Comparison of Renal and Hepatic Glutathione S-Transferases in the Rat*

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Renal and hepatic GSH (reduced glutathione) S-transferase were compared with respect to substrate and inhibitory kinetics and hormonal influences in vivo. An example of each of five classes of substrates (aryl, aralkyl, epoxide, alkyl and alkene) was used. In the gel filtration of renal or hepatic cytosol, an identical elution volume was found for all the transferase activities. Close correspondence in \( K_m \) values was found for aryl- and alkyl-transferase activity, with only the aralkyl activity significantly lower in kidney. Probenecid and \( p \)-aminophippurate were competitive inhibitors of renal aryl-, aralkyl-, epoxide- and alkyl-transferase activities and inhibited renal alkene activity. Close correspondence in \( K_i \) values for inhibition by probenecid of these activities in kidney and liver was found. In addition, furosemide was a potent competitive inhibitor of renal alkyltransferase activity. Hypophysectomy resulted in significant increases in aryl-, aralkyl-, and epoxide-transferase activities in liver and kidney. The hypophysectomy-induced increases in renal aryl- and aralkyl-transferase activities (approx. 100%) were more than twofold greater than increases in hepatic activities (approx. 40%). Administration of thyroxine prevented the hypophysectomy-induced increase in aryltransferase activity in both kidney and liver. The renal GSH S-transferases, in view of similarities to the hepatic activities, may play a role as cytoplasmic organic-anion receptors, as previously proposed for the hepatic enzymes.

The hepatocyte and kidney-tubular cell share the capacity to transport organic anions at physiological pH values. Organic anions are delivered to these cells principally bound to soluble plasma protein carriers such as albumin. In the translocation of these substances from blood to bile or urine, it is reasonable to suppose that cytosolic protein carriers may exist with an intracellular role comparable with that of albumin in plasma. Such a cytosolic transport protein has been identified in liver and was originally called 'Y protein', a designation which refers to organic-anion binding to the 45000-mol wt. protein fraction in gel filtration (Levi et al., 1969). Subsequently, a single protein was purified from the Y-protein fraction and was designated 'ligandin' (Litwack et al., 1971). Ligandin binds a variety of organic anions which are selectively extracted from plasma by the liver (Litwack et al., 1971). Ligandin has been identified as one (GSH\( _1 \)) S-transferase B) of a family of hepatic cytosol detoxifying enzymes, the GSH S-transferases (Habig et al., 1974a). Therefore it is believed that it functions both as an organic-anion binding receptor and as an enzyme (Habig et al., 1974a). However, ligandin is not unique as an organic-anion receptor and may share this role with the other GSH S-transferases, all of which bind organic anions (Kaplowitz et al., 1975b).

GSH S-transferase activities have been identified also in rat kidney and studied with respect to drug induction and sex differences (Clifton et al., 1975b). Ligandin has been shown to be immunologically identical in rat liver and kidney (Kirsch et al., 1975), but no comparison of the catalytic properties of this protein in the two organs was made. The present paper describes studies in the rat designed to compare the GSH S-transferases of liver and kidney with respect to Michaelis–Menten kinetics, inhibitory kinetics, and hormonal influences such as hypophysectomy, after which hepatic Y protein is known to increase (Reyes et al., 1971).

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‡ Abbreviation: GSH, reduced glutathione.
Section, American Federation for Clinical Research, Carmel, CA, U.S.A. (Clifton et al., 1975a).

Materials and Methods

Animals and preparation of cytosol

Male Sprague–Dawley rats (160–180 g) were killed after light ether anaesthesia. Hypophysectomized, adrenalectomized and gonadectomized male rats were obtained from Simonsen Laboratories, Gilroy, CA, U.S.A. All animals were maintained on usual laboratory chow and drinking water for 2 weeks before killing. Certain animals received L-thyroxine (Sigma Chemical Co., St. Louis, MO, U.S.A.) intraperitoneally in a daily dose of 14 μg/100 g body wt. The livers and kidneys were perfused in situ with 0.25 m sucrose containing sodium phosphate buffer, 0.01 m, pH 7.4, at 4°C. Homogenates (20%, w/v) were prepared in 0.01 m-sodium phosphate buffer, pH 7.4, and centrifuged for 10 min at 5000 g followed by 60 min at 105,000 g in a Beckman L2-65B ultracentrifuge (Beckman Instruments, Fullerton, CA, U.S.A.). The supernatants (cytosol) were carefully pipetted after removal of the lipid layer by suction.

