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INTER-ORGAN METABOLISM AND TRANSPORT OF A CYSTEINE-*S*-CONJUGATE OF XENOBIOTICS IN NORMAL AND MUTANT ANALBUMINEMIC RATS

MASAYASU INOUE,* KENJI OKAJIMA, SUMI NAGASE† and YOSHIMASA MORINO

Department of Biochemistry, Kumamoto University Medical School, Kumamoto 860, Japan; and
† Department of Chemistry, Sasaki Institute, Surugadai, Chiyodaku, Tokyo 101, Japan

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Abstract—Biosynthesis of *N*-acetylcysteine *S*-conjugates of toxic electrophiles, mercapturic acids, occurs via inter-organ metabolism and transport in which liver, small intestine and kidney play an important role. Since a mercapturic acid is a hydrophobic organic anion and strongly binds to plasma albumin *in vitro*, the ligand–albumin interaction may affect the metabolic fate of this final metabolite *in vivo*. To investigate the role of the circulating albumin in detoxication and elimination of a toxic electrophile, urinary occurrence of the final metabolite was determined in normal and mutant Nagase analbuminemic rats (NAR) after administration of *S*-benzylcysteine, a model compound of cysteine conjugates. *S*-Benzylcysteine intravenously administered was excreted rapidly into urine as its *N*-acetyl derivative in both animal groups. However, the urinary recovery of this mercapturic acid was significantly lower in NAR than in normal animals. The lower urinary recovery in NAR was due to a rapid and random distribution of the unbound metabolite in the circulation to extrarenal tissues. In contrast, no significant difference in the urinary recovery of the final metabolite was observed between the two animal groups if *S*-benzylcysteine was given orally. Kinetic analysis revealed that the major part of the orally administered *S*-benzylcysteine was transferred to the liver and acetylated predominantly in this organ in both animal groups; the mercapturic acid which was synthesized in the liver can be transferred to the kidney and excreted into urine even in the absence of the circulating albumin. These results indicate that albumin is important for a final elimination of a mercapturic acid when animals were extraorally challenged with a large dose of toxic electrophiles by which the rate of biosynthesis and the plasma level of the amphipathic metabolites were increased.

The liver possesses high activity of glutathione *S*-transferases which catalyze conjugation of electrophilic compounds with reduced glutathione (GSH). This reaction is the initial step in the events leading to the biosynthesis of the *N*-acetylcysteine *S*-conjugates, mercapturic acids [1–3]. Glutathione *S*-conjugates formed in the liver are excreted preferentially into bile at low concentrations of the metabolites [4]. Recent studies using isolated rat liver canalicular membrane vesicles revealed that a glutathione conjugate is excreted into bile as an organic anion via the membrane potential-dependent transport system [5]. The glutathione conjugate thus excreted is degraded partly within the bile ductular lumen [6], and the remaining part is degraded on the brush border membranes of the small intestine by γ-glutamyltransferase and peptidases that hydrolyze the cysteinylglycine bond [7]. In addition to these hydrolases, the small intestine also has an *N*-acetyltransferase specific for *S*-substituted cysteines [8] and, hence, participates in mercapturate biosynthesis *in vivo*.

Since *N*-acetyltransferase is bound to endoplasmic reticulum with its active site facing to the cytoplasmic space [9], a cysteine *S*-conjugate formed in the extracellular space must be translocated into cells for its *N*-acetylation. The resulting mercapturic acid may be transferred to the kidney and excreted into urine. Previous studies [10] revealed that, like phenolsulfophthalein (PSP) and *p*-aminohippuric acid (PAH) which bind to plasma albumin and undergo renal transtubular transport, a mercapturic acid in the circulation is excreted into urine via the probenecid-sensitive peritubular transport system for organic anions. Recent studies [11–13] in mutant analbuminemic rats revealed that binding of hydrophobic organic anions to the circulating albumin is important for directing these ligands to liver and kidney. Since a mercapturic acid is an amphipathic organic anion that binds to albumin with high affinity [14], its interaction with albumin may also be important for directing the ligand to the renal peritubular space where the transtubular secretory mechanism operates [13]. Despite extensive studies on the enzymes involved in mercapturate biosynthesis [1–3], the mode of its inter-organ metabolism and the role of albumin in the renal elimination of a final metabolite have remained unstudied. The present work compares the metabolic fate of *S*-benzylcysteine and *S*-benzyl-*N*-acetylcysteine, model compounds of a cysteine *S*-conjugate and its *N*-acetyl derivative, in normal and mutant Nagase analbuminemic rats (NAR).

