [P] \quad (7)$$

Because $[P_t]$, $[A_t]$, $[A_f]$, K_B, and $[B_f]$ are known, it is possible to calculate $[P]$ from Eq. 7. Inserting $[P]$ and the known values for K_A and $[A_f]$ in Eq. 5b gives x.

Results

Binding of Single Ligands to Defatted Human Serum Albumin

A prerequisite for a quantitative analysis of potential interactions between ligands bound to serum albumin is accurate information on n_i and K_i for each ligand. Therefore, as a preliminary step, the individual binding characteristics of the ligands azapropazone, phenylbutazone, chlorpropamide, and sulfathiazole were studied. The experimental data are shown in Figs. 1–4 and the n_i and K_i values calculated are included in Table 1.

As shown in Fig. 1, a steep increase in azapropazone binding takes place at low concentrations of free drug (corresponding to $\bar{\nu}$ values below 1). This phenomenon is especially apparent from Fig. 1, *inset* and indicates the existence of a high affinity binding site. At higher drug concentrations, the increase in binding is less pronounced, indicating the existence of sites to which azapropazone binds with smaller association constants. The computer-aided analysis resulted in $n_1 = 1$, $n_2 = 1$, and $n_3 = 2$ with binding constants in the order of 10^4–10^5 M^{-1} (Table 1). In comparison, Fehske *et al.* (16) found one high affinity and two low affinity binding sites for azapropazone with association constants of 1.2×10^6 M^{-1} and 5×10^4 M^{-1}, respectively. Kober and Sjöholm (14) reported $n_1 = 1.0$ and $K_1 = 5.0 \times 10^5$ M^{-1}. These authors did not determine the number of and the binding constants for weaker sites.

Binding of the related drug phenylbutazone is also characterized by one high affinity site (Fig. 2) for which an association constant of 7.0×10^5 M^{-1} was determined (Table 1). The number of secondary and tertiary sites were found to be 1 and 3 with association constants about 10 and 100 times lower than K_1, respectively (Table 1). Binding of phenylbutazone to human

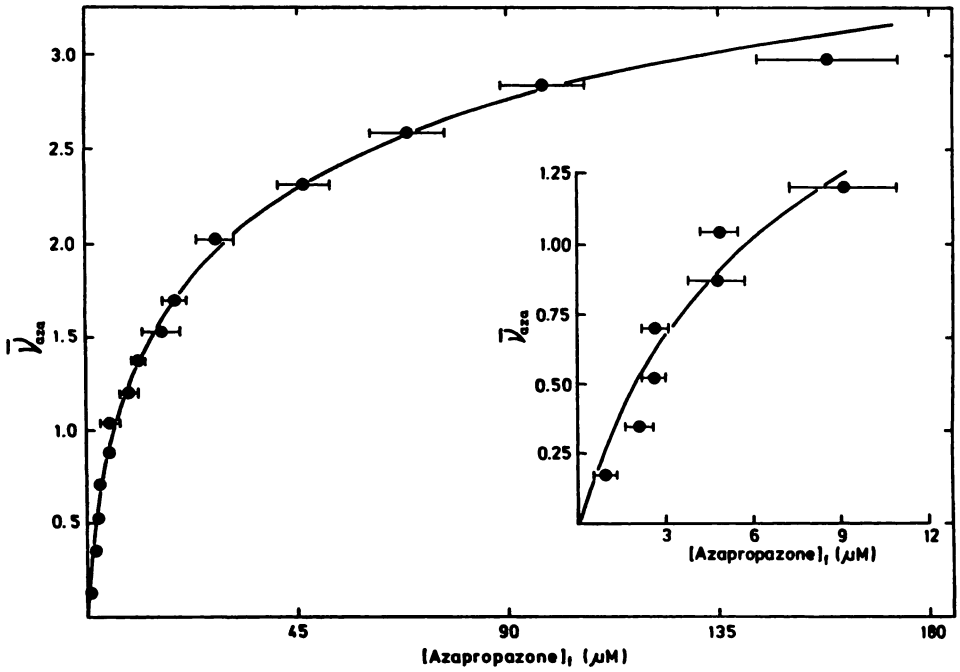


Fig. 1. Binding of azapropazone to defatted human serum albumin as determined by equilibrium dialysis. The *inset* shows the results at low drug-protein molar ratios in greater detail. The *binding curves* were computed by using the number of binding sites and the corresponding association constants given in Table 1. The *symbols* represent average values (± standard deviation) of five experiments carried out in duplicate.

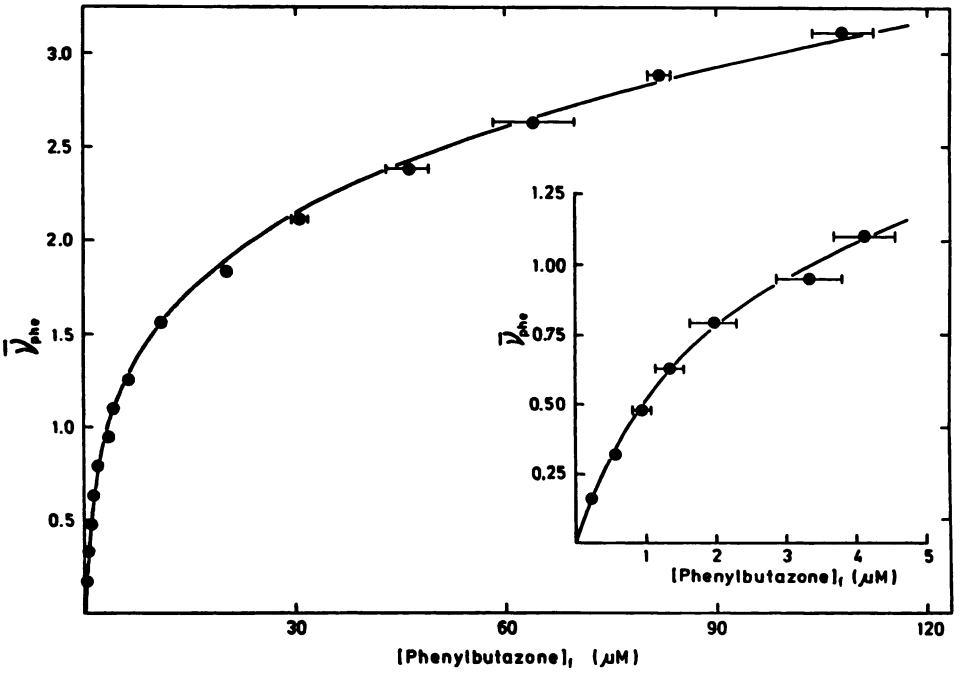


Fig. 2. Binding of phenylbutazone to human serum albumin. The *binding curves* were computed by using the number of binding sites and the corresponding association constants given in Table 1. The *symbols* represent average values (± standard deviation) of four experiments carried out in duplicate.

serum albumin has also been studied by equilibrium dialysis by other investigators (17–20). All authors agree on $n_1 = 1$. The primary association constant varies from a lower value of about 3×10^5 M^{-1} (18) to a higher value of 1.0×10^6 M^{-1} (17). Different values for n_2 were reported, namely 1 (19), 2 (17, 20), and 4 (18) with corresponding association constants of about 10^4 M^{-1}.

