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2-Bromo-(diglutathion-S-yl)hydroquinone Nephrotoxicity: Physiological, Biochemical, and Electrochemical Determinants

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SUMMARY

2-Bromo-(diglutathion-S-yl)hydroquinone [2-Br-(diGSyl)HQ] causes severe necrosis of the proximal renal tubules in the rat, elevations in blood urea nitrogen (BUN) and increased urinary excretion of protein, glucose, and lactate dehydrogenase. In contrast, 2-Br-3-(GSyl)HQ, 2-Br-5-(GSyl)HQ, and 2-Br-6-(GSyl)HQ caused differentially less toxicity than the diglutathionyl conjugate. None of these conjugates had any apparent effect on liver pathology and serum glutamate-pyruvate transaminase remained within the normal range. Pretreatment of rats with probenecid, an organic anion transport inhibitor, offered only slight protection against 2-Br-(diGSyl)HQ-mediated elevations in BUN, proteinuria, or glucosuria. In contrast, quinine, an organic cation transport inhibitor, potentiated the nephrotoxicity of 2-Br-(diGSyl)HQ. Thus, in contrast to other nephrotoxic sulfur conjugates, probenecid-sensitive organic ion transport systems do not contribute to the kidney-specific toxicity of 2-Br-(diGSyl)HQ. However, inhibition of renal γ-glutamyl transpeptidase by AT-125 completely protected rats from the nephrotoxic effects of 2-Br-(diGSyl)HQ. Aminooxyacetic acid, an inhibitor of cysteine conjugate β-lyase, caused a 20–25% decrease in 2-Br-(diGSyl)HQ-mediated elevations in BUN and urinary excretion parameters. The isomeric ^{35}S conjugates covalently bound to rat kidney 10,000 × *g* homogenate in the order 2-Br-6-(GSyl)HQ > 2-Br-5-(GSyl)HQ > 2-Br-3-(GSyl)HQ > 2-Br-(diGSyl)HQ. AT-125 (0.4 mM) decreased covalent binding by 25%, 17%, 33%, and 28%, respectively. Aminooxyacetic acid (0.1 mM) inhibited covalent binding by 26%, 10%, 17%, and 17% respectively. Ascorbic acid (1.0 mM) inhibited covalent binding by 63%, 87%, 62%, and 28%, respectively, and this inhibition correlated, inversely, with the redox potential of the conjugates. Thus, the covalent binding is mediated preferentially by oxidation of the quinol moiety, although the formation of reactive thiols cannot be excluded. In addition, the initial conjugation of 2-BrHQ with GSH does not result in the formation of a less redox-active species. However, the subsequent addition of a second molecule of GSH results in the formation of a more redox-stable compound, which, paradoxically, enhances toxicity. The metabolism of 2-Br-(diGSyl)HQ by renal proximal tubular γ-glutamyl transpeptidase and trans-membrane transport of the cysteine conjugate(s) followed by oxidation of the quinol moiety is probably responsible for the target organ toxicity of this compound.

Bromobenzene administration (9.3 mmol/kg, IP) to rats causes necrosis of the renal proximal convoluted tubules (1). Recently, we have shown that administration of *o*-bromophenol (1.9 mmol/kg, IP), a major metabolite of bromobenzene, to rats also caused a nephrotoxicity that was similar to that observed with bromobenzene (2). It was subsequently demonstrated that 2-BrHQ was a major metabolite of both bromobenzene and *o*-bromophenol and that it was a more potent nephrotoxicant than either of its precursors (3). Moreover, the results were consistent with the view that 2-BrHQ, or a metabolite thereof, might be formed in the liver and carried by the blood to the kidney, where it elicited toxicity. Microsomal incubation of 2-BrHQ in the presence of GSH resulted in the formation of several isomeric monosubstituted and disubstituted GSH conjugates (4). Administration of the chemically synthesized diglutathionyl conjugate to rats (30 μmol/kg, IV) caused elevations in BUN and histological alterations in the kidney that were indistinguishable from those observed after either bromobenzene (9.3 mmol/kg), *o*-bromophenol (1.9 mmol/kg), or 2-BrHQ (0.83 mmol/kg) administration. Thus, the dose of the diglutathionyl conjugate required to produce nephrotoxicity was 0.3% of that of bromobenzene. In contrast, 2-Br-3-(GSyl)HQ was far less toxic than the diglutathionyl conjugate. The reason(s) for the differential toxicity exhibited by the

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ABBREVIATIONS: IP, intraperitoneal; IV, intravenous; BUN, blood urea nitrogen; LDH, lactate dehydrogenase; AT-125, L-(α-5*S*)-α-amino-5-chloro-4,5-dihydro-5-isoxazoleacetic acid; 2-BrHQ, 2-bromohydroquinone; 2-Br-(diGSyl)HQ, 2-bromo-3,5- or 6-(diglutathionyl)hydroquinone; 2-Br-6-(GSyl)HQ, 2-bromo-6-(glutathionyl)hydroquinone; 2-Br-5-(GSyl)HQ, 2-bromo-5-(glutathionyl)hydroquinone; 2-Br-3-(GSyl)HQ, 2-bromo-3-(glutathionyl)hydroquinone; DCVC, S-(1,2-dichlorovinyl)-L-cysteine; HPLC, high performance liquid chromatography; SGPT, serum glutamate-pyruvate transaminase.

mono- and disubstituted conjugate is unclear. In the present study, we have, therefore, purified the two additional monosubstituted GSH conjugates, 2-Br-5-(GSyl)HQ and 2-Br-6-(GSyl)HQ, and determined their *in vivo* toxicity.

In contrast to the potent nephrotoxicity of the diglutathionyl conjugate of 2-BrHQ, it has no discernable effect on liver pathology. A number of both substrate- and tissue-related factors may contribute to the target organ toxicity of a compound. A major determinant of target organ toxicity is the manner in which a compound gains access to its target. In this respect, the kidneys remove a large proportion of plasma GSH (5–7) and as much as 70% of plasma GSH clearance is renal (8). One mechanism for the delivery of 2-BrHQ-GSH and other GSH *S*-conjugates to the kidneys is glomerular filtration. However, during a single pass through the renal circulation, only about 25% of the plasma conjugate is removed by this process. The fraction that enters the tubular lumen is metabolized by γ-glutamyl transpeptidase, via hydrolysis or transamination and transfer of the γ-glutamyl group to an appropriate acceptor (9–12). The product of this reaction is the *S*-substituted cysteinyl-glycine dipeptide, which in turn is subject to hydrolytic cleavage of the glycine residue by one of several membrane enzymes (13–15), after which transport of the cysteinyl conjugate into cells is effected. Both γ-glutamyl transpeptidase and the dipeptidase(s) are membrane-bound enzymes localized to the brush border membrane of the proximal tubule. It is the proximal tubules that appear to be particularly susceptible to the toxic effects of 2-Br-(diGSyl)HQ.

