MOLECULAR PHARMACOLOGY, 25:137–150

Albumin Binding of Anti-Inflammatory Drugs

Utility of a Site-Oriented versus a Stoichiometric Analysis

BENT HONORÉ AND ROLF BRODERSEN

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

Received May 10, 1983; Accepted September 14, 1983

SUMMARY

Binding equilibria of 12 nonsteroidal, anti-inflammatory substances, salicylic acid, diflunisal, phenylbutazone, azapropazone, fenbufen, biphenylacetic acid, naproxen, flurbiprofen, ibuprofin, diclofenac, indomethacin, and benoxaprofen, to defatted human serum albumin has been investigated at 37°, pH 7.4, in a sodium phosphate buffer, 66 mM, by means of equilibrium dialysis and, in case of salicyclic acid, by dialysis rate determinations. Cobinding of each of these drugs with monoacetyl-4,4′-diaminodiphenyl sulfone, warfarin, and diazepam has been studied by measuring dialysis rates of the last-mentioned ligands. Cobinding of each drug with bilirubuin was investigated by two techniques, equilibrium dialysis against albumin with and without bilirubin, and by measuring rates of oxidation of free bilirubin with hydrogen peroxide and peroxidase. Results were analyzed in quantitative terms. The use of a site-oriented description versus a stoichiometric analysis is discussed. The stoichiometric description is preferred for the following reasons: (a) Simple relations exist between the percentage of bound drug at low drug concentrations and the *first stoichiometric binding constant*. (b) The stoichiometric description does not imply that preformed binding sites are present in the albumin molecule. (c) A quantitative, stoichiometric analysis of multiple cobinding of two ligands is possible.

INTRODUCTION

Serum albumin binding equilibria of a ligand, X, are usually described by Scatchard's equation (1).

$$r_X = \sum_{i=1}^{n} \frac{xk_i}{1 + xk_i} \qquad (1)$$

where r_X is the average number of bound ligand molecules per molecule of albumin, x is the free ligand concentration, and n is the number of binding sites. The binding isotherm is characterized by numerical values of the site binding constants, k_i. Alternatively, the stoichiometric binding equation can be used (2),

$$r_X = \frac{xK_1 + 2x^2K_1K_2 + 3x^3K_1K_2K_3 + \cdots + Nx^NK_1K_2K_3\cdots K_N}{1 + xK_1 + x^2K_1K_2 + x^3K_1K_2K_3 + \cdots + x^NK_1K_2K_3\cdots K_N} \qquad (2)$$

reporting the stoichiometric binding constants, K_i. N is the maximal number of ligand molecules bound. Experience from numerous studies has shown that both equations are suitable for describing binding of a ligand to albumin in pure solution as well as in blood plasma, Scatchard's equation being more widely employed.

Scatchard's equation has been deduced for a carrier having a number of *independent sites*, while the stoichiometric equation is valid whether sites are present or not and irrespective of cooperativity or anticooperativity of binding. Most drugs appear to interact primarily with one of two loci. Site I and Site II (3, 4). Although a few exceptions are known, the concept of Site I and Site II has proven its value in classification of drugs according to binding characteristics and seems to be useful for prediction of drug competition, an otherwise bewildering area. This speaks in favor of using a site-oriented description.

The present paper reports serum albumin binding data for a group of nonsteroidal anti-inflammatory agents. Cobinding of these substances with bilirubin, MADDS,[1] warfarin, and diazepam has also been studied. Bilirubin, MADDS, and warfarin served as marker ligands for Site I and diazepam for Site II. The validity of the Site I and Site II models for characterization of primary binding of these drugs is tested, and the feasibility of a site-oriented analysis of multiple binding versus a stoichiometric description is discussed.

[1] The abbreviations used are: MADDS, monoacetyl-4,4′-diaminodiphenyl sulfone; TLC, thin-layer chromatography.

0026-895X/84/010137-14$02.00/0

STOICHIOMETRIC BINDING THEORY

Binding of one ligand. Multiple binding equilibria of a homogeneous ligand to a homogeneous carrier in the absence of other ligands are described by the stoichiometric binding equation, Eq. 2 (2).

The K values in this equation, with certain limitations as mentioned later, can be found by curve fitting to experimental binding data. The binding constants are related to the more familiar Scatchard's constants as described by Fletcher *et al.* (5).

Equation 2 can be simplified if we define

$$a_h = K_1 K_2 K_3 \cdots K_h$$

and

$$a_0 = 1$$

and obtain

$$r_X = \frac{\sum_{h=1}^{N} h x^h a_h}{\sum_{h=0}^{N} x^h a_h}$$

The concentration of *the stoichiometric complex*, PX_i, is defined as the total concentration of all complexes containing one molecule of carrier and i ligand molecules, irrespective of whether the ligand is bound to specific sites or is dissolved in or adsorbed to the carrier molecule. This concentration is

$$[PX_i] = \frac{x^i a_i}{\sum_{h=0}^{N} x^h a_h} P \qquad (3)$$

where P is the total carrier concentration.

If the amount of bound ligand is small compared with that of the carrier, and if the binding is not strongly cooperative, Eq. 2 simplifies to $r_X = xK_1$. The *first stoichiometric binding constant*, K_1, is thus equal to the ratio of bound, B, to free ligand, divided by the carrier concentration, $K_1 = (B/x)/P$ for small ligand concentrations, a useful relationship in practical pharmacology when binding of a drug is described by the percentage of bound substance in normal plasma. It should be noted that the relationship of Scatchard's binding constants to the percentage of bound drug is more complex since the first stoichiometric binding constant equals the sum of all of Scatchard's constants,

$$K_1 = (B/x)/P = \sum_{i=1}^{n} k_i \quad \text{for} \quad x \ll P \qquad (4)$$

Binding of two ligands. Multiple binding of two ligands, X and Y, to a carrier, P, occurs according to Scheme 1, where PX_iY_j denote stoichiometric complexes. The stoichiometric equilibrium constant, $K_{i(j)}$, is the binding constant for one molecule of X to the complex $PX_{i-1}Y_j$, and $K_{(i)j}$ is the binding constant for one molecule of Y to the complex PX_iY_{j-1}. The maximal numbers of molecules bound of the two ligands are N and M.

Scheme 1 contains $2NM+N+M$ binding constants. A cyclic process in one square, whereby a complex binds one molecule of X and one molecule of Y and then dissociates one X and finally one Y, must have the equilibrium constant 1. This means that

$$\frac{K_{i+1(j)} K_{(i+1)j+1}}{K_{i+1(j+1)} K_{(i)j+1}} = 1 \qquad (5)$$

If three binding constants in one square are known, the fourth can thus be calculated. The total equilibrium is therefore defined by $NM+N+M$ binding constants. In the following deduction, we choose to operate with the binding constants of ligand X to pure albumin, *i.e.*, the constants in the upper horizontal line of Scheme 1, $K_{1(0)} \ldots K_{N(0)}$ plus all of the constants for binding of ligand Y, *i.e.*, those in columns.

We define as above,

$$a_{i(j)} = K_{1(j)} K_{2(j)} K_{3(j)} \cdots K_{i(j)} \qquad a_{0(j)} = 1$$

$$a_{(i)j} = K_{(i)1} K_{(i)2} K_{(i)3} \cdots K_{(i)j} \qquad a_{(i)0} = 1$$

The concentration of the complex PX_i in the equilibrium mixture is then

$$[PX_i] = x^i [P] a_{i(0)}$$

and that of the complex PX_iY_j is

$$[PX_iY_j] = x^i [P] a_{i(0)} y^j a_{(i)j}$$

when x and y are the free concentrations of the ligands and [P] is the concentration of the unloaded carrier in the equilibrium mixture.

