Metabolism of Trifluralin in Rats

Figen Ünlü Erkog[1] and Robert E. Menzer*

The metabolism of the dinitroaniline herbicide trifluralin, α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine, was investigated in rats using [$^{14}CF_3$]-labeled trifluralin at two dosage levels, 1 and 10 mg/kg, and in a continuous feeding experiment. Rats excreted the herbicide rapidly in both the urine and especially feces. Residual radiocarbon in tissues was low. Seven trifluralin metabolites were positively identified from rat urine and six from feces. The major metabolic routes of trifluralin involve N-dealkylation, nitro reduction, hydroxylation, cyclization, and conjugation. Unchanged trifluralin was detected in the feces, comprising approximately 10% of the dose. Metabolites resulting from a combination of nitro reduction and N-dealkylations of one or both propyl groups represented the highest percentage of the dose. Products of cyclization reactions giving 2-ethylbenzimidazoles and 2-methylbenzimidazoles were abundant. Metabolites with one or both propyl groups removed were detected mostly in the feces. Aglycones found after β-glucuronidase treatment of rat urine were compounds with a hydroxylated propyl group and a 2-methyl-substituted benzimidazole.

Trifluralin, α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine, a prominent member of the substituted dinitroaniline herbicide family, is effective against most grasses and many broadleaf weeds. Trifluralin's consumption in 1975 exceeded 2×10^7 lb (Heck et al., 1977).

Metabolism of trifluralin has been studied in a number of biological systems: in artificial rumen fluid and ruminant animals (Golab et al., 1969; Fisher et al., 1965), in rat liver microsomes (Nelson et al., 1977), in rats (Emmerson and Anderson, 1966; Heck et al., 1977), and in the dog (Emmerson and Anderson, 1966). Emmerson and Anderson (1966) gave rats an oral dose of 100 mg/kg of [$^{14}CF_3$]trifluralin. Within 5 days after dosing, approximately 78% of the radiocarbon was eliminated in the feces and 22% in the urine. They also found that 19% of the ^{14}C given to rats as [*N-propyl*-^{14}C]trifluralin was expired as $^{14}CO_2$; thus, N-dealkylation clearly has occurred. They observed that 11–14% of the ^{14}C was excreted in the bile within 24 h. However, intact rats given an equal oral dose of trifluralin excreted nearly 60% of the ^{14}C in the feces during the same period. In this study, fecal metabolites from rats were isolated and identified as unchanged trifluralin and α,α,α-trifluoro-2-amino-6-nitro-*N*,*N*-dipropyl-*p*-toluidine. A metabolite arising from the removal of both propyl groups was identified from rat urine, accounting for 1.7% of the dose. The other two metabolites were products resulting from removal of one propyl group and the reduction of one nitro group (0.4% of the dose) and the diamino compound α,α,α-trifluoro-1,2-diamino-6-nitro-*p*-toluidine.

The same investigators studied the metabolism of unlabeled trifluralin in two female mongrel dogs. The dogs were dosed with cold trifluralin orally for 3 days. Unchanged trifluralin, 25% of the dose, and the metabolite with only one nitro group reduced to the amino form (9% of the dose) were isolated from feces. No work was done on the dog urinary metabolites in this study.

Golab et al. (1969) fed a lactating goat unlabeled trifluralin and a mixture of uniformly ring-labeled and [$^{14}CF_3$]trifluralin and reported that 17.8% of the radiocarbon was excreted in the urine and 81.2% in the feces within 15 days after dosing. The major metabolite identified from feces and urine of goats was the product of reduction of both nitro groups. The parent compound was not detected in the urine or feces of the goat. Other urinary metabolites resulted from N-dealkylation and/or reduction of one or both nitro groups. There was no indication of trifluralin or its metabolites in liver, kidney, fat, small intestine, large intestine and stomach.

Trifluralin was extensively metabolized by both phenobarbital-induced and noninduced microsomes of the rat (Nelson et al., 1977). Principal in vitro metabolites were the monodealkylated alcohols, namely α,α,α-trifluoro-2,6-dinitro-*N*-(3-hydroxypropyl)-*p*-toluidine and α,α,α-trifluoro-2,6-dinitro-*N*-(2-hydroxypropyl)-*p*-toluidine and the aniline resulting from removal of both propyl groups. Minor in vitro metabolites were products resulting from combinations of the reduction of one nitro group and N-dealkylation of both propyl groups and also an N-dealkylated cyclization product, 2-ethyl-7-nitro-5-(trifluoromethyl)benzimidazole.

The pharmacokinetics of trifluralin were studied in rats by Heck et al. (1977) using heptadeuterated trifluralin (trifluralin-d_7) for analysis in terms of a two-compartment model. Computer-generated curves plotted from the in vivo data were described by a model where a single exponential described the disappearance of the deuterated trifluralin from compartment 1, but compartment 2 exhibited both a first-order uptake and a first-order disappearance. The oral route of administration resulted in relatively little systemic absorption of the unchanged trifluralin. However, the intraperitoneal studies indicated that, once absorbed, the compound may persist in fat for longer periods of time.

Among the other dinitroanilines whose metabolism has been studied in mammals are benefin (Golab et al., 1970), nitralin (Gutenmann and Lisk, 1970), dinitramine (Kennedy et al., 1975), and ethalfluralin (Hanasono et al., 1978; Hanasono et al., 1979). However, these studies have not resulted in the comprehensive and detailed knowledge of metabolism necessary for this important group of herbicides. The object of the present study is to eluciate the metabolism of trifluralin, the prominent member of the dinitroanilines, in rats, with special emphasis on the more polar metabolites.

MATERIALS AND METHODS

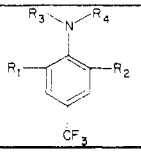
Synthesis of [*Trifluoromethyl*-^{14}C]trifluralin. To [*trifluoromethyl*-^{14}C]-α,α,α-trifluoro-2,6-dinitro-1-chloro-*p*-toluidine (29.7 mg, 0.11 mmol, 8.75 mCi/mmol; New

Department of Entomology, University of Maryland, College Park, Maryland 20742.

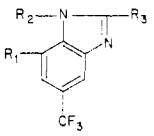
[1]Present address: Lalshan Hayvan Sağliği Nükleer Arastirma Enstitütsü, Lalahan, Ankara, Turkey.