A significant amount of circulating GSH is also removed by a nonfiltration mechanism (7), which involves transport into renal cells across the basal-lateral membrane (16–18). Lash and Jones (17, 18) have characterized an electrogenic, sodium-coupled, and probenecid-sensitive transport system for the uptake of intact GSH into renal basal-lateral membrane vesicles. This system may account for the ability of the kidney to extract most of the GSH passing through the renal circulation. Three lines of evidence, as follows, suggest that a similar mechanism for the delivery of GSH *S*-conjugates to the kidneys, in addition to glomerular filtration, may be operating: GSH and GSH *S*-conjugates undergo a similar pattern of interorgan metabolism (19–21); the same enzymes are responsible for the conversion of GSH and GSH *S*-conjugates to cysteine and cysteine *S*-conjugates respectively (22); and the basal-lateral membrane GSH transport system exhibits a broad substrate specificity for γ-glutamyl compounds (18). However, the relative contribution of sodium-coupled and γ-glutamyl transpeptidase-mediated GSH transport to total renal plasma GSH clearance is unclear. Thus, one reason for the specific nephrotoxicity of 2-Br-(diGSyl)HQ might be its preferential uptake into renal cells as a consequence of the normal physiological function of the kidneys.

A second important determinant of target organ- or cell-specific toxicity involves the ability of the target organ to activate otherwise innocuous compounds to toxic metabolites. In this respect, the presence of the enzymes involved in mercapturic acid formation (14) and those of the thiomethyl shunt (23, 24) are present in relatively high concentrations in the kidney and have been implicated in the generation of potentially reactive thiols from a number of GSH/cysteine conjugates. Thus, because of its physiological function and biochemical profile, the kidney may be particularly susceptible to the toxicity of GSH conjugates. In the present study we therefore investigate the differential nephrotoxicity caused by 2-Br-(diGSyl)HQ and three monosubstituted isomers and the role of both renal transport and metabolism in the production of this toxicity. The data suggest that a combination of physiological, biochemical, and electrochemical factors probably contribute to the potent nephrotoxicity of 2-Br-(diGSyl)HQ.

Methods

Reagents. [^{35}S]Glutathione (112.5 Ci/mmol; >98.5% pure) was purchased from New England Nuclear (Boston, MA). Glutathione, aminooxyacetic acid, ascorbic acid, quinine, and probenecid were obtained from Sigma Chemical Co. (St. Louis, MO). 2-BrHQ and silver oxide were products of ICN Biomedicals, Inc. (Plainview, NY). AT-125 (Acivicin; NSC163501; L-(α-5*S*)-α-amino-*S*-chloro-4,5-dihydro-5-isoxazoleacetic acid) was generously provided by the National Cancer Institute (Rockville, MD). All other chemicals were of the highest grade commercially available.

Animals. Male Sprague Dawley rats (Taconic Farms, Germantown, NY; 150–200 g) were used for all experiments and were allowed food and water *ad libitum* before the experiments.

NMR spectrometry. The spectra were determined on solutions of 5–10 mg of compounds in 0.5 ml of D_2O. The ^{1}H NMR spectra were recorded with a Varian XL200 spectrometer at 200 MHz under conditions previously described (25).

Synthesis of 2-BrHQ [^{35}S]GSH conjugates. [^{35}S]GSH conjugates of 2-BrHQ were synthesized according to our established method (4) with the following modification. 2-Bromoquinone was synthesized by mixing 1 g of 2-BrHQ and 1.8 g of silver oxide in 250 ml of methylene chloride with stirring at room temperature (25°) for 4 hr. The mixture was filtered under vacuum and the majority of the solvent was removed by rotary evaporation under vacuum. The remaining solvent was evaporated to dryness under nitrogen. 2-Bromoquinone (0.1 mmol in 0.5 ml of methanol) was added dropwise to 50 mM [^{35}S]GSH (4400 dpm/nmol) in 10 ml of 10 mM Tris·HCl buffer (pH 7.4). Aliquots of the mixture of 2-Br-([^{35}S]GSH)-HQ conjugates were injected onto a Partisil M9 10/25 ODS 3 reverse phase semipreparative column to isolate individual isomers. The column was eluted with water/methanol/acetic acid (90:9:1, v/v) at a flow rate of 3 ml/min. HPLC was performed with a model 6000A chromatograph (Water Associates, Milford, MA) equipped with a UV absorbance detector (254 nm) and Waters model U6K injector. Individual peaks corresponding to the synthesized reference compounds (4) were collected from several injections of the mixture of 2-Br-([^{35}S]GSH)-HQ conjugates. The methanol was then evaporated under nitrogen and the remaining aqueous fractions were frozen in dry ice/ethanol and lyophilized. This procedure yielded ^{35}S-labeled 2-Br-(diGSyl)HQ, 2-Br-3-(GSyl)HQ, 2-Br-5-(GSyl)HQ, and 2-Br-6-(GSyl)HQ (HPLC peaks 1, 5, 6, and 7 in order of elution from the HPLC column). Radiochemical purity was >99%.

Synthesis of unlabeled 2-BrHQ-GSH conjugates. Isolation and purification of the 2-Br-(diGSyl)HQ conjugate was achieved as follows. A total of 2 g of a mixture of isomeric 2-BrHQ-GSH conjugates was dissolved in 40 ml of distilled water, 2.5-ml fractions were applied to a Sephadex G-25M gel filtration column (PD-10; Pharmacia Fine Chemicals, Piscataway, NJ), and 3.0 ml of distilled water was used to elute the 2-Br-(diGSyl)HQ conjugate. The fractions containing the 2-Br-(diGSyl)HQ were pooled and lyophilized. The pure product had an identical HPLC retention time and a characteristic UV absorption spectrum when compared with the previously synthesized authentic standard (4). Isolation and purification of the monosubstituted GSH conjugates was carried out as previously described for 2-Br-3-(GSyl)HQ (4).

Preparation of 10,000 × *g* supernatant. Animals were euthanized by cervical dislocation and the kidneys were removed and homogenized with 0.1 M phosphate buffer, pH 7.4, at 4°. The homogenates

were centrifuged at 10,000 × *g* for 20 min and the supernatant was used for the *in vitro* covalent binding studies.

***In vitro* covalent binding of [^{35}S]GSH 2-BrHQ conjugates to renal 10,000 × *g* supernatant.** Mixtures containing 2 mg/ml kidney 10,000 × *g* supernatant protein, 100 μM of various 2-BrHQ [^{35}S]GSH conjugates in 0.1 M phosphate buffer, pH 7.4, in the presence or absence of AT-125 (0.4 mM), aminooxyacetic acid (0.1 mM), and ascorbic acid (1.0 mM), in a final volume of 1.5 ml, were incubated at 37° for 15 min. Control incubations were carried out in parallel at 4°. For experiments in which AT-125 and aminooxyacetic acid were used, the mixtures were preincubated in the presence of the inhibitor for 15 min before addition of the substrate. The incubations were terminated by placing the reaction vessel in a dry ice/acetone bath. Upon thawing, 1 ml of the incubation mixture was added to 0.1 ml of 10% trichloroacetic acid. The precipitated protein was washed with 3 ml of 1% trichloroacetic acid followed by exhaustive washing with methanol/ether (80:20, v/v) until no significant radioactivity (usually less than 100 dpm/ml) was detected in the washes. The pellets were then dissolved in 1 ml of 1 N NaOH and radioactivity was determined by liquid scintillation counting. An aliquot of the NaOH solution was taken for protein determination (Bio-Rad protein assay; Bio-Rad Laboratories, Richmond, California).

