

Initial Intraorgan Formation of Mercapturic Acid

Kazumi SANO,* Yoji IKEGAMI, and Takashi UESUGI

Department of Drug Metabolism and Disposition, Meiji Pharmaceutical University, Kiyose, Tokyo 204-8588, Japan.

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The disposition of *S*-benzyl-glutathione (BSG) in male Wistar rats was evaluated by the HPLC method to examine whether the kidney and liver contributed independently to the biosynthesis of *S*-benzyl-*N*-acetylcysteine (BNAc), a mercapturic acid (Chart 1). After intravenous injection, BSG was rapidly transported in both the kidney and the liver at a ratio of about 7:3. Simultaneously, a large amount of BNAc was found in both the kidney and the liver. In the kidney, *S*-benzyl-cysteine (BCys) reached a maximum concentration (C_{\max}) at 2 min after BSG injection, whereas BNAc reached C_{\max} within 3 to 5 min. The generation of BNAc was also observed in the liver. While renal BNAc reached C_{\max} within 3 to 5 min, hepatic BNAc reached C_{\max} around 5 min after BSG injection. Moreover, the elimination half-life of the BNAc after intravenous injection of the BSG was equivalent to that observed after intravenous injection of the BNAc itself. These results demonstrate that the kidney contributes to the initial intraorgan generation of BNAc and that this mercapturic acid is also synthesized in the liver and preferentially excreted into urine.

Key words *S*-benzyl glutathione; intraorgan; mercapturic acid; retrograde; renal excretion; hepatobiliary

Mercapturic acid biosynthesis is one of the important detoxification mechanisms for many xenobiotics. The mercapturic acid pathway is thought to involve interorgan cooperation of the liver and kidney. Inoue *et al.*¹⁾ reported that intravenous injection of *S*-carbamido (^{14}C) methyl glutathione in mice resulted in the rapid accumulation of radioactivity in the kidney. The subsequent decrease in renal radioactivity corresponded to an increase in hepatic radioactivity without excretion of radioactivity into urine. Based on these results, they proposed that mercapturic acid is formed by a process of interorgan cooperation involving these two organs. Lending support to such a notion of interorgan cooperation in the formation of mercapturic acid is the established fact that glutathione (GSH) conjugation occurs in the liver and GSH hydrolysis to cysteine takes place in the kidney before *N*-acetylation of cysteine occurs after transport back to the liver.

Hinchman *et al.*,²⁾ however, published findings that refuted such a theory of mercapturic acid formation. They demonstrated intrahepatic biosynthesis of mercapturic acids in isolated perfused livers from rats and guinea pigs using the model compound 1-chloro-2,4-dinitrobenzene (CDNB), which has a high molecular weight and lipophilicity. We have also confirmed the intra-hepatobiliary formation of mercapturic acid using sulphobromophthalein (BSP).³⁾

In the present study, *S*-benzyl-glutathione (BSG) was used as the model compound and its metabolites were determined by an ion-paired HPLC method. Using whole bodies of rats *in vivo*, we demonstrated that the liver and kidney have independent intraorganic pathways of mercapturic acid synthesis.

MATERIALS AND METHODS

Chemicals Cysteinylglycine and glutamylcysteine were purchased from Bachem Bioscience Inc. (Bubendorf, Switzerland). Acetyl-CoA and acivicin (AT-125) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents obtained were of the highest quality available.

Chemical Synthesis of *S*-Benzyl Mercaptides *S*-benzyl mercaptides were prepared from benzyl chloride as the de-

rivatizing reagent by the following modification of the method of Sokolovsky *et al.*⁴⁾ BSG, *S*-benzyl cysteine (BCys), *N*-acetyl-*S*-benzyl cysteine (BNAc), *S*-benzyl cysteinylglycine (BCysGly) and *S*-benzyl glutamylcysteine (BGlucys) were synthesized as follows: Benzyl chloride (1 mmol in 10 ml of methanol) was added slowly with stirring to a solution of 1 mmol of each mercaptide dissolved in 5 ml of 2.5% NH_4OH . The reaction was allowed to proceed for 15–20 min. The reaction mixture was concentrated *in vacuo* and acidified with dil. HCl. A fine white precipitate was collected by vacuum filtration and washed with ice cold water and methanol, then recrystallized from aqueous methanol. *N*-acetyl-*S*-benzyl glutathione (NABSG) was

