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METABOLISM OF 2-ACETAMIDO-4-(CHLOROMETHYL)THIAZOLE IN GERMFREE AND CONVENTIONAL RATS

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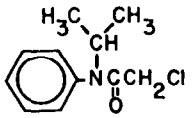
Abstract—In contrast with conventional rats, 2-acetamido-4-(chloromethyl)thiazole was not metabolized to the 4-(methylthiomethyl)-, 4-(methylsulfinylmethyl)- and 4-(methylsulfonylmethyl) analogues by germfree rats. Mechanisms for the formation of these metabolites from the mercapturate and the *S*-glucuronide are proposed. These mechanisms involve the biliary excretion of a mercapturic acid conjugate and an *S*-glucuronide conjugate which are metabolized in the intestine to metabolites that are reabsorbed, metabolized and excreted with the urine.

INTRODUCTION

In a previous study in this laboratory, we have shown that the herbicide propachlor (2-chloro-*N*-isopropylacetanilide) (Fig. 1) was metabolized via the mercapturic acid pathway [1]. In addition, metabolism studies with this compound in bile duct cannulated rats [2] and germfree rats [3] have shown that biliary secretion of the premercapturates and mercapturate of propachlor followed by metabolism by the intestinal microflora was necessary for the formation of the six methylsulfonyl-containing metabolites that were ultimately excreted in the urine.

Chatfield and Hunter [4] have shown that rats metabolize 2-acetamido-4-(chloromethyl)thiazole (I) (one of a series of compounds with anti-inflammatory activity) in part to the mercapturic acid (II); II is subsequently metabolized to the corresponding 4-(methylthiomethyl)-, 4-(methylsulfinylmethyl)-, and 4-(methylsulfonylmethyl)-analogues (III, IV and V, respectively). This metabolic pathway is outlined in Fig. 2. These authors also showed that I formed a glutathione conjugate *in vitro*.

Therefore, since both propachlor and I contain reactive aliphatic halogen functions, both are metabolized via the mercapturic acid pathway and both compounds give rise to methylsulfonyl-containing metabolites, it is possible that enterohepatic circulation of the mercapturic acid intermediates from I



propachlor

Fig. 1. Structure of 2-chloro-*N*-isopropylacetanilide.

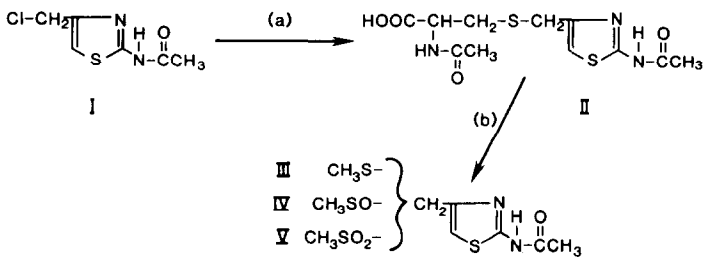


Fig. 2. The metabolism of 2-acetamido-4-(chloromethyl) thiazole (I) to 4-(methylthio)methyl-, 4-(methylsulfinyl)methyl- and 4-(methylsulfonyl) methylanalogues (III, IV, and V, respectively).

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and microbial metabolism were involved in the metabolic processes giving rise to III, IV and V.

Thus, with the general intention of establishing a role for intestinal microfloral metabolism in the overall metabolism of xenobiotic compounds and with the specific intention of determining whether the intestinal flora are involved in the metabolic formation of metabolites III, IV and V, we undertook to compare the metabolism of compound I in germfree and conventional rats.

