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Tetsuya Suga,*1 Isao Ohata,*2 Hiroshi Kumaoka,*3 and Masuo Akagi*3 : Studies on Mercapturic Acids. Investigation of Glutathione-conjugating Enzyme by the Method of Thin-layer Chromatography.*4

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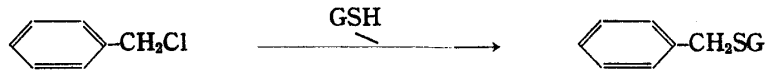
In previous work,[1] it was suggested that liver glutathione (GSH) was involved in mercapturic acid formation in animals. Since Booth, *et al.*[2] found the enzyme which catalyzed the conjugation of GSH with 3,4-dichloronitrobenzene, it has been found that there were several enzymes catalyzing the conjugation of GSH with some chemical compounds.

The enzyme activity, which catalyzed the conjugation of GSH with 3,4-dichloronitrobenzene, has been measured with a simple method; the formation of GSH-conjugated compound was measured by increase of extinction at 340 mμ.[2] This method, however, can not be applied to other substrates. So general method for assay of glutathione-conjugating enzymes has been required.

An assay method for GSH-conjugating enzyme was presented, by which GSH-conjugation with other compounds was able to be estimated. In the present work benzyl chloride (BzCl) was mainly used as substrate and S-benzylglutathione (BzSG) which was formed by conjugation was isolated by the method of thin-layer chromatography (TLC) and measured with Ninhydrin reagent.



Benzyl chloride (BzCl) S-Benzylglutathione (BzSG)

Chart 1. Enzymic Conjugation between BzCl and GSH

This method may be applied to all of mercapturic acid-forming compounds. Moreover, it was able to be used for the investigation of enzymic breakdown of GSH derivatives.[3]

Materials and Methods

Materials——GSH was purchased from Sigma Co., Ltd. VzSG was prepared by the method of Kermack, *et al.*[4] S-(2-Chloro-4-nitrophenyl)glutathione was prepared according to the method of Booth, *et al.*[5]

Assay Method for the Enzyme Activity——As the enzyme preparation, the supernatant fraction of rat tissue or partialy purified preparation was used. BzCl and GSH were used as substrates. Composition of the reaction mixture was as follows; 0.5 ml. of enzyme preparation, 20 m*M* of BzCl in 0.1 ml. of ethanol,

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*4 Part of this work was presented at the 38th Annual Meeting of the Japanese Biochemical Society (1965).

1) T. Suga, I. Ohata, M. Akagi : J. Biochem., **59**, 209 (1966).
2) J. Booth, E. Boyland, P. Sims : Biochem. J., **79**, 516 (1961).
3) T. Suga, H. Kumaoka, M. Akagi : J. Biochem., **60**, 133 (1966).
4) W. O. Kermack, N. A. Mantheson : Biochem. J., **65**, 45 (1957).
5) J. Booth, E. Boyland, P. Sims : *Ibid.*, **74**, 117 (1960).

20 mM of GSH, and 50 mM of phosphate buffer (pH 6.75) in 2.5 ml. of final volume. Incubations were carried out for 20 min. at 37° and terminated by the addition of 0.5 ml. of 18% metaphosphoric acid. After 15 min., the mixture was centrifuged and the supernatant fluid was subjected to the measurement of BzSG formed.

TLC of GSH Derivatives——GSH derivatives which formed by the conjugation of GSH with several compounds were detected on chromatogram of thin-layer (Fig. 1). TLC was carried out by use of silica gel plate (Wako gel B-5), 20×20 cm. in size, activated at 105° for 30 min. Sample solution (10~50 μl.) was spotted on the plate and developed with the solvent system of butanol-acetic acid-water (4:1:2, by vol.). After visualizing the GSH derivative with 0.5% Ninhydrin reagent in acetone-ethanol (1:1, by vol.), the corresponding area on the chromatogram was scraped up into a centrifuging tube and extracted with 4 ml. of a solvent was a mixture of ethanol (3 vol.) and 0.12% $CuSO_4$(1 vol.). The mixture was shaked to complete the elution and centrifuged for 10 min. at 3000 r.p.m. Extinction of the supernatant solution at 540 mμ was measured and the amount of the GSH derivative was calculated from the calibration curve of standard sample (Fig. 2). The calibration curve was found to be linear between 0 to 0.2 μmole.