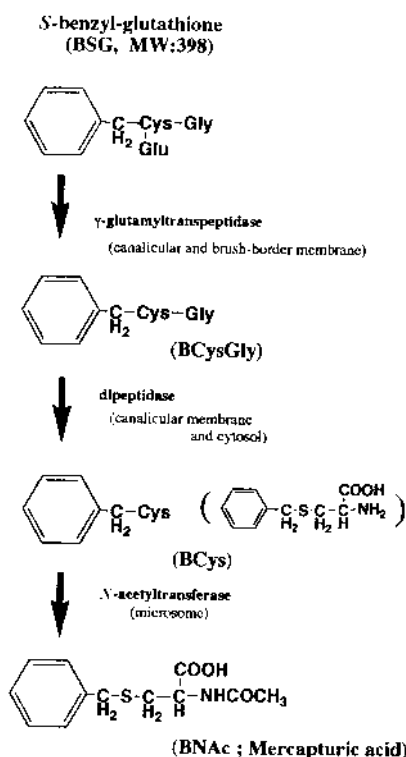


Chart 1

* To whom correspondence should be addressed. e-mail: ksano@my-pharm.ac.jp

synthesized according to the procedure of Zbarsky *et al.*⁵⁾ Each *S*-benzyl mercaptide was checked for purity by HPLC and confirmed to be more than 99% pure.

Animal Experiments Male Wistar rats (230–250 g, Tokyo Laboratory Animals Co., Ltd., Tokyo, Japan) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Through an abdominal incision, the bile duct and ureters were cannulated with polyethylene tubing (PE-10). Body temperature was maintained at $38 \pm 0.5^\circ\text{C}$ using a heating lamp. For the continuous collection of the urine, mannitol (20 mg/ml in saline) was infused into the femoral vein at a rate of 2.34 ml/h. BSG and BNAc were dissolved in saline and each solution containing 10 μmol of drug (2 ml/kg) was injected into the opposite femoral vein over a 15-s period.

Animals were pretreated by retrograde intrabiliary infusion of acivicin as follows: Acivicin (20 $\mu\text{mol/kg}$) dissolved in saline (50 μl) was introduced into the bile duct cannula over a 30-s period and followed by a 50 μl wash of normal saline. After 2 min, bile was allowed to flow freely. Ten minutes after the acivicin infusion, BSG solution was injected into rats.

Preparations of Liver, Kidney and Plasma Samples At fixed time periods (1, 2, 3, 5, 10, 15 min) after administration of BSG, the liver and kidneys were immediately excised and homogenized with 4 vol. of 0.1 M NaOH. An equal volume of 4.5% ZnSO_4 was added to the homogenates followed by centrifugation at $15000 \times g$ for 10 min. *S*-Benzyl mercaptides in the supernatants were determined by HPLC. Blood samples (0.1 ml) were collected from the femoral artery and centrifuged to obtain the plasma fractions.

HPLC Analysis *S*-benzyl mercaptides were detected by the modified HPLC method of Uesugi *et al.*⁶⁾ The HPLC system consisted of a Jasco model 2000 liquid chromatograph system utilizing a Jasco RP spectrometric detector (Nippon Bunko Inc., Tokyo, Japan) and a Shimadzu WX integrator (Shimadzu Bunko, Kyoto, Japan). Isocratic elutions were performed using a Capcell Pak C18 SG column (5 μm , $6 \times 150 \text{ mm}$; Shiseido Co., Ltd., Tokyo, Japan) with a mobile phase of 0.1 M (pH 7.0) triethylamine- H_3PO_4 buffer containing 1 mM tetra-*n*-butylammonium bromide : methanol : acetonitrile (75 : 25 : 8, v/v/v) at a flow rate of 1.0 ml/min. Compounds were detected at 240 nm and quantitated by the external standard method using the area under the peaks. The bile and urine samples were diluted with the mobile phase and injected into the column directly. The limit of detection was defined as the amounts of the compounds resulting in a signal-to-noise ratio of 5. The detection limit of BSG was *ca.* 20 pmol. The calibration lines for BSG resulting from a least-squares analysis for best fit was $y = 53.45x - 750$ ($r^2 = 0.998$). Five aliquots of each sample was analyzed on the same day, and the resulting coefficient variation (C.V.) indicated within-day reproducibility. Aliquots of the same samples were tested once a day for 5 d, and the resulting C.V. indicated between-day reproducibility. The C.V. of peak areas for each compound was less than 2%.