Binding of chlorpropamide (Fig. 3) can be described by one high affinity site and the same number of secondary and tertiary sites as in the case of phenylbutazone but with somewhat lower association constants. These are, on the average, half of those calculated for binding of phenylbutazone (Table 1). Binding of sulfathiazole is relatively weak (Fig. 4). The association constants are 1 order of magnitude lower than those of azapropazone, phenylbutazone, and chlorpropamide. However, one high affinity binding site is apparent, whereas the number of secondary binding sites is relatively high (Table 1). No studies on binding of chlorpropamide or sulfathiazole to human serum albumin, carried out in phosphate buffer and including molar drug to protein ratios at or below unity, could be found in the literature.

Mudge and co-workers (21, 22) have determined the Scatchard binding constants for the nine binding sites of human serum albumin with the highest affinities for iophenoxate. The association constants for the first three sites are included in Table 1. These data have been used in the present study, because, due to the very strong binding, it was not possible to determine free drug concentrations with an acceptable accuracy by spectrophotometry and because isotopically labeled iophenoxate was not available. The shortcoming of the spectrophotometric method can be illustrated as follows. From Astwood

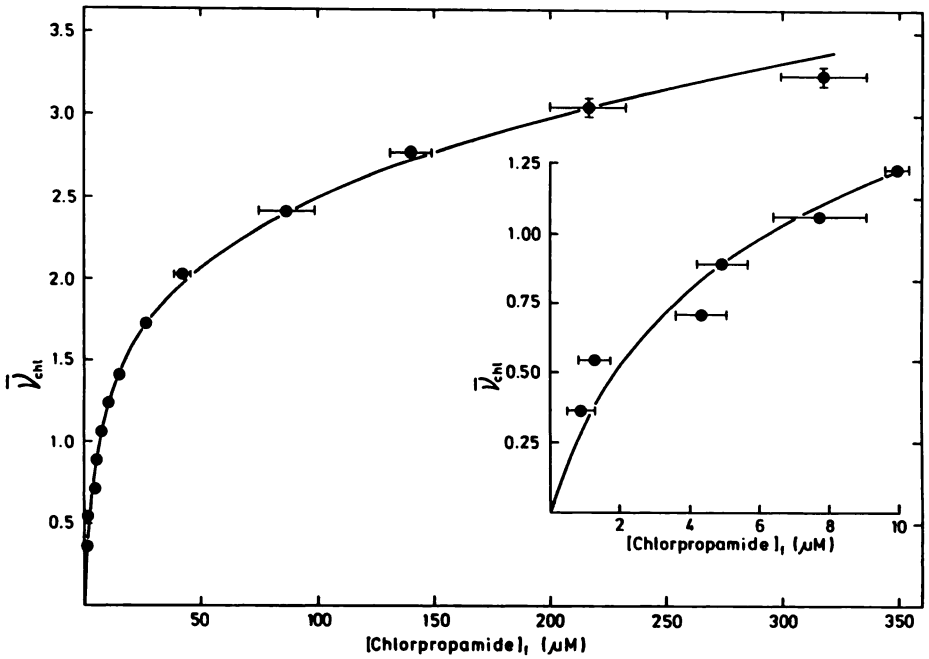


Fig. 3. Binding of chlorpropamide to human serum albumin. The *binding curves* were computed by using the number of binding sites and the corresponding association constants given in Table 1. The *symbols* represent average values (± standard deviation) of three experiments carried out in duplicate.

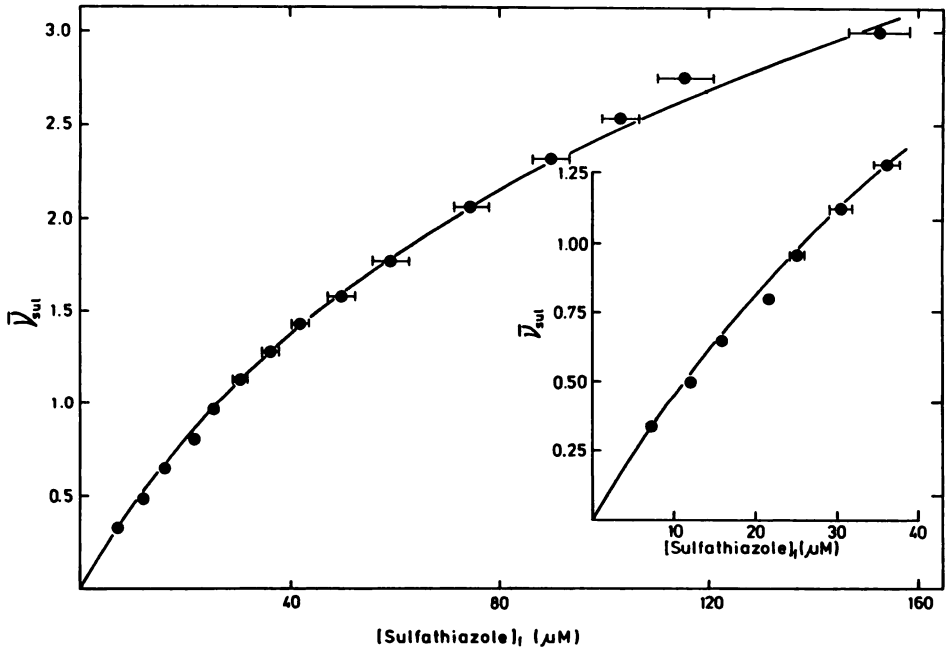


Fig. 4. Binding of sulfathiazole to human serum albumin. The *binding curves* were computed by using the number of binding sites and the corresponding association constants given in Table 1. The *symbols* represent average values (± standard deviation) of three experiments carried out in duplicate.

(23) a molar absorption coefficient of 6.98×10^3 (323 nm) can be calculated for iophenoxate dissolved in the most suitable medium for this purpose, namely alkali. Furthermore, it can be calculated, by the use of the K values given in Table 1, that at a drug-protein molar ratio of unity only about 0.03% of the ligand is unbound. Thus, the absorbancy attributable to unbound drug would only be about 0.001, when using the present albumin and iophenoxate concentrations.

The values given in Table 1 for binding of diazepam, phenol red, salicylate, and warfarin are the means of those calculated for the results shown in Figs. 5–9. n_1, n_2, and K_1 are all comparable to those previously reported (9, 24), whereas the K_2 values are somewhat higher in the present study.

Thus, binding of all nine ligands to human serum albumin can be characterized by only one high affinity site. This means that, despite the multiplicity of binding sites, it should be possible to analyze the relations between the high affinity sites by the procedures accounted for in Materials and Methods.