Gel-filtration experiments

Gel filtration with columns (38 cm × 2.5 cm) containing Sephadex G-75 or G-100 (Pharmacia, Uppsala, Sweden), flow rate 22–24 ml/h (10 fractions/h), was performed by using 0.01 m-sodium phosphate buffer, pH 7.4, as mobile phase in a pump-driven upward-flow system at 4°C. To 4.0 ml of cytosol was added 1.0 μCi of [2-3H]Gly glutathione ([3H]GSH; New England Nuclear Corp., Boston, MA, U.S.A.; 250 mCi/μmol), 5.0 μCi of [2-3H]Gly p-aminohippuric acid (Amersharm/Searle, Arlington Heights, IL, U.S.A.; 149 mCi/mm; or 5.0 μCi of [35S]fur osomide (a gift from Hoechst Pharmaceuticals, Somerville, NJ, U.S.A.; 662 μCi/mm).

Determination of enzymic activities

The GSH S-transferase activities were measured in cytosol and column fractions by using one example of five classes of substrates and previously described techniques (Kaplowitz et al., 1975a; Pabst et al., 1974; Habig et al., 1974b): 3,4-dichloronitrobenzene (GSH S-aryl-), p-nitrobenzyl chloride (S-aralkyl-), 2-epoxy-(3-p-nitrophenox)propene (S-epoxide-), ethacrynic acid (S-alkene-), and [14C]methyl iodide (S-aryl-transf). The latter was obtained from New England Nuclear Corp. (4.86 mCi/mm). All assays used excess of GSH (10 μm), except that for alk enetraftr activity, which used a lower GSH concentration (0.25 μm). Non-enzymic interaction of substrates was measured by using the same assay conditions without cytosol and was subtracted from reaction rates with cytosol. The enzymic reactions were linear with respect to time and protein concentrations. The substrates for enzymic reactions were obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. GSH was obtained from Sigma. Fur osomide was a gift from Hoechst Pharmaceuticals and ethacrynic acid was a gift from Merck, Sharp and Dohme, West Point, PA, U.S.A. Ovalbumin (mol wt. 45000) was obtained from Pharmacia, Piscataway, NJ, U.S.A. Probenecid [4-(dipropylsulphamyl)benzoic acid] and p-aminohippuric acid were obtained from Sigma and Aldrich respectively.

Enzyme kinetics

Each activity in kidney and liver was determined over a range of substrate concentrations. Data were expressed by the method of Lineweaver & Burk (1934). The Michaelis constant (Km) was calculated from the abscissa intercept of plots of least-squares-regression equations. An accurate determination of Km for ethacrynic acid substrate could not be obtained because of the limiting GSH concentration required to decrease the rate of the non-enzymic reaction.

Inhibitory kinetics were investigated by using three substrate concentrations and a range of inhibitor concentrations. The data were expressed by the method of Dixon (1953). The inhibitor constants were calculated from the least-squares-regression equations and represent the mean intersection of three lines.

Inhibition experiments were also performed by using [2-14C]ethacrynic acid (a gift from Merck, Sharp and Dohme Research Laboratories, Rahway, NJ, U.S.A.; 16 μCi/mg) as substrate. Duplicate 1 ml reaction mixtures contained 0.2 mM-ethacrynic acid (0.3 μCi), 0.25 mM-GSH, 0.1 mM-sodium phosphate buffer, pH 6.5, and inhibitor. Renal cytosol (50 μl) was added to initiate the reaction at 37°C and the reaction was stopped after 30 s by adding 1.0 ml of methanol and placing the sample on ice. Immediately, 25 μl was spotted on a pre-coated silica-gel t.l.c. plate (0.2 mm thickness) (Quanta gram, Quantum Industries, Fairfield, NJ, U.S.A.) and run overnight in a tank containing a butanol/acetic acid/water (4:1:2, by vol.) solvent system. Sections (1 cm wide) of the dried plates were scraped off and placed in counting vials to which were added 1.0 ml of water and 10 ml of Aquasol-II (New England Nuclear Corp.). Samples were counted for radioactivity in a Packard Tri-Carb liquid-scintillation spectrometer. The proportion of radioactivity (counts) running as the ethacrynic acid–GSH conjugate was calculated.

