Evidence for a common high affinity binding site on glutathione S-transferase B for lithocholic acid and bilirubin

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Abstract Binding of lithocholic acid, bilirubin, and gossypol to glutathione S-transferase B (ligandin or transferase Y_aY_c) was compared using four methods. Tryptophan quenching revealed a single high affinity site for bilirubin and gossypol but could not be used for lithocholic acid. Both displacement of the fluorescent probe, 1-anilino-8-naphthalenesulfonate, and spectral changes induced by bilirubin binding demonstrated a common high affinity site for which all three ligands compete. Similar results were obtained by equilibrium dialysis. The dissociation constants for the binding of both bilirubin and lithocholic acid were comparable with the various methods (range 0.2–0.7 μM). Thus, lithocholic acid and bilirubin share a high affinity binding site on gluthathione S-transferase B that appears to be separate from the binding site for substrates.—**Sugiyama, Y., A. Stolz, M. Sugimoto, and N. Kaplowitz.** Evidence for a common high affinity binding site on glutathione S-transferase B for lithocholic acid and bilirubin. *J. Lipid. Res.* 1984. **25:** 1177–1183.

Supplementary key words gossypol • fluorescence quenching • equilibrium dialysis • 1-anilino-8-naphthalenesulfonate displacement

Hepatic cytoplasmic proteins such as ligandin may have a physiologic role in intracellular transport of bile acids or may simply minimize potentially toxic free concentrations of bile acids in the cell. Ligandin, first identified as a bilirubin and sulfobromophthalein-binding protein (1), is also recognized as a glutathione S-transferase (2, 3). Some confusion and controversy exists as to which glutathione S-transferase should be designated as ligandin. Two rat enzymes, transferase "X" (subunit Y_aY_a) (4) and transferase B (subunit Y_aY_c) (5) are the principal candidates by virtue of their high affinity organic anion binding compared to other transferases having Y_b or Y_b' subunits (6). In fact, a special or distinct high affinity binding site for bilirubin has been characterized on the Y_a subunit (5, 7), whereas most transferases also have a low affinity site (5, 7).

Bile acids, such as lithocholic acid, have been shown to bind to various transferases (4). This has been demonstrated by inhibition of enzyme activity (8, 9) and by direct binding studies (10). Since multiple binding sites exist on ligandin (6, 7), we examined whether a bile acid, namely lithocholic acid, binds to the same site as bilirubin or its analogue, gossypol (11). For the purposes of these studies we used a purified preparation of rat glutathione S-transferase B (Y_aY_c), and compared binding using various techniques.

METHODS

Purification of ligandin

Ligandin (glutathione S-transferase B) was purified from rat liver cytosol according to Habig, Pabst, and Jakoby (12) and found in SDS-PAGE to be homogeneous showing the subunit composition Y_aY_c. Despite the confusion in nomenclature, transferase B is widely accepted as one form of ligandin. We will use these two designations interchangeably.

Binding studies: ANS fluorescence inhibition technique

Inhibition of the binding of 1-anilino-8-naphthalenesulfonate (ANS) to ligandin by bilirubin or gossypol was determined by following the decrease in the fluorescence of ANS. To cuvettes containing 2 ml of mixtures of 0.29 μM ligandin, 12.5 μM ANS, and 0.01 M sodium phosphate buffer, pH 7.4 (standard buffer), was added a maximum of 10 μl of the inhibitor in 1–2 μl aliquots. The solution was mixed after each addition and the fluorescence was measured at 480 nm during excitation at 400 nm (at room temperature). When necessary, the

Abbreviations: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ANS, 1-anilino-8-naphthalenesulfonate.

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fluorescence intensities were corrected for inner filter effect by the method of Chignell (13). The calculation of inhibition constants was as described previously (14) and is outlined in the Appendix.

To determine the type of inhibition, experiments were performed as follows. To cuvettes containing 2 ml of standard buffer and ligandin (0.29 μM) in the absence or presence of inhibitors, various amounts of ANS (up to 28 μl from a 5 mM stock solution) were added and fluorescence was determined as described above. The apparent dissociation constant (K_{dapp}) was calculated from the Scatchard plots (15, 16) i.e., change in fluorescence (ΔF)/ANS concentration against ΔF.

