0090-9556/94/2204-0503$02.00/0
DRUG METABOLISM AND DISPOSITION
Vol. 22, No. 4
Printed in U.S.A.

METABOLISM AND TOXICITY OF 2-BROMO-(DIGLUTATHION-S-YL)-HYDROQUINONE AND 2-BROMO-3-(GLUTATHION-S-YL)HYDROQUINONE IN THE *IN SITU* PERFUSED RAT KIDNEY

MARIA I. RIVERA,[1] LARA M. HINOJOSA, BARBARA A. HILL, SERRINE S. LAU, AND TERRENCE J. MONKS

Division of Pharmacology and Toxicology, College of Pharmacy, The University of Texas at Austin

(Received May 12, 1993; accepted February 17, 1994)

ABSTRACT:

2-Br-(diglutathion-S-yl)hydroquinone (2-Br-(diGSyl)HQ) is a potent nephrotoxicant, causing glucosuria, enzymuria, proteinuria, elevations in blood urea nitrogen, and severe histological alterations to renal proximal tubules at doses of 10–15 μmol/kg. In contrast, 2-Br-3-(glutathion-S-yl)hydroquinone (2-Br-3-(GSyl)HQ) is substantially less nephrotoxic than 2-Br-(diGSyl)HQ and requires a dose of at least 50 μmol/kg to cause modest elevations in blood urea nitrogen concentrations. The reason or reasons for this difference in potency is unclear, but since inhibition of renal γ-glutamyl transpeptidase (γ-GT) prevents 2-Br-(diGSyl)HQ-mediated nephrotoxicity, metabolism of these conjugates by the kidney must play an important role. To address this question we have compared the metabolism and toxicity of 2-Br-(diGSyl)HQ and 2-Br-3-(GSyl)HQ in the *in situ* perfused rat kidney (ISPRK). Following infusion of 20 μmol 2-Br-3-(GSyl)HQ into the right renal artery of male Sprague Dawley rats, a total of 23.5 ± 1.9% (mean ± SE) of the dose was accounted for in urine and bile over a period of 180 min. 2-Bromo-3-(cystein-S-yl)hydroquinone and 2-bromo-3-(*N*-acetylcystein-S-yl)hydroquinone were identified in urine, and unchanged 2-Br-3-(GSyl)HQ was identified in urine and bile. The product arising from the oxidative cyclization of 2-bromo-3-(cystein-S-glycine)hydroquinone, 2*H*-(3-glycine)-7-hydroxy-8-bromo-1,4-benzothiazine, was also identified in urine. In contrast, no known metabolites of 2-Br-(diGSyl)HQ were found in the urine or bile following its infusion (20 μmol) into the ISPRK, and only minor amounts of several unidentified metabolites, in addition to unchanged 2-Br-(diGSyl)HQ, were detected. Toxicity was assessed by determining mitochondrial function and the urinary excretion of γ-GT. Neither conjugate altered mitochondrial respiratory function or the activity of succinate dehydrogenase. The kinetics of γ-GT excretion into urine produced from the perfused and contralateral kidneys differed substantially following perfusion with either 2-Br-3-(GSyl)HQ or 2-Br-(diGSyl)HQ. The susceptibility of individual ISPRK preparations to the toxicity of 2-Br-3-(GSyl)HQ correlated with differences in the overall metabolism of 2-Br-3-(GSyl)HQ in that preparations that excreted more conjugate in the unmetabolized form excreted less γ-GT. Substantial differences in the intrarenal metabolism and disposition of 2-Br-(diGSyl)HQ and 2-Br-3-(GSyl)HQ may contribute to their differences in nephrotoxicity.

Administration of 2-BrHQ[2] (0.83 mmol/kg, ip) to rats causes extensive necrosis of renal proximal tubules (1). Recent evidence supports the hypothesis that the nephrotoxicity induced by 2-BrHQ is due to the formation of 2-Br-(diGSyl)HQ and perhaps, 2-Br-mono-(GSyl)HQ (1–3). Thus, 2-Br-(diGSyl)HQ is about 30 times more toxic than 2-BrHQ and between 5 to 10 fold more toxic (on a molar equivalent basis) than 2-Br-mono-(GSyl)HQ's. Although the reasons for the differences in potency exhibited by 2-Br-(diGSyl)HQ and 2-Br-mono-(GSyl)HQ's are unclear, it appears that a combination of physiological, biochemical, and electrochemical factors contribute to the toxicity of 2-Br-(diGSyl)HQ (3).

γ-GT catalyzes the first step in the metabolism of GSH conjugates to their corresponding mercapturic acids. Inhibition of renal γ-GT with acivicin (AT-125) protected animals against 2-BrHQ– and 2-Br-(diGSyl)HQ–induced nephrotoxicity (2, 3). Furthermore, the *in vivo* covalent binding of 2-Br[^{14}C]HQ to renal macromolecules was significantly inhibited by pretreatment of rats with acivicin (4). These data indicate that metabolism of 2-Br-(diGSyl)HQ by renal γ-GT is essential for the generation of reactive metabolites and for the expression of toxicity. The objectives of this study were therefore to determine whether differences in the renal metabolism and disposition of 2-Br-(diGSyl)HQ and 2-Br-3-(GSyl)HQ contribute to their differential toxicity. As a model for these studies we chose the ISPRK (5) in which the compound of interest is perfused into one kidney and urine collected from both the perfused and contralateral kidney, permitting an estimate of the first pass metabolism and clearance of the infused compound. That fraction of the perfused dose that is not cleared or metabolized by the perfused kidney during its first pass is available to the systemic circulation, and can subsequently be eliminated in the bile following hepatic extraction and metabolism and/or can be delivered in equal amounts to

This work was supported in part by U.S. Public Health Service grant ES 04662 (T.J.M.) and a National Institutes of Health Minority Access to Research Careers Fellowship to M.I.R. (GM 13270). B.A.H. was the recipient of a Pharmaceutical Manufacturers Association Foundation Predoctoral Fellowship. A preliminary account of this work was presented at the annual meeting of the Society of Toxicology, Seattle, February, 1992 (*Toxicologist*, 12, 345, Abstr. No. 1351, 1992).

[1] Present address: Laboratory of Drug Discovery Research and Development, National Cancer Institute, FCRDC, Frederick, Md 21702-1201.

[2] Abbreviations used are: 2-BrHQ, 2-bromohydroquinone; 2-Br-(diGSyl)HQ, 2-bromo-(diglutathion-S-yl)hydroquinone; 2-Br-(GSyl)HQ, 2-bromo-mono-(glutathion-S-yl)hydroquinones; γ-GT, γ-glutamyl transpeptidase; GSH, reduced glutathione; 2-Br-3-(GSyl)HQ, 2-bromo-3-(glutathion-S-yl)hydroquinone; ISPRK, *in situ* perfused rat kidney; 2-Br-(diCYS)HQ, 2-bromo-(dicystein-S-yl)-hydroquinone; 2-Br-(diNAC)HQ, 2-bromo-(diN-acetylcystein-S-yl)hydroquinone; 2-Br-3-(CYS)-HQ, 2-bromo-3-(cystein-S-yl)hydroquinone; 2-Br-3-(NAC)HQ, 2-bromo-3-(*N*-acetylcystein-S-yl)hydroquinone; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); EGTA, ethylene glycol-bis(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid; BSA, bovine serum albumin; RCR, respiratory control ratio.

