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STEREOSELECTIVE GLUTATHIONE CONJUGATION OF THE SEPARATE α-BROMOISOVALERYLUREA AND α-BROMOISOVALERIC ACID ENANTIOMERS IN THE RAT *IN VIVO* AND IN RAT LIVER SUBCELLULAR FRACTIONS

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Abstract—Glutathione (GSH) conjugation of the separate α-bromoisovalerylurea (BIU) enantiomers was studied in the rat. Administration of (*R*)-BIU resulted in excretion of a single glutathione conjugate in bile (IU-S-G/I) and a single mercapturate in urine (IU-S-MA/B). The other enantiomer, (*S*)-BIU, was exclusively metabolized to the other diastereomeric conjugates, IU-S-G/II and IU-S-MA/A. Thus, the conjugation of BIU with glutathione was completely stereospecific. Both the GSH conjugate and mercapturate derived from (*R*)-BIU were excreted two to three times more rapidly than their diastereomeric (*S*)-BIU counterparts. The enantiomers did not influence each others metabolism as reflected by identical metabolite excretion rates when the BIU enantiomers were administered either separately or as the racemic mixture. A similar rate difference for GSH conjugation of the separate BIU enantiomers was observed in incubations with rat liver cytosol as source of GSH transferases, suggesting that the stereoselectivity *in vivo* was due to glutathione conjugation properly. Similar results were obtained with a rat liver microsomal fraction, indicating that microsomal GSH transferases are active towards BIU and have a similar stereoselectivity as the cytosolic enzymes. Comparison of the GSH conjugation of BIU with that of its analogue α-bromoisovaleric acid (BI, which lacks the amide-linked urea group) revealed an opposite stereoselectivity: while (*R*)-BIU was conjugated faster than (*S*)-BIU, the (*R*) enantiomer of the acid was conjugated more slowly than (*S*)-BI. The α-bromocarbonyl compounds BI and BIU present a new type of substrate for the GSH transferases and allow studies of these enzymes in intact organisms as well as investigations on the stereoselectivity of GSH conjugation.

Glutathione (GSH)‡ conjugation is one of the major detoxication pathways of xenobiotics in the living cell. In most cases the products of this conjugation have lost their biological activities although there are some exceptions, as reviewed in Ref. 1. Therefore, the efficiency of this conjugation process is an important factor in the eventual toxicity resulting from the exposure to many xenobiotics. The efficiency of conjugation can be studied at several levels: from enzyme kinetics of purified enzymes to pharmacokinetics of GSH conjugation *in vivo*. In contrast to the wealth of data on GSH transferases studied *in vitro* with purified enzyme preparations or subcellular fractions, surprisingly little information is available about the pharmacokinetics of the conjugation process in intact cell systems *in vitro* and in intact animals [2]. The substrate studied in this paper, α-bromoisovalerylurea (BIU), is conjugated with GSH to a large extent in the rat [3–5], and allows investigation of the pharmacokinetics of GSH conjugation *in vivo*.

‡ Abbreviations used: BI, α-bromoisovaleric acid; BIU, α-bromoisovalerylurea; GSH, glutathione; I-S-G, α-(glutathion-*S*-yl)-isovaleric acid; IU-S-G/I and II, α-(glutathion-*S*-yl)-isovalerylurea, diastereomers I and II; IU-S-MA/A and B, α-(*N*-acetyl-(*R*)-cystein-*S*-yl)-isovalerylurea, diastereomers A and B.

Whereas the stereoselectivity (see Ref. 6 for a definition) of the handling of enantiomeric substrates by the GSH pathway has been extensively studied *in vitro* [7–13], little attention has been paid to stereoselectivity of GSH conjugation *in vivo*. This paper extends our previous work on BIU [3–5]; we now report the stereoselectivity of GSH conjugation, and subsequent excretion of the conjugates by the rat *in vivo* using the separate BIU enantiomers. A pronounced stereoselectivity in the excretion of the BIU conjugates was observed. The GSH conjugation *in vivo* was compared to that in rat liver cytosolic and microsomal fractions. Furthermore, the GSH conjugation of another α-bromocarbonyl compound, α-bromoisovaleric acid (BI) was studied; an interesting stereoselectivity of BI conjugation by rat liver subcellular fractions was observed.