The total carrier concentration, P, is then

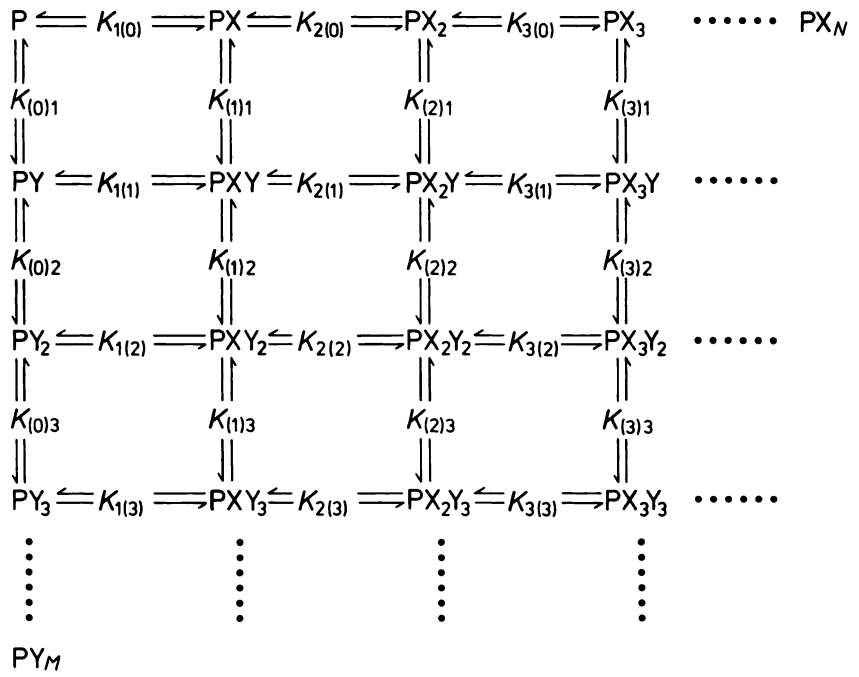
$$P = [P] \sum_{i=0}^{N} \left(x^i a_{i(0)} \sum_{j=0}^{M} y^j a_{(i)j} \right) \qquad (6)$$

and the concentration of bound ligand X,

$$X_{bound} = [P] \sum_{i=1}^{N} \left(i x^i a_{i(0)} \sum_{j=0}^{M} y^j a_{(i)j} \right) \qquad (7)$$

The binding isotherm for X is then described by

$$r_X = \frac{X_{bound}}{P} = \frac{\sum_{i=1}^{N} \left(i x^i a_{i(0)} \sum_{j=0}^{M} y^j a_{(i)j} \right)}{\sum_{i=0}^{N} \left(x^i a_{i(0)} \sum_{j=0}^{M} y^j a_{(i)j} \right)} \qquad (8)$$



SCHEME 1

and similarly for the other ligand, Y

$$r_Y = \frac{Y_{bound}}{P} = \frac{\sum_{i=0}^{N}\left(x^i a_{i(0)} \sum_{j=1}^{M} j y^j a_{(i)j}\right)}{\sum_{i=0}^{N}\left(x^i a_{i(0)} \sum_{j=0}^{M} y^j a_{(i)j}\right)} \qquad (9)$$

Equations 8 and 9 describe 3-dimensional binding isotherms for the ligands X and Y. Numerical values of the binding constants, with certain limitations as mentioned later, can be obtained by fitting these isotherms to experimental binding data.

Reserve carrier-equivalent concentration. A complete description of multiple-binding equilibria for two ligands to one carrier may be quite complicated, as seen from the above equations, Eqs. 8 and 9. If, however, the total concentration of one ligand, Y, is low compared with that of the carrier, and if the binding of Y is not strongly cooperative, cobinding equilibria of Y with multiple molecules of X can be characterized easily by determination of the *reserve carrier-equivalent concentration for binding of* Y in solutions containing the carrier and ligand X in varying proportions. The reserve carrier-equivalent concentration for binding of Y has been defined previously as the concentration of pure (standard) carrier, in a buffered solution without ligand X, which binds a trace amount of Y equally tightly as in the test solution containing X (6).

An equilibrium mixture of a carrier, P, with a multiply binding ligand, X, and a trace amount of ligand Y contains the following stoichiometric species, unloaded P, and PX, $PX_2 \cdots PX_N$ besides trace amounts of PY, PXY$\cdots$$PX_NY$. The concentration of bound Y is

$$Y_{bound} = y \sum_{i=0}^{N} [PX_i] K_{(i)1}$$

A solution of the standard preparation of carrier P in a concentration equal to the reserve carrier-equivalent concentration for binding of Y, to which ligand Y has been added in the same trace concentration as above, contains the same free and bound concentrations of Y, according to the definition of reserve carrier-equivalent,

$$Y_{bound} = ypK'_{(0)1}$$

where p is the reserve carrier-equivalent concentration for binding of Y and $K'_{(0)1}$ is the first stoichiometric binding constant for Y to the standard carrier preparation. We thus obtain

$$p = \sum_{i=0}^{N} [PX_i] \frac{K_{(i)1}}{K'_{(0)1}} \qquad (10)$$

indicating that the reserve carrier-equivalent concentration for binding of the trace ligand, Y, is equal to the sum of concentrations of all stoichiometric species in the equilibrium mixture, each concentration multiplied with the first stoichiometric binding constant of Y to the stoichiometric complex and divided by the first stoichiometric binding constant of Y to the standard preparation of the carrier.

Equation 10 can be used for determination of numerical values of $K_{(i)1}$, *i.e.*, the first stoichiometric binding constants of the trace ligand Y to the series of stoichio-

metric complexes of ligand X with the carrier. Binding data for X in the absence of Y are first obtained, and values of $K_{i(0)}$, the binding constants for ligand X alone, are found by fitting of Eq. 2. Concentrations of the stoichiometric complexes, PX_i, can then be found from Eq. 3. The reserve carrier-equivalent concentration for binding of Y is measured with a constant concentration of P and with varying amounts of X. Values of $K_{(i)1}/K'_{(0)1}$ can be found by fitting Eq. 10 to the data.

Energetic coupling of binding of two ligands. An alternative way of reporting the interaction of binding of two ligands is by energetic coupling (7). We presume that the stoichiometric binding constants for each of ligands X and Y are known. In Scheme 1 this means that the K values in the top line and left column are known. For each square, starting in the upper left corner, we now need one additional parameter in order to give a complete description of cobinding. This parameter may be one additional K value for each square; the fourth K in that square can then be calculated from Eq. 5. Alternatively, we can use the *energetic coupling*, as defined by Weber (7) for the first square

$$\Delta G_{X,Y} = RT \ln \frac{K_{1(1)}}{K_{1(0)}} = RT \ln \frac{K_{(1)1}}{K_{(0)1}} \qquad (11)$$

An energetic coupling value may be analogously defined for each square.

MATERIALS AND METHODS

Human serum albumin was obtained from AB Kabi (Stockholm, Sweden) and was defatted with charcoal in acid solution (8) and lyophilized. Albumin solutions of known molarity were prepared by weighing of the dry protein, taking the purity as 98% and the molecular weight as 66,000.

Twelve anti-inflammatory drugs were studied. Sodium salicylate p.a. was from E. Merck (Darmstadt, Germany). The following drugs were received as gifts: diflunisal and indomethacin from Dumex, Ltd. (Copenhagen); azapropazone from A/S Ferrosan (Copenhagen); fenbufen and its active metabolite, biphenylacetic acid, from Cyanamid Nordiska AB (Stockholm); naproxen from Syntex Research (Maidenhead, England); flurbiprofen and ibuprofen from Boots Company, PLC (Nottingham, England); diclofenac sodium from Ciba-Geigy A/S (Copenhagen); and benoxaprofen from Eli Lilly & Company, Denmark Aps (Copenhagen).

Bilirubin was obtained from Sigma Chemical Company (St. Louis, Mo.), and was purified according to the method of McDonagh and Assisi (9). The purified substance was dissolved in 0.5 M sodium hydroxide and added to a solution of albumin, at pH about 9 in order to avoid precipitation of bilirubin. The slightly alkaline solution of bilirubin-albumin was kept in the refrigerator for up to 5 days. Buffer was added before use. Experiments with bilirubin were conducted under orange light.

7-[^{14}C]Salicylic acid was obtained from New England Nuclear Corporation (Boston, Mass.). Radiochemical purity cannot be tested satisfactorily by TLC; it is almost impossible to exclude the presence of 1% radioactive impurities, as needed when free/bound salicylate ratios are between 0.02 and 1. However, binding isotherms obtained with varying albumin concentrations from 0.1 to 1 mM were identical (Fig. 1). Radiochemical purity of the labeled salicylate was thus sufficient for our purpose. It is estimated that nonbinding impurities account for less than 0.2% of the radioactivity, and tightly bound impurities for less than 4%.

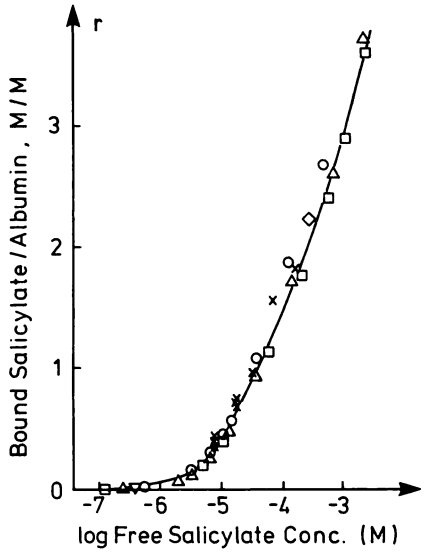


FIG. 1. *Binding isotherm for salicylate anion to defatted human serum albumin, sodium phosphate buffer, 66 mM, pH 7.4, 37°*

×, Points obtained by equilibrium dialysis, 30 μM albumin, and varying concentrations of sodium salicylate. All other points were found by dialysis rate determinations with the following albumin concentrations: ◇, 100 μM; ○, 200 μM; ▽, 300 μM; △, 500 μM; □, 1000 μM. The curve was drawn after least-squares fitting of the stoichiometric binding equation (Eq. 2). The binding constants shown in Table 1 were thus obtained.

