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FORMATION OF PHENOL AND THIOCATECHOL METABOLITES FROM BROMOBENZENE PREMERCAPTURIC ACIDS THROUGH PYRIDOXAL PHOSPHATE-DEPENDENT *C-S* LYASE ACTIVITY

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Abstract—When *N*-acetyl-*S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine (4-*S*-premercapturic acid) and *N*-acetyl-*S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine (3-*S*-premercapturic acid) were used as substrates in incubations with Hartley guinea pig kidney 9000 *g* supernatant preparations, the major products were the corresponding *S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine and *S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine. At the end of the incubation period, the percentage recovery of these *N*-deacetylate cysteine conjugates accounted for 77 ± 2% of the substrates, 3-*S*- and 4-*S*-premercapturic acids. Removal of the *N*-acetyl group from premercapturic acids to form the corresponding cysteine conjugates by kidney *N*-deacetylase(s) showed no preference with respect to the 3-*S*- and 4-*S*-positional isomeric conjugates. Other metabolites which included the known sulfur-containing acids, mercaptolactate and mercaptoacetate, were also detected. 3- and 4-Bromophenol and 3- and 4-bromothioanisole were also formed. The addition of pyridoxal-5′-phosphate to the kidney incubation mixture resulted in a 5-fold increase in the formation of phenols and thioanisoles, along with four different isomeric O- and S-methylated 3-*S*- and 4-*S*-bromothiocatechols and two *S*-methylated 3-*S*- and 4-*S*-bromodihydrobenzene thiolols. This result indicated that a pyridoxal phosphate-dependent *C-S* lyase(s) is involved in the formation of both phenol and thiophenolic metabolites from *S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine and *S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine. Guinea pig liver 9000 *g* supernatant preparations did not N-deacetylate the 3-*S*- and 4-*S*-premercapturic acids to the same extent as kidney preparations, and this may account for decreased conversion of 3-*S*- and 4-*S*-premercapturic acids to 3- and 4-bromophenol and to thiophenolic products by liver preparations.

As reported previously [1], both the Hartley guinea pig and the Sprague–Dawley rat metabolize *N*-acetyl-*S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine (4-*S*-premercapturic acid) (**A** in Scheme 1) and *N*-acetyl-S-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine (3-*S*-premercapturic acids) (3-*S*-isomer of **A** in Scheme 1) to 3- and 4-bromophenol. The extent of conversion, however, was found to be much greater in the guinea pig than in the rat. Besides 3- and 4-bromophenol, a series of thiophenolic products, which included S- and O-methylated 3-*S*- and 4-*S*-bromothiocatechols (**F**, **G** in Scheme 1, and their 3-*S*-isomers), and also *S*-methylated 3-*S*- and 4-*S*-bromodihydrobenzene thiolols (**D** in Scheme 1, and its 3-*S*-isomer), were found as guinea pig metabolites of 3-*S*- and 4-*S*-premercapturic acids. These arene oxide-derived thiophenolic products were found earlier as urinary metabolites of bromobenzene in the guinea pig [2], but they were not detected as urinary metabolites of bromobenzene [2] and of 3-*S*- and 4-*S*-premercapturic acids [1] in the Sprague–Dawley rat. In a recent investigation, they were also found as urinary metabolites of bromobenzene in the golden Syrian hamster.† 3-*S*- and 4-*S*-Bromothiocatechols are now known to be involved in bromobenzene-induced liver and kidney necrosis in the Syrian hamster, and their toxic effects can be prevented by an increase in O- and S-methylation processes. The formation of phenols from 3-*S*- and 4-*S*-premercapturic acids suggested that a *C-S* cleavage/aromatization must be involved and the most likely key intermediates in this mammalian metabolic pathway are the *S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine (**B** in Scheme 1) and *S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine (3-*S*-isomer of B in Scheme 1). A *C-S* cleavage at the β-carbon of the cysteinyl group of a number of aromatic and aliphatic cysteine conjugates leading to thiols is well known [3–13], but cleavage by *C-S* lyase(s) of a cysteine conjugate with a dihydrohydroxybenzene structure (as **B** in Scheme 1) to form phenol and thiophenolic conjugates is new.

The major objective of this study was to investigate the route of formation of phenol and thiocatechol metabolites of bromobenzene from 3-*S*- and 4-*S*-premercapturic acids, and an *in vitro* investigation was carried out to study the steps that are involved in this

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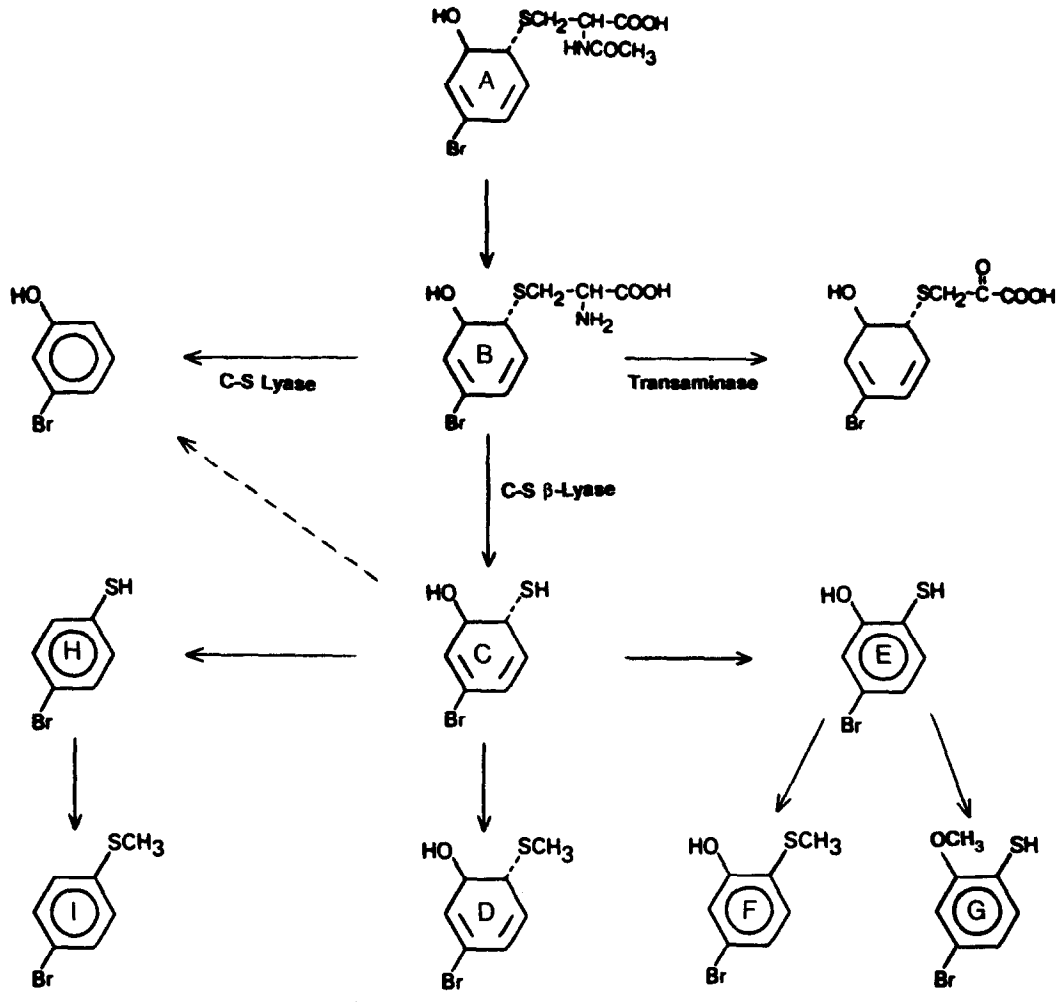