Binding of Pairs of Ligands to Defatted Human Serum Albumin

Azapropazone. Fig. 5A shows binding of various concentrations of diazepam in the absence and in the presence of a constant concentration of total azapropazone ($\bar{\nu} = 0.60$). Although the results are somewhat scattered, it seems safe to conclude that high affinity diazepam binding is not influenced by the presence of the other drug. The observation of independent binding of azapropazone and diazepam is in accordance with previous data by Kober and Sjöholm (14) and also with results published by Fehske *et al.* (20). These latter authors measured a mutual reduction in binding of only 0–1.1%, when the two drugs were added to human serum albumin in molar

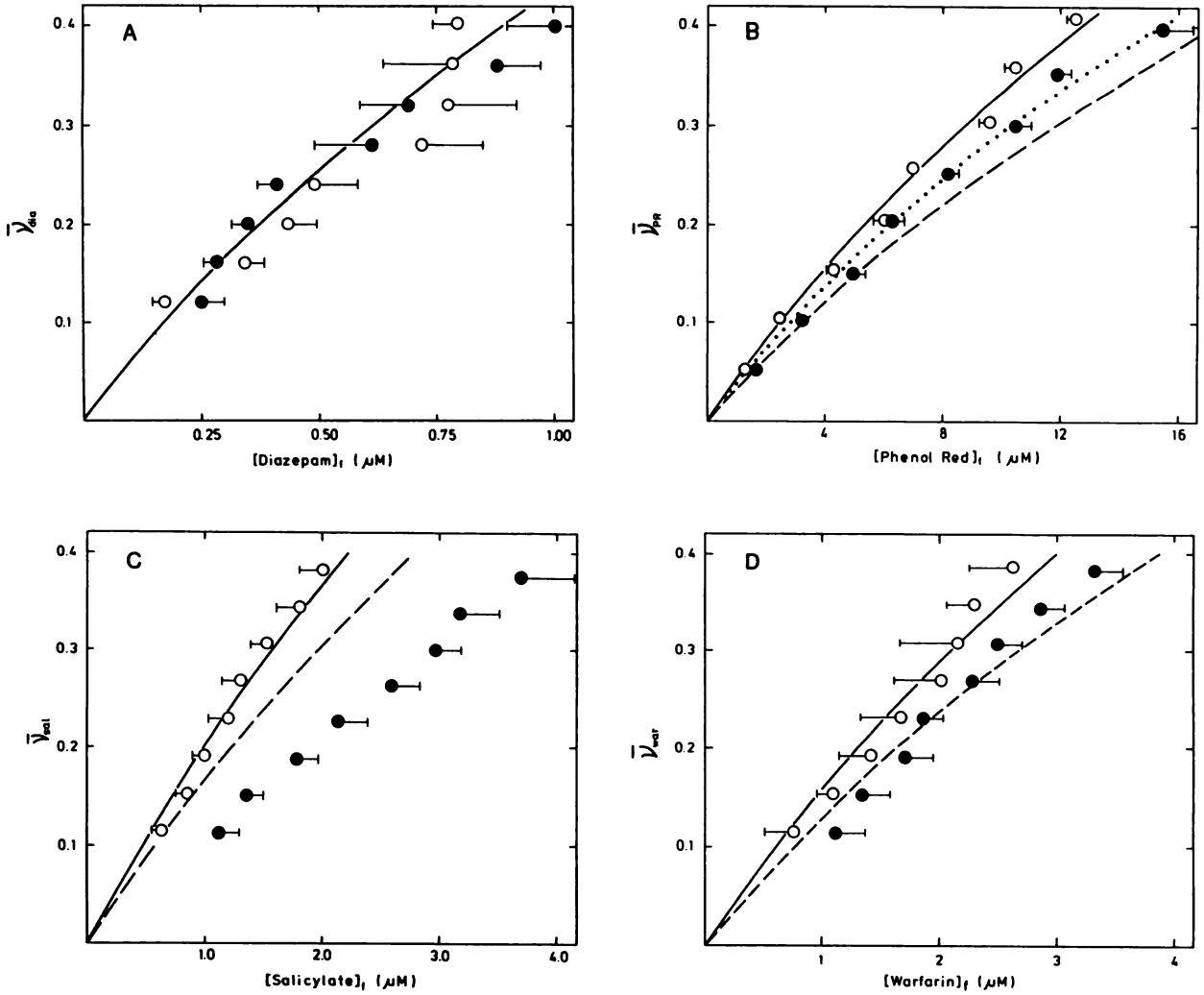


Fig. 5. Binding of diazepam (A), phenol red (B), salicylate (C), and warfarin (D) to human serum albumin in the absence (○) and in the presence (●) of azapropazone. When present, the concentration of total azapropazone was 0.184 mM (A) or 0.123 mM (B, C, D). The *unbroken curves* describe binding of marker ligand alone and were computed by using the *n* and *K* values given in Table 1. The *broken curves* in B, C, and D are theoretical curves calculated assuming competition for common high affinity binding sites. The *dotted curve* in B is a theoretical curve made assuming ligand binding to separate high affinity binding sites with mutual interactions. For further experimental and theoretical details, see the text. The data shown are average values (± standard deviation) of three to five experiments performed in duplicate.

TABLE 1

Ligand binding to defatted human serum albumin

n_1, n_2, and n_3 are the numbers of binding sites in the first, second, and third binding class, respectively, and K_1, K_2, and K_3 represent the corresponding association constants. Ligand binding was studied at pH 7.0 over the following concentration ranges: azapropazone, 0.053–1.050 mM; phenylbutazone, 0.048–1.035 mM; chlorpropamide, 0.054–1.285 mM; sulfathiazole, 0.105–1.049 mM; diazepam, 0.037–0.121 mM; phenol red, 0.017–0.134 mM; sodium salicylate, 0.035–0.116 mM; and warfarin, 0.035–0.119 mM. The protein concentration was 0.60 mM.

Ligand	n_1	$10^{-6} \times K_1$	n_2	$10^{-4} \times K_2$	n_3	$10^{-3} \times K_3$
		M^{-1}		M^{-1}		M^{-1}
Azapropazone	1	2.8	1	6.5	2	10.0
Phenylbutazone	1	7.0	1	9.0	3	6.0
Chlorpropamide	1	3.3	1	6.0	3	2.8
Sulfathiazole	1	0.25	5	0.53	ND[a]	ND
Iophenoxate[b]	1	770	1	38	1	30
Diazepam	1	6.6	2	4.5	ND	ND
Phenol Red	1	0.41	5	0.19	ND	ND
Salicylate	1	1.9	4	1.6	ND	ND
Warfarin	1	1.7	1	5.0	ND	ND

[a] ND, not determined.
[b] Taken from Refs. 21 and 22.

ratios of 1.1 to 1. Furthermore, as evidence of independent binding (not shown), the azapropazone binding was found to be independent of the presence of diazepam; the concentration of free azapropazone was 3.4 ± 0.5 μM, irrespective of the concentration of diazepam.

