0026-895X/94/061217-09$03.00/0

MOLECULAR PHARMACOLOGY, 46:1217-1225, 1994.

Glutathione- and Glutathione-S-Transferase-Dependent Oxidative Desulfuration of the Thione Xenobiotic Diethyldithiocarbamate Methyl Ester

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Received April 18, 1994; Accepted September 16, 1994

SUMMARY

Oxidative desulfuration of diethyldithiocarbamate methyl ester (DDTC-Me), a thione xenobiotic and a metabolite of disulfiram, was studied. Using a rat liver microsomal incubation system, DDTC-Me was oxidized at the thionosulfur group, forming DDTC-Me sulfine. Only minimal desulfuration of DDTC-Me to *S*-methyl-*N*,*N*-diethylthiolcarbamate (DETC-Me) occurred. Desulfuration of DDTC-Me increased 4-fold when the microsomal incubation was supplemented with reduced glutathione (GSH) and increased 8-fold when both GSH and glutathione-*S*-transferase (EC 2.5.1.18) were added. Similar results were obtained using a simplified system containing DDTC-Me sulfine, GSH, and glutathione-*S*-transferase. This suggested that DDTC-Me sulfine is a stable intermediate formed before DDTC-Me is desulfurated to DETC-Me. This unprecedented desulfuration process can be explained as follows. GSH attacks the oxithiirane isomer of DDTC-Me sulfine, resulting in ring opening followed by loss of glutathione hydrodisulfide, which is reduced by GSH to oxidized glutathione and H_2S. GSH can also reduce DDTC-Me sulfine to DDTC-Me. This mechanism is supported by *in vitro* studies. An approximately 1:1 stoichiometry was observed for the formation of H_2S and DETC-Me. A 1:1 stoichiometry was also observed for the consumption of DDTC-Me sulfine, formation of DETC-Me plus DDTC-Me, and formation of oxidized glutathione. Glutathione hydrodisulfide was trapped by derivatization *in situ* using 4-vinylpyridine. Oxidative desulfuration of a series of dithiocarbamate esters also followed a similar mechanism.

Sulfines (*S*-oxides) have been suggested to be intermediates during the oxidative desulfuration of various thione compounds (1). Examples include parathion (2), α-naphthylisothiocyanate (3), carbon disulfide (4), thioacetamide (5), and thiobenzamide (6). Several mechanisms have been proposed to explain the loss of the sulfur atom from these thione compounds. For example, thioacetamide sulfine (*S*-oxide) is postulated to be further oxidized by microsomal monooxygenases to the reactive and unstable intermediate thioacetamide sulfene (*S*,*S*-dioxide). The thioacetamide sulfene either can covalently bind to proteins (7) or can be attacked by water, resulting in the formation of acetamide and sulfoxylate (HSO_2^-) (5). A different mechanism has been proposed for the desulfuration of carbon disulfide (CS_2) (3), wherein the sulfine rearranges to an unstable oxithiirane thionosulfur, which subsequently degrades to carbonyl sulfide and a singlet sulfur atom. A mechanism similar to that of desulfuration of carbon disulfide has been postulated for the desulfuration of parathion (1). Although these hypothetical mechanisms provide an explanation for the desulfuration process, the exact nature of the leaving sulfur atom has not yet been established. The microsomal monooxygenase-mediated formation of sulfines or their further bioactivation to sulfene has been implicated in the toxic and adverse effects of thione compounds, including the inhibition of cytochrome P450, hepatotoxicity, pneumotoxicity, bone marrow depression, and induction of cancers (8).

DDTC-Me is a thione compound (Fig. 1) and a metabolite of disulfiram (9–11), a drug used clinically for the treatment of alcoholism. DDTC-Me is oxidatively desulfurated to DETC-Me in rats (12, 13) (Fig. 1). DETC-Me is found in human blood after the administration of disulfiram (14), suggesting that oxidative desulfuration of DDTC-Me also occurs in humans. Disulfiram has been reported to be hepatotoxic in humans (15) and also to inhibit cytochrome P450 (16). It is therefore possible that the toxic effects of disulfiram may be related to the oxidative desulfuration of DDTC-Me.

In an NADPH-dependent microsomal incubation, DDTC-

This work was supported in part by the National Institute on Alcohol Abuse and Alcoholism (Grant AA03577).

ABBREVIATIONS: DDTC-Me, diethyldithiocarbamate methyl ester; DETC-Me, *S*-methyl-*N*,*N*-diethylthiolcarbamate GSH, reduced glutathione; GSSG, oxidized glutathione; CAD, collision-activated decomposition; GSSH, glutathione hydrodisulfide; GSEP, *S*-[2-(4′-pyridyl)ethyl]glutathione; GSSEP, glutathione 2-(4′-pyridyl)ethyl disulfide; HPLC, high performance liquid chromatography; FAB, fast atom bombardment; EPTC, *S*-ethyl, *N*,*N*-di-*n*-propylthiolcarbamate.

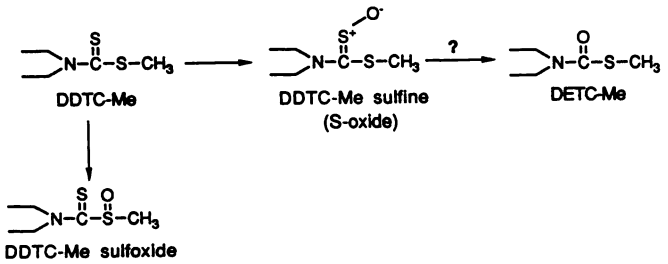

Fig. 1. *In vitro* metabolism of DDTC-Me in rat liver microsomes. DDTC-Me sulfoxide and DDTC-Me sulfine were identified as the major metabolites when DDTC-Me was incubated with rat liver microsomes and NADPH. DETC-Me was found only as a minor metabolite. ?, the mechanism of the conversion of DDTC-Me sulfine to DETC-Me is not clear.