Scheme 1. Proposed metabolic pathways leading from *N*-acetyl-*S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine (4-*S*-premercapturic acid) to 3-bromophenol and to 4-*S*-thiol-containing metabolites. These pathways are for 4-*S*-compounds; 3-*S*-positional isomers show analogous relationships. In this metabolic scheme, deacetylation of 4-*S*-premercapturic acid (**A**) leads to the formation of *S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine (**B**). This non-aromatic cysteine conjugate is substrate for lyase(s) and transaminase(s). (**A**) *N*-acetyl-*S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine (4-*S*-premercapturic acid); (**B**) *S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine; (**C**) 2-hydroxy-4-bromo-1,2-dihydrobenzenethiol (4-*S*-bromodihydrobenzene thiolol); (**D**) (*S*-methyl)-2-hydroxy-4-bromo-1,2-dihydrobenzenethiol (S-methylated 4-*S*-bromodihydrobenzene thiolol); (**E**) 2-hydroxy-4-bromobenzenethiol (4-*S*-bromothiocatechol); (**F**) (*S*-methyl)-2-hydroxy-4-bromobenzenethiol (S-methylated 4-*S*-bromothiocatechol); (**G**) 2-methoxy-4-bromobenzenethiol (O-methylated 4-*S*-bromothiocatechol); (**H**) 4-bromobenzenethiol (4-bromothiophenol); and (**I**) (*S*-methyl)-4-bromobenzenethiol (4-bromothioanisole).

mammalian metabolic pathway in the guinea pig. In initial studies, the metabolism of 3-*S*- and 4-*S*-premercapturic acids was studied with both kidney and liver 9000 *g* supernatant preparations. It was found, however, that N-deacetylation of 3-*S*- and 4-*S*-premercapturic acids occurred much more extensively in guinea pig kidney preparations than in liver preparations, and that the formation of 3- and 4-bromophenol was greater in kidney preparations than in liver preparations. Results from studies with liver preparations are included for comparison with results from studies with kidney preparations. Parallel experiments with kidney and liver 9000 *g* supernatant preparations were also carried out with *N* - acetyl - *S* - (4 - bromophenyl - L - cysteine (4-*S*-mercapturic acid). Results with the aromatic 4-*S*-mercapturic acid are used to compare with results observed with non-aromatic 3-*S*- and 4-*S*-premercapturic acids.

MATERIALS AND METHODS

Materials

Reference compounds, radioactive compounds, reagents and other materials were obtained from the following sources: uniformly labeled [^{14}C]-bromobenzene, 27 mCi/mmol (ICN Pharmaceuticals, Inc., Irvine, CA); bromobenzene, 4-bromophenol, aminooxyacetic acid (hemihydrochloride)

(AOAA*) pyridoxal-5′-phosphate, potassium chloride, magnesium chloride, Trizma-hydrochloride, bovine serum albumin, NADPH (tetrasodium salt), and NADH (disodium salt) (Sigma Chemical Co., St. Louis, MO); 3-bromophenol, 4-bromothioanisole, 3- and 4-bromothiophenol, and Diazald (Aldrich Chemical Co., Milwaukee, WI); bis(trimethylsilyl)acetamide (BSA) and *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (Pierce Chemical Co., Rockford, IL); and Aquasol (Dupont NEN Products, Boston, MA).

Animals

Young adult male Hartley guinea pigs (450–500 g) were obtained from Harlan Sprague–Dawley, Inc. (Indianapolis, IN). Animals were allowed food (Purina Lab Chow) and water *ad lib.* prior to experiments.

Biosynthesis and isolation of ^{14}C-*labeled* N-*acetyl*-S-(2-*hydroxy*-4-*bromocyclohexa*-3,5-*dienyl*)-L-*cysteine* (4-S-*premercapturic acid*) *and* N-*acetyl*-S-(2-*hydroxy*-5-*bromocyclohexa*-3,5-*dienyl*-L-*cysteine* (3-S-*premercapturic acid*)

These positional isomeric 3-*S*- and 4-*S*-premercapturic acids were biosynthesized by the rat after i.p. administration of a corn oil:ethanol (30:1) solution of bromobenzene (900 mg/kg) labeled with 5 μCi of [^{14}C]bromobenzene. The method of isolation and purification of 3-*S*- and 4-*S*-premercapturic acids was described in a previous publication [1]. Purification of these premercapturic acids was carried out by reverse-phase HPLC separations using linear gradient elution. A dual solvent delivery system (Waters model 510), a programmable multi-wavelength detector (Waters model 490) set at 254 nm, and a recorder (4500 Microscribe Strip chart recorder, The Recorder Co., San Marcos, TX) were used. Separations were carried out with a semi-preparative C_{18} μBondapak column (Waters, 30 × 0.78 cm). The flow rate was 1.2 mL/min. The solvent system consisted of: solvent A, methanol:water:acetic acid (20:80:0.1, by vol.); and solvent B, methanol:water:acetic acid (80:20:0.1, by vol.). Solvent B varied linearly from 10 to 90% in 40 min. The purified 3-*S*- and 4-*S*-premercapturic acids were converted to sodium salts and stored as dried lyophilized powder at −20° until used. To ascertain the purity of these premercapturic acids, all premercapturic acids were analyzed by HPLC immediately before use in incubations with kidney or liver 9000 *g* supernatant preparations.

Isolation of ^{14}C-*labeled* N-*acetyl*-S(4-*bromophenyl*)-L-*cysteine* (4-S-*mercapturic acid*)

4-*S*-Mercapturic acid was formed as a dehydration product during isolation of 3-*S*- and 4-*S*-premercapturic acids. This aromatic 4-*S*-mercapturic acid was also eluted from a semi-preparative HPLC column during purification of premercapturic acids. The 4-*S*-mercapturic acid was collected and converted to the sodium salt for storage as described for premercapturic acids. The purity of this product was also confirmed by HPLC immediately before use.

Kidney and liver 9000 g *supernatant preparations*

Guinea pigs were anesthetized with ether. The livers and kidneys were excised and immediately placed on ice, and each was homogenized at 4° in 3 vol. of ice-cold Tris–KCl (1.15% KCl; 20 mM Tris–HCl) buffer, pH 7.4, with a Potter–Elvehjem glass–Teflon homogenizer. The supernatant fraction was separated from the pellet after centrifugation at 9000 *g* for 20 min. Kidney and liver protein was determined by the Bio-Rad Protein Assay (Bio-Rad Chemical Division, Richmond, CA) with bovine serum albumin as reference protein standard. Protein concentrations of kidney and liver preparations were 12–14 and 15–20 mg/mL, respectively.

Incubation of kidney and liver 9000 g *supernatant with* ^{14}C-*labeled* 3-S- *and* 4-S-*premercapturic acids*

Kidney. A mixture of 9000 *g* supernatant containing 7–8 mg of kidney protein, 6 μmol magnesium chloride, 1.4 mg bovine serum albumin, 2.4 μmol NADPH, 1.8 μmol NADH, and 3 mg 3-*S*- and 4-*S*-premercapturic acids (as sodium salts) in a final volume of 2 mL of 0.1 M potassium phosphate buffer (pH 7.4) was incubated at 37° for 1.5 hr. The incubation was carried out in the presence and absence of added pyridoxal-5′-phosphate (Pyr-PO_4) (5 μmol) and in the presence and absence of added AOAA (5.6 μmol). When AOAA was used, it was added to the incubation mixture, and the mixture was incubated at 37° for 15 min before the addition of Pyr-PO_4 and substrate. At the end of the incubation period, the reaction was terminated by extraction with ammonium carbonate–ethyl acetate [14]. Experiments were carried out in triplicate from each kidney preparation, and five separate kidney preparations were used.

For control, parallel experiments were carried out with boiled kidney 9000 *g* supernatant preparations. Incubations were carried out in the presence and absence of added Pyr-PO_4.

Liver. Parallel experiments were carried out with liver 9000 *g* supernatant preparations. Only the incubations that were carried out in the presence of added Pyr-PO_4 are reported here. These results are compared with the results observed from kidney experiments.

Incubation of kidney and liver 9000 g *supernatant with* ^{14}C-*labeled* 4-S-*mercapturic acid*

Parallel experiments, as described above for liver and kidney incubations with 3-*S*- and 4-*S*-premercapturic acids, were carried out with 4-*S*-mercapturic acid and added Pyr-PO_4. These results are compared with the results observed from the 3-*S*- and 4-*S*-premercapturic acid experiments.

* Abbreviations: AOAA, aminooxyacetic acid; BSA, bis(trimethylsilyl)acetamide: DCI, direct chemical ionization; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; *m/z*, mass/charge ratio; ME-TMS, methyl ester-trimethylsilyl ether; 3-OH, 3-bromophenol; 4-OH, 4-bromophenol; Pyr-PO_4, pyridoxal-5′-phosphate; 3- and 4-SCH_3; 3- and 4-bromothioanisole; TBDMS, *tert*-butyldimethylsilyl ether; and TMS, trimethylsilyl ether.

