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Relations between high-affinity binding sites of markers for binding regions on human serum albumin

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Binding of warfarin, digitoxin, diazepam, salicylate and Phenol Red, individually or in different pair combinations, to defatted human serum albumin at ligand/protein molar ratios less than 1:1 was studied at pH 7.0. The binding was determined by ultrafiltration. Some of the experiments were repeated with the use of equilibrium dialysis in order to strengthen the results. Irrespective of the method used, all ligands bind to one high-affinity binding site with an association constant in the range 10^4–10^6 M^{-1}. High-affinity binding of the following pair of ligands took place independently: warfarin–Phenol Red, warfarin–diazepam, warfarin–digitoxin and digitoxin–diazepam. Simultaneous binding of warfarin and salicylate led to a mutual decrease in binding of one another, as did simultaneous binding of digitoxin and Phenol Red. Both effects could be accounted for by a coupling constant. The coupling constant is the factor by which the primary association constants are affected; in these examples of anti-co-operativity the factor has a value between 0 and 1. In the first example it was calculated to be 0.8 and in the latter 0.5. Finally, digitoxin and salicylate were found to compete for a common high-affinity binding site. The present findings support the proposal of four separate primary binding sites for warfarin, digitoxin (and salicylate), diazepam and Phenol Red. An attempt to correlate this partial binding model for serum albumin with other models in the literature is made.

Serum albumin is able to bind with high-affinity ligands as diverse as inorganic ions and aromatic and aliphatic molecules. A detailed account for the structural basis of this capability cannot be given at present. However, information on the secondary structure of albumin is sufficiently advanced that tentative identification of binding regions is within reach (Peters, 1975; Fehske *et al.*, 1981; Kragh-Hansen, 1981*b*; Brown & Shockley, 1982). Kragh-Hansen (1981*b*) suggested the existence of six or more binding regions on serum albumin to which various ligands can associate with a high affinity. Long-chain fatty acids, Cu^{2+} (and Ni^{2+}), L-tryptophan (and diazepam) and Phenol Red are markers for four of these regions, and perhaps haemin binds to a fifth binding region. Furthermore, it was argued that at least one additional drug-binding region is present on albumin. Such a region should be represented by the primary salicylate-binding site. This proposal has been supported by experimental findings showing that the high-affinity salicylate-binding site is different from those of L-tryptophan (and diazepam) and Phenol Red (Kragh-Hansen, 1983*a*). In the present study the relationship between the high-affinity binding sites of two widely used and strongly albumin-bound drugs, warfarin and digitoxin, and the primary sites of the following markers for binding regions was examined: diazepam, salicylate and Phenol Red. Warfarin and digitoxin were also chosen, because one of the drugs or both has been used by other investigators as markers for binding regions on albumin (see, e.g., Sjöholm *et al.*, 1979; Sudlow *et al.*, 1975, 1976). Therefore the results obtained in the present study also can be used to relate the albumin-binding model summarized above with other binding models in the literature.

Experimental

Materials

Human serum albumin (more than 95% pure according to the manufacturer) was obtained from AB Kabi, Stockholm, Sweden. Warfarin was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and digitoxin (puriss.) from Fluka A.G., Buchs, Switzerland. Diazepam (100% pure

according to the donor) was a gift from Dumex, Copenhagen, Denmark. Phenol Red and salicylic acid (sodium salt) were supplied by Merck, Darmstadt, Germany. The following isotopically-labelled compounds were bought from Amersham International, Amersham, Bucks., U.K.: [2-^{14}C]-diazepam (sp. radioactivity 57mCi/mmol) and [^{14}C]warfarin (sp. radioactivity 55mCi/mmol). Other isotopically-labelled ligands were obtained from New England Nuclear, Boston, MA, U.S.A.: [7-^{14}C]salicylic acid (sp. radioactivity 51.7mCi/mmol) and [G-^{3}H]digitoxin (sp. radioactivity 20Ci/mmol). All reagents were of analytical grade.

Albumin was defatted by the charcoal method of Chen (1967). The aqueous solutions of defatted albumin were freeze-dried, and the resulting powder was stored in a desiccator at 4°C until use.

Ultrafiltration studies

Samples (10ml) containing 0.019–0.188mM of warfarin+[^{14}C]warfarin, 0.019–0.189mM of diazepam+[^{14}C]diazepam, 0.035–0.177mM of Phenol Red or 0.039–0.156mM of salicylate+[^{14}C]salicylate, with and without albumin, were prepared. Samples containing the same different concentrations of warfarin and a constant total concentration of one of the other ligands and vice versa were also made, with and without the protein (for further details see the legends to Figs. 1–3). The media contained 0.033M-sodium phosphate buffer, pH7.0, at 20°C, and the albumin concentration was 0.38mM (2.5%, w/v). The samples without albumin were used as controls representing 100% free ligand in the ultrafiltration procedure. The ligands, except Phenol Red, were added to the solutions as small volumes (10–100μl) of a concentrated stock solution, consisting of both non-labelled and isotopically labelled ligand. The stock solutions were made in phosphate buffer (salicylate), 0.1M-NaOH (warfarin) or ethanol (diazepam). Addition of stock solutions of warfarin changed pH of the final solutions less than 0.05 pH unit, and pH was not re-adjusted. The final diazepam-containing samples contained 0.1–0.5% (v/v) ethanol.