Me sulfine and DDTC-Me sulfoxide were recently identified as major *in vitro* metabolites that are formed as a result of the regioselective *S*-oxidation of DDTC-Me (Fig. 1) (17). However, in those studies DETC-Me, the desulfurated form of DDTC-Me, was found to be only a minor metabolite. Studies were therefore carried out to investigate the *in vitro* desulfuration of DDTC-Me and DDTC-Me sulfine in greater detail. The results of these studies suggest that DDTC-Me sulfine is desulfurated by a novel mechanism that is dependent on GSH and glutathione-*S*-transferases (EC 2.5.1.18). Oxidative desulfuration of a series of dithiocarbamate esters also was studied, and these thione compounds also were found to undergo desulfuration by a similar mechanism. This novel GSH-dependent mechanism of desulfuration may help provide a better understanding of the various toxicological processes associated with thione compounds.

Materials and Methods

Chemicals. DDTC-Me (18), DETC-Me (12), and DDTC-Me sulfine (17) were synthesized as described previously. The series of dithiocarbamate esters (Table 1) were synthesized by reacting the appropriate amine with carbon disulfide under alkaline conditions, followed by alkylation of the sodium dithiocarbamate, using either ethyl iodide or propyl iodide, by a modification of the method of Koch (19), as described by Faiman *et al.* (18). Ethiolate and parathion were purchased from Crescent Chemical Co. (Hauppauge, NY). Molinate, butylate, EPTC, and vernolate were generous gifts from Dr. Thomas Castles (Stauffer Chemical Co., Richmond, CA). All other chemicals, including purified rat liver glutathione-*S*-transferase (mixture of the cytosolic and microsomal isozymes) (EC 2.5.1.18), were purchased from Sigma Chemical Co. (St. Louis, MO). C-18 Extract Clean solid-phase extraction columns were purchased from Alltech (Deerfield, IL).

Preparation of microsomes and cytosolic fraction. Sprague-Dawley-derived mature male rats (200–300 g, 7–8 weeks of age) were used for the preparation of microsomes. Rats were treated with 80 mg/kg sodium phenobarbital (dissolved in 1 ml/kg saline) intraperitoneally once daily for 3 days. The microsomes were prepared 24 hr after the last dose of phenobarbital. Both untreated and phenobarbital-treated fasted rats were anesthetized with CO_2 and killed by decapitation. The livers were removed and homogenized in 0.1 M potassium phosphate buffer, 7.4, containing 1.12% (w/v) KCl and 1 mM EDTA, at 4°. The homogenate was centrifuged for 15 min at 3000 × *g*, and the resulting supernatant was centrifuged for 20 min at 12,000 × *g*. The pellet was discarded and the supernatant was centrifuged for 60 min at 105,000 × *g*. The microsomal pellet was washed once and resuspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, as described previously (20). For the preparation of the cytosolic fraction, the rat livers were perfused with the 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, to remove all hemoglobin contamination in the cytosolic fraction. The remainder of the procedure was identical to that described for the preparation of microsomes, except that the supernatant obtained after the first 105,000 × *g* centrifugation was used as the cytosolic fraction. The cytosolic fraction and microsomes were assayed for protein content by the method of Lowry *et al.* (21). The microsomes and cytosolic fraction were frozen and stored at −70° until used.

Microsomal incubations. DDTC-Me or DDTC-Me sulfine was incubated at 37° for 30 min in a 1.5-ml incubation mixture, which contained 0.1 M potassium phosphate buffer, pH 7.4, 1 mM EDTA, microsomes from untreated rats (0.67 mg/ml), 0.67 mM $NADP^+$, 6.67 mM glucose-6-phosphate, and 0.67 unit/ml glucose-6-phosphate dehydrogenase, with or without other additions used as described in Results. The reactions were stopped by the addition of 20 μl of 800 mM $CaCl_2$. After the incubation was stopped, DDTC-Me sulfine, DETC-Me, and DDTC-Me in the reaction mixture were extracted by solid-phase extraction and quantitatively determined by reverse phase HPLC (column, Beckman/Altex, C-18, 250 × 4.6 mm, 5-μm particle size; mobile phase, 50% acetonitrile/50% water; flow rate, 1 ml/min; detection, 215 nm) by a method described in detail previously (17). Retention times of DDTC-Me sulfine, DETC-Me, and DDTC-Me were 3.7, 6.7, and 14.0 min, respectively.

Incubation of DDTC-Me sulfine with GSH and glutathione-*S*-transferase. DDTC-Me sulfine (250 μM) was incubated with GSH (10 mM) and varying amounts of purified glutathione-*S*-transferase (0–

TABLE 1
Some characteristics of the thione compounds studied

Thione compound [R-C(S)-R′] name	Structural features [R-C(X)-R′][a]		Desulfurated metabolite [R-C(O)-R′]		
	R	R′	Name	Acetonitrile in the mobile phase[b] %	Retention time[c] min
DDTC-Me	$(ET)_2N$	S-Me	DETC-Me	60	5.0
Ethiolate *S*-analog	$(Et)_2N$	S-Et	Ethiolate	60	6.6
EPTC *S*-analog	$(nPr)_2N$	S-ET	EPTC	70	8.2
Molinate *S*-analog	$(CH_2)_6N$[d]	S-Et	Molinate	70	6.5
Vernolate *S*-analog	$(nPr)_2N$	S-(nPr)	Vernolate	80	7.3
Butylate *S*-analog	$(iBu)_2N$	S-Et	Butylate	80	8.7
Thiobenzamide	Ph	NH_2	Benzamide	40	3.2

[a] The structural features of the thione compounds (X = S) and the desulfurated metabolites (X = O) are given. The thionosulfur group is replaced by a carbonyl group in the desulfurated metabolite. Ethiolate *S*-analog, diethyldithiocarbamate ethyl ester; EPTC *S*-analog, *n*-proply,*n*-propyldithiocarbamate ethyl ester; molinate S-analog, homopiperidyldithiocarbamate ethyl ester; vernolate *S*-analog, *n*-propyl, *n*-propyldithiocarbamate *n*-propyl ester; butylate S-analog, isobutyl,isobutylbsobuthyldithiocarbamate ethyl ester. Et, ethyl; Me, methyl; nPr, *n*-propyl; iBu, isobutyl; Ph, phenyl.
[b] The mobile phase used for HPLC analysis is expressed as percentage (v/v) of acetonitrile in water.
[c] The retention times of the desulfurated metabolites in HPLC analysis are given.
[d] The R group of molinate is homopiperidine.

