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Quantitative Assessment of the Competitive Binding of Anionic Ligands to Albumin

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SUMMARY

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A generalized model has been developed to test quantitatively the equilibrium displacement of ligands that bind to successive sites on bovine serum albumin with decreasing affinity. The equations, which are based on the site binding model of Scatchard, assume that competition exists at individual sites, that the affinity constants for ligand and competitor pertain to individual sites, and that the affinity constants for ligand or competitor at any given site are independent of ligand or competitor bound at other sites. The equations are unique in that they account for (a) variation in free competitor concentration as a function of ligand concentration and (b) the partitioning of a competitor into the organic phase. For the ligand–competitor pairs ^{14}C-octanoate–chlorophenoxyisobutyrate and ^{14}C-palmitate–stearate, the site binding model gives an excellent fit to the data, and apparent competitor constants (k_i') are generated that approximate the independently determined association constants (k_i) for the competitor, indicative of classical site–site competition. In the case of ^{14}C-stearate–chlorophenoxyisobutyrate and ^{3}H-anilinonapthalenesulfonate (ANS)–chlorophenoxyiosbutyrate, while the computer simulations for the site binding model fit the data well, the apparent k_1' of chlorophenoxyisobutyrate for the first site is 10- to 100-fold lower than the apparent k_1' value for the same site. In this instance, therefore, the equations assuming site–site competition do not account entirely for the data. A comparison was made of association constants of monomer ANS determined by fluorescence or radiolabeled ^{3}H-ANS binding. A single high-affinity site of 6.8×10^5 M^{-1} existed when ^{3}H-ANS binding was measured, compared to an apparent k_1 of 3.2×10^4 M^{-1} by fluorescence. Chlorophenoxyisobutyrate competitively displaced ANS with a k_1' of 1.4×10^4 M^{-1} measured by ^{3}H-ANS, but only 48 M^{-1} when fluorescence was determined. It is concluded that fluorescence is not a quantitative indicator of ANS binding to albumin and is not suitable for determination of competitive interactions.

INTRODUCTION

Long-chain free fatty acids (FFA)[1] and many drugs bind strongly to serum albumin. *In vivo*, under physiological conditions, the binding of such drugs occurs in the presence of 1–3 mol of endogenous long-chain FFA/mol of albumin and variable amounts of other drugs. In most cases that have been examined, the introduction of a second drug or FFA can displace anionic drugs (1–4), although some question exists as to whether the reaction is competitive or allosteric (1, 5). We have shown that a weakly bound drug, chlorophenoxyisobutyrate (CPIB), can displace FFA from albumin, provided that a hydrophobic phase is present to trap the displaced FFA (6, 7). Furthermore, simple graphical analysis indicates that the displacement from the high-affinity sites is apparently competitive in that complete displacement of each ligand by the other occurs.

Attempts to decribe quantitatively the nature of the binding of a single ligand have been limited by the nonlinearity of ligand binding. One interpretation of the nonlinearity is that high-affinity sites are occupied first, leaving only low-affinity sites for binding at higher ligand concentrations. Scatchard (8) has described this with a model, termed here the *site binding model*, which for-

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[1] Abbreviations used: FFA, free fatty acids; CPIB, chlorophenoxyisobutyrate; BSA, bovine serum albumin; ANS, 8-anilino-1-naphthalenesulfonate.

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mulates the mathematics in terms of affinities of individual sites. This model implicitly assumes that (a) all binding sites preexist and (b) these sites compete independently for available ligand. Both of these assumptions for ligand binding to albumin have been criticized (9–11), and a *stepwise equilibrium* (or stoichiometric) model has been proposed (12, 13). This model defines the equilibria in terms of individual species in solution, possessing n moles of ligand bound regardless of the specific physical site, or address, to which they are bound on each molecule and can easily accommodate cooperative interactions. Data for the binding of albumin to long-chain FFA (9, 14) and salicylate (11) have been fit to the stoichiometric model. However, the model requires complex computer solutions, thus limiting the utility of such a model.

The inclusion of two ligands which are capable of competing for the same sites has not been treated rigorously. The competitor might statistically bind to all sites in much the same fashion that the ligand does. In this case, the stoichiometric model, which sums over all protein species with the same number of competitors and ligands bound, would appear to be best. On the other hand, the specific sites at which ligand and competitor bind may be important, due to different inherent affinities for each, and thus the site binding model would be more appropriate. There is some evidence for the latter situation with proteolytic fragments of albumin, where each fragment contains sites having different ligand affinities (15–17). The site binding model is simpler, mathematically, so that it provides quicker, more reliable results in computer fitting algorithms, whereas the equations for the stoichiometric model tend to be ill conditioned either in the absence (11) or in the presence (Meisner and Neet, unpublished observations) of competing ligands.

In this report, we analyze the data for several pairs of ligands binding to defatted bovine serum albumin, assuming competitive displacement of one ligand at individual sites by another. The equations also take into account (a) variation in free competitor concentration as a function of ligand concentration, which is a problem that assumes major importance when the free competitor concentration approximates the competitor association constant; and (b) the partitioning of a competitor into the organic phase, with subsequent reduction in total competitor available.