Binding studies: quenching of tryptophan fluorescence

The decrease in intrinsic tryptophan fluorescence of ligandin after addition of bilirubin or gossypol was determined at 330 nm during excitation at 280 nm. To cuvettes containing 2 ml of ligandin (0.055 μM) in standard buffer, was added a maximum of 10 μl of stock solution of ligands in 1–2 μl aliquots (final concentration 0.25–3.0 μM). When necessary, the fluorescence intensities were corrected for inner-filter effect by the method of Chignell (13). The stock solutions of bilirubin (0.5 mM) were freshly prepared in 0.01 M NaOH. The analysis of the quenching data was performed according to the method of Chignell (13).

Binding studies: difference spectra

The binding of bilirubin was determined by a spectrophotometric method, which was slightly modified from the method of Tipping et al. (17). The difference spectrum between bound and unbound bilirubin showed a positive peak at 472 nm and a negative peak at 405 nm. Titrations were carried out by adding equal volumes of stock solutions of bilirubin to both sample and reference cuvettes; the former contained a protein solution in standard buffer (2.5 ml), the latter an equal volume of buffer. The concentration of bound bilirubin (C_b) was obtained from the following equation: $C_b = (A_{472} - A_{405})/(E_{472} - E_{405})$ where A_{472} and A_{405} are the absorbance differences (absorbance of total mixture of bilirubin and protein − absorbance of bilirubin alone) at 472 nm and 405 nm, respectively, and E_{472} and E_{405} are the differences in molar extinction coefficients of bound bilirubin and unbound bilirubin at 472 nm and 405 nm, respectively. The data were analyzed as discussed in the Appendix or in the form of Scatchard plots (15, 16).

Binding studies: equilibrium dialysis

Binding of lithocholic acid was measured by equilibrium dialysis at 4°C. A tracer amount of [^{14}C]lithocholic acid (0.005 μCi) (59 μCi/μmol, New England Nuclear) with various concentrations of unlabeled lithocholic acid (0.4 to 6 μM) was added to the protein compartment (ligandin concentration, 1.9 μM). After equilibrium was reached (40 hr), radioactivity on both sides of the membrane (Spectrapor membrane 12–14,000 dalton exclusion, Spectrum Medical Industries, Los Angeles, CA) was determined and expressed as C_b/C_f, where C_b is the bound concentration determined by subtracting the free concentration (C_f) in the protein-free side from the total concentration in the protein-containing side. The data were analyzed by the Scatchard method.

When the inhibition of the binding of lithocholic acid to ligandin by gossypol was measured, different concentrations of gossypol (5.7 to 43 μM) were initially added to the protein compartment. After 40 hr, the concentrations of gossypol on both sides of the dialysis chamber were determined from A_{390} (maximum absorbance of gossypol). The inhibition constant (K_i) was calculated according to the method of Sugiyama et al. (14) as outlined in the Appendix.

RESULTS AND DISCUSSION

Using the ANS technique, various concentrations of bilirubin and gossypol were found to inhibit the fluores-