* Address all correspondence to: Dr. M. Inoue, Department of Biochemistry, Kumamoto University Medical School, 2-2-1, Honjo 2-chome, Kumamoto 860, Japan.

MATERIALS AND METHODS

Materials. Cysteine, *N*-acetylcysteine and benzylchloride were obtained from the Nakarai Chemical

Co. (Kyoto, Japan). γ-Glutamyl-*p*-nitroanilide, acetyl-CoA, and defatted bovine serum albumin were obtained from the Sigma Chemical Co. (St. Louis, MO). [U-^{14}C]-Labeled cysteine was purchased from the Radiochemical Centre, Amersham. Radioactive *S*-benzylcysteine and *N*-acetylcysteine were synthesized as described previously [10, 15]. The specific radioactivity of the synthesized *S*-benzylcysteine and *N*-acetyl-*S*-benzylcysteine was 10 mCi/mol. Other reagents used were of analytical grade.

In vivo *experiments*. Male Sprague–Dawley rats and NAR, 180–200 g, were given laboratory chow and water *ad lib*. and were fasted overnight prior to experiments. Radioactive *S*-benzylcysteine and *N*-acetylcysteine which were dissolved in 1 ml of saline solution were administered orally under light ether anesthesia (for a few minutes). In some cases, animals were administered radioactive *S*-benzylcysteine in 1 ml of saline solution via the tail vein. Urine samples were collected from animals using metabolism cages over a period of 6–8 hr after the administration. Water was freely accessible to the animals during the collection of urine samples. *In vivo* experiments were performed between 9:00 a.m. and 5:00 p.m.

Under pentobarbital anesthesia (50 mg/kg of body wt), animals were bilaterally nephrectomized as described previously [10]. Ten minutes after the operation, animals were intravenously administered radioactive *S*-benzylcysteine. Bile samples were collected by bile duct cannulation as described previously [11]. The body temperature of anesthetized animals was kept constant at 37° under a tungsten lamp. At the indicated times after administration, animals were killed by bleeding from the right femoral artery.

The liver and kidneys were perfused simultaneously with 10 ml of ice-cold saline solution from the abdominal caval vein. Radioactive metabolites in serum, bile, liver and kidneys were analyzed by the ethyl acetate extraction method as described previously [15, 16].

Protein–ligand interaction. Binding of radioactive *S*-benzyl-*N*-acetylcysteine to plasma proteins was determined by an ultrafiltration technique using a micropartition system (Amicon, MPS-1) as described previously [14]. After incubation of the radioactive mercapturic acid with plasma samples obtained from NAR and normal animals, the mixtures were transferred to ultrafiltration cells and centrifuged at 700 *g* for 10 min. Amounts of protein-bound ligand were calculated from the differences in radioactivity between the incubated mixtures and the filtrates.

Assays. γ-Glutamyltransferase and *N*-acetyltransferase specific for *S*-substituted cysteines were determined in homogenates of the small intestinal mucosa of NAR and normal rats as described previously [16, 17]. One unit of the enzyme activity was defined as the amount of the enzyme required for the formation of 1 μmol product per min at 37°. Specific activity was expressed as mU per mg of protein. Protein concentration was determined by the method of Lowry *et al.* [18] using bovine serum albumin as the standard.