MATERIALS AND METHODS

Radiolabeled compound

2-Acetamido-4-(chloromethyl)[2-^{14}C] thiazole was prepared as outlined by Chatfield and Hunter [4]. To a solution of 76 mg (1 mmole, 0.25 mCi) of ^{14}C-labeled thiourea in 30 ml of acetone was added 30 μl of water (the use of dry acetone resulted in greatly reduced yields) and 127 mg (1 mmole) of 1,3-dichloroacetone in 5 ml of acetone. The reaction mixture was allowed to stand at room temperature for 20 hr, and the solvent was removed with a jet of nitrogen. Acetic anhydride (24 ml) and methanesulfonic acid (0.6 ml) were added, and the reaction mixture was stirred at room temperature for 48 hr. Water and ether were added, and the ether layer was removed and dried with magnesium sulfate. Most of the ether was removed, and hexane was added. The product was removed by filtration, the mother liquors were concentrated, and a second crop of product was collected. Total yield was 88 per cent. The purity of the radiolabeled compound was checked by gas chromatography on 3 per cent OV 101 on Gas Chrom Q, and by thin layer chromatography on silica gel using three solvent systems: (1) ethyl acetate; (2) chloroform; and (3) benzene/methanol (9:1). In each thin-layer chromatography system used, only one spot was obtained which had an identical R_f value with the authentic standard. The radiolabeled compound also gave only one peak on gas chromatography with a similar retention time to the authentic standard.

Synthesis of metabolites

Synthesis of *S*-(2-acetamido-4-thiazolylmethyl)-*N*-acetyl-4-cysteine (II, mercapturate), 2-acetamido-4-[(methylsulfonyl)methyl] thiazole (V), 2-acetamido-4-[(methylsulfinyl)methyl] thiazole (IV), and 2-acetamido-4-[(methylthio)methyl] thiazole (III), were performed according to the methods of Chatfield and Hunter [4]. 2-Acetamido-4-(hydroxymethyl) thiazole (VI) was also prepared as described by Chatfield and Hunter [5]. 2-Acetamido-4-[(*S*-ethanethioyl)methyl] thiazole (IX) was prepared from the synthetic mercapturate (II) by reaction of II with methanesulfonic acid in acetic anhydride (1:20; v/v) for 40 min at 100°. IX was isolated from the reaction mixture by g.l.c. and identified by mass spectral analysis.

Animal treatment

Three germfree rats, raised by the methods of Gustafsson [6, 7], and three conventional rats were given intraperitoneal doses of I-^{14}C (5 mg, 3.7 μCi/rat) as described by Chatfield and Hunter [4]. All rats were males of the Sprague–Dawley strain. Their weights were between 320 and 370 g, and they were fed a standardized semi synthetic diet [7] *ad lib*. The germfree rats were housed within an isolator in stainless steel cages that were designed for the separation of the urine from the feces. The conventional rats were housed in similar cages outside the isolator. The urine and feces were collected 24, 48 and 72 hr after dosing. All urine from germfree rats was pooled prior to metabolite cleanup as was urine from conventional animals. In addition, all germfree feces was pooled prior to metabolite cleanup as was conventional feces.

Chromatographic methods

Column A. Porapak Q (Waters Assoc. 200–325 mesh) was slurried with methanol and poured to form a 1 × 10 cm column. The column was washed with water and the samples were applied to the column in water. The unbound sample components were eluted from the column with water; the bound components were eluted with methanol.

Column B. Water-equilibrated Sephadex LH-20 (Pharmacia) was poured to form a 1 × 90 cm column. Samples (0.5–1 ml) were applied to the column in water; the radioactivity was eluted from the column with water.

High performance liquid chromatography (h.p.l.c.). The h.p.l.c. was performed with a 7.8 mm × 30 cm μ-Bondapack C_{18} column (Waters Assoc.). The samples were injected in water. The radioactivity was eluted from the column using a linear gradient of methanol in water.

Thin-layer chromatography (t.l.c.). The t.l.c. was conducted on 20 × 20 cm plates, 0.25 mm thick layers of silica gel 60 with fluorescent indicator (Merck, Darmstadt, Germany). Plates were developed with butan-1-ol-acetic acid-water (12:1;2, v/v/v) as previously described [4].

Separation, quantitation and identification of metabolites

The urinary metabolites were separated by the following procedure. The urine was applied to column A. Radioactivity was eluted from the column with both water and with methanol. Both of these fractions were processed by the same procedure. All solvents in all processes were removed by either freeze drying or evaporation under vacuum. These fractions were then applied to column B, followed by h.p.l.c.

