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The Metabolism of Benzyl Isothiocyanate and its Cysteine Conjugate

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1. The corresponding cysteine conjugate was formed when the GSH (reduced glutathione) or cysteinylglycine conjugates of benzyl isothiocyanate were incubated with rat liver or kidney homogenates. When the cysteine conjugate of benzyl isothiocyanate was similarly incubated in the presence of acetyl-CoA, the corresponding *N*-acetylcysteine conjugate (mercapturic acid) was formed. 2. The non-enzymic reaction of GSH with benzyl isothiocyanate was rapid and was catalysed by rat liver cytosol. 3. The mercapturic acid was excreted in the urine of rats dosed with benzyl isothiocyanate or its GSH, cysteinylglycine or cysteine conjugate, and was isolated as the dicyclohexylamine salt. 4. An oral dose of the cysteine conjugate of [^{14}C]benzyl isothiocyanate was rapidly absorbed and excreted by rats and dogs. After 3 days, rats had excreted a mean of 92.4 and 5.6% of the dose in the urine and faeces respectively, and dogs had excreted a mean of 86.3 and 13.2% respectively. 5. After an oral dose of the cysteine conjugate of [^{14}C]benzyl isothiocyanate, the major ^{14}C-labelled metabolite in rat urine was the corresponding mercapturic acid (62% of the dose), whereas in dog urine it was hippuric acid (40% of the dose). 6. Mercapturic acid biosynthesis may be an important route of metabolism of certain isothiocyanates in some mammalian species.

Benzyl isothiocyanate occurs naturally in several plant species, such as *Tropaeolum majus* L. (Gadamer, 1899*a*; Dannenberg *et al.*, 1956; Underhill & Chisholm, 1964), *Lepidium sativum* L. (Gadamer, 1899*b*; Virtanen & Saarivirta, 1962; Tapper & Butler, 1967), *Carica papaya* L. and other *Carica* species (Gmelin & Kjaer, 1970*a*) and *Capparis flexuosa* L. (Gmelin & Kjaer, 1970*b*), where it is present as the benzyl glucosinolate (glucotropaeolin). The isothiocyanate is readily liberated from the glucosinolate by enzymic hydrolysis (Ettlinger & Kjaer, 1968), and this could conceivably be part of a chemicobiological defence mechanism in plants against micro-organisms (Tang, 1971). Benzyl isothiocyanate shows good antibiotic activity against bacteria (Winter, 1954; Pulverer, 1969) and fungi (Drobnica *et al.*, 1967) *in vitro*, and consequently has been used therapeutically (Germer, 1954; Ebbinghaus, 1966; Borowski, 1966; Alexander & Krüger, 1965).

Mercapturic acid formation from benzyl isothiocyanate has been reported in a preliminary communication (Koch, 1973). Conjugation with GSH* is the initial stage in mercapturic acid biosynthesis for the elimination of foreign compounds from the body (Barnes *et al.*, 1959; Bray *et al.*, 1959*a*; Boyland & Chasseaud, 1969*b*; Chasseaud, 1976). In animals, the GSH conjugate is catabolized to the corresponding cysteinylglycine and cysteine conjugates and the latter is often *N*-acetylated to form a mercapturic acid, which may be excreted in the urine, or further metabolized. This paper describes the metabolic fate of benzyl isothiocyanate, which is a representative of a class of electrophilic compounds not previously reported to be metabolized by the mercapturic acid pathway.

Experimental

Materials

All melting points are uncorrected.

S-(N-Benzylthiocarbamoyl)-L-glutathione. GSH (0.033mol) was dissolved in aq. 50% (v/v) ethanol (250ml), and benzyl isothiocyanate (0.033mol) in aq. 80% (v/v) ethanol (125ml) was added. The mixture was stirred and allowed to react for 3 days at

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* Abbreviation: GSH, reduced glutathione.

ambient temperature (20°C). The yellow crystalline product was filtered, washed with ethanol (3×100ml) and dried at 60°C *in vacuo*. Recrystallization from water afforded the product, m.p. 195–198°C, $[\alpha]_D^{20}$ −3.0° (*c* 250mg in 1M-HCl) (Found: C, 47.2; H, 5.4; N, 12.1; S, 14.0; $C_{18}H_{24}O_6N_4S_2$ requires C, 47.4; H, 5.3; N, 12.3; S, 14.1%). N.m.r. ($[^2H_6]Me_2SO$): δ 10.5–10.8 (2H, CO_2H disappeared with ^2H_2O), δ 8.3–8.8 (4H, –N*H*– and N*H*$_2$ disappeared with ^2H_2O), δ 7.35 (5H, s, C_6H_5), δ 4.85 (2H, s, protons a), δ 4.3–4.7 (1H, m, proton c), δ 3.2–4.0 (5H, m, protons b, d and g), δ 1.8–2.8 (4H, m, protons e and f) (see Fig. 1).