Isolation of metabolites

Neutral and phenolic metabolites. These metabolites were extracted by an ammonium carbonate–ethyl acetate procedure [14]. An aliquot of the ethyl acetate extract was used for radioactive determination by liquid scintillation counting, and the remainder of the sample was evaporated under a gentle stream of nitrogen. The residue was dissolved in 0.5 mL of methanol for storage and transfer for derivative formation.

Acidic metabolites. After extraction of neutral and phenolic metabolites, the aqueous mixture was chilled in ice water and carefully acidified with 6 N sulfuric acid to pH 2–3. The mixture was extracted three times with ethyl acetate. The extracts were combined and washed with a small volume of glass-distilled water (0.5 mL). The ethyl acetate was evaporated with a nitrogen stream and the residue was dissolved in 1 mL of methanol and stored at −20° for analyses by HPLC and, after derivative formation, by gas chromatography (GC) and GC/mass spectrometry (GC/MS).

Water-soluble (ethyl acetate-insoluble) metabolites. The remaining aqueous phase was frozen in an acetone–dry ice bath and lyophilized. The dry residue was washed three times with a 90% methanol–water solution. The mixture was vortexed and centrifuged; the clear methanolic solution was separated from the residue and concentrated under nitrogen to a small volume (1 mL). An aliquot was used for analysis by HPLC, and the fractions that contained radioactivity were collected and used for structure identification by mass spectrometry.

Recovery studies with radioactive 3-S- *and* 4-S-*premercapturic acids and* 4-S-*mercapturic acid*

The percentage recovery of ^{14}C-labeled metabolites of 3-*S*- and 4-*S*-premercapturic acids and 4-*S*-mercapturic acid from kidney or liver 9000 *g* supernatant preparations was determined by liquid scintillation counting of an aliquot of the extract of the neutral, acidic, and water-soluble metabolites. Radioassays were carried out with a Beckman liquid scintillation counter model 3801. Aquasol scintillation fluid was used.

Quantification of neutral and phenolic metabolites

The neutral and phenolic metabolites that were isolated from incubations of kidney 9000 *g* supernatant preparations with 3-*S*- and 4-*S*-premercapturic acids were quantified by GC analyses. A measured amount of internal standard, *n*-tetradecane (in isooctane), was added to ethyl acetate extracts before removing an aliquot for liquid scintillation counting. Inasmuch as most of the metabolites are not available as reference standards, a response factor of unity was assumed for all metabolites in GC separations.

HPLC analyses of acidic and water-soluble (ethyl acetate-insoluble) metabolites

The same HPLC solvent system that was used for the separation of urinary acidic metabolites of bromobenzene was used for the analysis of both acidic and water-soluble metabolites.

For water-soluble metabolites, fractions that contained radioactivity were collected from a semi-preparative HPLC column. The solvents were removed from each of the collected fractions by Roto-evaporation followed by lyophilization. The residue was dissolved in a small volume of methanol for structure identification by mass spectrometry. The identification was carried out by both direct chemical ionization (DCI) mass spectrometry and by GC/MS analysis of *tert*-butyldimethylsilyl ether (TBDMS) derivatives as described in the following sections.

GC and GC/MS studies

(A) Preparation of derivatives

Neutral and phenolic metabolites. These metabolites were converted to trimethylsilyl ether (TMS) derivatives. The extracts were transferred with methanol to a 1-mL Reacti-Vial and dried under nitrogen. The dry residue was dissolved in 10 μL pyridine and silylated with 10 μL BSA. The mixture was heated at 60° for 1 hr. GC/MS studies were carried out with 0.5- to 1-μL samples.

Acidic (ethyl acetate-soluble) metabolites. For acidic metabolites, an aliquot of the acidic extract was used to prepare methyl ester-trimethylsilyl ether (ME-TMS) derivatives. The procedure described previously [2] was used. Comparisons were made with guinea pig urinary acids described earlier.

Water-soluble (ethyl acetate-insoluble) metabolites. To study the formation of *S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine and *S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine by kidney and liver 9000 *g* supernatant preparations, TBDMS derivatives were made. An aliquot of the purified HPLC fraction was dried in a Reacti-Vial under a nitrogen stream. The dried residue was dissolved in 10 μL of pyridine and 10 μL of *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide was added. The reaction mixture was heated at 60° for 1 hr, and an aliquot was analyzed by GC and GC/MS.

(B) GC analyses

GC analyses were carried out with a Hewlett Packard 5890 Gas Chromatograph equipped with a flame-ionization detector. A 30-meter DB-5 fused silica capillary column (0.32 mm i.d., 0.25 μm film thickness) (J & W Scientific, Folsom, CA) was used. All GC analyses were temperature programmed from 60° to 300° at the rate of 2°/min.

(C) GC/MS analyses

GC/MS analyses were carried out with a Nermag R10-10C (Delsi Nermag Instrument, Houston, TX) mass spectrometer coupled to a Varian 3400 gas chromatograph (Varian Instrument, Walnut Creek, CA). A PDP 11/73 data system (Digital Equipment Corp., Bedford, MA) was used. The mass spectrometry analyses were carried out in an electron impact ionization mode. A 30-meter DB-5 fused silica capillary column (0.25 mm i.d., 0.25 μm film thickness) (J & W Scientific) or a 30-meter SPB-1 column (0.32 mm i.d., 0.25 μm film thickness) (Supelco, Inc., Bellefonte, PA) was used. The ion source temperature was 200°, and the electron energy

was 70 eV. Helium was used as the carrier gas. The separation was carried out with temperature programming from 100° to 300° at 5° or 10°/min.

DCI mass spectrometry analyses

DCI analyses were carried out with a Nermag R10-10C mass spectrometer operated in a positive ionization mode with ammonia as a reagent gas. The reagent gas pressure was set at 10^{-1} Torr. The sample (usually 1–2 μL) was spotted on a DCI filament probe tip. The solvent was allowed to evaporate and the probe was inserted into the ion source. The probe was temperature programmed from 0 to 500 mA current at 20 mA/sec.

Statistical analyses

Student's *t*-test was used to determine the statistical significance of the effects of Pyr-PO_4 on the formation of phenols and thiophenolic metabolites of 3-*S*- and 4-*S*-premercapturic acids by the kidney 9000 *g* supernatant preparations.

RESULTS

In vitro *metabolism of* N-*acetyl*-S-*(2-hydroxy*-4-*bromocyclohexa* - 3,5 - *dienyl)* - L - *cysteine (*4-S-*premercapturic acid) and* N-*acetyl*-S-*(2-hydroxy*-5-*bromocyclohexa* - 3,5 - *dienyl)* - L - *cysteine (*3-S-*premercapturic acid)*

(A) By kidney 9000 g *supernatant preparations*

Recovery studies. When radioactive bromobenzene 3-*S*- and 4-*S*-premercapturic acids (**A** in Scheme 1, and its 3-*S*-isomer) were used as substrates in incubations with kidney 9000 *g* supernatant preparations (without added Pyr-PO_4), neutral and phenolic metabolites were formed. These metabolites accounted for 2.3 ± 0.1% of the total radioactivity present in the incubation mixture. Ethyl acetate-soluble acidic metabolites of 3-*S*- and 4-*S*-premercapturic acids accounted for 14.7 ± 1.4% of the original premercapturic acids. A total of 77.1 ± 1.8% of the radioactivity remained in the aqueous phase after extraction with ethyl acetate. These results are summarized in Table 1.

When similar experiments were carried out with boiled kidney 9000 *g* supernatant preparations, most of the 3-*S*- and 4-*S*-premercapturic acid substrates were detected unchanged at the end of the incubation period. This fraction accounted for 91.6 ± 1.8% of the original radioactivity present in the incubation mixture. A small amount of radioactivity (1.7 ± 2.5%) was recovered as water-soluble metabolites, whereas only 0.6 ± 0.7% was detected as neutral and phenolic metabolites.