METHODS

Equilibrium binding. The equilibrium partitioning and dialysis techniques have been described previously (6, 7, 18). Generally, partitioning was carried out in glass minivials with 1.0 ml of 0.1–0.2 mM BSA in 0.1 M NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, and 25 mM phosphate, pH 7.45, and 1.0 ml of hexane containing initially the ^{14}C-FFA. After equilibrating at 37°C for 16–20 h in a shaking bath, samples were obtained from both phases and radioactivity was counted by liquid scintillation, using a solution of 0.4% 2,5-diphenyloxazole in 2:1 toluene–Triton X-100. Equilibrium dialysis was performed at 37°C, in 1.0-ml-capacity lucite chambers separated by a dialysis membrane, with shaking for 16 h.

Chemicals. York *et al.* (19) have found that recrystallized ANS contains an intensely fluorescing dimer that interferes with protein binding assays. In our hands, recrystallized ANS (ammonium salt, Sigma) contained 23% bis-ANS, as measured by ϵ_{385} of 16,790 M^{-1}. Therefore, ANS was recrystallized routinely six times from water, then further purified to remove dimers by passing through a Sephadex LH-20 column (19). After elution of ANS with 0.1 M Tris, pH 8.0, the bis (ANS) dimer could be seen as a yellow band remaining near the origin. Eluted ANS was spotted on a silica gel plate and developed in a chloroform:methanol:acetic acid solvent (14:5:1, v/v). One fluorescent spot at R_f 0.75 was observed, corresponding to monomer ANS (19). No dimer, as measured by ϵ_{385} or thin-layer chromatography in the previous solvent, was present.

Tritiated ANS was kindly provided by Drs. Hans Cahnmann and Harold Edelhoch, National Institute of Arthritis, Metabolism and Digestive Diseases, NIH (20). When subjected to thin-layer chromatography in either chloroform:methanol:acetic acid or the upper phase of ethyl acetate:methanol:0.1 M NH_4OH (5:2:3, v/v), only one spot corresponding to monomer ANS was visible by autoradiography. The specific activity was 23,400 dpm/mol.

Radioactive FFA were purchased from New England Nuclear and purified before use (6). Chlorophenoxyisobutyrate (Na^+) was a gift from Ayerst Laboratories and was used without further purification. Radioactive ^{14}C-CPIB was synthesized as described (7, 17). Crystallized bovine serum albumin (BSA) was obtained from Armour Pharmaceutical Co., Kankakee, Ill., and defatted by the charcoal method (21) to remove any endogenous fatty acid.

Computer analysis and curve fitting. We have chosen in the present communication to fit data obtained with a variety of ligands and competitors over a broad concentration range to the site binding model as a phenomenological description of the results. The equation for such a site binding model for the measured, radioactive ligand, A, in the presence of the unmeasured competitor, C, may be readily derived from extensions of classical competition at individual "addressed" sites (22) to an n site model (8).

$$\bar{\nu}_A = \sum_{i=1}^{n} k_i(A)/[1 + k_i(A) + k_i'(C)] \qquad [1]$$

and

$$\bar{\nu}_C = \sum_{i=1}^{n} k_i'(C)/[1 + k_i'(C) + k_i(A)], \qquad [2]$$

where $\bar{\nu}_A$ and $\bar{\nu}_C$ are moles of ligand and competitor, respectively, bound per mole of albumin, and (A) and (C) are the concentrations of *free* ligand and *free* competitor, respectively.

In these equations, the apparent constants of k_i and k_i' should be considered as fitting parameters [equivalent to the k_α and k_β of Klotz and Hunston (23)] and, in general, are combinations of individual site association constants (23). In the special case where the sites are different and independent (noninteracting), the k_i and k_i' will be equal to the association constant for the site.

A special case of the site binding model exists when the values of more than one k_i or k_i' are essentially the same (8). In this degenerate situation for two classes of sites, the data are more easily fitted to an n_1 plus n_2 model (Eqs. [3] and [4]), where n_1 and n_2 are the number of sites in class one and class two, respectively. These equations are particularly useful for ligands in which there is one strong site ($n_1 \simeq 1$) and numerous weak sites (n_2 = 4 to 10) and will be utilized extensively in the following paper (17).

$$\bar{\nu}_A = \frac{n_1 k_1(A)}{1 + k_1(A) + k_i'(C)} + \frac{n_2 k_2(A)}{1 + k_2(A) + k_2'(C)}. \quad [3]$$

$$\bar{\nu}_C = \frac{n_1 k_1'(C)}{1 + k_1'(C) + k_1(A)} + \frac{n_2 k_2'(C)}{1 + k_2'(C) + k_2(A)}. \quad [4]$$

Eqs. [1–4] require that the concentrations of *free* ligand (A) and *free* competitor (C) be determined. In order to obtain reliable data, the concentration of protein and radiolabeled ligand in the equilibrium measurement must be of about the same order of magnitude as the reciprocal of the affinity constant that is being measured. If the affinity constant for the competitor is of the same order of magnitude as that of the ligand, or higher, then the free concentration of competitor cannot be assumed to be the same as the total concentration of competitor as is done, for example, in enzyme kinetics. Either an independent measure of the competitor must be made with a second isotopic label or a mathematical calculation of the concentration of free competitor must be made, as we have done here.