[^{14}C]-Warfarin (3-α-acetonyl[α-^{14}C]benzyl-4-hydroxycoumarin) was obtained from The Radiochemical Centre (Amersham, England), and was purified by TLC and tested. [5-^{14}C]Diazepam was a gift from Hoffmann-La Roche Ltd. (Basel, Switzerland) and was purified as previously described (6). [^{14}C]MADDS was kindly prepared by Dr. Chr. Jacobsen of this institute (6) and was purified by TLC.

A sodium phosphate buffer, final concentration 66 mM (pH 7.4), was used in all experiments. The temperature was 37°. Indomethacin was dissolved in ethanol and added to the aqueous solutions to obtain a final ethanol concentration of 1% (v/v). Other drugs were dissolved in buffer or in sodium hydroxide solution and then added to the aqueous buffer. The pH of the final solution was adjusted as necessary.

Binding of One Ligand

Equilibrium dialysis. Binding isotherms of the drugs were obtained by equilibrium dialysis, using cellophane membranes, cut from dialysis tubing manufactured by Union Carbide Corporation, Type 32/36.

The time necessary for equilibration was studied with sodium salicylate and with phenylbutazone. Constant concentrations were present in the time interval from 4 to 8 hr. Dialysis times of 6–8 hr were used. Equilibrium ligand concentrations were measured in the protein free solution, by spectrophotometry at light absorption maximum. Linearity of extinction with varying concentrations was verified with all substances. Binding of the drugs to chamber and membrane was investigated. It was found that, with diflunisal, 5% of the amount present as free drug was bound to chamber and membrane. The ratio of chamber- and membrane-bound drug to the free amount was constant with varying concentrations within the range studied. Corrections were made in calculating the albumin-bound drug concentrations. Salicylate anion was not bound measurably to chamber and membrane at free concentrations below 200 μM. At 1 mM salicylate, considerable binding to the membrane was observed. The binding was strongly cooperative, so that free salicylate concentrations a little higher than 1 mM were obtained after a 6-hr contact with the membrane, even with large amounts of salicylate added. Equilibrium dialysis experiments with salicylate accordingly were limited to concentrations below 200 μM,

and a different technique (dialysis rate determinations, *vide infra*) was resorted to. Remaining drugs did not bind measurably to chamber or membrane.

Other potential sources of error, *i.e.*, albumin decay, osmosis of water, leakage of albumin, UV-absorbing impurities from the membrane, unequal distribution of free ligand, or hydrogen ions due to the Donnan effect, were investigated and found negligible. The average number, r, of bound ligand molecules per molecule of albumin was calculated as $r = 2(x_0 - x_F)/P$, where x_0 is the initial ligand concentration, present in both chambers, x_F is the equilibrium ligand concentration in the protein-free chamber, and P is the albumin concentration. In experiments with ibuprofen, the albumin concentration was 300 μM; in all other cases 30 μM. Drug concentrations varied.

Dialysis rate method. The binding isotherm of salicylate was obtained by utilizing a recently developed technique for dialysis rate determinations (6). Twenty microliters of a solution of [^{14}C]salicylate and albumin in buffer were placed on one side of a cellophane membrane with 20 μl of an identical buffered albumin solution with unlabeled salicylate on the other side. Dialysis was allowed to proceed for 5 or 10 min, at 37 ± 0.3°. Procedures for entering and withdrawal of the fluids and rinsing were as previously described (6). Concentrations of ^{14}C on either side of the membrane were measured by liquid scintillation counting. It was found that the time course of the dialysis process could be described by the following equation:

$$\log\frac{x_1 - x_2}{x_1 + x_2} = -2k(t + t_0)$$

where x_1 and x_2 are the ^{14}C concentrations, t is the dialysis time, and t_0 is an empirically found constant, which in the present case was 1.92 ± 0.23 min. The value of t_0 is related to the procedure of filling, withdrawal, and rinsing and varies with the substance tested. The velocity constant, k, is proportional to the fraction of free/total salicylate. Concentrations of free salicylate can thus be calculated from measured values of k, after a standard experiment without albumin, dialysis times 5–15 min. Binding of salicylate to chamber and membrane could not be demonstrated in this technique, not even at free salicylate concentrations as high as 2 mM, in spite of the fact that chamber and membrane materials were the same as those used in the apparatus for equilibrium dialysis and had a higher surface to volume ratio in the dialysis rate technique. The process of cooperative salicylate binding to the membrane seems to progress with time, so that binding is insignificant within the 15 min of a dialysis rate determination while large amounts are bound in the 6 hr required for equilibrium dialysis. Results obtained with the two methods were in good agreement at free salicylate concentrations below 200 μM, where binding is insignificant in either technique.

Binding isotherms. Results of binding studies for the 12 drugs are pictured as *bound* versus *log free* drug concentration. The often-used Scatchard's plot, picturing *bound/free* versus *bound* drug, in all cases gave curved lines; extrapolation to intersection with either axis did not allow reasonably accurate determination of the binding parameters; accordingly, this procedure did not present any advantages and was not used.

Curve fitting. Binding constants for the various drugs to serum albumin were found by least squares fitting of K_i values in the stoichiometric binding equation (Eq. 2), minimizing s, calculated from the weighted deviations of f observed points, as follows

$$s = \left(\frac{\sum_{i=1}^{f} \left[\left(r_i(\text{obs}) - r_i(\text{calc}) \right) / r_i(\text{obs})^q \right]^2}{f - 1} \right)^{1/2}$$

The weighting exponent, q, was given a value between 0 and 1, the value 0 meaning that deviations were independent of r, and $q = 1$ indicating that deviations were proportional to r. The magnitude of q was chosen so that an approximately equal distribution of weighted deviations was obtained over the range of ligand concentrations studied. An HP 85 desk computer from Hewlett-Packard Company (Corvallis, Ore.) was programmed for a semiautomatic procedure; values of K_i and q were entered manually, and the weighted deviations were then returned automatically, plotted as a function of log x, together with the calculated value of s. A new set of K_i values, or, in case of obviously uneven distribution of weighted deviations, a new value of q, was then entered, and this procedure was continued until it was assumed that a minimum of s, with constant q, was obtained.

Cobinding of Two Ligands

Cobinding of drug and a trace ligand. In the presence of varying amounts of the anti-inflammatory drugs, we measured the reserve albumin-equivalent concentration for binding of either of three test ligands, MADDS, warfarin, and diazepam. The reserve albumin-equivalent concentration for binding of a test ligand in a sample is defined in the theoretical section. This was determined by adding the isotopically labeled test ligand in a small concentration to the sample and measuring its rate of dialysis into an identical sample without added test ligand. The rate of dialysis is compared with rates obtained with standard albumin solutions as the sample. Defatted human serum albumin from KABI was used as a standard.

Cobinding of drug and bilirubin. Binding of the drugs to human serum albumin in the presence of bilirubin, 0.5 mole/mole of albumin, was studied by equilibrium dialysis with a bilirubin-albumin mixture on one side of the membrane. Bilirubin is tightly bound to albumin and does not diffuse through the membrane.

The equilibrium concentration of free bilirubin in similar mixtures of albumin, bilirubin, and drug was measured by determining the rate of bilirubin oxidation with hydrogen peroxide and peroxidase (10). Free bilirubin is oxidized, not the bound.

Curve fitting. Reserve albumin data (Fig. 3) were fitted by Eq. 10. The albumin preparation used was the same as the standard albumin preparation, and hence $K_{(0)1}/K'_{(0)1} = 1$. The other ratios of test ligand binding constants, $K_{(i)1}/K'_{(0)1}$, were denoted Q_i, and Eq. 10 could then be written

$$p = [P] + \sum_{i=1}^{N} [PX_i]\, Q_i \qquad (12)$$

An iterative procedure was developed for least-squares fitting of this Eq. 12 to observed data. Interaction of multiple binding of each drug with a trace amount of each of the two trace ligands, MADDS and diazepam, was thus characterized by a set of Q values.

Data for cobinding of drugs and bilirubin were analyzed by means of Eqs. 8 and 9: The binding constants of bilirubin to albumin, $K_{(0)1}$ and $K_{(0)2}$, were calculated from previous work (11) as follows. Scatchard's binding constants under the conditions of the present experiments are $k_1 = 5.5 \times 10^7$ M^{-1} and $k_2 = 4.4 \times 10^6$ M^{-1}. A model with maximally two bound bilirubin molecules and these two binding constants gives an excellent fit to the formerly measured peroxidase oxidation rates. The first stoichiometric constant, $K_{(0)1}$, is then $k_1 + k_2 = 5.9 \times 10^7$ M^{-1} and the second, $K_{(0)2}$, is $k_1 k_2/(k_1 + k_2) = 4.1 \times 10^6$ M^{-1}. Binding constants for the drugs to albumin, $K_{1(0)} \ldots K_{N(0)}$, were known from the present equilibrium dialysis experiments, and the remaining constants in Scheme 1 were found by curve fitting, as follows.