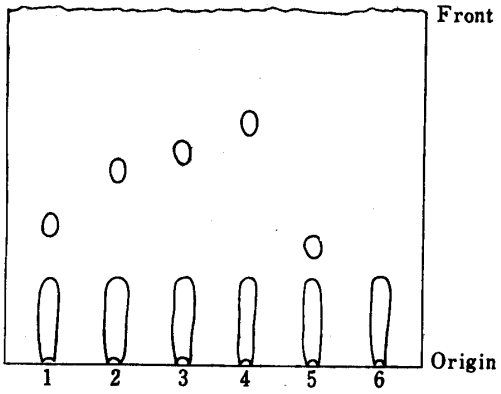

Fig. 1. Enzymic Conjugation of GSH with Several Compounds

Incubation were carried out with rat liver supernatant fraction, and substrate (10 mM) in 0.05M Tris buffer (pH 8.0) for 20 minutes at 37°. After the incubation, deproteinized samples were chromatographed by TLC (*n*-BuOH-AcOH-water, 9:1:2).
substrate
1. *n*-Butyl chloride
2. Benzyl chloride
3. *p*-Chlorobenzylchloride
4. *p*-Nitrobenzylchloride
5. 3,4-Dichloronitrobenzene
6. None

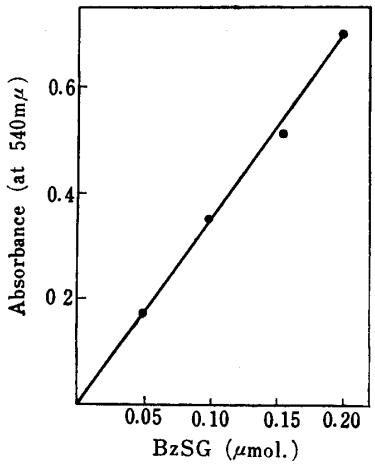

Fig. 2. Calibration Curves of BzSG

Measurement of Enzyme Activity Catalyzes the Conjugation of GSH with 3,4-Dichloronitrobenzene——S-(2-Chloro-4-nitrophenyl)glutathione which formed by the condensation of GSH and 3,4-dichloronitrobenzene by catalysis of the enzyme was determined by means of Booth, *et al.*[2] Composition of incubation mixture was as follows; 0.2 ml. of enzyme preparation, 20 mM of GSH, 20 mM of 3,4-dichloronitrobenzene in ethanol, and 50 mM of Tris buffer (pH 8.0) in 2.5 ml. of final volume. After the incubation at 37° for 10 min., 0.5 ml. of 18% metaphosphoric acid was added to the reaction mixture and centrifuged at 3000 r.p.m. for 15 min.

The supernatant fluid was diluted to a favorable volume, extinction of 340 mμ was measured and the amount of the GSH-conjugate was calcurated.

Enzyme Preparation——Generally the supernatant fraction of centrifugation at 105,000×g was used as an enzyme preparation. Rat was killed by decapitation and the liver was taken into ice-cold 0.15M KCl. Homogenization was carried out in a Potter-Elvehjem type homogenizer with a Teflon pestle for 2 min. All procedures were worked out below 2°.

Subcellular fractions were obtained by the homogenate successively centrifuging at 700×g for 10 min. (Nuclear fraction), at 1,300×g for 20 min. (Mitochondrial fraction), at 105,000×g for 60 min. (Microsomal fraction), and the remaining supernatant solution (Supernatant fraction). Each pellet was resuspended in 0.1M Tris (tris-(hydroxymethyl)amino methane) buffer and collected by centrifugation.

Partial Purification of the Enzyme——Procedures of enzyme purification were carried out by the method of Booth, *et al.*[2] (Chart 2).

Determination of Protein——The method of Lowry, *et al.*[6] was used for the determination of protein in enzyme preparations.

6) O. H. Lowry, N. J. Rosenbrough, A. L. Farr, R. J. Randall: J. Biol. Chem., **193**, 265 (1951).