The conservation equations for ligand and competitor are given by Eqs. [5] and [6].

$$(A)_{total} = (A) + \bar{\nu}_A[P] \quad [5]$$

and

$$(C)_{total} = (C) + \bar{\nu}_C[P], \quad [6]$$

where $[P]$ is the molar concentration of serum albumin. From the concentration of free ligand obtained by an experimental measurement, the total concentration of competitor, and the apparent affinity constants for ligand and competitor, the concentration of free competitor can be calculated from Eqs. [6] and [2] by numerical estimation.

The concentrations needed for the analysis are for ligand or competitor free in aqueous solution. Since the equilibrium partitioning method was used, an additional consideration had to be made in certain cases for the concentration of solute in the organic phase. For the radiolabeled ligand that partitions into the organic phase, such as long-chain fatty acids, the experimentally determined radioactivity in the organic phase directly yields the aqueous concentration of ligand from the partition coefficient and the bound concentration of ligand by difference. Thus, if the competitor is soluble in the organic phase, such as a long-chain fatty acid, Eq. [6] no longer holds and is replaced by Eq. [7], which includes a term for the concentration of competitor in the organic phase, $(C)_{org}$, as a function $[f(C)]$ of the estimated aqueous concentration. This Eq. [7] is valid provided that the volumes of the organic and aqueous phase are equal.

$$(C)_{total} = (C) + \bar{\nu}_C[P] + (C)_{org}. \quad [7]$$

In practice, the function was determined by a power series fit to the experimentally determined partition ratio over a range of FFA concentrations, according to Eq. [8] for stearate.

$$(C)_{org} = f(C) = 256(C)^4 - 505(C)^3 + 429(C)^2 + 467(C). \quad [8]$$

The computer-derived free competitor concentrations were used to estimate the apparent affinity constants for ligand and competitor by simultaneously fitting to Eqs. [1] and [2] on a Univac 1108 computer. The assumption was made, consistent with the site binding model, that the apparent affinity constant for ligand, k_i, or competitor k_i', at any given site was independent of ligand or competitor bound at other sites on albumin. An array of 50–100 data points of $(\bar{\nu}_A)$ and (A) at varying total concentrations of ligand or competitor was fit by a nonlinear least-squares analysis, weighted as a function of $1/\bar{\nu}$, to obtain simultaneously the best values of k_i and k_i'. The Fortran computer program consisted of a numerical estimation of free (C) from Eqs. [2] and [6] and the current values of k_i and k_i' computed from original estimates. The numerical approximation method was based upon the bisection method and was terminated when the calculated value of $(C)_{total}$ was within 0.01% of the experimentally added $(C)_{total}$. The data fitting program utilized the Marquardt algorithm and was normally terminated when χ^2 remained unchanged up to three significant figures in successive iterations. Initial estimates for the affinity constants had to be reasonably good in order for the program to converge on a solution in a reasonable period of time and were therefore made from the independently determined constants for ligand or competitor, alone. The results of the curve-fitting yield affinity constants for ligand and competitor, standard errors of the constants, bound and free competitor concentrations, and a measure of the goodness of fit, χ^2. Simulated binding profiles of ligand–competitor pairs were plotted with the constants obtained from the fit on a Hewlett-Packard 9815A calculator and 9862A plotter.

RESULTS

Octanoate vs. CPIB. Octanoate and CPIB represent ligands having a single high-affinity BSA binding site of approximately 5×10^5 M^{-1} (7) and multiple weak sites. The interaction between ^{14}C-octanoate and CPIB over a wide concentration range was measured by equilibrium dialysis and is shown in Fig. 1 in the form of a $\bar{\nu}$/A vs $\bar{\nu}$ plot. In this particular case, the individual site Scatchard model was not used, due to the apparent degeneracy of all of the weak sites having nearly identical association constants. Instead, simulated curves for the simultaneous best fits were obtained from an n_1 plus n_2 model, where n_1 = 1.0 and n_2 = 7. The calculated constants and resultant simulations agree very closely with the data, as evidenced by a weighted χ^2 of 0.022. Significantly, the computer-generated apparent k_1' constant (inset, Fig. 1)

for the CPIB high-affinity site of 1.4×10^5 M^{-1} is similar to the value of the apparent association constant, k_1, obtained with ^{14}C-CPIB in the absence of competing ligand [Fig. 2 (7)], emphasizing the competitive nature of the binding.

ANS binding. Binding of ANS to albumin has relied previously on fluorescence measurements (25, 26) which have led to the conclusion that the hydrophobic site probe binds to four independent and equal sites, with an affinity of approximately 1×10^6 M^{-1} (25). In order to measure more directly this interaction, the binding of ^{3}H-ANS to defatted BSA was examined by equilibrium dialysis, with CPIB as the competitor (Fig. 2). It is apparent that only one high-affinity site for ANS exists, with a k_1 of 6.8×10^5 M^{-1} at 37°C (see inset). Other studies with defatted human plasma albumin show a similar binding pattern (data not shown). Although the concentration of BSA is 0.05 mM, identical results were obtained at 0.2 mM. The solid lines, representing the computer-derived fit for a competitive interaction in a four site model, show that CPIB displaces ANS, but in this case the calculated apparent inhibition constant (k_1') for CPIB of 1.4×10^4 M^{-1} for the first site (inset, Fig. 2) is less than the apparent association constant of CPIB for its high-affinity site.