Send reprint requests to: Dr. Terrence J. Monks, Division of Pharmacology and Toxicology, College of Pharmacy, The University of Texas at Austin, Austin, TX 78712.

both the perfused and contralateral kidney. Results from experiments described in this article suggest that differences in the renal disposition of 2-Br-(diGSyl)HQ and 2-Br-3-(GSyl)HQ may play an important role in their differential nephrotoxicity.

Materials and Methods

Chemicals. 2-Br-(diGSyl)HQ, 2-Br-(diCYS)HQ, 2-Br-(diNAC)HQ, 2-Br-3-(GSyl)HQ, 2-Br-3-(CYS)HQ, 2-Br-3-(NAC)HQ, and 2*H*-(3-glycinyl)-7-hydroxy-8-bromo-1,4-benzothiazine were synthesized in our laboratory and were available from previously published studies (2, 6–8). Mannitol, magnesium sulfate, citric acid, ammonium acetate, EDTA, sucrose, propylene glycol, and potassium phosphate were products of Fisher Scientific (Houston, TX). HEPES, EGTA, BSA, L(−)malic acid, α-ketoglutarate, ADP, chloral hydrate, and sodium pentobarbital were obtained from the Sigma Chemical Co. (St. Louis, MO).

Preparation of Anesthetic (Equithesin). A solution (I) of 6 g chloral hydrate and 2 g $MgSO_4$ was prepared in 72 ml of sterile water. A second solution (II), containing 8 ml ethanol and 1.5 g sodium pentobarbital in 70 ml of propylene glycol, was stirred on low heat for 2 hr and cooled for 20 min. I was slowly added to II, gently stirred for 20 min, and filtered into sterile storage bottles until use.

***In Situ* Kidney Perfusion.** The technique of Davison (5), with modifications to the method of anesthesia and the composition of perfusing solution, was used as follows. Male Sprague Dawley rats (350–400 g; Harlan Sprague Dawley, Houston, TX) were fasted overnight and allowed water *ad libitum* prior to experiments. Animals were anesthetized with equithesin (0.35 ml/100 g, ip) and maintained under anesthesia with a 0.3 ml (im) booster dose every hour. The left jugular vein was cannulated with PE-10 tubing and 7% mannitol in phosphate-buffered saline was perfused through the vein at a flow rate of 0.1 ml/min. The abdomen was sterilized, opened, and the ureters and bile duct cannulated with PE-10 tubing. The right renal artery was cannulated with a 30 gauge hypodermic needle bent to a 90° angle and attached to PE-10 tubing. A drop of cyanoacrylate glue was applied at the point of needle insertion. Mannitol (7% in phosphate-buffered saline) perfusion was started immediately (0.1 ml/min) and the animal permitted to recover for 30 min, at which time continuous and normal urine flow rates were observed. 2-Br-(diGSyl)HQ (20 μmol in 2 ml 7% mannitol) or 2-Br-3-(GSyl)HQ (20 μmol in 2 ml 7% mannitol) were introduced through the renal cannula over a 20-min period at which time mannitol was perfused through the renal cannula for a further 160 min. Urine samples were collected on ice at 30-min intervals. Bile samples were collected at hourly intervals. Control rats were perfused with 7% mannitol in phosphate-buffered saline for 180 min without addition of conjugates.

Analysis of Metabolites. Urine and bile samples were analyzed by HPLC (Shimadzu LC6A, Columbia, MD) coupled to an electrochemical coulometric (EC) detector (ESA Coulochem, Model 5100A, Bedford, MA) (detector 1 = −0.20 V, detector 2 = +0.20 V) and an UV detector (280 nm; Shimadzu SPD-6AV) connected in series. The mobile phase was composed of 4 mM citrate, 8 mM ammonium acetate, 20 mg/liter EDTA, and 5 or 10% methanol, pH 4.0, for analysis of 2-Br-(diGSyl)HQ and 2-Br-3-(GSyl)HQ, respectively. Samples were diluted (1:20, v/v) with the mobile phase, and 20 μl aliquots injected onto an Optisil 5 μ C_{18} reverse phase analytical column (Phenomenex, Torrance, CA) and eluted at a flow rate of 1 ml/min. Urine collected from each animal prior to perfusion with the conjugates was used as the chromatographic control. Under these conditions, authentic 2-Br-(diGSyl)HQ and 2-Br-(diCYS)HQ eluted with retention times of 11.9 ± 0.2 and 27.6 ± 1.8 min, respectively (mean ± SD). 2-Br-(diNAC)HQ was assayed by increasing the methanol content of the mobile phase from 5 to 25% and was eluted at 12.5 ± 0.08 min. 2-Br-3-(CYS)HQ, 2-Br-3-(GSyl)HQ, and 2-Br-3-(NAC)HQ eluted with retention times of 12.6 ± 0.6, 16.3 ± 0.9, and 21.6 ± 1.2 min, respectively. In order to detect the 1,4-benzothiazine metabolite of 2-Br-3-(GSyl)HQ, undiluted urine was injected directly onto the analytical column and eluted with methanol/1% acetic acid in water (10:90 to 100:0 over 60 min) at a flow rate of 1 ml/min. 2*H*-(3-glycinyl)-7-hydroxy-8-bromo-1,4-benzothiazine eluted at 34.1 ± 0.23 min.

Assessment of Renal Mitochondrial Function. *Isolation of Mitochondria.* After completion of each experiment, kidneys were perfused, *via* the aorta, with buffer containing 220 mM mannitol, 70 mM sucrose, 2 mM HEPES, 1.0 mM EGTA and 0.5 mg/ml BSA, pH 7.4 (MSHEA buffer) (9). The kidneys were immediately removed and placed in ice-cold MSHEA buffer. All subsequent steps were carried out at 4°C. Kidneys were cut longitudinally and the papillae removed and discarded. The remainder of the kidney was minced and homogenized in MSHEA buffer (1:10, w/v) with a Potter-Elvehjem homogenizer using 4–5 strokes at approximately 1,200 rpm. Isolated renal mitochondria were prepared according to the method of Weinberg and Humes (10). Functional studies were performed only on freshly isolated mitochondria.