Owing to a poor ultrafiltrability of digitoxin in 0.033M-sodium phosphate buffer, all experiments involving this ligand were performed in 0.33M-sodium phosphate buffer, pH7.0, at 20°C. Furthermore, because of the relatively low solubility of digitoxin in aqueous media, these experiments were performed with relatively low concentrations of the drug and with an albumin concentration of 1.25% (w/v). Samples (10ml) containing digitoxin+[^{3}H]digitoxin of various concentrations within the range 0.010–0.112mM, 0.016–0.078mM of warfarin+[^{14}C]warfarin, 0.026–0.115mM of diazepam+[^{14}C]diazepam, 0.018–0.088mM of Phenol Red or 0.019–0.115mM of salicylate+[^{14}C]salicylate, with and without albumin, were prepared. Also, solutions containing both digitoxin and one of the other four ligands, with and without albumin, were prepared (for further details see the legends to Figs. 4–7). The stock solutions of digitoxin+[^{3}H]digitoxin were made in ethanol and resulted in ethanol concentrations of 0.20–1.25% (v/v) in the final solutions.

The percentages of ligand bound to albumin were determined as follows. Protein and reference solutions (4.5ml) were enclosed in cellophan bags (Visking, 18mm diameter) and placed in plastic tubes containing two compartments (Kragh-Hansen *et al.*, 1972). Ultrafiltrate (about 200μl), 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. 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 warfarin, digitoxin, salicylate and diazepam in the ultrafiltrates were determined by liquid scintillation counting by adding 150μl of filtrate to 2ml of Luma Gel (Lumac Systems, Basle, Switzerland). Radioactivity was counted in a Packard Tri-Carb spectrometer. The concentration of each radioactive ligand in ultrafiltrates containing mixtures of [^{3}H]digitoxin and ^{14}C-labelled warfarin, salicylate or diazepam was determined by using two channels of the spectrometer. Quenching of the radioactivity of [^{14}C]warfarin and of [^{3}H]digitoxin by Phenol Red was corrected for on the basis of the concentration of dye in the samples. In these filtrates, first the concentration of dye was determined, and afterwards the corrections were carried out by using quench curves determined in separate experiments. The concentration of Phenol Red was determined spectrophotometrically. A portion (typically 200μl) of the ultrafiltrate was diluted with a suitable volume of 0.1M-NaOH, and the absorbance was read at 559nm on a Zeiss PM2 DL Spektralphotometer. Control experiments revealed that neither warfarin nor digitoxin absorbs light at that wavelength.

Equilibrium-dialysis studies

Samples (10ml) containing 0.009–0.075mM of warfarin+[^{14}C]warfarin (with and without 0.088mM-Phenol Red), 0.035–0.106mM-Phenol Red (with and without 0.083mM-warfarin) or 0.019–0.095mM of diazepam+[^{14}C]diazepam (with and without 0.090mM-warfarin) were prepared in 0.033M-sodium phosphate buffer alone or in the buffer including 2.5% (w/v) albumin. Cellophan bags, containing 4.5ml of sample, were

placed in 4.5ml of the corresponding reference solution (solutions without albumin) in test tubes (diameter 2.5cm). The tubes were closed with Parafilm (American Can Co., Greenwich, CT, U.S.A.) and shaken gently for 20–24h at room temperature (approx. 20°C). Determination of the concentration of free ligand(s) in the solutions outside the bags after the dialysis, and the calculations of binding percentages, were performed as mentioned above. Control experiments with protein-free solutions showed that the cellophan bags are fully permeable for all three ligaads, and that equilibrium was established within the period of time.

Results

A prerequisite for an analysis of potential interactions between ligands bound to albumin is the knowledge of the number of binding sites (n) on the protein for the ligands and the corresponding association constants (K). Therefore, as a part of the present study, the individual binding of warfarin, digitoxin, diazepam, salicylate and Phenol Red to defatted human serum albumin was studied. The binding parameters n and K were calculated by using the double-reciprocal-plot method of Klotz (1946) as previously described (Kragh-Hansen, 1981*a*). In fitting the theoretical binding curves calculated on the basis of n and K to the experimentally obtained data, iteration computer programs were used.

Binding of warfarin to albumin

On the basis of results shown in Figs. 1–3, n and K for binding of warfarin to defatted human serum albumin were calculated. First, drug binding to only one binding site was assumed. However, binding curves constructed based on that assumption resulted in very poor fits to the experimental data. Afterwards, the existence of two binding sites with different association constants was suggested. As shown in the Figures, the experimental data are well described by such a proposal. The association constant for the first binding site (K_1) was calculated to be $1.6 \pm 0.2 \times 10^5 M^{-1}$. Comparable values for K_1 and n_1 (the number of binding sites in the first binding class) have been published by other workers: Tillement *et al.* (1974) found $n_1 = 0.95$ and $K_1 = 2.31 \times 10^5 M^{-1}$ (equilibrium-dialysis and ultrafiltration studies with media containing 0.2M-phosphate buffer, pH7.4, at 37°C). Sudlow *et al.* (1975) reported $n_1 = 0.9$ and $K_1 = 2.5 \times 10^5 M^{-1}$ (fluorimetry, 0.1M-sodium phosphate/0.9% NaCl, pH7.4, at 22°C). Sjöholm *et al.* (1979) determined $n_1 = 1$ and $K_1 = 2.1 \times 10^5 M^{-1}$ (equilibrium dialysis and albumin immobilized in microparticles, 0.005M-phosphate buffer/0.1M-KCl, pH7.4, at 22–24°C). Finally, Maes *et al.* (1982) calculated $n_1 = 0.94$ and $K_1 = 3.97 \times 10^5 M^{-1}$ (fluorimetry, 0.01M-sodium phosphate/0.9% NaCl, pH7.4, at 18°C). The slightly higher K values reported by the authors referred to probably are due to the higher pH in their media (cf. Wilting *et al.*, 1980).