Neutral and phenolic metabolites. The major neutral and phenolic metabolites of 3-*S*-and 4-*S*-premercapturic acids were 3- and 4-bromophenol, and 3- and 4-bromothioanisole (**I** in scheme 1, and its 3-*S*-isomer). A trace amount of 3,4-dihydroxybromobenzene was also detected. The structure of phenols and thioanisoles was confirmed by comparison of their GC and GC/MS properties (TMS derivatives of phenols) with those of reference standards. The confirmation for 3,4-dihydroxybromobenzene was based on the comparison of the GC and GC/MS properties (TMS derivative) with a previously identified urinary metabolite of bromobenzene [2].

Acidic metabolites. Several sulfur-containing acidic metabolites were detected. These metabolites were identified as previously observed urinary acids which included mercapturic, mercaptolactic and mercaptoacetic acids. GC and GC/MS properties of these sulfur-containing acids were described previously [2].

Water soluble (ethyl acetate-insoluble) metabolites. A typical HPLC separation is shown in Fig. 1. Two fractions (retention times of 14 and 30 min) were found to contain radioactivity. Each fraction was isolated through the use of a semi-preparative HPLC column, and the isolated materials were used for structure identification by mass spectrometry. Figure 2 shows a direct chemical ionization (ammonia reagent gas) mass spectrum of an underivatized

Table 1. Effects of Pyr-PO_4 on the metabolism of 3-*S*- and 4-*S*-premercapturic acids by guinea pig kidney 9000 *g* supernatant preparations

	% of initial radioactive 3-*S*- and 4-*S*-premercapturic acids	
	With Pyr-PO_4	Without Pyr-PO_4
Neutral and phenolic metabolites	10.0 ± 0.8*	2.3 ± 0.1
Acidic metabolites	24.3 ± 2.2	14.7 ± 1.4
Water-soluble metabolites†	57.6 ± 3.2	77.1 ± 1.8

The incubation mixtures contained 7–8 mg of kidney protein, 6 μmol magnesium chloride, 1.4 mg bovine serum albumin, 2.4 μmol NADPH, 1.8 μmol NADH, and 3 mg ^{14}C-labeled 3-*S*- and 4-*S*-premercapturic acids (as sodium salts) in a final volume of 2 mL of 0.1 M potassium phosphate buffer (pH 7.4). The incubation was carried out in the presence and absence of added Pyr-PO_4 (5 μmol) at 37° for 1.5 hr. At the end of the incubation period, the reaction was terminated by extraction with ammonium carbonate–ethyl acetate. Pyr-PO_4 markedly increased the metabolism of 3-*S*- and 4-*S*-premercapturic acids. The percentage conversion of ^{14}C-labeled 3-*S*- and 4-*S*-premercapturic acids to neutral/phenolic, acidic, and water-soluble metabolites was determined by liquid scintillation counting of an aliquot of the extract from each fraction. Values are means ± SEM of at least 12 incubations.

* $P < 0.01$, significantly different from the incubations that did not contain added Pyr-PO_4.

† This fraction was almost entirely *S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine, *S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine and *S*-(4-bromophenyl)-L-cysteine.

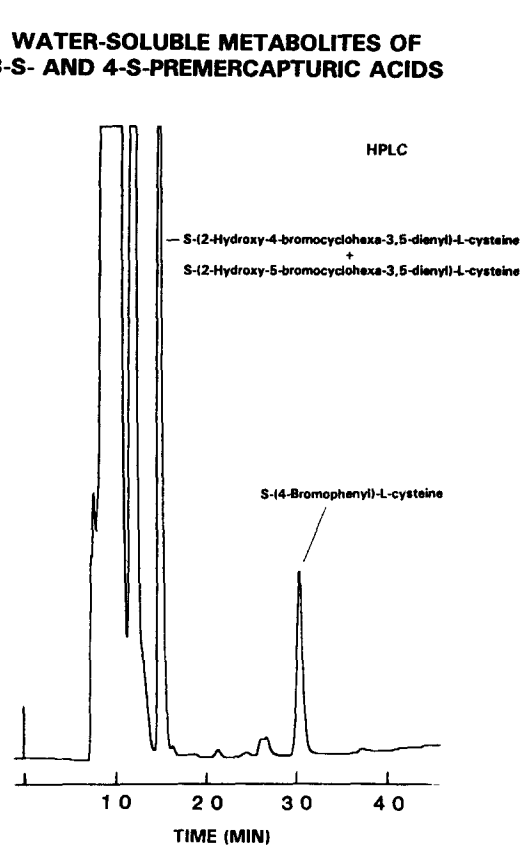

Fig. 1. HPLC analysis of water-soluble (ethyl acetate-insoluble) metabolites of *N*-acetyl-*S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine and *N*-acetyl-*S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine by guinea pig kidney 9000*g* supernatant preparation. Separation was carried out by a semi-preparative μBondapak C_{18} column. Two different chromatographic peaks with retention times of 14 and 30 min were isolated for structure identification by mass spectrometry. The peak with the retention time of 14 min was found to contain *S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine and *S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine. The mass spectrum (underivatized) of these isomeric cysteine conjugates is shown in Fig. 2. Gas chromatographic separation (TBDMS derivatives) of these isomeric products is shown in Fig. 3. The minor peak in this figure (retention time of 30 min) was identified as *S*-(4-bromophenyl)-L-cysteine.

sample of the major product (14 min retention time). An ion of high intensity was present at *m/z* 293 (the ion at *m/z* 295 was slightly more intense than the ion at *m/z* 293, and as a consequence the computer program labeled the ion at *m/z* 295) in the mass spectrum. This ion corresponds to $[(MH^+ + NH_3) - H_2O]$ for N-deacetylated products of 3-*S*- and 4-*S*-premercapturic acids. The base peak at *m/z* 276 (the computer program labeled the isotope ion at *m/z* 278) corresponds to MH^+ for a dehydration/aromatization product. An ion of high abundance was also present at *m/z* 122 which corresponds to MH^+ of cysteine. The ions at *m/z* 249 and 232 were due to the loss of CO_2 from the ions at *m/z* 293 and 276, respectively. The major peak in Fig. 1 was a mixture of *S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine (**B** in Scheme 1) and *S*-(2-hydroxy - 5 - bromocyclohexa - 3,5 - dienyl) - L - cysteine (3-*S*-isomer of **B** in Scheme 1). These dihydrohydroxybenzene cysteine conjugates were relatively unstable; the ratio of the major fraction to the minor fraction in Fig. 1 was found to vary among sample preparations (the major fraction can be dehydrated/aromatized during the isolation procedure to form the minor product).

To assign specific structures to these cysteine conjugates in Fig. 1, a combination of HPLC, GC and GC/MS techniques similar to those described earlier [1, 15] was used. Incubation of *N*-acetyl-*S*-(4-bromophenyl)-L-cysteine (4-*S*-mercapturic acid) with a guinea pig kidney 9000*g* supernatant preparation led to the formation of a water-soluble metabolite that had the same HPLC, GC and GC/MS (TBDMS derivative) properties as the minor product in Fig. 1. The minor product in Fig. 1 was *S*-(4-bromophenyl)-L-cysteine.

When the major fraction shown in Fig. 1 was partially silylated with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide, two major products were observed by GC and GC/MS analytical techniques (Fig. 3). These compounds, which have almost identical mass spectrometric properties (TBDMS derivatives), have carboxylic acid and amino groups that were derivatized with *tert*-butyldimethylsilyl groups. The hydroxyl groups of the conjugates were not derivatized and were eliminated by thermal dehydration in the vaporization zone of the gas chromatograph. A related reaction was described earlier [1, 15]. Incomplete silylation was probably due to steric hindrance caused by the cysteinyl group. This made it possible to identify the 3-*S*- and 4-*S*-isomers shown in Fig. 3. The peak labeled 4-*S*- had the same GC and GC/MS (TBDMS derivative) properties as those observed from the minor product in Fig. 1. It was therefore assigned the structure *S*-(4-bromophenyl)-L-cysteine. The other product in Fig. 3. was assigned the structure *S*-(3-bromophenyl)-L-cysteine.

It should be noted that the ratio of the 3-*S*- to 4-*S*-cysteine conjugate in Fig. 3 was identical to that observed earlier for the 3-*S*- and 4-*S*-premercapturic acids [see Fig. 4, in Ref. 1].