EXPERIMENTAL

Materials. Racemic BIU was obtained from Onderlinge Pharmaceutische Groothandel (Utrecht, The Netherlands, Ph. Ned. VI quality), GSH was obtained from Boehringer (Mannheim, F.R.G.) and sodium pentobarbital (Nembutal^{R}) from Ceva (Paris, France). Reference compounds for HPLC were prepared as described previously [3]. With the use of ^{14}C-labeled metabolites obtained after

administration of ^{14}C-BIU to rats, the chemical purity of the reference compounds was determined: the IU-S-G chemically synthesized from racemic BIU contained 46.8% IU-S-G/I and 39.5% IU-S-G/II while similarly prepared IU-S-MA contained 29.4 and 35.9% of the diastereomers A and B, respectively.

Synthesis of BI and BIU enantiomers. (*RS*)-α-bromoisovaleric acid (BI) was prepared according to ref. 14; m.p. 40–41°.

(*R*)- and (*S*)-BI were prepared from racemic α-bromoisovaleric acid by crystallization with optically active α-methylbenzylamine. After crystallization of one diastereomeric salt, the filtrate still contained substantial amounts of that same diastereomer. Therefore, the other enantiomer was prepared from racemic BI by crystallization with the other enantiomer of α-methylbenzylamine and not from the "enriched filtrate". To a warm solution of 71 g (*RS*)-α-bromoisovaleric acid (0.40 mol) in 1 l diethyl ether, slowly a solution of 50 g (*S*)-(−)-α-methylbenzylamine (0.41 mol) was added. The mixture was slowly cooled to −20°. After one night the crystalline solid was filtered. Repeated crystallizations from acetone (4 times; 1 g salt/5 ml acetone) resulted in a nearly optically pure salt (20 g; m.p. 130–131°, $[\alpha]_D^{20} = -22.0$ (c = 1, acetone)). The salt was dissolved in 100 ml 2 N HCl; the aqueous layer was extracted several times with diethyl ether. The combined diethyl ether layers were washed with water and dried on anhydric Na_2SO_4. After filtration and evaporation of the solvent colorless crystalline (*R*)-(+)-BI was isolated; m.p. 41–42°, $[\alpha]_D^{20} = 24.4$ (c = 1, methanol), chemical purity 92.4% (determined by Reversed Phase-HPLC with detection at 210 nm). The absolute configuration of (+)-BI was established as (*R*) by Gaffield and Galetto [15].

(*S*)-(−)-α-bromoisovaleric acid was prepared as described above, using (*R*)-(+)-α-methylbenzylamine; m.p. 39–40°, $[\alpha]_D^{20} = -22.4$ (c = 1, methanol), chemical purity 91.8%.

(*R*)-(+)- and (*S*)-(−)-α-bromoisovalerylurea were synthesized from (*R*)-(+)- and (*S*)-(−)-α-bromoisovaleric acid, respectively. A mixture of 20 g (0.11 mol) acid and 32 g (0.27 mol) thionylchloride was stirred at 75° for 14 hr. Most of the thionylchloride was evaporated; a remainder of 10% which improved the subsequent reaction, was left. After addition of 14 g (0.23 mol) of urea the reactants were heated until a homogeneous phase was formed (50°). After 1 hr the mixture solidified; heating was continued at 80° for 1 hr. Water (350 ml) was added and the pH was adjusted to 6 with an aqueous saturated $NaHCO_3$ solution. The crystalline precipitate was filtered off and washed with water. Recrystallization from 800 ml of water (pH 6) yielded 14.7 g (66%) white, crystalline α-bromoisovalerylurea. (*R*)-(+)-BIU: m.p. 158–160°, $[\alpha]_D^{20} = 25.7$ (c = 1, methanol). (*S*)-(−)-BIU: m.p. 159–160°, $[\alpha]_D^{20} = -25.5$ (c = 1, methanol). The chemical purities of (*R*)- and (*S*)-BIU were 88.5 and 97.1%, respectively (as determined by HPLC with detection at 210 nm); enantiomeric purities were 98.3 and 98.1%, respectively (see below).