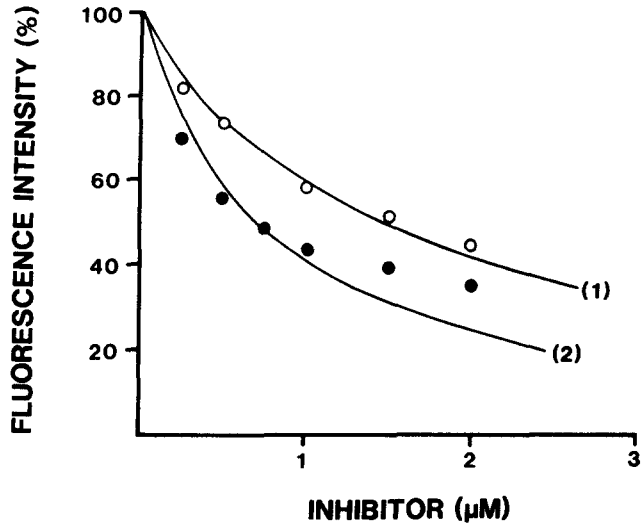

Fig. 1. Inhibition of the binding of ANS (intensity of fluorescence) by various concentrations of bilirubin (●) or gossypol (○). Concentrations of ANS and transferase B were fixed at 12.5 μM and 0.29 μM, respectively. Y axis represents percent of intensity of fluorescence with 100% representing that of bound ANS in the absence of inhibitors. The lines are theoretical curves calculated as described in the Appendix, based on competitive inhibition of the binding of ANS to ligandin. The binding parameters used in this calculation were K_d (dissociation constant for ANS) = 37 μM (from Fig. 2), and n (number of binding sites) = 1. Dissociation constants for inhibitors (K_i = 1.0 μM (1) and 0.4 μM (2)) were found to give best fits of the data.

TABLE 1. Dissociation constants (μM) for the binding of bilirubin, gossypol, and lithocholic acid to ligandin using various techniques

	Bilirubin			Lithocholic Acid	
	Present	Published	Gossypol	Present	Published
Direct methods					
[^{14}C]Lithocholic equilibrium dialysis				0.32 (n = 0.93)[a]	
Bilirubin difference spectrum	0.31 (n = 1.2)	0.14 (18)[b] 1.0 (18)			
Tryptophan quenching	0.61[c] (n = 0.68)	0.68 (20) 2.0 (21) 0.15 (UP)[c]	1.10 (n = 0.99)	NC[d]	
Inhibitory methods					
ANS method	0.4		1.0	0.7	0.3 (10)
Bilirubin difference spectrum				0.22	
[^{14}C]Lithocholic equilibrium dialysis			5.5		

[a] Number of binding sites per molecule of protein.
[b] Reference number in parentheses.
[c] Unpublished work of Sugiyama and Kaplowitz with glutathione S-transferase B prepared according to Mannervik and Jensson (22).
[d] No change in fluorescence.

cence of ANS in a concentration-dependent fashion (**Fig. 1**). Lithocholic acid also inhibited the binding of ANS (not shown), as we have previously described (10). Assuming that this inhibition was competitive, the K_i values for all three inhibitors were comparable (**Table 1**). The competitive nature of the inhibition of the binding of ANS by lithocholic acid, bilirubin, and gossypol was demonstrated in experiments where the concentrations of ANS were varied in the presence and absence of the inhibitors (**Fig. 2**). All the inhibitors

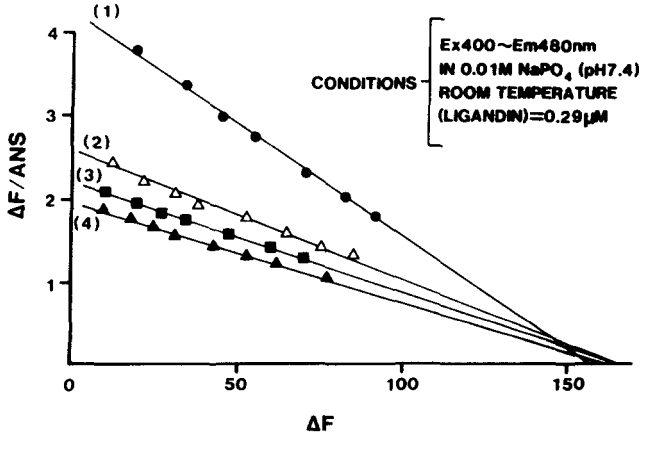

Fig. 2. Scatchard plot of the binding of ANS to ligandin in the absence (1) and presence of 0.5 μM lithocholic acid (2), 0.5 μM bilirubin (3), and 1.5 μM gossypol (4). The concentration of ANS was varied from 5 to 70 μM while concentrations of inhibitors and ligandin (0.29 μM) were held constant. The binding of ANS was determined by the change of fluorescence (ΔF) as described in the text.

increased K_{dANS} without affecting the maximum fluorescence change, suggesting competition by all three ligands for a binding site for ANS on ligandin.

