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RAPID COMMUNICATION

A NOVEL PATHWAY FOR FORMATION OF THIOL METABOLITES AND CYSTEINE CONJUGATES FROM CYSTEINE CONJUGATE SULPHOXIDES

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Abstract- *p*-Bromothiophenol and *S*-(*p*-bromophenyl)-*L*-cysteine were formed enzymatically from *S*-(*p*-bromophenyl)-*L*-cysteine sulphoxide in the *in vitro* systems with isolated rat hepatocytes or purified cysteine conjugate β-lyases. Isotope dilution study with non-radiolabelled carrier of each product suggested the initial liberation of the thiol and subsequent formation of the cysteine conjugate. C-S bond cleavage pathway to liberate sulphenic acid and thiol are postulated to play an important role in *in vivo* generation of toxic intermediates and products from cysteine conjugates.

A wide variety of organic compounds including drugs, herbicides, industrial materials, and endogenous compounds have been reported to be subjected to glutathione conjugation and subsequent metabolism (1). Conjugation with glutathione and further metabolism generate various kinds of sulphur-containing metabolites, e.g., mercapturic acid conjugates, cysteine conjugates, mercaptopyruvic acid conjugates, mercaptolactic acid conjugates, mercaptoacetic acid conjugates, sulphoxides of these conjugates, thiols, methyl sulphides, methyl sulphoxides, and methyl sulphones (2). In most cases, these biotransformations result in lowered biological activity and rapid elimination of the parent compounds. In the last decade, however, glutathione conjugation and related metabolic pathways have been shown to play a key role in the bioactivation of some nephrotoxic and mutagenic halogenated hydrocarbons (3-6). The implication of cysteine conjugate β-lyases are demonstrated in the biotransformation of cysteine conjugates to the penultimate toxicants, thiols, which are further converted to thionoacetyl fluorides and thioketenes as ultimate reactive intermediates (7-9).

Although the bioactivation pathway via thiols has extensively been investigated, contribution of other pathways to metabolic activation of cysteine conjugates remains to be elucidated. Among such pathways, sulphoxidation of cysteine conjugates and subsequent C-S cleavage to form reactive sulphenic acids have recently been postulated to activate cysteine conjugates (10-12). In the present study, the authors describe the first evidence for *in vitro* liberation of thiols from cysteine conjugate sulphoxides and subsequent transformation of the thiols into cysteine conjugates by the action of cysteine conjugate β-lyase, and discuss possible contribution of the pathway to the generation of toxic intermediates and products from the cysteine conjugates.

MATERIALS AND METHODS

Chemicals - Both [^{35}S]-labelled and non-radiolabelled *S*-(*p*-bromophenyl)-*L*-cysteine sulphoxide were prepared from the corresponding *S*-(*p*-bromophenyl)-*L*-cysteine by the method described by Oae et al.(13). *S*-(*p*-Bromophenyl)-*L*-cysteine was synthesized by the method of Saunders (14). Other reagents were commercially obtained. Cysteine conjugate β-lyase [EC 4.1.1.13] was purified from soluble fraction of rat liver and kidney by slightly modified methods previously described (15, 16). Each enzyme preparation gave a single band in SDS-polyacrylamide gel electrophoresis.
Identification of the Products - Metabolites of *S*-(*p*-bromophenyl)-*L*-cysteine sulphoxide, i.e. M1 (retention time, 4.7 min) and M2 (23 min), were separately collected by HPLC under the conditions

Abbreviations. GC, gas chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry; SDS, sodium dodecylsulphate.

described in Incubation with Isolated Rat Hepatocytes. M2 was directly analyzed by GC-MS, while M1 was treated serially with trifluoroacetic anhydride and trimethylsilyl diazomethane prior to GC-MS analysis. The analyses were carried out with a gas chromatograph (Model 5890, Hewlett-Packard, USA) coupled to a mass spectrometer (Model 5988A, Hewlett-Packard, USA). A capillary column (DB-17, 0.25 mm x 10 m, J&W Scientific, USA) was used for GC separation. The column temperature was elevated from 50° to 280° at a rate of 30°/min. Helium at a flow rate of 7.5 ml/min was used as carrier gas. The mass spectrometer was operated in the electron impact ionization mode with an ionization energy of 70 eV.