Cyclic voltammetry. Cyclic voltammograms were obtained using a BAS-100 electrochemical analyser equipped with a platinum electrode. All samples were dissolved in a solution of methanol/water/acetic acid (10:89:1; v/v/v) at pH 3.0. The cyclic voltammograms were obtained over a potential range of −500 to +1300 mV or −700 to +1300 mV at a scan rate of 100 mV/sec.

Toxicity studies. 2-Br-(diGSyl)HQ, dissolved in 200 μl of 0.85% phosphate-buffered saline (pH 7.4) was injected IV (tail vein) into rats at a dose of either 10, 30, or 50 μmol/kg. A dose of 30 μmol/kg was used for those studies illustrating the gross anatomical changes that occur in the kidney after 2-Br-(diGSyl)HQ administration, whereas the lower dose (10 μmol/kg) was employed to illustrate changes in the various biochemical parameters that occur after 2-Br-(diGSyl)HQ administration. Phosphate-buffered (0.85%) saline was injected into control rats. Some rats were pretreated with IP injections of either AT-125 (10 mg/kg), aminooxyacetic acid (0.62 mmol/kg), quinine (0.12 mmol/kg), or probenecid (0.7 mmol/kg) 1 hr before the administration of 2-Br-(diGSyl) conjugate (10 μmol/kg; IV). Both AT-125 and quinine were dissolved in phosphate-buffered saline by sonication. Probenecid was initially dissolved in 1 N NaOH and diluted with phosphate-buffered saline so that the final pH was between 6.8 and 7.0. Aminooxyacetic acid was dissolved in 0.2 M KH_2PO_4 and the final pH was between 6.3 and 6.8. Urine from individual rats was collected over 24 hr. The tubes into which the urine was collected were kept in darkness and maintained at 4°. The protein content of urine was determined with a Bio-Rad protein assay. Urinary glucose was determined with a Sigma Kit Glucose 15-UV. For urinary LDH activity, 2.5 ml of urine was applied to a Sephadex G-25M gel filtration column (9.1 ml; PD 10; Pharmacia) and the column was eluted with 3.5 ml of saline. This filtered urine was used to assay for LDH activity by measuring the disappearance of NADH (0.1 mg/ml) at 340 nm in the presence of pyruvate (2.0 mmol/l) according to Sigma Kit LDH 340-UV. Each of the isomeric 2-Br-mono-GSyl-HQ conjugates was injected IV into rats at doses of 30 and 50 μmol/kg. Twenty four hours after the administration of conjugates, a sample of blood (400 μl) was taken from the retroorbital sinus, plasma was separated by centrifugation, and the degree of renal and hepatic damage was assessed by measuring BUN and SGPT with Sigma Kits 535A and 505, respectively. Livers and kidneys were removed and histology slides were made and stained with eosin and hematoxylin by American Histolabs (Rockville, MD) and BUN concentrations were determined with a Sigma Kit BUN 535A.

Statistical analysis. Unless otherwise noted, all data are expressed as the mean ± standard deviation. Mean values were compared by Student-Newman-Kuel's test. Where appropriate, data were analyzed by analysis of variance, completely random design.

Results

Characterization of two monosubstituted 2-Br-HQ-GSH conjugates. Isolation and purification of the two chemically synthesised GSH conjugates were carried out by procedures described previously (4) for 2-Br-3(GSyl)HQ and 2-Br-(diGSyl)HQ. Analysis by ^{1}H NMR spectroscopy revealed the structure of the two compounds to be 2-Br-5-(GSyl)HQ and 2-Br-6-(GSyl)HQ (Figs. 1 and 2, respectively). These two conjugates correspond to HPLC peaks 6 and 7 as previously described (see Fig. 1 and Table 1, Ref. 4). In Figs. 1 and 2, the presence of GSH residues was shown by the characteristic aliphatic groupings at high field (4, 25–28). The position of the hydroquinone hydroxyl groups is already established, i.e., carbons 1 and 4. In Fig. 1 the two aromatic protons at 7.10 and 7.01 ppm exhibited a *para* coupling of $J = <1$ Hz and must therefore be the hydrogens attached to carbons 3 and 6. Thus, the position of the GSH substitution must have been at the remaining position 5 carbon atom. This compound was therefore identified as 2-bromo-5-(glutathion-Syl)hydroquinone. In Fig. 2 the two aromatic protons at 7.05 and 6.91 ppm exhibit a *meta* coupling of $J = 3$ Hz and by a similar interpretation as above, this compound was identified as 2-bromo-6-(glutathion-Syl) hydroquinone.

***In Vivo* Toxicity of 2-BrHQ-GSH Conjugates.** The *in vivo* toxicity of the isomeric 2-BrHQ-GSH conjugates is shown in Table 1. 2-Br(diGSyl)HQ caused substantial elevations in BUN at a dose of 30 μmol/kg, IV and alterations in renal pathology as previously described (4). Fig. 3 shows the effect of 2-Br-(diGSyl)HQ (30 μmol/kg; IV) on rat kidney histology. The histological changes are essentially localized to the corticomedullary junction, where extensive coagulative necrosis of the proximal tubules was observed. The most severe damage appeared in the S_3 segments of the proximal tubules, which contained eosinophilic cells with pyknotic nuclei. The collecting ducts and tubules showed occasional hyaline casts. Some of the tubules in the midcortex and outer cortex were markedly dilated and contained proteinaceous fluid. The glomeruli appeared unchanged. In contrast, none of the three monosubstituted 2-BrHQ-GSH conjugates showed any effect on BUN at a dose of 30 μmol/kg IV. However, elevations in BUN are observed when the dose of the monosubstituted conjugates is increased to 50 μmol/kg. Although the histological alterations were similar to those observed with 2-Br-(diGSyl)HQ, they were not as severe or as extensive. Neither the mono- nor disubstituted conjugates had any effect on SGPT values (Table 1) and liver pathology appeared normal.

Renal transport and 2-Br-(diGSyl)HQ nephrotoxicity. One of the main factors determining target-organ toxicity is the manner in which the substrate gains access to its target. Because the area of the nephron associated with organic ion transport appears particularly susceptible to the toxicity of 2-Br-(diGSyl)HQ, we investigated the effects of both organic anion and organic cation inhibitors on 2-Br-(diGSyl)HQ nephrotoxicity. Administration of 2-Br-(diGSyl)HQ to rats (10 μmol/kg; IV) caused substantial proteinuria, glucosuria, and elevations in the urinary excretion of LDH (Table 2). BUN concentrations were also significantly elevated. Probenecid (an organic anion transport inhibitor) caused only a slight decrease (10–20%) in 2-Br-(diGSyl)HQ-mediated elevations in urinary glucose and BUN and had no effect on urinary protein and LDH excretion. Quinine (an organic cation transport inhibitor)