Pharmacokinetic Analysis The plasma, hepatic and renal concentration–time data (0–15 min) from each subject was analyzed by a model-independent method using the MULTI computer program.⁷⁾ The area under the concentration–time curve (*AUC*) was calculated from the values obtained (0–15 min) using the trapezoidal rule. The half-life

($t_{1/2}$) was obtained by dividing natural logarithm of 2 by K_{el} , the apparent elimination rate constant, as obtained from the amount remaining to be excreted in urine.

Preparation of Microsomal Fractions After overnight starvation, the animals were anesthetized with ether and then killed by exsanguination. Tissues were homogenized in 4 vol. of ice-cold 1.15% KCl in Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at $20000 \times g$ for 20 min and then the supernatant was recentrifuged at $105000 \times g$ for 60 min. The microsomal pellet resuspended in the homogenizing medium containing 20 (v/v) % glycerol was used as an enzyme source of *N*-acetyltransferase. Protein concentrations were determined according to the method of Lowry *et al.*⁸⁾ using bovine serum albumin as a standard protein.

***N*-Acetyltransferase Activity** Microsomal *N*-acetyltransferase activity towards BCys was assayed by the modified procedure of Green *et al.*⁹⁾ in which cold acetyl-CoA was used instead of [^{14}C] acetyl-CoA. The addition of the same volume of dimethyl sulfoxide (DMSO) as the incubation mixture terminated the enzyme reaction, and the mixture was centrifuged at $15000 \times g$ for 5 min. The BNAc formed was directly determined by HPLC analysis of the supernatant. BNAc could be quantitatively recovered from the assay mixture at concentrations ranging from 20–2000 nmol/ml by DMSO extraction.

RESULTS AND DISCUSSION

Figure 1 shows the elution pattern of authentic compounds of BSG metabolites (A), chromatograms obtained from drug-free kidney homogenate (B) and a kidney homogenate sample at 3 min after BSG injection (C). The metabolites of BSG were separated and quantitated well, without disturbance to the physiological components.

When BSG (10 μmol) was injected intravenously, 76% of the dose over a 90-min period was excreted into urine, whereas only 6% was excreted in bile (Fig. 2). BNAc was the

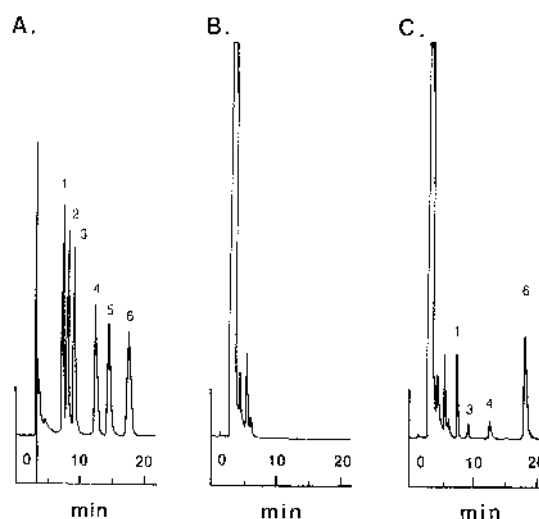


Fig. 1. Chromatograms of HPLC Pattern of A; Standard Mixture, B; Drug-Free Kidney Homogenate, and C; Kidney Homogenate at 3 min after Intravenous Injection of BSG (10 μmol)

HPLC conditions are given in Materials and Methods. Peaks: 1=*S*-benzyl cysteine (BCys); 2=*S*-benzyl glutamylcysteine (BGluCys); 3=*S*-benzyl-GSH (BSG); 4=*S*-benzyl-(*N*-acetyl)-glutathione (NAcBSG); 5=*S*-benzyl cysteinylglycine (BCysGly); 6=*S*-benzyl-(*N*-acetyl)-cysteine (BNAc).