The aglycone from metabolite VIII was characterized after treatment with glucuronidase. The metabolite was dissolved in 2.0 ml of 0.05 M sodium acetate buffer (pH 5.0) that contained 100 μl (10 units) of β-glucuronidase-aryl sulfatase (Calbiochem). The mixture was kept at 38° for 18 hr. After reaction, the radioactivity was extracted from the incubation mixture with column A and purified for mass spectrometry by h.p.l.c.

The feces were homogenized with water and centrifuged. The aqueous supernatant was then processed in the same manner as the urine.

The mercapturic acid (II) was methylated with diazomethane before mass spectrometry. Mass spec-

tra were obtained from III to VII without derivatization.

All radioactivity was quantitated by liquid scintillation spectrometry. Mass spectrometry was performed with either an LKB-2091 or a Varian MAT CH-5 DF mass spectrometer. All samples were introduced with a solid sample probe.

RESULTS

The excretion of the radioactivity from the doses of I-^{14}C given to both groups of rats are given in Table 1 along with the recovery that was reported by Chatfield and Hunter [4].

Eleven radiolabeled metabolites were separated from the urine from the conventional rats and four from the urine from the germfree rats. The quantitation of these metabolites, the structures assigned to the metabolites identified or characterized by mass spectrometry, and the chromatographic characteristics for each metabolite are given in Table 2. The mass spectra from metabolites II through VII, the aglycone from VIII, and the *S*-acetate of the aglycone (IX) are given in Table 3.

To confirm the assigned structures, the methyl ester of II, the *S*-acetate of VII (IX), and metabolites III, IV, V, and VI were synthesized and the mass spectra compared with those from the appropriate metabolite or metabolite derivative. We were unable to synthesize VII and therefore the *S*-glucuronide VIII. The structures of these metabolites were assigned from the following studies which are outlined in Fig. 3.

Table 1. Recoveries of ^{14}C from germfree and conventional rats given single intraperitoneal doses of 2-acetamido-4-(chloromethyl) [2-^{14}C] thiazole

	Recovery, per cent of dose		
Time (hr)	Urine	Feces	Total
Germfree rats			
0–24	66.3	2.8	69.1
24–96	5.4	2.0	7.4
Total	71.7	4.8	76.5
Conventional rats			
0–24	82.0	1.5	83.5
24–96	2.3	0.8	3.1
Total	84.3	2.3	86.6
Conventional rats [4]			
0–24	75	10	85
24–96	–	–	7
			92

The *S*-acetate of VII (XI) was prepared from II by reaction of II with methanesulfonic acid in acetic anhydride as shown in Fig. 3. Paulson and Portnoy [8] reported that reaction of sulfate ester conjugates with this reagent resulted in the replacement of the sulfate group with an acetyl group. In this case, the reagent replaced a 2-acetamidopropionyl-group with an acetate. Treatment of VIII with this reagent did not produce IX, however, IX was produced by acetylation of the glucuronidase hydrolysis product from

Table 2. Quantitation of the metabolites and the chromatographic data for the various metabolites

Metabolite designation		Per cent of dose		Chromatographic characteristics		
		Germfree urine*	Conventional urine†	Column A (solvent)	Column B elution vol.‡	HPLC % CH_3OH§
II	Mercapturate	56.6	72.2(42)	H_2O and CH_3OH	1	32–35
	Unknown	–	0.1	CH_3OH	1.6	42
VIII	Glucuronide of VII	14.3	6.9	H_2O	1.9	52–55
	Unknown	0.4	0.1	CH_3OH	2.1	43–46
	Unknown	–	0.01	CH_3OH	2.5	24
VII	2-Acetamido-4-(mercaptomethyl) thiazole	–	0.06	CH_3OH	2.5	33
V	2-Acetamido-4-[(methyl sulfonyl)methyl thiazole	–	0.2(2)	CH_3OH	2.5	48
VI	2-Acetamido-4-(hydroxymethyl) thiazole	0.5	0.1	CH_3OH	2.7	42–43
	Unknown	–	0.3	CH_3OH	4.7	51
IV	2-Acetamido-4-[(methyl sulfinyl)methyl] thiazole	–	0.1(11)	CH_3OH	4.7	53
III	2-Acetamido-4-[(methyl thio)methyl] thiazole	–	3.0(2)	CH_3OH	4.7	73

* A hyphen means none detectable.