A second approach was to study the binding of bilirubin directly by a spectrophotometric technique and then assess the effect of lithocholic acid on binding of bilirubin. The binding of bilirubin to ligandin is associated with a red shift in its absorption maximum and a large difference-spectrum, as first reported by Tipping et al. (17). Lithocholic acid inhibited the binding of bilirubin in a concentration-dependent fashion (**Fig. 3**). Support for the competitive nature of this interaction was obtained by comparing the binding spectra of bilirubin at various concentrations of bilirubin in the presence and absence of lithocholic acid (**Fig. 4**). The K_i for bilirubin and the K_i for lithocholic acid derived from these data (Table 1) agree with the findings using the ANS technique. Again, the results suggest competition for the same binding site on the protein.

A third approach was to directly assess the binding of lithocholic acid by equilibrium dialysis and to examine the effects of bilirubin and gossypol on this binding. With this technique a single high affinity binding site for lithocholic acid was demonstrated (**Fig. 5**) and the K_d value for lithocholic acid was in close agreement with that observed with the above two indirect techniques (Table 1). Attempts to study the effects of bilirubin on the binding of lithocholic acid to ligandin in this system were hampered by nonspecific absorption of bilirubin to the dialysis membrane and dialysis cells. Consequently, an analogue of bilirubin, gossypol (11), was used for the inhibitory experiment and the results are shown in **Fig.**

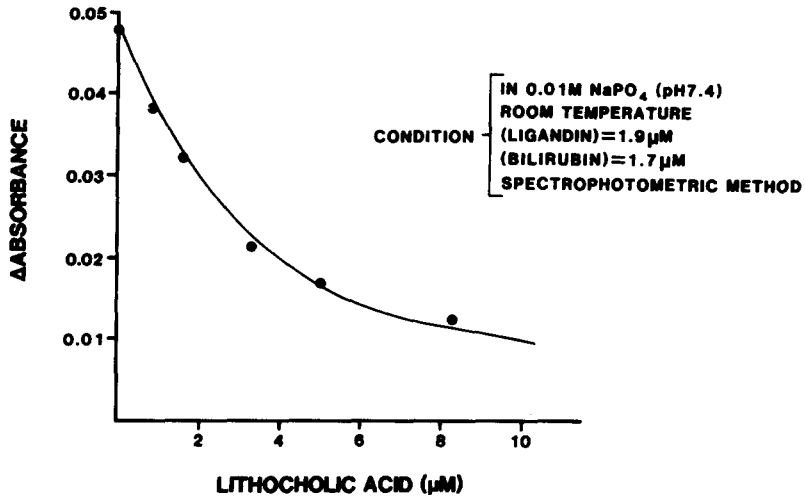

Fig. 3. Inhibition of the binding of bilirubin to ligandin by various concentrations of lithocholic acid. Concentrations of bilirubin and ligandin were fixed at 1.7 μM and 1.9 μM, respectively. Y axis represents the absorbance difference at 472 nm and 405 nm ($\Delta A = A_{472}-A_{405}$). The line is a theoretical curve calculated as described in the Appendix, based on the assumption that lithocholic acid competitively inhibits bilirubin binding to ligandin. The binding parameters used in this calculation were K_d (for bilirubin) = 0.31 μM, (from Fig. 4) and n (for bilirubin) = 1.2. K_i (for lithocholic acid) = 0.22 μM was found to give best fit of the data.

6. Gossypol also sticks to membrane and dialysis cells to some extent and therefore quantitative interpretation of this experiment is difficult. However, the results shown in Fig. 6 clearly indicate that gossypol does inhibit the binding of lithocholic acid to ligandin. The binding of gossypol to ligandin was independently confirmed by the quenching of tryptophan fluorescence. Both gossypol and bilirubin exhibited a single high affinity site (data not shown) and ligandin had comparable affinity for both ligands (Table 1). As previously reported, lithocholic acid did not quench the intrinsic fluorescence of the protein, presumably being unable to affect the energy transfer.