Incubation with Isolated Rat Hepatocytes - Hepatocytes were isolated by collagenase perfusion and subsequent isopycnic centrifugation (17). The cells at a final concentration of 1.0 x 10^7 cells/ml were incubated in Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 2% (w/v) bovine serum albumin and *S*-(*p*-bromophenyl)-*L*-cysteine sulphoxide (0.5 mM) at 37° for 30 min. Cell viability estimated by trypan blue staining before and after the incubation was 92% and 77%, respectively. Reaction was terminated by freezing the incubation mixture at -20.° After 30 min the medium was thawed and centrifuged, and the supernatant was subjected to HPLC equipped with Capcellpak C_{18} SG120 (4.6 x 250 mm, Shiseido, Japan). *p*-Bromothiophenol and *S*-(*p*-bromophenyl)-*L*-cysteine were eluted with methanol/H_2O/acetic acid (60:40:1, by vol.) at a flow rate of 0.9 ml/min and detected at 254 nm.

Enzymatic Reaction by Purified Cysteine Conjugate β-Lyase - The reaction mixture contained in a final volume of 0.1 ml: radiolabelled or non-radiolabelled *S*-(*p*-bromophenyl)-*L*-cysteine sulphoxide (0.5 mM) as substrate, reduced glutathione (1 mM), Tris-HCl buffer (100 mM, pH 7.4), and enzyme (30-70 µg). The reaction mixture was incubated at 37° for 30 min under anaerobic conditions. The reaction was terminated by an addition of 0.2 ml of methanol. After centrifugation, the supernatant was subjected to HPLC analysis as described above. When radiolabelled substrate was used, the eluate corresponding to each product was collected for measurement of radioactivity.

RESULTS AND DISCUSSION

The metabolites of *S*-(*p*-bromophenyl)-*L*-cysteine sulphoxide incubated with isolated rat hepatocytes were analyzed by HPLC. Metabolites emerging at 4.7 min (M1) and 23 min (M2) were isolated by HPLC (Fig. 1) and their structures were elucidated by GC-MS directly or after derivatization. The derivatized M1 and M2 metabolites showed a peak at 5.3 and 2.5 min, respectively. Characteristic ions were as follows: m/z 385, 387 [M^+, relative abundance of 12%],

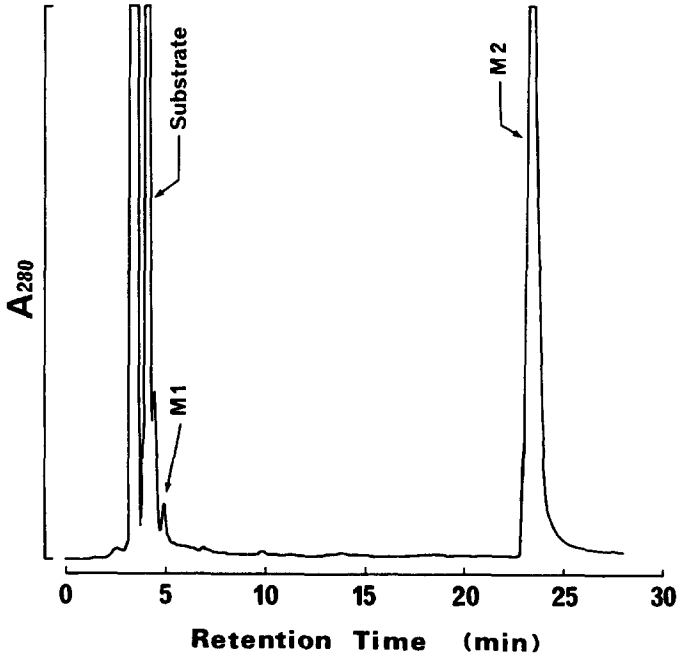
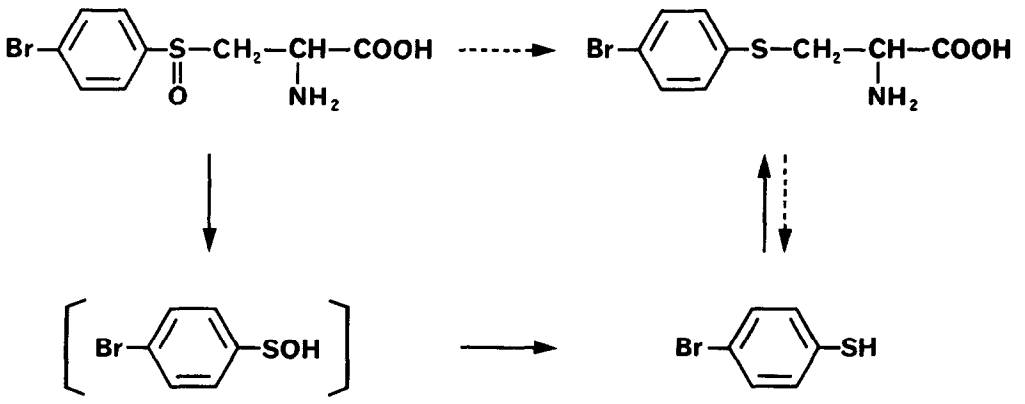