Fig. 5B shows that binding of phenol red was inhibited to some extent by azapropazone ($\bar{\nu} \approx 0.4$). Furthermore, in these experiments the concentration of free azapropazone rose to 1.1–1.8 μM as a function of the phenol red concentration. The possibility that the mutual reduction in ligand binding can be described by a competitive mechanism was analyzed by the use of Eqs. 3a–4. However, as seen in Fig. 5B, this is not the case—only a few of the *filled circles* are close to the *broken curve*. Therefore, the reduction in dye binding is best characterized by a coupling constant (x). By the use of Eqs. 5a–7, x was calculated to be 0.6. As a control of the relevance of the coupling constant, a theoretical curve for phenol red binding was constructed. As seen in Fig. 5B, the *dotted curve* is in accordance with the experimental findings.

Fig. 5C shows that binding of salicylate to serum albumin is

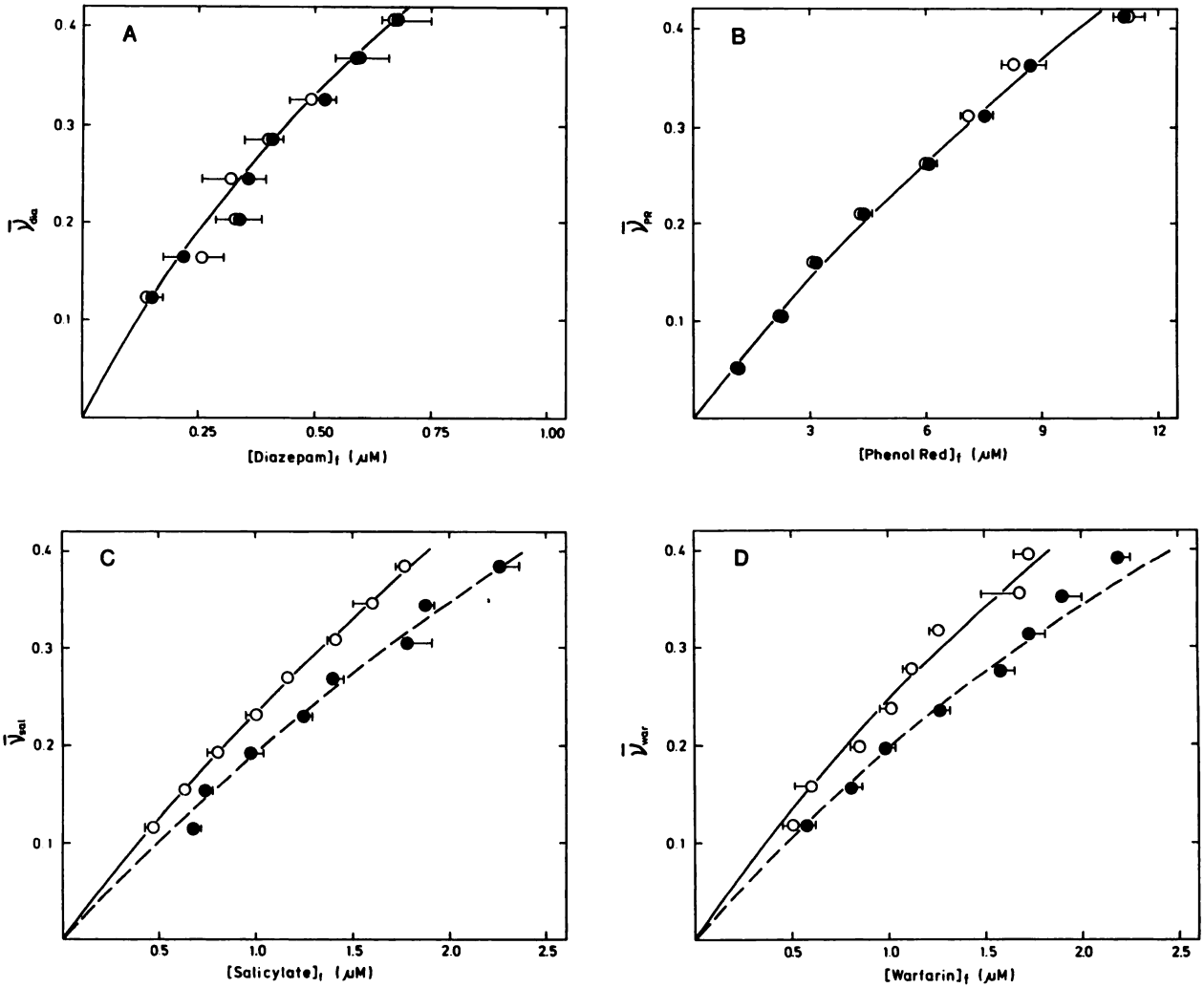


Fig. 6. Binding of marker ligands in the absence (○) and in the presence (●) of phenylbutazone. When present, the concentration of total phenylbutazone was 0.183 mM (A and B) or 0.120 mM (C and D). The *unbroken curves* describe binding of marker ligand alone and were computed by using the *n* and *K* values given in Table 1. The *broken curves* in C and D are theoretical curves calculated assuming competition for common high affinity binding sites. For further information, see legend to Fig. 5.

markedly reduced in the presence of a constant concentration of total azapropazone ($\bar{\nu} \approx 0.4$). The decrease in salicylate binding is even more pronounced than expected from a competitive mechanism, i.e., the binding represented by the *closed circles* is lower than that shown by the *broken curve* in the figure. Likewise, the concentration of free azapropazone rose from 3.5 to 4.7 μM as a function of the salicylate concentration. If a purely competitive binding mechanism between the high affinity sites had been operative, the concentration of free azapropazone should be about 1.4–1.9 μM. Thus, a surprisingly great mutual reduction of albumin binding was registered in this case.

Fig. 5D shows diminished warfarin binding that can be accounted for by a competitive scheme in the presence of a constant concentration of azapropazone ($\bar{\nu} \approx 0.4$). This is in agreement with Sjöholm and co-workers (8, 14), who found a pronounced displacing effect of azapropazone on warfarin binding and suggested common high affinity sites for the two drugs. Fehske *et al.* (20) also registered a mutual decrease in azapropazone-warfarin binding. Warfarin also reduced the binding of azapropazone, leading to a rise in the concentration of free azapropazone from 1.5 to 2.0 μM as a function of the warfarin concentration (expected for a competitive mechanism, from 1.4 to 1.8 μM).

Phenylbutazone. As shown in Fig. 6A, high affinity binding of diazepam was unaffected by the presence of a constant concentration of total phenylbutazone ($\bar{\nu} = 0.60$). Independent high affinity binding of the two drugs is supported by the finding that the concentration of free phenylbutazone was 1.5 ± 0.4 μM, irrespective of the concentration of diazepam. Fehske *et al.* (20) previously reported a reduction in diazepam binding (from 85.8 to 83.9%) after addition of phenylbutazone. However, this is a small decrease and these authors, in contrast to the present work, used drug to protein molar ratios above unity (1.1:1).

Fig. 6B shows that neither was high affinity binding of phenol red altered by addition of phenylbutazone ($\bar{\nu} = 0.59$). Conversely, the concentration of free phenylbutazone (1.8 ± 0.3 μM) was unaffected by the presence of phenol red.