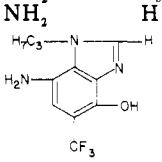
0021-8561/85/1433-1061$01.50/0

Table I. Structural Characterization and Chromatographic Characterization of [^{14}C]Trifluralin Metabolites and Standard Reference Compounds



compd	R_1	R_2	R_3	R_4	TLC R_f values[a] 1	2	3	HPLC[b] t_R, min	% methanol
trifluralin	NO_2	NO_2	C_3H_7	C_3H_7	0.72	0.85	0.86	62	60
I	NO_2	NO_2	H	C_3H_7				38	55
II	NO_2	NO_2	H	$CH_2CH_2CH_2OH$	0.55	0.72	0.70	18	23
III	NO_2	NO_2	H	$CH_2CH(OH)CH_3$	0.59	0.75	0.76	13	40
IV	NO_2	NO_2	H	H	0.67	0.79	0.80	33	50
V	NO_2	NH_2	C_3H_7	C_3H_7	0.71	0.82	0.83	44	60
VI	NO_2	NH_2	H	C_3H_7	0.61	0.78	0.80	32	55
VII	NO_2	NH_2	H	H	0.34	0.68	0.59	40	40
VIII	NH_2	NH_2	C_3H_7	C_3H_7	0.68	0.79	0.81	36	55



compd	R_1	R_2	R_3	TLC R_f values[a] 1	2	3	HPLC[b] t_R, min	% methanol
IX	NO_2	C_3H_7	C_2H_5	0.65	0.77	0.77	44	40
X	NO_2	H	C_2H_5	0.51	0.72	0.62	58	35
XI	NO_2	C_3H_7	CH_3	0.50	0.52	0.12	9	0
XII	NO_2	H	CH_3					
XIII	NH_2	C_3H_7	CH_3	0.47	0.62	0.18	28	42
XIV	NH_2	H	CH_3	0.11	0.31	0.09	56	42
XV				0.37	0.60	0.41	8	25
XVI	unknown			0.05	0.68	0.20	21[d]	0[d]
XVII	unknown				0.13	[c]	42	15

[a] TLC systems: 1, chloroform/ethanol (9:1, v/v); 2, chloroform/glacial acetic acid (5:4:1, v/v/v); 3, methylene chloride/dioxane/ethanol (90:30:1, v/v/v). [b] Conditions of HPLC analysis: instrument, Du Pont 830, column, 1 m × 2.1 mm (i.d.) packed with Permaphase ODS; eluant, methanol/water (v/v); pressure, 1000 psi; temperature, ambient; detector, 254 nm ultraviolet; attenuation, 4×10^{-2} or 8×10^{-2} AUFS. [c] Acetonitrile/methanol/water (2:1:1, v/v/v), R_f 0.90. [d] Pressure 1025 psi.

England Nuclear, Boston, MA) in 2.75 mL of ether (used as received) was added *N,N*-dipropylamine (0.06 mL, 0.47 mmol) with stirring. The reaction was run for 24 h at 24 °C in a round-bottom flask equipped with a reflux condenser. The reaction mixture was then spotted on a TLC plate and developed in carbon tetrachloride. The band corresponding to trifluralin (R_f 0.37) was scraped, eluted with ethanol, and crystallized from ethanol to which a few drops of water was added (82.8% yield). GLC analysis (described below) of the radiolabeled trifluralin indicated a single peak with the purity calculated to be greater than 98%.

Synthesis of Trifluralin Analogues. To a stirred solution of trifluralin (0.2 g, 0.5 mmol) in 7.0 mL of methanol were added sodium sulfide (0.56 g, 2.3 mmol) and sodium bicarbonate (0.19 g, 2.2 mmol) in 0.4 mL of distilled water. The reaction mixture was refluxed at 70 °C for 17 h. After 17 h, the mixture was poured over distilled water and the precipitated α,α,α-trifluoro-2-amino-6-nitro-*N,N*-dipropyl-*p*-toluidine (V) was collected by filtration. The precipitate was taken up in benzene and purified by TLC (benzene–carbon tetrachloride, 1:1): IR 3200–3600 (NH_2), 3000 (alkyl), 1540 cm^{-1} (NO_2).

α,α,α-Trifluoro-2,6-dinitro-1-chloro-*p*-toluidine was synthesized by the method of Hall and Giam (1972). α,α,α-Trifluoro-2,6-dinitro-*N*-(*n*-propyl)-*p*-toluidine (I), α,α,α-trifluoro-2,6-dinitro-*p*-toluidine (IV), α,α,α-trifluoro-2-amino-6-nitro-*N*-(*n*-propyl)-*p*-toluidine (VI), 2-ethyl-7-nitro-1-propyl-5-(trifluoromethyl)benzimidazole (IX), and 2-ethyl-7-nitro-5-(trifluoromethyl)benzimidazole (X) were prepared as described by Leitis and Crosby (1974). α,α,α-Trifluoro-2,6-dinitro-*N*-(3-hydroxypropyl)-*p*-toluidine (II) and α,α,α-trifluoro-2,6-dinitro-*N*-(2-hydroxypropyl)-*p*-toluidine (III) were synthesized according to the procedure of Nelson et al. (1977). The structures of the synthesized standard reference compounds were confirmed by obtaining their infrared spectra on a Beckman Acculab 4 spectrophotometer, KBr disks or thin films; 60-MHz nuclear magnetic resonance spectra, Varian EM-360; and 100-MHz NMR spectra, Varian XL 100 FT NMR, in deuterated solvents, Me_4Si internal standard.

Other Chemicals. α,α,α-Trifluoro-2,6-diamino-*N,N*-dipropyl-*p*-toluidine (VIII) was obtained from Eli Lilly and Co., Indianapolis, IN. α,α,α-Trifluoro-1,2-diamino-6-nitro-*p*-toluidine (VII), 2-methyl-7-nitro-5-(trifluoromethyl)benzimidazole (XII), 7-amino-2-methyl-1-propyl-5-(trifluoromethyl)benzimidazole (XIII), 2-methyl-7-nitro-1-propyl-5-(trifluoromethyl)benzimidazole (XI), and 7-amino-2-methyl-5-(trifluoromethyl)benzimidazole (XIV) were synthesized by Dr. J. O. Nelson of the Department of Entomology, University of Maryland (College Park, MD). The structures of trifluralin analogues are depicted in Table I.