The association constant calculated for the secondary warfarin binding site is 2.0×10^4–$2.5 \times 10^4 M^{-1}$. Other authors have also suggested the existence of secondary binding sites, e.g. Tillement *et al.* (1974) and Sudlow *et al.* (1975).

Warfarin–Phenol Red. Ultrafiltration studies revealed (Fig. 1) that binding of warfarin to albumin (□), at the drug/protein molar ratios

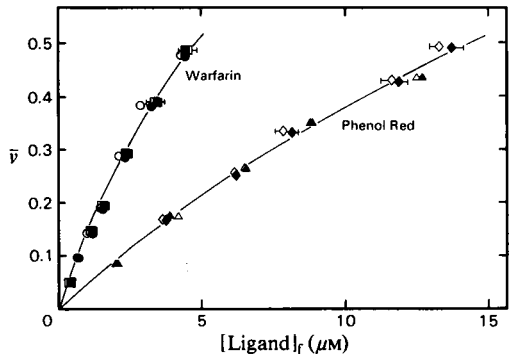

Fig. 1. *Binding of warfarin and Phenol Red to human serum albumin*

The ligand binding was determined by both ultrafiltration and equilibrium dialysis. □ and ○, Binding of warfarin alone; ■ and ●, binding of various concentrations of warfarin in the presence of a constant concentration of Phenol Red; □ and ■, results obtained by ultrafiltration ($\bar{\nu}_{PR} = 0.43$ in the case of ■); ○ and ●, results from equilibrium dialysis experiments ($\bar{\nu}_{PR} = 0.41$ in the case of ●); △ and ◇, binding of Phenol Red alone; ▲ and ◆, binding of various concentrations of Phenol Red in the presence of a constant concentration of warfarin; △ and ▲, results obtained by ultrafiltration ($\bar{\nu}_{war} = 0.46$ in the case of ▲); ◇ and ◆, results from equilibrium dialysis experiments ($\bar{\nu}_{war} = 0.43$ in the case of ◆). $\bar{\nu}_{war}$ and $\bar{\nu}_{PR}$ represent the average number of mol of warfarin and of Phenol Red bound per mol of albumin respectively. The concentration of albumin was 0.38mM (2.5%, w/v), and the media contained 0.033M-sodium phosphate buffer, pH7.0, at 20°C. The curves were constructed by using $n_1 = 1$, $K_1 = 1.5 \times 10^5 M^{-1}$, $n_2 = 1$ and $K_2 = 1.8 \times 10^4 M^{-1}$ for binding of warfarin, and $n_1 = 1$, $K_1 = 3.9 \times 10^4 M^{-1}$, $n_2 = 5$ and $K_2 = 1.5 \times 10^3 M^{-1}$ in the case of Phenol Red binding. In this and the following Figures the symbols represent the averages ± S.D. for three or four duplicate experiments.

studied, is not influenced by addition of a constant concentration of Phenol Red (■). These experiments were repeated with the use of equilibrium dialysis. As shown in Fig. 1 (○ and ●), the results obtained by this technique also showed that high-affinity binding of warfarin to albumin is not affected by the presence of the dye.

The converse experiments were also performed, i.e. determining the binding of Phenol Red to albumin in the absence and in the presence of a constant concentration of warfarin. As seen in Fig. 1, both the ultrafiltration data (△ and ▲) and the results obtained by equilibrium dialysis (◇ and ◆) indicate independent high-affinity binding of the two ligands to albumin.

Warfarin–diazepam. Binding of warfarin to albumin with and without the presence of diazepam is illustrated by the lower curve in Fig. 2. According to these results, obtained by ultrafiltration, binding of warfarin is not clearly reduced by addition of a constant concentration of diazepam. The steeper curve in Fig. 2 represents the results of converse experiments. The results obtained by ultrafiltration (□ and ■) are somewhat scattered (relatively great standard deviations) and could indicate that binding of diazepam is somewhat increased in the presence of warfarin. This apparent co-operativity is not in accordance with the results represented by the lower curve, which merely indicates independent ligand binding. Therefore these experiments were repeated with the use of equilibrium dialysis (△ and ▲). The results thereby obtained were much less scattered and support the proposal of independent high-affinity binding of warfarin and diazepam to albumin.