Mitochondrial Respiratory Function. Oxygen consumption was measured polarographically at 25°C using a YSI Model 53 Biological Oxygen Monitor (Yellow Springs, OH). Respiration was measured in a closed 1 ml glass chamber equipped with a 0.025″ platinum/silver electrode (YSI Model 5331 Oxygen Probe) and a magnetic stirring bar. The incubation media contained 220 mM mannitol, 70 mM sucrose, 10 mM potassium phosphate (pH 7.4), 0.5 mg/ml BSA, 1.0 mM EGTA, and the respiratory substrates, malic acid (5.0 mM) and α-ketoglutarate (5.0 mM). Experiments were initiated by adding 0.3–0.8 mg (25–50 μl) mitochondrial suspension to the incubation medium within the chamber. One min after the addition of mitochondria, 10 μl of ADP (25 mM) was added to initiate state 3 respiration. State 3 respiration, state 4 respiration, RCRs, and the ADP/O ratio were measured as determinants of the effects of 2-Br-(diGSyl)HQ and 2-Br-3-(GSyl)HQ on mitochondrial respiratory function. State 4 respiration was measured as the rate of oxygen consumption following ADP depletion. RCR is defined as state 3 respiration/state 4 respiration. The ADP/O ratio represents the ratio of added ADP to the quantity of oxygen consumed and provides an indirect estimate of the amount of ATP synthesized per gram-atom of oxygen consumed. Oxygen consumption calculations were based on oxygen solubility in the buffer at 25°C of 480 natom of oxygen/ml (11). Respiratory rates are reported as natom equivalents of oxygen consumed per mg of protein per min. The yield of mitochondrial protein was unaffected by perfusion of kidneys with either 2-Br-(diGSyl)HQ or 2-Br-3-(GSyl)HQ.

Succinate Dehydrogenase Activity. Succinate dehydrogenase was assayed by the procedure of Sottocasa *et al.* (12), in which enzyme activity is measured by following the reduction of cytochrome c at 550 nm in the presence of succinate. Protein was determined by the method of Lowry *et al.* (13) using BSA as the standard.

Determination of Proximal Tubular Toxicity. Excretion of the brush-border enzyme γ-GT is an early and sensitive indicator of quinone-thioether–mediated nephrotoxicity (6). Therefore, to assess the adverse effects 2-Br-(diGSyl)HQ and 2-Br-3-(GSyl)HQ in the ISPRK, 5 or 10 μl aliquot of each urine sample was assayed for γ-GT activity as described in *Sigma Technical Bulletin 545* γ-GT activity was measured with glutamyl-*p*-nitroaniline as the substrate. One unit of γ-GT is defined as 1 nmol of *p*-nitroaniline formed per min at 25°C.

Statistics. Statistical significance was determined by one-way analysis of variance followed by the Student Newman-Keuls test. For the ISPRK studies, differences in the excretion of γ-GT were analyzed (log data) as a randomized block factorial design. Individual comparisons were analyzed with an applied significance level of $p < 0.05$. Significance between time zero and subsequent time points, and between the perfused and contralateral kidneys at each time point, were determined by Dunn's Multiple Comparison test.

Results

Metabolism of 2-Br-(diGSyl)HQ in the ISPRK. Metabolites of 2-Br-(diGSyl)HQ were found in urine excreted from the infused kidney only within the first 30 min following infusion of the conjugate into the ISPRK (fig. 1). However, none of the electroactive peaks observed by HPLC-EC analysis eluted with retention times corresponding to either 2-Br-(diCYS)HQ or 2-

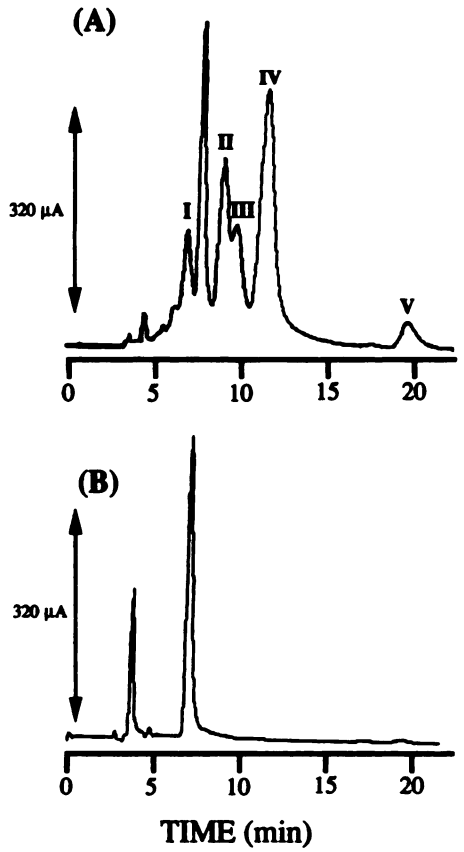

FIG. 1. *HPLC-EC profiles of 0–30 min urine produced from an ISPRK infused with (A) 20 μmol 2-Br(diGSyl)HQ or (B) 7% mannitol (control).*

EC active peaks I, II, III, and V represent metabolites of unknown identity. Peak IV corresponds to the parent compound, 2-Br-(diGSyl)HQ. Chromatographic conditions of the assay are described in *Materials and Methods.*

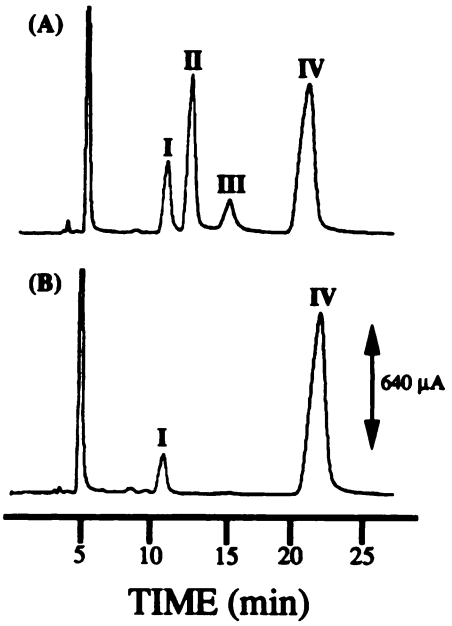

FIG. 2. *HPLC-EC profiles of 0–30 min urine produced from the (A) perfused, and (B) contralateral kidneys of the ISPRK infused with 20 μmol 2-Br-3(GSyl)HQ.*

Peak I represents a metabolite of unknown identity, but may be 2-Br-3-(cystein-*S*-ylglycine)hydroquinone (see text); II, 2-Br-3-(CYS)HQ; III, 2-Br-3-(GSyl)HQ; and IV, 2-Br-3-(NAC)HQ. Chromatographic conditions of the assay are described in *Materials and Methods.*

Br-(diNAC)HQ. The compound that eluted at 7.1 min was present in control urine. The four compounds eluting at 6.5 (I), 9.1 (II), 10.0 (III), and 20 min (V) were only present in urine from 2-Br-(diGSyl)HQ–infused kidneys and therefore probably represent metabolites of unknown structure. Neither 2-Br-(diGSyl)HQ nor any known or unknown metabolites were observed in urine excreted from the contralateral kidney, or from the bile. The amount of 2-Br-(diGSyl)HQ (IV) detected in the urine constituted only 3.12 ± 0.15% of the administered dose (mean ± SE, $N = 4$).