In the equilibrium dialysis experiments with binding of drug to bilirubin-albumin, a set of $K_{i(j)}$ values for $i = 1 \cdots N$ and $j = 1$ and 2 was guessed. For each experimental point, (x, r_X), where x is the free drug concentration, the concentration of free bilirubin, y, was determined by iteration in Eq. 6 and one analogous to Eq. 7 until the calculated concentration of bound bilirubin, Y_{bound}, plus the free approximated the known concentration of total bilirubin, 15 μM. A tentative value for the amount of bound drug, relative to the albumin concentration, was then calculated from Eq. 8 and was compared with

the observed value. All deviations thus calculated were weighted as described above, and the root mean-square weighted deviation was minimized by guessing new sets of $K_{i(j)}$ values until a good fit was obtained.

A similar iterative procedure was used for fitting binding constants to the data obtained by the peroxidase studies, calculating the total drug concentration as a function of free concentration of bilirubin and comparing the curve with experimental values.

RESULTS

Binding of anti-inflammatory drugs. Binding isotherms for the 12 drugs studied are shown in Fig. 2, and the stoichiometric binding constants, obtained by fitting Eq. 2 to the observed data, are given in Table 1. All of these substances are bound tightly to human serum albumin with first stoichiometric binding constants ranging from 4.8×10^4 M^{-1} for the salicylate anion to 1.9×10^6 M^{-1} for benoxaprofen. Multiple binding is seen in all cases, with as many as 11 bound molecules for diflunisal. Saturation of the albumin has not been achieved with any of the drugs, and indeed no signs of approaching saturation is seen in spite of the fact that experiments were generally carried to drug concentrations as high as solubility and

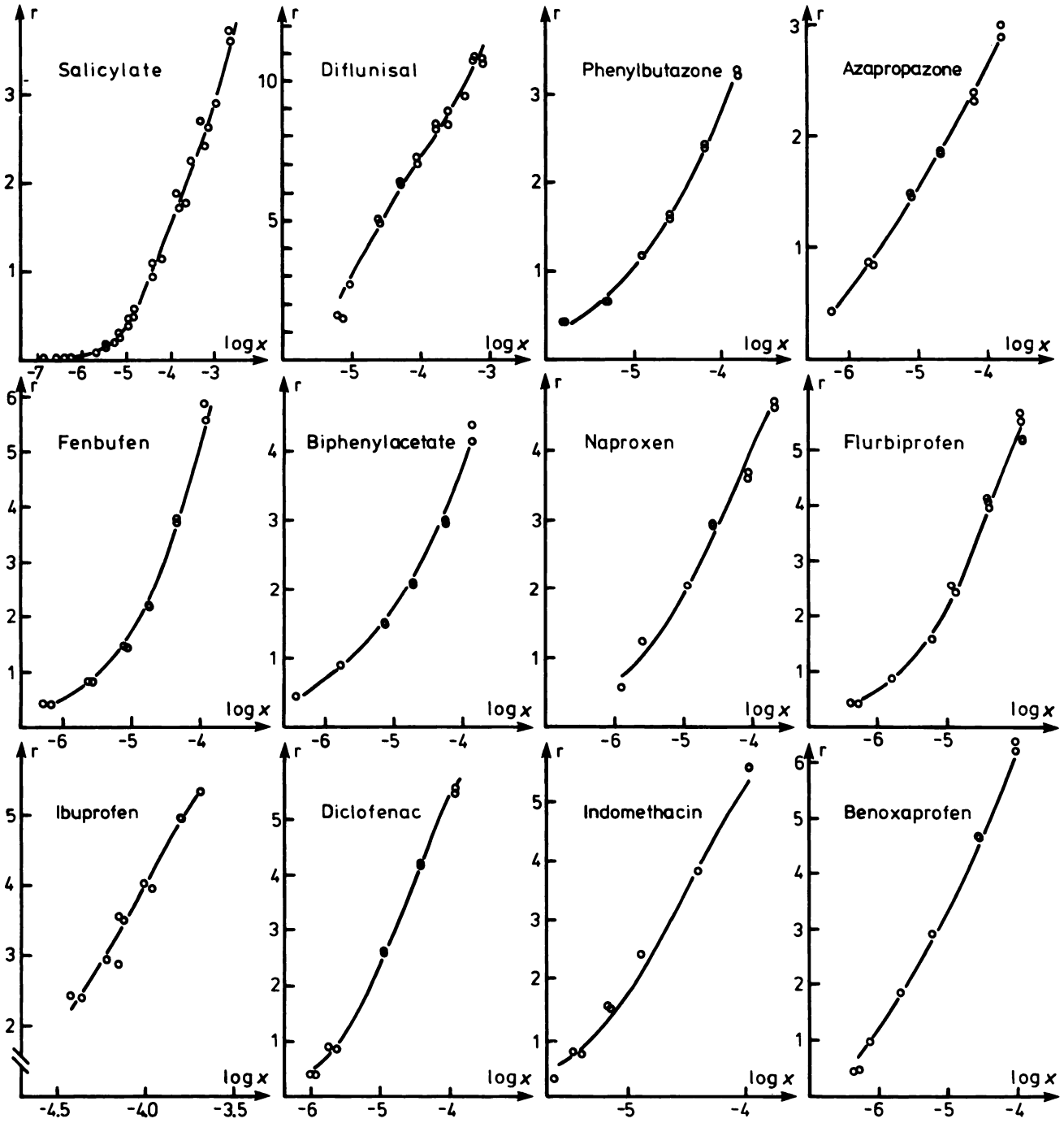


FIG. 2. *Binding isotherms for anti-inflammatory drugs to defatted human serum albumin under conditions as in Fig. 1*

The concentration of bound drug, divided by the albumin concentration, is pictured as a function of the logarithm of the free drug concentration. The observed points were obtained by equilibrium dialysis (for salicylate, by dialysis rate), and the curves represent the best fits of the stoichiometric binding equation (Eq. 2), giving the binding constants in Table 1.

TABLE 1

Stoichiometric binding constants of anti-inflammatory drugs to defatted human serum albumin (M^{-1})

Sodium phosphate buffer, 66 mM, pH 7.4, 37°

	Salicylate	Diflunisal	Phenylbutazone	Azapropazone	Fenbufen	Biphenylacetate
K_1	48,000	500,000	260,000	1,100,000	710,000	1,300,000
K_2	12,000	220,000	33,000	100,000	96,000	110,000
K_3	1,400	120,000	13,000	11,000	44,000	29,000
K_4	470	75,000	5,900	2,900	25,000	13,000
K_5	150	47,000	2,200		15,000	5,900
K_6		28,000			8,900	2,200
K_7		16,000			5,000	
K_8		7,700			2,100	
K_9		4,400				
K_{10}		2,700				
K_{11}		1,800				
K_{12}		1,200				
K_{13}		730				
K_{14}		420				
K_{15}		180				

	Naproxen	Flurbiprofen	Ibuprofen	Diclofenac	Indomethacin	Benoxaprofen
K_1	1,200,000	1,200,000	80,000	550,000	250,000	1,900,000
K_2	140,000	140,000	35,000	190,000	100,000	650,000
K_3	48,000	62,000	20,000	87,000	55,000	250,000
K_4	18,000	33,000	13,000	42,000	32,000	74,000
K_5	6,400	18,000	8,000	22,000	18,000	34,000
K_6	2,100	9,700	5,000	11,000	9,500	17,000
K_7		4,000	2,900	4,300	3,900	8,700
K_8			1,300			3,500

practical circumstances would allow. The meaning of the stoichiometric binding constants is discussed later.

Cobinding of drugs and traces of MADDS, warfarin, or diazepam. Reserve albumin-equivalent concentration (defined as in the theoretical section) for binding of MADDS and for binding of diazepam were measured in solutions of human serum albumin with various amounts of added anti-inflammatory drugs. The results are seen in Fig. 3. Reserve albumin for binding of diazepam is reduced on addition of small amounts of most of these agents, in some cases so that addition of a certain molar amount of the drug results in occupation of an equimolar amount of albumin, indicating that binding of one molecule of diazepam is competitive with binding of one molecule of the drug. In other instances interaction is less pronounced, and one drug, azapropazone, does not interfere with binding of diazepam until a molar excess of azapropazone over albumin has been added. Binding of one molecule of diazepam and the first molecule of azapropazone occurs independently.

MADDS is generally more prone to cobinding with these drugs. Binding of one molecule of MADDS and one of the drug ranges from independent for naproxen and a few other agents to a moderately negative interaction with azapropazone.

