Biochem. J. (1983) **209**, 135–142
Printed in Great Britain

Relations between high-affinity binding sites for L-tryptophan, diazepam, salicylate and Phenol Red on human serum albumin

Ulrich KRAGH-HANSEN
Institute of Medical Biochemistry, University of Aarhus, DK-8000 Aarhus C, Denmark

(*Received 14 June 1982/Accepted 21 September 1982*)

Binding of L-tryptophan, diazepam, salicylate and Phenol Red to defatted human serum albumin was studied by ultrafiltration at pH 7.0. All ligands bind to one high-affinity binding site with association constants of the order of 10^4–$10^5\,\text{M}^{-1}$. The number of secondary binding sites was found to vary from zero to five, with association constants about $10^3\,\text{M}^{-1}$. Competitive binding studies with different pairs of the ligands were performed. Binding of both ligands was determined simultaneously. L-Tryptophan and diazepam were found to compete for a common high-affinity binding site on albumin. The following combinations of ligands do not bind competitively to albumin: L-tryptophan–Phenol Red, L-tryptophan–salicylate and Phenol Red–salicylate. On the other hand, high-affinity bindings of the three ligands do not take place independently but in such a way that binding of one of the ligands results in a decrease in binding of the other ligands. The decreases in binding are reciprocal and can be accounted for by introducing a coupling constant. The magnitude of the constant is dependent on the ligands being bound. In the present study, the mutual decrease in binding was more pronounced with L-tryptophan–salicylate and Phenol Red–salicylate than with L-tryptophan–Phenol Red.

Serum albumin is characterized by a capability to bind extensively a great number of different compounds in a reversible manner. The unique binding properties of the protein are usually explained by the presence of several discrete binding regions (Peters, 1975; Sjöholm *et al.*, 1979; Fehske *et al.*, 1981; Kragh-Hansen, 1981*b*). However, modulatory effects among bound ligands are presumably also important for the binding properties of the protein. Before assignment of the high-affinity binding sites of two different ligands to the same binding region on the protein, it is essential to determine whether a decrease in binding of one of the ligands caused by simultaneous addition of the other ligand is the result of competition or of modulatory effects. Another important aspect, when ligand–serum albumin interactions are being studied, is that ligands usually bind not only to one or two high-affinity binding sites but also to a greater number of low-affinity binding sites.

In order to test the correctness of some important elements of the albumin binding model proposed previously (Kragh-Hansen, 1981*b*), binding studies involving L-tryptophan, diazepam, salicylate and Phenol Red, separately and in different combinations, and defatted human serum albumin were performed. To ensure an accurate analysis of the competitive binding studies, the following precautions were taken: (1) the number of mol of ligand bound per mol of protein was 0.4 or less, to minimize ligand binding to secondary sites, and (2) bindings of both ligands were determined simultaneously.

Experimental

Materials

Human serum albumin (98% pure) was obtained from AB Kabi, Stockholm, Sweden. L-Tryptophan was a product of Cambrian Chemicals, Croydon, Surrey, U.K. Phenol Red and salicylic acid (sodium salt) were purchased from Merck, Darmstadt, Germany. Diazepam (100% pure according to the manufacturer) was a gift from Dumex, Copenhagen, Denmark. L-[5-^{3}H]Tryptophan (sp. radioactivity 20 or 26 Ci/mmol) and [2-^{14}C]diazepam (sp. radioactivity 57 mCi/mmol) were supplied by Amersham International, Amersham, Bucks., U.K. [7-^{14}C]Salicylic acid (sp. radioactivity 51.7 mCi/mmol) was bought from New England Nuclear, Boston, MA, U.S.A. All other reagents were of A.R. grade.

Albumin was defatted by using the charcoal method described by Chen (1967). The aqueous

0306-3275/83/010135-08$02.00 © 1983 The Biochemical Society

solutions of defatted albumin were freeze-dried, and the resulting powder was stored in a desiccator at 4°C until use.

Binding of individual ligands to albumin

All experiments were performed in media containing 33mM-sodium phosphate buffer, pH 7.0, at 20°C. In most cases the concentration of human serum albumin (mol.wt. 66300) was 0.38mM (2.5%, w/v). Samples of volume 10ml containing L-tryptophan + [^{3}H]tryptophan, Phenol Red, salicylate + [^{14}C]salicylate or diazepam + [^{14}C]diazepam with and without albumin were prepared. The samples without albumin were controls representing 100% free ligand.

