Fluorescence spectroscopy and inhibition kinetics were used to quantify the affinity of nonsubstrate ligands for the rat liver glutathione S-transferases AA, A, B, and C in the presence of glutathione. The dissociation constants $K_d$ for ligands such as bilirubin, indocyanine green, and hematin were determined by measuring the decrease in the intrinsic fluorescence of the proteins attendant on the addition of ligand. A second technique, used for compounds which absorb strongly at the excitation maxima of tryptophan, was to utilize 8-anilinonaphthalenesulfonate in the formation of a protein complex fluorescing at a higher wavelength. The quenching of this complex allowed the determination of the dissociation constants for ligands such as 3,6-dibromosulfophthalein and cephalothin. These data indicate that all four proteins bind these ligands but do so with different affinities. The bilirubin-induced decrease in fluorescence was used to estimate the stoichiometry of binding as 1.2 mol of bilirubin bound/mol of transferase B and 0.7 mol/mol of transferase C. All of the ligands examined are inhibitors of catalytic activity, as tested in a standard assay with GSH and 1-chloro-2,4-dinitrobenzene as substrates. From these studies we conclude that these proteins have a broad specificity not only for their substrates, but for the binding of nonsubstrate ligands as well.

On the basis of affinity for bilirubin (1), for an azo dye carcinogen (2), and for a metabolite of cortisone (3), three laboratories isolated proteins from rat liver which later proved to react identically with an antibody prepared against one of them (4). The protein was effective in binding a variety of drugs and dyes and such compounds as hemin and bromosulphophthalein (4, 5). Because of this versatility in binding, the term ligandin was coined to designate the protein (4). From a different point of view, we had isolated a series of enzymes, the glutathione S-transferases of rat liver, that catalyzed with overlapping specificity the nucleophilic attack by GSH on a large number of compounds bearing a variety of functional groups containing an electrophilic center (6, 7). After purification to homogeneity by several criteria, the glutathione S-transferases were each found to be proteins of about 45,000 daltons, composed of two subunits of equal size, and possessing an isoelectric point in the alkaline range; the proteins differed in their amino acid composition and could be distinguished by their response to a spectrum of substrates (7-10). In their common physical properties they resemble ligandin and subsequent work characterized ligandin as identical with one of the enzyme species, that labeled as glutathione S-transferase B (11).

These observations introduce the question of the relationship of such broad binding capability to glutathione S-transferase activity. Specifically, do the transferases other than transferase B have the dual capacity for binding nonsubstrates such as bilirubin and hematin? And, is there any effect of ligand-protein interaction on the catalytic activity? This report presents evidence for an affirmative answer to both questions.

MATERIALS AND METHODS

Indocyanine green and 3,6-dibromosulfophthalein were products of Hynson, Westcott and Dunning; the latter compound was a gift from Norman Javitt and from the manufacturer. Rat liver glutathione S-transferases A, B, and C were purified to apparent homogeneity as previously described (7, 8); transferase AA is also a homogeneous protein. 8-Anilinonaphthalenesulfonate, recrystallized from water, was a gift from Harold Edelhoch.

Kinetic Data—Enzyme assays (7) were conducted at a constant temperature of 25° in a Cary model 15 spectrophotometer with an amount of enzyme resulting in an absorbance change of less than 0.05/min at 340 nm in a 1-cm light path. The incubation mixture, in a volume of 1 ml, included 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM GSH in 0.1 M potassium phosphate at pH 6.5. Inhibition constants are based on reciprocal plots of substrate and initial velocity obtained at a minimum of three concentrations of inhibitor. Curves for the experimental points were calculated by means of an interactive curve-fitting program, MLAB, developed at the National Institutes of Health for a PDP digital computer (19). Final estimates of kinetic constants were made by fitting the data points to the appropriate program.

Preparation of GSH-free Enzyme—The enzyme preparation was passed through a column of Sephadex G 25 (1.3 x 24 cm) equilibrated with 0.1 M potassium phosphate at pH 7.5 containing 0.5 mM EDTA and 15% (v/v) glycerol. For titration of fluorescence the resultant protein solution was diluted 2.5-fold with 0.1 M potassium phosphate at the same pH.