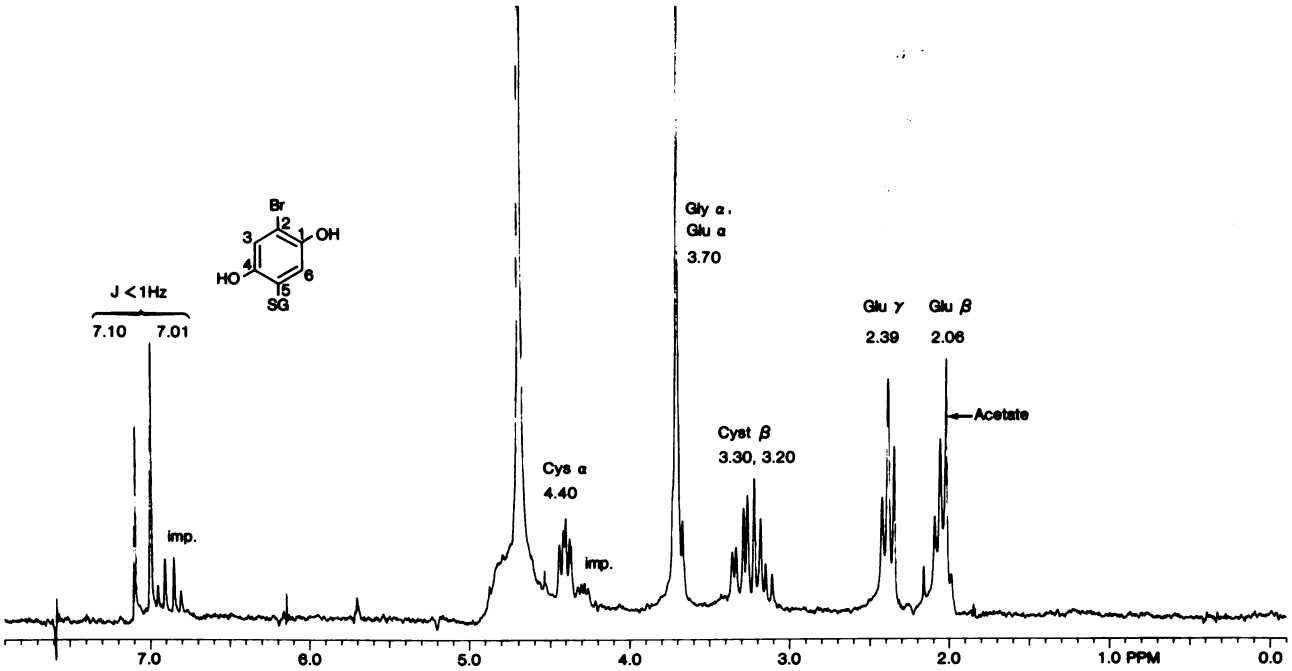

Fig. 1. 360 MHz ^{1}H NMR spectrum of a chemically synthesized monosubstituted GSH conjugate of 2-BrHQ. Isolation and purification of this compound was carried out by HPLC with a Partisil 5 ODS-3 reverse phase column eluted with water/methanol/acetic acid (90:9:1, v/v) at a flow rate of 1 ml/min. The retention time for this compound was 21 min. The eluate was frozen and lyophilized and a 1–2-mg sample was used for analysis.

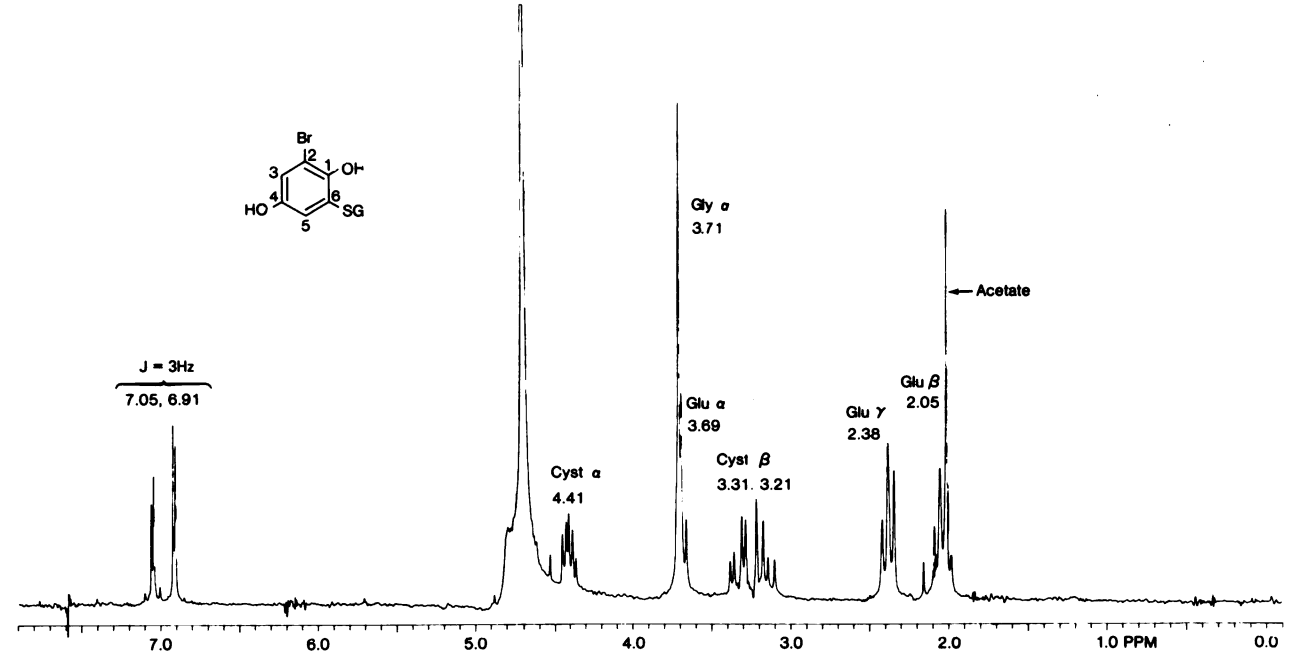

Fig. 2. 360 MHz ^{1}H NMR spectrum of a chemically synthesized monosubstituted glutathione conjugate of 2-BrHQ. Isolation and purification of this compound was carried out by HPLC with a Partisil 5 ODS-3 reverse phase column eluted with water/methanol/acetic acid (90:9:1; v/v) at a flow rate of 1 ml/min. The retention time for this compound was 23.5 min.

significantly potentiated the proteinuria, glucosuria, and urinary LDH leakage caused by 2-Br-(diGSyl)HQ but had no effect on BUN (Table 2).

Metabolism and 2-Br-(diGSyl)HQ nephrotoxicity. The first step in the metabolism of GSH conjugates is catalyzed by γ-glutamyl transpeptidase. In an effort to establish the possibility that the nephrotoxicity of 2-Br-(diGSyl)HQ is dependent upon metabolism, rats were treated *in vivo* with AT-125 (10 mg/kg; IP). This treatment has previously been shown to cause substantial inhibition of rat kidney γ-glutamyl transpeptidase (4). Such a treatment protected rats from the nephrotoxic effects of 2-Br-(diGSyl)HQ (Table 3). Thus, AT-125 pretreatment caused a significant inhibition of the proteinuria and glucosuria mediated by 2-Br-(diGSyl)HQ, decreased the urinary excretion of LDH, and decreased 2-Br-(diGSyl)HQ-mediated elevations in BUN. Histological examination of kidney slices prepared from AT-125-pretreated rats showed no discernable pathological alterations (data not shown). To deter-

TABLE 1

***In vivo* toxicity of isomeric 2-BrHQ-GSH Conjugates**

The isomeric GSH conjugates were given by IV injection in 200 μl of 0.85% phosphate-buffered saline. Control animals received vehicle only. BUN and SGPT levels were determined 24 hr later as described under Methods. Data represent the mean ± standard deviation (n = 4). Statistical analyses were performed by Student-Newman-Kuel's test. Values were statistically significantly different when compared with control animals at the following confidence levels: [a] $p < 0.01$, [b] $p < 0.05$.

