Cysteine Conjugate S-Oxidase

CHARACTERIZATION OF A NOVEL ENZYMATIC ACTIVITY IN RAT HEPATIC AND RENAL MICROSOMES*

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Cysteine conjugate S-oxidase activity, with S-benzyl-L-cysteine as substrate, was found mostly in the microsomal fractions of rat liver and kidney. In the presence of oxygen and NADPH, S-benzyl-L-cysteine is converted to S-benzyl-L-cysteine sulfoxide; no S-benzyl-L-cysteine sulfone was detected. The V_{\text{max}} for S-benzyl-L-cysteine sulfoxide formation by kidney microsomes was nearly 3-fold greater than the rate measured with liver microsomes. Inclusion of catalase, superoxide dismutase, glutathione, butylated hydroxyanisole, the peroxidase inhibitor, potassium cyanide, the cytochrome P-450 inhibitors, 1-benzylimidazole and metyrapone, or a monoclonal antibody to cytochrome P-450 reductase did not inhibit the metabolic reaction. Flavin-containing monooxygenase alternate activities may be associated with flavin-containing intermediates or cytochrome P-450 in the sulfoxidation evidence against the involvement of reactive oxygen intermediates or cytochrome P-450 reductase did not inhibit the metabolic reaction. Flavin-containing monooxygenase alternate substrates, N,N-dimethylaniline, n-octylamine, and methimazole inhibited the S-oxidase activities. Analogues of S-benzyl-L-cysteine, S-methyl-L-cysteine, and S-(1,2-dichlorovinyl)-L-cysteine inhibited the S-benzyl-L-cysteine S-oxidase activities, whereas S-carboxymethyl-L-cysteine and S-benzyl-L-cysteine methyl ester had no effect. These results provide clear evidence against the involvement of reactive oxygen intermediates or cytochrome P-450 reductase in the sulfoxidation of S-benzyl-L-cysteine and indicate that the S-oxidase activities may be associated with flavin-containing monooxygenases which exhibit selectivity in the interaction with cysteine S-conjugates.

Glutathione S-conjugate formation is an important mechanism for detoxication of many chemicals (1, 2). This metabolic reaction is catalyzed by glutathione S-transferases which are present in several tissues, with activity in the liver being much higher than in the kidney. Glutathione S-conjugates are eliminated in the bile or, after further metabolism by mercapturic acid, in the urine. The activities of the enzymes that catalyze mercapturic acid formation, namely, γ-glutamyl transpeptidase, cysteinylglycine dipeptidase, and cysteine conjugate N-acetyltransferase, are much higher in the kidney than in liver (1-5).

Recentl, the nephrotoxicity, mutagenicity, and carcinogenicity of halogenated hydrocarbons such as ethylene dichloride, ethylene dibromide, trichloroethylene, chlorotrifluoroethylene, and hexachlorobutadiene have been attributed to the formation of glutathione and cysteine S-conjugates (6-14). Evidence for the existence of two classes of nephrotoxic cysteine conjugates, i.e., direct acting through the formation of reactive episulfonium ions, and indirect acting which require activation by cysteine conjugate β-lyase to generate reactive intermediates, was obtained (12-14). Metabolism of cysteine S-conjugates by cysteine conjugate β-lyase may also result in the formation of stable thiol (1, 15). This metabolic reaction has been exploited recently for site selective delivery of 6-mercaptopurine, an anti-tumor drug, to the kidneys (16).

Evidence for the presence of another metabolic pathway which involves the conversion of cysteine S-conjugates to sulfoxides has been reported. For example, in vivo treatments of rats and mice with ethylene dibromide or ethylene dimethanesulfonate resulted in the excretion of S-(2-hydroxyethyl)-N-acetyl-L-cysteine sulfoxide in urine (17); ethyl mercapturic acid sulfoxide was identified as a urinary metabolite of bromoethane in rats (18). Furthermore, a number of cysteine S-conjugates such as S-methyl-L-cysteine, S-carboxymethyl-L-cysteine, S-ethyl-L-cysteine, S-[2-hydroxyethyl]-L-cysteine, S-propyl-L-cysteine, S-butyl-L-cysteine, and S-pentyl-L-cysteine were metabolized in vivo to sulfoxides (18-22). When rats were given S-ethyl-L-cysteine, approximately 18 and 30% of the dose was excreted as ethylmercapturic acid and the corresponding sulfoxide, respectively (22). A sulfenic acid species which could arise by the cysteine conjugate β-lyase-dependent metabolism of S-(pentachlorobutadienyl)-L-cysteine sulfoxide had also been identified in the urine of rats given hexachlorobutadiene (8).