Protein concentration was determined by the method of Lowry et al. (1951), with bovine albumin (Sigma) as standard.

Statistical analyses involving comparison of unpaired groups of data used Student's t test (Batson, 1956).
RENAL AND HEPATIC GLUTATHIONE S-TRANSFERASES

Results

Identification of GSH S-transferases in gel filtration

Gel filtration (on Sephadex G-100) was applied with kidney cytosol, and each fraction was assayed for five enzyme activities and binding of \(^{3}H\)GSH. An identical elution volume (90ml) was found for each activity and \(^{3}H\)GSH. Under the same column conditions, an identical elution volume (90ml) was found for the hepatic activities and ovalbumin (mol.wt. 45000). By using renal cytosol \(^{3}H\)p-aminohippuric acid and \(^{35}S\)furosemide bound to the same gel filtration fractions that exhibited GSH S-transferase activity.

Comparison of \(K_m\) values for renal and hepatic GSH S-transferases

\(K_m\) values for the four activities in liver and kidney listed in Table 1 represent means ± S.E.M. of determinations with renal or hepatic cytosol from four rats. No significant difference in \(K_m\) between kidney and liver was found for aryl-, epoxide, or alkyltransferase activity. The \(K_m\) for renal aralkyltransferase activity, however, was significantly lower than the hepatic value (P<0.005).

Table 1. Comparison of Michaelis constants for renal and hepatic glutathione S-transferases

<table>
<thead>
<tr>
<th>Transferase activity</th>
<th>Kidney (mm)</th>
<th>Liver (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aryl</td>
<td>3.1 ± 0.1</td>
<td>3.2 ± 0.6</td>
</tr>
<tr>
<td>Epoxide</td>
<td>0.69 ± 0.02</td>
<td>0.73 ± 0.03</td>
</tr>
<tr>
<td>Aralkyl</td>
<td>0.74 ± 0.03</td>
<td>1.15 ± 0.05</td>
</tr>
<tr>
<td>Alkyl</td>
<td>2.4 ± 0.4</td>
<td>2.8 ± 0.6</td>
</tr>
</tbody>
</table>

Inhibition by organic anions

Inhibitory kinetics of GSH S-transferases by organic anions were studied by using kidney supernatant as the source of enzyme activity. Both probenecid and \(p\)-aminohippurate were competitive inhibitors of all GSH S-transferase activities studied. The inhibitor constants for the competitive inhibition of the GSH S-transferases by probenecid and \(p\)-aminohippurate are given in Table 2. On the basis of the 2–40-fold lower \(K_i\) values, probenecid was a more potent inhibitor than \(p\)-aminohippurate. As a typical example, the competitive inhibition of GSH S-epoxidetransferase activity by probenecid is shown as a Dixon plot in Fig. 1. As indicated in Table 2, a close correspondence in \(K_i\) values for the competitive inhibition by probenecid of the hepatic and renal GSH S-transferases was found.

Inhibition of renal GSH S-alkenetransferase activity by organic anions was examined by using \([^{14}C]\)ethacrynic acid as substrate. The spectrophotometric assay was not used because of inhibitor interference and therefore a detailed kinetic study was not made. As indicated in Table 3, both probenecid and \(p\)-aminohippurate inhibited this GSH S-alkenetransferase activity. However, a more than 12-fold higher concentration of \(p\)-aminohippurate than probenecid was required to produce approx. 50% inhibition of the enzymic reaction.

Examination of kinetics by using furosemide as inhibitor was hampered by its strong interference with the spectrophotometric enzyme assays. Therefore only the inhibition of renal GSH S-alkyltransferase (\([^{14}C]\)methyl iodide as substrate) was studied in detail. Furosemide was found to be a potent competitive inhibitor of this activity, with a \(K_i\) value (0.19mm) one order of magnitude lower than that found for the probenecid inhibition (cf. Table 2).

Neither probenecid, \(p\)-aminohippurate, nor furosemide was a substrate for enzymic activity when incubated for 60min \(in vitro\) with cytosol and GSH.