2-BrHQ-GSH Conjugate	Dose	BUN	SGPT
	μmol/kg	mg/100 ml	units/liter
2-Br-(diGSyl)HQ	50	106.9 ± 6.3[a]	18.3 ± 2.4
	30	117.7 ± 9.3[a]	18.9 ± 5.4
2-Br-3-(GSyl)HQ	50	26.2 ± 3.5[b]	19.1 ± 7.2
	30	16.4 ± 1.8	18.0 ± 3.9
2-Br-5-(GSyl)HQ	50	33.5 ± 1.7[b]	19.3 ± 2.0
	30	17.1 ± 2.4	18.2 ± 2.0
2-Br-6-(GSyl)HQ	50	41.2 ± 10.6[b]	21.6 ± 2.2
	30	13.1 ± 1.5	22.1 ± 3.1
Control		15.5 ± 2.5	18.3 ± 6.1

mine the role of cysteine conjugate β-lyase in the metabolism and nephrotoxicity of 2-Br-(diGSyl)HQ, rats were pretreated with aminooxyacetic acid, a general inhibitor of pyridoxal phosphate-utilizing enzymes. Pretreatment with aminooxyacetic acid (0.62 mmol/kg; IP) caused a modest (20–23%) decrease in the urinary excretion of protein, glucose, and LDH and a similar (25%) decrease in 2-Br-(diGSyl)HQ-mediated elevations of BUN (Table 3).

***In vitro* metabolism of the isomeric [^{35}S]2-Br-(GSyl)HQ conjugates to reactive intermediates.** The formation of reactive metabolites was assayed by determining the covalent binding of the individual [^{35}S]2-Br-(GSyl)HQ conjugates to rat renal 10,000 × g homogenates. The order of covalent binding was 2-Br-6-(GSyl)HQ > 2-Br-5-(GSyl)HQ > 2-Br-3-(GSyl)HQ > 2-Br-(diGSyl)HQ (Table 4). Thus, 2-Br-(diGSyl)HQ, which is the most potent nephrotoxicant, gave rise to less covalently bound material than either of the three monosubstituted isomers. AT-125 (0.4 mM) decreased the covalent binding of the various isomers by 17–33% (Table 4) and aminooxyacetic acid (0.1 mM) inhibited covalent binding by 10–26% (Table 4). Ascorbic acid was more effective than either AT-125 or aminooxyacetic, inhibiting the covalent binding of the three monosubstituted isomers by 62–87%. In contrast, ascorbic acid inhibited 2-Br-(diGSyl)HQ covalent binding by only 28%. It is of interest to note that the inhibition of covalent binding of the 2-Br-(monoGSyl)HQ conjugates by AT-125 plus that inhibited by ascorbic acid totals 88–104%, whereas the more potent nephrotoxicant 2-Br-(diGSyl)HQ is inhibited only 56% by AT-125 and ascorbic acid.

Electrochemistry of the isomeric 2-Br-(GSyl)HQ conjugates. Cyclic voltammograms of 2-BrHQ, 2-Br-3-(GSyl)HQ, 2-Br-5-(GSyl)HQ, 2-Br-6-(GSyl)HQ, and 2-Br-(diGSyl)HQ are shown in Fig. 4. Each compound was dissolved in methanol/water/acetic acid (10:89:1) at pH 3.0. It is evident that the redox properties of the three monosubstituted GSH conjugates are similar to that of 2-BrHQ itself. On the first anodic sweep the quinols are oxidized to the corresponding quinones. 2-Br-3-(GSyl)HQ, 2-Br-5-(GSyl)HQ, and 2-Br-6-(GSyl)HQ exhibited redox potentials of 601, 502, and 600 mV, respectively, similar to that of 2-BrHQ (600 mV). In contrast, 2-Br-(diGSyl)HQ exhibited a redox potential of ~1,000 mV. The correlation between the redox potential of the GSH conjugates and the ascorbic acid-mediated inhibition of their covalent binding to renal 10,000 × g homogenate is illustrated in Fig. 5. The lower the redox potential of the GSH conjugates the more effective the inhibition of covalent binding by ascorbate.

Discussion

We have shown that 2-Br-(diGSyl)HQ is a more potent nephrotoxicant than either of three monosubstituted isomers (Table 1). 2-Br-(diGSyl)HQ administration to rats (30 μmol/

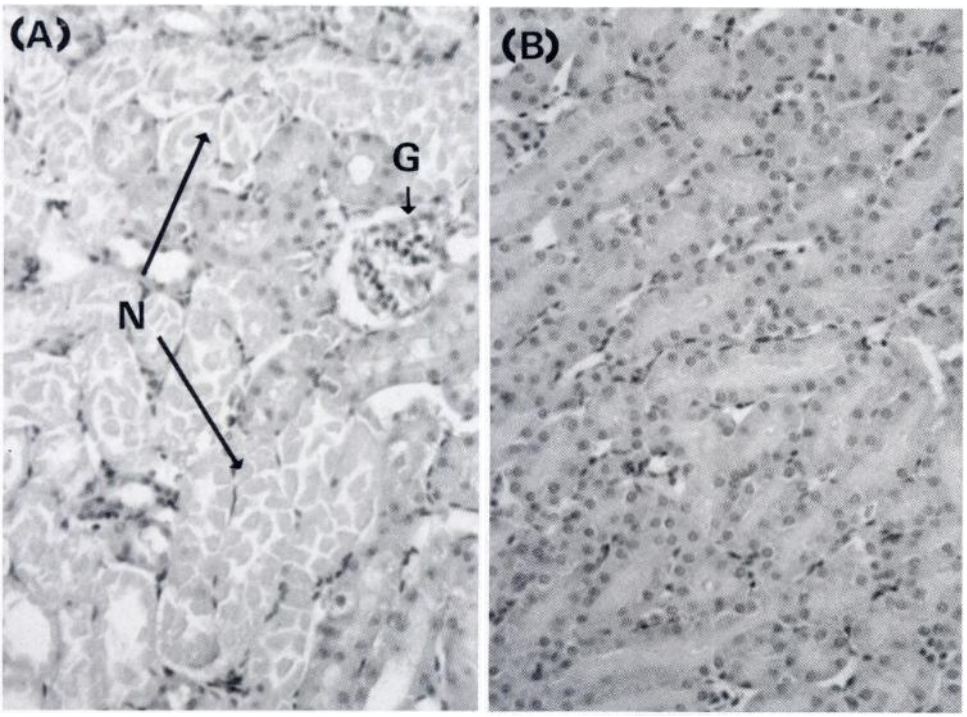

Fig. 3. Kidney sections obtained from rats 24 hr after (A) treatment with 2-Br-(diGSyl)HQ (30 μmol/kg, IV) or (B) treatment with phosphate-buffered saline (0.85%). The sections were stained with hematoxylin and eosin and the magnification was ×250. Each photograph was taken of the cortico-medullary junction. Severe proximal tubular necrosis (*N*) was observed in the S_3 segments. The glomerulus (*G*) appeared unaffected.