Although sulfoxidation appears to be a major metabolic pathway for cysteine S-conjugates in vivo, the nature of the enzymes involved and their role in the metabolism and toxicity of cysteine S-conjugates were not previously investigated. Therefore, the present study was performed to characterize the hepatic and renal S-oxidation of cysteine S-conjugates, using S-benzyl-L-cysteine (SBC) as substrate, in terms of the metabolites formed and the mechanisms involved. The results of these studies provide clear evidence against the involvement of reactive oxygen intermediates or cytochrome P-450 in this metabolic reaction. Furthermore, the results indicate the involvement of flavin-containing monooxygenases. The rate of SBC sulfoxide formation by kidney

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The abbreviations used are: SBC, S-benzyl-L-cysteine; S-oxidase, cysteine conjugate S-oxidase; HPLC, high pressure liquid chromatography.

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Microsomes was nearly 3-fold greater than the rate obtained with liver microsomes.

EXPERIMENTAL PROCEDURES

Materials—NADPH, catalase, superoxide dismutase, horse heart cytochrome c, S-methyl-L-cysteine, S-carboxymethyl-L-cysteine, S-benzyl-L-cysteine, methyl ether, glutathione, N,N-dimethylaminoethanol, butylated hydroxyanisole, and glucose 6-phosphate were obtained from Sigma. A monoclonal antibody to rat cytochrome P-450 reduc-tase was a generous gift of Dr. Charles Kasper, McArdle Labs, Madison, WI. SBC, N-octylamine, methimazole, 1-benzylimidazole, metyrapone, 2,4-dinitrofluorobenzene, and trifluoroacetic acid were obtained from Sigma (St. Louis, MO). Wistaria fruit, acetonitrile, and methanol were obtained from EM Science (Gibstown, NJ). All other chemicals were of the highest grade commercially available.

Synthesis, Purification, and Characterization of SBC Sulfoxide and SBC Sulfone—SBC sulfoxide and SBC sulfone were synthesized as described for the synthesis of S-ethyl-L-cysteine sulfoxide and S-benzyl-N-o-methylcysteine sulfone (20, 23). For SBC sulfoxide, 30% H$_2$O$_2$ (0.63 ml) was added to a solution of SBC (1.13 g) in water (5 ml). 30% H$_2$O$_2$ (5.8 ml) was added to the reaction mixture in small portions with care taken to keep the solution temperature near 4 °C during the addition. The slurry was stirred overnight, filtered, and the crude product (1.61 g) was filtered and recrystallized twice from water and ethanol to give a pure sample of SBC sulfoxide (0.43 g, melting point was 152–153 °C (uncorrected); in some crystallization batches a melting point of 156–157 °C (uncorrected) was observed, possibly due to varying proportions of the two possible diastereomers). For the synthesis of SBC sulfone, 0.63 ml of perchloric acid (60%) was added cautiously to a solution of ammonium molybdate (91 mg) in water (2.7 ml), and the mixture boiled for 5 min, allowed to cool to touch, filtered, and added slowly to a slurry of SBC (1.13 g) in water (5 ml). 30% H$_2$O$_2$ (6.8 ml) was added to the reaction mixture in small portions with care taken to keep the solution temperature near 4 °C during the addition. The slurry was stirred overnight, filtered, and the crude product (0.46 g) recrystallized from hot water to give a pure sample of SBC sulfone (0.081 g, melting point 162–163 °C (uncorrected); S-(1,2-Dichlorovinyl)-L-cysteine was synthesized by the method of McKinney et al. (24):