RESULTS

Fate of orally administered S-*benzylcysteine*. Figure 1 shows urinary excretion of *S*-benzyl-*N*-acetylcysteine, a mercapturic acid, after oral administration of radioactive *S*-benzylcysteine in normal rats and NAR. The urinary mercapturic acid increased with time; within 6 hr of administration, more than 80% of the dose appeared in urine of normal and analbuminemic rats as its *N*-acetyl metabolite. There was no significant difference in the excretory profiles of the metabolite between NAR and normal rats. Thus, the orally administered cysteine conjugate was similarly *N*-acetylated in some tissue(s) prior to its urinary excretion in both animal groups.

Urinary excretion of orally administered S-*benzyl*-N-*acetylcysteine*. To study the mechanism for processing a cysteine conjugate and its derivative in the intestine, urinary excretion of radioactive *S*-benzyl-

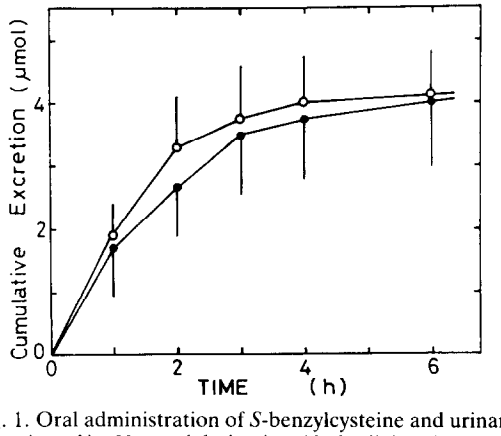

Fig. 1. Oral administration of *S*-benzylcysteine and urinary excretion of its *N*-acetyl derivative. Under light ether anestheia for a few minutes, animals were orally administered radioactive *S*-benzylcysteine (5 μmol/200 g rat) which was dissolved in 1 ml of saline solution. Urine samples were collected in a metabolism cage. Radioactive metabolites in urine samples were determined as described previously [15, 16]. Data are mean values ± SD derived from twelve animals. Key: (○) normal rats, and (●) NAR.

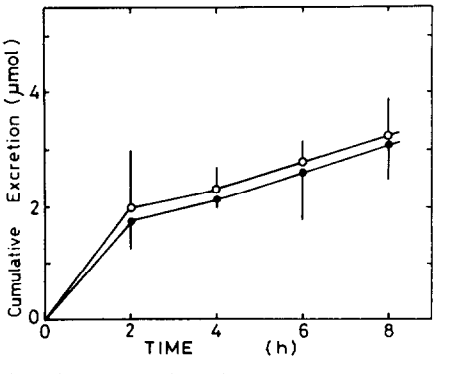

Fig. 2. Urinary excretion of orally administered *N*-acetyl-*S*-benzylcysteine. After oral administration of radioactive *N*-acetyl-*S*-benzylcysteine (5 μmol/200 g rat), urinary radioactivity was analyzed. Other conditions were the same as in Fig. 1. Data are mean values ± SD derived from twelve animals. Key: (○) normal rats, and (●) NAR.

N-acetylcysteine was examined after oral administration of the mercapturic acid. Urinary occurrence of the mercapturic acid increased with time; about 60% of the dose appeared unchanged in urine of normal rats within 8 hr of administration. There was no significant difference in the excretory profiles between the two animal groups (Fig. 2). Thus, the orally administered mercapturic acid as well as that generated from *S*-benzylcysteine *in vivo* can be transferred to the kidney similarly in both animals groups.

Enzymes involved in mercapturate biosynthesis. To know the quantitative aspects and the *in vivo* site(s) of *N*-acetylation of the orally administered cysteine conjugate, *N*-acetyltransferase activity was compared in various tissues of NAR and normal rats. As shown in Table 1, the small intestines of both animal groups possessed similar activities of *N*-acetyltransferase specific for *S*-substituted cysteines. Consistent with previous reports [15, 16, 19], liver and kidney of normal rats showed potent activity of *N*-acetyltransferase; liver and kidneys of NAR also exhibited activities of this enzyme similar to those of normal animals. It should be noted that *N*-acetyltransferase activity in the small intestine was less than 3% of that found in liver and kidneys of both animals groups. Thus, acetyltransferase activity of the small intestine toward acetylate cysteine conjugates appeared to be considerably lower than those of liver and kidney. In contrast to the low activity of *N*-acetyltransferase, the small intestines of both animal groups exhibited significantly higher activity of γ-glutamyltransferase, the enzyme that hydrolyzes peptide bonds of glutathione and its *S*-conjugates in cooperation with aminopeptidase M and dipeptidase [20–22]; 4063 ± 1764 and 4992 ± 1295 mU of the enzyme were localized in the small intestines of normal and analbuminemic rats respectively.