Animal Treatments. Rats were treated at two single administration dosage levels and in a 2-month continuous administration of 500 ppm in the feed. In the first ex-

periment, six male albino rats, Sprague–Dawley strain (Flow Labs, Inc., Bethesda, MD), were treated through a stomach tube with 1 mg/kg (4.1 μCi) of [*trifluoromethyl*-^{14}C]trifluralin in 0.1 mL of corn oil. At the second dosage level, six male albino rats, Sprague–Dawley strain (Camm Research Animals, Wayne, NJ), were treated through a stomach tube with 10 mg/kg (35.2 μCi) of the [^{14}C]trifluralin in 0.1 mL of corn oil.

Four male albino rats, same strain and source as the 10 mg/kg dosed animals, were fed 500 ppm of trifluralin in their diet (Purina Laboratory Chow, Ralston Purina Co., St. Louis, MO) for 53 days. Food consumption was approximately 14 g/day per rat. On the 54th day, 1.05 μCi of [^{14}C]trifluralin was added to their diet. The animals were continued on nonradioactive trifluralin in their feed for an additional 3 days.

Excreta Collection, Extraction, and Quantitation. In all three experiments described above, the animals were housed in individual metabolism cages that permitted the separate collection of urine and feces. Food and water were provided ad libitum. In the single dose treatments urine and feces were collected at 12-h intervals, except for the initial collection at 6 h, starting at the time of [^{14}C]trifluralin administration and continuing until sacrifice. In the continuous feeding experiment urine and feces were collected once daily until administration of radiolabeled trifluralin, after which collections were made every 12 h. Aliquots of collected rat urine were assayed for radioactivity, using the aqueous scintillation cocktail, and fecal radiocarbon content was quantitated.

Urine (6, 12, and 24 h), from rats treated at the 1 mg/kg dosage level, was extracted three times with equal volumes of ethylene dichloride at pH 7.0. The ethylene dichloride fraction was dried over anhydrous sodium sulfate. Aliquots of the organic and aqueous layers were assayed for radioactivity. Similarly, urine from 12- and 24-h sampling times at the higher dose was extracted first at pH 7.0, later at pH 3.0, with diethyl ether.

Rat urine from the feeding experiment was pooled and centrifuged for 20 min at 2000 rpm in a Model CS International centrifuge. The pH was adjusted to 7.0, and the supernatant urine was extracted in batches continuously for 24 h, in a liquid–liquid extraction apparatus with diethyl ether. After the neutral metabolites were extracted, the pH was adjusted to 3.0 and extracted in a similar way, with diethyl ether. The neutral and acidic extracts were dried over anhydrous sodium sulfate.

Rat feces were pooled, and a wet weight was obtained. Then, they were added to methanol (weight/volume, 1:1) and allowed to stand overnight before extracting by blending the excreta three times in a Waring Blender with an equal volume of methanol each time. The residue and methanol fractions were separated by centrifuging the extract at 2000 rpm in a Model CS International centrifuge for 15 min. The methanol phase was treated with sodium sulfate and concentrated to an oily residue in a rotary evaporator under reduced pressure. Then, the residue was partitioned between hexane and acetonitrile (1:1) and counted, and the acetonitrile fraction was subjected to silica gel column chromatography.

After 4 days, animals dosed at 1 and 10 mg/kg were sacrificed and samples of liver, kidney, stomach wall, small and large intestine, spleen, fat, and lung were removed. These were assayed for radioactivity as described below.

Radioassay Techniques. Radiolabeled materials were detected with a Packard TriCarb liquid scintillation spectrometer (Model 3375) using toluene-based, organic, and aqueous scintillators as described by Opdycke et al., 1982. [^{14}C]-*n*-Hexadecane (Radiochemical Centre, Amersham, England) was used for internal standardization of counting efficiency. Radioactivity was detected on thin-layer chromatograms using both the Varian Aerograph Series 6000 thin-layer scanner equipped with a dual rate meter integrator (Berthold LB 242K) and Kodak No-Screen medical X-ray film to which the plates were exposed for 3–15 days.

Radioactivity in feces was assayed by first blending the feces (dried with known amounts of anhydrous sodium sulfate) in a Sorvall Omni-Mixer and then incubating 40-mg samples in 0.1 mL of distilled water plus Soluene-100 (Packard Instrument Co., Inc., Downers Grove, IL) at 60 °C for 24 h. After solubilization, the samples were decolorized by adding 0.2 mL each of isopropyl alcohol and hydrogen peroxide and incubating at 50 °C for 24 h. The samples were counted for radioactivity in 10 mL of aqueous scintillation cocktail. [^{14}C]-*n*-Hexadecane was used for internal standardization of counting efficiency.

The samples from selected tissues were also assayed for radioactivity by using the wet solubilization technique described for feces. Soluene-100 was added to 20–50 mg of tissue sample in a ratio of 0.1 mL/10 mg of wet tissue.

Chromatographic Procedures. Precoated silica gel 60 F-254 (E. Merck Co., Elmsford, NY) plates were used for TLC. Normally thin layers of 0.25-mm thickness were used except when purifying large amounts of material in which case PLQF 1000 Quanta-gram plates (Quantum Industries, Fairfield, NJ) were found to be essential. R_f values for trifluralin and its analogues in the three most useful solvent systems are given in Table I.

For column chromatography the ether extracts of urine were concentrated to a minimum volume, mixed with column materials, and then chromatographed on 2.25 × 115 cm columns. Columns were dry-packed with silica gel for dry column chromatography (ICN Pharmaceuticals GmbH and Co., Eschwege, West Germany) and eluted with the eluotropic series of solvents starting with hexane, finally ending with 1% glacial acetic acid in methanol. Similarly, the acetonitrile fraction of feces was subjected to dry column chromatography as described for urine extracts. Eluants were chloroform–methanol mixtures; the first solvent was pure chloroform and the last 5% glacial acetic acid in methanol. Fractions of 20 mL were collected in all cases, at a flow rate of 0.8 mL/min.

The aqueous fraction from urine after ether extractions was mixed with Amerlite XAD-2 (Mallinckrodt Inc., St. Louis, Mo), in a batch fashion in order to remove inorganic salts and organic contaminants. The resin was soaked in methanol overnight and washed with distilled water several times to remove the salts. After application of the urine fraction, the resin was washed three times with distilled water. Then, radioactivity was recovered by washing with methanol, followed by Soxhlet extraction of the resin with methanol for 48 h.