The binding percentages were determined by ultrafiltration. Protein and reference solutions (4ml) were enclosed in cellophan bags (Visking, 18mm diameter) and placed in plastic tubes containing two compartments (Kragh-Hansen *et al.*, 1972). Ultrafiltrate (200–300μl), formed by centrifuging the tubes at 320*g* for 1–1½h at 20°C in a Christ IV KS refrigerating centrifuge, was collected in the lower part of the tubes. Control experiments indicated that all four ligands were fully filterable. The binding percentages of the ligands were calculated from the concentrations of the ligand in the ultrafiltrate from the albumin-containing solutions and the corresponding reference solutions (Kragh-Hansen *et al.*, 1972). The concentrations of L-tryptophan, salicylate and diazepam were determined by liquid-scintillation counting. Ultrafiltrate of volume 150μl was added to 10ml of Luma Gel (Lumac Systems, Basel, Switzerland) and its radioactivity counted in a Packard Tri-Carb spectrometer. The concentration of Phenol Red was determined spectrophotometrically. A portion (typically 200μl) of the ultrafiltrate was diluted with a suitable volume of 10mM-NaOH, and the absorbance was read at 559nm on a Zeiss PM2 DL Spektralphotometer.

Binding of pairs of ligands to albumin

Samples of volume 10ml, with and without albumin (0.38mM), were prepared. Simultaneous binding of L-tryptophan–Phenol Red and of salicylate–Phenol Red was studied. Depending on the dye concentration, the radioactivity of [^{3}H]tryptophan was quenched by 0–40% and that of [^{14}C]-salicylate by 0–10% in the presence of Phenol Red. Corrections for the quenching were made on the basis of the concentration of Phenol Red. The presence of L-tryptophan or salicylate did not influence the spectrophotometric determination of the Phenol Red concentration.

Simultaneous binding of L-tryptophan–diazepam and of L-tryptophan–salicylate to albumin was also studied. The diazepam-containing samples contained 0.1–0.5% ethanol originating from stock solutions of the drug. The concentration of each radioactive ligand in ultrafiltrates containing mixtures of ^{3}H- and ^{14}C-labelled compounds were determined by using two channels of a Packard Tri-Carb spectrometer.

Results

Binding of individual ligands to defatted human serum albumin

As a preliminary to the study of ligand interactions, the bindings of L-tryptophan, Phenol Red, salicylate and diazepam to defatted human serum albumin were studied individually. The number of binding sites (n) and the corresponding association constants (K) were calculated by use of the double-reciprocal-plot method of Klotz (1946) as previously described (Kragh-Hansen, 1981*a*). The results obtained are summarized in Table 1. All four ligands bind to only one high-affinity binding site on albumin, with association constants of the order of 10^4–10^5M^{-1}. n_1 and K_1 calculated for the binding of L-tryptophan are the same as those reported by McMenamy & Oncley (1958). These values (Table 1) are comparable with those published by other authors (Müller & Wollert, 1975; Kober *et al.*, 1978; Bruderlein & Bernstein, 1979). n_1 and K_1 for salicylate binding were found to be 1 and 4.6×10^5M^{-1} respectively (Table 1). Keresztes-Nagy *et al.* (1972), using equilibrium dialysis and frontal gel chromatography, and Brown & Crooks (1976), using dynamic dialysis, also reported $n_1 = 1$ for salicylate binding. In those studies K_1 was determined to be 7.1×10^4M^{-1} and 2.2×10^5M^{-1} respectively. By contrast, Hultmark *et al.* (1975), using equilibrium dialysis, calculated that n_1 for salicylate binding is 2 ($K_1 = 1.3 \times 10^5$M^{-1}). In the present study, n_2 was determined to be 4 and K_2 was found

Table 1. *Ligand binding to defatted human serum albumin*

n_1 and n_2 are the numbers of binding sites in the first and second binding class respectively, and K_1 and K_2 represent the corresponding association constants. The ligand binding was studied over the following concentration ranges: L-tryptophan, 0.047–0.188mM; Phenol Red, 0.033–8.475mM; salicylate, 0.038–1.885mM; diazepam, 0.069–0.710mM. The pH was 7.0. For full experimental details see the text. The values given in the Table are the averages for six determinations (± S.D.).

Ligand	n_1	$10^{-4} \times K_1$ (M^{-1})	n_2	$10^{-3} \times K_2$ (M^{-1})
L-Tryptophan	0.95 ± 0.15	1.6 ± 0.2		
Phenol Red	1.00 ± 0.02	3.9 ± 0.1	5.1 ± 0.1	1.0 ± 0.2
Salicylate	1.03 ± 0.04	46 ± 2	3.9 ± 0.2	4.9 ± 0.3
Diazepam	1.01 ± 0.05	47 ± 3	2.2 ± 0.2	1.9 ± 0.3