Fig. 1. HPLC profiles of metabolites of *S*-(*p*-bromophenyl)-*L*-cysteine sulphoxide after incubation with isolated rat hepatocytes. *S*-(*p*-Bromophenyl)-*L*-cysteine sulphoxide was incubated with isolated rat hepatocytes and metabolites were analyzed by HPLC. Conditions are described in MATERIALS AND METHODS.



Scheme 1. Possible pathway for formation of thiol and cysteine conjugate from cysteine conjugate sulphoxide

Table 1. Isotope dilution during enzymatic formation of thiol and cysteine conjugate from cysteine conjugate sulphoxide

	Specific radioactivity (μCi/μmole)		
Additives	Substrate	Thiol	Cysteine conjugate
None	1.7 ± 0.3	1.8 ± 0.1	1.8 ± 0.1
Thiol	1.9 ± 0.3	1.1 ± 0.1	0.8 ± 0.0
Cysteine conjugate	1.8 ± 0.1	1.6 ± 0.0	0.6 ± 0.1

[^{35}S]-*S*-(*p*-Bromophenyl)-*L*-cysteine sulphoxide was incubated with purified rat hepatic cysteine conjugate β-lyase with or without an addition of non-radiolabelled *p*-bromothiophenol or *S*-(*p*-bromophenyl)-*L*-cysteine as described in MATERIALS AND METHODS. After incubation, the supernatant of centrifuged reaction mixture was subjected to HPLC analysis and radiolabelled products were collected for measurement of radioactivity. Each value represents mean ± S.D. of three experiments.

m/z 272, 274 [(M-H_2NCOCF_3)$^+$, 63%], m/z 213, 215 [(M-H_2NCOCF_3-CH_3COO)$^+$, 21%], m/z 201, 203 [($C_6H_4BrSCH_2$)$^+$, 100%], m/z 187, 189 [(C_6H_4BrS)$^+$, 33%] for derivatized M1, and m/z 188, 190 [M$^+$, C_6H_4BrSH] for M2. These results were consistent with the proposed structure, i.e. *S*-(*p*-bromophenyl)-*L*-cysteine for M1 and *p*-bromothiophenol for M2. The mass spectra were identical with those of the corresponding authentic compounds. Addition of malononitrile (10 mM) (an inhibitor of pyridoxal enzymes which catalyze β-elimination (18)) to the incubation mixture blocked the formation of M1 and M2 by 100% and 75%, respectively.

Formation of thiol and cysteine conjugate from cysteine conjugate sulphoxide was further confirmed with cysteine conjugate β-lyase purified from rat liver. *S*-(*p*-Bromophenyl)-*L*-cysteine sulphoxide was incubated with the β-lyase in the presence of glutathione. HPLC analysis of the incubation media showed concomitant formation of *p*-bromothiophenol and *S*-(*p*-bromophenyl)-*L*-cysteine. The rate of formation of the thiol and cysteine conjugate was 12 and 1.4 nmole/min/mg, respectively, and was constant during the incubation time until 30 min and with the amount of the enzyme up to 70 μg. Exclusion of glutathione or addition of aminooxyacetic acid (1 mM), a potent inhibitor of cysteine conjugate β-lyase, caused complete inhibition of formation of the two products, although the substrate was consumed even in the absence of glutathione. Similar results were obtained when purified rat renal cysteine conjugate β-lyase was used in place of the hepatic enzyme. These results indicated an involvement of cysteine conjugate β-lyase in the formation of the two products.