S-(N-Benzylthiocarbamoyl)-L-cysteinylglycine. GSH (0.033mol) was dissolved in 1.2M-HCl (25ml), and the solution was stirred for 60min at 94°C in an atmosphere of N_2. This solution, containing cysteinylglycine, was cooled, adjusted to pH6.5 with 1M-NaOH, and ethanol (50ml) and benzyl isothiocyanate (0.033mol) were then added. The mixture was allowed to react and the product isolated as described above, m.p. 118–121°C, $[\alpha]_D^{20}$ +65.2° (*c* 46mg in aq. 50% (v/v) methanol) (Found: C, 47.6; H, 5.3; N, 12.9; S, 19.5; $C_{13}H_{17}O_3N_3S_2$ requires C, 47.7; H, 5.2; N, 12.8; S, 19.6%). N.m.r. ($[^2H_6]Me_2SO$) δ 8.2–8.5 (1H, CO_2H, disappeared with ^2H_2O), δ 7.35 (5H, s, C_6H_5), δ 6.0 (3H, s, –NH– and –NH_2 disappeared with ^2H_2O), δ 4.85 (2H, s, protons a), δ 3.5–4.0 (5H, m, protons b, c and d) (see Fig. 1).

S-(N-Benzylthiocarbamoyl)-L-cysteine. L-Cysteine hydrochloride monohydrate (7.3mol) was dissolved in water (450ml), and benzyl isothiocyanate (6.7mol) in aq. 80% (v/v) ethanol was added, followed by NaOH (29.1g) in water (300ml) to raise the pH to 6.2. The mixture was allowed to react for 3 days and occasionally stirred. The precipitate was filtered, washed with water (2×2000ml) and ethanol (2×4000ml), and dried at 60°C *in vacuo*. Recrystallization from water acidified with HCl afforded the product, m.p. 190–194°C, $[\alpha]_D^{20}$ −31.9° (*c* 500mg in 1M-HCl) (Found: C, 48.8; H, 5.2; O, 12.0; N, 10.5; S, 23.8; $C_{11}H_{14}O_2N_2S_2$ requires C, 48.9; H, 5.2; O, 11.8; N, 10.4; S, 23.7%). N.m.r. ($[^2H_6]Me_2SO$) δ 7.55–8.25 (3–4 H, disappeared with ^2H_2O), δ 7.28 (5H, s, C_6H_5), δ 4.83 (2H, s, protons a), δ 3.28–3.95 (3H, m, protons b and c) (see Fig. 1).

N-Acetyl-S-(N-benzylthiocarbamoyl)-L-cysteine (mercapturic acid). *N*-Acetylcysteine (0.04mol), dissolved in aq. 50% (v/v) pyridine (200ml), was heated to 40°C and the pH adjusted to 9 with 1M-NaOH. To this solution was added, dropwise, benzyl isothiocyanate (0.08mol). On cooling, the reaction mixture was extracted with benzene (5×100ml), and the extracts were discarded. On adjusting the remaining aqueous solution to pH1 with 1M-HCl at 0°C, the product was precipitated.

The product was recrystallized several times from light petroleum (b.p. range 60–80°C) and dried *in vacuo* at 30°C, m.p. 58–62°C, $[\alpha]_D^{20}$ −14.9° (*c* 500mg in methanol) Found: C, 50.2; H, 5.1; N, 9.0; S, 20.2; $C_{13}H_{16}O_3N_2S_2$ requires C, 50.0; H, 5.1; N, 9.0; S, 20.5%). N.m.r. (C^2HCl_3+trace of $[^2H_6]Me_2SO$) δ 9.6–9.8 (1H, CO_2H disappeared with ^2H_2O), δ 7.5–7.8 (1H, –N*H*– disappeared with ^2H_2O), δ 7.3 (5H, s, C_6H_5), δ 4.9 (2H, d, J = 5.5Hz, after ^2H_2O exchange s, protons a), δ 4.7 (1H, t, J = 6.5Hz, proton c), δ 3.8 (2H, d, J = 6.5Hz, protons b), δ 1.9 (3H, s, protons d) (see Fig. 1).

[7-^{14}C]Benzyl isothiocyanate. Benzylamine hydrochloride (552mg) and [7-^{14}C]benzylamine (205mg) were dissolved in water (7ml), and aq. 46.6% (w/v) NaOH (0.33ml) was added. The solution was cooled to about 0°C, when dichloroethane (5ml), $CaCO_3$ (575mg) and thiophosgene (0.34ml) were added with vigorous stirring. The mixture was stirred for 18h, heated to 35°C and diluted with 1M-HCl. The radioactivity was extracted with dichloroethane, and the extract washed with water, dried and concentrated to yield crude [^{14}C]benzyl isothiocyanate, which was identical on t.l.c. (hexane) with an authentic sample.