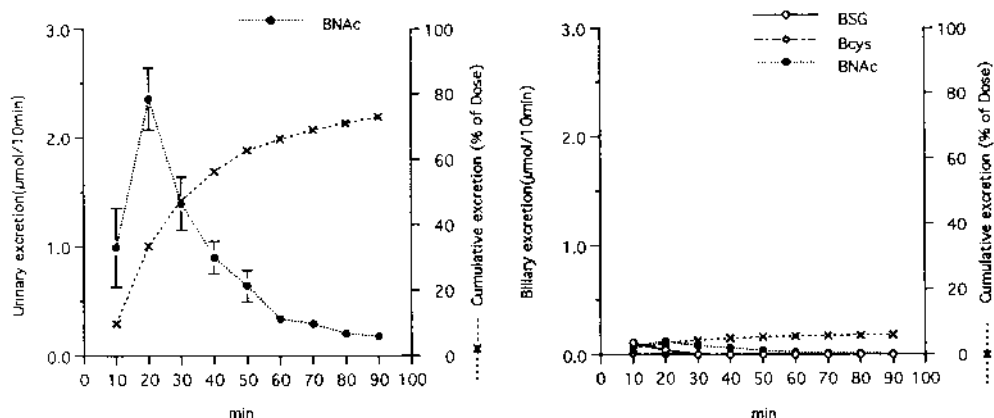


Fig. 2. Time Course of S-Benzyl-mercaptide Excretion after Intravenous Injection of BSG (10 μ mol) in Male Rats

Data are expressed as the mean \pm S.E. of 4 animals.

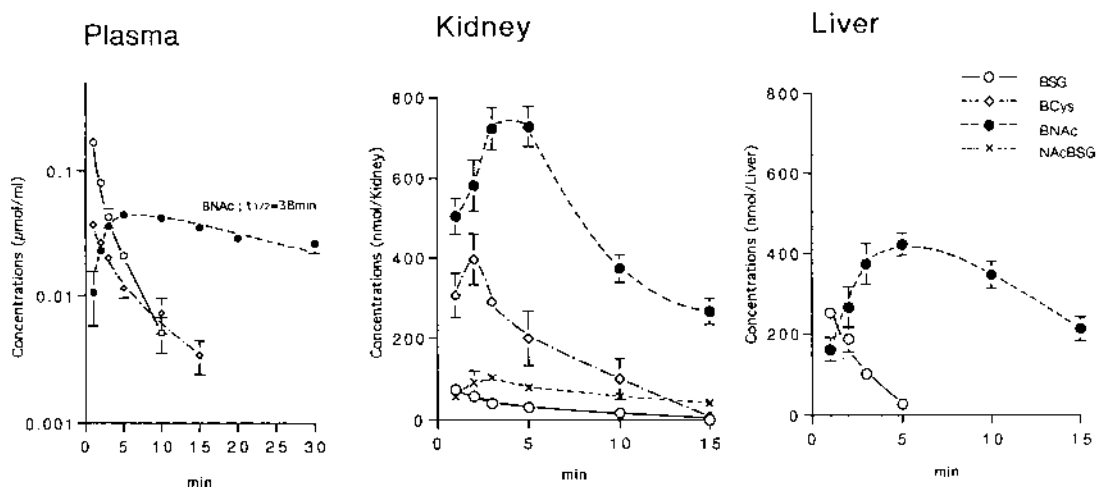


Fig. 3. Time Course of S-Benzyl-mercaptide Concentrations in Plasma, Kidney and Liver after i.v. Intravenous Injection of BSG (10 μ mol)