Enantioselective gas chromatography of BI and BIU. Enantiomeric purities of the synthesized BI and BIU enantiomers were determined by gas chromatography with a chiral stationary phase (XE-60-(*S*)-Val-α-phenethylamide [16]); a glass capillary column (length, 24 m; inside diameter, 0.3 mm) was preceded by a retention gap (8 m uncoated column). Operation temperatures of the injection port and (nitrogen selective) detector were 200 and 250°, respectively. Helium flow rates were 2 (carrier gas) and 30 ml/min (auxiliary gas). Hydrogen and air flow rates were 3 and 40 ml/min. The BIU solutions (see below) were extracted with an equal volume of ethyl acetate. Of the organic layer 2 μl was introduced in the gas chromatograph at a column temperature of 120°. BI was derivatized before gas chromatography could be applied. Therefore, aqueous BI solutions were acidified with an equal volume of 8 N HCl and extracted with a double volume of $CHCl_3$. In the organic phase, BI was derivatized with (*S*)-(+)-amphetamine to the corresponding amide [17]. Excess amine was removed with an acidified silica column. The resulting $CHCl_3$ solution was evaporated. After addition of ethyl acetate, 1 μl was injected in the gas chromatograph operating at a column temperature of 105°.

Animal experiments. Male Wistar rats of the Dept. of Pharmacology, Medical Faculty, University of Leiden (SPF; body weight 200 g) were used. They had free access to food (RMH-TM, Hope Farms, Woerden, The Netherlands) and water. Experiments started between 9 and 11 a.m. and were performed under pentobarbital anesthesia as described before [4]. The bile duct and urine bladder were catheterized to collect bile and (mannitol-driven [18]) urine fractions. During the experiments, the rats were kept at 38° by a heating pad.

Under heating (approx. 80°) and rigorous whirl mixing, BIU was dissolved in a solution of 2% (w/v) bovine serum albumin containing 75 mg/ml D-mannitol. The BIU concentration was 5 mM for the pure enantiomers and 10 mM for the racemate. The enantiomeric purity of these solutions was determined by gas chromatography with a chiral stationary phase (see above). The *in vivo* experiment was started by injection of the BIU solution into the jugular vein (5 ml/kg; injected in about 1 min; doses for (*R*)- and (*S*)-BIU were 25 μmol/kg, for (*RS*)-BIU 50 μmol/kg). At regular time intervals bile and urine samples were collected.

Incubations with rat liver subcellular fractions. A 33% (w/v) homogenate of rat liver in ice-cold 0.15 M KCl was prepared with a Potter-Elvehjem homogenizer. After centrifugation at 9000 *g* for 20 min, the post-mitochondrial supernatant was centrifuged for 60 min at 100,000 *g*. The resulting supernatant (cytosol) was collected. The microsomal pellet was resuspended in 0.15 M KCl and centrifuged again at 100,000 *g* for 60 min to remove cytosolic contaminations. All procedures were performed at 0–4°. Incubations were performed at 37° in 50 mM sodium phosphate buffer, pH 7.4, and contained (final concentration) 1.0 mM GSH and 500 μm racemic acceptor substrate (BI or BIU) or 250 μM of the separate enantiomers at a protein concentration of approximately 5 mg/ml. Total incubation volumes were 2.0 ml. After various incubation periods, samples (150 μl) were taken and added to a

double volume of methanol on ice. Samples were stored at −20° until analysis.

Assays. HPLC analysis of bile, urine or incubation samples to determine the concentration of the conjugates or mercapturates was performed as described elsewhere [3, 5, 19]. Data are presented as mean ± SEM. Statistical analysis of the difference between groups was done with the Wilcoxon significance test [20]; excretion half-lives were tested one-sided; a two-sided test was used for the cumulative excretion data.

RESULTS AND DISCUSSION

Enantioselective gas chromatography

To determine the enantiomeric purities of the synthetic BI and BIU enantiomers a chiral stationary phase was used. The diastereomeric amides from α-bromoisovaleric acid and (*S*)-(+)-amphetamine were satisfactorily separated (Fig. 1a); their capacity factors were 97 and 109 for the (*R*)- and (*S*)-enantiomer, respectively, with a resolution (R_s) of 1.49. The enantiomeric purities of (*R*)- and (*S*)-BI were found to be 98.3 and 96.6%, respectively (based on peak area measurements). An acceptable separation between the BIU enantiomer peaks was also achieved, albeit that long retention times were required to do so (Fig. 1b). Capacity factors of (*S*) and (*R*)-BIU were 28.2 and 30.3, respectively (R_s = 1.29). The enantiomeric purities of (*R*)- and (*S*)-BIU were found to be 98.3 and 98.1%, respectively.

