[Chem. Pharm. Bull. 18(1) 115—119 (1970)]

UDC 577.156.04

# Studies on Mercapturic Acids. Effect of Some Chemical Compounds on the Activities of Glutathione-conjugating Enzymes

TETSUYA SUGA[1a] and MASUO AKAGI[1b]

*Faculty of Pharmaceutical Sciences, Hokkaido University*[1]

(Received June 30, 1969)

1. For the purpose of clarifying the mechanism of mercapturic acid formation, properties of enzymes which catalyzed the conjugation of GSH with benzyl chloride (BzCl), bromobenzene (BrPh) and 3,4-dichloronitrobenzene (DCNB) were examined in the liver of rats.

2. In addition to the supernatant fraction, a microsomal fraction was necessary for the enzymic conjugation of GSH with BrPh.

3. GSH-conjugating activity with BzCl was depleted in the liver of rats treated with BzCl. Either GSH-conjugating activity toward DCNB or BrPh did not show a significant change after the administration of each compound.

4. GSH-conjugating activity toward BrPh markedly increased in the liver of rats treated with phenobarbital, and its ratio of the stimulation was paralleled with that of aniline-hydroxylating activity.

5. It was assumed that increase of the activity of GSH-conjugation with BrPh by the treatment with phenobarbital was due to the induction of a microsomal oxygenase system.

It is known that mercapturic acids, S-derivatives of N-acetyl-L-cysteine, are excreted in the urine of animals which have been treated with some chemical compounds, such as bromobenzene, naphthalene and benzyl chloride. Many experiments suggesting the participation of glutathione (GSH) for the formation of mercapturic acids have been presented.[2]

It has been assumed that an initial step in mercapturic acid formation was a conjugation of GSH with foreign compounds administered to animals. These reactions were catalyzed by GSH S-alkyltransferase,[3] catalyzing the conjugation of GSH with alkyl halogen compounds, GSH S-aryltransferase,[4] catalyzing the conjugation of GSH with labile aryl halogen or nitro compounds, and GSH S-epoxidetransferase,[5] catalyzing the conjugation of GSH with epoxide compounds. More recently, it was proposed that enzymes catalyzed the conjugation of GSH with $\alpha,\beta$-unsaturated carbonyl compounds and that these enzymes, named GSH S-alkene-transferase, differed from GSH S-transferases described above.[6]

In the present work, the activities of GSH–conjugating enzymes of the rat liver were measured after the treatment of rats with benzyl chloride (BzCl), bromobenzene (BrPh), 3,4-dichloronitrobenzene (DCNB) and phenobarbital. From these results, a possible mechanism of mercapturic acid formation from BrPh was discussed.

1) Location: *Sapporo, Hokkaido*; Present address: *a) Tokyo College of Pharmacy, Shinjuku-ku, Tokyo*; *b) Hoshi College of Pharmacy, Shinagawa-ku, Tokyo.*

2) *a)* M.M. Barnes, S.P. James, and P.B. Wood, *Biochem. J.*, **71**, 680 (1959); *b)* H.G. Bray, T.J. Franklin, and S.P. James, *Biochem. J.*, **71**, 690 (1959); *c)* T. Suga, I. Ohata, and M. Akagi, *J. Biochem.*, **59**, 209 (1966).

3) M.K. Johnson, *Biochem. J.*, **98**, 44 (1966).

4) J. Booth, E. Boyland, and P. Sims, *Biochem. J.*, **79**, 516 (1961).

5) E. Boyland and K. Williams, *Biochem. J.*, **94**, 190 (1965).

6) E. Boyland and L.F. Chasseaud, *Biochem. J.*, **109**, 651 (1968).

## Experimental

**Materials**——GSH was purchased from Sigma Chemical Co., U.S.A. S-(4-Bromophenyl)glutathione (BrPhSG) and S-(2-chloro-4-nitrophenyl)glutathione were prepared by the method of Booth, *et al.*[7]

**Animals and Dosages**——Female rats (Wistar strain, 150—200 g) were used. BrPh, DCNB and BzCl were administered by metal stomach tube as a suspension in acacia in a dose of 25 mg/100 g body weight. Phenobarbital was administered by the intraperitoneal injection in a dose of 5 mg/100 g.