† Values in parenthesis are those reported by Chatfield and Hunter [4].

‡ Elution volumes and R_{II} values are relative to the values obtained for the mercapturate (II).

§ Values are for the per cent of methanol in the solvent at the pumps when the radioactivity eluted from the column.

Table 3. Mass spectra from metabolites

m-equiv	Relative abundance (per cent)	m-equiv	Relative abundance (per cent)
Metabolite II (methyl ester)		**Metabolite V**	
331 (2S)	8	234 (2S)	7
272	0.5	192	5
213	3	155	55
188	10	113	65
187	20	43	100
171	5		
159	12	**Metabolite VI**	
158	7	172	31
157	12	155	8
156	100	130	99
155	20	129	24
146	13	113	13
145	21	112	19
144	11	101	37
143	5	43	100
114	50		
113	56	**Metabolite VII and aglycone from VIII**	
43	90	188 (2S)	62
Metabolite III		156	15
202 (2S)	11	155	5
156	54	146	87
114	57	145	10
113	49	114	31
71	17	113	84
45	40	59	83
43	100	44	100
Metabolite IV		43	30
218	0.5	**Compound IX (acetylated VII)**	
202	3.5	230 (2S)	8
156	17	188	5
155	32	187	31
114	23	155	2
113	52	146	16
71	17	145	26
45	36	113	23
43	100	43	100

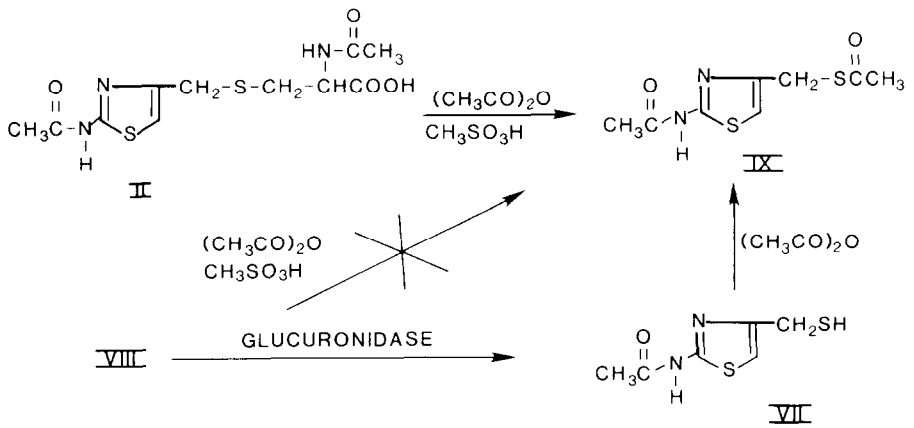

Fig. 3. Chemical formation of the *S*-acetate of IIV (IX) from the mercapturic acid and the *S*-glucuronide.

VIII with acetic anhydride, VIII was therefore concluded to be the *S*-glucuronide of VII. VIII was at first assumed to be 2-acetamidothiazole-4-carboxylic acid as reported previously [4], however we were unable to form the corresponding methyl ester with either diazomethane or methanolic-HCl.

To our knowledge, the metabolites discussed above are thermally and chemically stable and directly reflect those metabolites excreted by the animal.

DISCUSSION

Chatfield and Hunter [4] have shown that the mercapture (II) is a precursor to III, IV, and V. These three metabolites were detected in the excreta from the conventional rats but were not detected in the excreta from the germfree rats (minimum detectability, <0.01 per cent of the dose), therefore, the conversion of II to III (step b, Fig. 2) must not take place in the germfree rat. The actual sequence of the metabolic process from II to III and the reactions mediated by the flora have not been determined.