Determination of Binding by Fluorescence Change—Binding studies were performed with a Farrand Mark 1 spectrofluorimeter equipped

with a temperature-controlled cell compartment maintained at 25°. Dust was removed from the solutions by passage through a GSW0.22μM Millipore filter. To small cuvettes (3 mm in cross-section) containing 100μl of 0.9 to 1.3 μM protein, 6% (v/v) glycerol, 0.1 m potassium phosphate at pH 7.5, 0.2 mM EDTA, and 1 mM GSH, were added a maximum of 10 μl of the ligand in 0.5 or 1.0 μl aliquots. The solution was stirred with a Teflon rod after each addition and the fluorescence recorded. Corrections were made for dilution and, where necessary, for self-absorption of incident or fluorescent light (13). The decrease in the intrinsic fluorescence of each transferase upon addition of ligand was measured at 330 nm while exciting at 285 nm. In those instances in which a ligand absorbed significantly in the region of 280 to 330 nm, an alternate method, competition of the ligand with ANS, was used. Changes in the fluorescence of ANS bound to protein were measured at 470 nm for transferases AA and B and at 490 nm for transferases A and C during excitation at 465 nm. The K<sub>a</sub>, as determined by intrinsic fluorescence of the transferases, was calculated from a plot of the reciprocal of the change in fluorescence (ΔF<sub>0</sub>) against the reciprocal of the total ligand concentration. The K<sub>a</sub>, as determined by competition with ANS, was calculated from a plot of ΔF/([total ligand]) against ΔF<sub>0</sub>. In each instance this relationship resulted in a straight line, the slope of which is equal to -K<sub>a</sub> (1 - [ANS]/K<sub>a</sub>) (15). The value for K<sub>a</sub> was determined separately for each protein.

The stoichiometry for binding of bilirubin was determined from fluorescence data obtained at two widely different protein concentrations according to the method of Halfman and Nishida (16). These concentrations were 1.39 μM and 13.9 μM for transferase B and 1.48 μM and 14.8 μM for transferase C. The ratio of the molar concentration of bound ligand to that of protein, v, was calculated from the concentration of total ligand added/m01 of protein (C/P) according to Equation 1. Each of the values chosen for C/P was that which gave the same ratio of ΔF to ΔF<sub>0</sub> max at each of the two protein concentrations, P<sub>a</sub> and P<sub>b</sub> (16).

\[ \frac{v}{P} = \frac{P_a (C/P)_a}{P_b (C/P)_b} \]

\[ C = P (C/P - v) \]

The equilibrium free ligand concentration C, corresponding to any value of v was determined with Equation 2 in which P represents the lowest protein concentration tested.

RESULTS

Dissociation Constants—In a series of preliminary titrations of the tryptophanyl fluorescence of transferase B with indocyanine green, we observed that GSH was necessary for optimum affinity of protein for ligand. K<sub>D</sub> for indocyanine green was 2.8 μM in the presence of 1 mM GSH and 13 μM in the absence of GSH. Upon substitution of equimolar dithiothreitol for GSH, a K<sub>D</sub> of 2.6 μM was obtained, indicating that the requirement for binding is for a mercaptan rather than specifically for GSH.

As with many proteins bearing a hydrophobic binding site (14, 17), addition of GSH to the transferases produced increased fluorescence and a blue shift in the emission maxima from 515 nm to 470 nm for transferases AA and B, and to 490 nm for transferases A and C. Addition of a ligand to the ANS-protein complex results in quenching of the fluorescence. Such quenching is due to competition of ligand for the ANS-binding site, and not to secondary effects of ligand bound at a distant site, is suggested by the competitive relationship shown in Fig. 1. Titrations with ANS and transferase B were performed in the presence of 0, 1, 2, and 3 mM cephalothin and the reciprocal of the change in fluorescence was plotted against the reciprocal of the ANS concentration. Both the strictly competitive nature and the linearity of this plot can be taken as evidence that cephalothin binds at an ANS site.