By contrast, salicylate binding is diminished in the presence of a constant concentration of total phenylbutazone ($\bar{\nu} \approx 0.4$)—cf. Fig. 6C. As shown by the *broken curve*, the phenylbutazone-induced reduction in salicylate binding can be described by competition for a common high affinity binding site. Further-

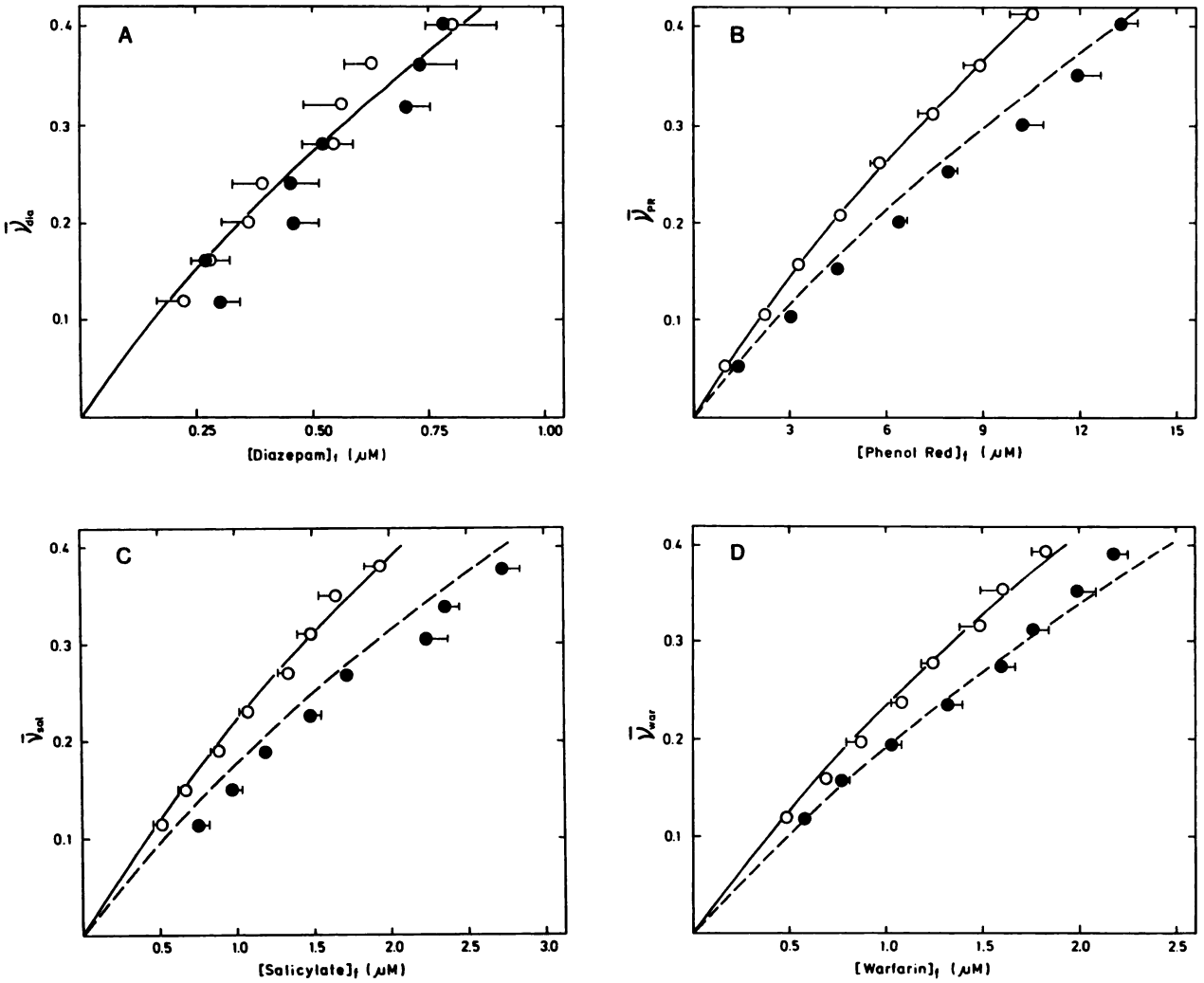


Fig. 7. Binding of marker ligands in the absence (○) and in the presence (●) of chlorpropamide. When present, the concentration of total chlorpropamide was 0.181 mM (A) or 0.121 mM (B, C, and D). The *unbroken curves* describe binding of marker ligand alone and were computed by using the *n* and *K* values given in Table 1. The *broken curves* in B, C, and D are theoretical curves calculated assuming competition for common high affinity binding sites. For further information, see legend to Fig. 5.

more, the concentration of free phenylbutazone varied from 0.8 to 1.2 μM as a function of the salicylate concentration (expected, 0.5–0.8 μM). In agreement with these findings Otagiri and Perrin (25) in circular dichroism studies registered a mutual displacement of albumin-bound phenylbutazone and salicylate.

In agreement with several previous studies (e.g., Refs. 6–8, 20, 26, and 27), Fig. 6D shows depressed warfarin binding in the presence of a constant concentration of total phenylbutazone ($\bar{\nu} \approx 0.4$). The present detailed calculations showed that the reduction in warfarin binding is compatible with a competitive mechanism. It is in accordance with this finding that the free phenylbutazone concentration rose to 0.7–0.9 μM as a function of the warfarin concentration (expected, 0.6–0.8 μM).

Chlorpropamide. On the basis of the results depicted in Fig. 7A, independent high affinity binding of diazepam and chlorpropamide ($\bar{\nu} = 0.56$) is proposed. In accordance with this proposal, it was found that the concentration of free chlorpropamide was independent of the diazepam concentration; 2.5 ± 1.6 μM. By contrast, addition of a constant concentration of total chlorpropamide ($\bar{\nu} \approx 0.4$) markedly diminished the binding of all the other marker ligands: phenol red (Fig. 7B), salicylate (Fig. 7C), and warfarin (Fig. 7D). In all situations the reduction in marker ligand binding corresponded to, or was slightly more pronounced than, that calculated on the assumption of competitive ligand binding at the high affinity sites alone. The concentrations of free chlorpropamide were also increased in the presence of these marker ligands to an extent compatible with competitive binding, in the presence of phenol red from 1.3 to 2.0 μM, in salicylate from 1.2 to 2.1 μM, and in warfarin from 1.1 to 1.5 μM. Sjöholm *et al.* (8) have carried out displacement studies with chlorpropamide, diazepam, and warfarin and suggested that chlorpropamide is bound to the same site as that for warfarin.

Sulfathiazole. Fig. 8A shows independent high affinity binding of diazepam and sulfathiazole. In support of this finding, the concentration of free sulfonamide was 13.7 ± 0.7 μM ($\bar{\nu} = 0.58$), irrespective of the concentration of marker ligand.