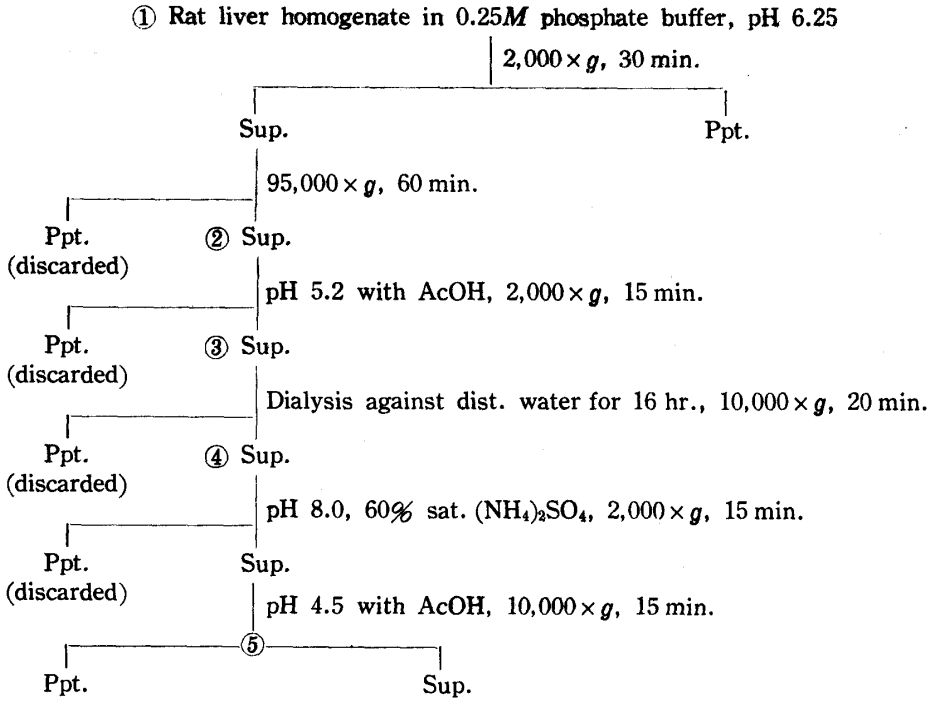

Chart 2. Partial Purification of GSH-conjugating Enzyme

Results

1. The Behavior of the Enzyme Reaction

Condensation of BzCl and GSH undergoes non-enzymatically. The reaction was accelerated by the addition of enzyme preparation and its rate was linear to the amount

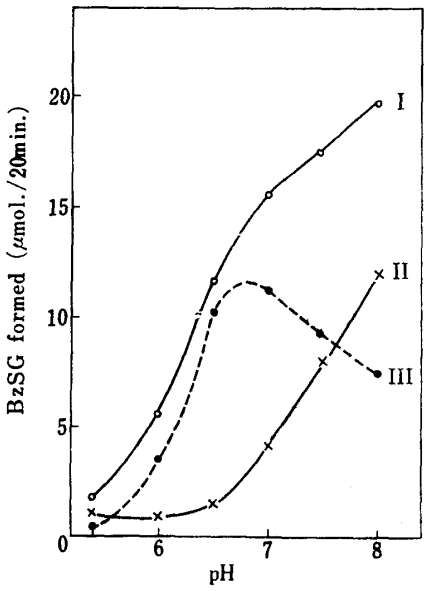

Fig. 3. Effect of pH on Formation of BzSG

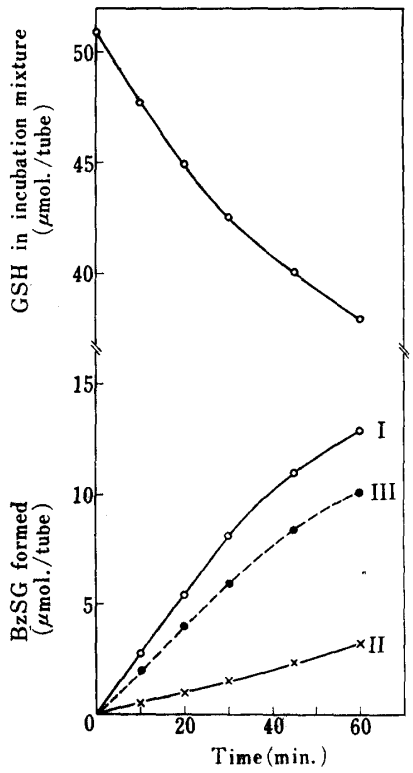

Fig. 4. Formation of BzSG and Consumption of GSH

I. Complete system II. Boiled enzyme system III. I − II

of enzyme. As shown in Fig. 3, non-enzymic reaction was not significant below pH 6.5. Enzymic activity was shown with curve Ⅲ and its optimal pH was about 6.75.