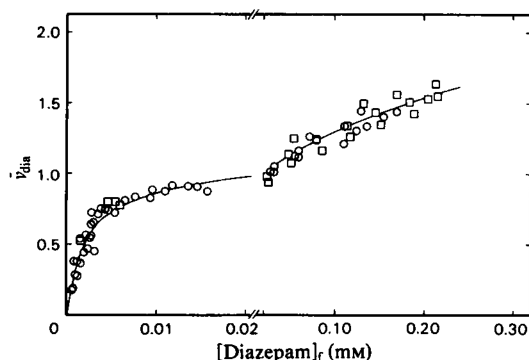


Fig. 1. *Binding of diazepam to human serum albumin*
The concentration of albumin was 0.38mM (○) or 0.19mM (□). The experiments were performed in 33mM-sodium phosphate buffer, pH 7.0, at 20°C. For full experimental details see the text. The binding curve is constructed on the basis of the number of binding sites and the corresponding association constants given in Table 1. $\bar{\nu}_{dia}$ and $[\text{Diazepam}]_f$ represent the average number of mol of diazepam bound per mol of albumin and the concentration of free diazepam respectively. Each point represents the result of a duplicate experiment.

to be two orders of magnitude lower than K_1 (Table 1). This is also essentially in agreement with other data found in the literature. n_1 and K_1 calculated for the binding of Phenol Red to albumin are identical with the values previously presented (Kragh-Hansen, 1981*a*).

Fig. 1 shows the binding curve for diazepam. Because of the relatively low solubility of diazepam in aqueous media the drug was added to the test solutions as small portions of a diazepam/ethanol stock solution. The possibility that diazepam is not completely dissolved in the final solutions was tested by determining the drug binding at fixed diazepam/albumin molar ratios but at different concentrations. As shown in Fig. 1, the binding curves obtained with 1.25% and 2.5% albumin solutions are identical, indicating that the drug is fully dissolved under the present conditions. n_1 and K_1 were calculated to be 1 and $4.7 \times 10^5\,\text{M}^{-1}$ respectively (Table 1). These values are very close to those reported by Müller & Wollert (1973), who used gel filtration: $n_1 = 1$ and $K_1 = 4.9 \times 10^5\,\text{M}^{-1}$. Sjödin *et al.* (1976) and Brodersen *et al.* (1977), using circular dichroism, and Kober *et al.* (1978), using microparticles and equilibrium dialysis, found $n_1 = 1$ and $K_1 = 1.8 \times 10^5\,\text{M}^{-1}$. Müller & Wollert (1973) used a medium containing 67mM-phosphate buffer, pH 7.4, whereas the other authors mentioned used 5mM-sodium phosphate buffer, pH 7.4, containing 100mM-KCl. The relatively low K_1 values reported by Sjödin *et al.* (1976), Brodersen *et al.* (1977) and Kober *et al.* (1978) could be caused by displacement of diazepam by the high concentration of Cl^- ions (cf. Kragh-Hansen, 1981*b*).

On the basis of the results shown in Fig. 1, n_2 and K_2 were calculated to be 2 and $1.9 \times 10^3\,\text{M}^{-1}$ respectively. Müller & Wollert (1973) and Sjödin *et al.* (1976) did not comment on the existence of secondary binding sites. However, Brodersen *et al.* (1977) suggested the existence of such binding sites but did not determine n_2 and K_2.

Binding of pairs of ligands to defatted human serum albumin

L-*Tryptophan–diazepam.* Binding of various concentrations of L-tryptophan to albumin in the presence of a constant concentration of diazepam is shown in Fig. 2(*a*) (▼ symbols). In these experiments the concentration of free drug varied from 0.40μM to 0.59μM, depending on the L-tryptophan concentration. Fig. 2(*b*) shows the converse experiments (▽ symbols), i.e. binding of various concentrations of diazepam in the presence of a constant concentration of L-tryptophan. The free concentration of the amino acid varied from 0.135mM to 0.189mM, depending on the diazepam concentration. In Figs. 2(*a*) and 2(*b*) the dotted curves represent binding of L-tryptophan and diazepam respectively in the absence of the other ligand (Table 1). If the two ligands bind independently to albumin, the experimental points should follow these curves. This is obviously not the case; binding of L-tryptophan decreases the binding of diazepam and vice versa.