**Enzyme Preparations**——Rats were killed by decapitation. The liver was immediately washed with cold 0.15M KCl and homogenized in 0.1M tris–HCl buffer, pH 8.0, containing 0.15M KCl. Homogenates were centrifuged at 13000 $\times g$ for 20 minutes. The supernatant fluid was centrifuged at 105000 $\times g$ for 60 minutes. The supernatant fraction was separated and the remained precipitates were suspended in 0.1M tris–HCl buffer (microsomal fraction). These fractions were used as enzyme preparations.

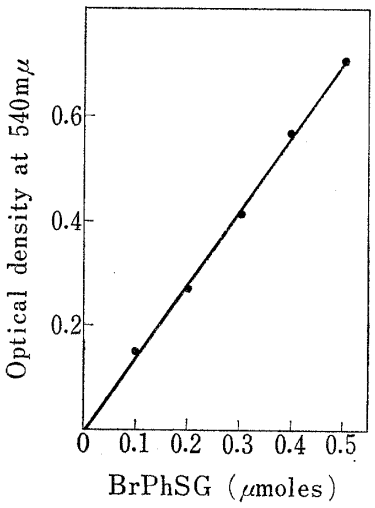


Fig. 1. Calibration Curve of S-(4-Bromophenyl)glutathione (BrPhSG)

**Assay Methods for Enzyme Activities**——The activity of the conjugation of GSH with BzCl was measured according to the thin–layer chromatography method (TLC-method) described previously.[8] The supernatant of rat liver was used as enzyme preparation in above experiment.

The activity of the conjugation of GSH with BrPh was determined by the TLC–method with a slight modification. Incubation mixture contained 1 ml enzyme preparation (microsomal fraction plus the supernatant fraction containing 20—40 mg protein), 8.0 mM GSH, 5.0 mM BrPh, 8.0 mM nicotinamide, 0.1 mM NADP, 8.0 mM G-6-P, 4.0 mM $Mg(OAc)_2$, 1.5 unit G-6-P dehydrogenase and 50.0 mM tris-HCl buffer, pH 8.0, in a total volume of 2.5 ml. After incubation at 37°, 0.5 ml of 18% metaphosphoric acid was added and the protein precipitated was centrifuged off. An aliquot of the supernatant fluid was used for the determination of BrPhSG. Calibration curve of BrPhSG was shown in Fig. 1.

Assay for the activity of aniline–hydroxylating enzyme was carried out according to the method of Imai, *et al.*[9] Incubation mixture contained 0.5 ml microsomal fraction (containing about 15 mg protein), 5.0 mM aniline, 8.0 mM nicotinamide, 4.0 mM $Mg(OAc)_2$, 0.1 mM NADP, 8.0 mM G-6-P, 1.6 unit G-6-P dehydrogenase and 50.0 mM tris–HCl buffer, pH 8.0, in a total volume of 2.5 ml.

After the incubation, 0.5 ml of 30% of trichloroacetic acid was added to the reaction mixture and the precipitate was centrifuged off. The supernatant fluid was used for the determination of *p*-aminophenol produced.