From Fig. 8B it is seen that sulfathiazole ($\bar{\nu} \approx 0.4$) decreases phenol red binding to a level well described by a competitive mechanism. The concentration of free sulfathiazole is also increased to an extent compatible with such a binding mechanism, from 13.6 to 21.2 μM, depending on the dye concentration (expected, 13.6 to 18.5 μM).

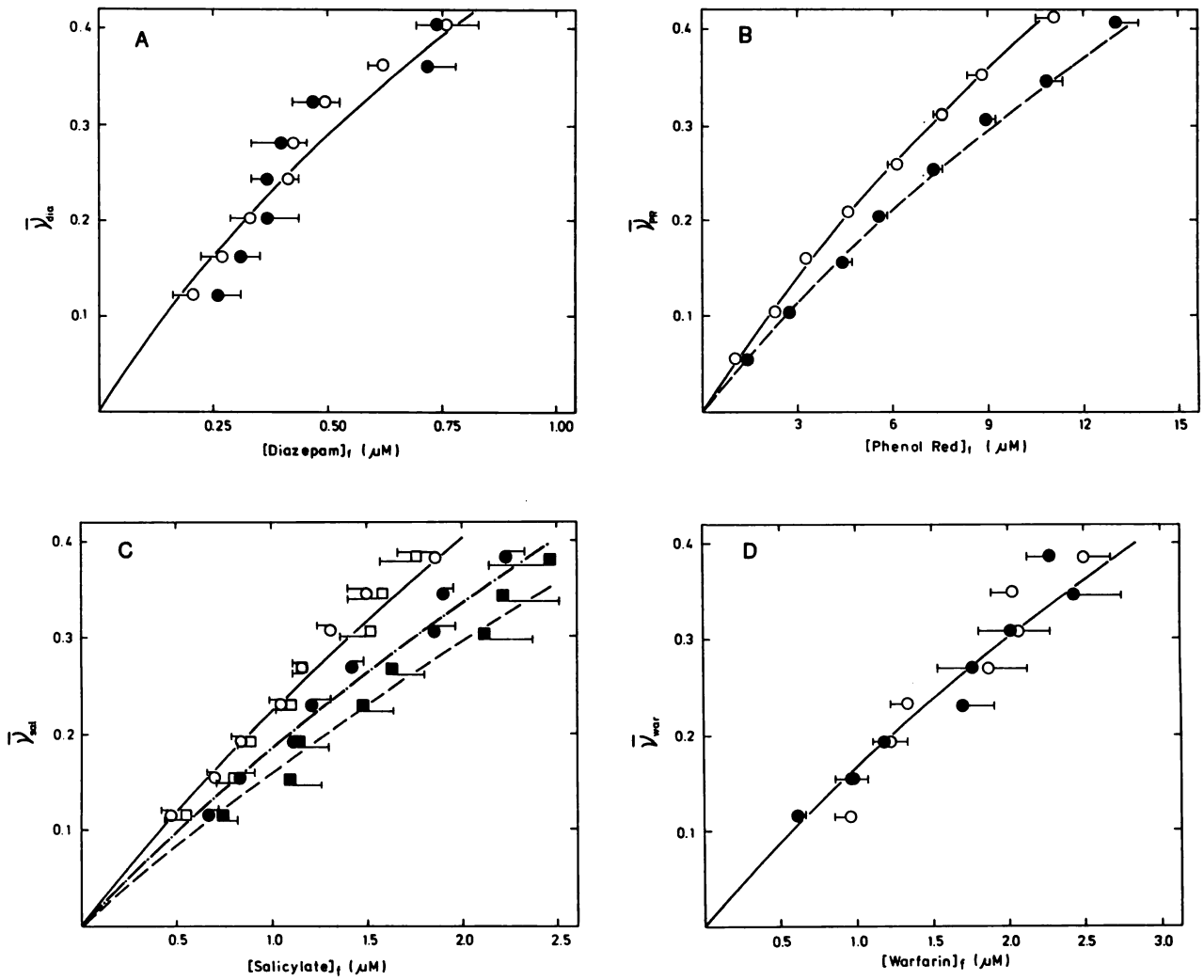


Fig. 8. Binding of marker ligands in the absence (○, □) and in the presence (●, ■) of sulfathiazole. When present, the concentration of total sulfathiazole was 0.200 mM [A, D, and C (■)] or 0.130 mM [B and C (●)]. The *unbroken curves* describe binding of marker ligand alone and were computed by using the *n* and *K* values given in Table 1. The *broken curve* in B and those of C, calculated for addition of 0.200 mM (– – –) and 0.130 mM sulfathiazole (– · –), are theoretical curves calculated assuming competition for common high affinity binding sites. For further information, see legend to Fig. 5.

The results represented by the *circles* in Fig. 8C reveal that sulfathiazole ($\bar{\nu} \approx 0.4$) also reduces salicylate binding to its primary site in accordance with a competitive scheme. Due to the relatively large scatter of these data, the experiments were repeated with an additional concentration of total sulfathiazole ($\bar{\nu} \approx 0.6$). As shown by the *squares* in the figure, the results of these binding studies also support the suggestion of competitive binding. The concentration of free sulfonamide in the two sets of experiments were 17.4–20.7 and 31.4–34.2 μM, respectively. These figures are comparable to those calculated theoretically, assuming a competitive binding mechanism (14.3–17.6 and 30.3–36.7 μM, respectively). The work of Perrin and Nelson (28) suggests, although in an indirect way, that sulfathiazole and salicylate bind to a common primary site.

On the other hand, Fig. 8D clearly shows that warfarin binding is independent of the presence of sulfathiazole ($\bar{\nu} = 0.60$). It is in accordance with this observation that the concentration of free sulfathiazole (14.4 ± 1.1 μM) was unchanged by the presence of warfarin.

Iophenoxate. Fig. 9A shows binding of diazepam to serum albumin both in the absence and in the presence of a constant concentration of total iophenoxate ($\bar{\nu} \approx 0.6$). For the first time during the course of this study, we observed reduced diazepam binding. This finding seems to be in accordance with the data of Fehske *et al.* (20), who measured a reduction in diazepam binding from 85.8 to 81.1%, when both ligands were added to human serum albumin in molar ratios of 1.1 to 1. However, calculations revealed that the reduction in diazepam binding was not as pronounced as expected for a competitive binding mechanism (Fig. 9A). Therefore, although the concentration of free iophenoxate was not determined (cf. the arguments given in the section dealing with single ligand binding), the diminished diazepam binding was sought quantitated by using a coupling constant. In doing so, it was assumed that all iophenoxate added to the albumin-containing solutions was bound to the high affinity drug binding site. (Calculations, carried out by using the binding data given in Table 1, indicated that only about 0.02% of the drug is unbound under these conditions. Furthermore, K_1 is more than 200-fold greater than K_2). As seen by the *dotted curve* in Fig. 9A, a coupling constant of 0.8 is compatible with the experimental findings.