S-(N-[7-^{14}C]Benzylthiocarbamoyl)-L-cysteine. L-Cysteine hydrochloride monohydrate (656mg) was dissolved in water (2ml) containing NaOH (150mg), and crude [7-^{14}C]benzyl isothiocyanate (566mg) in aq. 80% (v/v) ethanol (12ml) was added dropwise with stirring. The mixture was allowed to react for 2 days with occasional stirring, and the solid product was filtered, washed with water and ethanol, and dried. The radiochemical purity of the product exceeded 98% on t.l.c. and the specific radioactivity was 3.4μCi/mg. The identity of the product was demonstrated by chromatographic comparison with an authentic sample.

[^{35}S]S-(N-Benzylthiocarbamoyl)-L-cysteine. This compound was prepared under conditions identical with those for the ^{14}C-labelled compound, except that L-[^{35}S]cysteine hydrochloride monohydrate was used. The radiochemical purity of the product exceeded 96% on t.l.c. and the specific radioactivity was 4.2μCi/mg. The identity of the product was demonstrated by chromatographic comparison with an authentic sample.

Buffers. Phosphate buffers were prepared from KH_2PO_4 or NaH_2PO_4 and Na_2HPO_4.

Thin-layer chromatography

T.l.c. (Table 1) was carried out on pre-layered Kieselgel GF_{254} plates (Merck A.-G., Darmstadt, Germany) of layer thickness 0.25mm or 2mm for preparative chromatography, in the following solvent systems: A, butan-1-ol saturated with water/acetic acid (10:1, v/v); B, butan-1-ol saturated with water/acetic acid (100:1, v/v); C, ethyl acetate/water/formic acid (12:7:1, by vol., upper phase); D, light petroleum/chloroform (9:1, v/v); E, chloroform/

Table 1. *R_F values of reference compounds*
For the full chemical names of the reference compounds see the Experimental section.

Compound	Solvent system ...	$10^{-2} \times R_F$ A	B	C	D	E	F
Benzyl isothiocyanate		—	—	97	52	97	83
GSH conjugate		35	16	3	0	2	33
Cysteinylglycine conjugate		48	35	13	0	3	47
Cysteine conjugate		56	53	15	0	5	51
Mercapturic acid		80	55	70	0	45	62
Hippuric acid		—	—	70	0	48	62

ethanol/acetic acid (18:6:1, by vol.); F, butan-1-ol/acetic acid/water (2:1:1, by vol.). Butan-1-ol used in solvent systems A and B was first saturated with water. Dried chromatograms were sprayed with 0.1 M-$AgNO_3$ or 0.3% (w/v) ninhydrin in methanol/acetic acid (100:3, by vol.).

Non-radioactive reference compounds were also detected by their quenching of silica-gel GF_{254} fluorescence in u.v. light, and radioactive components by apposition radioautography on X-ray film and measurement as described by Chasseaud *et al.* (1972).

Tissue preparations

Rat liver supernatant was prepared as described by Boyland & Chasseaud (1969*b*).

Rat livers or kidneys were homogenized in 5 vol. of 0.5 M-sodium orthophosphate buffer, pH 7.4, in a Potter & Elvehjem (1936)-type homogenizer (obtained from Braun, D-3508 Melsungen, Germany). The resulting whole-tissue homogenates were used for incubation.

Metabolism in vitro

Action of rat liver supernatant. Equimolar amounts (3 mM) of benzyl isothiocyanate and GSH were incubated in the presence and absence of rat liver supernatant in 0.1 M-orthophosphate buffer, pH 7.4, at 25°C for 1 min. The reaction was stopped by the addition of an equal volume of aq. 4% (w/v) sulphosalicylic acid, and the precipitated protein removed by centrifugation. Unchanged GSH was determined by titration with iodate in the presence of iodide in sulphosalicylic acid (Woodward & Fry, 1932); sodium starch glycollate was used as an indicator. Quantitative recoveries of GSH were obtained by this method. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Action of rat whole-liver or -kidney homogenates. The GSH, cysteinylglycine or cysteine conjugates of benzyl isothiocyanate (6 μmol) were added to liver or kidney homogenate. When the cysteine conjugate of benzyl isothiocyanate was the substrate, acetyl-CoA (10 μmol) was also added. The homogenates were incubated for 30 min at 37°C and acidified with acetic acid. The precipitate was removed by centrifugation (1000*g* for 15 min). The supernatant was treated with 5 vol. of methanol, and the precipitate removed by centrifugation (1000*g* for 15 min). The supernatant was concentrated *in vacuo* to a small volume (3 ml) and subjected to t.l.c. in solvent system B (GSH or cysteinylglycine conjugates as substrates) or C (cysteine conjugate as substrate). Zones corresponding to the relevant reference compounds, located under u.v. light (254 nm) were scraped off, added to water (50 ml) and steam-distilled in a Karlsruher apparatus (Stahl, 1953) for 1 h to liberate benzyl isothiocyanate, which was extracted with cyclohexane (1–2 ml). The cyclohexane extract was subjected to t.l.c. in solvent system D, and the benzyl isothiocyanate was located by spraying the plates with 0.1 M-$AgNO_3$. Also, immediately after development, other thin-layer plates were sprayed with 0.1 M-$AgNO_3$ or 0.3% (w/v) ninhydrin in methanol/acetic acid (100:3, v/v) to detect products corresponding to the reference compounds. These experiments demonstrated the formation of conjugates corresponding chromatographically to the reference conjugates and the presence of the benzyl isothiocyanate moiety in these conjugates.