Metabolites formed from BIU enantiomers in vivo

Previously we have shown that in the rat BIU gives rise to the formation of two different GSH conjugates in bile and two different mercapturates in urine [4]. Identification of the compounds showed that they were diastereomeric conjugates, presumably derived from the two BIU enantiomers [3]. In order to establish which of the diastereomeric GSH conjugates (IU-S-G/I and II) and mercapturic acids (IU-S-MA/A and B) originated from which enantiomer, we administered the separate BIU enantiomers. The results show that (*R*)-BIU resulted in excretion of one diastereomeric GSH conjugate in bile, IU-S-G/I, and one mercapturate, IU-S-MA/B, in urine (Table 1). The (*S*)-enantiomer results in the exclusive formation of the conjugates IU-S-G/II in bile and IU-S-MA/A in urine. In either case, less than 3% of the dose was converted to the other diastereomers (Table 1). Bearing in mind, that the synthesized BIU enantiomers contained about 2% of the other enantiomer, the results strongly indicate that the conjugation of BIU with glutathione proceeded with complete stereospecificity.

Rates and amounts of GSH conjugate formation

Administration of racemic BIU to rats results in different excretion rates of the two diastereomeric GSH conjugates in bile, and for the mercapturic acid diastereomers in urine [4]. In this study we found that the separately administered (*R*)-enantiomer of BIU resulted in the rapid excretion of IU-S-G/I (in

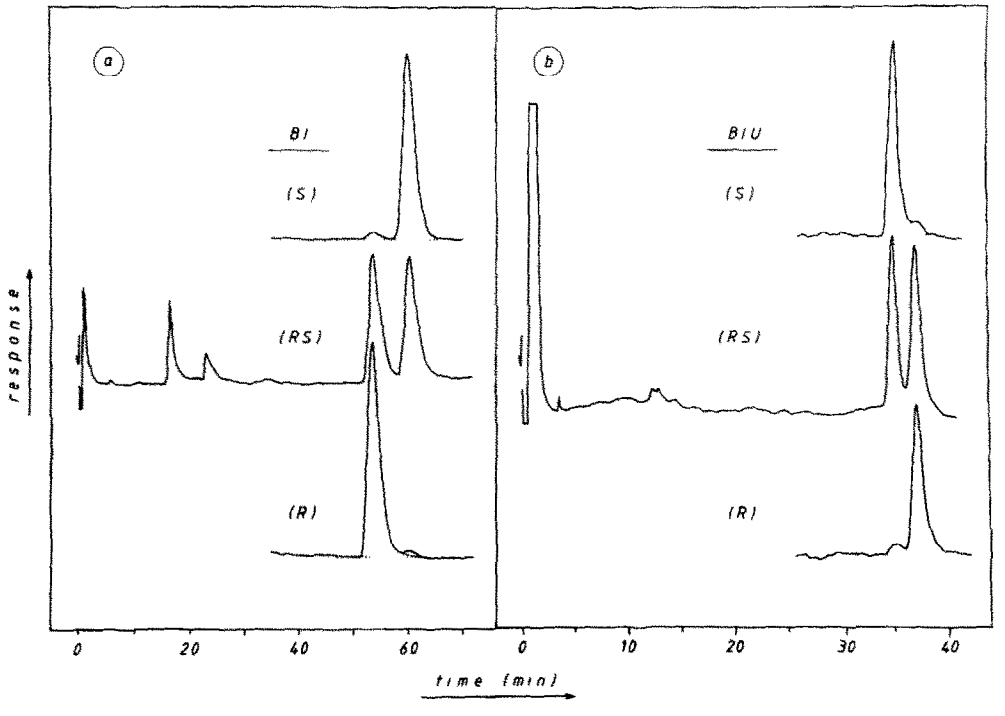

Fig. 1. Gas chromatograms of (a) BI and (b) BIU racemate and synthesized enantiomers using a chiral stationary phase (XE-60-(S)-Val-α-phenethylamide [16]). BIU was injected unchanged, BI was derivatized with (*S*)-amphetamine before gas chromatography. Dotted lines express the baselines used to determine the enantiomeric purities.