Metabolism of 2-Br-3(GSyl)HQ in the ISPRK: Urinary Metabolites. Under the conditions of the HPLC-EC analysis, four electroactive compounds with retention times of 10.3 (I), 12.4 (II), 14.5 (III), and 20.4 (IV) min were observed in urine excreted from the perfused (right) kidney of rats treated with 20 μmol 2-Br-3-(GSyl)HQ (fig. 2). The component eluting at 5 min was a constituent of control, untreated rat urine. By comparing the retention times and EC properties of the urinary metabolites to those of authentic standards, metabolites II, III, and IV were identified as 2-Br-3-(CYS)HQ, 2-Br-3-(GSyl)HQ, and 2-Br-3-(NAC)HQ, respectively. HPLC-UV analysis of urine confirmed the presence of the cysteine and mercapturate metabolites (I and II, fig. 3) but lacked sufficient sensitivity to detect 2-Br-3-(GSyl)HQ. In addition, the UV absorbing compound that eluted at 33.4 min (V, fig. 3) did not exhibit a response on the electrochemical detector. The retention time and UV spectrum of this compound was identical to that of authentic 2*H*-(3-glycinyl)-7-hydroxy-8-bromo-1,4-benzothiazine (8). Additional minor UV absorbing peaks eluting between 50–55 min (VI) were observed and probably represent polymers formed from the oxidative coupling of the 1,4-benzothiazine (fig. 3). The "unknown" electroactive compound eluting at 10.3 min (I, fig. 2) was unstable, the intensity of the signal slowly decreased upon consecutive reanalysis. This metabolite probably represents 2-Br-3-(cystein-*S*-ylglycine)hydroquinone since concomitant with the decrease in the intensity of this compound, an increase in the amount of the corresponding 1,4-benzothiazine was observed.

Quantitation of metabolites excreted in the urine produced by both the perfused and contralateral kidneys is shown in table 1. The mercapturic acid was identified as the major metabolite of 2-Br-3-(GSyl)HQ excreted in urine, with smaller amounts of 2-Br-3-(CYS)HQ, 2-Br-3-(GSyl)HQ, and the 1,4-benzothiazine excreted principally *via* the perfused kidney. There was no difference in the time course of 2-Br-3-(NAC)HQ excretion into urine produced from either the perfused or contralateral kidneys (fig. 4). Only 2-Br-3-(NAC)HQ was excreted in urine beyond the first 60 min of the perfusion; neither 2-Br-3-(CYS)HQ, 2-Br-3-(GSyl)HQ, nor the 1,4-benzothiazine were observed in urine samples collected between 60–180 min. Between 20–27% of the administered dose of 2-Br-3-(GSyl)HQ was identified as known metabolites. 2-Br-3-(GSyl)HQ was the major compound excreted in bile (I, fig. 5). The metabolites eluting at 19.2 min (II) and 23.2 min (III) did not cochromatograph with any of the authentic standards. The major difference between individual preparations of the ISPRK was in the overall metabolism of 2-Br-3-(GSyl)HQ, as indicated by the fraction of 2-Br-3-(GSyl)HQ excreted unchanged (see below).

Toxicity of 2-Br-(diGSyl)HQ and 2-Br-3-(GSyl)HQ in the ISPRK. Urine was produced by control, mannitol-perfused kid-

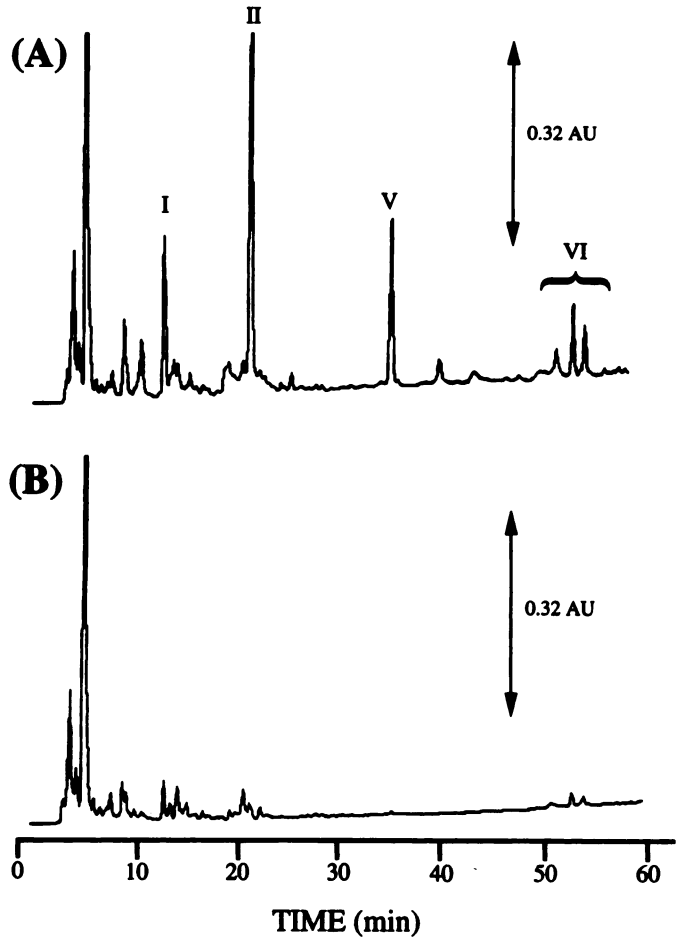

FIG. 3. *HPLC-UV profile of urine produced from an ISPRK infused with (A) 20 μmol 2-Br-3-(GSyl)HQ or (B) 7% mannitol (control).*

Peak I represents 2-Br-3-(CYS)HQ; II, 2-Br-3-(NAC)HQ; V, 2*H*-(3-glycinyl)-7-hydroxy-8-bromo-1,4-benzothiazine; and VI, polymers derived from the oxidative coupling of 2*H*-(3-glycinyl)-7-hydroxy-8-bromo-1,4-benzothiazine. Chromatographic conditions of the assay are described in *Materials and Methods.*

neys at a rate of 57 ± 5 and 46 ± 4 μl/min (mean ± SE, $N = 3$, collection time 180 min) for the perfused and contralateral kidneys, respectively. Urine flow rate decreased slightly to 40 ± 7 and 40 ± 7 μl/min (mean ± SE, $N = 4$) in 2-Br-3-(GSyl)HQ-perfused kidneys and to 45 ± 4 and 29 ± 6 μl/min (mean ± SE, $N = 4$) in 2-Br-(diGSyl)HQ perfused kidneys. We have previously shown that the urinary excretion of the brush-border enzyme γ-GT is a sensitive indicator of 2-Br-(diGSyl)HQ–mediated damage to renal proximal tubular epithelium (6). We therefore monitored the excretion of this enzyme into urine as a marker of proximal tubular injury to the ISPRK. The time course of γ-GT excretion was dependent upon the conjugate infused. Thus, equivalent amounts of γ-GT were excreted into urine produced by both the perfused and contralateral kidneys following infusion of 2-Br-(diGSyl)HQ (fig. 6). In contrast, infusion of 2-Br-3-(GSyl)HQ resulted in significantly greater excretion of γ-GT into urine produced from the right (perfused) kidney (fig. 6). In addition, the total amount of γ-GT excreted by both kidneys was greater in response to 2-Br-(diGSyl)HQ than to 2-Br-3-(GSyl)HQ, indicating that the former was more toxic to the ISPRK than the latter. Finally, there was an inverse correlation ($y = 40.7 - 4.4\,\epsilon^{-2}$, $r^2 = 1.0$) between the total amount of 2-Br-3-(GSyl)HQ excreted "unchanged" by the ISPRK and the total amount of γ-GT excreted in urine (fig. 7).