In Fig. 3, the interaction of ANS plus CPIB with BSA was measured by fluorescence, under identical conditions, except the BSA concentration was 10^{-8} M, so that $ANS_{free} = ANS_{total}$. A double-reciprocal plot is employed, since the mole fraction of bound ANS/BSA is unknown. From Fig. 3, the k_1 of ANS for the high-affinity site(s) is 3.2×10^4 M^{-1}. The addition of unlabeled CPIB displaces ANS, but the k_1' of CPIB equals 48 M^{-1}, which is considerably less than that obtained by direct binding assays. Thus, different apparent binding constants of ANS for BSA are obtained, depending on the method employed. Furthermore, the apparent inhibition constants for CPIB are quite different when measured by either method.

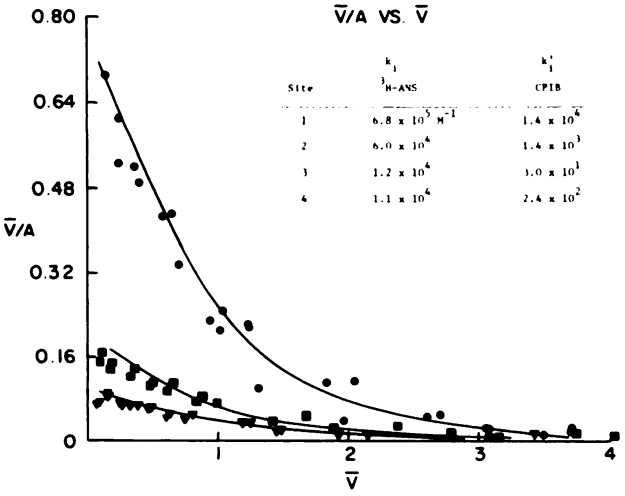

FIG. 2. *Displacement of ^{3}H-ANS from bovine serum albumin by chlorophenoxyisobutyrate*

Solid lines represent simulations of the data based on computer-derived estimates of k_i and k_i' values. The inset gives the k_i and k_i' estimates using a five-site model. BSA, 0.05 mM. (■) 2 mM CPIB; (▼) 5 mM CPIB. Ordinate, bound/free ANS ($\bar{\nu}/A \times 10^6$ M); abscissa, moles bound ANS/mole BSA.

In a counterpart to Fig. 2, where the effects of CPIB on ^{3}H-ANS binding were measured, the interaction of unlabeled ANS with ^{14}C-CPIB binding was studied (Fig. 4). The solid lines indicate a good fit to a competitive interaction, but in this case the apparent inhibition constants for ANS (see inset) are similar to the apparent association constants obtained directly (Fig. 2).

FFA binding. The effect of CPIB on the binding of

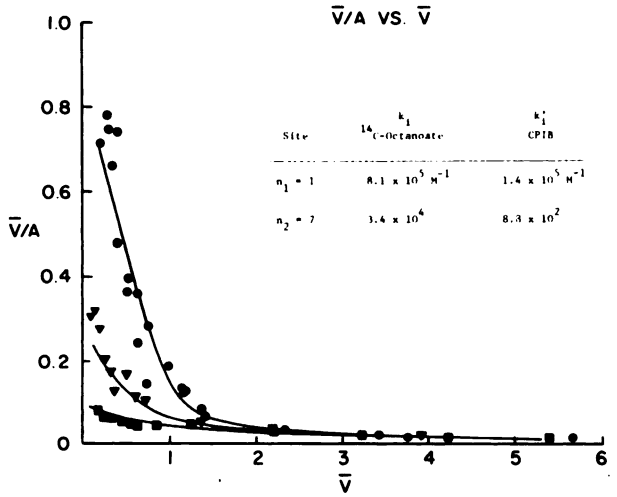

FIG. 1. *Displacement of ^{14}C-octanoate from bovine serum albumin by chlorophenoxyisobutyrate*

Solid lines represent simulation of the data based on computer-derived k_i and k_i' values, using a two-site Scatchard model, where $n_1 = 1$ and $n_2 = 7$. (●) Control; (▼) 0.2 mM CPIB; (■) 0.4 mM CPIB. BSA, 0.2 mM. Ordinate, bound/free octanoate ($\bar{\nu}/A \times 10^6$ M); abscissa, moles octanoate bound/mole BSA ($\bar{\nu}$). The inset gives the computer-derived ligand and competitor constants for the high- and low-affinity sites.

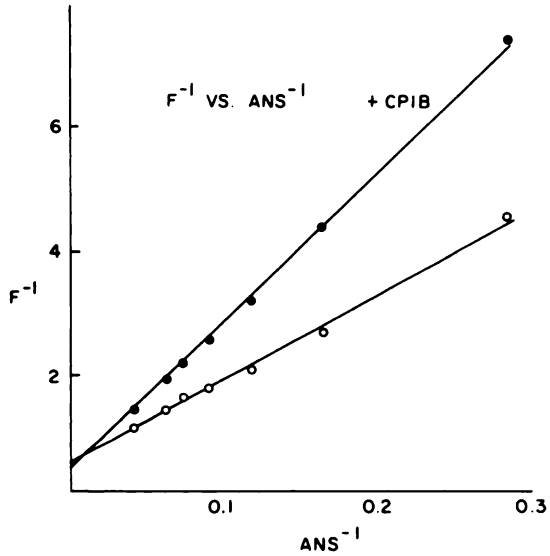

FIG. 3. *Double-reciprocal plot of the effect of chlorophenoxyisobutyrate on the fluorescence of ANS bound to bovine serum albumin*

BSA, 10^{-8} M, was incubated at 37°C in salts–phosphate buffer, pH 7.45. ANS was added, and fluorescence measured in an Eppendorf fluorimeter, with the excitation filter at 313–366 nm and emission at 400–3000 nm. (○) Control; (●) 20 mM CPIB. Ordinate, fluorescence^{-1} in arbitrary units; abscissa, unbound ANS, × 10^6 M.