Table 1. Cumulative excretion of BIU metabolites in anesthetized rats

	(*R*)-BIU	(*RS*)-BIU*		(*S*)-BIU
		(*R*)	(*S*)	
Bile: IU-S-G/I	32.0 ± 5.4	37.4 ± 1.6		2.3†
IU-S-G/II	1.9†		58.5 ± 3.7	53.0 ± 3.1
Urine: IU-S-MA/A	—‡		32.0 ± 3.0	31.0 ± 1.5
IU-S-MA/B	45.0 ± 6.0	33.8 ± 2.5		—‡
Total: I + B	77.0 ± 1.9	71.2 ± 3.0		
II + A			91.2 ± 4.6	84.0 ± 2.2

(*S*)-, (*RS*)-, or (*S*)-BIU were administered i.v. Bile and urine were collected for 5 hr. Data are expressed as mean ± SEM (N = 4) and are percentages of the dose.

* For comparative purposes the dose was expressed on the basis of the dose of the single enantiomers: 25 μmol/kg = 100%. Therefore, the dose of the racemate (50 μmol/kg) is in fact 200% (100% (*R*)-BIU + 100% (*S*)-BIU).

† Only detectable in 2 of the 4 animals.

‡ Not detectable.

bile) and IU-S-MA/B (in urine). The conjugates derived from (*S*)-BIU, IU-S-G/II and IU-S-MA/A, were excreted at half to one third the rate of their (*R*)-BIU counterparts (Fig. 2 and 3, Table 2). Identical half-lives were found when the racemate was administered ($P \geq 0.05$). Thus, at a dose of 25 μmol per enantiomer, the (*R*) and (*S*)-enantiomers have no influence upon each other. Although the rates of metabolite excretion were quite different for the two BIU enantiomers, in each case after 5 hr about 80%

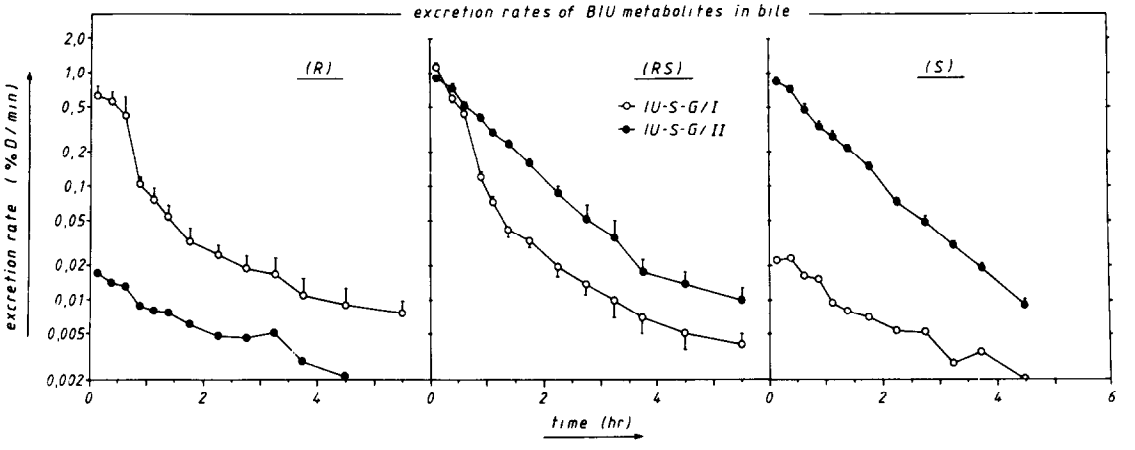

Fig. 2. Excretion rates of glutathione conjugates in bile after i.v. administration of (*R*)-, (*RS*), or (*S*)-BIU to anesthetized rats (mean ± SEM; N = 4). D, dose.