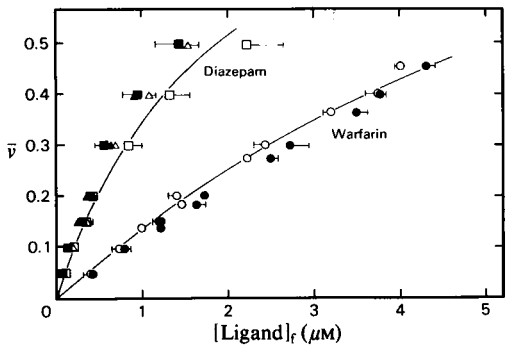

Fig. 2. *Binding of warfarin and diazepam to human serum albumin*

Binding of various concentrations of warfarin to albumin was determined by ultrafiltration in the absence (○) and in the presence (●) of diazepam ($\bar{\nu}_{dia} = 0.40$). Binding of diazepam was studied by both ultrafiltration (□ and ■) and equilibrium dialysis (△ and ▲). □ and △, Binding of diazepam in the absence of warfarin; ■ and ▲, binding of diazepam in the presence of warfarin ($\bar{\nu}_{war} = 0.46$ in both types of experiments). $\bar{\nu}_{war}$ and $\bar{\nu}_{dia}$ represent the average number of mol of warfarin and of diazepam bound per mol of albumin respectively. The curves were constructed by using $n_1 = 1$, $K_1 = 1.3 \times 10^5\,M^{-1}$, $n_2 = 1$ and $K_2 = 2.3 \times 10^4\,M^{-1}$ for binding of warfarin, and $n_1 = 1$, $K_1 = 5.1 \times 10^5\,M^{-1}$, $n_2 = 2$ and $K_2 = 2.2 \times 10^3\,M^{-1}$ in the case of diazepam binding. For further information see the legend to Fig. 1.

Warfarin–salicylate. Ultrafiltration data show (Fig. 3*a*) that binding of warfarin to albumin (○) is diminished in the presence of a constant concentration of salicylate (●). In Fig. 3(*b*) the results of converse experiments are illustrated, and they show that binding of salicylate is decreased by addition of warfarin. Thus the two drugs mutually decrease the binding of one another to albumin.

The possibility that the diminished drug 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 warfarin and salicylate respectively. The procedure for calculating $\bar{\nu}_{war}$ (in the presence of salicylate) as a function of the concentration of free warfarin (Fig. 3*a*) and to calculate $\bar{\nu}_{sal}$ (in the presence of warfarin) as a function of the concentration of free salicylate (Fig. 3*b*) has been previously described (Kragh-Hansen, 1981*a*). It is seen from the broken curves shown in Fig. 3 that binding is not diminished to the extent expected for competitive binding, indicating that both drugs may be bound simultaneously to albumin. The following approach is an attempt to quantify the mutual decrease in binding.

In Fig. 3(*a*) the binding to albumin of various concentrations of warfarin+[^{14}C]warfarin in the presence of a constant concentration of non-labelled salicylate is shown. In these experiments only the concentration of free warfarin was determined. I have also performed experiments with the same concentrations of warfarin (non-

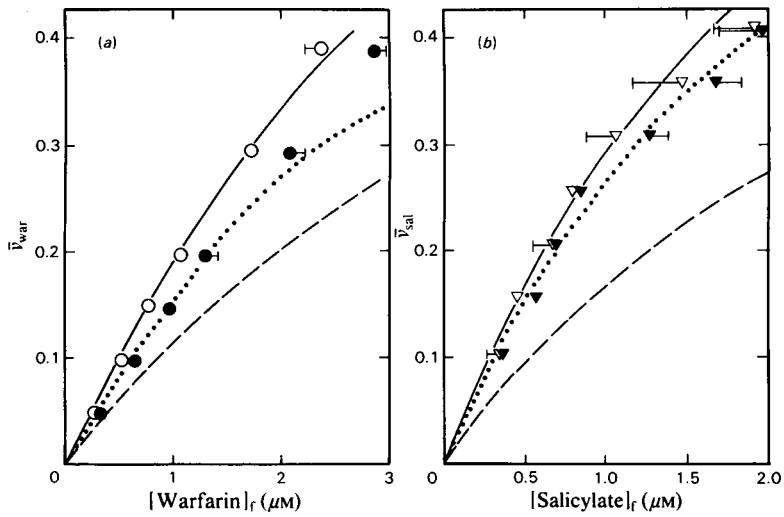

Fig. 3. *Binding of warfarin and salicylate to human serum albumin*

(*a*) Binding of various concentrations of warfarin to albumin in the absence (○) and in the presence (●) of a constant concentration of salicylate (0.177 mM). The unbroken curve describes binding of warfarin alone and is constructed by using $n_1 = 1$, $K_1 = 2.0 \times 10^5\,M^{-1}$, $n_2 = 1$ and $K_2 = 2.5 \times 10^4\,M^{-1}$. (*b*) Binding of various concentrations of salicylate in the absence (▽) and in the presence (▼) of a constant concentration of warfarin (0.177 mM). The unbroken curve characterizes binding of salicylate alone and is calculated by using $n_1 = 1$, $K_1 = 3.8 \times 10^5\,M^{-1}$, $n_2 = 4$ and $K_2 = 3.9 \times 10^3\,M^{-1}$. The broken curves are theoretical curves for binding of warfarin (*a*) and salicylate (*b*) assuming competition of the ligands for a common high-affinity binding site on the protein. The small contributions of drug binding to secondary sites have been added. The dotted curves in both (*a*) and (*b*) are theoretical curves made assuming ligand binding to separate high-affinity binding sites with mutual interactions. For further details see the text. $\bar{\nu}_{war}$ and $\bar{\nu}_{sal}$ represent the average number of mol of warfarin and of salicylate bound per mol of albumin respectively. The binding was determined by ultrafiltration. For further information see the legend to Fig. 1.