Table 2. Inhibition of glutathione S-transferase by organic anions

<table>
<thead>
<tr>
<th>Inhibitor Transferase activity</th>
<th>Kidney (K_i) (mm)</th>
<th>Liver (K_i) (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(p)-Aminohippurate</td>
<td>Probenecid</td>
</tr>
<tr>
<td>Aryl</td>
<td>16.3 ± 1.2</td>
<td>7.9 ± 0.8</td>
</tr>
<tr>
<td>Aralkyl</td>
<td>N.D.</td>
<td>4.4 ± 0.6</td>
</tr>
<tr>
<td>Epoxide</td>
<td>44.7 ± 5.4</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Alkyl</td>
<td>10.6 ± 2.2</td>
<td>1.5 ± 0.2</td>
</tr>
</tbody>
</table>

Vol. 158
Effect of hypophysectomy on renal and hepatic GSH S-transferases

The GSH S-transferases were measured in groups of eight hypophysectomized and eight sham-operated control rats 14 days after surgery. No change in liver wt./body wt. or kidney wt./body wt. ratios was found after surgery. Significant increases in both renal and hepatic aryl-, aralkyl-, and epoxide-transferase activities ($P<0.01$ for hepatic aryltransferase; $P<0.001$ for the others) were found after hypophysectomy (Fig. 2). Increases in aryl- and aralkyltransferase activities, however, were significantly greater (more than twofold) in the kidney ($P<0.001$) than in liver, whereas the increase in epoxidetransferase activity was comparable in the two organs. There was no significant change in alkyl- and alkenetransferase activities in either organ. Groups of six animals that had been subjected to gonadectomy or adrenalec- tomy showed no significant change in hepatic or renal GSH S-transferases activities compared with sham-operated controls assayed after a 14-day interval. Thyroxine was administered daily to eight sham-operated controls and eight hypophysectomized rats from 8–14 days after surgery by the procedure of Reyes et al. (1971). The hypophysectomy-induced increase in hepatic and renal aryltransferase activity was prevented by thyroxine administration so that activities were the same in both groups. Thyroxine did not, however, prevent the hypophysectomy-induced increases in aralkyl- and epoxide-transferase activities in kidney and liver. Thyroxine administration to controls did not alter the transferase activities.

Discussion

The GSH S-transferases are a group of soluble enzymes (mol.wt. 45000) which catalyse the detoxification of electrophilic drugs and carcinogens through conjugation with nucleophilic GSH (Boyland & Chasseaud, 1969; Habig et al., 1974b; Pabst et al., 1974; Kaplowitz et al., 1975a). In the liver, four enzymes have been identified, referred to as glutathione S-transferases A, B, C and E (Habig et al., 1974b; Pabst et al., 1974). Overlap has been found in the ability of the individual enzymes to catalyse reactions with the different substrate classes. However, transferase E alone seems to have epoxide-transferase activity (Habig et al., 1974b), and transferase B, which is identical with ligandin, has the bulk of activity towards ethacrynic acid (alkene substrate) (Habig et al., 1974b). According to these workers, although transferase E has higher activity towards methyl iodide (alkyl substrate), the more abundant transferase B accounts for the bulk of activity towards this substrate in liver.

Transferases A and C, which are immunologically related, share virtually all activity towards 3,4-
RENAL AND HEPATIC GLUTATHIONE S-TRANSFERASES

Fig. 2. Effect of hypophysectomy on hepatic and renal GSH S-transferase specific activities

* Indicates a significant difference (P < 0.001) between the change in enzyme activity per mg of protein in kidney compared with liver. S.E.M. values are indicated. For experimental details, see the Materials and Methods section. N.S., not significant.

dichloronitrobenzene (aryl substrate) (Habig et al., 1974b; Pabst et al., 1974). Activity towards aralkyl substrates, represented by p-nitrobenzyl chloride, is low for transferase B but does reside in all four proteins (Habig et al., 1974b), although its hepatic induction by phenobarbital seems to parallel increases in aryltransferase activity (Kaplowitz et al., 1975a).