3 units/ml), in 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, for 30 min at 37°. The incubation volume was 1 ml. The cytosolic fraction (2 mg/ml), when present, was used as an alternate source of glutathione-*S*-transferase. Incubations were stopped by freezing in a dry ice/2-propanol bath and were analyzed for DDTC-Me sulfine, DETC-Me, DDTC-Me, H_2S, and GSSG. The amounts of DDTC-Me sulfine, DETC-Me, and DDTC-Me were determined as described above.

For the determination of H_2S, the reaction was carried out in vials that were sealed with rubber septa to prevent loss of H_2S. The H_2S formed was detected and quantified as the amount of methylene blue formed after derivatization of H_2S with *N*,*N*-dimethyl-*p*-phenyldiamine and ferric nitrate (22). The amount of methylene blue in each reaction was determined by reverse phase HPLC (column, Beckman/Altex, C-18, 250 × 4.6 mm, 5-μm particle size; mobile phase, 0.5%, v/v, glacial acetic acid/0.1%, w/v, pentanesulfonic acid/80% acetonitrile/19.5% water; flow rate, 1.5 ml/min; detection, 664 nm; retention time of methylene blue, 2.4 min). A multiple-point calibration curve was generated with sodium sulfide (98% pure) as the source of standard for H_2S.

GSSG was determined by reverse phase HPLC (column, Beckman/Altex, C-18, 250 × 4.6 mm, 5-μm particle size; mobile phase, 2.5%, v/v, acetonitrile/0.1%, v/v, trifluoroacetic acid/97.4%, v/v, water; flow rate, 1 ml/min; detection, 215 nm; retention time of GSSG, 6.5 min).

Derivatization of GSH and GSSH. Incubation of DDTC-Me sulfine (1 mM), GSH (5 mM), and purified glutathione-*S*-transferase (1 unit/ml) was carried out in 0.1 M potassium phosphate buffer, pH 7.4, in a final volume of 5 ml, at room temperature. After 1 min of incubation, 4-vinylpyridine (final concentration, 10 mM) was added to the reaction mixture. Thirty minutes after addition of 4-vinylpyridine, the pH of the reaction mixture was adjusted to 3.0 using 1 N HCl, and the entire sample was applied to a C-18 Extract Clean column that had been washed with methanol and equilibrated with 0.1 M potassium phosphate buffer, pH 3.0. The column was then washed with 1 ml of deionized water and the glutathione derivatives were eluted with 1 ml of acetonitrile. The acetonitrile eluant was evaporated to dryness and the residue obtained was subjected to mass spectral analysis.

Mass spectral analysis of glutathione derivatives. Mass spectra were obtained with an AUTOSPEC-Q tandem hybrid mass spectrometer (Fisons/VG Analytical Ltd., Manchester, UK) equipped with an OPUS data system. FAB experiments were performed using a cesium gun operated at 20-keV energy and 2-μA emission. Samples derived from the vinyl pyridine-trapping experiment were dissolved in a 1:1 mixture of acetonitrile and 0.1% (v/v) trifluoroacetic acid in water and were added to the thioglycerol/glycerol (3:1) matrix. CAD experiments were performed with precursor ions attenuated to 45% with xenon in the collision octopole (GB Scientific, Navato, CA, and Dr. Julie Leary, University of California at Berkely, Berkely, CA). The collision energy used was 140 eV (laboratory frame of reference). The analyzer quadrupole was tuned to 1.5-AM unit full width at half-height and the precursor ion was transmitted with mass spectrometer 1 tuned to 1500 resolution. The scan rate was 1 sec/100 units, and five scans were integrated.

Microsomal incubation of other thione-containing compounds. Liver microsomes (1 mg/ml) obtained from phenobarbital-treated rats were incubated with each of the various thione compounds (1 mM) (Table 1) at 37° for 30 min. The incubation mixtures also included 0.1 M potassium phosphate buffer, pH 7.4, 1 mM EDTA, 0.67 mM $NADP^+$, 6.67 mM glucose-6-phosphate, and 0.67 unit/ml glucose-6-phosphate dehydrogenase, with or without 10 mM GSH and 2 units/ml glutathione-*S*-transferase. The incubation volume was 0.5 ml. The reaction was stopped by the addition of 0.5 ml of ice-cold acetonitrile, the precipitated proteins were removed by centrifugation, and the amount of desulfurated metabolite in the supernatant was determined by reverse phase HPLC, without additional sample preparation. The column and conditions used were as follows: column, Beckman/Altex, C-18, 250 × 4.6 mm, 5-μm particle size; flow rate, 1 ml/min; detection, 215 nm. The mobile phase and retention time for each metabolite studied are described in Table 1.

Results and Discussion

Ample evidence exists that suggests that formation of sulfines (*S*-oxides) is required before oxidative desulfuration of thione compounds occurs (1). Rearrangement of the sulfine group and further oxidation of the sulfine to a sulfene are two possible mechanisms that lead to the formation of intermediates through which the loss of sulfur from the sulfine can be rationalized (1). When DDTC-Me was incubated with rat liver microsomes and NADPH, DDTC-Me sulfine was found to be the major metabolite (254 nmol formed/30-min incubation/mg of protein), but the extent of desulfuration was minimal (3.5 nmol of DETC-Me formed/30-min incubation/mg of protein) (Table 2). In these incubations, the substrate DDTC-Me was present in excess because the rate of formation of metabolites was being studied. Therefore, the possibility existed that unoxidized DDTC-Me was competing with DDTC-Me sulfine for the same monooxygenase, thus preventing further oxidation of DDTC-Me sulfine to the sulfene and also reducing the extent of desulfuration. However, even when DDTC-Me sulfine was directly incubated with microsomes and NADPH, desulfuration to DETC-Me was not observed (Table 2). These data, taken together with the observations that DDTC-Me is desulfurated to DETC-Me *in vivo* in rats (12) and also in the postmitochondrial, 9000 × *g* supernatant fraction obtained from rat liver homogenates (data not shown), suggested that a nonmicrosomal enzyme system and/or cofactors may be required for the desulfuration of DDTC-Me sulfine to DETC-Me.