labelled) and the same constant concentration of salicylate, but used salicylate + [^{14}C]salicylate, and determined the concentration of free salicylate (results not shown). Therefore in these experiments the following quantities are now known: the concentrations of total warfarin, salicylate and protein, the concentrations of free warfarin and salicylate and the association constants of the two drugs (the constants for the drug–albumin binding determined in the absence of the other drug). With the knowledge of the magnitude of these parameters it is possible to calculate a coupling constant (x) as previously described (Kragh-Hansen, 1983*a*). The coupling constant is the factor by which the binding of one ligand to its high-affinity binding site affects K_1 for the binding of the other ligand. The factor x is greater than 1 in the case of co-operative binding, equal to 1 in the case of independent ligand binding, less than 1 when anti-co-operative binding takes place, and equal to 0 in the case of competitive binding. In the present example (Fig. 3*a*) x is calculated to be 0.80 ± 0.27.

I have performed analogous supplementary experiments to the studies (Fig. 3*b*) on salicylate binding in the presence of warfarin (results not shown). On the basis of these two sets of data, x was calculated to be 0.79 ± 0.17. The conclusion of these calculations is that the simultaneous high-affinity binding of warfarin and salicylate to albumin is characterized by a moderate repulsive interaction.

As a control on the relevance of x, theoretical binding curves, using a common value of $x = 0.80$, for the two ligands were constructed. As shown in Figs. 3(*a*) and 3(*b*), both curves (·····) are in accordance with the experimental findings.

Binding of digitoxin to albumin

Calculations on ultrafiltration data for binding of digitoxin to albumin alone (Figs. 4–7) revealed that the drug–protein interaction can be described adequately by assuming one high-affinity binding site with a K value of $1.5 \pm 0.1 \times 10^5\,M^{-1}$. Results found in the literature also propose that digitoxin binds to only one binding site on human serum albumin. Lukas & De Martino (1969) calculated $n = 1.02$ and $K = 8.74 \times 10^4\,M^{-1}$ (equilibrium dialysis, 0.05 M-Tris/HCl/0.1 M-KCl, pH 7.4, at 20°C). Brock (1975) found $n = 1$ with an 'apparent'

association constant of $4.3 \times 10^4 M^{-1}$ (equilibrium dialysis, 0.01 M-Tris/HCl, pH 7.4, at 37°C). Finally, Sjöholm *et al.* (1979) reported $n = 1$ and $K = 9 \times 10^4 M^{-1}$ (equilibrium dialysis and albumin immobilized in microparticles, 0.005 M-phosphate/0.1 M-KCl, pH 7.4, at 22–24°C). Thus the n and K values estimated in the present study are essentially in agreement with the results found in the literature. The possibility of secondary digitoxin-binding sites cannot be excluded. However, if such sites exist, they probably are characterized by very low association constants. Owing to the relatively low solubility of digitoxin in aqueous media, that possibility has not been investigated either in the present work or in the studies cited.

Digitoxin–Phenol Red. In the experiments investigating the simultaneous binding of digitoxin and Phenol Red to albumin the concentration of the free forms of both ligands was determined. In Fig. 4(*a*) it is seen that binding of digitoxin to albumin (○) is diminished in the presence of a constant concentration of Phenol Red (●). In these experiments it was also found that the concentration of free dye varied from 19.9 μM to 24.0 μM, depending on the drug concentration. In Fig. 4(*b*) the results of converse experiments are illustrated. Binding of Phenol Red is diminished in the presence of digitoxin, and, although the concentration of total digitoxin was constant, the concentration of free drug varied from 3.0 μM to 4.1 μM, depending on the dye concentration. Thus binding of digitoxin decreases the binding of Phenol Red and vice versa.

The possibility that the two ligands compete for a common high-affinity binding site on albumin was tested by using eqns. (1) and (1a). It is seen that the experimental data do not follow these curves, i.e. the ligands bind to two different high-affinity binding sites on the protein, and the mutual decrease in binding is caused by indirect means. Therefore coupling constants were calculated. The results underlying Fig. 4(*a*) lead to $x = 0.50 \pm 0.11$. For the converse experiments (Fig. 4*b*) calculations resulted in $x = 0.54 \pm 0.12$. As shown in Figs. 4(*a*)