The possibility that the diminished binding represents competition for a common high-affinity binding site on albumin was analysed by the following equations:

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

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

In these general formulations $\bar{\nu}_A$ and $\bar{\nu}_B$ are the average numbers of mol of A and B bound per mol of protein respectively, K_A and K_B are the association constants of ligand A and B respectively, and $[A_f]$ and $[B_f]$ are the concentrations of the free forms of the ligands. In the present context A and B stand for L-tryptophan and diazepam respectively. With the knowledge of the concentration of total diazepam (Fig. 2*a*) or of L-tryptophan (Fig. 2*b*), the association constants of the ligands (Table 1) and the concentration of albumin, it is possible, by the use of the above-mentioned relationships, to calculate $\bar{\nu}_{Trp}$ as a function of the concentration of free L-tryptophan (Fig. 2*a*) and to calculate $\bar{\nu}_{dia}$ as a function of the concentration of free diazepam (Fig. 2*b*). Fig. 2 shows that the experimental points are well described by the calculated binding curves

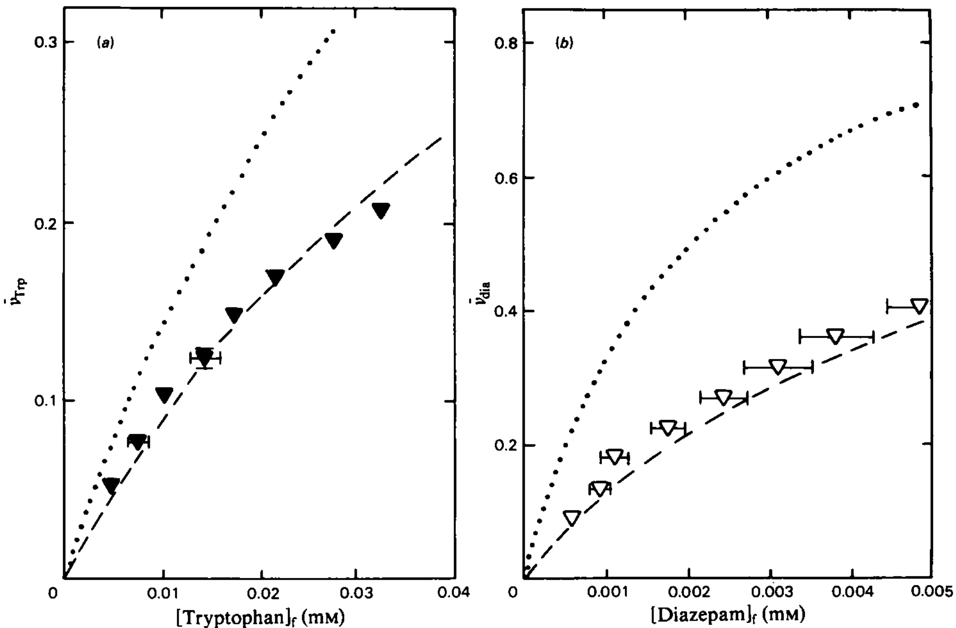
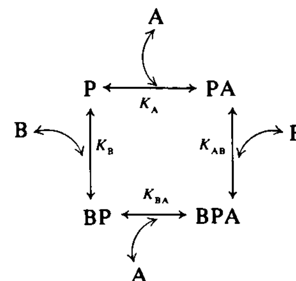


Fig. 2. *Binding of* L*-tryptophan and diazepam to human serum albumin*
(*a*) Binding of L-tryptophan (0.025–0.110mM) to albumin (▼) in the presence of diazepam (0.137mM). (*b*) Binding of diazepam (0.035–0.156mM) to albumin (▽) in the presence of L-tryptophan (0.376mM). The concentration of albumin was 0.38mM, and the media contained 33mM-sodium phosphate buffer, pH7.0, at 20°C. For full experimental details see the text. The dotted curves (••••••) represent binding of L-tryptophan (*a*) and diazepam (*b*) without addition of the other ligand. The broken curves (––––) are theoretically calculated curves for binding of L-tryptophan (*a*) and diazepam (*b*) assuming competition of the ligands for a common high-affinity binding site on the protein. In the case of binding of diazepam the small contribution from ligand binding to low-affinity sites is added. $\bar{\nu}_{Trp}$ and $\bar{\nu}_{dia}$ represent the average numbers of mol of L-tryptophan and diazepam bound per mol of albumin respectively, and [Tryptophan]$_f$ and [Diazepam]$_f$ the concentrations of free L-tryptophan and free diazepam respectively. The points show the average of three duplicate experiments.

(broken curves). These findings indicate that L-tryptophan and diazepam compete for a common high-affinity binding site on albumin.

L-*Tryptophan–salicylate.* Binding of various concentrations of L-tryptophan to albumin in the presence of a constant concentration of salicylate is shown in Fig. 3(*a*) (▲). The concentration of free drug varied only slightly in these experiments. In Fig. 3(*b*) the results of the converse experiments are shown (△), i.e. binding of various concentrations of salicylate to albumin in the presence of a constant concentration of L-tryptophan. The concentration of free amino acid varied from 0.145mM to 0.162mM. The Figures show that the two ligands do not bind independently to albumin. The broken curves in Figs. 3(*a*) and 3(*b*) are the theoretical binding curves for L-tryptophan and salicylate respectively, if the ligands compete for a common high-affinity binding site on albumin calculated as described in the preceding subsection. It is seen that the experimentally obtained data do not follow these theoretical curves. This means that the ligands bind to two different high-affinity binding sites on the protein. However, a mutual decrease in binding of the two ligands is observed. The following approach is an attempt to quantify this decrease.