Effect of pyridoxal phosphate on the formation of neutral and phenolic metabolites from 3-S- *and* 4-S-*premercapturic acids.* The addition of Pyr-PO_4 to kidney incubations resulted in significant changes in the metabolism of 3-*S*- and 4-*S*-premercapturic acids. These results are shown in Table 1. The percentage recovery of water-soluble metabolites, which are *S*-(2 - hydroxy - 4 - bromocyclohexa - 3,5 - dienyl) - L - cysteine and *S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine, was decreased from 77.1 ± 1.8% (without added Pyr-PO_4) to 57.6 ± 3.2% (with added Pyr-PO_4). For acidic metabolites, the percentage recovery in this fraction was increased from 14.7 ± 1.4 to 24.3 ± 2.2%. When these metabolites were analyzed by GC and GC/MS (ME-TMS derivatives), the percentage increase was due mainly to an increase in the formation of mercaptolactic acids. The formation of neutral and phenolic metabolites was also affected by the presence of

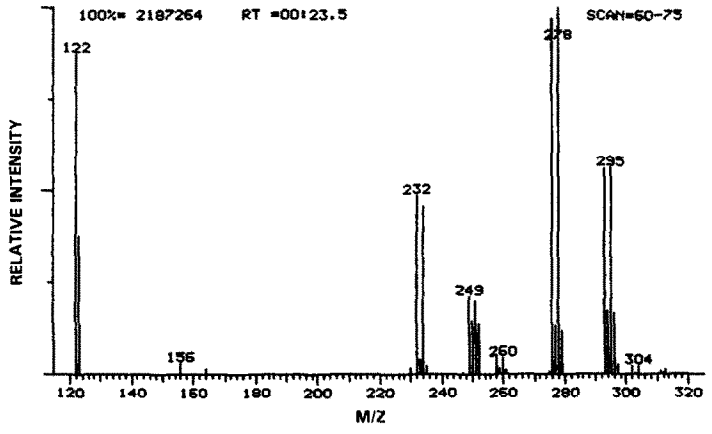

Fig. 2. Mass spectrum of underivatized *S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine and *S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine. Analysis was carried out by direct chemical ionization mass spectrometry, operated in a positive ionization mode with ammonia as the reagent gas.

added Pyr-PO_4; this accounted for an increase in the percentage recovery from 2.3 ± 0.1 to 10.0 ± 0.8%. The following changes were found.

3- *and* 4-*Bromophenol.* The addition of Pyr-PO_4 led to an approximately 5-fold increase in neutral and phenolic products (Fig. 4A). These changes were due mainly to increased formation of 3- and 4-bromophenol (Fig. 4B). Figure 5 shows a typical GC/MS analysis of neutral and phenolic metabolites (TMS derivatives) isolated from a kidney 9000 *g* supernatant preparation with added Pyr-PO_4. The identification of 3- and 4-bromophenol was based

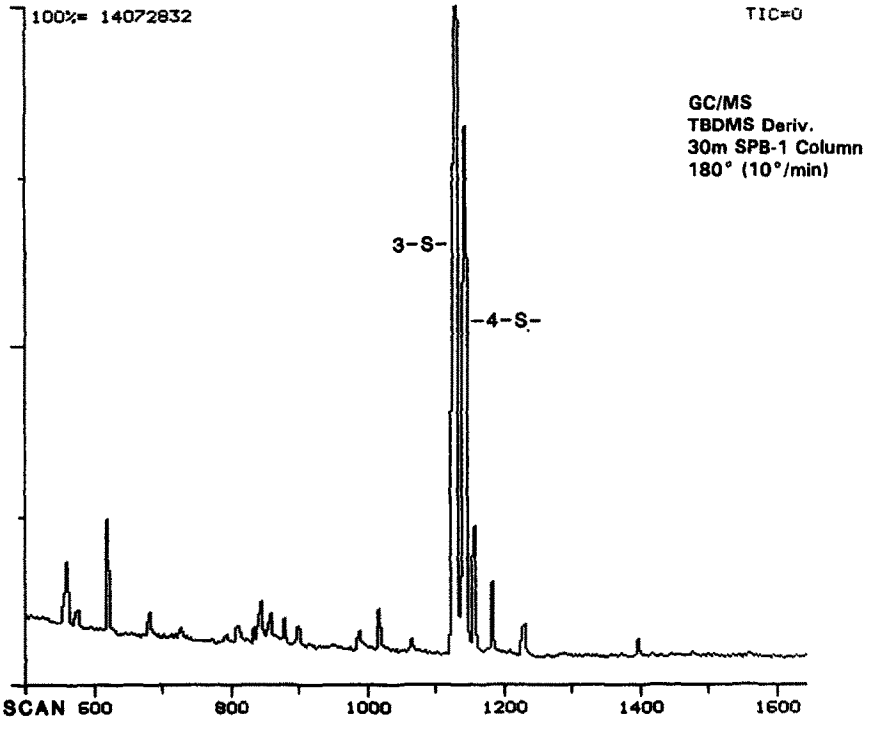

Fig. 3. GC/MS analysis of TBDMS derivatives of *S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine and *S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine. The compounds shown in this figure (as 3-*S*- and 4-*S*-) are thermal/dehydration/aromatization products of partially silylated *S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine and *S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine. They are di-substituted (carboxylic acid and amino groups) derivatives (TBDMS) of the corresponding *S*-(3-bromophenyl)-L-cysteine (labeled as 3-*S*-) and *S*-(4-bromophenyl)-L-cysteine (labeled as 4-*S*). Thermal dehydration/aromatization occurred in the injector zone of the gas chromatograph.

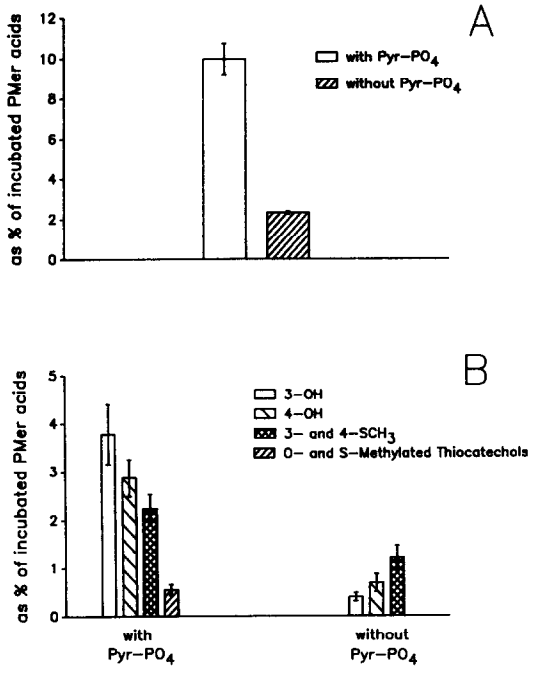

Fig. 4. Comparison of the effect of Pyr-PO_4 on the metabolism of *N*-acetyl-*S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine and *N*-acetyl-*S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine by guinea pig kidney 9000 *g* supernatant preparations. Panel A shows a comparison of the recovery of radioactive neutral and phenolic metabolites. Panel B shows a comparison of phenolic and neutral thiol-containing metabolites (TMS derivatives). PMer acids = 3-*S*- and 4-*S*-premercapturic acids; 3- and 4-SCH_3 = 3- and 4-bromothioanisole. Values are means ± SEM of at least 12 different incubations.

on comparisons of their GC and GC/MS properties (TMS derivatives) with those of reference compounds.

O- and S-Methylated derivatives of 2-hydroxy-4-bromobenzenethiol (4-S-bromothiocatechol) and 2-hydroxy-5-bromobenzenethiol (3-S-bromothiocatechol) and related metabolites. Six sulfur-containing guinea pig urinary metabolites of bromobenzene found earlier [2] were identified as kidney products of 3-*S*- and 4-*S*-premercapturic acids. These metabolites were not detected in the incubation without added Pyr-PO_4 (Fig. 4B). They were identified as four different S- and O-methylated* (**F** and **G** in Scheme 1, and their 3-*S*-isomers) 3-*S*- and 4-*S*-bromothiocatechols (**E** in Scheme 1, and its 3-*S*-isomer) and two isomeric *S*-methylated 3-*S*- and 4-*S*-bromodihydrobenzene thiolols (**D** in Scheme 1 and its 3-*S*-isomer). A typical GC separation (TMS derivatives) of the four O- and S-methylated 3-*S*- and 4-*S*-bromothiocatechols is shown in Fig. 5. The middle and bottom chromatographic separations showed limited mass search for *m/z* 290 and 292 which corresponded to molecular ions (TMS derivatives) of O- and S-methylated 3-*S*- and 4-*S*-bromothiocatechol. Their mass spectrometric properties have been described previously [2].