Effects of 2-Br-(diGSyl)HQ and 2-Br-3-(GSyl)HQ on Mitochondrial Respiratory Function in the ISPRK. Neither 2-Br-(diGSyl)HQ nor 2-Br-3-(GSyl)HQ had any adverse effects on renal mitochondrial respiratory function during the time course of these experiments. State 3 and state 4 respiration, RCRs, and ADP/O ratios were all unaffected in mitochondria isolated from the ISPRKs (table 2). Succinate dehydrogenase activity determined in mitochondria isolated from both the perfused and contralateral kidneys remained at control values (table 2).

Discussion

2-Br-(diGSyl)HQ is an extremely potent renal proximal tubular toxicant, whereas 2-Br-mono-(GSyl)HQ's are significantly less toxic (2, 3). The reason or reasons for this difference in potency are unclear. In the present study we utilized the ISPRK model to determine whether differences in the renal metabolism and disposition of 2-Br-(diGSyl)HQ and 2-Br-3-(GSyl)HQ contribute to their differential nephrotoxicity. Following infusion of 20 μmol 2-Br-3-(GSyl)HQ into the ISPRK, 2-Br-3-(CYS)HQ, 2-Br-3-(NAC)HQ, and 2*H*-(3-glycinyl)-7-hydroxy-8-bromo-1,4-benzothiazine were all detected within 30 min as urinary metabolites, along with unchanged 2-Br-3-(GSyl)HQ. Beyond 60 min, only the mercapturate could be identified in urine. 1,4-Benzothiazine formation represents a novel pathway of quinone-thioether metabolism that diverges from the classical route of mercapturic acid biosynthesis following the conjugation of xenobiotics with GSH (8). The identification of 2*H*-(3-glycinyl)-7-hydroxy-8-bromo-1,4-benzothiazine in urine produced from kidneys perfused with 2-Br-3-(GSyl)HQ provides the first evidence for the occurrence of this pathway *in vivo*. It appears that removal of

TABLE 1

Quantitation of 2-Br-3-(GSyl)HQ and its metabolites excreted in urine produced by the perfused and contralateral kidneys and 2-Br-3-(GSyl)HQ in bile

Figures are expressed in nmol and represent the mean ± SE of four perfusions. Metabolites and unchanged 2-Br-3-(GSyl)HQ were quantified in urine collected at 30 min intervals for 180 min. Since no material was excreted in the final 60 min the data represent the amount of 2-Br-3-(GSyl)HQ and its metabolites excreted within 120 min.

2-Br-3-(GSyl)HQ and Metabolites	Perfused	Contralateral	Bile	Dose
				%
2-Br-3-(CYS)HQ	484 ± 46	4 ± 4	0	2.4 ± 0.3
2-Br-3-(NAC)HQ	1711 ± 162	1948 ± 354	0	18.3 ± 2.5
2*H*-(3-glycinyl)-7-hydroxy-8-bromo-1,4-benzothiazine	147 ± 22	7 ± 3	0	0.8 ± 0.1
2-Br-3-(GSyl)HQ	131 ± 52	19 ± 8	243 ± 88	2.0 ± 0.3
Total				23.5 ± 1.9

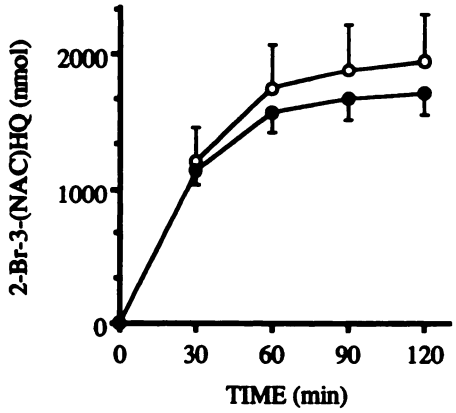

FIG. 4. *Time course of 2-Br-3-(NAC)HQ excretion in urine produced from the perfused (●) and contralateral (○) kidneys following infusion of 20 μmol 2-Br-3-(GSyl)HQ in the ISPRK.*

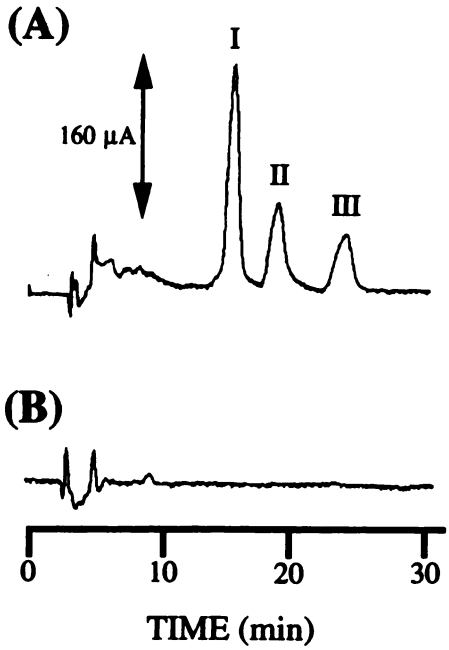

FIG. 5. *HPLC-EC profiles of bile (0–30 min) produced from an ISPRK infused with (A) 20 μmol 2-Br-3-(GSyl)HQ or (B) 7% mannitol (control).*

Peak I represents 2-Br-3-(GSyl)HQ, and *peaks* II and III represent metabolites of unknown identity. Chromatographic conditions of the assay are described in *Materials and Methods.*

the reactive quinone function *via* intramolecular cyclization constitutes a detoxication reaction (14). Thus, the corresponding homocysteine conjugates of 2-BrHO, which contain an additional methylene group to effectively prevent cyclization, remain nephrotoxic (14). In support of this view, only the *N*-acetylcysteine conjugate of menadione and not the GSH conjugate was cytotoxic when incubated with rat kidney cortical epithelial cells (15). The differences between the mercapturate and GSH conjugate of menadione are probably due to the ability of the former to undergo cyclization and 1,4-benzothiazine formation following metabolism by γ-GT (15). Since this reaction eliminates the reactive quinone function from the molecule it prevents redox cycling of the conjugate. In contrast, the presence of the *N*-acetyl group in the mercapturic acid conjugate prevents condensation of the cysteinyl amino group with the quinone carbonyl group,