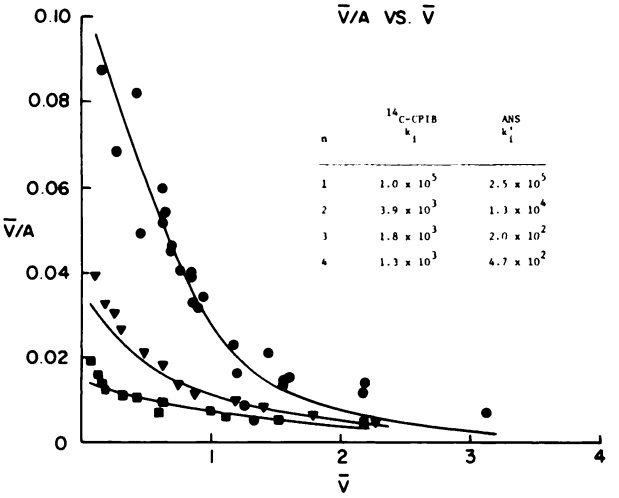

FIG. 4. *Displacement of ^{14}C-chlorophenoxyisobutyrate from bovine serum albumin by ANS*

Solid lines represent simulations of the data based on computer-derived estimates of k_i and k_i' values. The inset gives the k_i and k_i' estimates, using a four-site model. BSA, 0.05 mM. (●) Control; (▲) 0.05 mM ANS; (■) 0.1 mM ANS. Ordinate, bound/free CPIB ($\bar{\nu} \times 10^6$ M); abscissa, moles bound CPIB/mole BSA.

^{14}C-stearate to defatted BSA was measured by equilibrium partitioning (Fig. 5). The solid curves are simulations for a simultaneous best fit of three competitor concentrations plus a control, based on the site binding model with five ligand and five competitor sites. An excellent fit to all data can be seen, as evidenced by a χ^2 of 0.0066. At any concentration of ligand–competitor, the data show a deviation no greater than 10–16% from the simulated line. The inset shows the k values that were generated for each site to obtain the best fit. The apparent competitor constant of the high-affinity site for CPIB as determined by stearate displacement is 1–2 × 10^4 M^{-1}, which is 10^{-1} less than the apparent association constant determined in the absence of stearate (7).

The case of an inhibitor that partitions in the organic phase presents an additional complexity, namely, that $C_{TOT} \neq C_{free} + C_{bound}$, but is also a function of the organic/aqueous partition ratio. The insertion of the partitioning term in the computer simulations enables the determination of free competitor concentrations, based on the actual amount present in the aqueous phase. Since the partition ratio increases with total [FFA], the partitioning term becomes more important as the total competitor concentration that is added initially to the hexane phase is increased. Figure 6 shows an experiment in which stearate was employed as a competitor for ^{14}C-palmitate. A reasonably good fit is obtained, as indicated by a χ^2 of 0.0168. The inset to Fig. 6 shows that the calculated values for k_i' of stearate range from 0.2 × 10^8 to 0.2 × 10^6 M^{-1}, which closely approximate the k_i for stearate determined independently (Fig. 5).

Computer estimates were made of changes in free stearate concentration as a function of free ligand and were compared to experimentally derived values of free stearate based on an equilibrium partitioning experiment with ^{3}H-palmitate and ^{14}C-stearate (Fig. 7). As free ^{3}H-palmitate increases from 0.1 to 10.0 μM, radioactivity measurements reveal that free ^{14}C-stearate increases from 0.052 to 0.309 μM, respectively. The computer simulation for a seven-site model shows that, over the same concentration range of free palmitate, free stearate is calculated to change from 0.054 to 0.337 μM, which agrees closely with the experimental value and supports the validity of the computer equations. For the five-site model, the computer-generated free stearate concentration is higher, increasing from 0.089 to 0.369 μM. Apparently, as the number of binding sites employed in the computer model is reduced, the calculated free competitor levels increase. One explanation is that the competitor

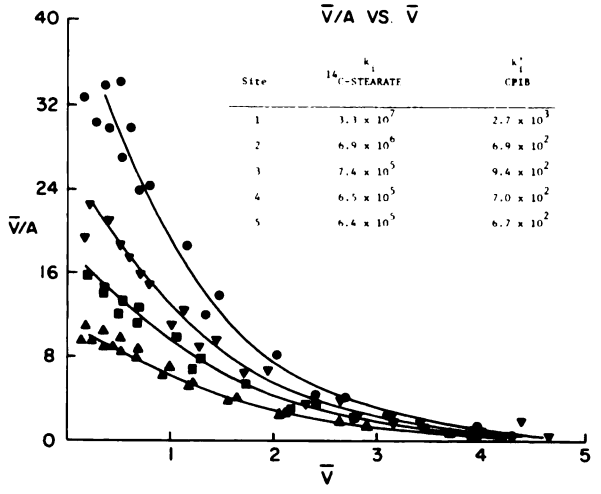

FIG. 5. *Displacement of ^{14}C-stearate from bovine serum albumin by chlorophenoxyisobutyrate*

Solid lines represent simulations of the data based on computer-derived estimates of k_i and k_i' values (see inset), using a five-site site binding model. BSA, 0.2 mM. Ordinate, bound/free stearate ($\bar{\nu} \times 10^6$ M); abscissa, moles stearate/mole BSA. (●) Control; (▼) 0.5 mM CPIB; (■) 1.0 mM CPIB; (▲) 2.0 mM CPIB.