GSH is the most abundant cofactor present in the S9 fraction of liver homogenates. Therefore, the possible role of GSH and glutathione-*S*-transferase in the desulfuration of DDTC-Me was investigated. When GSH was included in the microsomal incubation containing DDTC-Me and NADPH, no DDTC-Me sulfine was detected and the formation of DETC-Me was increased 4-fold (Table 2). Furthermore, DETC-Me formation was increased approximately 8-fold when both GSH and glutathione-*S*-transferase were added to the incubation containing DDTC-Me, microsomes, and NADPH (Table 2). The desulfuration of DDTC-Me was also dependent on the presence of NADPH (Table 2), which suggested that the formation of DDTC-Me sulfine was required for desulfuration to occur. These data therefore suggested that desulfuration of DDTC-Me sulfine occurred by a mechanism that required GSH and was further enhanced by glutathione-*S*-transferase.

To investigate this unprecedented mechanism of desulfuration in additional detail, studies were carried out in a simplified *in vitro* system that contained DDTC-Me sulfine, GSH, and a purified preparation of glutathione-*S*-transferases (containing a mixture of all isozymes of glutathione-*S*-transferase found in rat liver) (Table 3). When GSH was mixed with DDTC-Me sulfine, all DDTC-Me sulfine was converted to DETC-Me or DDTC-Me. Furthermore, this reaction was complete within 1 min of the addition of GSH (data not shown). In the presence of GSH, ~8% of the DDTC-Me sulfine was converted to DETC-Me (20 nmol/incubation), with the remaining DDTC-Me sulfine being reduced to DDTC-Me (189 nmol/incubation) (Table 3). However, when glutathione-*S*-transferase and GSH were incubated with DDTC-Me sulfine, formation of DETC-Me was favored such that ~40% of the DDTC-Me sulfine was converted

TABLE 2

Desulfuration of DDTC-Me and DDTC-Me sulfine in rat microsomes

The incubation system consisted of microsomes (0.67 mg/ml), DDTC-Me (1 mM), and an NADPH-generating system. GSH and glutathione-*S*-transferase, when added, were present at final concentrations of 10 mM and 0.17 unit/ml, respectively. The incubation volume was 1.5 ml. DDTC-Me sulfine, when used as a substrate, was present at a final concentration of 250 μM. The incubation was initiated by the addition of DDTC-Me or DDTC-Me sulfine and was maintained at 37° for 30 min, and the reactions were stopped by the addition of $CaCl_2$. The amounts of DDTC-Me sulfine, DETC-Me, and DDTC-Me in each incubation were determined by HPLC, as described in Materials and Methods. The data are expressed as means ± standard errors of four incubations.

Incubation system	Amount		
	DDTC-Me sulfine	DETC-Me	DDTC-Me
	nmol formed or nmol remaining/30-min incubation/mg of protein		
DDTC-Me + NADPH	0 ± 0	1.1 ± 0.0	1300 ± 23
Microsomes + DDTC-Me + NADPH	254 ± 13	3.5 ± 0.4	1040 ± 29
Microsomes + DDTC-Me + NADPH + GSH	0 ± 0	14.5 ± 0.5 (4-fold)[a]	1147 ± 39
Microsomes + DDTC-Me + NADPH + GSH + glutathione-*S*-transferase	0 ± 0	26.0 ± 0.7 (8-fold)	1209 ± 33
Microsomes + DDTC-Me + GSH + glutathione-*S*-transferase	0 ± 0	1.4 ± 0.4	1232 ± 17
Microsomes + DDTC-Me sulfine	266 ± 7.5	0 ± 0	0 ± 0
Microsomes + DDTC-Me sulfine + NADPH	265 ± 9.6	0 ± 0	0 ± 0

[a] Values in parentheses, fold increase in formation of DETC-Me, compared with the group containing microsomes, DDTC-Me, and NADPH only.

TABLE 3

GSH-dependent desulfuration of DDTC-Me sulfine

The incubation system consisted of DDTC-Me sulfine (250 μM), GSH (10 mM), and purified rat glutathione-*S*-transferase (0.75 unit/ml) or the cytosolic fraction (2 mg/ml) obtained from untreated rat liver. The incubation volume was 1 ml. The boiled glutathione-*S*-transferase (final concentration, 1.5 unit/ml) or the boiled cytosolic fraction (final concentration, 2 mg/ml) were separately placed in a boiling water bath for 2 min, cooled, and then added to the incubation system. The incubation was started by the addition of DDTC-Me sulfine and was maintained at 37° for 30 min, at the end of which the reactions were stopped and the DDTC-Me sulfine remaining and DETC-Me and DDTC-Me formed were determined by HPLC, as described in Materials and Method. The data are expressed as means ± standard errors of four incubations.