Previous quantitative studies of the binding of bile acids using purified ligandin have only been reported by Tipping et al. (18) and our laboratory (10). The

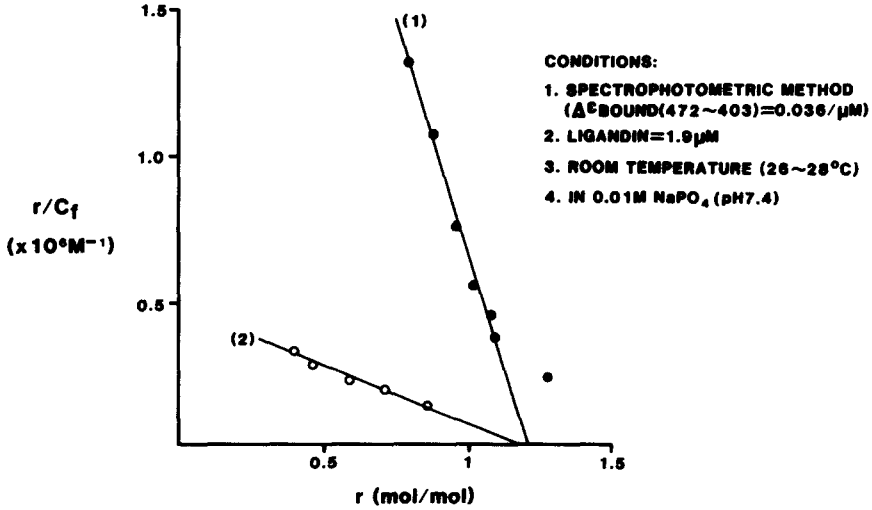

Fig. 4. Scatchard plot of the binding of bilirubin to ligandin in the absence (1) and presence (2) of lithocholic acid. The concentration of bilirubin was varied from 2 μM to 7.5 μM, while concentrations of lithocholic acid (5 μM) and ligandin (1.9 μM) were held constant. The binding of bilirubin was determined by the difference of absorbance as described in the text.

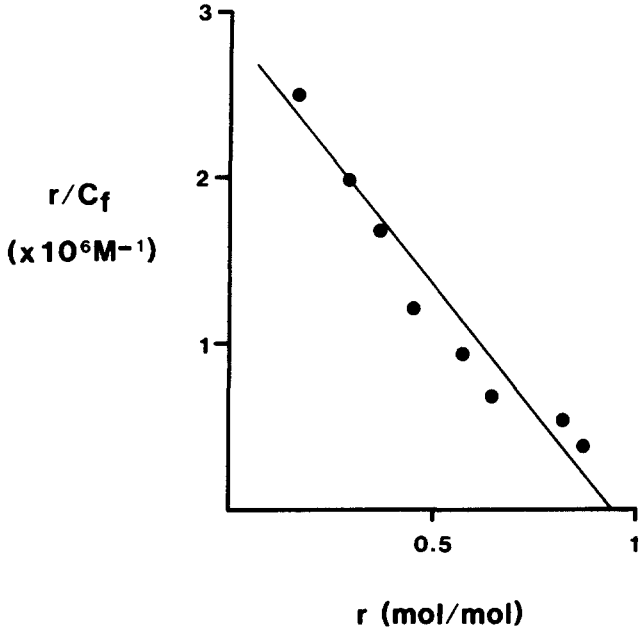

Fig. 5. Scatchard plot for the binding of lithocholic acid to ligandin. Binding was measured by equilibrium dialysis at 4°C. The concentration of ligandin (1.0 μM) was held constant and the initial concentration of lithocholic acid was varied from 0.4 μM to 6 μM. Calculations from these data revealed n = 0.93 and K_d = 0.32 μM.