Control incubations were carried out in the absence of either the substrate or of the tissue homogenate.

Determination of the influence of pH on the liberation of benzyl isothiocyanate from N-acetyl-S-(N-benzylthiocarbamoyl)-L-cysteine (mercapturic acid).

Portions of the mercapturic acid (100 mg) were dissolved at 37°C in 0.15 M-sodium phosphate buffer (100 ml) at various pH values ranging from pH 5 to pH 8. Cyclohexane (50 ml) was added and the two-phase system carefully stirred for 10 min without destroying the boundary between the phases. After removal of the organic phase, the buffer was extracted with cyclohexane (2 × 20 ml). The combined organic extracts containing liberated benzyl isothiocyanate were made up to 100 ml with cyclohexane and the A_{250} was measured against a blank of cyclohexane saturated with water, to measure the amount of free benzyl isothiocyanate present.

Metabolism in vivo

Non-radioactive compounds administered to rats. Wistar rats (body weight about 200–300 g, obtained from Mus and Rattus AG, D-8011, Brunnthal, Germany) were given benzyl isothiocyanate and its

Table 2. *Administration of benzyl isothiocyanate and its conjugates to rats by different routes*

For oral dosing, the conjugates were suspended in aq. 1% (w/v) gum tragacanth (3ml). For intraperitoneal dosing, the conjugates were suspended in 0.9% NaCl (2ml). For intravenous dosing, the dosage forms were prepared in 0.9% NaCl as described in the text.

Compound \ Route ...	Dose (mg/kg) Oral	Intraperitoneal	Intravenous
Benzyl isothiocyanate	10*	—	—
GSH conjugate	200	40	40
Cysteinylglycine conjugate	200	—	20
Cysteine conjugate	20 or 80	40	20
Mercapturic acid	400	—	40

* In arachis oil.

conjugates by different routes (Table 2). For intravenous dosing, the GSH, cysteinylglycine or cysteine conjugates were suspended in aq. 0.9% NaCl and acidified with 1M-HCl to effect dissolution. The pH was re-adjusted to 2 with 1M-NaOH, and the preparation centrifuged to remove any precipitate; the supernatant was used for injection. The mercapturic acid of benzyl isothiocyanate was dissolved in aq. 0.9% NaCl. Control animals received the dose vehicle only, The animals were kept in metabolism cages so that urine and faeces could be separately collected. Samples of urine were subjected to t.l.c. together with the reference compounds, in solvent systems A, B or C. Areas of the silica gel corresponding to the reference compounds were located under u.v. light (254nm), scraped off and subjected to steam-distillation for detection of benzyl isothiocyanate as described above. It was considered to be present if a positive response to the 0.1M-$AgNO_3$ spray reagent was obtained within 2min.

Benzyl isothiocyanate was administered to male rats (10mg/rat), and urine was collected for up to 20h. The pooled urines were acidified with 0.1M-HCl (pH3) and extracted three times with a threefold excess of ethyl acetate. The pooled ethyl acetate extracts were washed with water, frozen at −30°C to remove traces of water, and concentrated *in vacuo* to yield a dark-brown residue. The residue (60mg) was dissolved in ethyl acetate (2ml), and dicyclohexylamine (30mg) was added. The turbid mixture was left at −30°C for 30min. The clear pale-yellow solution was decanted, and kept at −30°C, when colourless crystals were precipitated. This product (29mg) was recrystallized from ethyl acetate, m.p. 141–145°C and had an i.r. spectrum identical with that of the authentic dicyclohexylamine salt of the mercapturic acid of benzyl isothiocyanate (Found: C, 60.7; H, 8.0; O, 9.9; N, 8.5; S, 12.8; $C_{25}H_{39}O_3N_3S_2$ requires C, 60.8; H, 8.0; O, 9.7; N, 8.5; S, 12.0%). This mercapturic acid was similarly isolated as the dicyclohexylamine salt from the urine of rats dosed with the corresponding cysteine conjugate of benzyl isothiocyanate.