Metabolic fate of intravenously administered S-benzylcysteine. As shown in Table 1, *N*-acetyltransferase in the small intestine was low; about 0.34 and 0.47 μmol of *S*-benzylcysteine were maximally N-acetylated per hr by the small intestines of normal and the mutant rats respectively. Orally administered *S*-benzylcysteine, however, was efficiently excreted in urine as its *N*-acetyl metabolite in both animal groups. Within 1 hr of oral administration of *S*-benzylcysteine, 1.9 and 1.7 μmol of the mercapturic acid appeared in urine of normal and the mutant rats respectively. These values are 5.6- and 3.6-fold

Table 1. Tissue *N*-acetyltransferase activity

Tissues	Enzyme activity	
	Normal rats	Analbuminemic rats
Liver	508.8 ± 49.8	585.5 ± 54.8
	(0.46 ± 0.05)	(0.45 ± 0.04)
Kidney	306.9 ± 50.7	257.9 ± 12.8
	(2.92 ± 0.48)	(1.78 ± 0.09)
Small intestine	5.6 ± 2.5	7.8 ± 3.3
	(0.04 ± 0.02)	(0.05 ± 0.02)

Enzyme activity is expressed as mU/organ. Values in parentheses show the specific activity of the enzyme (mU/mg protein). Data are means ± SD derived from six animals. See the text for details.

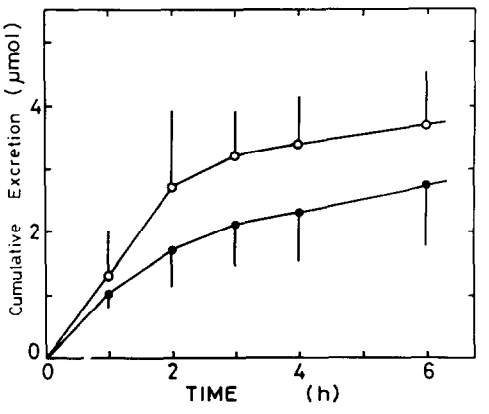

Fig. 3. Intravenous administration of *S*-benzylcysteine and urinary excretion of its *N*-acetyl derivative. Under ether anesthesia, animals were intravenously injected over a period of 5 sec through the tail vein with radioactive *S*-benzylcysteine (5 μmol/200 g rat) which was dissolved in 0.5 ml of saline solution. Urinary radioactivity was analyzed. Other conditions were the same as in Fig. 1. Data are means ± SD derived from twelve rats. Key: (○) normal rats, and (●) NAR.