As seen from Fig. 9B, addition of iophenoxate ($\bar{\nu} \approx 0.6$) reduced the binding of phenol red, but also not to the extent

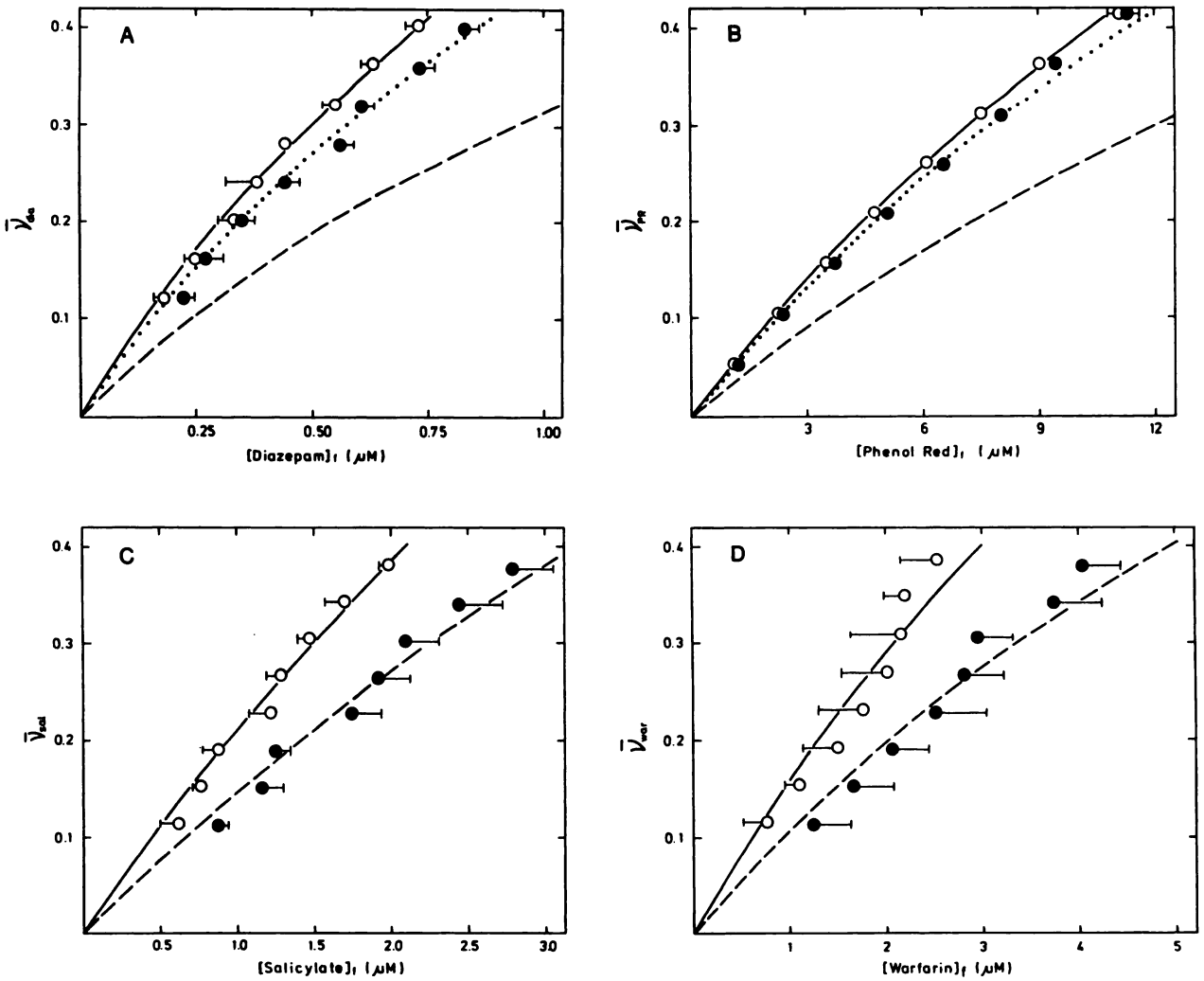


Fig. 9. Binding of marker ligands in the absence (○) and in the presence of iophenoxate (●). When present, the concentration of total iophenoxate was 0.184 mM. The *unbroken curves* describe binding of marker ligand alone and were computed by using the *n* and *K* values given in Table 1. The *broken curves* are theoretical curves calculated assuming competition for common high affinity binding sites, and the *dotted curves* in A and B are other theoretical curves made assuming ligand binding to separate high affinity binding sites with mutual interactions. For further information, see legend to Fig. 5.

expected for competitive binding. A coupling constant of 0.9 was calculated, and it is seen in Fig. 9B that the theoretical curve constructed using this value is in accordance with the experimental data.

By contrast, iophenoxate reduced binding of both salicylate (Fig. 9C) and warfarin (Fig. 9D) according to competitive mechanisms. In these experiments the concentration of competitor (iophenoxate) was greater ($\bar{\nu} \approx 0.6$) than usual ($\bar{\nu} \approx 0.4$). This could be done, because the problem of ligand binding to secondary sites is negligible even at this elevated concentration in the case of this ligand.

Sudlow *et al.* (6), using a fluorescence technique, as well as Fehske *et al.* (20), using equilibrium dialysis, registered a pronounced displacement of albumin-bound warfarin after addition of iophenoxate. Both groups of investigators suggested a common high affinity binding site for the two ligands.

Discussion

In the present work, a series of competition experiments was performed in order to investigate the relations between the high affinity binding sites for azapropazone, phenylbutazone, chlorpropamide, sulfathiazole, and iophenoxate and the binding regions of human serum albumin represented by the marker ligands diazepam, phenol red, salicylate, and warfarin (9, 24). The results of the studies involving diazepam clearly showed the existence of at least two different binding regions for high affinity drug binding, namely one to which diazepam binds and at least one with which the other ligands interact. The existence of a discrete binding region including the high affinity site for diazepam has previously been proposed. That is the region called the indole and benzodiazepine binding site (see, e.g., Refs. 1 and 9) or site II (6). Often high affinity binding of diazepam to that region takes place independently of high affinity binding of ligands to other regions. This is the case for simultaneous binding with, for example, azapropazone (Fig. 5A), phenylbutazone (Fig. 6A), chlorpropamide (Fig. 7A), sulfathiazole (Fig. 8A), warfarin (8, 9), digitoxin (8, 9), and bilirubin (29). However, exceptions exist, e.g., diazepam-iophenoxate (Fig. 9A). The explanation for the last-mentioned finding is probably ligand-induced conformational changes of the protein molecule.