3- and 4-Bromothioanisole. The formation of these metabolites (**I** in Scheme 1, and its 3-*S*-isomer) was enhanced in the presence of added Pyr-PO_4 (Fig. 4B). These metabolites were identified by comparison of their GC and GC/MS properties with those of reference samples.

For control experiments, the presence of added Pyr-PO_4 did not change the metabolism of 3-*S*- and 4-*S*-premercapturic acids by boiled kidney 9000 *g* supernatant preparations. The percentage recoveries of radioactive neutral/phenolic, acidic and water-soluble metabolites were identical to those observed from incubations that did not contain added Pyr-PO_4.

Effect of AOAA on the formation of neutral and phenolic metabolites of 3-S- and 4-S-premercapturic acids. When AOAA was added to kidney incubations (without added Pyr-PO_4), no significant inhibition was observed in terms of the formation of neutral/phenolic metabolites (right, Fig. 6). This result was different from incubations that contained added Pyr-PO_4 (left, Fig. 6). The time-course of addition of Pyr-PO_4 and AOAA to the incubation mixture, however, was relatively important. A strong inhibition was observed when AOAA was added to kidney 9000 *g* supernatant preparations 15 min prior to the addition of Pyr-PO_4 and substrates (as shown in Fig. 6), whereas only a small degree of inhibition was observed when AOAA was added after the addition of Pyr-PO_4 (data not shown). The formation of 3- and 4-bromophenol, O- and S-methylated 3-*S*- and 4-*S*-bromothiocatechols, and 3- and 4-bromothioanisole was suppressed in the presence of added AOAA (Fig. 7).

(B) By liver 9000 g supernatant preparations

The metabolism of 3-*S*- and 4-*S*-premercapturic acids by liver preparations was compared with the results observed from kidney preparations. Panels A and C of Fig. 8 show a comparison of the percentage recovery of radioactive neutral/phenolic, acidic, and water-soluble metabolites isolated from kidney and liver preparations, respectively. All these experiments were carried out in the presence of added Pyr-PO_4. For liver preparations, the fraction that contained most of the radioactivity was the acidic (ethyl acetate-soluble) metabolites. This fraction accounted for 80 ± 2% of the substrates, 3-*S*- and 4-*S*-premercapturic acids. When the metabolites in this fraction were analyzed by GC and GC/MS (ME-TMS derivatives), the major products were identified as unchanged 3-*S*- and 4-*S*-premercapturic acids. Of the original 3-*S*- and 4-*S*-premercapturic acid substrates, 17 ± 1% was recovered as water-soluble (ethyl acetate-insoluble) metabolites, and this fraction contained the *S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine and *S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine. The neutral and phenolic metabolite fraction accounted for 3 ± 1%. All of the neutral and phenolic metabolites that were detected as kidney metabolites were also detected as liver metabolites.

* Lertratanangkoon K, Manuscript submitted for publication.

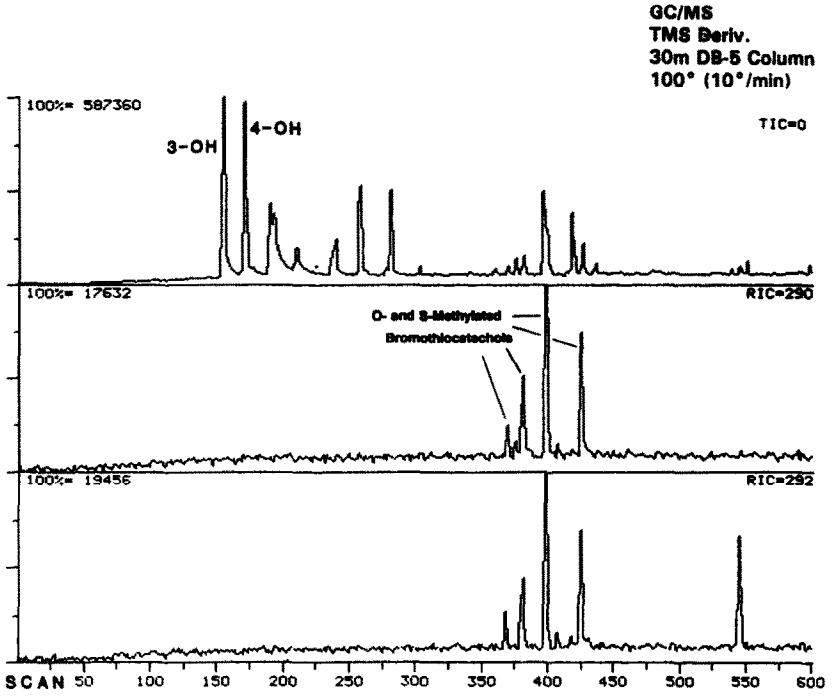

Fig. 5. GC/MS analysis of neutral and phenolic metabolites of *N*-acetyl-*S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine and *N*-acetyl-*S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine by guinea pig kidney 9000 *g* supernatant preparation. The incubation was carried out in the presence of added pyridoxal-5'-phosphate. Upper: scan of total ion current of neutral and phenolic metabolites, as TMS derivatives. Middle and lower: scans for ions at 290 and 292 amu, corresponding to TMS derivatives of O- and S-methylated 3-*S*- and 4-*S*-bromothiocatechols.

In vitro *metabolism of* N-*acetyl*-S-*(4-bromophenyl)*-L-*cysteine (*4-S-*mercapturic acid)*

The metabolism of 4-*S*-mercapturic acid by kidney (Fig. 8B) and liver (Fig. 8D) 9000 *g* supernatant preparations showed a much simpler pattern of products than that observed for 3-*S*- and 4-*S*-premercapturic acids (Fig. 8A and 8C). Both the kidney and liver could N-deacetylate the aromatic 4-*S*-mercapturic acid. This accounted for a high percentage recovery of radioactive metabolites in the water-soluble fraction. With the same amounts of substrate and kidney or liver protein employed in studies with 3-*S*- and 4-*S*-premercapturic acids,

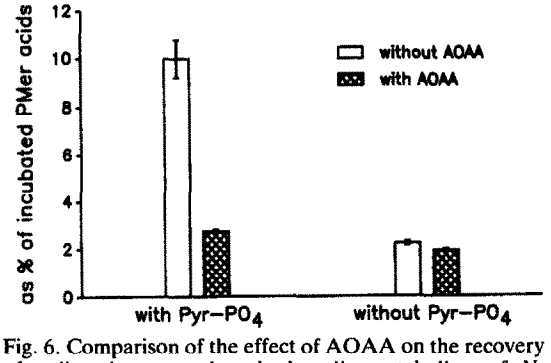

Fig. 6. Comparison of the effect of AOAA on the recovery of radioactive neutral and phenolic metabolites of *N*-acetyl-*S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine and *N*-acetyl-*S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine by guinea pig kidney 9000 *g* supernatant preparations. Left: comparison of the recovery from the incubations that were carried out in the presence of added pyridoxal-5'-phosphate (Pyr-PO_4). AOAA (5.6 μmol) was added to the kidney incubation mixture 15 min prior to the addition of Pyr-PO_4 and substrates. Right: comparison of the recovery from the incubations that were carried out in the absence of added Pyr-PO_4. PMer acids = 3-*S*- and 4-*S*-premercapturic acids. Values are means ± SEM of at least 12 different incubations.