Fractionation of Hepatic and Renal Homogenates—Male Sprague-Dawley rats (250–500 g; Charles River Laboratories, Wilmington, MA) were killed by decapitation and the liver and kidneys removed. For localization of hepatic and renal S-oxidase activities, the tissues were washed, homogenized (Potter-Elvehjem) in 3 ml of buffer (0.1 M KC1, 0.1 M KH$_2$PO$_4$, 5 mM EDTA, pH 7.4)/g tissue. Subcellular fractions were prepared by the method of Doen and Anders (15) and were used as the S-oxidase source without further purification. Glu cose-6-phosphatase activity, as measured by the method of Swanson (25), was used as the microsomal marker enzyme; inorganic phosphate was measured by the method of Fiske and Subbarow (26). For characterization of hepatic and renal microsomal S-oxidase activities, the whole tissue homogenates were centrifuged directly at 48,000 X g for 30 min. The supernatant was centrifuged for 90 min at 105,000 X g and the pellet resuspended in buffer and spun again at 105,000 X g for 60 min resulting in "washed microsomes" which were used for enzymatic incubations.

Analysis of SBC Sulfoxide as an Enzymatic Metabolite of SBC—Typical enzymatic incubations of SBC were carried out at 37 °C in a Dubnoff metabolic incubator with continuous shaking. Standard assays were carried out in 0.1 M potassium phosphate buffer pH 7.4, containing 0.1 M KC1, 5 mM EDTA, and 0.9–1.6 mg of protein (liver) or 0.6–1.0 mg of protein (kidney) in a 0.5-ml working volume. Microsomes were incubated for 6 min at 37 °C before SBC was added to begin the enzymatic reaction. After 15 min ice-cold ethanol (0.5 ml) was added, samples were vortexed, and placed on ice until they were centrifuged for 30 min at 4 °C in a Beckman model TJ-6R benchtop centrifuge to remove precipitated proteins. A 0.5-ml aliquot of the supernatant was removed and derivatized with 0.5 ml of 2.4- dinitrophenylhydrazine, incubated at 100 °C for 30 min, cooled in an ice bath, and sodium bisulfite added (0.5%) to reduce any dinitrophenylhydrazones which might form during derivatization. Approximately 1 mg of sample was dissolved in a water/glycerol mixture with the aid of a few drops of concentrated HCl prior to being placed on the target. The spectrum was recorded over the range of m/z 100-500; m/z (relative intensity): 241 (2), 239 (2), 204 (15), 202 (20), 199 (23), 226 (12), 228 (M + 1, 100), 229 (M + 2, 14.7).

Elemental analysis of SBC sulfoxide was performed (Midwest Analytical Instruments), an Anspec SP4270 integrator (Kratos Analytical Instruments), and a Beckman model 167 Scanning Absorbance Detector on a Waters Nova-Pak (3.9 x 75 mm) reverse-phase C-18 column. The mobile phase, acetonitrile and water (pump-A 25%; pump B 75% (v/v)), was run at a flow rate of 1 ml/min. Reference samples of SBC, SBC sulfoxide, and SBC sulfone had retention times of 11.3, 6.6, and 7.5 min, respectively, as determined by HPLC.

HPLC analyses of N-2,4-dinitrophenyl derivatives of SBC sulfoxide and SBC sulfone were performed with a Gilson Gradient Controlled HPLC System (model 302 pumps) equipped with a Rhodyne model 7125 Injector (20-µl loop), a 3-cm Brownie ODS guard column, and a Beckman model 167 Scanning Absorbance Detector on a Waters Nova-Pak (3.9 x 75 mm) reverse-phase C-18 column. The mobile phase, acetonitrile and water (pump A 25%; pump B 75%) was run at a flow rate of 1 ml/min. The gradient went from 100% pump A over 4 min, remained constant at 40% pump A for 2 min, and then went back to 100% pump A over 2 min for a total run time of 8 min. UV detection was performed at 365 nm, and the electronic absorption spectrum of the N-2,4-dinitrophenyl derivative of SBC sulfoxide was recorded between 220-400 nm (Fig. 1C).