Let us consider the following general scheme:



where P represents a protein possessing one binding site for ligand A and one binding site for ligand B. The various K values stand for association constants. If A and B bind independently to P, then K_{BA} is the same as K_A, and K_{AB} is equal to K_B. In all other situations $K_{BA} = x \cdot K_A$ and $K_{AB} = x \cdot K_B$, i.e. the

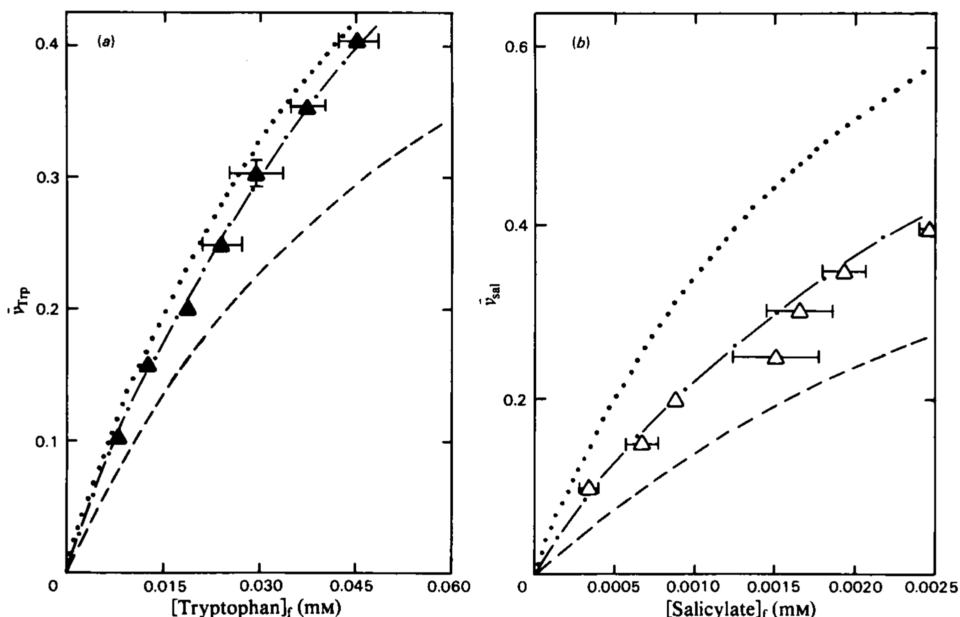


Fig. 3. *Binding of* L-*tryptophan and salicylate to human serum albumin*

(*a*) Binding of L-tryptophan (0.047–0.188 mM) to albumin (▲) in the presence of salicylate (0.113 mM). (*b*) Binding of salicylate (0.038–0.151 mM) to albumin (△) in the presence of L-tryptophan (0.376 mM). The concentration of albumin was 0.38 mM, and the media contained 33 mM-sodium phosphate buffer, pH 7.0, at 20°C. For full experimental details see the text. The dotted curves (······) represent binding of L-tryptophan (*a*) and salicylate (*b*) without addition of the other ligand. The broken curves (– – – –) are theoretically calculated curves for binding of L-tryptophan (*a*) and salicylate (*b*) assuming competition of the ligands for a common high-affinity binding site on the protein. —·—, Theoretical binding curves assuming ligand binding to separate high-affinity binding sites with mutual interactions (for further details see the text). In drawing the theoretical curves for salicylate binding the small contribution from ligand binding to low-affinity sites is added. $\bar{\nu}_{Trp}$ and $\bar{\nu}_{sal}$ represent the average numbers of mol of L-tryptophan and salicylate bound per mol of albumin respectively, and [Tryptophan]$_f$ and [Salicylate]$_f$ the concentrations of free L-tryptophan and free salicylate respectively. The points show the average of three duplicate experiments.

association constants are affected to the same extent. The factor x could be called a coupling constant. Co-operative binding and anti-co-operative binding are characterized by $x > 1$ and $x < 1$ respectively. In the case of binding of L-tryptophan and salicylate to albumin, $x < 1$ (cf. Fig. 3).

