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REVERSIBLE INHIBITION OF RAT HEPATIC GLUTATHIONE *S*-TRANSFERASE 1-2 BY BILIRUBIN

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Abstract—The inhibition of rat hepatic glutathione (GSH) *S*-transferase 1-2 by bilirubin exhibited pseudo first-order kinetics with k_{obs} values of 0.0214 ± 0.0005 and $0.040 \pm 0.008\ sec^{-1}$ at 4 and 8 μM bilirubin, when followed to 72 and 84% completion respectively. These correspond to calculated second-order rate constants of $5.3 \pm 0.1 \times 10^3$ and $5.0 \pm 1.0 \times 10^3/M \cdot sec$. The extent of inhibition of the transferase increased with bilirubin concentration, with half-maximal inhibition at 4 μM bilirubin. Inhibition was reversed by 10-fold dilution of bilirubin or by increasing the pH from 6.0 to 7.4. Premixing 0.2 to 0.5 μM albumin, hemoglobin or aldolase with bilirubin prevented inhibition of GSH *S*-transferase 1-2. Protection by these proteins occurred at a selected high concentration (0.2 to 0.4 μM) at which they reduced free bilirubin to concentrations (<0.5 μM) that did not inhibit isoenzyme 1-2 significantly. No protection was afforded by a selected low protein concentration (0.001 to 0.01 μM) which did not strikingly reduce bilirubin levels in solution. We conclude that bilirubin inhibition of GSH *S*-transferase 1-2 appears to be a second-order process; the reaction is clearly first-order with respect to GSH *S*-transferase and appears also to be first-order with respect to bilirubin. It is proposed that (a) inhibition of GSH *S*-transferase 1-2 results from slow, reversible bilirubin binding, and (b) added proteins appear to prevent GSH *S*-transferase inhibition by binding high molar ratios of bilirubin.

The multi-functional glutathione *S*-transferases (GSTs)§ (EC 2.5.1.18) catalyze the conjugation of glutathione (GSH) to organic electrophiles; these proteins also non-covalently bind and transport non-substrate ligands such as bilirubin. In view of the physiological relevance, bilirubin binding by the GSTs and the resultant effect on transferase activity have been studied extensively. GST isoenzymes reversibly bind bilirubin at high ($K_d = 0.1\ \mu M$) and/or low ($K_d = 5$–$100\ \mu M$) affinity sites [1–7], with only the latter apparently decreasing GST activity [2–4, 6].

Bilirubin inhibition of several GST isoenzymes has been reported by Vander Jagt and co-workers [3, 4, 8] to be a relatively slow process which exhibits complex, non-first-order kinetics when [bilirubin] ≫ [GST]. Bilirubin inhibition was not reversed by increased pH or added protein, although the extent of GST inhibition varied with pH and order of addition. Kinetically controlled conformational states of GST–bilirubin complexes and enzyme memory were proposed to explain these observations [8].

We have investigated the inhibition of rat hepatic GST 1-2 by bilirubin and the prevention of this inhibition by proteins. We found that the loss of enzyme activity was reversible and followed pseudo first-order kinetics when [bilirubin] ≫ [GST]. Prevention of transferase inhibition by proteins appeared to correlate with their ability to lower significantly the concentration of bilirubin in solution.

METHODS

Materials were obtained as follows: GSH, bilirubin, human serum albumin, human hemoglobin, bovine pancreatic ribonuclease A, and aldolase were from the Sigma Chemical Co., St. Louis, MO, U.S.A.; 1-chloro-2,4-dinitrobenzene (CDNB) was from Merck Chemicals, Darmstadt, F.R.G. Rat liver cytosolic GST 1-2 was purified by affinity and ion exchange chromatography [9, 10]. The purity was assessed by elution profiles, specific activity toward the substrates CDNB, 1,2-dichloro-4-nitrobenzene, cumene hydroperoxide, *trans*-4-phenyl-3-buten-2-one and 1,2-epoxy-3-(*p*-nitrophenoxy)propane [11], and by gel electrophoresis. GST 1-2 was stored at −80°, without loss of activity.