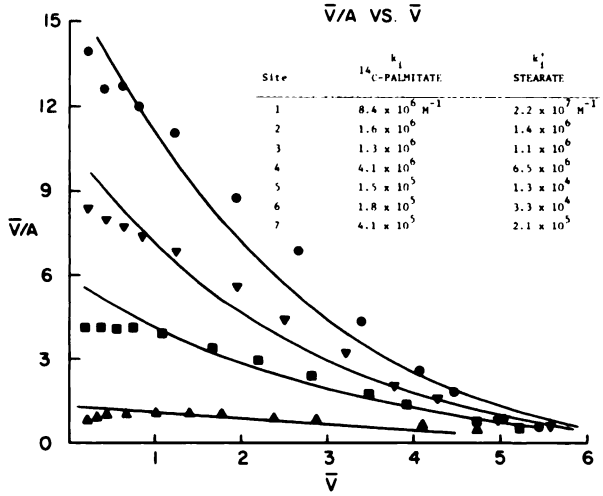

FIG. 6. *Displacement of ^{14}C-palmitate from bovine serum albumin by stearate*

Free stearate was determined from the partitioning function, Eq. [8]. Solid lines show simulations based on k_i and k_i' values using a seven-site binding model. Computer-derived estimates of the k_i and k_i' values are shown in the inset. (●) Control; (▼) 0.2 mM; (■) 0.5 mM; (▲) 2.5 mM total stearate.

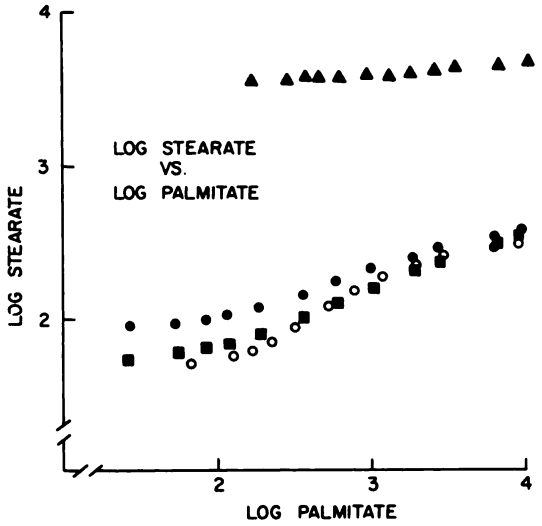

FIG. 7. *Effect of ligand (^{3}H-palmitate) on the free concentration of a competitor, ^{14}C-stearate*

Both radioligands were added to the hexane phase of a partitioning system and allowed to equilibrate with 0.2 mM bovine serum albumin at 37°C for 20 h. Free palmitate was calculated from the hexane phase concentration and partition ratios. Free stearate was estimated by two methods: (a) directly from the hexane phase concentration of ^{14}C-stearate and the partition ratio and (b) calculated from Eq. [7]. (■) 0.2 mM total stearate, free concentration estimated directly from ^{14}C-stearate. (●) 0.2 mM total stearate, free concentration calculated from Eq. [7], with a five-site model; $\chi^2 = 0.050$. (○) 0.2 mM total stearate, free concentration calculated from Eq. [7], with a seven-site model; $\chi^2 = 0.017$. (▲) 2.5 mM total stearate, free concentration calculated from Eq. [7], with either a five- or a seven-site model. Ordinate, log stearate ($\times 10^9$ M); abscissa, log palmitate ($\times 10^9$ M).

is afforded fewer possible binding sites, which results in artificially forcing more competitor into the aqueous phase. It follows that the differences observed in Fig. 7 between calculated and observed free competitor levels can be minimized further by raising the number of sites employed in the model. However, the decrease in χ^2 that we have found is slight and is outweighed by the increased variation in k_i and k_i' values, which results from the lack of data beyond $\bar{\nu} = 5$–6.