greater than the amounts of the mercapturic acid which were synthesized in the small intestines of normal and the mutant rats respectively. This suggests that a significant part of the orally administered *S*-benzylcysteine may have been transferred to the portal circulation, acetylated in extraintestinal tissue(s), and then excreted in urine. Since the liver is the major organ to metabolize various compounds that are absorbed by the small intestine and has high *N*-acetyltransferase activity, this organ may be the predominant site for N-acetylation. To test this possibility, *S*-benzylcysteine was injected intravenously, and the urine was analyzed for the occurrence of its metabolite(s). As shown in Fig. 3, intravenously administered *S*-benzylcysteine was excreted as the *N*-acetyl metabolite in the urine of both animal groups. The rate of its urinary excretion, however, was markedly lower in NAR than in normal rats. This is in marked contrast to the finding that the rates of urinary excretion of the *N*-acetyl metabolite were similar in the two animal groups when *S*-benzylcysteine was administered orally. Previous studies [15, 19] have shown that a cysteine conjugate in the circulation is taken up preferentially by the livers of normal rats and acetylated *in situ*, and the resulting mercapturic acid is transferred to the kidney where a probenecid-sensitive transtubular secretory system for the *N*-acetyl metabolite operates. To determine the reason for the difference in the urinary recoveries of the *N*-acetyl metabolite of intravenously administered *S*-benzylcysteine of the two animal groups, changes in the mercapturate levels in liver and plasma were determined in bilaterally nephrectomized animals. The hepatic level of *N*-acetyl-*S*-benzylcysteine transiently increased in both normal and analbuminemic rats after intravenous administration of *S*-benzylcysteine (Fig. 4). It should be noted that the hepatic level of the *N*-acetyl metabolite was higher in NAR than in normal rats. In contrast, the plasma level of the mercapturic acid

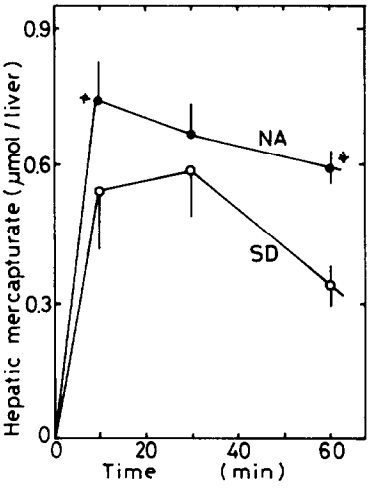

Fig. 4. Hepatic level of *N*-acetyl-*S*-benzylcysteine in nephrectomized animals. Under pentobarbital anesthesia, bilaterally nephrectomized animals were intravenously injected with 0.5 ml of radioactive *S*-benzylcysteine (20 μmol/kg of body wt). At indicated times after injection, animals were killed by bleeding from the left femoral artery. The liver was perfused with 10 ml of ice-cold saline. The excised liver was homogenized in 3 vol. of saline solution. The homogenates thus obtained were acidified with 1 N HCl, and the radioactive *N*-acetyl metabolite was extracted with 3 ml of ethyl acetate. Ethyl acetate fractions thus obtained were analyzed for radioactivity as described previously [15, 16]. Key: (○) normal rats, (●) NAR and (★) significantly different ($P < 0.01$) from normal rats.

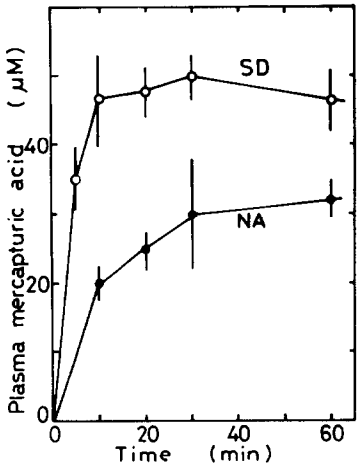

Fig. 5. Plasma level of *N*-acetyl-*S*-benzylcysteine in nephrectomized animals. Five minutes before each experiment, animals were bilaterally nephrectomized under pentobarbital anesthesia (50 mg/kg body wt). Radioactive *S*-benzylcysteine (20 μmol/kg body wt), which was dissolved in 0.5 ml saline, was injected into the right femoral vein. At indicated times, blood samples (0.3 ml) were obtained from the left femoral vein, diluted with 1 ml saline solution, and centrifuged at 12,000 *g* for 1 min in an Eppendorf centrifuge 542. Supernatant fractions thus obtained were acidified with 1 N HCl. Radioactive *N*-acetyl metabolite in acidified plasma was extracted with 3 ml of ethyl acetate and radioactivity was determined as described in Fig. 4. Other conditions were the same as in Fig. 4. Data are means ± SD derived from six animals. Key: (○) normal rats, and (●) NAR.