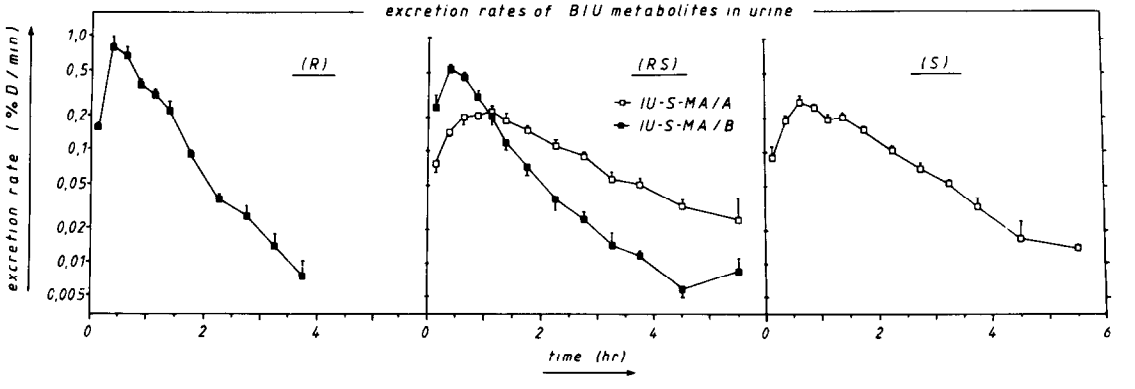

Fig. 3. Excretion rates of mercapturates in (mannitol driven) urine after i.v. administration of (*R*)-, (*RS*)-, or (*S*)-BIU to anesthetized rats (mean ± SEM; N = 4). D, dose.

Table 2. Excretion half-lives of BIU metabolites in bile and urine of anesthetized rats

	Half-lives of excretion (min)			
		(*RS*)-BIU		
	(*R*)-BIU	(*R*)	(*S*)	(*S*)-BIU
Bile: IU-S-G/I	11 ± 2*	12 ± 1*		—
IU-S-G/II	—		38 ± 3	38 ± 3
Urine: IU-S-MA/A	—		70 ± 8	56 ± 6
IU-S-MA/B	26 ± 1	29 ± 4		—

(*R*)-, (*RS*)- or (*S*)-BIU were administered i.v. Data are expressed as mean ± SEM (N = 4).
* Corrected for the slow terminal phase by feathering.

of the dose had been metabolized by GSH conjugation (Table 1; no significant differences were observed for corresponding enantiomers, $P \geq 0.05$).

Competing metabolism of BIU: amidase-catalyzed hydrolysis

Next to the GSH conjugation, amidase-catalyzed hydrolysis of BIU could also occur, resulting in the formation of α-bromoisovaleric acid (BI) and urea. It was shown previously that BI can be conjugated with GSH [5]. After administration of BIU, the GSH conjugate of the BI hydrolytically formed, I-S-G, was excreted in bile, and a mercapturate derivative was found in urine. In the present experiments with the separate enantiomers in all cases these metabolites represented only a minor fraction of the dose; for (*R*)-BIU about 7% of the dose was converted to the glutathione conjugate I-S-G, while for the (*S*) enantiomer this was only 4%. In accordance with previous findings [5], this shows that amidase-catalyzed hydrolysis is only a minor metabolic pathway of BIU *in vivo* compared to its conjugation with GSH.

GSH conjugation of BIU in a rat liver cytosolic fraction

Incubation of the separate BIU enantiomers with rat liver cytosol showed completely stereospecific GSH conjugation: (*R*)-BIU was converted exclusively to IU-S-G/I and from (*S*)-BIU only IU-S-G/II was formed. With respect to the rates of GSH conjugation, a similar stereoselectivity was observed as *in vivo*: the initial formation rate of IU-S-G/I from (*R*)-BIU was 3-fold that of IU-S-G/II formation from (*S*)-BIU (Fig. 4). Thus, the stereoselective metabolite excretion of the BIU enantiomers *in vivo* most likely results from rate differences in GSH conjugation properly, although we cannot yet exclude that differences in cellular uptake or transport of the diastereomeric conjugates out of the cell play a role.

GSH conjugate formation from (*R*)-BIU was not influenced by the presence of an equimolar amount of (*S*)-BIU (as in the racemate, Fig. 4). Conjugation of (*S*)-BIU, however, was depressed by the presence of the (*R*) antipode. This most likely is due to a (competitive) inhibition of the GSH conjugation of (*S*)-BIU by (*R*)-BIU (unpublished results). No such inhibition was observed *in vivo* which was probably due to the much lower acceptor substrate concentrations (blood concentrations of BIU are less than 20 μM at a dose of 25 μmol/kg [4]).