Deoxygenated aqueous stock solutions of 50 mM GSH (pH 6–7) and of 2.1 mM bilirubin in 10 mM NaOH were prepared daily, with the latter being protected from light. Stock solutions of 30 mM CDNB in 99% ethanol were prepared weekly and protected from light. All solutions were stored at 4°.

Typical reaction mixtures contained, in 3 mL total volume, 20 mM potassium phosphate/100 mM NaCl (pH 6.5), GST 1-2 (4 nM), and, where indicated, bilirubin (0–35 μM added in 0–50 μL) or an equivalent volume of 10 mM NaOH (0–50 μL), and protein (0–15 μM). Incubations were performed in cuvettes of 1 cm path length at 25 ± 1° without shaking. Incubation time and order of addition of components are described in Results.

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§ Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; GSH, glutathione; and GST, glutathione *S*-transferase.

GST activity toward CDNB was measured spectrophotometrically at 340 nm in 20 mM potassium phosphate/100 mM NaCl buffer containing 2.5 mM GSH and 1 mM CDNB [12]. Normally, the reaction was initiated with the addition of GSH. The final concentration of ethanol in the assay solution was 3.3%. Initial reaction rates, which were measured continuously for 1 min using a Beckman 5230 spectrophotometer, were less than 0.05 AU_{340}/min in all cases. Typically, initial rates were measured in four to eight replicates.

To assess the extent of bilirubin binding, each protein was mixed with 3–4 μM bilirubin in 20 mM potassium phosphate/100 mM NaCl (pH 6.5). One milliliter was applied to a Sephadex G-25 column (0.8 cm diameter × 1 cm height) equilibrated with the above buffer. Protein (including protein–bilirubin complexes) was eluted with 7 mL of buffer. The recovery of protein chromatographed on the above column in the absence of bilirubin was assessed spectrally at 280 nm. The recovery of protein in the absence of bilirubin was found to be 84–108%. Bilirubin not bound to protein was eluted with 10 mL of 10 mM NaOH (93–136% recovery assessed spectrally at 468 nm). For proteins lacking strong visible absorbance bands, protein-bound bilirubin was measured on the protein eluate at 468 nm in 4 cm path length cuvettes. Standard curves for free bilirubin were prepared following chromatography of bilirubin solutions of known concentration. Standard curves for protein-bound bilirubin in buffer were prepared from the absorbance of known concentrations of bilirubin in buffer.

Results are expressed as means ± SD. Standard deviations of percentages were computed by standard methods of error propagation [13]. The significance of differences between means was assessed with Student's *t*-test. GST inactivation curves were fitted with a three-parameter single exponential decay equation using a non-linear least squares regression data analysis program (Enzfitter, Elsevier 1987). Experiments (N = 3–8) were typically performed once with GST 1-2 in view of the difficulty in obtaining significant amounts of pure enzyme. However, the results of each experiment were confirmed by one or more experiments with the mixture of rat liver cytosolic GST isoenzymes, in which results similar to those reported for GST 1-2 were obtained.

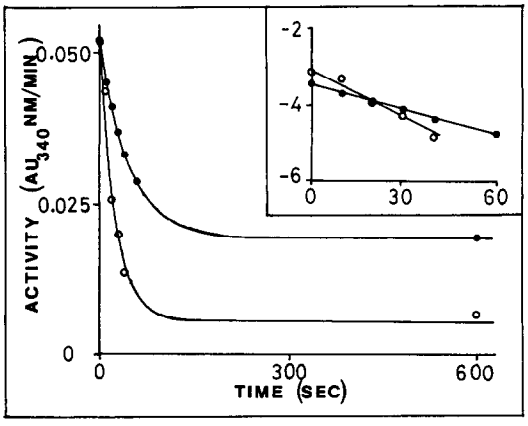

Fig. 1. Activity of GST 1-2 (4 nM) versus time of incubation at 25° in 0.02 M potassium phosphate/0.1 M NaCl (pH 6.5) with 4 μM (●) and 8 μM (○) bilirubin. Assays at each time point were performed in quadruplicate. Reported activities are corrected for loss of enzyme activity in buffer. Curves were drawn by fitting the data to the three-parameter first-order decay equation: $A_t = A_\infty - (A_0 - A_\infty)e^{-k_1 t}$. Inset contains a plot of $\ln(A_t - A_\infty)$ vs time.