Radioactive compounds. Starved CFHB Wistar rats (body weight about 200g, obtained from Anglia Laboratory Animals, Huntingdon, U.K.) were dosed orally with the cysteine conjugate of [^{14}C]benzyl isothiocyanate (20mg/kg) as a suspension in aq. 0.5% (w/v) gum tragacanth (2ml) followed by water (3ml).

Beagle dogs (body weight about 9kg) were dosed orally with the cysteine conjugate of [^{14}C]benzyl isothiocyanate (5mg/kg) contained in a gelatin capsule.

The excretion (balance studies), studies of the kinetics of radioactivity in plasma, and biliary-secretion studies were carried out as described by Chasseaud *et al.* (1974). Urines were collected in HCl (pH2) to prevent decomposition of excreted conjugates to free benzyl isothiocyanate. Concentrations of radioactivity in the various biological samples were measured as described by Down *et al.* (1974).

Urine collected in the excretion studies was applied to columns of Amberlite XAD-2 resin as described by Cameron *et al.* (1975), and 95% of the applied radioactivity was recovered by this technique and subjected to t.l.c. in solvent systems C, E or F.

Experiments with ^{35}S-labelled compounds

Two rats were orally dosed with L-[^{35}S]cysteine hydrochloride (20μCi/day) in water (1ml) for 4 days to radiolabel the body sulphur pool. On the fourth day, one rat received additionally the non-radioactive cysteine conjugate of benzyl isothiocyanate and the other was used as a control. At the same time a third rat was dosed with the [^{35}S]cysteine conjugate of benzyl isothiocyanate and a fourth rat with the cysteine conjugate of [^{14}C]benzyl isothiocyanate. Also the [^{35}S]cysteine conjugate of benzyl isothiocyanate (5mg/kg) was administered to one dog. The animals were kept for 24h in metabolism cages and the urines collected separately and processed on Amberlite XAD-2 resin as previously described.

Mass spectrometry

Samples of metabolites for mass spectrometry were isolated by preparative t.l.c. in solvent system C. Silica gel containing the radioactive component of interest was eluted with chloroform/ethanol (3:1, v/v). The eluate was evaporated to a small volume and applied to the mass-spectrometer probe. Electron-impact mass spectra were recorded on a Hitachi Perkin-Elmer RMS-4 instrument operating at an

ionization potential of 70eV and an ionizing current of 100μA. Samples were introduced by the direct insertion probe at a chamber temperature of approx. 100°C. Spectra were normalized by using an Elliott 903 computer, and histograms were produced on an IBM 360/44 by using a computer-controlled graph plotter.

Results

Metabolism of benzyl isothiocyanate conjugates by rat whole-tissue homogenates

The cysteine conjugate of benzyl isothiocyanate was detected by t.l.c. when the corresponding GSH conjugate was incubated with liver or kidney homogenates (Table 3). The GSH conjugate was converted completely into the cysteine conjugate only in the presence of kidney homogenates, showing that catabolism of the GSH conjugate occurred more readily in kidney than in liver homogenates. This result is expected because the activity of γ-glutamyltranspeptidase towards GSH conjugates is apparently low in rat liver (Bray *et al.*, 1959*a*; Revel & Ball, 1959).

The corresponding GSH and cysteine conjugates were both detected by t.l.c. when the cysteinylglycine conjugate of benzyl isothiocyanate was incubated with liver homogenates (Table 3). The formation of the GSH conjugate was unexpected, but may be due to the lability of the cysteinylglycine conjugate to yield free benzyl isothiocyanate, which would react with GSH in the presence (or absence) of liver homogenates. When the cysteinylglycine conjugate was incubated with kidney homogenates, only the corresponding cysteine conjugate was detected (Table 3). The corresponding mercapturic acid was detected by t.l.c. when the cysteine conjugate of benzyl isothiocyanate was incubated with liver or kidney homogenates in the presence of acetyl-CoA. Both the liver and kidneys of rats have been shown to catalyse the *N*-acetylation of cysteine conjugates (Green & Elce, 1975). Limited acetylation also occurred in the absence of the homogenates or in the absence of acetyl-CoA, indicating respectively non-enzymic acetylation and the presence of sufficient acetyl-CoA in the homogenates to produce detectable amounts of mercapturic acid.

Non-enzymic reaction of GSH with benzyl isothiocyanate was fairly rapid, since about 10% conjugation occurred during 1min at pH7.4 and 25°C. Addition of rat liver supernatant increased the proportion of GSH conjugated by at least twofold. No increase in conjugation occurred after addition of boiled rat liver supernatant. These results suggest that conjugation of GSH with benzyl isothiocyanate was enzyme-catalysed.