Stereoselective GSH conjugation of BI versus BIU

Incubation of α-bromoisovaleric acid with (GSH transferase containing) rat liver cytosol resulted in the formation of a GSH conjguate, I-S-G. Substantial amounts of I-S-G were formed when racemic BI or (*S*)-BI were incubated with a cytosolic fraction (Fig. 4). In contrast, (*R*)-BI was hardly conjugated with GSH. Interestingly, the pronounced stereoselectivity in GSH conjugation of bromoisovaleric acid is opposite to that observed for BIU (Fig. 4); (*R*)-BIU is conjugated more rapidly than (*S*)-BIU while (*R*)-BI is conjugated more slowly than (*S*)-BI. In order to elucidate this somewhat unexpected finding, studies on the GSH conjugation of BI and BIU enantiomers with purified GSH transferase isoenzymes from rat liver are presently in progress.

Incubations of BI and BIU with a rat liver microsomal fraction

Incubation of racemic BIU with rat liver microsomes resulted in the formation of substantial amounts of GSH conjugates. Correspondingly, (*R*)-BIU was converted exclusively to IU-S-G/I and (S)-BIU resulted in IU-S-G/II, so that the microsomal conjugation of BIU with glutathione also is completely stereospecific. Furthermore, the rates of GSH conjugation were different: (*R*)-BIU was conjugated 4 times faster than (*S*)-BIU (Fig. 5). The rate of conjugation was 5 to 10 times faster in cytosol than in microsomes. The total capacity of microsomal BIU-conjugation can be calculated as approximately 7% of that of the total cytosolic GSH transferase activity towards BIU. Since the amount of (adsorbed) cytosolic GSH transferases in once washed microsomes is approximately 1% (K. H. Tan, B. Ketterer, personal communication, London) the observed microsomal conjugation of BIU is most likely due to microsomal GSH transferases, and not to adsorbed cytosolic transferases.

BI was poorly conjugated by the microsomal fraction. The results show, that again (*S*)-BI was conjugated faster than (*R*)-BI; no conjugate formation of the latter was observed (Fig. 5). Thus, the microsomal fraction showed a stereoselectivity in GSH conjugation of BI and BIU enantiomers similar to that observed in rat liver cytosol. However, the conjugation of (*S*)-BI in microsomes was very low,