The methanol eluate from the XAD-2 cleanup was further purified by chromatography on a CGA 540 anion-exchange column, 100–200 mesh, ionic form Cl^- (J. T. Baker Chemical Co., Phillipsburg, NJ). The column was prepared by soaking 300 g of the resin in 0.1 M Tris-HCl buffer, pH 8.3, for 24 h at 4 °C. The slurry was poured into a 2.25 × 80 cm column and equilibrated with 10 column void volumes of the same buffer; the sample was applied in a minimum volume. The column was first eluted with 200 mL of the same buffer. Then, a linear gradient of 0.1–1.0 N HCl was applied using 2 L of each concentration, followed by a mixture of 2 L of 2 N HCl plus methanol (1:1), at a flow rate of 0.7 mL/min. Frac-

tions of 20 mL were collected and assayed for radioactivity. Radioactive fractions were lyophilized in a Virtis Automatic freeze-dryer (Model 10-010). All solvents were glass distilled or reagent grade.

A Du Pont 830 high-pressure liquid chromatograph (HPLC) fitted with a 254-nm ultraviolet detector and a 1-m (2.1-mm i.d.) column packed with Permaphase ODS (octadecyltrimethyloxysilane) was used to compare retention times with authentic standards and purify certain metabolites. Mixtures of methanol and water were used as the mobile phase. Retention times of trifluralin and its analogues are given in Table I.

Gas–liquid chromatographic analysis of the synthesized [^{14}C]trifluralin was performed on a Packard Model 803 gas chromatograph equipped with an electron capture detector and fitted with borosilicate glass columns packed with 10% DC 200 on gas chrom Q, 100/120 mesh. Trifluralin was identified from an authentic standard and quantitated by peak height measurements. Instrument conditions were as follows: column, 190 °C; injector, 200 °C; carrier gas, nitrogen at 60 mL/min.

Trimethylsilyl (Me_3Si) derivatives of trifluralin metabolites were prepared according to Brimfield et al. (1978) and Poole (1977). To the dried samples was added 1.0 mL of hexamethyldisilazane in pyridine (Tri-Sil, Pierce Chemical Co., Rockford, IL). The tubes were stoppered and shaken at room temperature for 1 h. After termination of the reaction, the solvent was evaporated and the contents were diluted with spectral grade methanol for HPLC analysis. The reaction was checked with glucose as a positive control.

Methyl esters of trifluralin metabolites were prepared by using ethereal diazomethane. To the dried samples was added 0.2 mL of a solution of ethereal diazomethane at room temperature prepared from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co., Inc., Milwaukee, WI) as described by Fales et al. (1973). The ether was evaporated, and the product was diluted with spectral grade methanol for HPLC analysis. The reaction was checked with α,α,α-trifluoro-2,6-dinitro-*p*-cresol as a positive control.

Detection and Identification of Metabolites. [^{14}C]Trifluralin and its metabolites were identified by cochromatography on TLC and HPLC of standard reference compounds with the radioactive extracts or isolated metabolites, with, in some cases, subsequent spectral analyses. When possible, and particularly when authentic reference compounds were unavailable, metabolites were isolated for spectral analyses. Mass spectra were obtained on a Du Pont Model 21-491B gas chromatography/mass spectrometer with a direct probe inlet and interfaced with a Hewlett-Packard Model 2100A computer. Infrared spectra were taken on a Beckman Acculab 4 spectrophotometer using KBr disks or thin films. With metabolites that could not be isolated in sufficient quantities, IR spectra were obtained using AgCl Mini Cells (Wilks Scientific Corp., South Norwalk, CT) in carbon tetrachloride or carbon disulfide.

Analysis of Conjugates. To determine the presence of possible conjugates in rat urine, the aqueous fractions were incubated with β-glucuronidase, aryl sulfatase, and hydrochloric acid. The rat urine was extracted with ether, and the remaining ether in the aqueous phase was removed under reduced pressure in preparation for incubations with enzyme or acid. For incubations with β-glucuronidase, 1×10^6 cpm from the aqueous urine fraction was mixed with 10 mg of β-glucuronidase (70 000–100 000 units/g; Nutritional Biochemicals Corp., Cleveland, OH) in 5.0 mL of 0.2 M sodium acetate buffer, pH 5.0. To test aryl sulfatase activity, 5 mg of the enzyme (type V from limpets, 11.6 units/mg; Sigma Chemical Co., St. Louis, MO) was mixed with 1×10^6 cpm from the aqueous urine fraction for incubation in the presence of 19.2 mg of 1,4-saccharolactone (20 mM; Sigma Chemical Co.) as a β-glucuronidase inhibitor in 5.0 mL of 0.2 M sodium acetate buffer, pH 5.0. The acid hydrolysis assay was run with a 1×10^6 cpm aliquot of the same aqueous urine fraction in 5 mL of 0.1 N HCl. The control consisted of the same amount of radioactive aliquot in 5 mL of buffer.

Each incubation was conducted at 38 °C for 24 h, with shaking. At the termination of incubation, the pH was readjusted to 7.0 and the incubates were extracted twice with equal amounts of ether. The aqueous fraction remaining after the neutral ether extraction was adjusted to pH 3.0 and extracted in a similar manner. Aliquots of both phases were assayed for radioactivity. The organic phases were spotted on TLC plates and analyzed for aglycones.