The reaction was linear to 30 min. and 8.1 μmoles of BzSG was formed after 30 min. and 5.9 μmoles among them was enzymic product (Fig. 4). The supernatant fraction of rat liver was used for the enzyme preparation and incubation was carried out at 37° for favorable time intervals. Reaction was terminated by the addition of 3% (final concentration) metaphosphoric acid and 10~50 μl. of deproteinized supernatant fluid was applied on thin-layer chromatogram. After developing for 3 hr. with a solvent system, visualized spots by Ninhydrin reagent were eluted in the solvent as described above. The residual GSH in the reaction mixture was estimated during the incubation by the method previously described.[1]

For first 30 minutes, the amount of GSH decrease was parallel with the amount of BzSG formation. Enzymic conjugation of BzCl with cysteine was not observed. This enzyme was stable at 37° for 30 min. and at 0° or −20° for 4 days, but 63% of the activity was lost by allowing to stand for 10 min. at 50°.

Effect of substrate concentration on the enzyme activity was as shown in Fig 5.

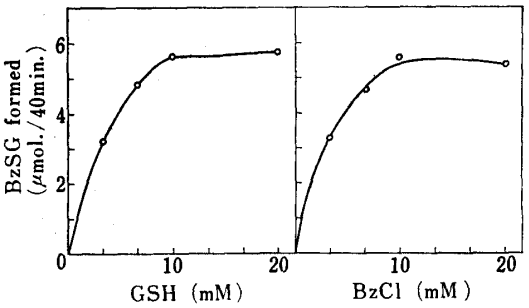

Fig. 5. Effect of Substrate-concentration on the Enzyme Activity

2. Distribution of the Enzyme

Distribution of the enzyme was examined in tissues. Ten per cent homogenate in 0.15*M* KCl was prepared and centrifuged at 105,000×*g* for an hour. The supernatant fraction was used as enzyme preparation. Among tissues tested, the liver, was the richest in the enzyme and its specific activity was 4.03 μmol. BzSG mg. protein/10 min. That of the kidney was 2.94 μmol. and it was corresponded to 73% of the liver.

Table I. Distribution of the Activity of GSH-conjugating Enzyme in Tissues

Tissue	Specific activity (μmol./mg. protein/10 min.)	Ratio (%)
Liver	4.03	(100)
Kidney	2.94	73
Spleen	0.54	13
Brain	0.18	4
Intestine	0.16	4
Lung	0.15	4
Heart	0.11	3

Table II. Specific Activity of GSH-conjugating Enzyme in Subcellular Fractions

Fraction	Specific activity (μmol./mg. protein/20 min.)	
	Liver	Kidney
Nuclear fraction	0.50	0.41
Mitochondrial fraction	0.18	0.10
Microsomal fraction	0.45	0.43
Supernatant fraction	7.31	4.91

Subcellular distributions of GSH-conjugating enzyme in the liver and kidney were examined (Table II). The specific activity was the highest in the supernatant fraction of the liver as well as the kidney.

Total activity of this fraction was more than 75% in the whole homogenate.

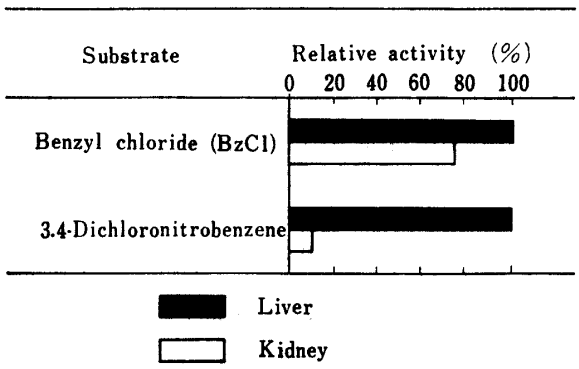

Fig. 6. Effect of Substrate on the Distribution Pattern of GSH-conjugating Enzyme in Tissues