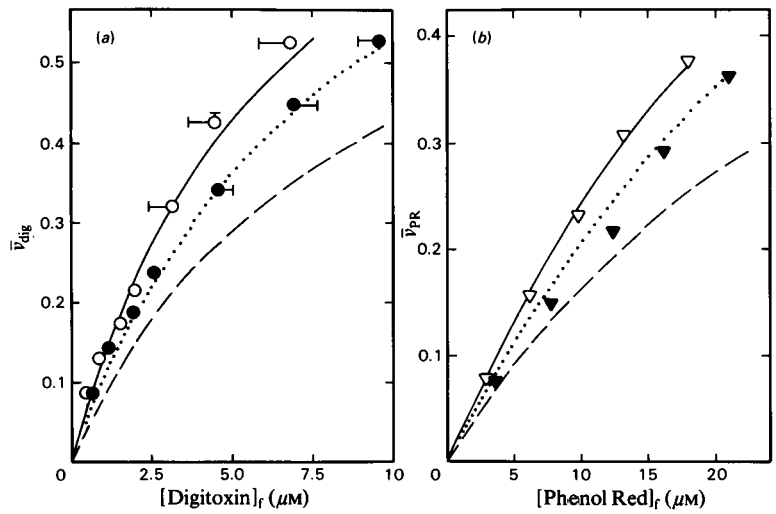

Fig. 4. *Binding of digitoxin and Phenol Red to human serum albumin*
(*a*) Binding of various concentrations of digitoxin to albumin in the absence (○) and in the presence (●) of a constant concentration of Phenol Red (0.088 mM). The unbroken curve describes binding of digitoxin alone and is constructed by using $n = 1$ and $K = 1.5 \times 10^5 M^{-1}$. (*b*) Binding of various concentrations of Phenol Red in the absence (▽) and in the presence (▼) of a constant concentration of digitoxin (0.066 mM). The unbroken curve characterizes binding of Phenol Red alone and is calculated by using $n_1 = 1$, $K_1 = 3.0 \times 10^4 M^{-1}$, $n_2 = 5$ and $K_2 = 2.0 \times 10^2 M^{-1}$. The broken curves are theoretical curves for binding of digitoxin (*a*) and Phenol Red (*b*) assuming competition of the ligands for a common high-affinity binding site on the protein. In the case of Phenol Red binding, the small contribution of dye binding to secondary sites has been added. The dotted curves in both (*a*) and (*b*) are theoretical curves made assuming ligand binding to separate high-affinity binding sites with mutual interactions. For further details see the text. $\bar{\nu}_{PR}$ and $\bar{\nu}_{dig}$ represent the average number of mol of Phenol Red and of digitoxin bound per mol of albumin respectively. Common experimental conditions: the binding was determined by ultrafiltration, the concentration of albumin was 0.19 M (1.25%, w/v), and the media contained 0.33 M-sodium phosphate buffer, pH 7.0, at 20°C.

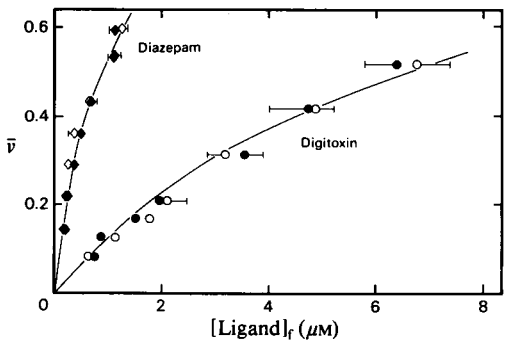

Fig. 5. *Binding of digitoxin and diazepam to human serum albumin*

Binding of various concentrations of digitoxin to albumin in the absence (○) and in the presence of a constant concentration (●) of diazepam ($\bar{v}_{dia} = 0.41$). The results of converse experiments are also shown: ◇ and ◆, binding of various concentrations of diazepam without and with a constant concentration of digitoxin ($\bar{v}_{dig} = 0.35$). $\bar{v}_{dia}$ and $\bar{v}_{dig}$ represent the average number of mol of diazepam and of digitoxin bound per mol of albumin respectively. The curves were constructed by using $n = 1$ and $K = 1.5 \times 10^5 M^{-1}$ for binding of digitoxin and $n_1 = 1$, $K_1 = 1.1 \times 10^6 M^{-1}$, $n_2 = 2$ and $K_2 = 4.0 \times 10^3 M^{-1}$ in the case of diazepam binding. For further information see the legend to Fig. 4.

and 4(*b*), binding curves constructed by using a common value of $x = 0.50$ (·····) are in accordance with the experimental findings.

Digitoxin–diazepam. In Fig. 5 (lower curve) it is seen that binding of digitoxin is not influenced by addition of diazepam. The steeper curve in the Figure shows the results of converse experiments. The results of these investigations also show that digitoxin and diazepam bind independently to different high-affinity binding sites on albumin.

Digitoxin–salicylate. In Fig. 6(*a*) it is seen that salicylate diminishes the binding of digitoxin to albumin. From Fig. 6(*b*) it is apparent that the effect is reciprocal. The experimental data, obtained when both drugs are added to albumin, are adequately described by the broken curves, which are constructed as outlined above, assuming that digitoxin and salicylate compete for a common high-affinity binding site on albumin.

Digitoxin–warfarin. Fig. 7 shows the binding of digitoxin (lower curve) with and without addition of warfarin and the binding of warfarin (upper curve) in the absence and in the presence of digitoxin. In both situations it is seen that ligand binding is not affected by addition of the second ligand, i.e. digitoxin and warfarin bind independently to different high-affinity binding sites on albumin.