Incubation system	Amount			
	DDTC-Me sulfine	DETC-Me	DDTC-Me	Total metabolites[a]
	nmol formed or nmol remaining/30-min incubation			
DDTC-Me sulfine	258 ± 6.3	0 ± 0	0 ± 0	258
DDTC-Me sulfine + GSH	0 ± 0	20 ± 0.8	189 ± 6.9	219
DDTC-Me sulfine + GSH + glutathione-*S*-transferase	0 ± 0	100 ± 0.7	138 ± 10	238
DDTC-Me sulfine + GSH + boiled glutathione-*S*-transferase	0 ± 0	19 ± 0.4	199 ± 4.4	218
DDTCMe sulfine + GSH + cytosolic fraction	0 ± 0	91 ± 2.7	97 ± 7.0	188
DDTC-Me sulfine + GSH + boiled cytosolic fraction	0 ± 0	21 ± 0.0	178 ± 1.6	199

[a] Total metabolites are the sum total of the means (nmol) of DDTC-Me sulfine, DETC-Me, and DDTC-Me for each group.

to DETC-Me (100 nmol/incubation), whereas ~53% was reduced to DDTC-Me (138 nmol/incubation) (Table 3). The increase in the formation of DETC-Me in the presence of GSH and glutathione-*S*-transferase was dependent on the amount of glutathione-*S*-transferase added to the incubation mixture (Fig. 2). The increase in desulfuration from 8% to 40% in the presence of glutathione-*S*-transferase could be abolished if the purified glutathione-*S*-transferase enzyme was boiled before being added to the incubation (Table 3). Similar findings were observed when the cytosolic fraction from rat liver homogenates was used instead of purified glutathione-*S*-transferase (Table 3). Because the purified glutathione-*S*-transferase contained a mixture of both cytosolic and microsomal isozymes, it is not known which transferases are involved in this reaction.

To explain the GSH-dependent desulfuration of DDTC-Me sulfine, and the change in the product ratio when glutathione-*S*-transferase is also added, several mechanisms of desulfuration can be proposed (Fig. 3B). According to one such mechanism, attack of GSH on the oxithiirane ring (II) can result in ring opening, leading to the formation of an intermediate (III) that can break down to DETC-Me and GSSH. The GSSH can then react with another GSH molecule, resulting in the formation of GSSG and H_2S. The intermediate III can also be degraded to methanethiol and VI. Alternatively, attack of GSH

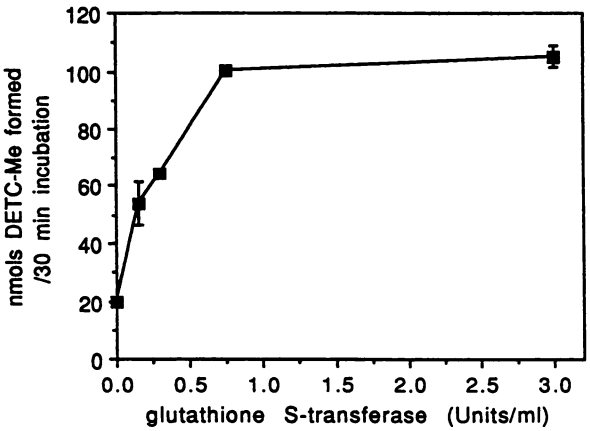

Fig. 2. GSH-dependent desulfuration of DDTC-Me sulfine in the presence of glutathione-*S*-transferase. The incubation system consisted of DDTC-Me sulfine (250 μM), GSH (10 mM), and various concentrations of rat glutathione-*S*-transferase. The incubations were started by the addition of DDTC-Me sulfine and were maintained at 37° for 30 min. The reaction was then stopped and the amount of DETC-Me formed was determined by HPLC, as described in Materials and Methods. The data are expressed as means ± standard errors of three or four incubations. When the standard error bars are not visible, they fall within the data points.

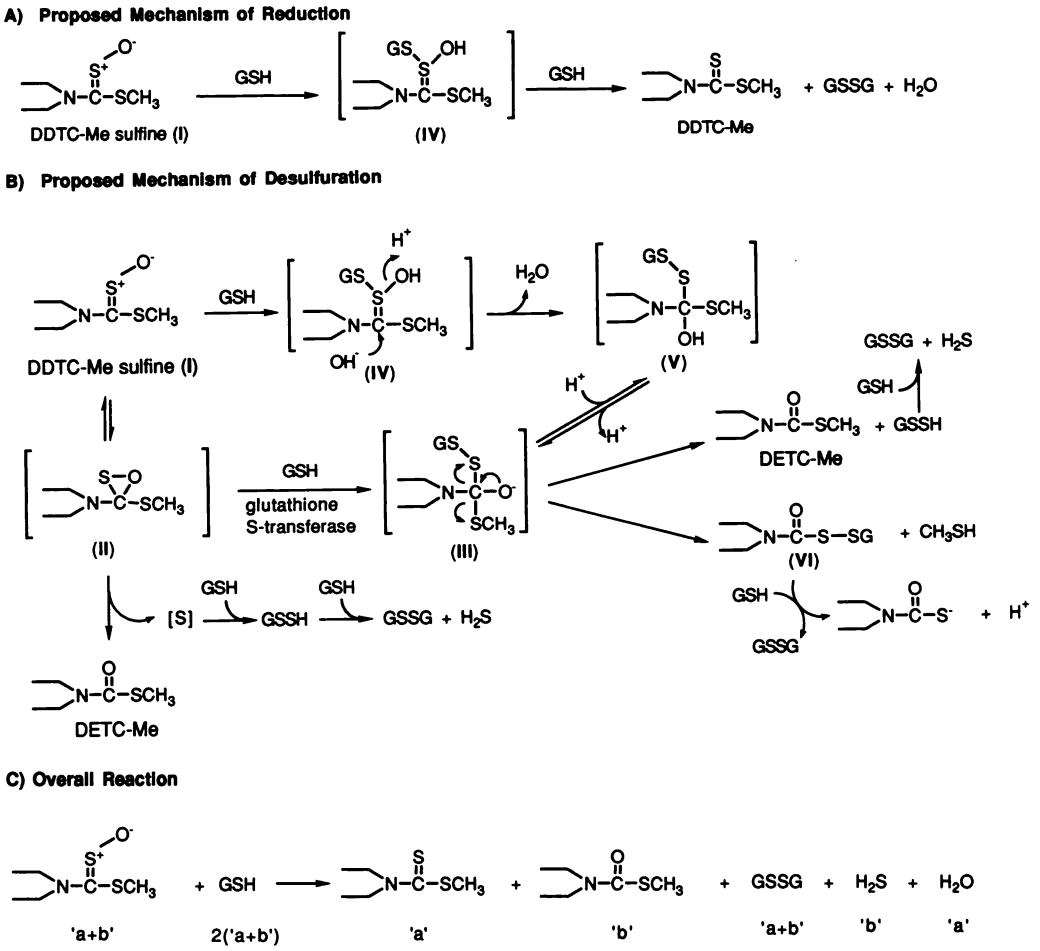

Fig. 3. A, Proposed mechanism for GSH-dependent reduction of DDTC-Me sulfine. B, Proposed mechanism for GSH- and glutathione-*S*-transferase-dependent desulfuration of DDTC-Me sulfine. C, Stoichiometry of the proposed reaction. *a* and *b*, number of moles of each product formed in the overall reaction.