To which region(s) and to what part(s) of the albumin

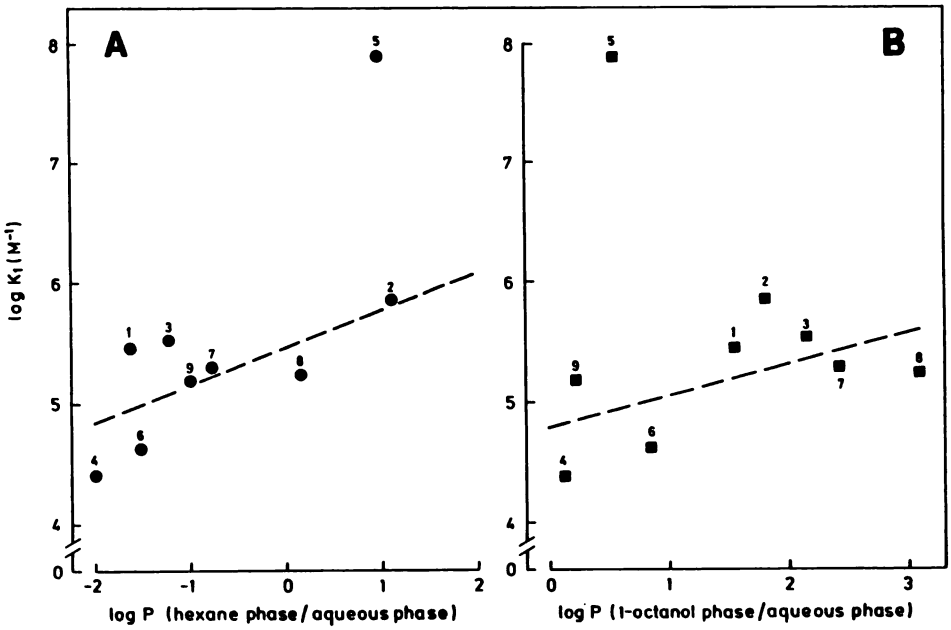


Fig. 10. Correlations between primary association constants and partition coefficients for hexane-aqueous media (A) and octanol-aqueous media (B). The acidity of the aqueous media was such that the various ligands were in their unionized form. The results are presented according to Eq. 8 and can be described by the following regression lines, when excluding the results of iophenoxate: *A*, log K_1 = 0.313 log P + 5.458 (r = 0.678); and *B*, log K_1 = 0.263 log P + 4.790 (r = 0.586). In order to make the analyses more complete, the results of digitoxin are included (K_1 is taken from Ref. 9). The ligands are numbered as follows. **1**, azapropazone; **2**, phenylbutazone; **3**, chlorpropamide; **4**, sulfathiazole; **5**, iophenoxic acid; **6**, phenol red; **7**, salicylic acid; **8**, warfarin; and **9**, digitoxin.

molecule can the primary site for the other ligands be assigned? It is very unlikely that the drugs bind with a high affinity to the primary site for long chain fatty acid ions or to that for divalent cations (Cu^{2+} and Ni^{2+}) (2). It is generally agreed that the fatty acids bind to a special region in the C-terminal domain of serum albumin (1, 2, 4, 30) and that Cu^{2+} and Ni^{2+} are primarily bound to a special site in the N-terminal end of the protein (2, 4, 31).

In a previous publication (9), it was possible to account for the ligand binding data published until then from this and other laboratories in terms of three binding regions in addition to those just mentioned above. Marker ligands for these regions were phenol red (or bilirubin), salicylate (or digitoxin), and warfarin. The scope of the present study was to investigate whether these three binding regions could also accommodate the drugs studied here. This was affirmed by ample evidence for competitive binding of the drugs and the three marker ligands. Although instances of independent binding were noted, the most striking observation was the pronounced interdependence between binding of the drugs and one or more marker ligands. This was in contrast to the diazepam site which, as already mentioned, was only affected to a slight extent by one of the ligands, namely iophenoxate. For example, phenylbutazone, azapropazone, and iophenoxate all competitively displaced salicylate as well as warfarin from their respective binding sites, and addition of chlorpropamide led to competitive displacement of all three marker ligands from the albumin. Although competitive binding does not necessarily denote binding to the same site, but merely expresses the absence of coexisting binding of pairs of ligands (2), it seems highly unlikely that displacement could have occurred to this extent by conformational changes and electrostatic interactions alone. This would have to be assumed if phenol red, warfarin, and salicylate were bound at entirely different locations in the albumin molecule. Instead the present data suggest that the high affinity binding sites for phenol red, warfarin, and salicylate are situated quite close to each other in a common binding region. The proposed region is sufficiently large to accommodate at least two ligands at the same time inasmuch as examples of independent high affinity binding of pairs of ligands can be given. A detailed consideration suggests that the data could be explained by sets of overlapping binding sites, e.g., the site for phenylbutazone overlapping with the sites for salicylate and warfarin, and that for sulfathiazole with those for phenol red and salicylate. However, such a detailed interpretation is probably not warranted because of the likelihood of conformational and other interactions between closely spaced ligands. Another reason to caution against strict competition for well defined binding sites is the chemical diversity of ligands bound in this region.

What then is the structural basis and by what means are ligands bound in this region? According to studies of affinity labeling and of ligand binding by chemically modified albumins and serum albumin fragments, several high affinity sites for aromatic ligands seem to be associated with subdomains 1C and 2A-B in the protein model of Brown (2). Therefore it is tempting to suggest that these subdomains are essential components in the formation of the hypothetical binding region.

Given the chemical diversity of ligands bound to this region, it is of interest to evaluate the importance of hydrophobic forces in the binding processes. Therefore, we have determined partition coefficients for the ligands under study both in the very hydrophobic solvent *n*-hexane (Fig. 10A) and in 1-octanol (Fig. 10B). In the figures the data are presented according to the following relationship (32, 33):

$$\log K_1 = a \log P + b \qquad (8)$$

where K_1 and P are the primary association constants (taken from Table 1 and Ref. 9) and the partition coefficients, respectively, and a and b are constants. In Fig. 10, both A and B, is seen a general increase of log K_1 as log P increases. This finding illustrates that hydrophobic forces are important for the ligand binding processes. However, because the correlations between log K_1 and log P are relatively poor, other binding forces must be operative as well (the correlation coefficients, r, for the regression lines in Fig. 10, A and B are, when excluding the results of iophenoxate, 0.678 and 0.586, respectively). Because the partition coefficients for octanol-aqueous media were al-

ways higher than those for hexane-aqueous media, hydrogen-bonding and dipolar effects are probably also of importance for ligand binding. However, because the correlation between log K_1 and log P for octanol-aqueous media is also poor, still other binding forces must exist between the ligands in question and the protein. These are probably of a steric and an electrostatic nature.

Helmer *et al.* (33) found a good correlation ($r \approx 0.95$) when plotting binding parameters for the interaction between a great number of different organic ligands and bovine serum albumin as a function of their octanol-water partition coefficients. On the basis of this finding, it was proposed that ligand binding to bovine serum albumin is of a hydrophobic and nonspecific nature (33). However, the study of these authors, in contrast to the present one, was mainly based on low affinity ligands.

In conclusion, human serum albumin seems to possess four well separated binding regions, namely one for high affinity binding of long chain fatty acid ions, one for diazepam (an example only), and one for Cu^{2+} and Ni^{2+}. In addition a large and rather flexible binding region for high affinity binding of several diverse ligands seems to exist with a location in the albumin molecule that can tentatively be assigned to subdomains 1C and 2A-B.

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