HPLC analyses of N-2,4-dinitrophenyl derivatives of SBC sulfoxide and SBC sulfone were performed with a Gilson Gradient Controlled HPLC System (model 302 pumps) equipped with a Rhodyne model 7125 Injector (20-µl loop), a 3-cm Brownie ODS guard column, and a Beckman model 167 Scanning Absorbance Detector on a Waters Nova-Pak (3.9 x 75 mm) reverse-phase C-18 column. The mobile phase, acetonitrile and water (pump A 25%; pump B 75%) was run at a flow rate of 1 ml/min. Reference samples of SBC, SBC sulfoxide, and SBC sulfone had detection limits of nearly 1 part/million.

In Vivo Characterization of SBC S-Oxidase Activity—Hepatic and renal microsomal S-oxidase activities were determined with SBC as substrate, and the amount of SBC sulfoxide formed was determined by HPLC analysis, as described above. All incubations contained SBC (0.13 mM), NADPH (2.0 mM), and NADPH (2.0 mM) in the absence or presence of the selected substrate analogue (5 mM liver; 10 mM kidney).

The ability of the monoclonal antibody to rat cytochrome P-450 reductase (28) to inhibit cytochrome P-450 reductase activity in rat hepatic and renal microsomes was determined using cytochrome c reduction as a marker for cytochrome P-450 activity. The electronic absorption spectrum of electrons to cytochrome P-450. Cytochrome c reduction assays were carried out as described by Lake (29). Briefly, cytochrome c (0.13 mM in 1 ml of 0.1 mM phosphate buffer, pH 7.4), 0.2 ml KCN (15 mM) and 0.1 ml of microsomal suspension containing varying concentrations of antibody were placed into the cuvette. The absorbance was brought up to 2.4 ml with 0.1 mM phosphate buffer. This mixture was vortexed and placed in a Beckman DU-7 spectrophotometer equipped with a kinetics unit set at 37 °C. After 2 min, 0.1 ml of NADPH (10 mM) was added and absorbance at 550 nm versus time was monitored for 3 min. The decreasing rate of cytochrome c reduction due to the increasing concentration of antibody was used to estimate the amount of antibody needed to inhibit the reduction of cytochrome P-450. Experiments to determine the effect of the cytochrome P-450 reductase activity on hepatic and renal S-oxidase activities were conducted using antibody concentrations of 9.3 (liver) and 4.5 (kidney).
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mg antibody/mg microsomal protein. The microsomal protein was mixed with the antibody and kept on ice for 15 min prior to the addition of SBC (2.5 mM, liver; 5.0 mM, kidney) and NADPH (2 mM) and incubating at 37 °C for 20 min.

Protein concentrations were determined by the method of Lowry et al. (30) using bovine serum albumin as standard. Antibody protein concentrations were determined by using $E_{1%}^{1cm} = 1.35$ cm$^{-1}$ (31).

RESULTS

A highly sensitive HPLC assay, which involves the formation, separation, and detection of nanomolar amounts of N-2,4-dinitrophenyl derivatives of SBC and its potential metabolites, SBC sulfoxide and SBC sulfone, was developed to characterize hepatic and renal cysteine conjugate S-oxidases. HPLC analyses of SBC incubation mixtures with rat hepatic and renal microsomes in the presence of NADPH and oxygen resulted in the appearance of a new component on the HPLC chromatogram (Fig. 1B), which had an electronic absorption spectrum (Fig. 1C) and retention time similar to that obtained with a reference sample of SBC sulfoxide (data not shown). These results demonstrate the presence of cysteine conjugate S-oxidases in rat liver and kidney. Whereas no enzymatic formation of SBC sulfoxide was detected, SBC sulfoxide formation was dependent on the presence of NADPH, and oxygen (Fig. 1). Sulfoxidation of SBC by hepatic and renal microsomes was also dependent upon incubation time and protein concentrations (Fig. 2). Kidney microsomal SBC sulfoxidation exhibited a temperature optimum of 45 °C and a pH optimum of 7.2; liver SBC S-oxidase exhibited a similar trend (Fig. 2).