These findings are expressed in quantitative terms by the Q values of Eq. 12, obtained by curve fitting (Table 2). From Eqs. 10, 11, and 12, it is seen that the energetic coupling of binding of small amounts of the drugs with small amounts of the test ligands can be calculated as

$$\Delta G_{XY} = RT \ln Q_1$$

The values of $\Delta G_{X,Y}$ for cobinding of each drug with MADDS, warfarin, and diazepam are listed in Table 3. A zero value indicates that a complex of albumin and one molecule of each of the two ligands can be formed in which the ligands are bound independently. Competition of binding among the first molecule of each of the two ligands is signified by the value $-\infty$, indicating that cobinding of the ligands at low concentrations could not be demonstrated.

Cobinding of drugs and bilirubin. Free bilirubin concentrations in equilibrium mixtures of bilirubin (15 μM) and albumin (30 μM) usually increase on addition of cobinding drugs. This was studied by measuring the relative concentration of free bilirubin by the rate of oxidation with hydrogen peroxide and peroxidase. Results for the 12 drugs are seen in Fig. 4. The reverse process, displacement of bound drug on addition of bilirubin, was investigated by recording binding isotherms for the drugs to albumin (30 μM) with or without added bilirubin (15 μM). Examples of these curves are shown in Fig. 5. Displacement of drug by bilirubin was in most cases slight, as shown for phenylbutazone, but was considerable with azapropazone.

Curve fitting of the cobinding equations, Eqs. 8 and 9, showed that a set of binding constants could be found for 10 of the 12 drugs, describing binding of drug and bilirubin and producing excellent fits in equilibrium dialysis as well as in peroxidase experiments, as illustrated by the curves in Fig. 4.

Two of the drugs, phenylbutazone and azapropazone showed marked acceleration of bilirubin oxidation, considerably more than was calculated from the reverse

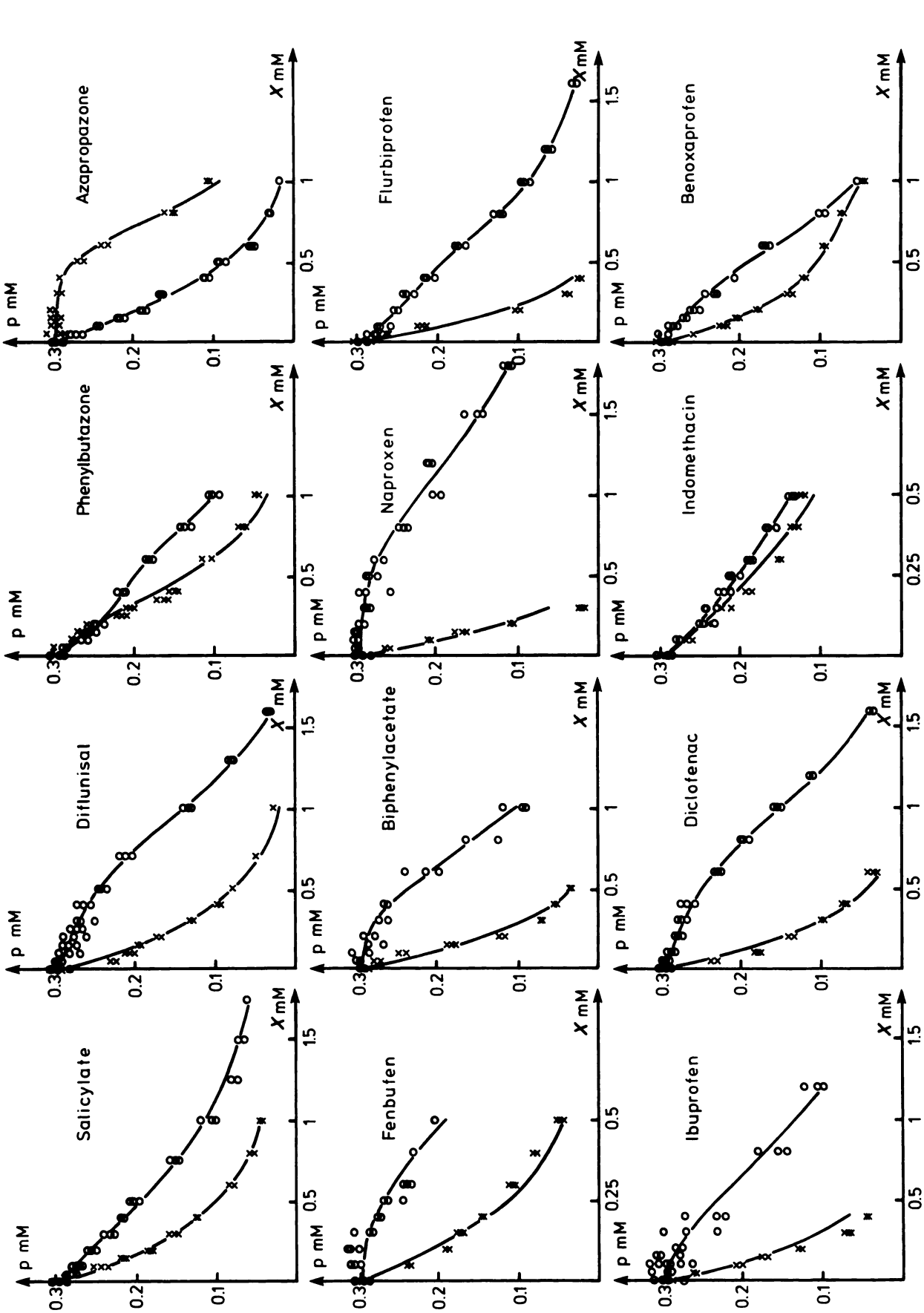


FIG. 3. *Reserve albumin-equivalent concentration, defined as in the theoretical section, for binding of MADDS (○) and for binding of diazepam (×) in a solution of 300 μM defatted human serum albumin with varying concentrations of anti-inflammatory agents (abscissa), measured by the dialysis rate method, under conditions as in Fig. 1*
The curves were obtained by least-squares fitting of the *Q* values in Eq. 12, giving the numerical results reported in Table 2.

TABLE 2

Values of Q_i

Q_i, the test ligand binding constant to the stoichiometric complex, PX_i, containing i molecules of bound drug, divided by the binding constant for the test ligand to albumin, was obtained by fitting Eq. 12 to the reserve albumin data in Fig. 3, using the stoichiometric binding constants from Table 1. Conditions as in Table 1.

Drug	Test ligand	Q_1	Q_2	Q_3	Q_4	Q_5	$Q_6 \rightarrow Q_N$
Salicylate	MADDS	0.80	0.50	0	0	0	
	Diazepam	0.37	0.18	0	0	0	
Diflunisal	MADDS	0.90	0.90	0.60	0.25	0	0
	Diazepam	0.18	0.18	0	0	0	0
Phenylbutazone	MADDS	0.71	0.71	0.30	0	0	
	Diazepam	0.80	0.10	0.10	0	0	
Azapropazone	MADDS	0.47	0.15	0	0		
	Diazepam	1.00	1.00	0	0		
Fenbufen	MADDS	1.00	0.40	0.20	0	0	0
	Diazepam	0.11	0.11	0	0	0	0
Biphenylacetate	MADDS	1.00	0.70	0.45	0	0	0
	Diazepam	0.20	0	0	0	0	0
Naproxen	MADDS	1.00	1.00	0.80	0.60	0.50	0
	Diazepam	0	0	0	0	0	0
Flurbiprofen	MADDS	0.75	0.70	0.20	0.15	0.15	0
	Diazepam	0	0	0	0	0	0
Ibuprofen	MADDS	1.00	0.50	0.50	0.50	0	0
	Diazepam	0.15	0	0	0	0	0
Diclofenac	MADDS	0.95	0.85	0.63	0.32	0.10	0
	Diazepam	0.10	0	0	0	0	0
Indomethacin	MADDS	0.55	0.45	0	0	0	0
	Diazepam	0.50	0.20	0	0	0	0
Benoxaprofen	MADDS	0.90	0.55	0.20	0	0	0
	Diazepam	0.29	0.29	0.29	0	0	0

displacement of drug by bilirubin, as seen in Fig. 4, where the curves represent the calculated course as obtained from the isotherms in Fig. 5. Two groups of substances, phenol and some phenol derivatives, and phenothiazines, have previously been found to increase the rate of the peroxidase process out of proportion to the increase in free bilirubin (12). These substances form free radicals, oxidizing bound bilirubin, and the same mechanism is probably responsible for the aberrant findings with phenylbutazone and azapropazone.

Most of the drugs are characterized by not displacing bilirubin when the drugs are present in small amounts and, vice versa, by not being displaced by one molecule of bilirubin. One or a few molecules of the drugs can thus be cobound independently to albumin together with one of bilirubin. Two drugs, naproxen and flurbiprofen, even increased the binding of bilirubin to a slight but significant extent. When larger amounts of drug are added, bilirubin is displaced in all cases.