Data are expressed as the mean \pm S.E. of 4 animals.

sole metabolite in urinary excretion. In biliary excretion, however, small amounts of unchanged BSG, BCys and BNAC were detected simultaneously. Subsequent measurement of the concentration transitions of plasma, and the kidney and liver are shown in Fig. 3. Following the rapid disappearance of BSG from the blood, an efflux of BNAC from the liver into blood was observed. The elimination half-life ($t_{1/2}$) of BNAC was 38 min. The BSG was rapidly distributed into both the kidney and the liver at a ratio of about 7:3. This distribution ratio was estimated from the amount of total metabolites in the kidney and liver at 1 min postinjection.

Although a small amount of BSG was detected in the kidney in the initial stage, it subsequently disappeared, and NAcBSG was also detected. BCys reached a maximum concentration (C_{\max}) at 2 min after BSG injection, whereas BNAC reached C_{\max} within 3 to 5 min. The peak value in the kidney was about 1.7 times of that detected in the liver. In addition, a high concentration of BNAC was observed in the liver. The AUC (0–15 min) of BNAC in the liver (4.73 μ mol/tissue/min) was two-thirds of that in the kidney (7.23 μ mol/tissue/min). While renal BNAC reached C_{\max} within 3 to 5 min, hepatic BNAC reached C_{\max} around 5 min after BSG injection.

The elimination rate of mercapturic acid was estimated from urinary excretion after the BSG injection. The $t_{1/2}$ was 14.7 min, which was essentially equivalent to that observed after intravenous injection of mercapturic acid itself (14.4 min) (Fig. 4). This result strongly suggests that BSG was very rapidly converted to BNAC. Moreover, it suggests that the BNAC generation rate in the liver and the efflux rate from the liver into blood occurs more rapidly than the urinary excretion rate. Given these results, we could not deny that BCys was indeed shuttled from the kidney to the liver, but it was also clear that BNAC was generated in each organ independently in the early term (1–5 min). However, we observed an efflux of BNAC, and not BCys, into blood following the rapid disappearance of BSG. The elimination half-life of BNAC was 38 min. The most efflux of BNAC to plasma appears to be derived from the liver, because the elimination rate of BNAC estimated from urinary excretion was rapid ($t_{1/2}$, 15 min) and BNAC was not excreted at all into bile.

BSG transported in the liver may be rapidly secreted into the bile canaliculi and then broken down to a cysteine conjugate by biliary γ -glutamyltranspeptidase (γ -GT) and dipeptidases.^{10,11} Rats pretreated with acivicin (an inhibitor of γ -GT)¹² demonstrated increased biliary excretion of BSG and

a concomitant decrease in the biliary excretion of mercapturic acid in relation to that of the control animals (Fig. 5). This is clear evidence that BSG distributed in liver is degraded to BNAC by biliary γ -GT and dipeptidases.

Next, we compared the enzymatic activity related to mercapturic acid generation in the liver and kidney to confirm that this generation occurs in these two organs independently. The kidney is known to have high γ -GT activity^{13,14)} and the liver to have high *N*-acetyltransferase activity. Table 1 shows the specific activities of microsomal *N*-acetyltransferase of the liver and kidney toward BCys. The renal activity of *N*-acetyltransferase was sufficient to synthesize BNAC in the kidney.

Mercapturic acid biosynthesis is believed to occur through an interorgan process, with the liver as the major site for GSH conjugation and the kidney as the primary site for conversion of GSH conjugates into cysteine conjugates. The cysteine conjugates formed in the kidney appear to be transported back to the liver for *N*-acetylation, and mercapturic acids are ultimately excreted mainly in urine.¹⁾ This interorgan cooperation model originated from a study on the meta-