An energy transfer between the aromatic amino acids of the transferases and the bound dye exists. At an excitation wavelength of 285 nm and with increasing concentrations of ANS, the fluorescence of bound ANS increased, whereas protein fluorescence (λ<sub>max</sub> 330 nm) decreased. Thus, the ligand is bound in a region of the protein which also contains a fluorescing residue (17). The isoemissive points for transferases AA, A, B, and C are 430 nm, 425 nm, 405 nm, and 430 nm, respectively.

Dissociation constants for the ligands tested are shown in Table I. It is apparent that the tightness of binding is dependent on both the nature of the ligand and the specific transferase. For example, indocyanine green binds strongly to transferases A, B, and C and poorly to transferase AA, whereas hematin binds strongly to all four transferases. Cephalothin, morphine sulfate, and polymixin B have a low affinity; the limited solubility of the last two compounds did not allow a determination of K<sub>D</sub>.

Stoichiometry of Binding—The bilirubin induced decrease in intrinsic fluorescence may be used to determine the stoichiometry of binding (16). The number of binding sites was determined from the change in fluorescence with two solutions of the same transferase differing by a factor of 10 in protein concentration (Fig. 2). As may be seen in the inset of Fig. 2, extrapolation of the Scatchard plot gave 1.2 mol of bilirubin bound/m01 of transferase B. In a similar experiment with

![Fig. 1. Reciprocal plots of the binding of ANS to glutathione S-transferase B in the presence of 0, 1, 2, and 3 mM cephalothin.](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Table I</th>
<th>Binding to glutathione S-transferase</th>
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<tbody>
<tr>
<td>Ligand</td>
<td>ANS&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>AA</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>100</td>
</tr>
<tr>
<td>Indocyanine green</td>
<td>100</td>
</tr>
<tr>
<td>8-Anilinonaphthalene sulfonate</td>
<td>+</td>
</tr>
<tr>
<td>3,6-Dibromosulfo-phthalein</td>
<td>+</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>+</td>
</tr>
<tr>
<td>Morphin sulfate</td>
<td>+</td>
</tr>
<tr>
<td>Polymixin B</td>
<td>+</td>
</tr>
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</table>

<sup>a</sup>The notation, plus (+), indicates that K<sub>D</sub> was determined from the intrinsic fluorescence; the notation, minus (−), indicates that K<sub>D</sub> was determined by following changes in the fluorescence of ANS upon titration with ligand.
FIG. 2. The fraction of maximal increase, $\frac{\Delta F}{\Delta F_{\text{max}}}$, as a function of added bilirubin to protein for transferase B. The inset shows a Scatchard plot derived from these data. Protein concentrations: A, 13.9 $\mu$M; B, 1.39 $\mu$M.

Transferase C, 0.7 mol of bilirubin is bound/mol of enzyme.

Inhibition of Enzymatic Activity—As another parameter of binding we measured inhibition of catalytic activity by ligand in the standard assay system with 1-chloro-2,4-dinitrobenzene and glutathione. Each of the ligands inhibited catalytic activity (Table II). Representative data were selected for MLAB computer analysis (Fig. 3). When surveyed for best fit to Cleland’s program for determining the type of inhibition (18), indocyanine green exhibits linear competitive kinetics with a $K_i$ value of 2.99 ± 0.32 $\mu$M and hyperbolic noncompetitive kinetics with transferase A with a $K_i$ value of 0.015 ± .003 $\mu$M, a $K_i$ value of 0.034 ± 0.007 $\mu$M, and a $K_i$ value of 0.015 ± 0.003 $\mu$M.

As with the fluorescence studies, the tightness of binding and the type of kinetics seen depended on both the ligand and the transferase (Table II). With transferase B, all of the ligands are competitive inhibitors. With the exception of 3,6-dibromosulphophthalein, transferase AA also shows the same competitive kinetics as transferase B although the affinity is lower. In contrast, transferases A and C, are similar to each other in their kinetic response to ligands and display mainly noncompetitive inhibition with substrate.