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

$$P_t = p + pa + bp + bpa \quad (2)$$

This relationship is readily transformed to:

$$P_t = p + K_A \cdot p \cdot a + K_B \cdot b \cdot p + x \cdot K_A \cdot K_B \cdot a \cdot b \cdot p \quad (2a)$$

where a and b stand for the concentration of free A and B respectively. Furthermore, the concentration of bound A (A_b) is given by:

$$A_b = A_t - A_f = K_A \cdot p \cdot a + x \cdot K_A \cdot K_B \cdot a \cdot b \cdot p \quad (3)$$

where A_t and A_f represent the concentration of total A and free A respectively. Subtracting eqn. (3) from eqn. (2a) gives:

$$P_t - A_b = p + K_B \cdot b \cdot p \quad (4)$$

Since P_t, A_t, A_f, K_B and b are known, it is possible to calculate p. Inserting p, and the known values for K_A and a, in eqn. (2a) gives x.

On the basis of the experimentally obtained data shown in Fig. 3(*a*), x was determined to be 0.21 ± 0.04 (S.D.), i.e. binding of L-tryptophan to its high-affinity binding site decreased the association constant for salicylate by the factor 0.21. Calculations performed with the data shown in Fig. 3(*b*) resulted in $x = 0.24 \pm 0.07$ (Table 2). A statistical analysis showed that the accordance between the two sets of coupling constants is highly significant ($P > 0.40$). As a control on the relevance of the coupling constants, theoretical binding curves for the two ligands were constructed. As seen in Figs. 3(*a*)

and 3(*b*), both curves (—•—) are in accordance with the experimental findings.

Phenol Red–salicylate. Binding of various concentrations of Phenol Red to albumin in the presence of a constant concentration of salicylate, and the converse experiments, was also investigated (not shown). The binding of both ligands was determined simultaneously in all the experiments. It was found that the two ligands bind neither independently nor competitively to albumin. As with the simultaneous binding of L-tryptophan and salicylate, the results were best described by ligand binding to two different high-affinity sites with mutual interactions. Calculations, made as outlined above, gave x values of about 0.3 (Table 2).

Table 2. *Coupling constants* (x) *characterizing mutual interactions between albumin-bound ligands*

Ligand A (various total concentrations)	Ligand B (constant total concentration)	x (±S.D.)	
L-Tryptophan	Salicylate	0.21±0.04	0.50 > P > 0.40
Salicylate	L-Tryptophan	0.24±0.07	
Phenol Red	Salicylate	0.30±0.08	0.20 > P > 0.10
Salicylate	Phenol Red	0.35±0.10	
L-Tryptophan	Phenol Red	0.60±0.07	0.10 > P > 0.05
Phenol Red	L-Tryptophan	0.52±0.04	

L-Tryptophan–Phenol Red. Binding of various concentrations of L-tryptophan to albumin in the presence of a constant concentration of Phenol Red is shown in Fig. 4(*a*) (■). The concentration of free dye was determined to vary from 0.015 mM to 0.018 mM. In Fig. 4(*b*) the results of the converse experiments are shown (□). The concentration of free amino acid varied from 0.173 mM to 0.279 mM. The Figures show that the two ligands bind neither independently nor competitively to albumin. As in