Table 1. Effect of bilirubin concentration on GST 1-2 activity

Bilirubin (μM)	% Residual activity
0	88.2 ± 0.3
0.5	84.1 ± 2.4
1.0	73.5 ± 2.4*
2.0	62.3 ± 0.4*
4.0	43.5 ± 6.7*
8.0	6.2 ± 2.3*

GST 1-2 activity was measured after a 20-min incubation with bilirubin in 20 mM potassium phosphate/100 mM NaCl (pH 6.5) at 25°. The substrates CDNB (1 mM) and GSH (2.5 mM) were added in rapid succession immediately prior to assay. Residual activity at 20 min is reported relative to identically constituted unincubated samples. Control assay mixtures contained *ca.* 0.005 units/mL of GST 1-20. Values are means ± SD, N = 4–8.

* Differs significantly from activity in the absence of bilirubin, $P < 0.001$.

RESULTS

Kinetics of bilirubin inhibition of GST 1-2. The kinetics of the inhibition of GST 1-2 in the presence of 4 and 8 μM bilirubin at pH 6.5 were followed to 72 and 84% completion respectively. The data in Fig. 1 have been corrected for bilirubin-independent inactivation of GST in buffer. The inhibition exhibited pseudo first-order kinetics, with first-order rate constants of 0.0214 ± 0.0005 and 0.040 ± 0.008 sec^{-1} at 4 and 8 μM bilirubin. The observed first-order constants correspond to calculated second-order rate constants of 5.3 ± 0.1 × 10^3 and 5.0 ± 1.0 × 10^3/M·sec respectively.

The extent of inhibition of GST 1-2 increased with bilirubin concentration (Table 1). Inhibition was essentially quantitative after a 20-min incubation with 8 μM bilirubin. Half-maximal inhibition occurred at approximately 4 μM bilirubin.

Reversibility of bilirubin inhibition of GST 1-2. The inhibition of GST 1-2 by bilirubin was reversed on dilution. Incubation of GST 1-2 with 8 μM bilirubin at pH 6.5 for 3 min followed by a 10-fold dilution into buffer containing 8 μM bilirubin resulted in nonmeasurable GST activity. Ten-fold dilution of the incubation mixture into buffer and assay after 5 min led to a 74% recovery of GST activity.

Inhibition of GST 1-2 was reversed by increasing the pH. Incubation of GST 1-2 with 8 μM bilirubin at pH 6.0 and 7.4 for 3 min reduced enzyme activity to 0 and 55 ± 1%, respectively, relative to enzyme

Table 2. Effects of bilirubin and albumin on GST 1-2 activity

Additions	% Residual activity
GST	89.0 ± 0.3
CDNB + GST	88.4 ± 1.3
HSA + GST	98.4 ± 1.8
(CDNB + HSA) + GST	100.4 ± 0.3
(8 μM BR + CDNB) + GST	0.0*
(8 μM BR + CDNB + HSA) + GST	93.3 ± 0.1†
(CDNB + HSA + GST) + 8 μM BR	75.2 ± 2.0*†‡
(35 μM BR + CDNB + HSA) + GST	69.6 ± 1.9*†
(CDNB + HSA + GST) + 35 μM BR	26.5 ± 1.8*†‡

GST 1-2 activity after a 20-min incubation with or without CDNB (1 mM), bilirubin (BR) and albumin (HSA) (2 μM) is reported relative to identical unincubated samples. Control assay mixtures contained *ca.* 0.005 units/mL of GST 1-2. The components were added in the order indicated. Components in brackets were will mixed prior to subsequent additions. The reaction was initiated with GSH. Values are means ± SD, N = 4–8. Experimental conditions are as in Table 1.

* Differs significantly from activity for GST or CDNB plus GST, $P < 0.001$.

† Differs significantly from activity for (BR plus CDNB) plus GST, $P < 0.001$.