**DISCUSSION**

The glutathione S-transferases are primarily enzymes of detoxification in that they catalyze the reaction of GSH with a wide variety of noxious electrophilic substrates and yield, among other products (9), the theoethers (19) that are further metabolized and excreted as mercapturic acids (20). From the studies reported here, the detoxification function must be extended to a role for these enzymes as carriers for such endogenously produced toxins as bilirubin and for an even larger group of ingested compounds. The data obtained with the four transferases available in amounts sufficient for binding studies, suggest that glutathione transferase activity and the capability for binding of a diverse group of ligands, are functions held in common by each of this group of proteins. An estimate of the amount of these proteins in rat liver is that transferases A, C, and AA are together equal to that of transferase B. Since transferase B is normally present as 5% of the total extractable protein of liver (21), it is evident that the transferases represent major constituents.

The binding studies which we report are limited to compounds which are not substrates. Since each of the substrates that we have studied reacts with mercaptans in the absence of enzyme, and since a mercaptan appears to be necessary for optimum binding, it has not been possible to determine the affinity of enzyme for substrate by direct measurement. Although transferase B, i.e. ligandin, has been examined by circular dichroism and by equilibrium dialysis techniques with a variety of ligands (22), such work was conducted in the absence of a mercaptan and is subject to re-evaluation. Nevertheless, there is qualitative agreement with respect to the ligands effective for ligandin and those for which $K_i$ values are presented here. Furthermore, we have shown 1 mol of bilirubin as binding/mol of transferses B or C and the data obtained by circular dichroism studies of bilirubin with ligandin, in the absence of mercaptan (22), are in agreement.

In each instance, exposure to nonsubstrate ligands is inhibitory to the enzyme in the reaction of GSH and 1-chloro-2,4-dinitrobenzene (Table II). With the exception of the system, bilirubin-transferase AA, and for ANS with each of the

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$K_i$ for Transferase A</th>
<th>$K_i$ for Transferase B</th>
<th>$K_i$ for Transferase C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin</td>
<td>0.002</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Indocyanine green</td>
<td>1.8</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>3,6-Dibromosulphophthalein</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
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<tr>
<td>Cephalothin</td>
<td>0.002</td>
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**Fig. 3.** Initial velocity patterns for the transferases. The lines drawn through the experimental points are those obtained from fits to the equation shown. Top, reciprocal plot of inhibition by indocyanine green of transferase B; bottom, reciprocal plot of inhibition by indocyanine green of transferase A.
transferases, $K_d$ values are essentially the same as $K_i$ in cases of competitive inhibition. We expect that more than 1 mol of ANS is bound/mol of enzyme and have noted that only one half of protein bound ANS fluorescence is quenched by saturating concentrations of competing ligand. The other $K_d$ are more complex and represent noncompetitive inhibition; $K_i$ and $K_d$ cannot, therefore, be strictly equated under such circumstances. A difference between a $K_i$ and $K_d$ value for the same ligand could be a reflection of a major difference in the two experimental methods. Both determinations were made in the presence of ligand and glutathione. However, in the measurements of $K_i$, the second substrate, 1,2-dichloro-4-nitrobenzene was present and might induce changes in the protein such that a greater or lesser affinity for ligand results.

The four transferases appear to fall into two classes with regard to their affinity for small molecules. For transferase B, cephalothin and ANS are both competitive inhibitors with the substrate, 1,2-dichloro-4-nitrobenzene, and compete with each other when fluorescent quenching is measured. This suggests that these compounds are binding at the same site and that the site’s specificity requirements are broad enough to accommodate the different molecules known to interact with this protein. On the other hand, transferases A and C share in common the property of binding many ligands which are noncompetitive with substrate and also have fluorescence maxima for ANS which are different from those shown by transferases AA and B. That one of the substrates, benzyl chloride, is noncompetitive with both 1,2-dichloro-4-nitrobenzene and GSH suggests that there is a site on these proteins sufficiently diverse that two different compounds can bind and be acted on catalytically in a fashion such that they do not exert major influence on one another (10).

The unusually broad specificity for binding of the transferases can be taken as support for a proposed mechanism of catalysis (9) in which these enzymes activate GSH to the glutathione thiolate ion; this very reactive species can then attack any substrate bound within a limited radius of its formation.

Acknowledgments—We thank Jack Folk for his help with the computer analysis of the enzyme kinetic data, Harold Edelhoch for his advice on the fluorescence titrations, and A. Donald Ketley for helpful discussions.

REFERENCES