TABLE 2

The effect of organic ion transport inhibitors on 2-Br-(diGSyl)HQ nephrotoxicity

Quinine (0.12 mmol/kg; IP) or probenecid (0.7 mmol/kg; IP) in phosphate-buffered saline were given to rats either alone or 1 hr before the 2-Br-(diGSyl)HQ conjugate (10 μmol/kg; IV). Control animals were given vehicle (phosphate-buffered saline) only. Urine was collected over 24 hr, at which time blood was obtained via the retro-orbital sinus for BUN determination. Urinary protein, LDH, and glucose were determined as described in Methods. One unit of LDH is defined as reduction of 1.0 μmol of pyruvate to L-lactate per min at pH 7.5 at 37°. Figures represent the mean ± standard deviation ($n = 4$). Values a-c are statistically significantly different when compared with control animals at the following confidence levels: [a] $p < 0.01$, [c] $p < 0.05$; [b] significantly different from rats that received 2-BrHQ-GSH.

	Control	2-BrHQ-GSH	Quinine + 2-BrHQ-GSH	Probenecid + 2-BrHQ-GSH	Quinine	Probenecid
Urinary protein (mg/24 hr)	1.6 ± 0.05	8.2 ± 1.8[a]	18.9 ± 3.8[a,b]	9.6 ± 1.1	1.6 ± 0.1	1.7 ± 0.1
Urinary LDH (unit/24 hr)	0.04 ± 0.02	1.3 ± 0.3[a]	2.8 ± 0.2[b,c]	0.9 ± 0.3	0.2 ± 0.1	0.04 ± 0.02
Urinary glucose (mg/24 hr)	0.4 ± 0.02	19.4 ± 0.3[a]	35.3 ± 0.5[a,b]	17.4 ± 0.8	0.2 ± 0.1	0.4 ± 0.1
BUN (mg/100 ml/24 hr)	10.2 ± 0.4	24.3 ± 0.8[a]	22.2 ± 0.5	19.4 ± 0.2	9.3 ± 0.9	11.8 ± 0.6

TABLE 3

The effect of γ-glutamyl transpeptidase and cysteine conjugate β-lyase inhibitors on 2-Br-(diGSyl)HQ nephrotoxicity

AT-125 (10 mg/kg; IP) and aminooxyacetic acid (55 mg/kg; IP) in phosphate-buffered saline were given to rats either alone or 1 hr before the 2-Br-(diGSyl)HQ conjugate (10 μmol/kg; IV). Control animals were given vehicle (phosphate-buffered saline) only. Urine was collected over 24 hr, at which time blood was obtained via the retro-orbital sinus for BUN determination. Urinary protein, LDH, and glucose were determined as described in Methods. One unit of LDH is defined as reduction of 1.0 μmol of pyruvate to L-lactate per min at pH 7.5 at 37°. Figures represent the mean ± standard deviation ($n = 4$). Values are statistically significantly different when compared with control animals at the following confidence levels: [a] $p < 0.01$, [c] $p < 0.05$; [b] significantly different from rats that received 2-BrHQ-GSH.

	Control	2-BrHQ-GSH	AT-125 + 2-BrHQ-GSH	Aminooxyacetic acid + 2-BrHQ-GSH	AT-125	Aminooxyacetic acid
Urinary protein (mg/24 hr)	1.6 ± 0.05	8.2 ± 1.8[a]	0.8 ± 0.3[a,b]	6.4 ± 0.8	1.6 ± 0.2	1.6 ± 0.7
Urinary LDH (unit/24 hr)	0.04 ± 0.02	1.3 ± 0.3[a]	0.2 ± 0.1[a,b]	1.0 ± 0.3	0.16 ± 0.08	0.13 ± 0.02
Urinary glucose (mg/24 hr)	0.4 ± 0.02	19.4 ± 0.3[a]	0.6 ± 0.1[a,b]	15.5 ± 1.2	0.4 ± 0.1	0.3 ± 0.1
BUN (mg/100 ml/24 hr)	10.2 ± 0.4	24.3 ± 0.8[a]	18.3 ± 0.2[b,c]	19.4 ± 0.2	10.4 ± 0.5	12.4 ± 1.2

TABLE 4

The covalent binding of purified 2-BrHQ[^{35}S]GSH conjugates to rat kidney 10,000 × *g* supernatant

Incubations contained 2 mg/ml kidney protein in 0.1 M phosphate (pH 7.4) and 100 μM ^{35}S-labeled conjugate in the presence or absence (controls) of the various inhibitors in a total volume of 1.5 ml. The figures represent the mean ± standard deviation of triplicate incubations. Values in parentheses represent the per cent inhibition when compared with control incubations that lack the presence of the various inhibitors. AT-125 and aminooxyacetic acid were preincubated with 10,000 × *g* supernatant for 15 min before the addition of substrate. Ascorbic acid was added immediately before substrate. Incubations were performed at 37° for 15 min. Values were statistically significantly different when compared with incubations containing the ^{35}S-labeled conjugates alone at the following confidence levels. [a] $p < 0.05$, [b] $p < 0.01$.

	Covalent binding			
	2-Br-(diGSyl)HQ	2-Br-3-(GSyl)HQ	2-Br-5-(GSyl)HQ	2-Br-6-(GSyl)HQ
	nmol/mg/15 min			
No addition	1.00 ± 0.04	2.15 ± 0.37	2.23 ± 0.27	3.34 ± 0.11
AT-125 (0.4 mM)	0.71 ± 0.02 (28)[a]	1.45 ± 0.08 (33)[a]	1.85 ± 0.17 (17)	2.51 ± 0.06 (25)[b]
Aminooxyacetic acid (0.1 mM)	0.83 ± 0.11 (17)	1.78 ± 0.12 (17)	2.00 ± 0.42 (10)	2.46 ± 0.64 (26)
Ascorbic acid (1.0 mM)	0.72 ± 0.11 (28)[a]	0.82 ± 0.18 (62)[a]	0.28 ± 0.07 (87)[b]	1.22 ± 0.11 (63)[b]

kg IV) caused a striking and extensive coagulative necrosis of renal tubular cells in the cortico-medullary region (Fig. 3). Moreover, this effect of 2-Br-(diGSyl)HQ appeared to be organ specific, inasmuch as it caused no discernable alterations to the liver and SGPT values remained in the normal range over the dose range employed (Table 1). However, the reason(s) for this tissue-selective toxicity is unknown. In this respect, a major determinant of target organ toxicity is the manner in which a compound gains access to its target. The renal toxicity of a number of GSH, cysteine, and *N*-acetyl cysteine conjugates has been shown to be localized to the pars recta segment of the proximal tubule, that area of the nephron associated with organic anion secretion (29). Pretreatment of rats with probenecid (0.7 mmol/kg; IP), a selective organic anion transport inhibitor (30), offered only slight protection against 2-Br-(diGSyl)HQ mediated nephrotoxicity, as evidenced by a slight decrease in BUN and the urinary excretion of glucose (Table 2). In contrast, Lock and Ishmael (31) have provided convincing evidence that the renal cortical accumulation and selective proximal tubular toxicity of hexachloro-1,3-butadiene and its sulfur conjugates is related to a carrier-mediated transport system that can be inhibited by probenecid. Administration of probenecid (0.175 mmol/kg) has also been shown to protect against DCVC mediated nephrotoxicity as indicated by decreased elevations in urine glucose excretion rates (32). Protection against the renal necrosis produced by *S*-(2-chloroethyl)cysteine is also effected by prior administration of probenecid (33). The relative lack of effect of probenecid in the present studies is intriguing but it is becoming increasingly apparent that multiple transport processes exist for the proximal tubular accumulation of GSH and cysteine conjugates (34).