Distribution of enzyme activity of GSH-conjugation in using BzCl as substrate was compared with that of 3,4-dichloronitrobenzene in liver and kidney. Supernatant fractions of liver and kidney were used for the enzyme preparation. Enzyme activity was measured by means of TLC-Ninhydrin method and the method of Booth, *et al.*,[2] respectively. As shown in Fig. 5., ratio of distribution of enzyme activity in liver and kidney was 100:73 when BzCl was used as substrate. On the other hand, the ratio was 100:8 when 3,4-dichloronitrobenzene was used.

3. Attempt to Purify the GSH-conjugating Enzyme

In order to separate two enzymes which require BzCl and 3,4-dichloronitrobenzene as substrates, purification procedures were carried out by the method of Booth, *et al.*[2] (Chart 2).

As shown in Table III, the enzyme which catalyzed the conjugation of GSH with BzCl was 9 times purified from the original homogenate. Relatively high specific activity of the enzyme was lost significantly between Step 4 and Step 5.

This enzyme may, therefore, be unstable at pH 4.5. Specific activity of the enzyme was 2.40 μmol./mg. protein/20 min. and recovery of total activity was 10%.

Table III. Partial Purification of GSH-conjugating Enzyme

Step	Protein (mg.)	Total act. $\left(\frac{\mu mol.}{20\ min.}\right)$	Sp. act. $\left(\frac{\mu mol.}{mg.\ prot.}\right)$	Purity	Recovery
1. Homogenate	6700	1750	0.26	(1)	(100)
2. Sup. fr.	3250	1150	0.35	1.3	66
3. pH 5.2, sup.	1640	810	0.49	1.9	46
4. Dialysis, sup.	1220	700	0.62	2.4	43
5. pH 4.5, p.p.t.	31.9	45	1.40	5.4	3
pH 4.5, sup.	77.0	182	2.40	9.2	10

Discussion

In earlier works,[1,7,8] it was found that GSH level in animals received some foreign compounds was depressed and "GSH-conjugating enzymes" were found in animal tissues, which catalyzed conjugation of GSH with those compounds. In view of these findings, it may be true that GSH is involved in mercapturic acid formation.

The enzyme activity of GSH-conjugation has been determined by several methods. When 3,4-dichloronitrobenzene was used as substrate, absorbance of the GSH-

7) H. Waelsch: Arch. exp. Path. Pharm., **156**, 356 (1930).
8) K. Yamamoto: Mitt. med. Akad. Kioto, **29**, 431 (1940).

conjugate (340 mμ) was measured with higher sensitivity. This method, however, was not applicable to other compounds. In the conjugation of GSH and nitro compounds, NO_2^- ion is liverated for each GSH molecule conjugations. Al-Kassab, *et al.*[9] measured NO_2^- ion to determine the enzyme activity of those conjugations. Johnson[10,11] determined the activity of enzyme which catalyzed the conjugation of GSH with halogen compounds.

In the present work, a TLC-Ninhydrin method for the determination of the activity of GSH-conjugating enzymes was presented and properties of the enzyme which catalyzed the conjugation of GSH with BzCl were examined. This method may be suitable not only for the determination of GSH-conjugation but for the quantitative investigation for the conversion of GSH derivatives to cysteine derivatives which is proposed to the intermediate pathway of mercapturic acid formation.

Conjugation of GSH with BzCl was seen to proceed nonenzymatically and to be accelerated by the addition of the supernatant fraction of rat liver. Its reaction rate was proportional to the amount of the enzyme preparation. Optimal pH of this enzyme reaction was 6.75. Liver and kidney was rich in this enzyme. The ratio of distribution of the activity in liver and kidney was 100:73 when BzCl was used as substrate, while the ratio using 3,4-dichloronitrobenzene was 100:8. Differency of the distribution of activities for those two compounds seems to support a consideration that separated enzymes catalyze GSH-conjugation to each compound.

9) S. Al-Kassab, E. Boyland, K. Williams : Biochem. J., **87**, 4 (1963).
10) M. K. Johnson : Biochem., **87**, 9 P (1963).
11) *Idem* : Biochem. J., **98**, 44 (1966).