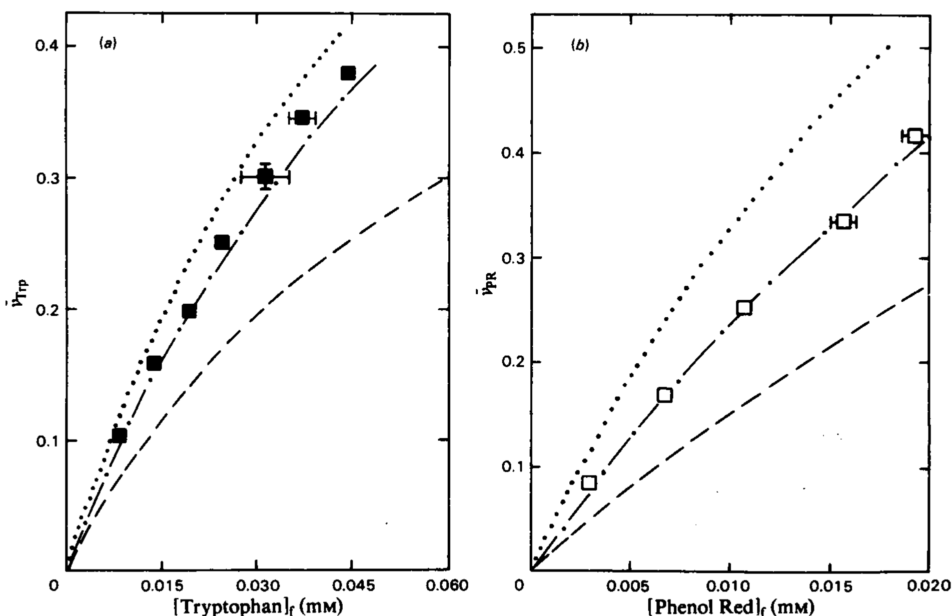


Fig. 4. *Binding of L-tryptophan and Phenol Red to human serum albumin*
(*a*) Binding of L-tryptophan (0.047–0.188 mM) to albumin (■) in the presence of Phenol Red (0.177 mM). (*b*) Binding of Phenol Red (0.035–0.177 mM) to albumin (□) in the presence of L-tryptophan (0.376 mM). The concentration of albumin was 0.38 mM, and the media contained 33 mM-sodium phosphate buffer, pH 7.0, at 20°C. For full experimental details see the text. The dotted binding curves (••••••) represent binding of L-tryptophan (*a*) and Phenol Red (*b*) without addition of the other ligand. The broken binding curves (– – – –) are theoretically calculated curves for binding of L-tryptophan (*a*) and Phenol Red (*b*) assuming competition of the ligands for a common high-affinity binding site on the protein. —•—, Theoretical binding curves assuming ligand binding to separate high-affinity binding sites with mutual interactions (for further details see the text). In drawing the theoretical curves for Phenol Red binding the small contribution from ligand binding to low-affinity sites is added. $\bar{\nu}_{Trp}$ and $\bar{\nu}_{PR}$ represent the average numbers of mol of L-tryptophan and Phenol Red bound per mol of albumin respectively, and [Tryptophan]$_f$ and [Phenol Red]$_f$ the concentrations of free L-tryptophan and free Phenol Red respectively. The points show the average of three duplicate experiments.

the two foregoing examples, the results are best described by ligand binding to different high-affinity sites with mutual interactions. The average coupling constants calculated on the basis of the experimentally obtained data are 0.60 and 0.52 respectively (Table 2). The statistical analysis showed that the accordance between the two sets of constants is relatively poor. Nevertheless, theoretical binding curves, constructed by using a common x value of 0.55, fit the experimentally obtained data in Fig. 4(*a*) and 4(*b*) quite well, especially when compared with a competitive binding scheme.

Discussion

The binding model for serum albumin previously put forward (Kragh-Hansen, 1981*b*) can be summarized briefly as shown in Table 3. Binding regions numbers 1, 4 and probably also 5 are very specific, whereas binding regions numbers 2, 3 and 6 seem to be less specific and to be capable of high-affinity interaction with several different ligands.

In the present study I have investigated the relation of the high-affinity binding site of diazepam to the binding scheme represented by Table 3. Competitive binding studies with L-tryptophan and diazepam (Fig. 2) revealed that the two ligands compete for a common high-affinity binding site. This finding indicates that the high-affinity binding site of the drug is placed in region 2. Various findings, of a more indirect nature, published by others are in accordance with this conclusion. For example, Müller & Wollert (1975) and Sjöholm *et al.* (1979) observed that the presence of diazepam diminished the binding of L-tryptophan to albumin. Fehske *et al.* (1979), who studied binding of the same two ligands to chemically modified albumin, also suggested the existence of a common high-affinity binding site for L-tryptophan and diazepam.

The results presented in Fig. 4 are best described by assuming, in accordance with the model, that L-tryptophan and Phenol Red interact with two different high-affinity binding sites. This finding is consistent with other results from this and other laboratories. Results reported by Kragh-Hansen *et al.* (1974) suggested that Phenol Red and bilirubin share a common high-affinity binding site on albumin. The high-affinity binding sites for L-tryptophan and diazepam are probably placed in the same region (cf. above). Finally, Brodersen *et al.* (1977) observed independent high-affinity binding of diazepam and bilirubin.

Table 3 also suggests that albumin possesses one, or possibly more, drug-binding region(s). The high-affinity binding site for salicylate seems to be a representative of such a region. The results obtained in the present study support such a proposal by indicating that L-tryptophan, Phenol Red and salicylate bind to three different high-affinity binding sites. Moreover, the literature contains supporting evidence for a separate binding site for salicylate. Brodersen (1974) concluded that the high-affinity binding sites of bilirubin and salicylate are not in common. Competitive binding studies by Tabachnick (1964) indicated that the high-affinity binding site for salicylate is not the same as that for L-thyroxine [which probably is the same as that for L-tryptophan (Tritsch & Tritsch, 1963)]. Furthermore, the effects of adding long-chain fatty acid ions to different albumin/salicylate combinations indicated that salicylate does not share a high-affinity binding site either with these acids or with L-tryptophan or diazepam (Rudman *et al.*, 1971). It also seems unlikely that salicylate binds with a high affinity to regions 4 and/or 5 (Kragh-Hansen, 1981*b*). All the studies cited in this and the foregoing sections were performed with human serum albumin. Table 3 also includes the names of some other drugs that probably bind competitively to the high-affinity salicylate-binding site.