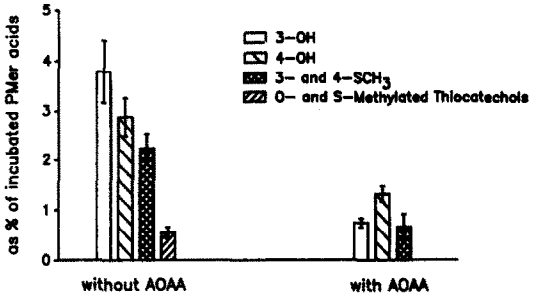

Fig. 7. Comparison of the effect of AOAA (5 μmol) on the formation of phenolic and thiol-containing metabolites of *N*-acetyl-*S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine and *N*-acetyl-*S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine by guinea pig kidney 9000 *g* supernatant preparations. All incubations were carried out in the presence of added Pyr-PO_4. PMer acids = 3-*S*- and 4-*S*-premercapturic acids; 3- and 4-SCH_3 = 3- and 4-bromothioanisole. Values are means ± SEM of at least 12 different incubations.

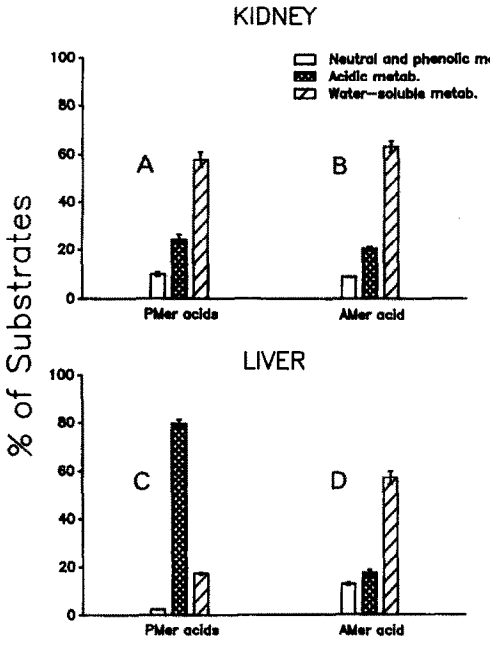

Fig. 8. Comparison of the metabolism of non-aromatic *N*-acetyl-*S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine (4-*S*-premercapturic acid) and *N*-acetyl-*S*-2-(hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine (3-*S*-premercapturic acid) (A and C) and aromatic *S*-(4-bromophenyl)-L-cysteine (4-*S*-mercapturic acid) (B and D) by guinea pig kidney (A and B) and liver (C and D) 9000 *g* supernatant preparations. All incubations were carried out in the presence of added Pyr-PO_4. The guinea pig kidney contains an *N*-deacetylase(s) for both the non-aromatic 3-*S*- and 4-*S*-premercapturic acids (PMer acids) and aromatic 4-*S*-mercapturic acid (AMer acid), whereas an *N*-deacetylase(s) in the liver acts mainly on the aromatic 4-*S*-mercapturic acid. The acidic metabolite fraction in A, B and D contains mainly mercaptolactic acids, whereas the corresponding fraction in C contains mainly recovered 3-*S*- and 4-*S*-premercapturic acids. Values are means ± SEM of at least 12 different incubations.

almost all of the 4-*S*-mercapturic acid was N-deacetylated to *S*-(4-bromophenyl)-L-cysteine at the end of the experiment. These results were quite different from those observed when 3-*S*- and 4-*S*-premercapturic acids were used as substrates. With the non-aromatic premercapturic acids, almost all of the substrates were N-deacetylated by kidney preparations (Fig. 8A), but only a small fraction of the original premercapturic acids was N-deacetylated by the liver (Fig. 8C).

The *S*-(4-bromophenyl)-L-cysteine was further metabolized by kidney and liver β-lyase(s) and transaminase(s) as expected. The major acidic metabolite detected in the acidic fraction was the 4-*S*-mercaptolactic acid (a trace amount of 4-*S*-mercaptoacetic acid was also detected). For neutral metabolites, 4-bromothioanisole was the major product. The formation of 4-bromothioanisole by kidney 9000 *g* supernatant preparations was enhanced in the presence of added Pyr-PO_4 when compared with the incubations that did not contain added Pyr-PO_4 (data not shown).

DISCUSSION

The incubation of *N*-acetyl-*S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine (4-*S*-premercapturic acid) and *N*-acetyl-*S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine (3-*S*-premercapturic acid) with Hartley guinea pig kidney and liver 9000 *g* supernatant preparations led to a series of neutral/phenolic, acidic and water-soluble metabolites. Most of these metabolites, excluding *S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine and *S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine and 3- and 4-bromothioanisole, had been identified previously as urinary metabolites of bromobenzene in the guinea pig [2].

(A) Water-soluble metabolites of 3-S- *and* 4-S-*premercapturic acids*

The kidney 9000 *g* supernatant preparations could N-deacetylate both the non-aromatic 3-*S*- and 4-*S*-premercapturic acids (Fig. 8A) and the aromatic *N*-acetyl-*S*-(4-bromophenyl)-L-cysteine (4-*S*-mercapturic acid) (Fig. 8B). With the same amounts of substrate employed, N-deacetylation was almost completed at the end of the experiment. In contrast with the kidney preparations, the liver preparations did not *N*-deacetylate the 3-*S*- and 4-*S*-premercapturic acids to the same extent (Fig. 8C). Significant amounts of 3-*S*- and 4-*S*-premercapturic acid substrates were recovered at the end of experiments, whereas only 17 ± 1% was converted to *S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine and *S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine. The liver, however, showed no difficulty in removal of the *N*-acetyl group from the aromatic 4-*S*-mercapturic acid (Fig. 8D). These results suggest that the *N*-deacetylase that catalyzes the removal of the *N*-acetyl group present in the 3-*S*- and 4-*S*-premercapturic acids may not be the same as the *N*-deacetylase that utilizes the 4-*S*-mercapturic acid as a substrate as those described earlier for rat liver enzyme [16]. Mammalian liver and kidney *N*-deacetylases that are specific for either aromatic mercapturic acids or aliphatic compounds are known [16–18], but no report was found in the literature that describes the removal of an *N*-acetyl group from a premercapturic acid to form a dihydrohydroxybenzene cysteine conjugate (in this instance, **A**→**B** in Scheme 1). The present observation of substrate specificity of liver *N*-deacetylases suggests the existence of a family of enzymes that catalyze the biotransformation of non-aromatic dihydrohydroxybenzene sulfur-containing conjugates, and this class of enzymes may be distinct from the enzymes that are known to act on aromatic and aliphatic types of compounds.

N-Deacetylation of the 3-*S*- and 4-*S*-premercapturic acids leads to the formation of positional isomeric *S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine and *S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine. The ratio of the 3-*S*- to 4-*S*-cysteine conjugate (Fig. 3) is identical to the ratio of the 3-*S*- to 4-*S*-premercapturic acids [see Fig. 4, in Ref. 1]. This indicates that removal of the *N*-acetyl group by kidney *N*-

deacetylase shows no preference with respect to 3-*S*- and 4-*S*-positional isomers of premercapturic acids.

(B) Neutral/phenolic metabolites of 3-S- *and* 4-S-*premercapturic acids*

O- and S-Methylated 3-S *and* 4-S-*bromothiocatechols and S-methylated* 3-S- *and* 4-S-*bromodihydrobenzene thiolols.* In the metabolic pathways shown in Scheme 1, four different isomeric *S*- and *O*-methylated products (**F**, **G** and their 3-*S*-isomers) of 3-*S*- and 4-*S*-bromothiocatechols (**E** and its 3-*S*-isomer) were detected as kidney and liver products of 3-*S*- and 4-*S*-premercapturic acids. They were found earlier as urinary metabolites of bromobenzene in the guinea pig [2], and recently they were also found as urinary metabolites of bromobenzene in the golden Syrian hamster*. The extent of their formation *in vitro* is dependent on the addition of Pyr-PO_4 to the incubation mixture, and their formation is sensitive to the presence of AOAA. This result suggests that a Pyr-PO_4-dependent *C-S* β-lyase is involved and that the required substrates must be *S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine and *S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine. Although this is a characteristic of *C-S* β-lyases that is known to act on aliphatic and aromatic cysteine conjugates, we believe that the *C-S* β-lyase responsible for the cleavage of the non-aromatic cysteine conjugates (**B** and its 3-*S*-isomer) reported here is not the β-lyase that is known to cleave the *C—S* bond of the *S*-(4-bromophenyl)-L-cysteine [4, 5]. Evidence for this suggestion came from the comparison of β-lyase products formed from 3-*S*- 4-*S*- premercapturic acids (Fig. 8C) and from 4-*S*-mercapturic acid by liver protein (Fig. 8D). 4-Bromothioanisole was a major product from the 4-*S*-mercapturic acid experiment (12 ± 1% of initial radioactivity), but only very small amounts of *C-S* β-lyase products were found for the 3-*S*- and 4-*S*-premercapturic acids.