Two pathways are conceivable for formation of the thiol and cysteine conjugate (Scheme 1): 1. Initial reduction of the sulphoxide to the cysteine conjugate and subsequent C-S bond cleavage by cysteine conjugate β-lyase to yield the thiol. 2. Initial C-S bond cleavage of the sulphoxide to give a putative intermediate, sulphenic acid, followed by non-enzymatic conversion to the thiol and subsequent transformation of the thiol to the cysteine conjugate by the action of cysteine conjugate β-lyase (reverse reaction). In order to elucidate the operating pathway, we incubated [^{35}S]-*S*-(*p*-bromophenyl)-*L*-cysteine sulphoxide with purified rat hepatic cysteine conjugate β-lyase with or without an addition of non-radiolabelled *p*-bromothiophenol or *S*-(*p*-bromophenyl)-*L*-cysteine (Table 1). Addition of non-radiolabelled thiol decreased the specific radioactivity of the cysteine conjugate formed, while addition of non-radiolabelled *S*-(*p*-bromophenyl)-*L*-cysteine slightly decreased the specific radioactivity of the liberated thiol. In addition, incubation of *S*-phenyl-*L*-cysteine with the β-lyase in the presence of *p*-bromothiophenol yielded *S*-(*p*-bromophenyl)-*L*-cysteine (data not shown). These results indicated that the formation of thiol and cysteine conjugate from the sulphoxide apparently followed the latter pathway and also that the C-S bond formation was dominant in the reversible enzymic reaction between the thiol and the cysteine conjugate.

The intermediacy of sulphenic acid derived from naturally-occurred cysteine conjugate sulphoxide was described in the biotransformation of marasmine (19) and alliin (20) in plants. The first evidence presented for xenobiotic sulphenic acid formation in mammals was the urinary excretion of 1,1,2,3,4-pentachloro-1,3-butadienyl sulphenic acid in rats dosed with hexachloro-1,3-butadiene (21). Sausen and Elfarra (12) reported that *S*-(1,2-dichlorovinyl)-*L*-cysteine sulphoxide was much more toxic than *S*-(1,2-dichlorovinyl)-*L*-cysteine *per se in vivo*. In view of the relatively higher chemical reactivity of sulphenic acids compared to the thiols, these findings suggested the conversion of sulphenic acid to thiols may be detoxication pathway. Thus the present study demonstrates a possible alternative pathway for detoxication of sulphenic acids. The highly reactive sulphenic acids may readily be converted by an intermolecular dehydration to give thiosulphinates, which subsequently liberate thiols in the presence of glutathione by thiol-disulphide exchange. The thiols thus generated most probably react with the aminoacrylate-enzyme intermediate derived from the enzyme-substrate Schiff base by loss of the α-proton of cysteine conjugate sulphoxide. Similar reaction mechanism was reported for tryptophan synthase, in which indole reacted with the highly reactive aminoacrylate-enzyme intermediate to form tryptophan (22). Together with the lower glutathione concentration in the kidney (11), the essential requirement of glutathione for detoxication of cysteine conjugate sulphoxide found in the present study may explain the kidney specific toxicity of cysteine conjugate sulphoxide. Contribution of the thiol and cysteine conjugate to toxicity as a result of re-involvement in the pathway for sulphenic acid formation still remains to be elucidated.