on the sulfine sulfur (**I**) can result in the formation of **IV**, which is susceptible to attack by water, accompanied by loss of a water molecule and the rearrangement of **IV** to a tetrahedral intermediate (**V**). Deprotonation of **V** results in the formation of **III**, followed by formation of DETC-Me and GSSH similarly to the mechanism described above. Another possible mechanism is the spontaneous decomposition of the oxithiirane ring (**II**) to DETC-Me and elemental sulfur, as suggested previously for other thione compounds (1).

DDTC-Me sulfine was also found to be reduced in the presence of GSH. The mechanism of reduction can be rationalized by attack of GSH on DDTC-Me sulfine (**I**), which neutralizes the formal positive charge on the sulfur center, resulting in the formation of **IV** (Fig. 3A). In the next step, another molecule of GSH attacks **IV**, ultimately forming GSSG, DDTC-Me, and water. The rupture of the S-O bond in **IV** constitutes the reduction of DDTC-Me sulfine to DETC-Me, coupled with the oxidation of GSH to GSSG. The reduction of sulfines by thiols has previously been observed with thiobenzophenone-*S*-oxide and thiobenzamides (23, 24).

According to the proposed mechanisms and the overall reaction (Fig. 3C), there should be a 1:1 stoichiometry between the formation of DETC-Me and H_2S and between the consumption of DDTC-Me sulfine, the formation of DETC-Me plus DDTC-Me, and the formation of GSSG. Mass balance studies were therefore carried out to rationalize the desulfuration of DDTC-Me sulfine by one or more of the proposed mechanisms. The amount of DETC-Me formed was found to be approximately equal to the amount of H_2S produced in the incubations (Table 4). In addition, the amount of GSSG formed was approximately equal to the amount of DETC-Me plus DDTC-Me formed (Table 4). Mass balance was observed in both groups, that is, with and without glutathione-*S*-transferase. These mass balances are approximate, possibly because of some variability in the determination of absolute amounts of H_2S, GSSG, DETC-Me, and DDTC-Me by HPLC. The stoichiometry is better delineated when the ratios of the values for the group containing GSH only to the values for the group containing both GSH and glutathione-*S*-transferase are compared (Table 4). Comparison of these ratios negates possible variations associated with each HPLC assay. These data thus provided evidence that H_2S and GSSG were formed in amounts supporting the mechanisms of desulfuration and reduction of DDTC-Me illustrated in Fig. 3.

To explore the mechanism of desulfuration further, we attempted to trap GSSH before it could react with GSH to form H_2S. GSSH was trapped by derivatization *in situ* with 4-vinylpyridine. The derivatized products were extracted by solid-

TABLE 4

Stoichiometry of the formation of DETC-Me, DDTC-Me, H_2S, and GSSG during desulfuration of DDTC-Me sulfine

The incubation system consisted of DDTC-Me sulfine (250 μM), GSH (10 mM), and purified rat liver glutathione-*S*-transferase (2 units/ml). The incubation volume was 1 ml. The incubation was started by the addition of DDTC-Me sulfine and was maintained as 37° for 30 min. The reaction was stopped and the DDTC-Me sulfine remaining and DETC-Me and DDTC-Me formed were determined by HPLC, as described in Materials and Methods. The amounts of H_2S and GSSG formed in the incubations were determined by HPLC assays as described in Materials and Methods. The data are expressed as means ± standard errors of four incubations.

Incubation system	Amount					
	DDTC-Me sulfine	DETC-Me	DDTC-Me	DETC-Me + DDTC-Me	H_2S	GSSG
	nmol formed or nmol remaining/30-min incubation					
DDTC-Me sulfine	224 ± 3.6	0 ± 0	0 ± 0	0	0 ± 0	0 ± 0
DDTC-Me sulfine + GSH	0 ± 0	13 ± 0.4	149 ± 1.1	162	8.2 ± 0.4	146 ± 0.8
DDTC-Me sulfine + GSH + glutathione-*S*-transferase	0 ± 0	83 ± 1.8 (6.4)[a]	76 ± 2.3 (0.5)	158 (1.0)	64 ± 3.4 (7.8)	181 ± 4.6 (1.2)

[a] Values in parentheses, ratio of DDTC-Me sulfine plus GSH with glutathione-*S*-transferase without glutathione-*S*-transferase.

phase extraction, and the GSSEP in the extract was identified by FAB/CAD mass spectrometry. Because GSH also reacts with 4-vinylpyridine, GSEP was formed and used as a standard to determine the fragmentations to be expected for a 4-vinylpyridine derivative of GSH or GSSH. The FAB mass spectra of the 4-vinylpyridine-treated incubation extract showed abundant protonated molecular ion (MH^+) of GSEP (m/z 413) and a modest signal at m/z 445 [$(GSSEP+H)^+$]. The CAD spectra of m/z 413 and 445 ions are shown in Fig. 4. Fig. 4A, *inset*, describes the possible identity of product ions of the m/z 413 ion. The CAD spectrum of the m/z 445 ion (Fig. 4B) yielded ions shifted by 32 units, consistent with an extra sulfur on the cysteine in GSH. The ions expected to shift by 32 units are marked on the structure (Fig. 4A, *inset*). These data therefore provide evidence that GSSH is formed during the desulfuration of DDTC-Me sulfine. Although it is apparant that GSSH is the most likely source of H_2S, this cannot be determined with certainty without the use of ^{35}S-labeled DDTC-Me sulfine.