In Scheme 1, detection of **F** and **G** in urine samples from the Hartley guinea pig [2], the Syrian hamster, and in these *in vitro* studies was always accompanied with the detection of **D**. Similar results were also followed for the 3-*S*-isomers. These observations strongly suggest that their pathways of formation may be related. In our view, the most likely intermediate in the formation of 4-*S*-bromothiocatechol (**E**) is the 4-*S*-bromodihydrobenzene thiolol (**C**) (sulfur-series analogs of the 3,4-dihydro-3,4-diol). Dehydrogenation of **C** would lead to the formation of **E**; the mechanism of dehydrogenation of a dihydrobenzene thiolol to thiocatechol presumably occurred as an analogous reaction to catechol formation from the 3,4-dihydro-3,4-diol [19].

3- *and* 4-*Bromothioanisole.* Products found in the neutral/phenolic fraction included 3- and 4-bromothioanisole (Scheme 1, **I** and its 3-*S*-isomer). These *S*-methylated bromothiophenols were not detected in earlier studies of urinary metabolites of bromobenzene in the Hartley guinea pig and in the Sprague–Dawley rat [2], and it is unlikely that these lipophilic products would be excreted in urine. It was reported by Mizutani *et al.* [20] that 2-, 3- and 4-bromothioanisole were found as minor metabolites of bromobenzene after the addition of sodium hydroxide to urine samples from Wistar rats, and it was proposed that the excretory products were sulfonium compounds. There may be a minor route of metabolism of this kind. In our view (Scheme 1), dehydration of **C** would lead to the formation of 4-bromothiophenol (**H**) which subsequently S-methylated to form 4-bromothioanisole (**I**). The mode of elimination of the hydroxyl group from **C** is not certain, but it may be related to the aromatization process that converts the 3,4-dihydro-3,4-diol to both 3- and 4-bromophenol [2].

Incomplete silylation of a dihydrohydroxybenzene sulfur-containing conjugate (for example, 3-*S*- and 4-*S*-premercapturic acids) results in thermal dehydration during GC and GC/MS analyses [1]. This leads to the formation of both the 3-*S*- and 4-*S*-isomeric aromatic conjugates. Detection of both 3- and 4-bromothioanisole as kidney and liver products raises a question concerning the mechanism of their formation (biological vs experimental artifacts). If the hydroxyl groups of S-methylated 3-*S*- and 4-*S*-bromodihydrobenzene thiolol (**D** in Scheme 1, and its 3-*S*-isomer) were only partially silylated, thermal dehydration would occur during GC and GC/MS analyses, and the resulting products would be 3- and 4-bromothioanisole. This possibility, however, is not supported by previous analytical studies of the metabolism of bromobenzene in the guinea pig [2]. With the same analytical procedures employed for both *in vivo* and *in vitro* studies, the *in vivo* studies show no evidence that **D** and its 3-*S*-isomer are difficult to derivatize by our usual silylation procedure (60° in pyridine with BSA); 3- and 4-bromothioanisole have not been detected in any urine sample that has been examined in this way [2]. This suggests that 3- and 4-bromothioanisole found as kidney and liver products in this investigation are more likely a result of a biological process than a consequence of thermal dehydration of incompleted derivatization of S-methylated 3-*S*- and 4-*S*-bromodihydrobenzene thiolols.

3-*and* 4-*Bromophenol.* Both 3- and 4-bromophenol are *in vivo* guinea pig and rat metabolites of 3-*S* and 4-*S*-premercapturic acids [1]; this conversion is now known to occur both in the kidney and liver, but when 3-*S*- and 4-*S*-premercapturic acids are used as precursors, an N-deacetylation reaction is required. This reaction, however, is not the sole requirement for the generation of phenol by this sulfur-series route. Besides N-deacetylation, the action by a *C-S* lyase is also needed. Results in Table 1 show that regardless of the presence or absence of added Pyr-PO_4, N-deacetylated products were generated. Nevertheless, the availability of *S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine and *S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine could not readily metabolize to 3- and 4-bromophenol unless Pyr-PO_4 is added to the incubation mixture. The addition of Pyr-PO_4 resulted in a 5-fold increase in neutral/phenolic products (Fig. 4A), and the formation of 3- and 4-bromophenol was increased

* Lertratanangkoon K and Scimeca JM, Manuscript submitted for publication.

when compared with incubations that did not contain added Pyr-PO_4 (Fig. 4B).

Our observations clearly demonstrated that a Pyr-PO_4-dependent *C-S* lyase is required for the generation of phenols by the sulfur-series route and that the required substrates are the *S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine and *S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine. In Scheme 1, the exact mechanism for *C-S* lyase reaction that results in removal of the entire cysteinyl group of **B** and is followed by ring aromatization to form 3-bromophenol is not yet certain, and a hypothetical reaction has been proposed.* It is possible that 3-bromophenol is also formed from **C**. A broken line is indicated in Scheme 1 to show this unproved but possible relationship.

(C) Acidic metabolites of 3-S- *and* 4-S-*premercapturic acids*

The detection of urinary 3-*S*- and 4-*S*-premercapturic acids or 4-*S*-mercapturic acid is evidence for the presence of arene oxide-glutathione conjugation reactions; the major metabolic steps in the mercapturate pathway for aromatic compounds are arene oxide → glutathione conjugate → → cysteine conjugate → *N*-acetylcysteine conjugate. This route is generally regarded as a detoxication pathway, and it is a major route of bromobenzene metabolism in the Sprague–Dawley rat [2].

In the guinea pig, this metabolic sequence is greatly modified, apparently due both to the presence of an active *N*-deacetylase(s) in the kidney (and to some extent in the liver) and to a *C-S* lyase(s)/transaminase(s). As a consequence, guinea pig urinary acids derived from bromobenzene include the usual products from *in vivo* transamination of *S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine and *S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine. These are the corresponding lactates and acetates, and if the urine (or an *in vitro* preparation containing these acids) is acidified, the resulting dehydration/rearrangement/aromatization reactions lead to the corresponding aromatic 4-*S*-acids [1].

It should be noted that a mammalian species like the guinea pig which possesses active transaminase(s) also has active *C-S* β-lyase(s), while transamination reactions of cysteine conjugates have never appeared to be a prominent reaction in the Sprague–Dawley rat [2, 21, 22]. The Sprague–Dawley rat has also proved to be a species that did not metabolize bromobenzene [2] or 3-*S*- and 4-*S*-premercapturic acids [1] to the same β-lyase products as those observed in the guinea pig. This suggests that transaminase(s) and β-lyase(s) in the Hartley guinea pig may be related, as in another instance observed earlier by Stevens and his colleagues [9]. The addition of α-ketoglutarate to a kidney incubation with 3-*S*- and 4-*S*-premercapturic acids, which contained added Pyr-PO_4, resulted in an increase in transamination products as expected, and a slight decrease in *C-S* lyase products was observed (Lertratanangkoon K, unpublished observations). This effect was not investigated in greater detail; it may represent a shift in *C-S* lyase/transaminase products in the direction of transamination, with a corresponding decrease in *C-S* lyase products.

Conclusions

Hartley guinea pig kidney and liver 9000 *g* supernatant preparations contain all the necessary enzymes to convert 3-*S*- and 4-*S*-premercapturic acids to phenols. This sulfur-series pathway, which also leads to thiocatechols, dihydrobenzene thiolols and thiophenols, is a major metabolic pathway of bromobenzene that initially involves the conjugation of bromobenzene 3,4-oxide with glutathione. The formation of phenols, thiocatechols, dihydrobenzene thiolols and thiophenols by this pathway is dependent both upon the availability of *S*-(2-hydroxy-4-bromocyclohexa-3,5-dienyl)-L-cysteine and *S*-(2-hydroxy-5-bromocyclohexa-3,5-dienyl)-L-cysteine as substrates as well as Pyr-PO_4-dependent *C-S* lyase(s).

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