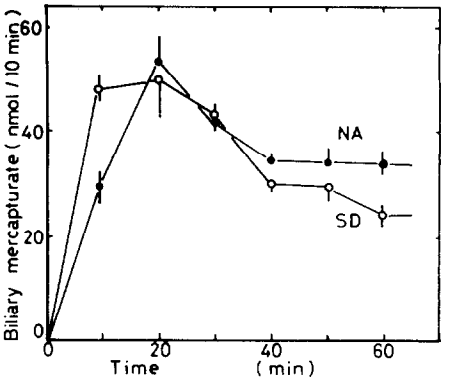

Fig. 6. Biliary excretion of *N*-acetyl-*S*-benzylcysteine in bilaterally nephrectomized rats. Under pentobarbital anesthesia, biliary excretion of *N*-acetyl-*S*-benzylcysteine was determined in bilaterally nephrectomized rats after intravenous administration of radioactive *S*-benzylcysteine (20 μmol/kg body wt). Radioactive metabolite(s) in the bile was determined for samples of six consecutive 10-min collection periods for 60 min. Sampling of rat bile was carried out as described previously [12]. Data are means ± SD derived from six animals. Key: (○) normal rats, and (●) NAR.

was markedly higher in normal rats than in NAR (Fig. 5). Since the liver excretes various metabolites into bile and plasma, the low level of plasma mercapturic acid in nephrectomized NAR may reflect increased secretion of this metabolite into bile of the mutant rats. To test this possibility, biliary excretion of mercapturic acid was measured in nephrectomized animals after administration of *S*-benzylcysteine. As shown in Fig. 6, the intravenously injected *S*-benzylcysteine appeared in the bile as its *N*-acetyl metabolite. Biliary excretion of the mercapturic acid was similar in both animal groups; about 4–6% of the dose was recovered in bile samples within 60 min of administration.

Binding of a mercapturic acid to plasma proteins. Previous studies [14] reveal that *S*-benzyl-*N*-acetylcysteine binds to bovine serum albumin with an association constant of $2.24 \times 10^5\ M^{-1}$. Since the mutant rats lack albumin, the lower level of plasma mercapturate in NAR may reflect a low capacity of plasma protein(s) to retain the amphipathic metabolite in the circulation. To test this possibility, binding of the mercapturic acid to plasma protein(s) was

Table 2. Binding of *N*-acetyl-*S*-benzylcysteine to serum proteins

Mercapturic acid (mM)	Protein bound fraction (%)	
	Normal rats	Analbuminemic rats
0.1	82.0	26.8
0.5	50.0	5.8

After incubation with radioactive *N*-acetyl-*S*-benzylcysteine at 25° for 10 min, plasma samples were analyzed for the protein-bound fraction of the ligand by an ultrafiltration method as described under Materials and Methods.

compared in the two animal groups. Ultrafiltration analysis revealed that the extent of binding of the mercapturic acid was markedly higher in serum samples from normal rats than in those of NAR (Table 2). At the ligand concentration of 0.5 mM, as much as 94% of the ligand remained unbound in the sera from NAR, whereas only one-half was free in the sera from normal rats.

DISCUSSION

The present work demonstrates that orally administered *S*-benzylcysteine was metabolized to its *N*-acetyl derivative, and the resulting mercapturate was excreted in urine of both normal and mutant analbuminemic rats. The rate of urinary excretion of the *N*-acetyl metabolite in both animal group apparently exceeded the capacity of the intestines to synthesize this metabolite (compare Fig. 1 and Table 1). Thus, it seems likely that the major part of a cysteine *S*-conjugate would enter into the circulation and undergo N-acetylation in some extraintestinal tissue(s), such as the liver. Consistent with the present findings, Grafström *et al.* [8] reported that hydrolysis of glutathione *S*-paracetamol into cysteine *S*-paracetamol occurs rapidly in isolated rat intestinal cells, whereas the subsequent N-acetylation proceeds very slowly.