Subcellular localizations of the NADPH-dependent SBC S-oxidase activities in both rat liver and kidney showed that all of the S-oxidase activity was present in the particulate fractions (Table I). Because the renal S-oxidase activity was found in both the microsomal and mitochondrial fractions, the possibility of contamination of the kidney mitochondrial fraction with microsomes was investigated by studying the distribution of glucose-6-phosphatase, a microsomal marker enzyme, in kidney subcellular fractions. The results show that the distribution of glucose-6-phosphatase activity paralleled that of the S-oxidase activity (data not shown). These results indicate that the renal mitochondrial fraction was contaminated significantly with microsomes. Attempts to carry out the S-oxidase assay using subcellular fractions which were prepared by a method reported to yield a pure kidney mitochondrial fraction (32) were not successful. This fractionation method required the use of sucrose, and triethanolamine, which was found to inhibit the hepatic and renal S-oxidase activities (data not shown). The addition of only sucrose to the fractionation buffer, described under "Experimental Procedures," did not improve the purity of the mitochondrial fraction (data not shown). The total recovery of SBC S-oxidase in liver or kidney subcellular fractions was nearly 100% with the nuclear and cell debris fractions containing approximately 30% of the total activity (data not shown).

The kinetics of SBC sulfoxidation in hepatic and renal microsomes were studied using double-reciprocal plots. Renal microsomal SBC S-oxidase exhibited a nearly 3-fold higher $V_{max}$ than hepatic microsomal S-oxidase (Table II).

To determine the type of enzyme catalyzing SBC oxidation, the effects of selected cofactors or inhibitors on SBC S-oxidase activities were determined in liver and kidney microsomes (Table III). The absence of NADPH or removal of oxygen, by a 5- or 10-min nitrogen purge, significantly inhibited SBC S-oxidation in both liver and kidney microsomes. Inclusion of 1-benzylimidazole, a selective cytochrome P-450 inhibitor which does not affect flavin-containing monooxygenase activities (35), or metyrapone, a commonly used inhibitor of the cytochrome P-450 (33-36), had no significant effect on hepatic or renal microsomal SBC S-oxidation. n-Octylamine, a chemical known to inhibit cytochrome P-450, and activate, inhibit, or have no effect on different flavin-containing monooxygenase isoenzymes (35-41), significantly inhibited SBC S-oxidase activities in both liver and kidney microsomes. The flavin-containing monooxygenase alternate substrates, N,N-dimethylaniline and methimazole (35-41), also inhibited SBC S-oxidase. Since methimazole appeared to be such a potent inhibitor of SBC S-oxidase, the kinetics of this inhibition were studied. Using methimazole (18-140 μM) and SBC (1.0 and 4.1 mM, liver; 2.6 and 5.1 mM, kidney), Dixon plots (42) revealed that methimazole is a competitive inhibitor of hepatic and renal microsomal SBC S-oxidases with $K_i$ of 60 and 40 μM, respectively (data not shown).

Because SBC is readily oxidized by hydrogen peroxide,
TABLE I
Distribution of NADPH-dependent S-benzyl-L-cysteine S-oxidase activities in various subcellular fractions of rat liver and kidney

Rat liver and kidney fractions were incubated with S-benzyl-L-cysteine (2.5 mM liver, 5.0 mM kidney), and NADPH (2.0 mM) for 15 min at 37 °C. S-Benzyl-L-cysteine sulfoxide formation was monitored by HPLC after derivatization with 2,4-dinitrofluorobenzene. Results are presented as mean ± S.D. for four preparations.