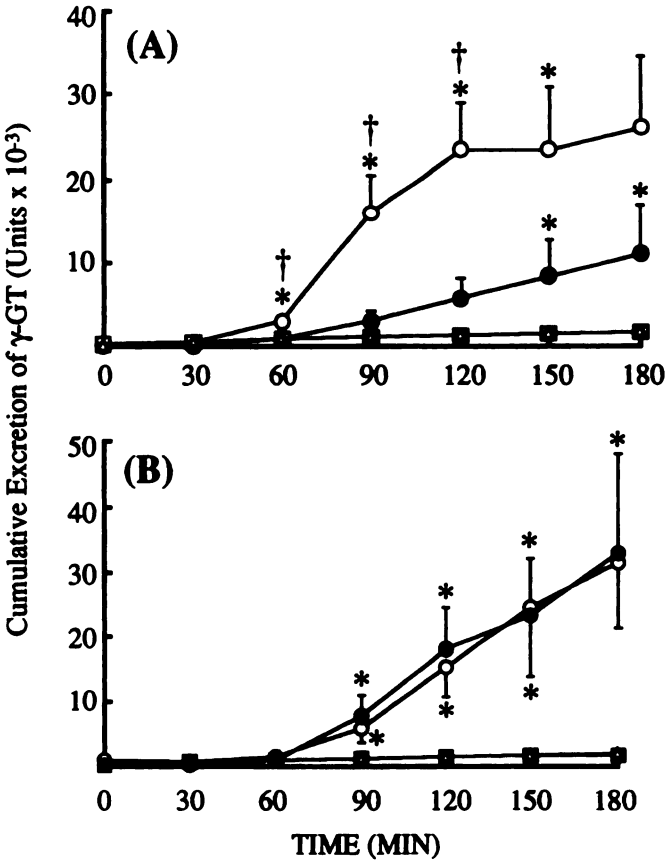

FIG. 6. *Time course of the urinary excretion of γ-GT following infusion of the ISPRK with 20 μmol of either (A) 2-Br-3-(GSyl)HQ or (B) 2-Br-(diGSyl)HQ*

γ-GT activity was determined in urine produced from both the perfused (○) and contralateral (●) kidneys. γ-GT excretion from control (7% mannitol perfused) kidneys is also presented for comparison (△, □). *Dagger* indicates rate of γ-GT excretion during 30-min collection period significantly different ($p < 0.05$) from contralateral kidney. *Asterisk* indicates rate of γ-GT excretion during 30-min collection period significantly different ($p < 0.05$) from mannitol-perfused control kidneys.

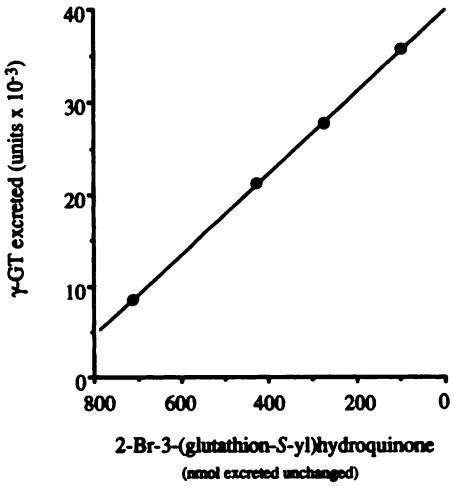

FIG. 7. *Correlation between the amount of 2-Br-3-(GSyl)HQ excreted unchanged by each preparation of the ISPRK, and toxicity as assessed by the excretion of γ-GT by the directly perfused kidney.*

TABLE 2

Effects of 2-Br-(diGSyl)HQ (20 μmol) and 2-Br-3-(GSyl)HQ (20 μmol) on mitochondrial respiratory and function and succinate dehydrogenase activity

All values represent the mean ± SE (N = 3). No statistically significant difference between mitochondria isolated from control and perfused or contralateral kidneys were observed.

	RCR	State 3 Respiration[a]	State 4 Respiration	ADP/O Ratio	SDH[b]
2-Br-(diGSyl)HQ					
Control kidney	4.83 ± 0.6	90.4 ± 4.84	19.2 ± 1.52	2.97 ± 0.05	167 ± 20
Perfused kidney	4.96 ± 1.18	86.7 ± 0.58	19.2 ± 3.58	3.03 ± 0.10	175 ± 10
Contralateral kidney	4.86 ± 0.99	95.1 ± 2.49	20.9 ± 3.10	3.10 ± 0.09	188 ± 34
2-Br-3-(GSyl)HQ					
Control kidney	5.11 ± 0.12	78.0 ± 10.0	15.3 ± 1.8	3.23 ± 0.05	129 ± 7
Perfused kidney	4.83 ± 0.50	67.3 ± 11.6	13.7 ± 1.3	3.17 ± 0.07	102 ± 7
Contralateral kidney	5.09 ± 0.59	77.0 ± 6.35	16.0 ± 3.1	3.20 ± 0.09	114 ± 8

[a] State 3 and state 4 respiration are expressed as natom equivalents of oxygen/mg protein/min.

[b] Succinate dehydrogenase (SDH) activity is expressed as nmol cytochrome c reduced/min/mg protein.

and this conjugate therefore retains the ability to redox cycle with the concomitant generation of reactive oxygen species (15).

2-Br-3-(NAC)HQ was the major metabolite excreted in urine. Approximately equal amounts were excreted by both the perfused and contralateral kidneys (table 1). In contrast, 2-Br-3-(GSyl)HQ, 2-Br-3-(CYS)HQ, and the 1,4-benzothiazine were excreted primarily by the perfused kidney. These data suggest that most of the 2-Br-3-(CYS)HQ and 1,4-benzothiazine present in urine produced from the directly perfused kidney arise *via* first pass metabolism of 2-Br-3-(GSyl)HQ. In contrast, 2-Br-3-(NAC)HQ is formed predominantly from systemic metabolism. Indeed, 2-Br-3-(GSyl)HQ was detected in bile (fig. 3) indicating that 2-Br-3-(GSyl)HQ, which escapes first pass metabolism and clearance by the perfused kidney, may be metabolized to 2-Br-3-(NAC)HQ in extrarenal tissues (liver, intestines, etc.) and eventually delivered by the blood to both kidneys. Only small quantities of 2-Br-3-(GSyl)HQ appear to reach the contralateral (nonperfused) kidney, since only minor amounts of metabolites are detected in urine produced from this kidney (table 1).

The identification of 2-Br-3-(GSyl)HQ in bile deserves further comment. The ability of the liver to conjugate GSH to electrophiles is well established (16, 17). However, it is not clear whether the liver is capable of extracting intact GSH conjugates from the blood. The presence of 2-Br-3-(GSyl)HQ in the bile indicates that the liver can certainly extract GSH conjugates from the blood. Additional evidence for this was provided by Davison *et al.* (18) who recovered about 8% of the dose of propachlor-GSH in the bile after portal administration of the conjugate to rats. Koob and Dekant (19) have also reported the excretion of 1-(glutathion-*S*-yl)-pentachlorobutadiene in bile following its perfusion into the isolated rat liver.