Table II. Elimination of Radiocarbon in the Urine and Feces of Rats Treated with 1 mg/kg of [^{14}C]Trifluralin as a Single Oral Dose

h after administrn	% of administered dose (mean, n = 6), ±SEM		cum % of dose
	urine	feces	
0–6	15.3 ± 3.9	1.6 ± 0.4	16.9
6–12	14.1 ± 4.1	8.9 ± 2.9	39.9
12–24	7.3 ± 2.1	22.1 ± 5.9	69.2
24–36	2.1 ± 0.8	5.5 ± 1.2	76.8
36–48	0.9 ± 0.3	2.9 ± 0.9	80.7
48–60	0.6 ± 0.2	5.8 ± 2.6	87.1
cagewash		13.4 ± 1.2	
totl rec		100.5% of dose	

Table III. Elimination of Radiocarbon in the Urine and Feces of Rats Treated with 10 mg/kg of [^{14}C]Trifluralin as a Single Oral Dose

h after administrn	% of administered dose (mean, n = 6), ±SEM		cum % of dose
	urine	feces	
0–6	2.6 ± 0.9	0.2 ± 0.02	2.8
6–12	4.9 ± 1.5	4.3[a]	12.1
12–24	5.0 ± 1.4	30.3[a]	47.4
14–36	2.0 ± 0.6	6.1 ± 2.7	55.4
36–48	1.4 ± 0.5	2.9 ± 1.2	59.7
48–60	0.6 ± 0.1	5.7 ± 3.2	65.9
60–72	0.4 ± 0.2	3.7 ± 2.3	70.1
72–84	0.3 ± 0.1	0.1 ± 0.05	70.5
84–106	0.3 ± 0.1	2.9 ± 1.6	73.7
cagewash		0.6 ± 0.2	
total rec		74.3%	

[a] Estimated; see explanation in text.

RESULTS

Excretion and Recovery of Administered Radioactivity. Table II shows the excretion of [^{14}C]trifluralin equivalents in the urine and feces following administration of 1 mg/kg of trifluralin. A high percentage of the administered dose, 87%, was excreted within the 60-h sampling period, nearly 70% within the first 24 h. The trifluralin equivalents excreted during that period were approximately equally divided between the urine and feces: 40.3% in the urine and 46.8% in the feces after 60 h. The maximum excretion of trifluralin equivalents in the urine peaked in the first 12 h after administration, while excretion in the feces was highest at 24 h. Recovery totaled 100.5% of the administered dose.

Table III shows the excretion of [^{14}C]trifluralin equivalents in the urine following administration of 10 mg/kg

Table IV. Residual Radiocarbon in the Tissues of Rats after Oral Doses of 1 and 10 mg/kg of [^{14}C]Trifluralin

tissue	ppm of [^{14}C]trifluralin equiv (n = 6), ±SEM	
	1 mg/kg	10 mg/kg
liver	0.22 ± 0.082	0.37 ± 0.094
kidney	0.10 ± 0.796	0.22 ± 0.057
stomach	0.04 ± 0.007	0.09 ± 0.037
intestine	0.05 ± 0.001	0.11 ± 0.029
spleen	0.07 ± 0.012	0.11 ± 0.026
lung	0.08 ± 0.189	0.17 ± 0.053
adrenal fat		0.19 ± 0.056

Table V. Percentage Distribution of Radioactivity following Fractionation of Urine Samples from Rats Treated with [^{14}C]Trifluralin

h after administrn	org solvent fraction[a]		aq fraction	sodium sulfate res	total rec
	pH 7.0	pH 3.0			
	Dose of 1.0 mg/kg				
0–6	25.4		34.4		59.8
6–12	15.7		43.9		59.7
12–24	19.6		62.8		82.4
	Dose of 10 mg/kg				
6–12	25.0	24.6	37.3	13.1	100.0
12–24	29.0	16.1	41.8	15.8	102.7

[a] Solvent: ethylene dichloride in 1.0 mg/kg treatment and diethyl ether in 10 mg/kg treatment.

of trifluralin. After 106 h total excretion in the urine was only 17.5% of the administered dose, only approximately one-third of the amount at the comparable time period in the 1 mg/kg dose. Furthermore, the peak excretion in the urine was later at the higher dose. Excretion of radioactivity in the feces followed much the same pattern as in the lower dose; after 106 h, 56.2% of the administered dose was excreted in the feces. This figure may be inaccurately low since the values obtained for 12 and 24 h are estimates. These samples had been extracted prior to analysis for radioactivity, and the values reported are based on extrapolations from the amounts of radioactivity in the extracts. Total recovery of radioactivity at this higher dosage was 74.3%, somewhat lower than at the 1 mg/kg level. The actual recovery may be somewhat higher than reported because of the estimated values for feces at 12 and 24 h, but it is certain that excretion of trifluralin equivalents at the higher dose was more prolonged and less complete.

Residual radioactivity in several tissues was measured following the sacrifice of the rats dosed with 1 and 10 mg/kg of [^{14}C]trifluralin. The results are presented in Table IV. The highest residues in both instances were in the liver, with significant amounts in the kidneys. Total recovery of trifluralin equivalents in the tissues analyzed amounted to 2.09% of the administered dose at 1 mg/kg and 0.22 at 10 mg/kg.

Characterization of Excreted Radioactivity. Preliminary separations of [^{14}C]trifluralin metabolites in the urine were effected by extraction with either ethylene dichloride or diethyl ether. The partitioning of the radioactivity between the organic solvent and aqueous fractions is reported in Table V. Low recoveries in the 1 mg/kg experiment probably result from failure to account for the radioactivity absorbed by sodium sulfate during the process of drying the organic extract. In the 10 mg/kg experiment recoveries were satisfactory when the sodium sulfate was taken into account. At the higher dose an additional organic solvent extraction was done after adjustment to pH 3.0, in an attempt to recover possible acidic metabolites. The results of these experiments were used to design the extraction procedure for the urine collected during the continuous feeding experiment.

Table VI. Quantitation of Rat Urine Metabolites of [^{14}C]Trifluralin Separated by TLC of the Ether Fractions after an Oral Dose of 10 mg/kg

metab	% of dose		R_f[a]
	pH 7.0	pH 3.0	
III	0.19	0.20	0.59
IV	0.19	1.21	0.67
VII	0.08	0.40	0.34
X	0.22	0.12	0.52
XI	0.08		0.51
XIII	0.54	0.18	0.10
XV	0.07	0.18	0.37
unknowns	3.04	1.97	*b*

[a] TLC system: chloroform/ethanol (9:1). [b] At least seven unknowns were present, at R_f 0.0–0.10, 0.12, 0.16, 0.18, 0.21, 0.26, and 0.29.

Table VII. Distribution of Radioactivity in Different Fractions after Enzyme or Acid Treatment of Rat Urine for the Detection of Possible Aglycones

incubn	% of total		
	org phase		
	pH 7.0	pH 3.0	aq phase
control	5.7	6.2	88.1
β-glucuronidase	17.2	12.9	69.8
aryl sulfatase[a]	8.5	8.2	83.3
HCl (0.1 N)	19.0	8.8	72.3

[a] Contains 20 mM 1,4-saccharolactone.