bolic fate of *S*-carbamido(¹⁴C)methyl glutathione (*Mw*: 364) in mice.¹⁾ The authors found a rapid accumulation (within 1 to 2 min) of radioactivity, accounted for by the presence of cysteine and cysteinylglycine conjugates, predominantly in the kidney, that subsequently decreased gradually with a concomitant increase in hepatic radioactivity. After intravenous administration of *S*-carbamido(¹⁴C)methyl cysteine (*Mw*: 178), a rapid accumulation of radioactivity in the liver was also observed. It is important to note that the shuttling of the cysteine conjugates back to the liver is analogous to that of amino acids and dipeptides released after the renal degradation of GSH,¹⁾ where the amino acids and dipeptides, such as glutamic acid and cysteinylglycine formed from GSH by renal γ -GT and dipeptidases, can be rapidly transported back to the liver for synthesis of new GSH.^{15,16)} Since the kidney has enough *N*-acetyltransferase activity (243 nmol/min/kidney towards BCys) to *N*-acetylation of cysteine conjugates (BCys *AUC* after BSG administration; 160 nmol/min/kidney) and a specific activity nearly the same as (Table 1) or twice that of the liver,^{9,17)} the shuttling of *S*-carbamidomethyl cysteine back to the liver for acetylation is puzzling. Given these findings, such a small cysteine conjugate as *S*-carbamidomethyl cysteine would probably be incorporated into the interorgan metabolism of GSH.

Other research groups have also suggested the intraorganic generation of mercapturic acid. Moldèus *et al.*¹⁸⁾ used acetaminophen as a substrate to demonstrate that conversion of the GSH conjugate to mercapturic acid was primarily catalyzed by isolated rat kidney cells, whereas isolated liver cells were quite efficient at catalyzing the formation of the GSH conjugate. Moreover, intrahepatic conversion of dini-

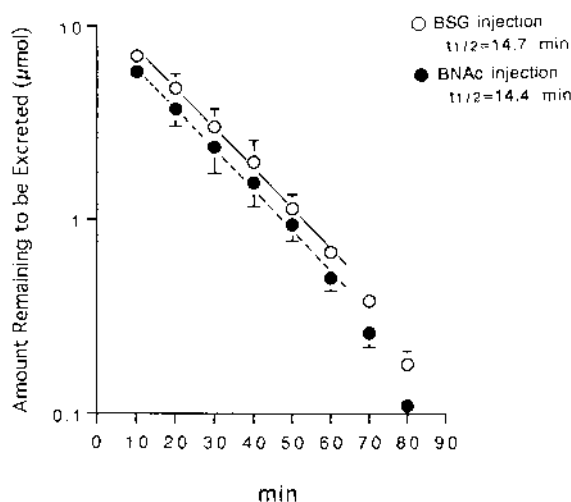


Fig. 4. Urinary Excretion of *S*-Benzyl Mercapturic Acid after i.v. Intravenous Injection of Drug (BSG: ○, BNAC: ●) (10 μ mol, respectively)

Table 1. *N*-Acetyltransferase towards *S*-Benzyl Cysteine (BCys) in the Microsomes of Male Wistar Rats

<i>N</i> -Acetyltransferase activity (nmol/min/mg protein)	
Liver	16.5 \pm 2.0 (14.5 \pm 0.9 ^{a)})
Kidney	16.2 \pm 0.6 (33.6 \pm 1.3 ^{a)})

N-Acetyl-*S*-benzyl cysteine (BNAC) formed were determined by the HPLC method as described in the text. Data are expressed as the mean \pm S.E. of 4 experiments. ^{a)} Values determined by the radio-assay method are cited from the data of Green *et al.*⁹⁾