TABLE 3

Energetic coupling, $\Delta G_{X,Y}$ (kcal/mole) of binding of two ligands at low ligand concentrations to defatted human serum albumin

Conditions as in Table 1.

	Bilirubin	MADDS	Warfarin	Diazepam
Salicylate	−0.28	−0.14	−0.30	−0.61
Diflunisal	0.00	−0.06	−0.34	−1.06
Phenylbutazone	−0.02	−0.21	−0.88	−0.14
Azapropazone	−1.12	−0.47	−0.56	0.00
Fenbufen	−0.04	0.00	−0.02	−1.36
Biphenylacetate	−0.01	0.00	−0.01	−0.99
Naproxen	+0.16	0.00	−0.06	$-\infty^a$
Flurbiprofen	+0.16	−0.18	0.00	$-\infty^a$
Ibuprofen		0.00	−0.02	−1.17
Diclofenac	−0.19	−0.03	−0.19	−1.42
Indomethacin	−0.20	−0.37	−0.02	−0.43
Benoxaprofen	−0.18	−0.06	+0.12	−0.76

[a] $\Delta G_{X,Y} < -2$ kcal/mole. Cobinding at low ligand concentrations could not be demonstrated.

Two examples of sets of binding constants for bilirubin and drug are given in Table 4. The numerical values of the constants are rather indeterminate, especially in cases of binding of several molecules of each ligand. The values given are not to be understood as true binding constants but serve the sole purpose of reconstruction of the binding and cobinding isotherms when entered in Eqs. 8 and 9. The best accuracy is found in the upper left corner, where the first square contains the first stoichiometric binding constants for bilirubin and for the drug, when each is bound alone, and the constants for binding of one molecule of bilirubin when one of the drugs is already bound. These figures describe the affinity of binding and mutual interaction of bilirubin and drug when both are present in small amounts. The mutual interactions at low bilirubin and drug levels are more easily rated by energetic couplings, calculated as described in the theoretical section and reported in Table 3, first column.

DISCUSSION

Primary binding of one drug. The simplest way of rating drug binding to serum albumin is by the percent-

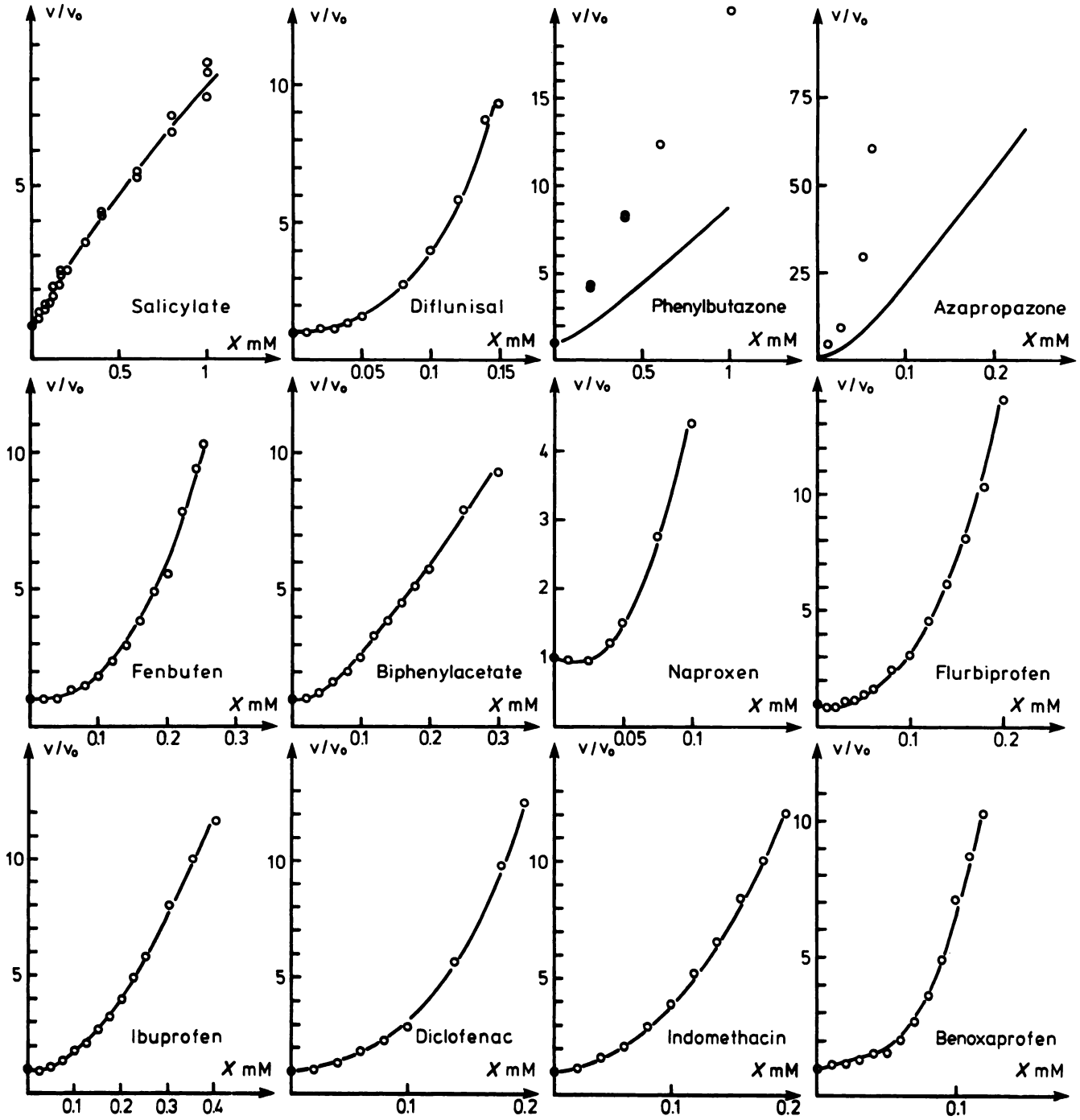


FIG. 4. *Cobinding of bilirubin and anti-inflammatory drugs, studied by the rate of free bilirubin oxidation with hydrogen peroxide and peroxidase*
The concentration of defatted human serum albumin was 30 μM with 15 μM bilirubin; other conditions were as in Fig. 1. Observed oxidation rates (O) are plotted as a function of drug concentration. The curves were obtained by least-squares fitting of Eqs. 8 and 9 to the present peroxidase kinetics data as well as to observed binding isotherms for the drugs to serum albumin with and without added bilirubin, as exemplified in Fig. 5. With 10 of the drugs, this resulted in good agreement with both types of data and gave sets of cobinding constants, as exemplified in Table 4. Two drugs, phenylbutazone and azapropazone, gave biliruin oxidation rates considerably higher than could be explained by the free bilirubin concentration; in these cases, the curves were obtained from fitting to the binding isotherms with and without bilirubin and calculating the free bilirubin concentrations, using Eq. 6 and an equation analogous to Eq. 7.

age of bound drug. This practice is meaningful when the drug concentration is low as compared with that of albumin, and when variations of binding affinity and albumin concentration as well as interaction with binding of other substances can be neglected. The first *stoichiometric* binding constant has a specific meaning in relation to the ratio of bound drug at low drug concentrations (Eq. 4). A similar, simple relationship of Scatchard's binding constants does not exist.

Multiple binding of one drug. At higher ratios of bound ligand to albumin, significant amounts of complexes with more than one ligand molecule are formed, and the

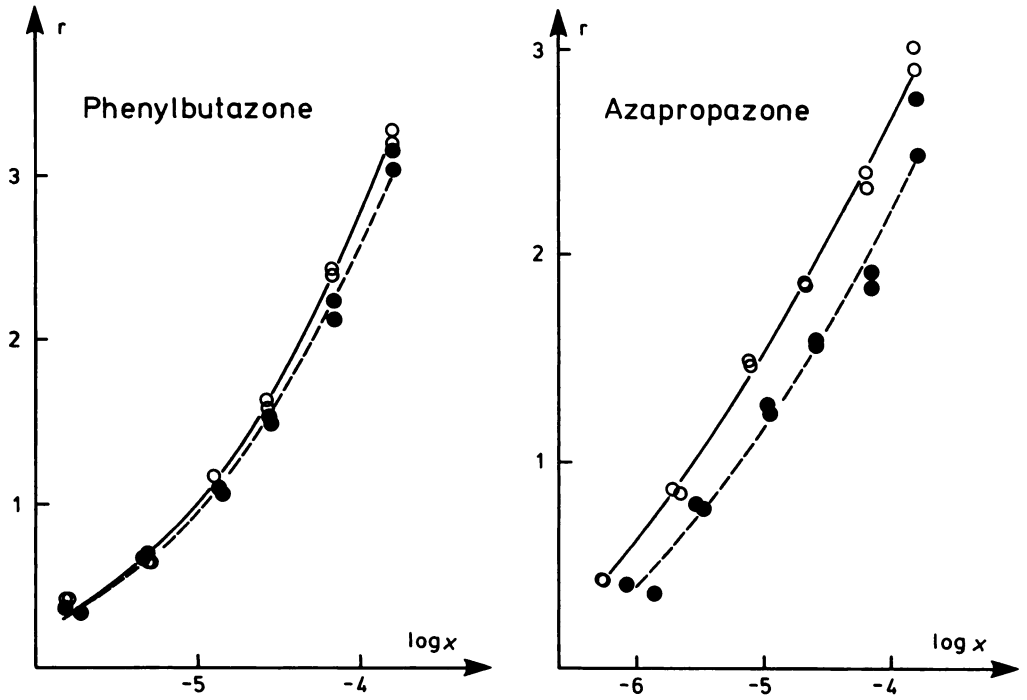


FIG. 5. *Examples of binding isotherms, obtained by equilibrium dialysis, for anti-inflammatory drugs to human serum albumin, (30 μM) with 15 μM bilirubin (●) and without bilirubin (○) under conditions as in Fig. 1*