Table VIII. Quantitation of Rat Fecal Metabolites of [^{14}C]Trifluralin Separated by TLC of the Methanol Fraction after an Oral Dose of 10 mg/kg

metab	% of dose	R_f[a]	metab	% of dose	R_f[a]
unknown	9.84	0.00	XIII	3.12	0.58
unknown	5.04	0.05	trifluralin[b]	5.04	0.73
possible XV	1.28	0.13			

[a] TLC system: chloroform/ethanol (9:1). [b] This solvent system does not resolve trifluralin from I and V.

The procedure finally developed utilized diethyl ether extractions at pH 7.0 and 3.0 followed by chromatographic separations of metabolites in each of the organic extracts. The final aqueous fraction was also subjected to further chromatographic separations. At least 10 metabolites appeared in the acidic ether extracts, and only one in the final aqueous fraction. Quantitation of the metabolites in the ether fraction is given in Table VI. None of the metabolites in the urine comprised more than 2% of the administered dose.

Further analysis of the aqueous fraction involved treatment with β-glucuronidase, aryl sulfatase, or HCl. Table VII shows the percentages of radioactivity recovered in each of the control, β-glucuronidase, aryl sulfatase, and HCl incubations. The ether-extractable radioactivity was greater than the control for the β-glucuronidase and HCl incubations but was not greater in the aryl sulfatase incubations. Thin-layer chromatographic analysis of the organic extract following β-glucuronidase treatment indicated the presence of metabolites III and XIII.

Extractions of feces with methanol removed 50% of the radioactivity. Preliminary TLC of the methanol gave five zones as shown in Table VIII. The radioactivity in each of the zones was less than 10% of the administered dose. The material at R_f 0.73 was definitely characterized as the parent compound. The material at R_f 0.58 was likewise

a single material and was characterized as metabolite XIII. It was not possible to obtain a single pure material from the radioactivity at R_f 0.13. It probably contained metabolite XV contaminated with at least one other material. In addition two zones at R_f 0.05 and at the origin could be separated, but characterization of the nature of the materials present was not possible.

Since approximately 80% of the methanolic radioactivity from feces appeared in the final acetronitrile following fractionation, this material was subjected to further chromatographic analysis, resulting in preliminary separations of seven distinct peaks on silica gel columns, each of which contained one or more metabolites. Since the hexane fraction contained so little radioactivity, no further analysis was attempted. The residues remaining after extractions could not be further fractionated.

Nature of Metabolites. *Urinary Metabolites.* Unchanged [^{14}C]trifluralin was not detected in the urine in any experiment. Of the metabolites isolated from the urine, positive identifications can be established for seven compounds.

The presence of compounds III, IV, VII, X, and XIII (Table I) in the neutral ether extracts of urine was determined by cochromatography of the metabolites (three replicates of each, at least) with the synthesized standard reference compounds on HPLC and checking the corresponding peaks for radioactivity. In addition, metabolites XI and XV and one unknown metabolite (designated metabolite XVI) were isolated and purified from the neutral ether extract. Additional materials were present in very minor amounts and could not be further characterized.

The ether extraction of the acidic urine (pH 3.0) resulted in the isolation of four more materials as well as additional quantities of the metabolites isolated at neutral pH. Only one of these metabolites could be obtained in sufficient quantity for characterization (designated metabolite XVII).

Metabolites III, IV, and VII cochromatographed with standard reference materials on HPLC and at least two TLC systems (Table I). Quantities of these metabolites were insufficient for spectral analysis, but mass spectral data on these compounds have been reported by other workers (Kearney et al., 1976; Nelson et al., 1977).

Metabolite X cochromatographed on HPLC and TLC, as above. The metabolite was not available in sufficient quantity for infrared spectral analysis; however, mass spectra obtained on the isolated metabolite showed a molecular ion at m/e 259 (base); other abundant peaks were at m/e 258 (M − 1), 230 (M − 29), 217 (M − 42), 213 (M − 46), and 187 (M − 72) (Table IX).

Metabolite XI cochromatographed on TLC with standard reference compound. Its mass spectrum showed a molecular ion at m/e 287; other abundant ions were at m/e 272 (M − 15), 245 (M − 42), and 228 (M − 59) and base at m/e 227 (M − 60) (Table IX). IR: 3000–2860 (CH), 1530 and 1320 (NO_2), typical benzimidazole absorption bands at 1460, 1390, 1310, 1270, 1250, 1160, 1100, and 1020 cm^{-1}. The bands mentioned for the benzimidazole ring system were reported to be very characteristic (O'Sullivan, 1960). The standard reference compound was shown by IR to be identical with the metabolite.

Metabolite XIII cochromatographed on HPLC and TLC with standard reference compounds. Its mass spectrum gave a molecular ion at m/e 257; other prominent peaks were at m/e 242 (M − 15), 238 (M − 19), and 215 (M − 42) as base (Table IX). IR [3450–3300 (NH), 1460 (CH_2, CH_3) (O'Sullivan, 1960), 1320 (CN, primary aromatic amines) cm^{-1}] and absorption bands supporting the benzimidazole structure were present [1390, 1310, 1250, 1160, 1100, 1060 cm^{-1}].

Table IX. Mass Spectral Data of Isolated Trifluralin Metabolites[a]

	isolated metab		synth metab	
metab	m/e	rel abund, %	m/e	rel abund, %
VI	263	17.9	263	98.5
	245	10.0	244	21.1
	244	3.8	235	11.9
	234	12.1	234	100.0
	228	8.8	228	17.4
			216	64.2
X	259	100.0	259	100.0
	258	9.0	258	72.2
	244	8.2	244	2.8
	230	85.4	240	16.9
	229	7.9	229	1.8
	217	94.9	213	36.0
	213	5.7	212	40.3
	187	6.6		
XI	287	59.8	287	100.0
	272	3.9	272	1.2
	245	42.9	271	4.6
	228	23.7	245	3.8
	227	100.0	228	15.4
			227	5.0
XIII	257	51.5		
	242	3.6		
	238	6.0		
	216	16.4		
	215	100.0		
	214	12.9		
XV	259	92.2		
	242	14.7		
	231	17.7		
	230	87.8		
	217	100.0		
	202	27.0		
XVI	507	37.5		
	384	16.7		
	252	16.7		
	211	20.8		
	181	16.7		
	169	29.2		
	145	16.7		
XVII	561	100.0		
(methylated)	485	42.7		
	448	8.5		
	446	78.0		
	367	74.4		

[a] See Nelson et al. (1977) for mass spectra of other metabolites.