On the basis of these results it can be concluded that the metabolism of benzyl isothiocyanate in rat tissues probably follows the pathway (Fig. 1) known for mercapturic acid biosynthesis (Barnes *et al.*, 1959; Boyland & Chasseaud, 1969*b*).

Metabolism of benzyl isothiocyanate and its conjugates in rats

With qualitative procedures, only one product was detected in urine after the oral administration of benzyl isothiocyanate to rats (Table 1). This product was chromatographically identical with the corresponding mercapturic acid and contained the benzyl isothiocyanate moiety.

Two products were detected after the administration by various routes (Table 1) of the GSH, cysteinylglycine, cysteine and *N*-acetylcysteine conjugates of benzyl isothiocyanate to rats. These

Table 3. *Metabolism of benzyl isothiocyanate conjugates by rat tissue whole homogenates*

Tissue homogenates, prepared as described in the text, were incubated with GSH, cysteinylglycine and cysteine conjugates of benzyl isothiocyanate (B–GSH, B–Cys-Gly and B–Cys respectively) at pH7.4 and 37°C for 30min. The reaction products were detected by t.l.c. as described in the text. Benzyl isothiocyanate was also formed when the conjugate was incubated with buffer alone at pH7.4. Small amounts were always detected, presumably because of the instability of these conjugates at non-acid pH values.

	Products formed in the presence of liver homogenates				
Substrate (6μmol)	B–GSH	B–Cys-Gly	B–Cys	Mercapturate	Benzyl isothiocyanate
GSH conjugate	+	−	+	−	+
Cysteinylglycine conjugate	+	−	+	−	+
Cysteine conjugate	−	−	−	+	+
	Products formed in the presence of kidney homogenates				
GSH conjugate	−	Trace	+	−	+
Cysteinylglycine conjugate	−	−	+	−	+
Cysteine conjugate	−	−	−	+	+

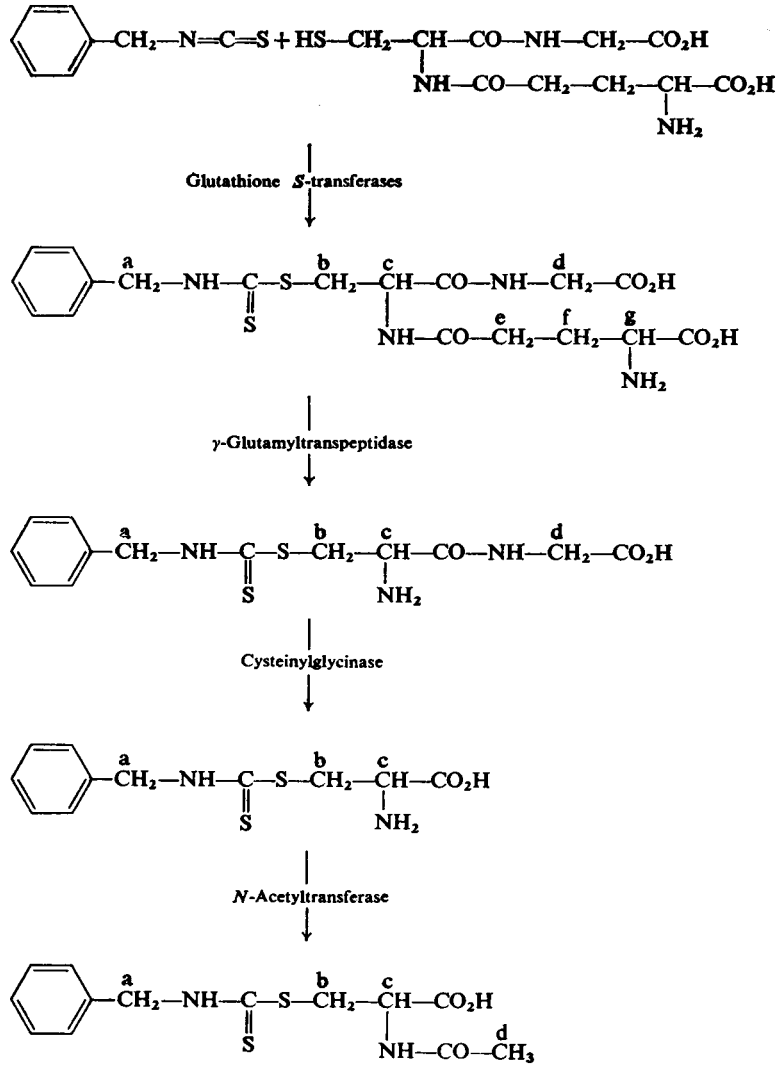

Fig. 1. *Possible route of metabolism of benzyl isothiocyanate in rats and other species*
The superscript letters refer to protons detected by n.m.r.

products were chromatographically identical with free benzyl isothiocyanate and its corresponding mercapturic acid. None of the other conjugates was detected. Free benzyl isothiocyanate could have originated through catabolism of the administered conjugate to its constituent compounds or alternatively through cleavage of the mercapturic acid to benzyl isothiocyanate and *N*-acetylcystine, which occurs to the extent of about 1% at pH5, 7% at pH6, 11% at pH7 and 14% at pH8.