Table 3. *Scheme for binding regions located on serum albumin*

Binding region	Ligand bound with high affinity
1	Long-chain fatty acid ions
2	L- and D-Tryptophan L-Thyroxine Octanoate Chlorazepate *p*-Iodobenzoate Cl^- ion
3	Bilirubin Iopanoate Certain dyes (e.g. phenolsulphonphthalein dyes)
4	Cu^{2+} and Ni^{2+} ions
5	Haemin
6	Salicylate Sulfaethidole Sulfathiazole Chlorpropamide Tolbutamide Indomethacin

High-affinity bindings of L-tryptophan, Phenol Red and salicylate, however, do not take place independently. Binding of one of the ligands to albumin diminishes the simultaneous high-affinity binding of the other two ligands. The decrease in binding can be accounted for by the introduction of a coupling constant, i.e. the factor by which two different ligands mutually affect their association constants during simultaneous binding to albumin. The decrease in binding was found to be reciprocal, as expected from thermodynamic considerations

[see, e.g., Wyman (1964) and Weber (1972)]. Furthermore, the magnitude of the coupling constant is dependent on the ligands being bound. In the present study, the mutual decrease in binding was more pronounced with L-tryptophan–salicylate and Phenol Red–salicylate than with L-tryptophan–Phenol Red. The present study emphasizes that a detailed investigation has to be made before a decrease in binding of a ligand caused by the presence of another ligand can be interpreted as binding to a common binding site.

I am grateful to Dr. Jesper V. Møller for helpful discussions and to Ms. Connie Hylander for technical assistance. Thanks are also due to Dumex, Copenhagen, Denmark, for the gift of diazepam. This work was supported by the Danish Medical Research Council, Aarhus Universitets Forskningsfond and P. Carl Petersens Fond.

References

Brodersen, R. (1974) *J. Clin. Invest.* **54**, 1353–1364

Brodersen, R., Sjödin, T. & Sjöholm, I. (1977) *J. Biol. Chem.* **252**, 5067–5072

Brown, K. F. & Crooks, M. J. (1976) *Biochem. Pharmacol.* **25**, 1175–1178

Bruderlein, H. & Bernstein, J. (1979) *J. Biol. Chem.* **254**, 11570–11576

Chen, R. F. (1967) *J. Biol. Chem.* **242**, 173–181

Fehske, K. J., Müller, W. E. & Wollert, U. (1979) *Biochim. Biophys. Acta* **577**, 346–359

Fehske, K. J., Müller, W. E. & Wollert, U. (1981) *Biochem. Pharmacol.* **30**, 687–692

Hultmark, D., Borg, K. O., Elofsson, R. & Palmer, L. (1975) *Acta Pharm. Suec.* **12**, 259–276

Keresztes-Nagy, S., Mais, R. F., Oester, Y. T. & Zaroslinski, J. F. (1972) *Anal. Biochem.* **48**, 80–89

Klotz, I. M. (1946) *Arch. Biochem.* **9**, 109–117

Kober, A., Ekman, B. & Sjöholm, I. (1978) *J. Pharm. Sci.* **67**, 107–109

Kragh-Hansen, U. (1981*a*) *Biochem. J.* **195**, 603–613

Kragh-Hansen, U. (1981*b*) *Pharmacol. Rev.* **33**, 17–53

Kragh-Hansen, U., Møller, J. V. & Sheikh, M. I. (1972) *Pflügers Arch.* **337**, 163–176

Kragh-Hansen, U., Møller, J. V. & Lind, K. E. (1974) *Biochim. Biophys. Acta* **365**, 360–371

McMenamy, R. H. & Oncley, J. L. (1958) *J. Biol. Chem.* **233**, 1436–1447

Müller, W. E. & Wollert, U. (1973) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **280**, 229–237

Müller, W. E. & Wollert, U. (1975) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **288**, 17–27

Peters, T., Jr. (1975) in *The Plasma Proteins* (Putnam, F. W., ed.), 2nd edn., vol. 1, pp. 133–181, Academic Press, New York

Rudman, D., Bixler, T. J. & Del Rio, A. E. (1971) *J. Pharmacol. Exp. Ther.* **176**, 261–272

Sjödin, T., Roosdorp, N. & Sjöholm, I. (1976) *Biochem. Pharmacol.* **25**, 2131–2140

Sjöholm, I., Ekman, B., Kober, A., Ljungstedt-Påhlman, I., Seiving, B. & Sjödin, T. (1979) *Mol. Pharmacol.* **16**, 767–777

Tabachnick, M. (1964) *Arch. Biochem. Biophys.* **106**, 415–421

Tritsch, G. L. & Tritsch, N. E. (1963) *J. Biol. Chem.* **238**, 138–142

Weber, G. (1972) *Biochemistry* **11**, 864–878

Wyman, J. (1964) *Adv. Protein Chem.* **19**, 223–286