The curves were obtained by least-squares fitting of Eq. 8, giving the sets of cobinding constants in Table 3.

description of binding by a certain percentage becomes insufficient. Both Scatchard's equation and the stoichiometric binding equation can be used. The two equations are mathematically identical in the sense that one can be transformed into the other. The stoichiometric binding equation is thus obtained by placing all of the added terms of Scatchard's equation on a common denominator. Both equations can be used for describing albumin binding equilibria with multile molecules of a ligand. In case of strongly cooperative binding, however, it would be necessary to use non-real values of Scatchard's binding constants. This is not needed in the stoichiometric equation, where real K values can describe any type of interaction. Strong cooperativity has probably not been

TABLE 4

Stoichiometric binding constants for cobinding of two ligands to defatted human serum albumin (M^{-1})

Ref. Scheme 1; Conditions as in Table 1.

Phenylbutazone →

Bilirubin ↓

59,000,000	260,000	57,000,000	33,000	44,000,000	13,000
4,100,000	250,000	3,900,000	25,000	2,700,000	8,800
	240,000		17,000		4,400

29,000,000	5,900	15,000,000	2,200		0
1,300,000	3,000	0	0		0
	0		0		0

Azapropazone →

Bilirubin ↓

59,000,000	1,100,000	9,600,000	100,000	1,300,000	11,000
4,100,000	180,000	270,000	14,000	58,000	3,000
	12,000		3,000		0

360,000	2,900		0
0	0		0
	0		0

observed for ligand binding to albumin, and Scatchard's equation has in fact been used almost universally.

On the other hand, it should be emphasized that successfully fitting binding data by Scatchard's equation does not allow the conclusion that independent binding to a number of preformed sites is dealt with. Generally speaking, binding to albumin is heterogeneous, as seen from the fact that binding classes of diverse affinity are incurred (13–24). Under these circumstances, the binding data do not indicate that independent sites are present (25, 26). It is not even indicated that preformed sites exist. Any binding phenomenon, whether the mechanism is adsorption to the protein surface, dissolution of the ligand in the interior of the carrier molecule, or binding to multiple, heterogeneous sites, would give binding curves of the type seen with albumin and could be described by Scatchard's equation. The very common conclusion of the presence of *sites*, with affinities as described by the Scatchard constants, is unjustified and should be reserved for cases in which actual site binding is indicated or considered.

In the present study we have preferred to analyze our data for multiple binding of one ligand in stoichiometric terms, implying that information on the presence of sites or of site-to-site interaction cannot be obtained from simple binding studies.

A considerable variability of binding constants is experienced during the curve-fitting procedure, usually so that the first stoichiometric constant is fairly well defined while an increasing degree of indetermination is found for the following constants. The figures reported should be understood as *examples* of binding constant sets which will reliably serve for the purpose of reconstruction of the binding isotherms. An independent physical meaning cannot be attached to single constants (27).

Cobinding of two ligands. An often used equation for cobinding of two ligands was derived by Klotz *et al.* in 1948 (28). This equation is valid if all binding sites are equal and independent and interaction is purely competitive. If these conditions are fulfilled, Scatchard's plot for binding of each ligand is a straight line. This was not the case with any of the ligands studied here.

Analysis in stoichiometric terms of simultaneous binding of two interacting ligands to one carrier is fairly simple if small amounts are present of both ligands. Three parameters, the first stoichiometric binding constant for each ligand and the energetic coupling, are sufficient to define the binding equilibria, and these parameters can usually be determined with relative ease.

If one ligand is present in larger amounts, producing multiple complexes with the carrier, and the other ligand is limited to a trace amount, a relatively easy analysis is still possible, if based on a stoichiometric model, using the concept of reserve carrier-equivalent concentration (Tables 2 and 3).

With larger amounts of both ligands, multiply interacting with the carrier, it is feasible, although tedious, to describe the equilibria by a set of stoichiometric binding constants as in Scheme 1, using Eqs. 8 and 9 (Table 4).

The complexity of the analysis of multiple cobinding raises the question of whether a simple rule of thumb can be established for description of drug binding interactions, suitable for practical purposes when an accurate calculation of binding equilibria is unnecessary. The concept of Site I and II is promising in this context.

Do sites I and II exist? In Table 3, two cases of negative, infinite energetic coupling are reported, for diazepam and naproxen, and for diazepam and flurbiprofen. This means that cobinding of these ligands could not be demonstrated in the dialysis rate experiments with small concentrations of the ligands. Competition of binding in these cases is complete, one molecule of diazepam replacing one molecule of the anti-inflammatory drug, and vice versa. This finding is best explained by an albumin model with one site where the primary binding of diazepam, naproxen, and flurbiprofen takes place and where only one ligand molecule can be bound. Similarly, it has been reported that bilirubin and MADDS bind competitively in the same complete sense (6), and we may reasonably conclude that one site for binding of bilirubin or MADDS exists. It has further been shown that binding of bilirubin and diazepam, one molecule of each, occurs independently (29). The bilirubin site is thus different from the site of diazepam, and these two can exist in the same albumin molecule. It is very likely that these sites are identical with Sites I and II of Sudlow *et al.* (3).

Sjöholm and co-workers (4, 30) have studied competition of a large number of drugs with warfarin and diazepam for binding to human serum albumin. Warfarin in small amounts competes with bilirubin, and MADDS and is bound independently of diazepam. In a site model, we may thus assume that warfarin is bound to Site I. With respect to anti-inflammatory drugs, our results with warfarin and diazepam competition (Table 3) largely confirm those of Sjöholm *et al.*, qualitatively indicating that azapropazone is bound primarily to Site I, indomethacin to both sites, and the other drugs to Site II. It seems safe to conclude from Sjöholm's extensive studies that most drugs bind predominantly to one or the other of these two loci. This simple rule may generally be used for prediction of drug interactions, when specific data are not at hand.

On the other hand, limitations of the site model are obvious. Competition among multiply bound drugs is more complicated. Also, Bruderlein and Bernstein (31) found that binding of tryptophan analogues appears to overlap with both Site I and Site II. Among the presently investigated drugs, only naproxen and flurbiprofen bind solely to Site II at low drug concentrations. The picture is less clear for the other substances. Salicylate, diflunisal, fenbufen, biphenylacetate, ibuprofen, diclofenac, and benoxaprofen also seem to prefer Site II when present in small amounts. Competition of these ligands with diazepam is not absolute, however, and this means that in a stoichiometric 1:1 complex of albumin and one of these ligands, some albumin molecules carry the drug on Site II and some in one or several other places. Allosteric site-to-site interactions must further occur with the binding of bilirubin and MADDS, since these are influenced differently by most of the drugs. Allosteric interaction is particularly evident with naproxen-bilirubin and with flurbiprofen-bilirubin, since it is impossible to explain the positive interactions except by an allosteric mechanism.

A remarkable deviation from the simple Site I-II rule is noted for phenylbutazone (Table 3). This ligand com-

petes primarily with warfarin. The numerical magnitude of the energetic coupling, −0.88 kcal/mole, in fact indicates that simultaneous binding of warfarin and phenylbutazone could barely be demonstrated. In spite of this, phenylbutazone does not compete primarily with binding of bilirubin and little with binding of MADDS. It is therefore not possible to maintain that phenylbutazone, warfarin, MADDS, and bilirubin bind primarily to the same site, Site I.

It is finally important to stress that the evidence available for the reality of Sites I and II does not indicate that these are present as preformed sites in the albumin molecule. All we seem to know is that such sites are present in the complexes of albumin with the respective ligands. The sites may well be formed by extensive conformational changes during the binding process. It seems likely that the albumin molecule is highly flexible, containing several binding units which can be brought together for formation of *ad hoc* sites, in variable combinations according to the needs of individual ligands. "Mapping" of the albumin molecule as having a number of localized sites for various ligands does not seem possible, not even if overlapping is allowed and sites are replaced by binding areas of a less specific nature. Site models of the albumin molecule should incorporate a pronounced flexibility (32) and do not necessarily have to contain preformed sites (33).