The corresponding mercapturic acid was isolated as the dicyclohexylamine salt from the urines of rats after the administration of benzyl isothiocyanate and its cysteine conjugate.

Metabolism of the radioactively labelled cysteine conjugate of benzyl isothiocyanate in rats

After oral administration of the cysteine conjugate of [^{14}C]benzyl isothiocyanate to rats, the radioactivity was well absorbed and rapidly excreted. Peak mean plasma concentrations of radioactivity (43 and 24 μg-equiv./ml in males and females respectively) occurred within 45 min, and the mean area under the plasma concentration–time relationship for male rats (2.7% of the dose × h per ml) was significantly higher ($P<0.001$) than that for females (1.0% of the dose × h per ml). Rapid excretion of radioactivity is implied by its rapid decline (half-life about 1–2h) in the plasma.

Over 3 days, a mean (±S.E.M.) of 92.4±5.8% of the radioactivity was excreted in the urine, 5.6±1.7% in the faeces and 0.4% in the expired air. Less than 0.5±0.04% was still retained in the carcases. In two rats with cannulated bile ducts, a mean of 3.9% was excreted in the bile, showing that biliary secretion was not an important route of excretion for the cysteine conjugate and/or its metabolites.

One major radioactive component, which accounted for about 62% of the dose, was shown by t.l.c. of rat urine extracts in solvent systems C, E and F to correspond to the mercapturic acid of benzyl isothiocyanate. Components corresponding to the unchanged cysteine conjugate or free benzyl isothiocyanate each did not account for more than about 1% of the administered dose and other components accounted for less than 10% of the dose. Benzyl isothiocyanate conjugates were unstable in the mass spectrometer undergoing thermal or electron-impact degradation and in all cases, no molecular ion was detected, but a major fragment (*m/e* 149) corresponded to the benzyl isothiocyanate ion.

Administration of the [^{35}S]cysteine conjugate of benzyl isothiocyanate to a rat or administration of the non-radioactive cysteine conjugate to a rat pretreated with [^{35}S]cysteine to radiolabel the body sulphur pool, resulted in the excretion of one major radioactive component ([^{35}S]mercapturic acid of benzyl isothiocyanate) chromatographically identical with the mercapturic acid in both cases. Excretion of the [^{35}S]mercapturic acid by the rat dosed with the non-radioactive cysteine conjugate showed that the latter was partly converted into free benzyl isothiocyanate *in vivo*, which presumably was reconjugated with [^{35}S]GSH to undergo mercapturic acid biosynthesis and yield the [^{35}S]mercapturic acid. Had this occurred to any great extent, however, greater biliary excretion of radioactivity in the rat would have been expected, since the initial GSH conjugate of benzyl isothiocyanate has the requisite physicochemical properties for secretion into bile (Chasseaud, 1973). On the other hand, excretion of [^{35}S]mercapturic acid by the rat dosed with the [^{35}S]cysteine conjugate showed that the cysteine conjugate was partly absorbed unchanged and *N*-acetylated to yield the [^{35}S]mercapturic acid.

Metabolism of the radioactively labelled cysteine conjugate of benzyl isothiocyanate in dogs

After oral administration of single doses of the cysteine conjugate of [^{14}C]benzyl isothiocyanate to dogs, the radioactivity was well absorbed and excreted fairly rapidly. Absorption of the cysteine conjugate in dogs appeared to occur more slowly than in rats, as suggested by peak mean plasma concentrations of radioactivity (3.4 μg-equiv./ml) maintained during 1.5–6h. After 3 days, a mean of 86.3% was excreted in the urine and 13.2% in the faeces.