The GSH S-transferase activities for the above substrate classes have also been identified in the kidney (Clifton et al., 1975a,b). The specific activities of the GSH S-transferases in kidney as compared with liver for aryl, aralkyl, epoxide, alkyl and alkene substrates are 2, 17, 68, 92 and 109% respectively (Clifton et al., 1975b). Purification of all enzyme proteins in kidney responsible for these activities has not been accomplished. In gel filtration, the elution volume of the enzyme activities is the same for kidney or liver. Further, ligandin (transferase B) has been identified by an immunofluorescence technique in the proximal tubular cell and is immunologically identical with liver ligandin (Kirsch et al., 1975). Thus there is little doubt that the group of enzymes present in the kidney is closely related to those in the liver. The similarity between the enzymes in both organs is now further supported by comparisons of substrate-dependence and response to inhibitors, and the effects of hypophysectomy.

Close correspondence in $K_r$ values for probenecid inhibition of both hepatic and renal activities was noted. In addition, apparent $K_m$ values were found to be very close for all but the aralkyltransferase activity, which had a $K_m$ significantly different in the two organs. The finding of a difference in the activity with $p$-nitrobenzylchloride is particularly noteworthy, because of the previously reported selective induction of this renal activity by phenobarbital in the rat (Clifton et al., 1975b).

We have previously described sex differences in activities of both hepatic and renal GSH S-transferases (Clifton et al., 1975b; Kaplowitz et al., 1975a). The effect of hypophysectomy to increase certain of these enzyme activities in both kidney and liver further emphasizes the complex hormonal regulation of this enzyme system. Measurement of these activities after adrenalectomy and gonadectomy failed to reproduce the increases in enzyme specific activities seen with hypophysectomy. In addition, the administration of thyroxine by the same procedure shown to affect Y protein (Reyes et al., 1971) blocked only the increase in renal and hepatic aryltransferase activity, not affecting the hypophysectomy-induced increases in epoxide- or aralkyl-transferase activities. These observations, in addition to contrasting the renal and hepatic responses to changes in hormonal milieu, may help clarify previous studies of the hormonal influences on hepatic Y protein. Hepatic Y protein, characterized by sulphobromophthalein binding in gel filtration of the cytosol, has been reported to increase in response to hypophysectomy, and this effect has been blocked by thyroxine replacement.
(Reyes et al., 1971). The gel-filtration fractions exhibiting sulphobromophthalene binding, however, have been shown to contain all of the GSH S-transferases (Kaplowitz et al., 1975b). The effect of hypophysectomy on content of ligandin (GSH S-transferase B), which has activity with iodomethane and the bulk of activity with ethacrynic acid but trace activity for 3,4-dichloronitrobenzene, has not been reported. In the present studies, the lack of response to hypophysectomy for the hepatic and renal alkyltransferase and alkenetranferase activities strongly suggests that, in fact, ligandin is not affected by hypophysectomy and/or thyroxine administration. All the present findings are consistent with the suggestion that the previously reported increase in Y protein after hypophysectomy may reflect enhanced sulphobromophthaline binding as a result of a relatively specific increase, namely, in the enzyme(s) having the bulk of S-aryltransferase activity.

Our observations are not entirely consistent, however, with the existence of only four GSH S-transferase enzymes as characterized by Habig et al. (1974b). As a consequence of hypophysectomy, epoxide activity (associated almost exclusively with transferase E) is increased but not alkyl activity (transferases E and B). These observations would suggest that transferase E is heterogeneous; that the epoxide and alkyl activities attributed to transferase E belong to different enzymes.

The binding to the enzymes of organic anions that are not substrates has been demonstrated at least qualitatively; probencid, p-aminom-phurate and furosemide are competitive inhibitors of the transferases. The hepatic activities are also inhibited by bilirubin and Indocyanine Green (Kaplowitz et al., 1975b). As previously demonstrated for the liver (Kaplowitz et al., 1975b), it is likely that several different glutathione S-transferase enzymes bind organic anions. The more potent inhibition by probenecid as compared with p-aminophpurate parallels evidence in vitro regarding the relative rate of renal uptake of the two compounds (Huang & Lin, 1965; Park et al., 1971). The identification of ligandin in the proximal tubule (Kirsch et al., 1975) is consistent with a physiological role of the transferases in organic-anion transport in this portion of the nephron.

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