The metabolism and disposition of 2-Br-(diGSyl)HQ was substantially different from that of 2-Br-3-(GSyl)HQ since neither 2-Br-(diCYS)HQ nor 2-Br-(diNAC)HQ were found in the urine or bile following infusion of 2-Br-(diGSyl)HQ into the ISPRK. Minor amounts of several unidentified metabolites and some unchanged 2-Br-(diGSyl)HQ were found in urine. It is likely that the unknown metabolites represent mixed thioethers of 2-Br-(diGSyl)HQ in which one of the GSH moieties undergoes selective metabolism in preference to the second GSH moiety. However, since we do not possess authentic samples of such potential mixed thioethers, the identity of these unknown metabolites remains to be determined. Only 3.1 ± 0.2% and 2.0 ± 0.3% of the administered dose of 2-Br-(diGSyl)HQ and 2-Br-3-(GSyl)HQ respectively, was recovered unchanged, indicating that nearly all of the administered dose of both conjugates was metabolized. These data are consistent with the kinetics of the γ-GT–mediated metabolism of 2-Br-3-(GSyl)HQ and 2-Br-(diGSyl)HQ. Thus, each conjugate was infused into the kidney over a 20-min period. This results in the delivery of 2-Br-3-(GSyl)HQ and 2-Br-(diGSyl)HQ to the kidney at a rate of 1 μmol/min. The V_{max} for the γ-GT-catalyzed metabolism of 2-Br-3-(GSyl)HQ and 2-Br-(diGSyl)HQ are 14 and 9 pmol/min/unit respectively for the hydrolysis reaction, and 277 and 11 pmol/min/unit respectively for the transpeptidation reaction (7). Since each kidney contains roughly 4×10^5 units γ-GT, the perfused kidney has the capacity to metabolize between 5.6–110 μmol/min of 2-Br-3-(GSyl)HQ, and 3.6–4.4 μmol/min of 2-Br-(diGSyl)HQ. The lower value for both these estimates is several fold higher than the infused dose. Although nearly all of the conjugates are metabolized by the ISPRK, only 21.5% of the dose of 2-Br-3-(GSyl)HQ was recovered as known metabolites (table 1), whereas no known mercapturic acid metabolites of 2-Br-(diGSyl)HQ were identified. Moreover, since unknown metabolites of 2-Br-(diGSyl)HQ in urine could only be observed following HPLC-EC analysis, which is approximately an order of magnitude more sensitive than HPLC-UV analysis, this suggests that the fraction of the dose of 2-Br-(diGSyl)HQ that is metabolized and retained by the kidney is higher than the fraction of 2-Br-3-(GSyl)HQ retained by the kidney. Studies with radiolabeled material will be required to identify metabolites retained by the kidney. However, our findings with 2-Br-3-(GSyl)HQ and 2-Br-(diGSyl)HQ are consistent with those reported by Elce (20). Following the administration of radiolabeled 2-hydroxy-1-(glutathion-*S*-yl)-estradiol to rats, only 15% of the dose was recovered in urine and 5% in the feces after several days (20).

The urinary excretion of the brush-border (luminal) enzyme γ-GT is a sensitive indicator of quinone-thioether–mediated nephrotoxicity (6). The kinetics of γ-GT excretion into the urine differed substantially in response to 2-Br-(diGSyl)HQ and 2-Br-3-(GSyl)HQ (fig. 6). Following perfusion with 2-Br-3-(GSyl)HQ, γ-GT excretion was higher in urine produced from the perfused (right) than the contralateral (left) kidney. This was not unexpected since 2-Br-3-(GSyl)HQ was infused directly through the right renal artery, and the effective dose delivered to the contralateral kidney would therefore be lower than the dose delivered to the perfused kidney. Preparations of the ISPRK, that metabolized higher amounts of 2-Br-3-(GSyl)HQ (as determined by

the amount of the conjugate excreted intact) exhibited greater toxicity (fig. 7). In contrast to 2-Br-3-(GSyl)HQ, perfusion with 2-Br-(diGSyl)HQ resulted in similar amounts of γ-GT excretion by both the perfused and contralateral kidney (fig. 6). This suggests that either sufficient amounts of 2-Br-(diGSyl)HQ escape metabolism and excretion by the perfused kidney to produce toxicity in the contralateral kidney, or that metabolites of 2-Br-(diGSyl)HQ are delivered via the systemic circulation to the contralateral kidney in sufficient quantities to induce toxicity. Consistent with this finding, metabolism of 2-Br-3-(GSyl)HQ in the ISPRK appears rapid. As noted above, only the mercapturate is excreted in urine beyond 60 min of the initiation of perfusion and the limiting factor for the elimination of the mercapturate is likely to be its affinity for the organic anion transporter. Moreover, based upon the kinetics of the γ-GT–mediated metabolism of the conjugates (see above) less 2-Br-3-(GSyl)HQ should escape passage through the perfused kidney and subsequent delivery to the contralateral kidney than would equimolar concentrations of 2-Br-(diGSyl)HQ. No metabolites of 2-Br-(diGSyl)HQ were detected in either the bile or the urine excreted from the contralateral kidney, and only 3% of the dose was found in the urine excreted by the perfused kidney. Therefore, most of the dose of 2-Br-(diGSyl)HQ is retained in the animal. This is consistent with the finding that 2-Br-(diCYS)HQ has a lower oxidation potential than 2-Br-3-(CYS)HQ and is therefore more likely to be oxidized to a reactive quinone than 2-Br-3-(CYS)HQ (7). The amount of 2-Br-(diGSyl)HQ and/or its reactive metabolite or metabolites delivered to the systemic circulation is unknown but appears sufficient to cause toxicity to the nonperfused kidney.

The relatively rapid excretion of γ-GT into urine after perfusion with either 2-Br-(diGSyl)HQ or 2-Br-3-(GSyl)HQ suggests that disruption of the brush-border membrane might be important in the development of toxicity. In contrast, mitochondria have been identified as a primary target for nephrotoxic aliphatic GSH and cysteine conjugates (21–23) and mitochondrial function was inhibited in *in vitro* incubations of 2-BrHQ with rabbit renal proximal tubule suspensions (24–26). We therefore measured the effect of 2-Br-(diGSyl)HQ and 2-Br-3-(GSyl)HQ on mitochondria isolated after completion of the perfusions. Neither 2-Br-(diGSyl)HQ or 2-Br-3-(GSyl)HQ had any adverse effect on renal mitochondrial respiratory function or succinate dehydrogenase activity (table 2). These results are consistent with those obtained *in vivo*. Mitochondria isolated after administration of nephrotoxic doses of 2-Br-(diGSyl)HQ to rats maintain respiratory function despite significant elevations in blood urea nitrogen concentrations (27). In contrast, the urinary excretion of γ-GT is a rapid event following 2-Br-(di-GSyl)HQ administration (27).

In summary, we have found differences in both the metabolism and disposition of 2-Br-(diGSyl)HQ and 2-Br-3-(GSyl)HQ in the ISPRK. Such differences may contribute to the differential toxicity of these conjugates observed both *in vivo* and in the ISPRK reported herein. The toxicity of 2-Br-3-(GSyl)HQ to each preparation of the ISPRK correlated inversely with the amount of parent compound recovered in the urine and bile. The identification of 2*H*-(3-glycinyl)-7-hydroxy-8-bromo-1,4-benzothiazine in the urine of kidneys perfused with 2-Br-3-(GSyl)HQ also provides the first evidence for the occurrence of this metabolic pathway *in vivo*.