former group assumed competitive displacement of [^{3}H]estrone sulfate and were able to only approximate the binding of chenodeoxycholate and deoxycholate ($K_d 10^{-4}$ to 10^{-5}M). We previously demonstrated that a variety of bile acids competitively inhibit the binding of ANS to transferase B (10). Kamisaka et al. (19) attempted to study lithocholic acid as an inhibitor of the binding of bilirubin using circular dichroism but obtained anomalous spectral shifts which led them to conclude that the binding of bile acids may not be competitive (same site) with bilirubin. Other laboratories have studied the binding of bile acids indirectly by inhibition of enzyme activity (8, 9). However, kinetics are complex, rarely competitive, and the concentrations required to produce 50% inhibition of enzyme activity are considerably higher than K_d values determined by direct binding studies (10). These findings suggest that the substrate binding site differs from the bile acid high affinity site. Moreover, a distinct high affinity binding site for bilirubin has been determined on ligandin (6, 7). Thus our demonstration of the competitive interaction of lithocholic acid and bilirubin indicates that lithocholic acid binds to the same high affinity site as bilirubin. As we have previously shown that other bile acids competitively displace ANS

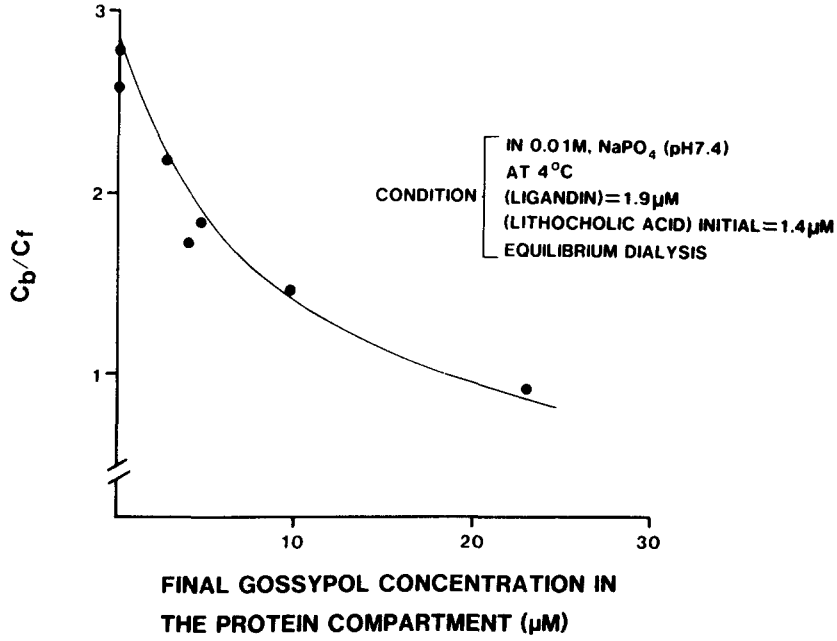

Fig. 6. Inhibition of the binding of lithocholic acid to ligandin by various concentrations of gossypol. Binding of lithocholic acid was measured by equilibrium dialysis at 4°C. The concentration of ligandin (1.9 μM) and initial concentration of lithocholic acid (1.4 μM) were held constant. Various concentrations of gossypol initially were added to the protein-containing compartment. After equilibrium for the binding of lithocholic acid was achieved (40 hr), radioactive lithocholic acid and A_{390} of gossypol were determined on both sides of the dialysis chamber. The line is a theoretical curve calculated as described in the Appendix, based on the assumption that gossypol competitively inhibits the binding of lithocholic acid to ligandin. The binding parameters used in this calculation were K_d for lithocholic acid = 0.32 μM and n = 0.93. K_i for gossypol which best fit the data was 5.5 μM.

from transferase B, it seems reasonable to conclude that the bilirubin-lithocholic acid binding site is common for other bile acids.

APPENDIX

Assuming two ligands, C and I compete for a single class of n binding sites on a protein, the following equations hold (14, 23):

$$\frac{C_b}{C_f} = \frac{nP - C_b - I_b}{K_c} \qquad Eq.\ 1)$$

$$\frac{I_b}{I_f} = \frac{nP - I_b - C_b}{K_I}. \qquad Eq.\ 2)$$

Where C_f and C_b are the free and bound concentrations of C, respectively, I_f and I_b are the free and bound concentrations of I (competitor), respectively, P is the protein concentration, and K_c and K_I are the dissociation constants of C and I, respectively.