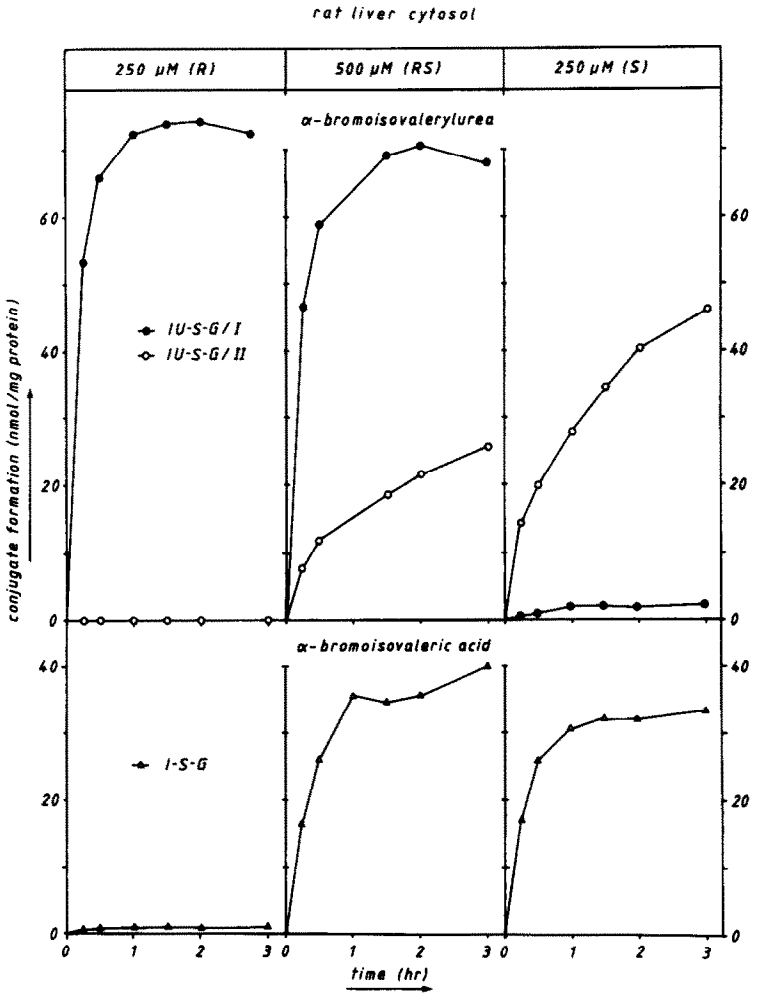

Fig. 4. Representative example of the glutathione conjugation of BIU and BI by a rat liver cytosolic fraction. Incubations were performed with (*R*)- and (*S*)-enantiomers (250 μM) and the racemic mixture (500 μM total concentration) and contained 1 mM GSH and 4.2 mg cytosolic protein/ml.

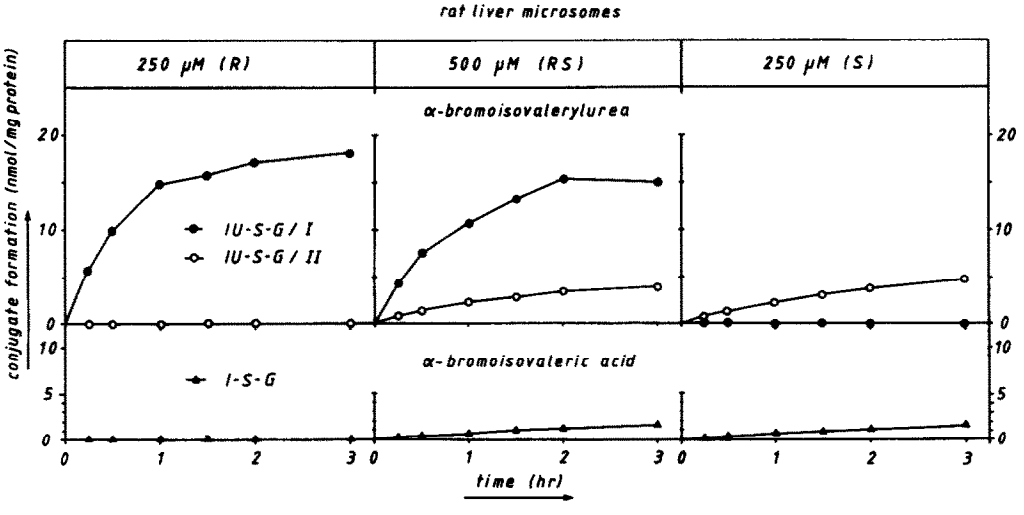

Fig. 5. Representative example of the formation of GSH conjugation of BIU and BI by a rat liver microsomal fraction. The separate enantiomers and the racemate were incubated with 5.2 mg of microsomal protein/ml in the presence of 1 mM GSH.

so that it may be due to adsorbed cytosolic GSH transferases.

As described previously [5], the microsomal fraction was responsible for the hydrolysis of BIU to BI and urea. Therefore, BIU can undergo competing metabolism when incubated with a microsomal fraction. No I-S-G formation was observed in the microsomal incubations of BIU which corresponds to the very poor GSH conjugation of the hydrolytic product BI with a microsomal fraction (see above).

CONCLUSIONS

Stereospecific conjugation of BIU enantiomers with glutathione, resulting in the exclusive formation of IU-S-G/I from (*R*)-BIU and IU-S-G/II from (*S*)-BIU was consistently found *in vitro* and *in vivo*. Furthermore, the difference in excretion rates between the diastereomeric GSH conjugates and mercapturates in the intact animal were parallel to differences in the rates of formation of diastereomeric GSH conjugates in rat liver cytosol. In cytosolic as well as microsomal fractions from rat liver a distinct, opposite stereoselectivity in the rate of GSH conjugation of BI and BIU enantiomers was observed. Presently this phenomenon is studied with purified GSH transferase isoenzymes. Further, investigations on the GSH conjugation of α-bromoisovaleric acid in the intact rat are in progress.

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