These data provide the first evidence for the involvement of GSH and glutathione-*S*-transferases in the desulfuration of a thione compound. The formation of GSSH during such a desulfuration process is also unprecedented. The mechanisms that had been proposed previously (1) suggested that desulfuration of sulfines occurred by 1) cyclization of the sulfine to form an oxithiirane ring, which loses the sulfur in its singlet state, resulting in the formation of the desulfurated product, and 2) further oxidation of the sulfine to the reactive sulfene and the loss of sulfur as HSO_2^-. DDTC-Me sulfine, however, was not found to be desulfurated when it was incubated with microsomes with or without the NADPH-generating system. This suggested that DDTC-Me sulfine is desulfurated neither by spontaneous decomposition of the oxithiirane ring nor by further oxidation of DDTC-Me sulfine to a sulfene, thereby ruling out the aforementioned mechanisms of desulfuration. The addition of GSH to DDTC-Me sulfine, resulting in the formation of intermediate **IV**, can explain the desulfuration as well as the reduction in the presence of glutathione (Fig. 3B). However, this mechanism does not explain why the presence of glutathione-*S*-transferases should increase the extent of desulfuration, because **IV** is formed regardless of whether desulfuration occurs or reduction occurs. It is the attack by water or by another GSH molecule that apparantly determines whether desulfuration or reduction occurs. Therefore, at this stage it can only be speculated that during desulfuration of DDTC-Me sulfine the oxithiirane ring (**II**) undergoes nucleophilic attack by GSH (a process that may be facilitated by glutathione-*S*-transferase). Although there is no direct evidence for the existence of oxithiirane rings, their formation has been speculated (1, 25, 26). The attack of GSH on the oxithiirane rings leads to ring opening and formation of a tetrahedral intermediate with two potential leaving groups (CH_3S^- and GSS^-). However, mass balance results imply that CH_3S^- loss, if it occurred, must have been very minor (Fig. 3B).

According to previously described mechanisms of toxicity of carbon disulfide or thioacetamide, the singlet sulfur or the sulfenes are thought to attack nucleophilic groups on macromolecules, such as free sulfhydryl groups on cysteines, resulting in covalent binding and toxicity. However, the data presented in this study also suggest that GSH may serve a protective function *in vivo*, by trapping the sulfur and preventing modification of essential macromolecules.

Studies were also carried out to determine whether this GSH-dependent mechanism for DDTC-Me can be generalized to other dithiocarbamate esters, as well as thiobenzamide. A series of dithiocarbamate esters were synthesized (Table 1) and their metabolism was studied by using a microsomal system supplemented with GSH, with or without glutathione-*S*-transferase. Desulfuration was found to be minimal for all thione compounds when they were incubated in the presence of microsomes from phenobarbital-treated rats and an NADPH-generating system (Table 5). Phenobarbital treatment induces cytochrome P4502B1/2 in rat liver (27), and several studies have suggested that this enzyme actively catalyzes the oxidation of the thionosulfur group (28). When the incubation mixture of microsomes from phenobarbital-treated rats and the NADPH-generating system was supplemented with GSH, a significant increase in desulfuration was observed for all thione compounds studied, except for the vernolate *S*-analog and the butylate *S*-analog. Similarly, when the microsomal incubation system was supplemented with both GSH and glutathione-*S*-transferase, oxidative desulfuration was further increased significantly in most cases, except for the vernolate *S*-analog, the butylate *S*-analog, and thiobenzamide (Table 5). Although formation of sulfine was not measured, it can be speculated that the vernolate *S*-analog and the butylate *S*-analog were not extensively oxidized at the thionosulfur, and as a result only a small increase in desulfuration was observed. However, thiobenzamide is known to be extensively oxidized at the thionosulfur (29), and the increase in desulfuration observed in the presence of GSH was unaffected by the presence of glutathione-*S*-transferase. These data suggested that, although the mechanism of oxidative desulfuration may be identical for DDTC-Me and other dithiocarbamate esters, thiobenzamide may be desulfurated by a different mechanism, possibly one not requiring