‡ Differs significantly from activity for (BR plus CDNB plus HSA) plus GST, $P < 0.001$.

assayed at the same pH in the absence of bilirubin.* Incubation of GST 1-2 with 8 μM bilirubin at pH 6.0 for 3 min, followed by adjustment of the pH to 7.4, led to recovery of activity; at 0, 2 and 4 min after the pH increase, the activity was 69 ± 2, 92 ± 18 and 118 ± 33%, respectively, of the activity in the presence of 8 μM bilirubin at pH 7.4.

Effect of albumin on bilirubin inhibition of GST 1-2. Incubation of GST 1-2 with 1 mM CDNB in buffer for 20 min resulted in *ca.* 10% inhibition. Incubation with 8 μM bilirubin and 1 mM CDNB fully inhibited GST activity (Table 2). Inhibition of GST 1-2 by bilirubin was diminished by albumin. The extent of protection by albumin depended on the order of addition; premixing albumin and GST 1-2 was less effective than premixing albumin and bilirubin. The difference in residual activity with the order of mixing was more striking at 35 μM than at 8 μM bilirubin (Table 2).

Bilirubin binding by proteins. The capacity of four proteins to bind bilirubin was assessed by gel filtration. The levels of albumin-, aldose- and ribonuclease-bound bilirubin increased with protein concentration, while the levels of free bilirubin fell (Fig. 2). Hemoglobin-bound bilirubin was not analyzed since this hemoprotein strongly absorbs in the visible region of the spectrum used for bilirubin quantification. At concentrations greater than 0.05 μM, these proteins diminished the concentration of free bilirubin by ≥75%, namely from 3–4 μM to below 0.5 μM (Fig. 2). Maximally, *ca.* 100 moles of bilirubin were bound per mole of protein. Ribonuclease (up to 16 μM) appeared to bind ≤15% of the bilirubin.

Prevention of bilirubin inhibition of GST 1-2 *by added proteins.* The effects of proteins on transferase activity in the absence or presence of bilirubin (4 μM) are presented in Table 3. Low concentrations of albumin, hemoglobin and aldolase (0.001 to 0.01 μM) which did not strikingly lower free bilirubin concentration (Fig. 2), did not prevent inhibition of GST 1-2 (Table 3). At higher concentrations (0.2 to 0.4 μM), these proteins effectively removed bilirubin from solution (Fig. 2) and completely prevented inhibition of GST 1-2 (Table 3). Ribonuclease (>0.5 μM) fully protected transferase activity, but had no effect on transferase activity at low concentration (0.004 μM) (Table 3).

* Using the rate constant determined at pH 6.5, the half-life for inhibition at 8 μM bilirubin is 15 sec. The 3-min reaction time corresponds to *ca.* 12 half-lives or over 99% completion.

DISCUSSION

Bilirubin inhibition of GST 1-2 was found to be time and concentration dependent (Fig. 2, Tables 1 and 2), in agreement with previous reports [3, 4, 8]. However, our results indicate that the reaction follows a single-exponential decay (Fig. 1), up to 2.7 half-lives, when [bilirubin] ≫ [GST]. Our data indicate that the reaction is thus first-order with respect to enzyme. The reaction also appears to be first-order with respect to bilirubin, and thus overall appears to be second-order. This conclusion and the calculated second-order rate constant of *ca.* $5.0 \times 10^3/M \cdot sec$ (see Results) are based, however, on only two concentrations of bilirubin.

In view of the above kinetic data, we considered the possibility that the complex, non-first-order kinetics previously reported for human liver GST and rat liver GST 1-2 and 3-3 when [bilirubin] ≫ [GST] [3, 4] might reflect multiple inhibition processes with different rate constants. One known competing reaction is the pseudo first-order GST inactivation in buffer ($k_{obs} \approx 0.003\ sec^{-1}$) previously reported for a number of rat and human GST isoenzymes [14], that