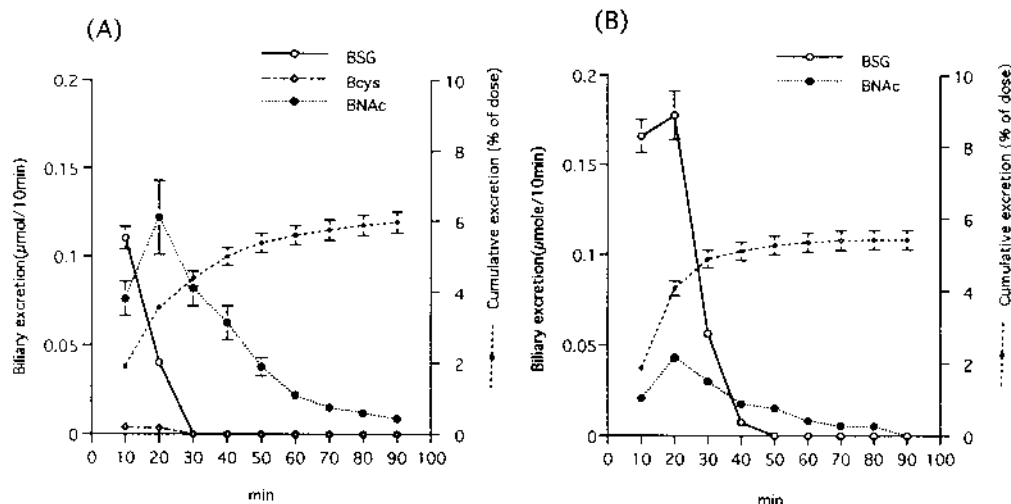


Fig. 5. Time Course of Biliary Metabolites after i.v. Intravenous Injection of BSG (10 μ mol)

(A) Control animals, (B) animals treated by retrograde infusion of acivicin (20 μ mol/kg) into bile duct. Data are expressed as the mean \pm S.E. of 4 animals.

trophenyl GSH (DNPSG) to mercapturic acid has been confirmed in isolated perfused rat and guinea pig livers.²⁾ Hinchman *et al.* indicated that cysteinylglycine, cysteine and *N*-acetylcysteine (NAC) conjugates were detected in bile and a noticeably larger amount of mercapturic acids were found in guinea pigs, which have higher γ -GT activities than rats. Recently they reported that the liver plays a significant role in the clearance of a representative mercapturic acid, DNP-NAC, from the bloodstream.¹⁹⁾ The study indicated that DNP-NAC is a substrate for an oatp-related sinusoidal organic anion solute transporter and a substrate for mrp2, the ATP-dependent canalicular organic solute transporter. At the same time, Jösch *et al.* reported the existence of substantial cysteinylglycine *S*-conjugate dipeptidase activity in the cytosol of the liver using biman *S*-conjugates as a model compound.²⁰⁾ They propose that this cysteinylglycine *S*-conjugate dipeptidase is involved in hepatic mercapturic acid formation from cysteinylglycine *S*-conjugate generated in the sinusoidal domain of the liver due to the presence of high γ -GT activity, as found in guinea pigs and humans. Taken together, these findings do indeed appear to confirm the presence of an intrahepatic mercapturic acid pathway.

The present study demonstrates that the kidney contributes significantly to the degradation of BSG and the subsequent intraorgan conversion to mercapturic acid, while mercapturic acid is also synthesized in the liver and preferentially excreted into the urine. Although the present study provides evidence for the intraorgan formation of mercapturic acid from GSH conjugates in the kidney and the liver, additional studies are needed to examine the extent to which mercapturic acid is excreted in the urine or bile of rats. The urinary and biliary excretion of the metabolites after intravenous injection of BSG (*Mw*: 398) was compared to those of *p*-nitrobenzyl-, DNP- and BSP-GSH conjugates in rats. *p*-nitrobenzyl-GSH (*Mw*: 443) was excreted as mercapturic acid into urine more than bile, 67.1 and 22.4% of dose, respectively. While biliary metabolite of DNP-GSH (*Mw*: 473) was GSH conjugate (21% of dose) and mercapturic acid (18%), urinary metabolite is only mercapturic acid (32%). BSP-GSH (*Mw*: 1064) conjugate was excreted only in the bile as unchanged GSH conjugate mainly (82% of dose). The excretion data of these GSH conjugates shows that urine and bile are comple-

mentary pathways; urinary excretion is greater for conjugates with lower molecular weights, and tends to decrease as molecular weight increases. This finding suggests that a balance of the orientation of distribution and excretion of each glutathione conjugate would decide the proportion of intraorgan and interorgan mercapturic acid synthesis. How to determine the orientation of distribution of GSH conjugates is under current investigation.

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