**Protein**——Protein contents were determined by the method of Lowry, *et al.*[10]

## Results

### Effect of Various Conditions of S-(4-Bromophenyl)glutathione (BrPhSG) *in Vitro*

Microsomal fraction (1.45 mg protein per 0.5 ml) and the supernatant fraction (21.2 mg per 0.5 ml) of rat liver were used as enzyme preparations. This reaction showed the lineality with in cubation time for first 2 hours. As shown in Table I, microsomal fraction, the supernatant fraction and NADPH–generating system were necessary for the formation of BrPhSG. No reaction was observed when one of those components was omitted from complete system. A slight formation of BrPhSG in the system without GSH was assumed to be due to the presence of GSH in the supernatant fraction used.[3c]

7) J. Booth, E. Boyland, and P. Sims, *Biochem. J.*, **74**, 117 (1960).
8) T. Suga, I. Ohata, H. Kumaoka, and M. Akagi, *Chem. Pharm. Bull.* (Tokyo), **15**, 1059 (1967).
9) Y. Imai and R. Sato, *Biochim. Biophys. Acta*, **42**, 164 (1960).
10) O.H. Lowry, N.J. Rosenbrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

Table I. Effect of Various Conditions on the Formation of S-(4-Bromophenyl)glutathione

| Conditions | Enzyme activity ($\mu$mole/tube/hr) |
|---|---|
| Complete system | 1.18 |
| Complete system minus microsomal fraction | 0 |
| Complete system minus the supernatant fraction | 0 |
| Complete system minus GSH | 0.04 |
| Complete system minus NADPH-generating system | 0 |
| Complete system with boiled enzyme preparations | 0 |

In the complete system, reaction mixture contained enzyme preparation (0.5 ml of microsomal fraction plus 0.5 ml of the supernatant fraction), 5.0 mM BrPh, 8.0 mM GSH, 8.0 mM nicotinamide, 0.1 mM NADP, 8.0 mM G-6-P, 4.0 mM $Mg(OAc)_2$, 1.5 unit G-6-P dehydrogenase and 50.0 mM Tris buffer, pH 8.0, in a total volume of 25 ml.

## Effects of Pretreatment of Benzyl Chloride (BzCl), 3,4-Dichloronitrobenzene (DCNB), Bromobenzene (BrPh) and Phenobarbital on GSH-conjugating Enzymes in Rat Liver

Rats were administered with BzCl, DCNB or BrPh orally (25 mg/100 g/day) for 7 days, and enzyme activities of the liver which conjugated each substarte with GSH were determined. Enzyme activities were expressed as per cent of the activities of control rats. As shown in Fig. 2, enzyme activities of rats pretreated with DCNB or BrPh did not show a significant change. After rats had been pretreated with BzCl, the enzyme activity which conjugated BzCl with GSH fell to 75% of the control rats.

After rats were dosed with BrPh (25 mg/100 g/day; orally) or with phenobarbital (5 mg/100 g/day; intraperitoneally) for 3 days, enzyme activities which conjugated GSH with BzCl, DCNB or BrPh and aniline–hydroxylating activities of the liver were determined. As the enzyme preparation, the supernatant fraction was used when BzCl or DCNB was substrate, and the supernatant plus microsomal fraction was used when BrPh was substrate. These enzyme activities were not significantly affected by the treatment with BrPh (Table II). By pretreatment of rats with phenobarbital, however, the activity of formation of BrPhSG was considerably stimulated. The ratio of the stimulation (228.4%) was on the same degree as that of aniline–hydroxylation (230.8%).

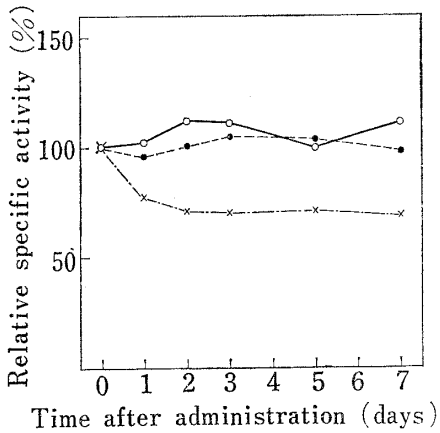


Fig. 2. Effects of Benzyl Chloride (BzCl), 3,4-Dichloronitrobenzene (DCNB) and Bromobenzene (BrPh) on GSH-conjugating Enzyme Activities in the Liver of Rats

Enzyme activity was measured using:
---×---: BzCl as substrate after administration of BzCl
---●---: DCNB as substrate after administration of DCNB
—○—: BrPh as substrate after administration stration of BrPh