Conclusion. Site models of ligand-binding to albumin are useful for colloquial purposes since they are easily perceived. If we say that bilirubin and azapropazone compete for one site on the albumin molecule, we indicate a mutual, negative interaction of bilirubin and azapropazone binding and thus refer azapropazone to the group of drugs primarily binding to Site I, as distinguished from another group mainly interacting with Site II. This is a meaningful statement in qualitative terms, provided that we deal with low concentrations of both ligands, avoiding multiple binding. Scatchard's equation, based on a model with several independent sites, can further be used for characterization of multiple binding of a single ligand. An actual site-binding mechanism should not be implied, however, unless knowledge of sites has been obtained from sources other than simple binding and cobinding measurements.

On the other hand, it is not really necessary to talk of sites when relating results of binding studies. It is a valid and sufficient statement if we say that azapropazone primarily competes with bilirubin and not with diazepam. Isotherms for multiple binding of single ligands are characterized just as well by the stoichiometric binding equation as by a site model, and multiple binding of two ligands to albumin can only be described in stoichiometric terms. Furthermore, stoichiometric descriptions contain all of the information available from binding experiments and have an advantage in avoiding dubious claims of the existence of sites. The term *site*, when used in the strict sense, should be reserved for those cases in which an actual knowledge or consideration of sites is implied.

ACKNOWLEDGMENTS

The authors wish to thank Signe Andersen, Nina Jørgensen, and Birthe Lindgaard for technical work, and Frede Nielsen for the drawings. An HP 85 desk computer was obtained from the Danish Medical Research Foundation through Grant 12-2365.

REFERENCES

1. Scatchard, G. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51**:660–672 (1949).
2. Klotz, I. M. The application of the law of mass action to binding by proteins: interactions with calcium. *Arch. Biochem.* **9**:109–117 (1946).
3. Sudlow, G., D. J. Birkett, and D. N. Wade. The characterization of two specific drug binding sites on human serum albumin. *Mol. Pharmacol.* **11**:824–832 (1975).
4. Sjöholm, I., B. Ekman, A. Kober, I. Ljungstedt-Påhlman, B. Seiving, and T. Sjödin. Binding of drugs to human serum albumin. XI. The specificity of three binding sites as studied with albumin immobilized in microparticles. *Mol. Pharmacol.* **16**:767–777 (1979).
5. Fletcher, J. E., A. A. Spector, and J. D. Ashbrook. Analysis of macromolecule-ligand binding by determination of stepwise equilibrium constants. *Biochemistry* **9**:4580–4587 (1970).
6. Brodersen, R., S. Andersen, C. Jacobsen, O. Sønderskov, F. Ebbesen, W. J. Cashore, and S. Larsen. Determination of reserve albumin-equivalent for ligand binding, probing two distinct binding functions of the protein. *Anal. Biochem.* **121**:395–408 (1982).
7. Weber, G. Energetics of ligand binding to proteins. *Adv. Protein Chem.* **29**:1–83 (1975).
8. Chen, R. F. Removal of fatty acids from serum albumin by charcoal treatment. *J. Biol. Chem.* **242**:173–181 (1967).
9. McDonagh, A. F., and F. Assisi. The ready isomerization of bilirubin IX-α in aqueous solution. *Biochem. J.* **129**:797–800 (1972).
10. Brodersen, R. Competitive binding of bilirubin and drugs to human serum albumin studied by enzymatic oxidation. *J. Clin. Invest.* **54**:1353–1364 (1974).
11. Brodersen, R. Bilirubin: solubility and interaction with albumin and phospholipid. *J. Biol. Chem.* **254**:2364–2369 (1979).
12. Brodersen, R., W. J. Cashore, R. P. Wennberg, C. E. Ahlfors, L. F. Rasmussen, and D. Shusterman. Kinetics of bilirubin oxidation with peroxidase, as applied to studies of bilirubin-albumin binding. *Scand. J. Clin. Lab. Invest.* **39**:143–150 (1979).
13. Zaroslinski, J. F., S. Keresztes-Nagy, R. F. Mais, and Y. T. Oester. Effect of temperature on the binding of salicylate by human serum albumin. *Biochem. Pharmacol.* **23**:1767–1776 (1974).
14. Otagiri, M., and J. H. Perrin. Circular dichroic investigations of the binding of salicylate and related compounds to human serum albumin. *Biochem. Pharmacol.* **26**:283–288 (1977).
15. Chignell, C. F. Optical studies of drug-protein complexes. II. Interaction of phenylbutazone and its analogues with human serum albumin. *Mol. Pharmacol.* **5**:244–252 (1969).
16. Rosen, A. The measurement of binding constants using circular dichroism: binding of phenylbutazone and oxyphenbutazone. *Biochem. Pharmacol.* **19**:2075–2081 (1970).
17. Brown, K. F., and M. J. Crooks. Displacement of tolbutamide, glibenclamide and chlorpropamide from serum albumin by anionic drugs. *Biochem. Pharmacol.* **25**:1175–1178 (1976).
18. Tillement, J.-P., R. Zini, P. d'Athis, and G. Vassent. Binding of certain acidic drugs to human albumin: theoretical and practical estimation of fundamental parameters. *Eur. J. Clin. Pharmacol.* **7**:307–313 (1974).
19. Fehske, K. J., E. Jähnchen, W. E. Müller, and A. Stillbauer. Azapropazone binding to human serum albumin. *Naunyn Schmiedebergs Arch. Pharmacol.* **313**:159–163 (1980).
20. Mortensen, A., E. B. Jensen, P. B. Petersen, S. Husted, and F. Andreasen. The determination of naproxen by spectrofluorometry and its binding to serum proteins. *Acta Pharmacol. Toxicol.* **44**:277–283 (1979).
21. Risdall, P. C., S. S. Adams, E. L. Crampton, and B. Marchant. The disposition and metabolism of flurbiprofen in several species including man. *Xenobiotica* **8**:691–704 (1978).
22. Whitlam, J. B., M. J. Crooks, K. F. Brown, and P. V. Pedersen. Binding of nonsteroidal anti-inflammatory agents to proteins. I. Ibuprofen-serum albumin interaction. *Biochem. Pharmacol.* **28**:675–678 (1979).
23. Wagner, J., and M. Sulc. Binding of diclofenac-Na (Voltaren®) to serum proteins of different species and interactions with other drugs in protein binding. *Aktuel. Rheumatol.* **4**:153–162 (1979).
24. Ekman, B., T. Sjödin, and I. Sjöholm. Binding of drugs to human serum albumin. XV. Characterization and identification of the binding sites of indomethacin. *Biochem. Pharmacol.* **29**:1759–1765 (1980).
25. Klotz, I. M., and D. L. Hunston. Protein interactions with small molecules: relationships between stoichiometric binding constants, site binding constants, and empirical binding parameters. *J. Biol. Chem.* **250**:3001–3009 (1975).
26. Klotz, I. M., and D. L. Hunston. Protein affinities for small molecules: conceptions and misconceptions. *Arch. Biochem. Biophys.* **193**:314–328 (1979).
27. Winslow, R. M., M.-L. Swenberg, R. L. Berger, R. I. Shrager, M. Luzzana, M. Samaja, and L. Rossi-Bernardi. Oxygen equilibrium curve of normal human blood and its evaluation by Adair's equation. *J. Biol. Chem.* **252**:2331–2337 (1977).
28. Klotz, I. M., H. Triwush, and F. M. Walker. The binding of organic ions by proteins: competition phenomena and denaturation effects. *J. Am. Chem.*

Soc. **70**:2935–2941 (1948).
29. Brodersen, R., T. Sjödin, and I. Sjöholm. Independent binding of ligands to human serum albumin. *J. Biol. Chem.* **252**:5067–5072 (1977).
30. Kober, A., and I. Sjöholm. The binding sites on human serum albumin for some non-steroidal anti-inflammatory drugs. *Mol. Pharmacol.* **18**:421–426 (1980).
31. Bruderlein, H., and J. Bernstein. An investigation of the L-tryptophan binding site on serum albumin, using cyclic analogs and fluorescent probes. *J. Biol. Chem.* **254**:11570–11576 (1979).
32. Jacobsen, J., and R. Brodersen. Albumin-bilirubin binding mechanism: kinetic and spectroscopic studies of binding of bilirubin and xanthobilirubic acid to human serum albumin. *J. Biol. Chem.* **258**:6319–6326 (1983).
33. Fletcher, J. E., J. D. Ashbrook, and A. A. Spector. Computer analysis of drug-protein binding data. *Ann. N. Y. Acad. Sci.* **226**:69–81 (1973).

Send reprint requests to: Dr. Rolf Brodersen, Institute of Medical Biochemistry, University of Aarhus, DK-8000 Aarhus C, Denmark.