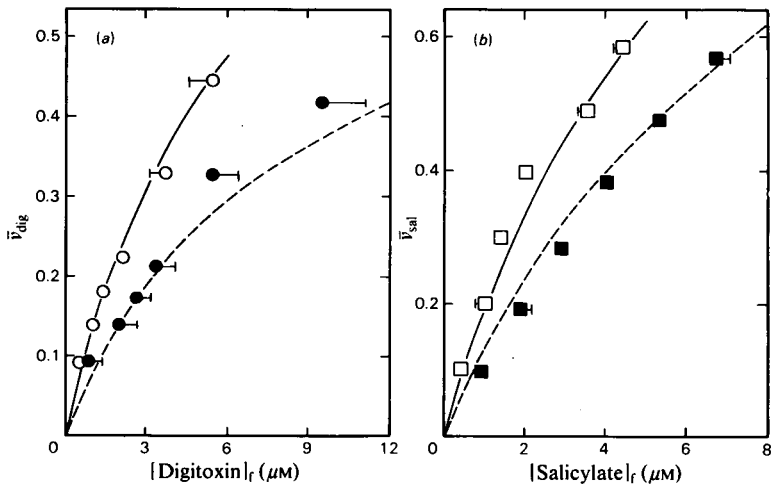

Fig. 6. *Binding of digitoxin and salicylate to human serum albumin*

(*a*) Binding of various concentrations of digitoxin to albumin in the absence (○) and in the presence (●) of a constant concentration of salicylate (0.078 mM). The unbroken curve describes binding of digitoxin alone and is constructed by using $n = 1$ and $K = 1.5 \times 10^5 M^{-1}$. (*b*) Binding of various concentrations of salicylate in the absence (□) and in the presence (■) of a constant concentration of digitoxin (0.065 mM). The unbroken curve characterizes binding of salicylate alone and is calculated by using $n_1 = 1$, $K_1 = 2.0 \times 10^5 M^{-1}$, $n_2 = 4$ and $K_2 = 6.0 \times 10^3 M^{-1}$. The broken curves are theoretical curves for binding of digitoxin (*a*) and salicylate (*b*) assuming competition of the ligands for a common high-affinity binding site on the protein. In the case of salicylate binding, the small contribution of drug binding to secondary sites has been added. $\bar{v}_{dig}$ and $\bar{v}_{sal}$ represent the average number of mol of digitoxin and of salicylate bound per mol of albumin respectively. For further information see the legend to Fig. 4.

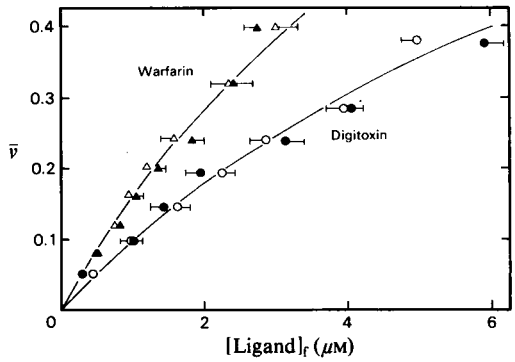

Fig. 7. *Binding of digitoxin and warfarin to human serum albumin*

Binding of various concentrations of digitoxin to albumin in the absence (○) and in the presence of a constant concentration (●) of warfarin ($\bar{\nu}_{war} = 0.39$). The results of converse experiments are also shown: △ and ▲, binding of various concentrations of warfarin without and with a constant concentration of digitoxin ($\bar{\nu}_{dig} = 0.39$). $\bar{\nu}_{war}$ and $\bar{\nu}_{dig}$ represent the average number of mol of warfarin and of digitoxin bound per mol of albumin respectively. The curves were constructed by using $n = 1$ and $K = 1.2 \times 10^5 M^{-1}$ for binding of digitoxin and $n_1 = 1$, $K_1 = 1.6 \times 10^5 M^{-1}$, $n_2 = 1$ and $K_2 = 2.0 \times 10^4 M^{-1}$ in the case of warfarin binding. For further information see the legend to Fig. 4.

Discussion

In the present work high-affinity binding of ligands to defatted human serum albumin was studied. The results can be summarized as follows. (1) Diazepam, Phenol Red, digitoxin and warfarin interact with the protein at four separate primary binding sites. (2) Salicylate competes with digitoxin for binding at its binding site. (3) Digitoxin–Phenol Red and salicylate–warfarin mutually decrease the binding of one another to albumin but not by a competitive mechanism. This anti-cooperativity is characterized by coupling constants of 0.5 and 0.8 respectively, and may be caused by conformational changes of the protein upon ligand binding, by electrostatic interactions, or by a combination of both mechanisms. The coupling constants indicate that the repulsive interactions between albumin-bound digitoxin and Phenol Red are more pronounced than those between albumin-bound salicylate and warfarin.

It should be noted that several examples of independent high-affinity ligand binding was observed in the present study: warfarin–Phenol Red (Fig. 1), warfarin–diazepam (Fig. 2), digitoxin–diazepam (Fig. 5) and digitoxin–warfarin (Fig. 7). Only a few examples of independent ligand binding to albumin have been published. Sjöholm *et al.* (1979) reported that the binding of neither diazepam nor warfarin should be affected by the presence of the other at low ligand/albumin molar ratios. Furthermore, these authors mentioned that neither diazepam nor warfarin diminished the binding of digitoxin to albumin. These observations are in agreement with the results of the present, and more extensive, studies (Figs. 2, 5 and 7). Brodersen *et al.* (1977) registered independent high-affinity binding of diazepam and bilirubin. Santos & Spector (1972) found that the interaction between equimolar concentrations of 8-anilinonaphthalene-1-sulphonate and bovine serum albumin was unaffected by addition of 1 mol of palmitate/mol of protein. The examples of independent high-affinity binding of ligands to albumin support the proposal of albumin as a binding protein possessing several discrete binding regions (sites).