## Site of Enzyme Stimulation of GSH-conjugation with Bromobenzene (BrPh) in Rat Liver Treated with Phenobarbital

Rats were treated with phenobarbital (5 mg/100 g/day; intraperitoneally) for 3 days and liver GSH–conjugating activities with BrPh were determined. Microsomal and the supernatant fraction of liver of those rats were fractionated and enzyme activities were estimated under conditions as shown in Table III. Protein concentrations of microsomal fraction of control and treated rats were adjusted to 14.1 mg/ml and those of the supernatant fraction were adjusted to 21.8 mg/ml. Activity for the conjugation of GSH with BrPh in the control

Table II. Effect of Pretreatment of Bromobenzene (BrPh) and Phenobarbital on Activities of GSH-conjugating Enzymes and Aniline-hydroxylating Enzyme in the Liver of Rats

| System | Relative specific activities (%)[a] | |
|---|---|---|
| | BrPh-treated rat | Phenobarbital-treated rat |
| GSH-conjugating activities | | |
| BzCl | 96.5±6.1[b] | 97.2± 4.3[b] |
| DCNB | 105.0±5.2 | 114.0± 9.3 |
| BrPh | 110.2±8.7 | 228.4±12.3 |
| Hydroxylating activity | | |
| Aniline | 108.7±8.8 | 230.8±14.1 |

a) Values are represented as per cent of the control rat.
b) mean ±standard error

rat was 0.56 μmole/tube/hr. That of phenobarbital-treated rat was apparently stimulated (1.23 μmole/tube/hr, 220% of the control). The activity in the system with treated microsomal fraction plus control supernatant fraction was stimulated (1.18 μmole/tube/hr, 312% of the control), although that with control microsomal fraction plus treated supernatant fraction was not stimulated (0.59 μmole/tube/hr).

Table III. Stimulation of the Enzyme Activity for the Conjugation of GSH with Bromobenzene

| Enzyme preparation used | | Enzyme activity (μmole/tube/hr) | Ratio (%) |
|---|---|---|---|
| Microsomal fraction | Supernatant fraction | | |
| Control | control | 0.56 | (100) |
| Treated | treated | 1.23 | 220 |
| Control | treated | 0.59 | 105 |
| Treated | control | 1.18 | 213 |

The microsomal fraction (0.5 ml)and the supernatant fraction (0.5 ml) were used as enzyme preparations. Bromobenzene and GSH were used as substrates. Protein concentrations were 14.1 mg/ml and 21.8 mg/ml, respectively.

## Discussion

It was shown by Grover and Sims[11] that the enzyme activity of GSH-conjugation with 3,4-dichloronitrobenzene was not changed but was decreased by the pretreatment of rats with aromatic hydrocarbons. In the present experiment, after rats were pretreated with bromobenzene, benzylchloride or 3,4-dichloronitrobenzene, the activities of GSH-conjugating system with those compounds were determined in the liver. The enzyme activity toward bromobenzene or 3,4-dichloronitrobenzene was not significantly enhanced but the activity toward benzylchloride was decreased by the pretreatment of each compound. These results support the view by Grover and Sims[11] that the enzyme catalyzes the GSH-conjugation with benzyl chloride is different from enzymes toward 3,4-dichloronitrobenzene and bromobenzene.

The enzyme activities toward benzylchloride and 3,4-dichloronitrobenzene did not show the significant changes by the pretreatment of rats with bromobenzene or phenobarbital.

Although the activity toward bromobenzene was also not enhanced by the pretreatment of rats with bromobenzene, the activity was stimulated by the pretreatment with phenobarbital (about 2 times of the control). It was found that this stimulation was due to increase

11) P.L. Grover and P. Sims, *Biochem. J.*, **90**, 603 (1964).

of microsomal enzyme system and the ratio of the stimulation was on the same degree as that of aniline–hydroxylation in microsomes. From these results, it was assumed that the stimulation of the activity by the treatment with phenobarbital was due to the enzyme induction in the liver microsomes of the rat.