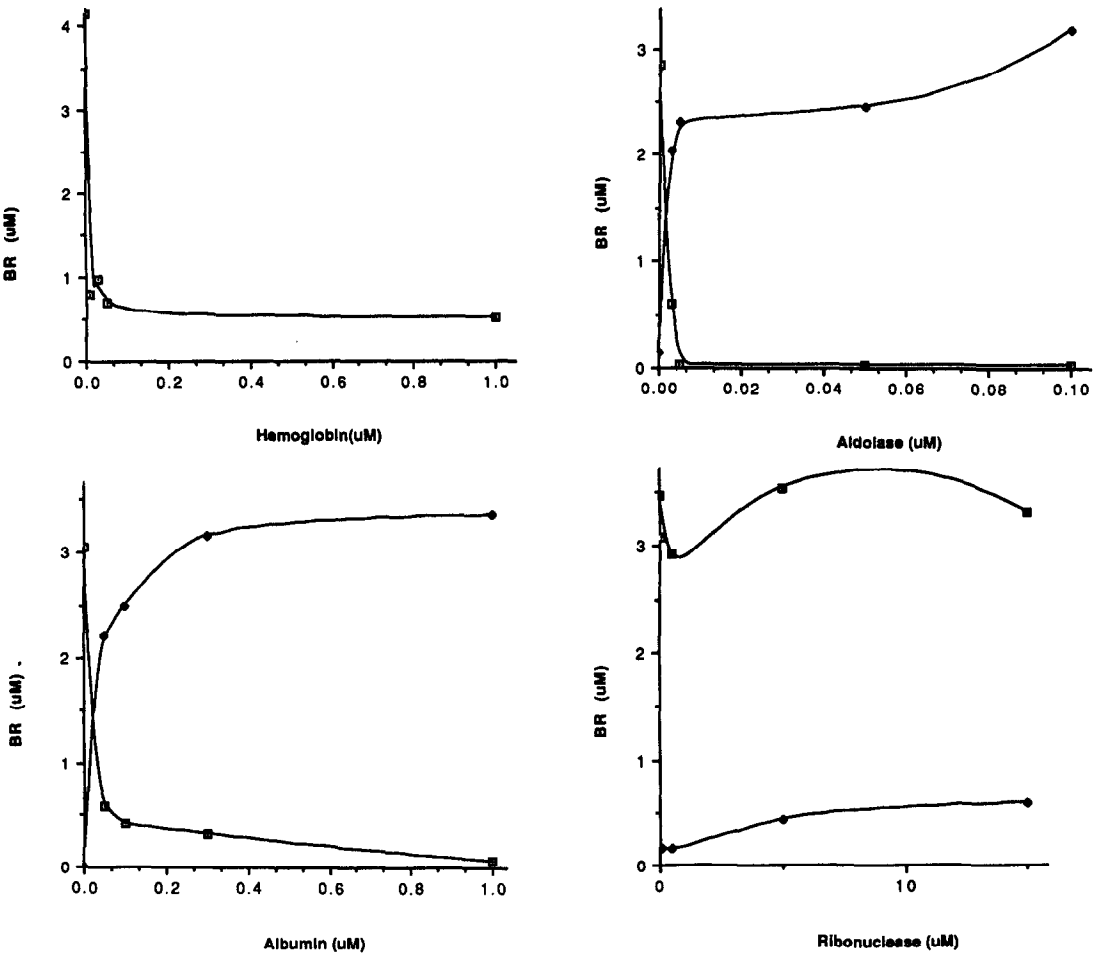

Fig. 2. Concentration of free (□) and protein-bound (◆) bilirubin (BR) in eluate from Sephadex G-25 columns as a function of protein concentration. Protein was premixed with 3–4 μM bilirubin prior to application to the column.

Table 3. Effects of proteins on bilirubin inhibition of GST 1-2

Additions*	% Residual activity	Additions*	% Residual activity
None	84.1 ± 0.2	None	88.2 ± 3.7
0.01 μM HSA	83.4 ± 2.4	0.001 μM HB	94.3 ± 3.5
0.4 μM HSA	90.9 ± 1.9	0.2 μM HB	97.9 ± 3.0
BR	54.2 ± 0.5†	BR	48.7 ± 0.9†
BR + 0.01 μM HSA	54.0 ± 0.1†	BR + 0.001 μM HB	51.5 ± 5.2†
BR + 0.4 μM HSA	78.4 ± 0.4‡	BR + 0.2 μM HB	95.2 ± 1.2‡
None	98.3 ± 0.4	None	94.3 ± 2.7
0.001 μM ALD	80.3 ± 5.9	0.004 μM RIB	76.6 ± 2.7
0.3 μM ALD	93.3 ± 0.4	0.5 μM RIB	91.2 ± 3.1
BR	53.6 ± 3.7†	BR	55.2 ± 0.7†
BR + 0.001 μM ALD	55.8 ± 1.0†	BR + 0.004 μM RIB	56.8 ± 2.8†
BR + 0.3 μM ALD	89.4 ± 0.5‡	BR + 0.5 μM RIB	97.1 ± 1.2‡