In an attempt to explain the unique binding capabilities of serum albumin, more or less complete binding models, operating with discrete binding regions, have been published. It is commonly accepted (Peters, 1975; Kragh-Hansen, 1981*b*; Fehske *et al.*, 1981) that long-chain fatty acids, probably the first two molecules (Berde *et al.*, 1979), bind with high association constants to a specific binding area, which is located in the *C*-terminal third of the protein (Peters, 1977). Furthermore, the Cu^{2+} ion, and probably also other metal ions such as Ni^{2+} (Glennon & Sarkar, 1982), can bind with a high affinity to a well-defined binding site formed by the first three amino acid residues from the *N*-terminal end of albumin (for reviews see Peters, 1975; Kragh-Hansen, 1981*b*). However, the number, specificity and molecular localization of additional binding regions is still undefined, and has been the subject of several studies. The following is an attempt to correlate the findings of the present study with other results and proposals published from this and other laboratories (cf. Table 1). The present data define a minimum of four sites represented by the high-affinity binding sites for diazepam, Phenol Red, digitoxin (salicylate) and warfarin respectively. Previous data of a similar kind showed that there is a good evidence that L-tryptophan interacts with albumin at the diazepam-binding site (Kragh-Hansen, 1983*a*,*b*). In addition, we have reported results proposing that Phenol Red and bilirubin bind at the same region on albumin (Kragh-Hansen *et al.*, 1974). Furthermore, this assignment of binding regions (Table 1, first column) is in agreement with previous results showing that L-tryptophan, Phenol Red and salicylate bind, with mutual interactions, to three different high-affinity binding sites (Kragh-Hansen, 1983*a*).

Table 1. *Partial ligand-binding models of serum albumin*

Binding region/ binding area	Markers for binding regions as proposed by: Present work	Peters (1975)	Fehske *et al.* (1981)	Sjöholm *et al.* (1979)	Sudlow *et al.* (1975, 1976)
I	Diazepam (L-tryptophan)	L-Tryptophan	Indoles + benzodiazepines	Diazepam	5-Dimethylamino-naphthalene-1-sulphonyl-sarcosine
II	Phenol Red (bilirubin)	Bilirubin	Bilirubin		
III	Digitoxin Salicylate		Digitoxin	Digitoxin	
IV	Warfarin		Warfarin–azapropazone	[Warfarin]	5-Dimethylamino-naphthalene-1-sulphonamide

Peters (1975) forwarded the idea that albumin, besides separate high-affinity binding sites for long-chain fatty acids and Cu^{2+}/Ni^{2+}, possesses two additional high-affinity binding sites with L-tryptophan and bilirubin as representative ligands. Fehske *et al.* (1981) suggested the existence of three discrete high-affinity binding sites for indoles plus benzodiazepines, bilirubin and digitoxin respectively, and a binding area consisting of partially overlapping binding sites for warfarin and azapropazone. This model is comparable with that suggested above (Table 1, first column), except that the relationship between the azapropazone-binding site and the binding regions in my model has not been examined.

Sjöholm *et al.* (1979) proposed diazepam, digitoxin and warfarin as markers of three separate binding sites on albumin, but mentioned that some of their results point to the possibility of more drug-binding sites on the protein. These authors also suggested that L-tryptophan binds with a high affinity to the diazepam-binding site. Sjöholm *et al.* (1979) considered warfarin and azapropazone to compete for a common high-affinity binding site. This is not in contradiction with Fehske *et al.* (1981), who suggested partially overlapping binding sites for these two ligands. However, the relationship between the high-affinity warfarin-binding and bilirubin-binding sites is different in the various models. Sjöholm *et al.* (1979) suggested that the sites are placed in the same region. This is not in agreement with results from this laboratory. Analyses carried out by Kragh-Hansen *et al.* (1974) showed that Phenol Red and bilirubin probably bind to the same area on albumin. Therefore, if both warfarin and Phenol Red compete with bilirubin for a common high-affinity binding site, Phenol Red and warfarin should be expected to do the same. However, these two ligands bind independently to the protein (Fig. 1). It should be noted that this finding was confirmed by two different techniques, namely ultrafiltration and equilibrium dialysis. Fehske *et al.* (1981) thought that the high-affinity binding site of warfarin is identical not with the primary binding site of bilirubin but with a secondary bilirubin-binding site. Our findings would be in better accordance with such a proposal than with the suggestion made by Sjöholm *et al.* (1979).

Finally, Sudlow *et al.* (1975, 1976) used the flourescent probes 5-dimethylaminonaphthalene-1-sulphonamide (site I) and 5-dimethylamino-naphthalene-1-sulphonylsarcosine (site II) to characterize two drug-binding sites on albumin. The authors suggested that there must be at least one more strong drug-binding site on the protein. Furthermore, they reported that warfarin binds with a high affinity to site I. In a further paper from the same laboratory (Wanwimolruk *et al.*, 1983) it was shown that diazepam binds with a high affinity to site II. Sjöholm *et al.* (1979) have also suggested, on the basis of their own experimental findings, that 5-dimethylaminonaphthalene-1-sulphonylsarcosine and 5-dimethylamino-naphthalene-1-sulphonamide bind with a high affinity to the high-affinity binding sites for diazepam and warfarin respectively.

It looks like the unique ligand-binding capability of serum albumin can be explained by assuming the existence of four separate high-affinity binding regions, in addition to special sites for long-chain fatty acids and for Cu^{2+} and Ni^{2+} ions. However, the presence of other regions to which ligands can bind with a high affinity should not be excluded at present. For example, it has not yet been possible to relate the high-affinity binding site for haemin to the regions presented in Table 1 (Kragh-Hansen, 1981*b*).

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