DISCUSSION

ANS binding. Reported association constants of ANS to albumin have been based on fluorescence measurements (25), in which four sites of equal affinity have been calculated. In the present study, the use of ^{3}H-ANS to measure binding directly has revealed that the probe binds to albumin with a single high-affinity site of 6.8×10^5 M^{-1} and to several weaker sites. The apparent discrepancy can be explained by (i) the method used to measure binding, i.e., fluorescence vs equilibrium dialysis or (ii) the purity of ANS. In the latter case, Ferguson *et al.* (20) present evidence that a dimer, which is abundant in recrystallized ANS, binds with a higher affinity than the monomer. The fluorescence studies reported elsewhere, carried out with recrystallized but not monomer ANS, may have measured the interaction of the dimer/monomer mixture to albumin. Regarding the first and more likely possibility, the fluorescence intensity of ANS when adsorbed to albumin may not be a sensitive indicator of the strength of the hydrophobic interaction, as had been tacitly assumed (25, 26). We have measured the affinity of ANS for BSA by fluorescence and found that the apparent k_1 of ANS is 3×10^4 M^{-1}, which is less than 10^{-1} the affinity determined directly by ^{3}H-ANS binding. The displacement of ANS by CPIB was competitive, when measured by either method. However, the apparent k_1' of CPIB differed greatly, being 1.4×10^4 M^{-1} in the case of radioligand binding and 0.49×10^2 M^{-1} determined by fluorescence. Certainly, the measurement of ^{3}H-ANS binding reported here is the most direct approach and indicates the true association constant more accurately than fluorescence.

Mechanism of ligand displacement from albumin. Mechanistically, the use of a Scatchard or site binding model has been questioned (9, 10, 23), due to the underlying assumptions of noninteracting sites with inherent affinities at each site, which excludes the possibility that conformational changes (27) are responsible for the observed phenomena. The stepwise equilibrium model (13) is not restricted to these limitations, and is more general in that negative cooperativity can be accounted for. Nevertheless, the site binding model we have used does, in certain cases, completely account for and fit the data very well. Good overall fits to Eqs. [1] and [2] were obtained with the ligand–competitor pairs palmitate–stearate (Fig. 6) and octanoate–CPIB (Fig. 1), with apparent k_i' values determined for a given competitor essentially equal to apparent k_i values determined in the absence of ligand, regardless of which of the pairs was the competitor. This equality would be expected for competition between ligand and competitor at a common set of sites which have different inherent affinities and are noninteracting (no cooperativity). This same condition provides for equivalency between the k_i or k_i' values of Eqs. [1] and [2] and the true site binding constants (9, 28). Thus, we conclude that for these examples, the binding of ligand behaves as if the sites are *independent* but that specific "addresses" exist with classical competition between different ligands for identical sites.

This type of classical competitive behavior, however, is limited to ligand–competitor pairs having similar apparent association constants. For ligand–competitor pairs having markedly different binding constants, true classical competition does not occur, but depends upon which compound is used as the competitor. On the one hand, the more tightly bound ligand (i.e., FFA) displaces classically the weaker ligand (i.e., octanoate), with $k_i = k_i'$ for the tightly bound ligand. Our data differ in this respect from several other reports claiming that long-chain FFA are bound to sites distinct from weaker bound ligands and displace these ligands by "noncompetitive" mechanisms (2, 3, 5, 10, 26, 29). For the converse experiment, the weakly bound ligands displace tightly bound ligands and appear to fit the competitive equations, but the k_i (from direct binding) and k_i' (from competition studies) values for the weakly bound competitor are *not* equivalent, particularly for the high-affinity binding site. The examples of ligand–competitor pairs included in this category are palmitate–CPIB, (Meisner and Neet, unpublished), stearate–CPIB (Fig. 5), palmitate–octanoate (7), and ANS–CPIB (Fig. 2). The competitive Scatchard

model is not, therefore, applicable generally to all ligands with albumin and must be modified to explain the unequal k and k' terms, e.g., with cooperative interactions. Other reports showing an enhancement of ligand binding by a second ligand (3, 5, 26) also cannot be explained by the simple site binding model. However, the alternative, stoichiometric model with competitor forms included was ill conditioned mathematically (Meisner and Neet, unpublished), with no improvement in agreement of competitor constants. Thus, the advantage of the stoichiometric model in accounting for cooperativity of binding may be lost in nondiscrimination between individual binding sites on the albumin molecule, as well as the more complex computer equations required, when competition between ligands occurs. A rigorous, mathematical treatment of the site binding and the stoichiometric models with ligand and competitor binding, a comparison of the adequacy of fits, and a mechanistic interpretation of ligand–competitor binding to albumin will be the subject of a subsequent report.

Taken with our previous studies, it appears that a "hierarchy" exists, at least among anionic ligands, with respect to the ability to displace another ligand in a classical competitive manner, i.e., where $k_i = k_i'$. Competition appears only when the competitor has an equal or greater association constant for the first few sites on albumin than the ligand. It should be possible to tabulate a hierarchy of competitors, based upon the association constants of the first few sites, at least in the case of acidic ligands. Although basic ligands do not bind to the same sites as acidic ligands (30), there is no reason why a similar list cannot be drawn for this group as well.

The pharmacological importance of drug (or FFA) displacement by FFA (or drug) has not been addressed. Meisner (6) has shown previously that CPIB displaced significant quantities of long-chain FFA at drug/albumin molar ratios of 1–2, which are considered to be within therapeutic levels. Palmitate was also shown to competitively displace CPIB at low FFA/albumin ratios. In the present study, drug/albumin molar ratios in certain experiments (Figs. 1, 5, and 6) are within the limits found *in vivo*, but in others (Figs. 2 and 3), competition is observed only at high ligand/albumin ratios. Nonetheless, the mechanism of ligand–ligand interaction as described in this study is valid, regardless of the physicological concentration of the competitor.