Lyle and LaMattina (1975) have reported cyclization reactions for the synthesis of methylbenzimidazoles from *N'*-(*n*-propyl)-2,6-diamino-*p*-toluidine. The possibility of metabolite XIII being formed from the diamino compound during ethyl acetate elution of the silica plates or thin layer chromatography employing an acetic acid solvent system was checked. Compounds VI, VII, V, and VIII were treated accordingly and analyzed for the possible formation of acetylated compounds. After analysis, no spots corresponding to the methylbenzimidazoles could be detected from the amines.

Another metabolite isolated from urine was XV. It was tentatively assigned the structure 7-amino-4-hydroxyl-1-propyl-5-(trifluoromethyl)benzimidazole. The mass spectrum of the isolated compound gave a molecular ion at m/e 259, base at 217 (M − 42), 242 (M − 17), 231 (M − 28), 230 (M − 28), and 202 (M − 57) (Table IX). The mass spectrum agrees with a phenolic structure: prominent molecular ion, small M − 1 peak and a strong M − 29 and M − 28 peaks. Metabolite XV gave positive results in tests for phenols with $FeCl_3/K_3Fe(CN)_6$ (blue spot) and

the Millon reagent (yellowish pink spot). IR: 3500–3200 (OH), 3000–2800 (CH_2, CH_3), 1370 and 1130 (CO) cm^{-1}.

Unknown metabolite XVI isolated from the neutral urine ether fraction did not cochromatograph with any of the known standard reference compounds listed in Table I. These data indicate that the compound is quite polar. An interpretable mass spectrum could not be obtained, and the probe capillary of the instrument had a large amount of char after analysis of the sample (Table IX). The metabolite gave negative results with $FeCl_3/K_3Fe(CN)_6$ and Millon's test for phenols.

Metabolite XVII isolated by ether extraction of acidified urine did not cochromatograph with any standard reference compound on TLC or HPLC. R_f values and retention times are reported in Table I. Its methylated derivative showed a molecular weight of 561, but it was not possible to assign a structure for this metabolite from the mass spectrum since the other prominant ions were resulting from big fragment and/or ion losses (Table IX). However, the presence of ions at m/e 446 and 448 suggested the presence of sulfur; however, the ninhydrin test was negative.

The aqueous fraction of the urine remaining after extraction with ether at pH 7.0 and 3.0 contained a substantial amount of radioactivity. Only one metabolite could be isolated in sufficient quantity for characterization studies from the aqueous fraction. Following chromatographic and mass spectral analysis this metabolite appeared to be identical with compound XIII. However, it is likely that the elution from the ion-exchange column with dilute HCl hydrolyzed a conjugated metabolite to its aglycone.

Fecal Metabolites. The radioactivity in the various peaks of the silica gel column of the acetonitrile fraction were further analyzed by TLC, HPLC, and mass spectrometry. Of the metabolites isolated from the feces, positive identifications can be established for six compounds. Unchanged trifluralin was isolated and identified. Its mass spectrum showed a strong molecular ion at m/e 335 with a base peak at m/e 306 (M – 29). Other aspects of the spectrum were identical with that of the standard reference compound. Due to the compound's instability to light, a sufficient amount could not be purified for an infrared spectrum. Another fecal metabolite eluted after the parent compound from the column and cochromatographed with compound VI. Its mass spectrum indicated a molecular weight of 263 (Table IX). The next two compounds eluting from the silica column corresponded to TLC with compounds I and V.

The final fractions resulted from much increased polarity of the eluting solvent systems. Three of these compounds do not correspond with any standard reference materials in subsequent chromatographic analysis. Each appeared to be a single compound. The next fraction was composed of a number of minor constituents, some of which also appeared in the previous peaks. The last fraction was a single material and cochromatographed on TLC and HPLC with compound XIII. Its identity was confirmed by mass spectral and infrared spectral analyses. One major metabolite and a number of minor metabolites eluted at the very end of the column and turned black upon standing. The possibility of a polymerization reaction was considered since such a reaction has been reported earlier for 3- and 5-OH derivatives of 2-amino-4-(acetylamino)toluene (Waring and Pheasant, 1976).

DISCUSSION

Excretion Patterns. Quantitation of radiocarbon recoveries from the excreta of rats following the administration of 1 and 10 mg/kg of trifluralin showed similarities in the fecal excretion, while urinary excretion was somewhat different. A 10-fold increase in the administered dose decreased the amount excreted in urine after 60 h to approximately 16%. However, fecal excretion totalled approximately 49% of the dose during the same time interval. When quantitation of the excreta was continued up to 118 h, at the 10 mg/kg dose, an additional 2% was recovered in urine. At 106 h after dosing, fecal recovery reached 56%. It is clear that urinary excretion of the administered radioactivity decreased by almost 3-fold by a 10-fold increase in the dosage. At the higher dosage, the excretion of fecal radioactivity continued for a longer period of time. The peak excretion was shifted 6 h later for urine while both fecal peaks were at 24 h.

In a study where 100 mg/kg of [$^{14}CF_3$]trifluralin was administered to rats (Emmerson and Anderson, 1966) in a single oral dose, approximately 22% of the dose was excreted in the urine and 78% in the feces, with both excretion peaks at 24 h. Saturation of the rats urinary excretory systems can be seen clearly by comparing the results from three dosages reported. The increased percentage excreted in the feces as the dosage is increased reflects the inability of the rats to absorb and metabolize the compound at the higher dosages. This is confirmed by the fact that substantial quantities of parent compound are isolated from the feces at 100 mg/kg.

Residual radiocarbon recoveries from tissues were low at both dosage levels (1 and 10 mg/kg). This is in agreement with studies reported earlier for a lactating goat and a cow (Golab et al., 1969), where trifluralin residues were detected only in cow fat in trace amounts. Similarly, low tissue residues were reported for other dinitroanilines (Golab et al., 1970; Kennedy et al., 1975; Hanasono et al., 1978) in goat, cow, and rats.