In contrast to the effects of probenecid, quinine, an organic cation transport inhibitor (35), actually potentiated the nephrotoxicity of 2-Br-(diGSyl)HQ (Table 2). In this instance the urinary excretion of protein, LDH, and glucose were all further significantly elevated when rats were pretreated with quinine (0.12 mmol/kg; IP) before 2-Br-(diGSyl)HQ administration. Although BUN concentrations were the same in both treatment groups, it should be noted that BUN is a less sensitive indicator of renal damage than are the urinary parameters. Although the mechanism of potentiation of toxicity by quinine is not known, quinine is a marked local irritant. These irritant properties

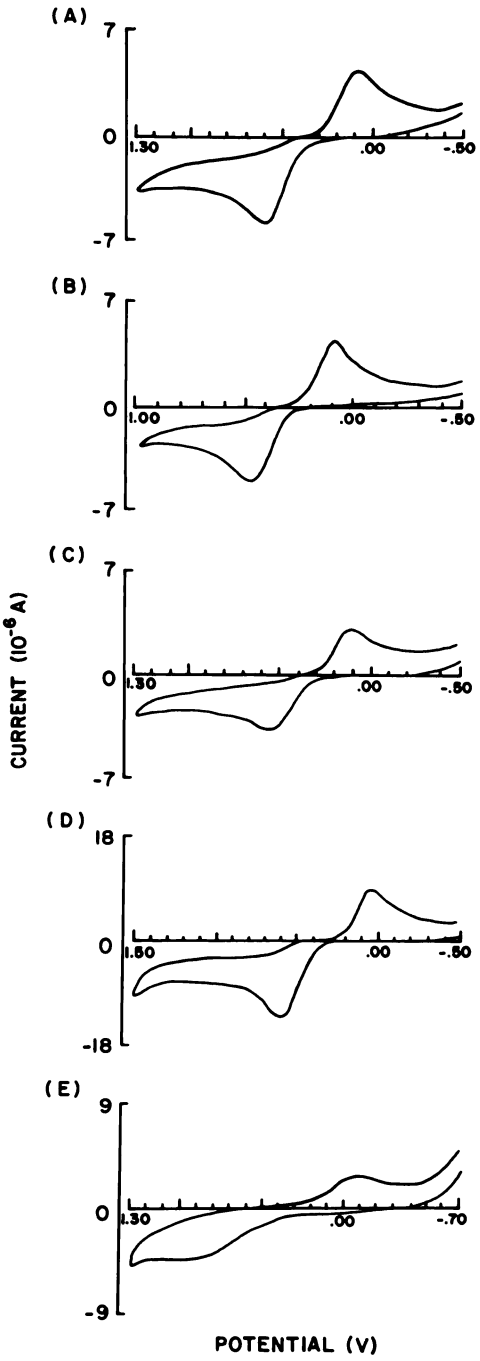

Fig. 4. Cyclic voltammograms of 2-BrHQ and 2-BrHQ-GSH conjugates. Cyclic voltammograms were obtained on a BAS-100 Electrochemical Analyser equipped with a platinum electrode, over a potential range of −500 or −700 to +1300 mV at a scan rate of 100 mV/sec. Voltammograms were obtained from (A) 2-Br-3-(GSyl)HQ; (B) 2-Br-5-(GSyl)HQ; (C) 2-Br-6-(GSyl)HQ; (D) 2-BrHQ, and (E) 2-Br-(diGSyl)HQ.

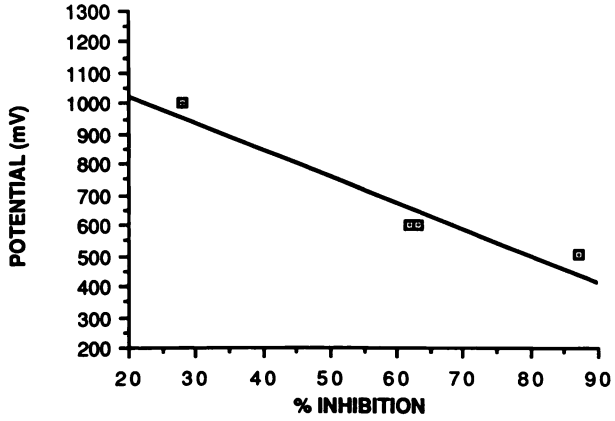

Fig. 5. Correlation between the inhibition of 2-Br-([^{35}S]GSH)HQ covalent binding to rat kidney 10,000 × *g* homogenate by 1 mM ascorbic acid and the redox potentials of 2-BrHQ-GSH conjugates.

become manifest where the drug is concentrated, such as in the kidney tubule, and renal injury may ensue (36). Thus, although the dose of quinine administered alone in the present studies was insufficient to produce any signs of toxicity (Table 2), it may act synergistically when administered in combination with 2-Br-(diGSyl)HQ.

The first step in the metabolism of GSH conjugates to their corresponding cysteine conjugates involves either hydrolysis or transamination by γ-glutamyl transpeptidase and transfer of the γ-glutamyl moiety to an appropriate acceptor. AT-125 is an inhibitor of several glutamine-utilizing enzymes (37) including γ-glutamyl transpeptidase (4, 38–40) and has been used as such to probe the possible role of this enzyme in the nephrotoxicity of a number of GSH conjugates. In the present studies, pretreatment of rats with AT-125 (10 mg/kg; IP) caused a significant reduction in 2-Br-(diGSyl)HQ-mediated proteinuria, glucosuria, and BUN (Table 3). These findings are in accord with *in vitro* studies, which showed that accumulation of the various isomeric 2-BrHQ-GSH conjugates into renal slices was also significantly inhibited by AT-125 (41). These data therefore indicate that the activity of γ-glutamyl transpeptidase is essential for the development of 2-Br-(diGSyl)HQ-mediated nephrotoxicity.

γ-Glutamyl transpeptidase activity may be required for one or both of two possible functions. The enzyme may be functioning indirectly in a transport capacity, in which conversion to the cysteine conjugate is a prerequisite for cellular uptake (Fig. 6), or in a metabolic capacity, in which generation of the cysteine conjugate and its subsequent metabolic processing by cysteine conjugate β-lyase, are the critical events leading to toxicity. In terms of the latter possibility, DCVC is an excellent substrate for β-lyase (42) and is converted to a sulfur-containing alkylating metabolite that probably reacts covalently with tissue components (43–49). β-Lyase has also been suggested to be involved in the nephrotoxicity of the cysteine conjugate of hexachloro-1,3-butadiene (50). β-Lyase is a pyridoxal phosphate-dependent enzyme that can be inhibited by aminooxyacetic acid, a general inhibitor of pyridoxal phosphate-utilizing enzymes. However, in our experiments, pretreatment of rats with aminooxyacetic acid (0.62 mmol/kg; IP) offered only slight protection against 2-Br-(diGSyl)HQ nephrotoxicity (Table 3). This result suggests that γ-glutamyl transpeptidase activity is required preferentially to facilitate transport of 2-Br-(diGSyl)HQ into cells as the corresponding cysteine conjugate(s), rather than for catalyzing its conversion to toxic metabolites per se. This interpretation is substantiated by examining the effects of these inhibitors on the *in vitro* generation of reactive metabolites from the isomeric 2-BrHQ-GSH conjugates (Table 4). For example, AT-125 and aminooxyacetic acid inhibited 2-Br-(diGSyl)HQ covalent binding in rat kidney 10,000 × *g* supernatant by only 28 and 17%, respectively. Similarly, the covalent binding of the three monosubstituted GSH conjugates