Total concentrations of C (C_t) and I (I_t) can be expressed:

$$C_t = C_b + C_f \qquad Eq.\ 3)$$

$$I_t = I_b + I_f \qquad Eq.\ 4)$$

Combining equations *1* and *3*:

$$I_b = nP - C_b - \left\{\frac{C_b \cdot K_c}{(C_t - C_b)}\right\}. \qquad Eq.\ 5)$$

Combining equations *1*, *2*, *3*, and *5*:

$$I_f = \left\{nP - C_b - \frac{C_b \cdot K_c}{(C_t - C_b)}\right\} \cdot \left\{\frac{K_I \cdot (C_t - C_b)}{C_b \cdot K_c}\right\}. \qquad Eq.\ 6)$$

Combining equations *4*, *5*, and *6*:

$$I_t = \left\{nP - C_b - \frac{C_b \cdot K_c}{(C_t - C_b)}\right\} \cdot \left\{1 + \frac{K_I \cdot (C_t - C_b)}{C_b \cdot K_c}\right\}. \qquad Eq.\ 7)$$

Equation *7* is the basic equation used to analyze the data in Figs. 1, 3, and 6. It can be solved for a series of chosen values of C_b to give the corresponding series of I_t values. By comparing inhibition curves thus calculated for various K_I values with the experimental data, the K_I values are estimated.

In some cases C_b was not directly measured (e.g., ANS or bilirubin), but instead, fluorescence charge (ΔF) or absorbance difference (ΔA), which is considered to be proportional to C_b, was measured. In these situations:

$$\Delta F \text{ (or } \Delta A) = \alpha \cdot C_b \qquad Eq.\ 8)$$

where α is the proportionality constant. In the absence of competitor:

$$C_b = \frac{nP \cdot C_f}{K_c + C_f} = \frac{nP(C_t - C_b)}{K_c + (C_t - C_b)} \qquad Eq.\ 9)$$

consequently,

$$C_b = \frac{(nP + K_c + C_t) - \sqrt{(nP + K_c + C_t)^2 - 4nPC_t}}{2}. \qquad Eq.\ 10)$$

Since C_t, nP, and K_c are known, C_b can be calculated from equation *10*. Then α can be calculated from equation *8*. Knowing the α value, the inhibition curve can be simulated by equation *7* using various K_I values so as to find the K_I value which best fits the data.

As an example, the procedure used to estimate the K_I for inhibition by gossypol of binding of ANS (Fig. 1) is described below where C = ANS and I = gossypol. The approach with data in Figs. 3 and 6 is identical. The following are known: P = 0.29 μM; C_t = 12.5 μM; n = 1; K_c = 37 μM.

Experimental data

[I]	Relative Fluorescence
0	100
0.25	82
0.5	74
1.0	59
1.5	52
2.0	46

In the absence of gossypol, from equation *10*:

$$C_b = \frac{(0.29 + 37 + 12.5) - \sqrt{(0.29 + 37 + 12.5)^2 - 4(0.29)12.5}}{2}$$

$$= 0.073\ \mu M.$$

$$\alpha = \frac{\Delta F}{C_b} = \frac{100}{0.073} = 1370.$$

Derivation of data for simulations

Simulations were then performed with equation *7* using K_I values from 0.5–10 μM. Shown below are values calculated when K_I = 1.0, which gave the best fit. C_b values for ANS were estimated from equation *8*. I_t was calculated from equation *7*.

ΔF	C_b (μM)	I_t
100	0.073	0
92	0.067	0.15
84	0.061	0.31
76	0.056	0.50
68	0.050	0.74
60	0.044	1.03
52	0.038	1.39
44	0.032	1.89
36	0.026	2.60
28	0.021	3.69
20	0.015	5.64
12	0.009	10.17

This work was supported by VA Medical Research Funds. A. Stolz was supported by NIH Training Grant AM07180 and an award from the American Liver Foundation.

Manuscript received 13 February 1984.

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