Gillham and Young[12] isolated 4-bromophenylpremercapturic acid from the urine of rats and rabbits treated with bromobenzene. The structure of the premercapturic acid was identified to N-acetyl-S-(4-bromo-1,2-dihydro-2-hydroxyphenyl)-L-cysteine (V in Fig. 3). It is expected that liver enzyme catalyzes the conjugation of GSH and Bromobenzene (I) in earlier step of the mercapturic acid formation. In the present experiment, it was concluded that microsomes and NADPH in addition to the supernatant fraction and GSH were necessary for this conjugation of GSH and Bromobenzene. This conjugation activity was stimulated by the pretreatment of rats with phenobarbital. This enzyme induction was found to occur in the microsomal enzyme but not in the supernatant enzyme. These results propose the possibility that the conjugation of GSH with bromobenzene proceeds in two step reaction; (i) bromobenzene was oxidized by the microsomal mixed function oxygenase system to give rise to an unidentified oxidized intermediate and (ii) the supernatant enzyme catalyzes the conjugation of GSH and the intermediate (Fig. 3).

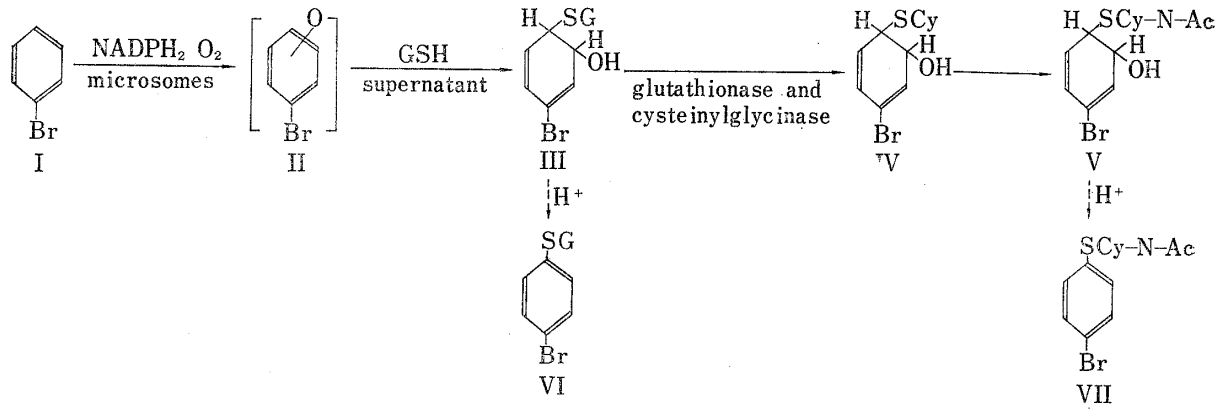


Fig. 3. Possible Pathway of 4-Bromophenylmercapturic Acid Formation in the Rat

Boyland and Williams[5] established that the supernatant enzyme of the rat liver catalyzed the GSH-conjugation with epoxides and proposed that the epoxide derivative of naphthalene was produced as the oxidized intermediate by the catalysis of the microsomal oxygenase system and that it conjugated with GSH to yield S-(1,2-dihydro-2-hydroxynaphthyl)glutathione by the action of the supernatant enzyme. Therefore, the oxidized intermediate (II) of bromobenzene may possibly be thought to be an epoxide-type compound. It was established by Suga, Kumaoka, and Akagi[13] that the conversion of GSH derivatives (III) to cysteine derivatives (IV) were catalyzed by constitutive enzymes; glutathionase and cysteinylglycinase. These findings may support the proposed pathway of the mercapturic acid formation.

12) B. Gillham and L. Young, *Biochem. J.*, **109**, 143 (1968).
13) T. Suga, H. Kumaoka, and M. Akagi, *J. Biochem.*, **60**, 133 (1966).