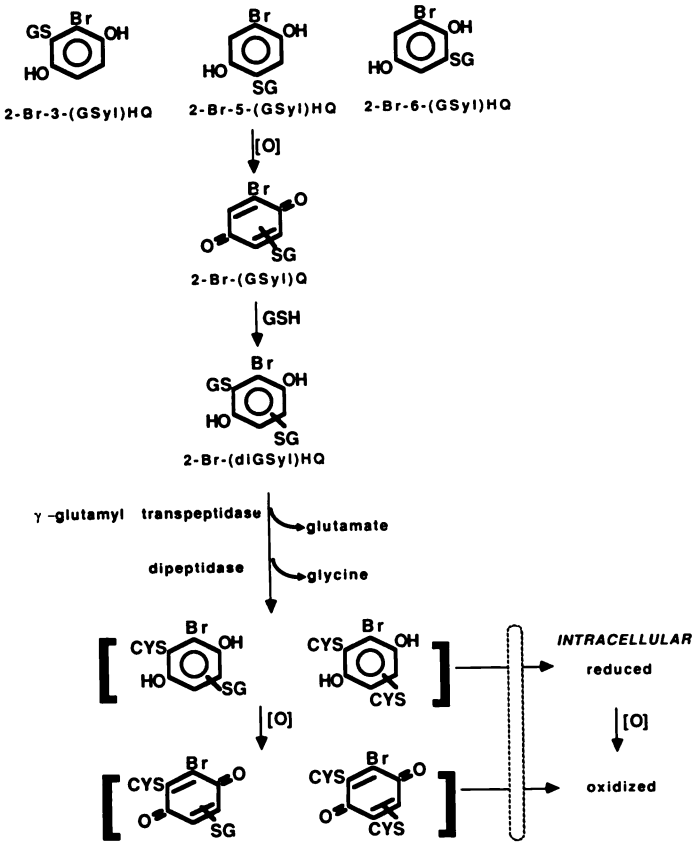

Fig. 6. Metabolism of the isomeric 2-Br-(GSyl)HQ conjugates to their corresponding cysteine adducts followed by their uptake into renal proximal tubular cells.

is inhibited by 10–33% by these agents. These data support the idea that the activities of γ-glutamyl transpeptidase and β-lyase are not required for the generation of the majority of reactive metabolites formed from these conjugates. These data should not be taken as absolute proof for the lack of involvement of reactive thiols in the nephrotoxicity of 2-Br-(diGSyl)HQ for at least two reasons. Firstly, AT-125 does not completely inhibit γ-glutamyl transpeptidase activity either *in vivo* or *in vitro* and the residual activity (~4%) may be sufficient to enable substantial metabolism of the GSH conjugates. Secondly, mechanisms other than β-lyase may contribute to the generation of potentially reactive thiols from cysteine conjugates (51). However, further evidence against the role of reactive thiols in the nephrotoxicity of these quinol-linked GSH conjugates has been obtained. We have shown that the selective nephrotoxicity of 6-bromo-2,5-dihydroxy-thiophenol, a putative metabolite of 2-Br-(GSyl)HQ conjugates, was dependent upon the quinone function (52). 2-, 3- and 4-bromothiophenols, which lack the dihydroxy (hydroquinone) moiety, did not produce any signs of renal damage when administered to rats at doses of 0.2–0.8 mmol/kg (52).

In contrast to the effects of AT-125 and aminooxyacetic acid, ascorbic acid substantially inhibited (62–87%) the covalant binding of the three monosubstituted GSH conjugates (Table 4). Because ascorbate reduces both semiquinones and quinones to quinols (53, 54) it seemed likely that the covalently bound material may have arisen from oxidation of the 2-BrHQ-GSH conjugates. Interestingly 2-Br-(diGSyl)HQ covalent binding is inhibited only 28% by ascorbic acid, which suggests that either 1) this conjugate is less susceptible to oxidation or 2) the quinone derived from this conjugate is more difficult to reduce than the monosubstituted isomers. Indeed, cyclic voltammetry indicates that 2-Br-(diGSyl)HQ is more redox stable than either 2-BrHQ or its three monosubstituted GSH conjugates (Fig. 4). The 2-Br-(monoGSyl)HQ conjugates exhibit redox potentials similar to that of 2-BrHQ (Fig. 4), indicating that the glutathionyl substituent on 2-BrHQ has little effect on the redox properties. A similar observation has been made with menadione and its GSH conjugate, 2-methyl-3-(GSyl)-1,4-naphthoquinone (55), and the addition of a thiol substituent to *tert*-butylhydroquinone actually lowers the oxidation potential (56). In contrast, 2-Br-(diGSyl)HQ exhibits a much higher redox potential than either 2-BrHQ or its 3-monosubstituted GSH conjugates, which may contribute to both its relatively low covalent binding and the lack of effect of ascorbic acid. It should be noted that the oxidation potential of these conjugates is dependent upon pH and that the values obtained by cyclic voltammetry (pH 3.0) will probably differ within the kidney. For example, differences between the pH of tubular fluid and the intracellular pH will determine the balance between the reduced and oxidized forms of the conjugate in each compartment. However, the inverse correlation between the redox potentials of these conjugates and the ability of ascorbic acid to inhibit their covalent binding to kidney homogenate at pH 7.4 (Fig. 5) suggests that the electrochemical properties of these compounds are important determinants of their biological reactivity. Because the initial conjugation of 2-BrHQ with GSH does not result in the formation of a less redox-active species this reaction is not one of detoxification. Rather, it is the conjugation with the second molecule of GSH that results in the formation of a more redox-stable compound. Paradoxically, however, 2-Br-(diGSyl)HQ is a far more potent nephrotoxicant than either of the more redox-active monosubstituted isomers.

Finally, steric factors might also contribute to the more potent nephrotoxicity of 2-Br-(diGSyl)HQ in that the heavily substituted nature of the molecule may preclude its effective reduction, particularly by enzymatic mechanisms but also by antioxidants such as ascorbate, GSH, and NAD(P)H. A combination of physiological, biochemical, and electrochemical factors therefore probably contribute to the potent nephrotoxicity of 2-Br-(diGSyl)HQ.

In conclusion, we have demonstrated that 1) in contrast to other nephrotoxic sulfur conjugates, 2-Br-(diGSyl)HQ is probably not transported into renal tubular cells by a probenecid-sensitive carrier system; 2) the activity of γ-glutamyl transpeptidase is essential for the expression of 2-Br-(diGSyl)HQ nephrotoxicity, probably in order to facilitate uptake into renal cells; 3) oxidation of 2-BrHQ-GSH conjugates appears to be responsible for the generation of the majority of reactive metabolites, although reactive thiol formation cannot be ruled out; and 4) contrary to expectation, the stability of 2-Br-(diGSyl)HQ to oxidation and reduction enhances its toxicity.

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