| Fraction                  | Total protein (mg/g tissue) | Total activity (nmol/min/g tissue) | Yield (%) |
|---------------------------|----------------------------|------------------------------------|-----------|
| Liver                     |                            |                                    |           |
| Whole homogenate          | 130 ± 45                   | 75 ± 51                            | 100       |
| 750 × g, 10-min pellet    | 4 ± 2                      | 4 ± 5                              | 6 ± 7     |
| 9,800 × g, 20-min pellet  | 74 ± 91                    | 0 ± 0                              | 0 ± 0     |
| 105,000 × g, 90-min supernatant | 17 ± 4               | 30 ± 16                            | 58 ± 35   |
| 105,000 × g, 90-min pellet |                            |                                    |           |
| Kidney                    |                            |                                    |           |
| Whole homogenate          | 88 ± 65                    | 134 ± 41                           | 100       |
| 750 × g, 10-min pellet    | 16 ± 3                     | 45 ± 8                             | 38 ± 18   |
| 9,800 × g, 20-min pellet  | 56 ± 20                    | 0 ± 0                              | 0 ± 0     |
| 105,000 × g, 90-min supernatant | 14 ± 7                | 66 ± 42                            | 54 ± 35   |
| 105,000 × g, 90-min pellet |                            |                                    |           |

* Values were corrected for protein recovery.

In one preparation this fraction contained nearly 30% of the total activity.

Chemical inhibition data indicated that cytochrome P-450 might not be involved in SBC sulfoxidation. A monoclonal antibody to cytochrome P-450 reductase has been shown to be effective in determining the relative contribution of cytochrome P-450 and flavin-containing monoxygenases in S- and N-oxidations of various chemicals in microsomes (37, 38, 41). Therefore, to provide further evidence against cytochrome P-450 involvement in SBC S-oxidase activity, a monoclonal antibody to rat cytochrome P-450 reductase was incubated with hepatic or renal microsomes and its effect on SBC sulfoxidation was compared with its effect on microsomal cytochrome c reduction. A concentration of cytochrome P-450 reductase antibody which inhibited nearly 70-80% of hepatic or renal microsome-catalyzed cytochrome c reduction had no effect on SBC sulfoxidation (Fig. 3). The addition of an equal amount of bovine serum albumin to incubations without antibody also had no effect on SBC S-oxidase activities, thereby ruling out the possibility that the increase in protein concentration due to the presence of the antibody did not affect on SBC S-oxidase activities. Further experiments were conducted to determine whether reactive oxygen intermediates contribute significantly to the net sulfoxidation of SBC observed with microsomes. Inclusion of superoxide diamutase, catalase, butylated hydroxyanisole, glutathione, or the peroxidase inhibitor, KCN (43-45), had no effect on hepatic and renal SBC S-oxidase activities. Furthermore, when microsomes were incubated with NADPH for 20 min and ethanol and SBC were added simultaneously, no SBC sulfoxide was detected. This indicates that NADPH does not simply initiate lipid peroxidation or generation of reactive oxygen species (35, 46) which lead to SBC sulfoxidation, but rather is required as a cofactor.

DISCUSSION

In the present study, the presence of cysteine conjugate S-oxidases which catalyzed the conversion of SBC to SBC sulfoxide was demonstrated in rat liver and kidney homoge-
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Rat liver and kidney microsomes were incubated with S-benzyl-L-cysteine and NADPH for 15 min at 37 °C. S-Benzyl-L-cysteine sulfoxide formation was monitored by HPLC after derivatization with 2,4-dinitrofluorobenzene. Results are presented as mean ± S.D. for three preparations.

| Variable substrate | Fixed substrate | $k_{-}$ | $V_{-}$ |
|-------------------|----------------|--------|--------|
| SBC (0.94-3.77 mM) | NADPH (2.5 mM) | 1.6 ± 0.6 | 4.3 ± 1.0 |
| Kidney SPC (0.25-3.77 mM) | NADPH (2.5 mM) | 3.5 ± 0.5 | 11.7 ± 1.4 |