Within 2 hr after the oral administration of *N*-acetyl-*S*-benzylcysteine, 35–40% of the dose was recovered in its intact form in the urines of both animal groups. Under identical conditions, 53–67% of the orally administered *S*-benzylcysteine was recovered in urine as its *N*-acetyl metabolite. Thus, the apparent rate of urinary excretion of the mercapturic acid was higher in both animal groups when the animals were orally administered *S*-benzylcysteine than when its *N*-acetyl derivative was given. This may reflect the difference in the rate of transintestinal transport of the two compounds.

Since the hepatic activity of *N*-acetyltransferase was high and the orally administered *S*-benzylcysteine was excreted similarly in urine of NAR and normal rats, N-acetylation of intravenously injected *S*-benzylcysteine would have occurred similarly in both animal groups. Consistent with this notion, biliary excretion of the intrahepatically synthesized mercapturic acid occurred similarly in both animal groups (Fig. 6). Despite lack of albumin, a small amount of *S*-benzyl-*N*-acetylcysteine bound to plasma protein(s) of NAR; the unbound fraction increased with increased concentration of the ligand. Thus, if high concentrations of the mercapturic acid entered into the circulation of NAR, the unbound fraction of the ligand would be increased significantly. We previously demonstrated that values for the plasma clearance and the volume of distribution of intravenously injected amphipathic organic anions, such as sulfobromophthalein [11], bilirubin [12], and phenolsulfophthalein [13], are significantly larger in NAR than in normal animals due to random distribution of the unbound ligands to many tissues. The present finding that, when *S*-benzylcysteine was injected intravenously, both the plasma level and the urinary recovery of its *N*-acetyl metabolite were lower in NAR than in normal rats could be explained on this basis, since *N*-acetyl-*S*-benzylcysteine represents an amphipathic organic anion. Because of high activity of hepatic *N*-acetyltransferase in both animal groups, intravenously administered *S*-benzylcysteine would be converted rapidly to its *N*-acetyl metabolite which readily leaves the liver and enters the circulation. The mercapturic acid, thus rapidly transferred into the plasma, normally binds to albumin and undergoes renal transtubular secretion. By contrast, the low binding capacity of plasma proteins in NAR (Table 2) would leave a significant fraction of the *N*-acetyl metabolite in the unbound form which tends to be randomly distributed into extrarenal tissues. Recent studies revealed that plasma clearance of *N*-acetyl-*S*-benzylcysteine is two times greater in NAR than in normal animals [23]. About 20 and 46% of the intravenously administered mercapturic acid (10 μmol/kg of body wt) was recovered from urine of NAR and normal rats, respectively, within 20 min of injection. At that time, the *N*-acetyl metabolite that associated with extrarenal tissues, such as liver, small intestine and lung, was increased significantly in NAR (3- to 10-fold) compared with that in normal rats. Intravenous administration of the mercapturic acid with equimolar albumin markedly increased the urinary recovery of the ligand; about 31% of the dose was found in the urine of NAR after 20 min of injection. These observations also suggest that, in processing the orally administered cysteine *S*-conjugate, transintestinal transport of this compound into the portal circulation may be the rate-determining step. Accordingly, the amount of cysteine *S*-conjugate available for *in vivo* N-acetylation in extraintestinal tissues, such as the liver, must be limited. In fact, preliminary experiments revealed that the mercapturate level was significantly lower both in liver and plasma of the two animal groups after oral administration of *S*-benzylcysteine. In this situation, the low plasma level of the mercapturate would keep most of it in the bound form even in the plasma of NAR, thus equalizing the apparent metabolic fate of the mercapturate in both animal groups. When NAR were injected intravenously, however, with a cysteine *S*-conjugate or challenged with a large dose of an electrophilic compound to increase the rate of mercapturate biosynthesis, the unbound form of an *N*-acetyl metabolite would increase markedly in the circulation and rapidly distribute among extrarenal tissues, resulting in its low recovery in the urine [23]. This may explain why both plasma level and urinary recovery of the mercapturic acid were lower in NAR than in normal rats when *S*-benzylcysteine or its *N*-acetyl metabolite was injected intravenously.

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