One major radioactive component, which accounted for at least 40% of the dose, was shown by t.l.c. of dog urine extracts in solvent systems C and F to correspond to the mercapturic acid of benzyl isothiocyanate. However, in solvent system E, it possessed a slightly greater R_F value, which implied that it was less polar than the mercapturic acid. The major metabolite was isolated and shown to have t.l.c. properties and a mass spectrum (Fig. 2) identical with that of hippuric acid. Further, this radiolabelled metabolite was not detected in the urine of a dog dosed with the [^{35}S]cysteine conjugate of benzyl isothiocyanate, showing that it had lost the radiolabel located in the cysteine moiety. Components in dog urine corresponding on t.l.c. to the unchanged cysteine conjugate, free benzyl isothiocyanate or

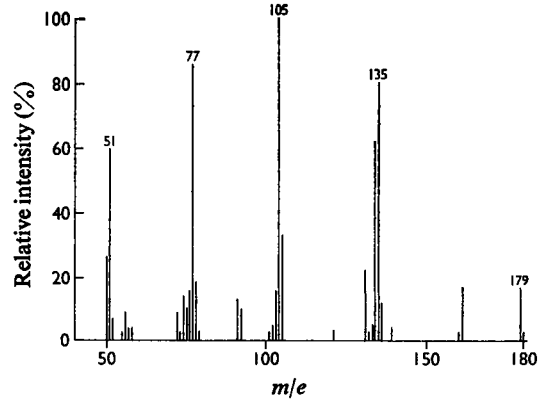

Fig. 2. *Mass spectrum of the major metabolite present in dog urine*
This spectrum was identical with that obtained from hippuric acid.

other substances only represented small proportions (<5%) of the administered dose. No evidence was obtained for the presence of the mercapturic acid in the urine of these dogs.

Discussion

Benzyl isothiocyanate is representative of a class of compounds about which little is reported with respect to metabolism. The present studies show that a major route of metabolism of isothiocyanates, at least in rats, may be mercapturic acid biosynthesis (Fig. 1). However, the position of the isothiocyanate moiety relative to other reactive positions may be important, since preliminary studies have shown that a mercapturic acid was produced by rats dosed with allyl isothiocyanate, but not with α-naphthyl isothiocyanate (G. Krumbiegel, K. Görler & W. H. Mennicke, unpublished work). An explanation for this finding is that mercapturic acid biosynthesis may only occur when the isothiocyanate moiety is attached to reactive positions such as benzylic or allylic centres.

The use of qualitative and quantitative procedures demonstrated that in the rat, the cysteine conjugate of benzyl isothiocyanate was mainly converted into the corresponding mercapturic acid. Other cysteine conjugates have been shown to be converted into the corresponding mercapturic acid (Stekol, 1938; Zbarsky & Young, 1943; West & Mathura, 1954; Bray *et al.*, 1959*b*; Barnsley *et al.*, 1969). After administration of *S*-pentyl-L-cysteine to animals, the urinary excretion of the corresponding mercapturic acid ranged from about 2% of the dose by guinea pigs to 12% by rabbits, 47% by rats and 73% by hamsters (James & Needham, 1973).

The difference in the major urinary excretion product in rats and dogs administered a single oral dose of the cysteine conjugate of benzyl isothiocyanate is a notable example of species differences. Rats mainly excreted the mercapturic acid, whereas dogs excreted the glycine conjugate, hippuric acid. Possibly in dogs the benzylic position is more readily hydroxylated and then oxidized to benzoic acid, which is known to be excreted as the glycine conjugate.

By using qualitative procedures, it was possible to detect mercapturic acid formation in dogs after higher and repeated oral doses of the cysteine conjugate of benzyl isothiocyanate, but the proportion of the dose excreted as the mercapturic acid was not established. The mercapturic acid has also been detected in the urines of humans and pigs after oral doses of the cysteine conjugate, but not in the urines of guinea pigs or rabbits (K. Görler, H. Koch, G. Krumbiegel & W. H. Mennicke, unpublished work). The guinea pig has been shown to be a poor excretor of mercapturic acids (Bray *et al.*, 1959*b*) although enzyme-catalysed conjugation with GSH occurs readily (Boyland & Chasseaud, 1969*a*).

An interesting feature of the disposition of the cysteine conjugate of [^{14}C]benzyl isothiocyanate in rats and dogs was the relatively large amounts of radioactivity, representing the unchanged conjugate and/or its metabolites, in the total plasma. Indeed, whole-body radioautographic studies have suggested that apart from the gastrointestinal tract, liver and kidneys, which are organs concerned with the absorption and elimination of the cysteine conjugate, the blood contained relatively higher concentrations than other organs or tissues (E. R. Franklin, unpublished work).

The appearance of free benzyl isothiocyanate in the urine and in the expired air after oral dosing with the corresponding cysteine conjugate or mercapturic acid needs further comment. Increasing spontaneous hydrolysis of these derivatives has been demonstrated *in vitro* to correlate with increasing pH, and the effective antibacterial properties *in vivo* of these conjugates can therefore be attributed to release of benzyl isothiocyanate from them.

These studies extend the list of substances (Chasseaud, 1976) that are known to be metabolized by conjugation with GSH in the mercapturic acid pathway and provide further evidence of the importance of this pathway (Boyland & Chasseaud, 1969*a*) and its associated GSH *S*-transferases (Chasseaud, 1973; Pabst *et al.*, 1973) for the removal of electrophilic compounds from the body.

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