### Table III

| Cofactor or inhibitor | Activity (% of control)* |
|-----------------------|-------------------------|
| Liver                 | Kidney                  |
| −NADPH                | 0                       | 0       |
| −O$_2$ (5 min N$_2$ purge) | 16 ± 14$^a$ | 35 ± 4$^a$ |
| +Methionine (1 mM)    | 99 ± 2                  | 97 ± 4  |
| +1-Benzylimidazole (1 mM) | 83 ± 12 | 81 ± 15  |
| +α-Octylamine (6 mM)  | 48 ± 9$^a$              | 52 ± 9$^a$ |
| +N,N-Dimethylglycine (2 mM) | 31 ± 20$^b$ | 38 ± 11$^b$ |
| +Methimazole (1 mM)   | 5 ± 9$^b$               | 13 ± 12$^b$ |
| Butylated hydroxyanisole (100 μM) | 92 ± 7 | 88 ± 3  |
| +Glutathione (2 mM)   | 96 ± 14                 | 73 ± 13 |
| +Superoxide dismutase (1075 units/ml) | 100,100 | 100,100 |
| +Catalase (5600 units/ml) | 100,100 | 83,93  |
| +KCN (1 mM)           | 87 ± 12                 | 93 ± 7  |

* Cysteine conjugate S-oxidase activity was determined by measuring NADPH-dependent conversion of S-benzyl-L-cysteine (2.5 mM) to S-benzyl-L-cysteine sulfoxide. The results are presented as mean ± S.D. for three experiments. Control values for liver and kidney microsomal S-oxidase activities were 1.9 ± 0.4 and 2.0 ± 0.3 nmol S-benzyl-L-cysteine sulfoxide/mg protein/min, respectively.

### Table IV

| Substrate analogue | Activity (% of control)* |
|-------------------|-------------------------|
| Liver             | Kidney                  |
| S-Methyl-L-cysteine | 53 ± 7$^a$ | 58 ± 9$^a$ |
| S-Carboxymethyl-L-cysteine | 99 ± 2 | 95 ± 5 |
| S-(1,2-Dichlorovinyl)-L-cysteine | 52 ± 7$^a$ | 44 ± 11$^b$ |
| S-Benzyl-L-cysteine methyl ester | 163 ± 55 | 114 ± 6 |

* Cysteine conjugate S-oxidase activity was determined by measuring NADPH-dependent conversion of S-benzyl-L-cysteine (2.5 mM liver; 5 mM kidney) to S-benzyl-L-cysteine sulfoxide in the absence or presence of various substrate analogues (5 mM liver, 10 mM kidney). The results are presented as mean ± S.D. for three experiments. Control values for liver and kidney microsomal S-oxidase activities were 1.3 ± 0.4 and 2.7 ± 0.6 nmol S-benzyl-L-cysteine sulfoxide/mg protein/min, respectively.

### Notes

The cysteine conjugate S-oxidases were characterized by studying the distribution of the activities in rat hepatic and renal subcellular fractions, and by studying the biochemical mechanism and other selected properties of the reaction. As shown in Tables I-III and Figs. 1 and 2, the S-oxidase activity which was present mostly in the microsomal fractions of both liver and kidney, was dependent on the presence of oxygen, NADPH, incubation temperature, time, pH, and protein concentration. S-Methyl-L-cysteine and S-(1,2-dichlorovinyl)-L-cysteine inhibited the hepatic and renal SBC S-oxidase activities, whereas S-carboxymethyl-L-cysteine and S-benzyl-L-cysteine methyl ester had no effect. (Table IV). These results suggest that cysteine S-conjugates which have a non-ionizable alkyl group on the sulfur atom, or which do not have an esterified carboxyl group on the cysteine moiety, are likely to act as substrates for the S-oxidases. Renal microsomes catalyzed SBC S-oxidation at a rate that was nearly 3-fold higher than the rate obtained with liver microsomes (Tables I and II, Fig. 2). Although the reason for this rate difference is not clear, inclusion of the cytochrome P-450 reductase antibody did not alter the preferential sulfoxidation of SBC in kidney microsomes compared with liver microsomes (Fig. 3). This indicates that reduction of SBC sulfoxide by cytochrome P-450 reductase did not contribute to the observed rate difference. SBC sulfoxidation is, to our knowledge, the first example of a sulfoxidation reaction that is preferentially catalyzed by kidney microsomes compared with liver microsomes. It should be noted, however, that metabolism of...
cysteine S-conjugates to mercapturic acids is also preferentially catalyzed by kidney microsomes compared with liver microsomes (5, 6).