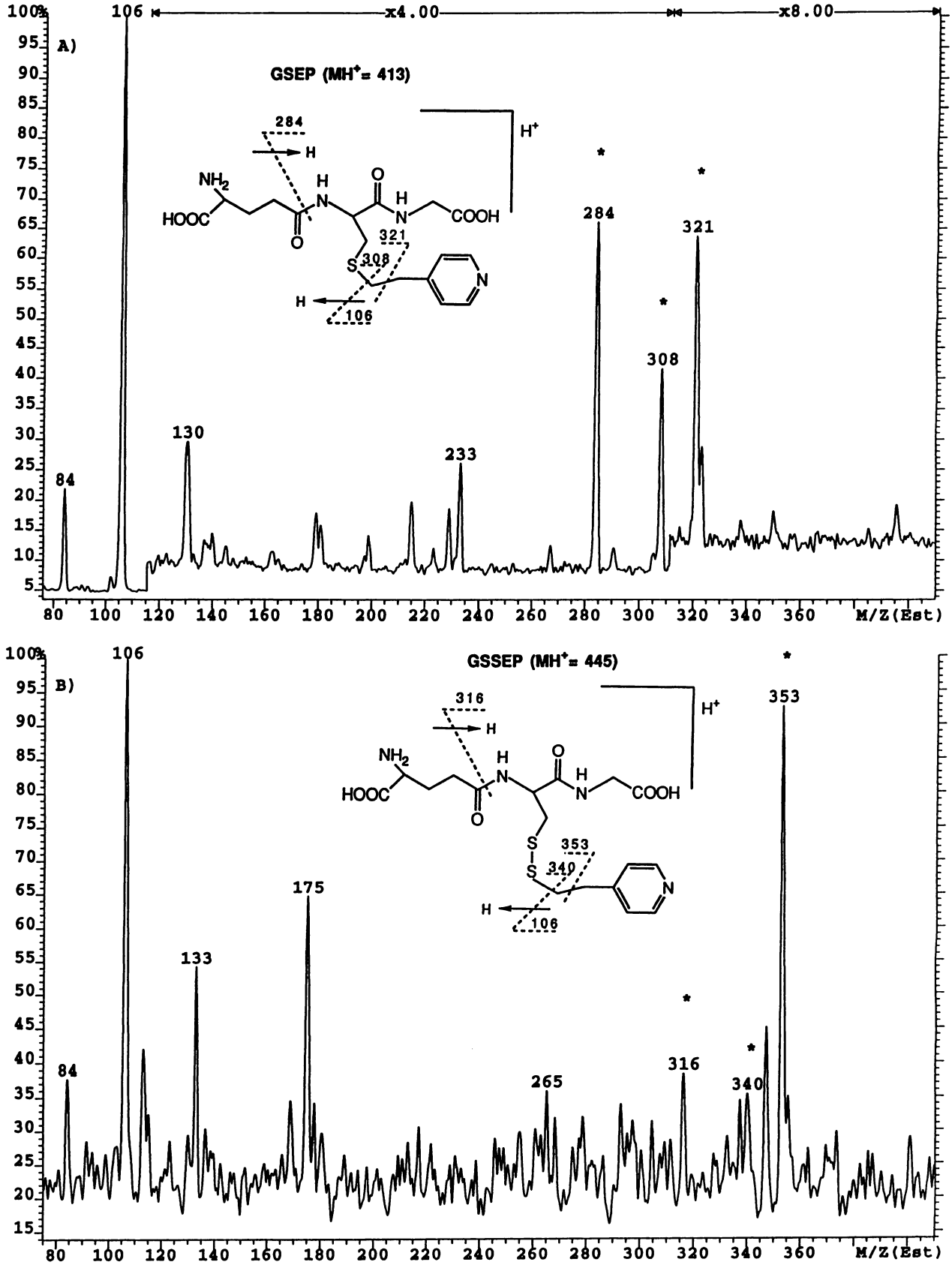

Fig. 4. FAB/CAD spectra of the *m/z* 413 (A) and *m/z* 445 (B) ions derived from the GSSH-trapping reaction described in Materials and Methods. A, The *m/z* 413 ion is assumed to be the MH^+ ion for GSEP, and the interpretation of the major product ions is shown in the *inset*. *, Product ions from the *m/z* 445 precursor that have a +32-unit shift from the fragment ions in A. B, Major product ions from the *m/z* 445 ion are interpreted as indicated in the *inset*, assuming that the *m/z* 445 ion is the MH^+ ion for GSSEP.

TABLE 5

Oxidative desulfuration of other thione compounds

The incubation system consisted of microsomes (1 mg/ml), substrate (1 mM), and an NADPH-generating system. GSH and purified glutathione-*S*-transferase, when added, were present at final concentration of 10 mM and 2 units/ml, respectively. The incubation volume was 1 ml. The incubation was initiated by the addition of the substrate and was maintained at 37° for 30 min. The reaction was stopped by the addition of 0.5 ml of cold acetonitrile. The amount of desulfurated metabolite formed in each incubation was determined by HPLC, as described in Materials and Methods. The data are expressed as means ± standard errors of four incubations.

Substrate[a]	Incubation System		
	Microsomes + substrate + NADPH	Microsomes + substrate + NADPH + GSH	Microsomes + substrate NADPH + GSH + glutathione-*S*-transferase
	nmol of desulfurated metabolite formed/30-min incubation/mg of protein		
DDTC-Me	4 ± 0.1[b]	27 ± 0.4	117 ± 1.4[b]
Ethiolate *S*-analog	0.7 ± 0.06[b]	17 ± 0.8	62 ± 0.7[b]
EPTC *S*-analog	0 ± 0[b]	1.2 ± 0.3	3.8 ± 0.02[b]
Vernolate *S*-analog	0 ± 0	0.1 ± 0.05	0.35 ± 0.19
Molinate *S*-analog	0.05 ± 0.05[b]	1.12 ± 0.06	2.24 ± 0.1[b]
Butylate *S*-analog	0.06 ± 0.003	0.11 ± 0.013	0.11 ± 0.013
Thiobenzamide	0.47 ± 0.12[b]	4.93 ± 0.31	4.83 ± 0.21[c]

[a] The thione compound used as a substrate. See Table 1 for structures.
[b] $p < 0.05$ by analysis of variance, compared with the group with microsomes plus substrate plus substrate plus NADPH plus GSH.
[c] $p < 0.05$ by analysis of variance, compared with the group with microsomes plus substrate plus NADPH.

glutathione-*S*-transferase. It is possible that glutathione-*S*-transferase has no importance in the attack of GSH on the oxithiirane ring system in thiobenzamide-*S*-oxide (if such a cyclization occurs with thiobenzamide-*S*-oxide) but is important for other thione-containing compounds, for example, thiocarbamate esters.

In conclusion, using DDTC-Me as a model substrate, we have observed an apparantly novel mechanism for the desulfuration of thione compounds, which in turn suggests a new and possibly protective role for GSH. During oxidative desulfuration, the possible fate of the sulfur may be to bind to GSH by forming a GSSH linkage, in addition to binding to proteins as proposed previously (1). It is known that DDTC-Me is desulfurated to DETC-Me *in vivo* (12). Whether desulfuration of DDTC-Me occurs *in vivo* by this mechanism is not known at this time. Also, it is not certain whether this GSH-dependent mechanism of desulfuration is common to other thione xenobiotics, such as parathion, thioacetamide, and carbon disulfide. Because GSH is present in all mammalian tissues, this GSH-dependent desulfuration mechanism adds a new dimension to the mechanisms of bioactivation and toxicity of thione compounds. If this mechanism is common to thione compounds, then H_2S may be implicated in some of the toxicities of these compounds.

Acknowledgments

The authors appreciate the helpful comments and suggestions provided by Dr. R. P. Hanzlik. We also wish to thank Mr. Robert Drake for his efforts in acquiring the FAB spectra. The tandem mass spectrometer was purchased with the aid of National Institutes of Health Grant S10-RR0-6294-01 (T.D.W.) and the University of Kansas. The laboratory assistance of Liang Wong is also acknowledged.

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