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REFERENCES

1. Solomon, H., J. Schrogie and D. Williams. The displacement of phenylbutazone-^{14}C and warfarin-^{14}C from human albumin by various drugs and fatty acids. *Biochem. Pharmacol.* **17:** 143–146 (1967).
2. Spector, A., E. Santos, J. Ashbrook and J. Fletcher. Influence of free fatty acid concentration on drug binding to plasma albumin. *Ann. N.Y. Acad. Sci.* **226:** 247–258 (1973).
3. Santos, E., and A. Spector. Effects of fatty acids on the interaction of 1-anilino-8-naphthalenesulfonate with human plasma albumin. *Mol. Pharmacol.* **10:** 519–528 (1974).
4. Rudman, D., T. Bixler and A. Del Rio. Effect of free fatty acids on binding of drugs by bovine serum albumin, by human serum albumin, and by rabbit serum. *J. Pharmacol. Exp. Ther.* **176:** 261–272 (1971).
5. Birkett, D., S. Myers and G. Sudlow. Effect of fatty acids on two specific drug binding sites on human serum albumin. *Mol. Pharmacol.* **13:** 987–992 (1977).
6. Meisner, H. Displacement of free fatty acids from albumin by chlorophenoxyisobutyrate. *Mol. Pharmacol.* **13:** 224–231 (1977).
7. Meisner, H., and K. Neet. Competitive binding of long chain free fatty acids, octanoate, and chlorophenoxyisobutyrate to albumin. *Mol. Pharmacol.* **14:** 337–346 (1978).
8. Scatchard, G. The attraction of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51:** 660–672 (1949).
9. Fletcher, J., A. Spector and J. Ashbrook. Analysis of macromolecule-ligand binding by determination of stepwise equilibrium constants. *Biochemistry* **9:** 4580–4587 (1970).
10. Spector, A. Fatty acid binding to plasma albumin. *J. Lipid Res.* **16:** 165–179 (1975).
11. Fletcher, J., and A. Spector. Alternative models for the analysis of drug-protein binding. *Mol. Pharmacol.* **13:** 387–399 (1977).
12. Adair, G. The hemoglobin system: The oxygen dissociation curve of hemoglobin. *J. Biol. Chem.* **63:** 529–545 (1925).
13. Klotz, I., F. Walker and R. Pivan. The binding of organic ions by proteins. *J. Am. Chem. Soc.* **68:** 1486–1490 (1946).
14. Ashbrook, J., A. Spector, E. Santos and J. Fletcher. Long chain fatty acid binding to human plasma albumin. *J. Biol. Chem.* **250:** 2333–2338 (1975).
15. King, T. On the location of the primary ligand binding site of bovine plasma albumin. *Ann. N.Y. Acad. Sci.* **226:** 94–100 (1973).
16. Reed, R., R. Feldhoff and T. Peters. Fragments of bovine serum albumin produced by limited proteolysis: complementary behavior of the large fragments. *Biochemistry* **15:** 5394–5398 (1976).
17. Meisner, H. Binding of anionic ligands to peptic fragments of bovine serum albumin. *Mol. Pharmacol.* **18:** 224–229 (1980).
18. Goodman, D. The interaction of human serum albumin with long chain fatty acid anions. *J. Am. Chem. Soc.* **80:** 3892–3898 (1958).
19. York, S., R. Lawson and D. Worah. Binding of recrystallized chromatographically purified 8-anilino-1-napthalenesulfonate to Escherichia coli lac repressor. *Biochemistry* **17:** 4480–4486 (1978).
20. Ferguson, R., H. Edelhoch, H. Saroff and J. Robbins. Negative cooperativity in the binding of thyroxine to human serum prealbumin. *Biochemistry* **14:** 282–289 (1975).
21. Chen, R. Removal of fatty acids from serum albumin by charcoal treatment. *J. Biol. Chem.* **242:** 173–181 (1967).
22. Segal, I. *Enzyme Kinetics.* Wiley Interscience, New York (1975).
23. Klotz, I., and D. Hunston. Protein affinities for single molecules: Conceptions and misconceptions. *Arch. Biochem. Biophys.* **193:** 314–328 (1979).
24. Bevington, P. R. *Data Reduction and Error Analysis for the Physical Sciences.* McGraw-Hill, New York. (1969).
25. Kolb, D., and G. Weber. Cooperativity of binding of anilinonaphthalenesulfonate to serum albumin induced by a second ligand. *Biochemistry* **14:** 4476–4481 (1975).
26. Muller, U. The use of 8-anilino-1-napthalenesulfonic acid as a reporter group molecule for circular dichroism and fluorescence measurements. *Hoppe-Seyler's Z. Physiol. Chem.* **357:** 1487–1494.
27. Karush, R. Heterogeneity of binding sites of bovine serum albumin. *J. Am. Chem. Soc.* **72:** 2705–2713 (1950).
28. Klotz, I., and D. Hunston. Protein interactions with small molecules. Relationships between stoichiometric binding constants, site binding constants. *J. Biol. Chem.* **250:** 3001–3009 (1975).
29. Sudlow, G., D. Birkett and D. Wade. The characterization of two specific drug binding sites on human serum albumin. *Mol. Pharmacol.* **11:** 824–832 (1975).
30. Brodersen, R., T. Sjodin and I. Sjoholm. Independent binding of ligands to human serum albumin. *J. Biol. Chem.* **252:** 5067–5072 (1977).

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