Microsomal enzymes such as the cytochrome P-450 family of monoxygenases, the flavin-containing monoxygenases, and prostaglandin synthetase are known to catalyze the conversion of many different sulfides to sulfoxides (38, 40–52). The findings that the S-oxidase activities were dependent on NADPH, and that inclusion of catalase or KCN in the incubation mixture did not affect the SBC S-oxidase activities, provide evidence against the participation of peroxides as prostaglandin synthetase in the NADPH-dependent S-oxidase activities. Similarly, involvement of reactive oxygen intermediates, such as hydrogen peroxide and superoxide anion, which can be generated from the cytochrome P-450 containing monoxygenases (46) or lipid peroxides, generated from peroxidative processes of microsomes (35, 43–46) was ruled out by demonstrating that SBC S-oxidase activities were not inhibited by catalase, superoxide dismutase, butylated hydroxyanisole, or glutathione (Table III). In addition, the findings that the S-oxidase activities were not inhibited by the cytochrome P-450 inhibitors, metyrapone and 1-benzylimidazole (Table III), or by a monoclonal antibody against cytochrome P-450 reductase (Fig. 3) provide clear evidence against the involvement of cytochrome P-450 in the metabolic reaction.

The flavin-containing monoxygenase alternate substrates, N,N-dimethylaniline, n-octylamine, and methimazole, significantly inhibited SBC S-oxidase activities in both hepatic and renal microsomes (Table III). These results indicate that SBC S-oxidase activities may be associated with flavin-containing monoxygenases. Methimazole saturates the flavin-containing monoxygenases at concentrations less than 200 μM, and at concentrations less than 2 mM, methimazole is S-oxygenated at measurable rates only by the flavin-containing monoxygenases (35). Thus, the finding that methimazole is an effective competitive inhibitor of the hepatic and renal S-oxidase activities with K values of 60 and 40 μM, respectively, provides further evidence for the involvement of flavin-containing monoxygenases in S-oxidation.

The results presented in this report indicate that cysteine S-conjugates may act as substrates for flavin-dependent monoxygenase enzymes. It should be noted, however, that a conclusive statement regarding this apparent new type of S-conjugate oxidation is not clear. The products of cysteine conjugate S-oxidase, i.e., the sulfoxides, would be expected to be more polar than the sulfides, and hence S-oxidase may affect the distribution and excretion of cysteine S-conjugates. Oxidation of the sulfur atom of S-(2-haloethyl)-L-cysteine, a potential metabolite of 1,2-dihaloethane, is expected to prevent the non-enzymatic rearrangement of the molecule to form a reactive episulfonium ion. Thus, S-oxidation of S-(2-haloethyl)-L-cysteine may represent a detoxication reaction. Sulfoxidation may also affect the activities of enzymes, e.g., cysteine conjugate N-acetyltransferase and cysteine conjugate β-lyase, which are involved in the detoxication and bioactivation of cysteine S-conjugates (5–14). Thus, it is also possible that sulfoxides of cysteine S-conjugates may be more toxic than the sulfide forms.

In conclusion, the results presented in this report describe the development of a highly sensitive HPLC assay which was used to characterize cysteine conjugate S-oxidase activities in rat liver and kidney microsomes. The usefulness of cofactor dependence, selective inhibitors, and monoclonal antibodies in studying the mechanism of SBC S-oxidase was demonstrated. Future studies, which examine the metabolism and toxicity of nephrotoxic and mutagenic cysteine S-conjugates and their corresponding sulfoxides, should elucidate the role of this enzyme in the bioactivation of cysteine S-conjugates. Enzyme purification studies and immunochemical comparison with known forms of flavin-containing monoxygenases should also reveal the characteristics of the hepatic and renal microsomal cysteine conjugate S-oxidases.

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