Experimental details are as in Table 1, except that bilirubin was at 4 μM. Control assay mixtures contained *ca.* 0.005 units/mL of GST 1-2. Values are means ± SD, N = 4–8.

* HSA, human serum albumin; HB, hemoglobin; BR, bilirubin; ALD, aldolase; and RIB, ribonuclease; † Differs significantly from activity for no additions, $P < 0.001$; ‡ Differs significantly from activity for BR, $P < 0.001$.

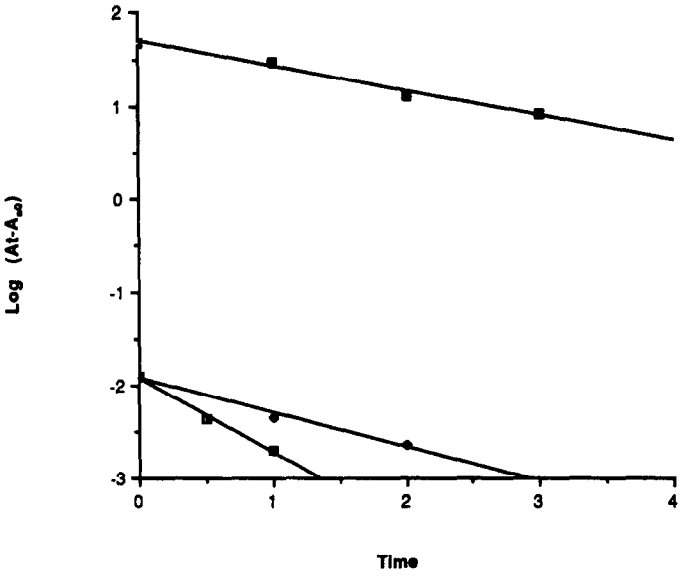

Fig. 3. Effects of bilirubin and gossypol on activity of GST isoenzymes. Data are from Refs 4 and 8. Key: (□) GST A inhibition by bilirubin (data from Fig. 4A, Ref. 4); (◆) GST B inhibition by bilirubin (data from Fig. 4B, Ref. 4); (■) GST H inhibition by gossypol (data from Fig. 7, Ref. 8).

has been proposed to result from active site solvation [14]. However, at least for GST 1-2, the extent of inhibition by CDNB or in buffer, by any mechanism, appears to be minimal over periods of up to 20 min (Table 2).

We therefore re-examined the basis on which the kinetics of BR binding to GST isoenzymes were described as complex and non-first-order. This was apparently based on plots of log A_t (activity) vs time. First-order plots require correction for infinity values, and thus must take the form of log $(A_t - A_\infty)$ versus time. Non-linearity of plots of log A vs time is not necessarily indicative of complex or non-first-order kinetics. When we extracted the data from the seven figures presented in three manuscripts reporting complex kinetics [3, 4, 8], we found the following: The majority of the data points were too close to the infinity value to be of assistance in accurate determination of kinetic properties or reaction order. For only 1 to 3 data points per figure was the $\Delta(A_t - A_\infty)$ greater than 12% of the maximal $\Delta(A_t - A_\infty)$. Typical first-order plots of $\log(A_t - A_\infty)$ for bilirubin inhibition of GST isoenzymes with three accurate points from the data of Vander Jagt *et al.* [4] are given in Fig. 3. These plots are linear with r values of 0.985 to 0.994. Similarly, for gossypol, which according to Vander Jagt *et al.* [8] behaves in an identical manner to bilirubin with respect to binding and kinetics of inhibition of GST H, we reconstructed a plot of $\log(A_t - A_\infty)$ versus time with the four data points sufficiently different from the infinity value; there was no evidence of non-linearity in this plot ($r = 0.986$) (see Fig. 3). We conclude that based on the kinetic data in the manuscripts of Vander Jagt and coworkers [3, 4], there is insufficient kinetic evidence to support the proposal that the kinetics of bilirubin inhibition of GST isoenzymes are more complex than pseudo first-order when [bilirubin] $\gg$ [GST].