References

1. S. S. Lau, T. J. Monks, and J. R. Gillette: Identification of 2-bromohydroquinone as a metabolite of bromobenzene and *o*-bromophenol: Implications for bromobenzene-induced nephrotoxicity. *J. Pharmacol. Exp. Ther.* **230**, 369–366 (1984).
2. T. J. Monks, S. S. Lau, R. J. Highet, and J. R. Gillette: Glutathione conjugates of 2-bromohydroquinone are nephrotoxic. *Drug Metab. Dispos.* **13**, 553–559 (1985).
3. T. J. Monks, R. J. Highet, and S. S. Lau: 2-Bromo-(diglutathion-*S*-yl)hydroquinone nephrotoxicity: physiological, biochemical, and electrochemical determinants. *Mol. Pharmacol.* **34**, 492–500 (1988).
4. S. S. Lau and T. J. Monks: The *in vivo* disposition of 2-bromo-[^{14}C]-hydroquinone and the effect of γ-glutamyl transpeptidase inhibition. *Toxicol. Appl. Pharmacol.* **103**, 121–132 (1990).
5. K. L. Davison: *In situ* perfusion and collection techniques for studying xenobiotic metabolism in animals. In "Intermediary Xenobiotic Metabolism in Animals; Methodology, Mechanism and Significance" (D.H. Hutson, J. Caldwell, and G. D. Paulson, eds.), pp. 315–333. Taylor & Francis, London, 1989.
6. S. S. Lau, T. W. Jones, R. Sioco, B. A. Hill, R. K. Pinon, and T. J. Monks: Species differences in renal γ-glutamyl transpeptidase activity do not correlate with susceptibility to 2-bromo-(diglutathion-*S*-yl)hydroquinone nephrotoxicity. *Toxicology* **64**, 291–311 (1990).
7. T. J. Monks, H.-H. Lo and S. S. Lau: Oxidation and acetylation as determinants of 2-bromo-(cystein-*S*-yl)hydroquinone mediated nephrotoxicity. *Chem. Res. Toxicol.*, in press.
8. T. J. Monks, R. J. Highet, and S. S. Lau: Oxidative cyclization, 1,4-benzothiazine formation and dimerization of 2-bromo-3-(glutathion-*S*-yl)hydroquinone. *Mol. Pharmacol.* **38**, 121–127 (1990).
9. P. J. Hayden and J. L. Stevens: Cysteine conjugate toxicity, metabolism and binding to macromolecules in isolated rat kidney mitochondria. *Mol. Pharmacol.* **37**, 468–476 (1990).
10. J. M. Weinberg and H. D. Hume: Calcium transport and inner mitochondrial damage in renal cortical mitochondria. *Amer. J. Physiol.* **248**, F876-F889 (1985).
11. P. L. Pedersen, J. W. Greenawalt, B. Reynatarje, J. Hullihen, G. L. Decker, J. W. Soper, and E. Bustamente: Preparation and characterization of mitochondria and submitochondrial particles of rat liver and liver-derived tissues. In "Methods of Cell Biology" (D. M. Prescott, ed.), pp. 411–481. Academic Press, New York, 1978.
12. G. L. Sottocasa, B. Kulensterna, L. Ernster, and A. Bergstrand: An electron-transport system associated with the outer membrane of liver mitochondria. A biochemical and morphological study. *J. Cell Biol.* **32**, 415–438 (1967).
13. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall: Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265–275 (1951).
14. S. S. Lau and T. J. Monks: Glutathione conjugation as a mechanism of targeting latent quinones to the kidney. In "Biological Reactive Intermediates IV: Molecular and Cellular Effects and their Impact on Human Health" (C. M. Witmer, R. R. Snyder, D. J. Jollow, G. F. Kalf, J. J. Kocsis, and I. G. Sipes, eds.), pp. 457–464. Plenum Press, New York, 1991.
15. P. C. Brown, D. M. Dulik, and T. W. Jones: The toxicity of menadione (2-methyl-1,4-naphthoquinone) and two thioether conjugates studied with isolated renal epithelial cells. *Arch. Biochem. Biophys.* **285**, 187–196 (1991).
16. H. Sies and B. Ketterer (eds.): "Glutathione Conjugation Mechanisms and Biological Significance." Academic Press, New York, 1988.
17. D. J. Reed: Cellular defense mechanisms against reactive metabolites. In "Bioactivation of Foreign Compounds" (M. W. Anders, ed.), pp. 71–108. Academic Press, New York, 1985.
18. K. L. Davison, J. E. Bakke, and G. L. Larsen: Metabolism of the glutathione conjugate of propachlor by *in situ* perfused kidneys and livers of rats. *Xenobiotica* **20**, 375–383 (1990).
19. M. Koob and W. Dekant: Biotransformation of the hexachlorobutadiene metabolites 1-(glutathion-*S*-yl)-pentachlorobutadiene and

1-(cystein-*S*-yl)-pentachlorobutadiene in the isolated perfused rat liver. *Xenobiotica* **22**, 125–138 (1992).
20. J. S. Elce: Metabolism of a glutathione conjugate of 2-hydroxyoestradiol-17β in the adult male rat. *Biochem. J.* **126**, 1067–1071 (1972).
21. P. J. Hayden and J. L. Stevens: Cysteine conjugate toxicity, metabolism and binding to macromolecules in isolated rat kidney mitochondria. *Mol. Pharmacol.* **37**, 468–476 (1990).
22. L. H. Lash and M. W. Anders: Mechanism of *S*-(1,2-dichlorovinyl)-L-cysteine and *S*-(1,2-dichlorovinyl)-L-homocysteine–induced renal mitochondrial toxicity. *Mol. Pharmacol.* **32**, 549–556 (1987).
23. A. Wallin, T. W. Jones, A. E. Vercesi, I. Cotgreave, K. Ormstad, and S. S. Orrenius: Toxicity of *S*-pentachlorobutadienyl-L-cysteine studied with isolated rat renal cortical mitochondria. *Arch. Biochem. Biophys.* **258**, 365–372 (1987).
24. R. G. Schnellman and L. J. Mandel: Cellular toxicity of bromobenzene and bromobenzene metabolites to rabbit proximal tubules: the role and mechanism of 2-bromohydroquinone. *J. Pharmacol. Exp. Ther.* **237**, 456–461 (1986).
25. R. G. Schnellman, F. P. Q. Ewell, M. Sgambati, and L. J. Mandel: Mitochondrial toxicity of 2-bromohydroquinone in rabbit proximal tubules. *Toxicol. Appl. Pharmacol.* **90**, 420–426 (1987).
26. R. G. Schnellman, T. J. Monks, L. J. Mandel, and S. S. Lau: 2-Bromohydroquinone–induced toxicity to rabbit proximal tubules: the role of biotransformation, glutathione, and covalent binding. *Toxicol. Appl. Pharmacol.* **99**, 19–27 (1989).
27. M. I. Rivera, T. W. Jones, S. S. Lau, and T. J. Monks: Early morphological and biochemical changes during 2-br-(di-glutathion-*S*-yl)hydroquinone–induced nephrotoxicity. *Toxicol. Appl. Pharmacol.*, in press.