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REFERENCES

1. Tateishi M and Tomisawa H, Cysteine conjugate β-lyase: Toxic thiol production. *Rev Pesticide Toxicol* **1**: 149-160, 1991.
2. Tomisawa H and Tateishi M, Metabolism and toxication of cysteine conjugates. *Xenobiotic Metab Dispos* **7**: 279-290, 1992.
3. Lash LH and Anders MW, Bioactivation and cytotoxicity of nephrotoxic amino acid and glutathione S-conjugates. *Comments Toxicol* **1**: 87-106, 1986.
4. Anders MW, Dekant W, Elfarra, AA and Dohn DR, Biosynthesis and biotransformation of glutathione S-conjugates to toxic metabolites. *CRC Crit Rev Toxicol* **18**: 311-341, 1988.
5. Bakke JE, Biochemical and physiological dispositions of glutathione conjugates. *Drug Metab Rev* **22**: 637-647, 1990.
6. Koob M and Dekant W, Bioactivation of xenobiotics by formation of toxic glutathione conjugates. *Chem-Biol Interact* **77**: 107-136, 1991.
7. Dekant W, Lash LH and Anders MW, Bioactivation of the cytotoxic and nephrotoxic S-conjugate S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine. *Proc Natl Acad Sci USA* **84**: 7443-7447, 1987.
8. Dekant W, Berthold K, Vamvakas S, Henschler D and Anders MW, Thioacylating intermediates as metabolites of S-(1,2-dichlorovinyl)-L-cysteine and S-(1,2,2-trichlorovinyl)-L-cysteine formed by cysteine conjugate β-lyase. *Chem Res Toxicol* **1**: 175-178, 1988.
9. Dekant W, Berthold K, Vamvakas S and Henschler D, Thioacylating agents as ultimate

intermediates in the β-lyase catalysed metabolism of S-(pentachlorobutadienyl)-L-cysteine. *Chem-Biol Interact* **67**: 139-148, 1988.
10. Tomisawa H and Tateishi M, Novel metabolic pathways of glutathione conjugates and their relation to toxicity. *Jpn J Toxicol Environ Health* **36**: 359-372, 1990.
11. Sausen PJ and Elfarra AA, Reactivity of cysteine S-conjugate sulfoxides: Formation of S-[1-chloro-2-(S-glutathionyl)vinyl]-L-cysteine sulfoxide by the reaction of S-(1,2-dichlorovinyl)-L-cysteine sulfoxide with glutathione. *Chem Res Toxicol* **4**: 655-660, 1991.
12. Sausen PJ and Elfarra AA, S-(1,2-dichlorovinyl)-L-cysteine sulfoxide, a putative metabolite of S-(1,2-dichlorovinyl)-L-cysteine (DCVC), is a potent nephrotoxin. *Toxicologist* **10**: 267, 1990.
13. Oae S, Ohnishi Y, Kozuka S and Tagaki W, The synthesis of sulfoxides by the oxidation of sulfide with the bromine complex of 1,4-diazabicyclo(2,2,2)octane. *Bull Chem Soc Jap* **39**: 364-366, 1966.
14. Saunders BC, Glutathione. Its reaction with alkali and some N and S derivatives. *Biochem J* **28**: 1977-1981, 1934.
15. Tateishi M and Shimizu H, Cysteine conjugate β-lyase. *Method Enzymol* **77**: 253-256, 1981.
16. Stevens JL, Robbins JD and Byrd RA, A purified cysteine conjugate β-lyase from rat kidney cystosol, requirement for an α-keto acid or an amino acid oxidase for activity and identity with soluble glutamine transaminase K. *J Biol Chem* **261**: 15529-15537, 1986.
17. Moldeus P, Högberg J and Orrenius S, Isolation and use of liver cells. *Method Enzymol* **52**: 60-71, 1978.
18. Pfeffer, M and Ressler, C, β-Cyanoalamine, an inhibitor of rat liver cystathionase. *Biochem Pharmacol* **16**: 2299-2308, 1967.
19. Van den Broek LAGM, Delbressine LPC and Ottenheijm HCJ, Biochemistry and metabolic pathways of sulfenic acids and their derivatives. In: The Chemistry of Sulphenic Acids and Their Derivatives, Patai S (ed), John Wiley & Sons (Chichester), pp 701-721, 1990.
20. Block E, The organosulfur chemistry of the genus allium - implications for the organic chemistry of sulfur. *Angew Chem Int Ed Engl* **31**: 1135-1178, 1992.
21. Nash JA, King LJ, Lock EA and Green T, The metabolism and disposition of hexachloro-1:3-butadiene in the rat and its relevance to nephrotoxicity. *Toxicol Appl Pharmacol* **73**: 124-137, 1984.
22. Anderson KS, Miles EW and Johnson KA, Serine modulates substrate channeling in tryptophan synthase, a novel intersubunit triggering mechanism. *J Biol Chem* **266**: 8020-8033, 1991.