Our results demonstrate the reversibility of bilirubin inhibition of GST 1-2 upon dilution and with pH (see Results). The former is in agreement with a report by Vander Jagt *et al.* [8] and is consistent with the proposed hepatic function of GST in binding and transporting bilirubin *in vivo* [15]. The latter is consistent with the reported reversal of biliverdin or indocyanine green inhibition of GST 1-2 by increasing pH [16]. Furthermore, bilirubin has been characterized extensively as a reversible type inhibitor of the GSTs [1–7]. This body of results contrasts to reports that bilirubin inhibition of GST 1-2 or 3-3 is not reversible by pH [4]. We have no explanation for this discrepancy, although we do note that Vander Jagt's experimental conditions differed from all of the other studies, in that CDNB was present in all incubations.

The prevention of bilirubin inhibition of GSTs by proteins has been reported [6, 8] and is confirmed by our data. The phenomenon has been proposed to result from stabilization of kinetically controlled states of GST–bilirubin complexes via enzyme memory [6, 8]. However, our data are consistent with a simpler mechanism. We demonstrated that the protection of GST activity by proteins occurred only when they remove bilirubin from solution. A single high concentration of albumin, hemoglobin and aldolase which lowered free bilirubin concentrations to below 0.5 μM prevented bilirubin inhibition of GST 1-2 (Table 3, Fig. 2). In contrast, a lower protein concentration, which did not remove bilirubin effectively from solution (Fig. 2), did not prevent GST inhibition (Table 3).

Ribonuclease did not appear to bind bilirubin effectively, as assessed by column chromatography (Fig. 2), although this protein at relatively high concentrations protected against bilirubin inhibition of GST 1-2 (Table 3). We suggest that ribonuclease may bind bilirubin with lower affinity than the Sephadex

resin and consequently release protein-bound ligand to the resin during chromatography. There is precedent for this suggestion, namely such a phenomenon reportedly interferes with measurement of the ligand affinity of weak binding proteins and results in anomalously low apparent affinity [17, 18].

Our observation that the extent of inhibition depended on the order of addition and premixing of albumin, GST and bilirubin (Table 1, Results) further supports the conclusion that added proteins prevent inhibition by binding bilirubin. We propose that (a) premixing albumin and GST 1-2 results in the partitioning of bilirubin between the two proteins, with significant transferase binding and inhibition, and (b) premixing albumin and bilirubin results in bilirubin binding or sequestration by albumin and prevents bilirubin from binding to and inhibiting the GST. The capacity of hemoglobin, albumin and aldolase to remove bilirubin from solution is high, with a molar ratio of [bound bilirubin]/[protein] of up to 100 at $\approx 0.04\ \mu M$ protein (Fig. 2). We have no evidence to infer the mechanism by which such high molar ratios of bilirubin are bound by these three proteins. However, the binding of equivalent molar ratios of bilirubin by albumin has been reported by many investigators [19, 20]. Brodersen *et al.* have proposed that the mechanism of this process reflects the co-aggregation of several albumin molecules with several hundred bilirubin acid molecules [19, 20]. The mechanism of binding or sequestrating of high molar ratios of bilirubin by albumin, and possibly hemoglobin and aldolase, reported here may reflect a similar mechanism or the sequestration or internalization of bilirubin or some other mechanism.

In conclusion, our results are consistent with slow, reversible pseudo first-order inhibition of GST 1-2 by micromolar concentrations of bilirubin. The protection against bilirubin inhibition of GST correlates with and appears to result from the binding and/or sequestration of high molar ratios of bilirubin by protein. Our results favor this simple mechanism for prevention of bilirubin inhibition by protein, rather than a more complex mechanism that requires GST–bilirubin–protein ternary complexes or the phenomenon of enzyme memory.

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