Figure 4 shows two possible pathways, both involving the intestinal microflora, for the production of III, IV and V from II. In one route, the mercapturate and/or precursors of the mercapturate formed in the liver are secreted, in part, with the bile. In the intestine a microfloral C-S lyase produces the sulfide (VII) that is subsequently methylated. The thiol *S*-methyltransferase which is active in the methylation of aliphatic mercaptans and has been shown to be present in the intestinal mucosa by Weisiger *et al.* [9] may be responsible for this latter step. The isolation of II (2 per cent of the dose) from the germfree feces as the only soluble fecal metabolite is evidence for the biliary secretion of II. A radioactive metabolite that migrated on thin layer plates with the same R_f as that of II was also extracted from the conventional feces (>1 per cent), but we were unable to methylate the material and obtain a mass spectrum.

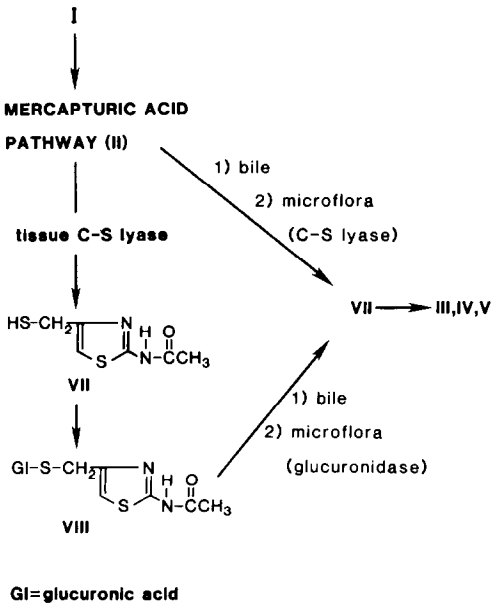

Fig. 4. Proposed pathways for the metabolism of II to III, IV and V that involve the intestinal flora.

The other route would involve a tissue C-S lyase such as that reported by Colucci and Buske [10]. In this pathway, the mercapturate and/or premercapturates are cleaved by a liver C-S lyase to yield VII. VII is then conjugated with glucuronic acid and secreted, in part, with the bile. The biliary metabolite would be hydrolyzed by microfloral glucuronidase to yield VII, which is subsequently methylated. The presence of this pathway is postulated only because the glucuronide of VII (VIII) was isolated from the urines from both germfree and conventional rats, and the aglycone (VII) was detected in small amounts in the urine from the conventional rats only. The *S*-glucuronide was not detected in the feces from either group of rats.

Two major differences were observed in the results of this study and those reported by Chatfield and Hunter [4]. We were unable to show the presence of 2-acetamidothiazole-4-carboxylic acid as a metabolite, but did characterize the *S*-glucuronide (VIII) of VII as a major metabolite. These differences could be a result of the different strain of rats and/or diets used in the experiments. A better explanation is that the reverse isotope dilution analysis used for identification did not discriminate between the two compounds. Feil *et al.* [11] have shown that isotope dilution analysis for diethylstilbestrol (DES) in mixture with hexestrol could not discriminate between the two compounds. If the metabolite that Chatfield and Hunter found was the *S*-glucuronide, it would better explain why it was present as a urinary metabolite from the rats to which they gave oral doses of the mercapturate (II).

Results in this study have established that the metabolic activity of the intestinal microflora is an essential component in the metabolism of compound I by the conventional rat. It has also been shown that the thiol produced from I by the tissue and microfloral C-S lyase systems are subsequently metabolized by different pathways. The thiol produced in the tissue is conjugated with glucuronic acid, and the thiol produced by the flora is subsequently methylated.

In conclusion, it appears, from studies being performed in our laboratory, that the intestinal microflora are involved in the metabolism of mercapturic acid pathway intermediates. This phenomenon, we feel, is significant, in view of the very large number of xenobiotic compounds which are known to be metabolized via the mercapturic acid pathway.

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