Metabolic Pattern. In this study, particular attention was given to polar trifluralin metabolites rather than the apolar products already identified by other workers (Fisher et al., 1965; Emmerson and Anderson, 1966; Golab et al., 1969). Differences in the chemical nature of metabolites were observed when the administered dose was increased. The number of metabolites decreased with an increase in dosage. A lower number of intermediary metabolites were detected at the higher dosages, with the isolation primarily of terminal metabolites.

Analysis of trifluralin metabolites is complicated by the ease of photodecomposition of the compound. Even though care was taken to minimize exposure to light during the processing of materials isolated from animals, it is still possible that decomposition of nonreduced and noncyclized products occurred to some extent.

Metabolites of trifluralin isolated from rats reflect several metabolic routes that operate to degrade the compound. A number of dealkylated products were identified. In addition, nitro reduction occurred, followed in some instances by cyclization to benzimidazoles.

Dealkylation. Dealkylation of one or both propyl groups was the primary metabolic route resulting in products in urine and feces. Although there was chromatographic evidence for the presence of the N-dealkylation product I, in urine, the unsubstituted aniline IV was detected as the terminal metabolite. The compound resulting from hydroxylation on the *N*-propyl group was detected in rat urine, metabolite III. The presence of hydroxylated products indicated that the dealkylation of trifluralin is an oxidative process.

Reduction. Reduction of nitro groups to the corresponding amines is a well-established metabolic route for

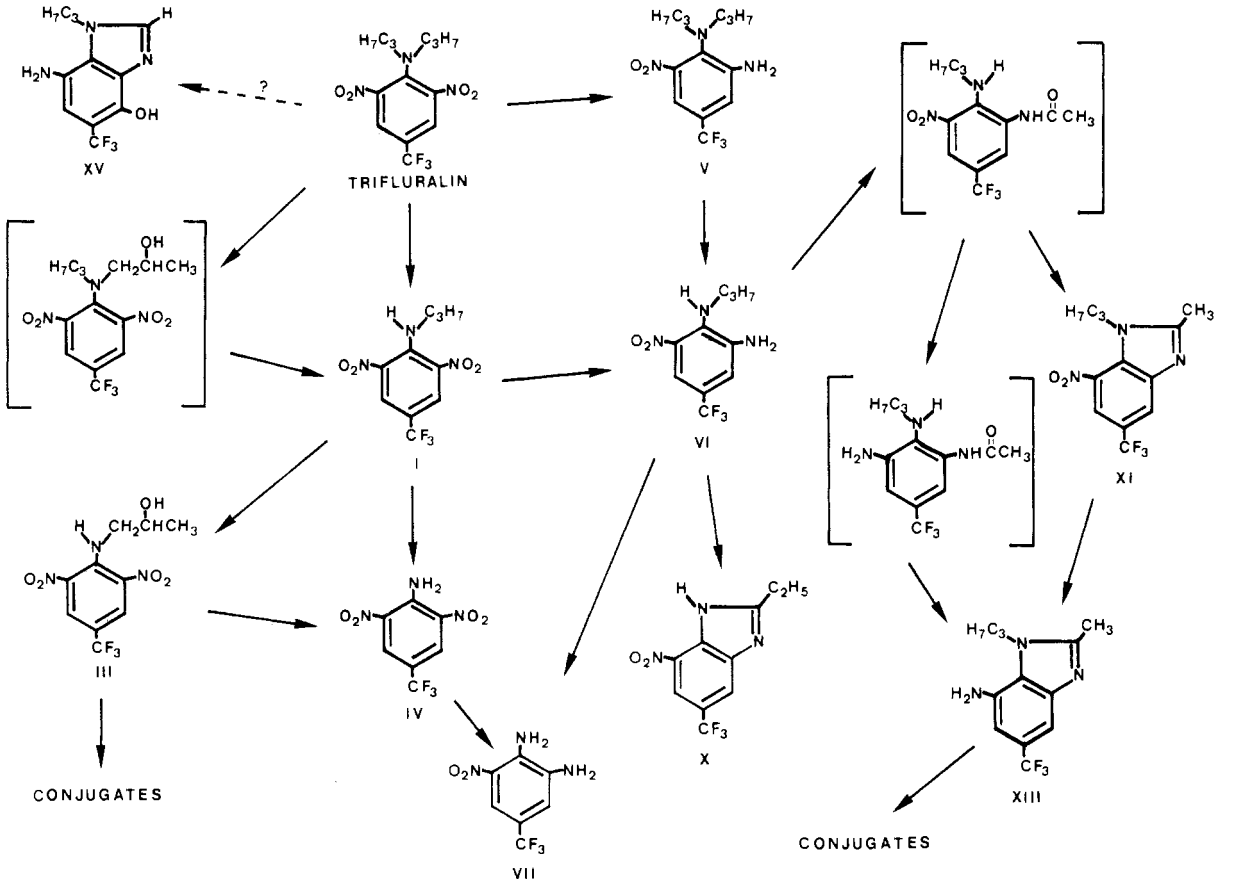


Figure 1. Proposed metabolic scheme for trifluralin in rats (compounds shown in brackets are probable intermediates but were not detected).

xenobiotics. Such metabolites were detected in this study, in both urine and feces. Evidence was present for compounds V and VI in feces. Products arising from N-dealkylations with subsequent nitro reductions were also observed, for example, the presence of the diamino compound VII in urine. However, compound VIII could not be detected in this study; it has been reported in a previous investigation by Golab et al. (1969) in goat feces. The same compound has been detected in rumen fluid (Golab et al., 1969) and rumen microbial cultures (Williams and Feil, 1971), indicating a microbial contribution to the nitro reduction reactions. No attempt was made to estimate such a contribution quantitatively in this study.

Benzimidazoles. Benzimidazoles arising from cyclization reactions have been reported as in vitro metabolites of trifluralin from rat liver microsomal incubations (Nelson et al., 1977) and soil (Kearney et al., 1976; Golab et al., 1979). In the previous studies, the identified benzimidazoles were 2-ethylbenzimidazoles in each case.

Ethylbenzimidazole. Compound X was unequivocably identified from rat urine. Most probably the N-dealkylation reaction occurs readily either prior to or simultaneously with the cyclization, since compound IX was not isolated in sufficient quantities for unequivocable characterization. There was evidence for the presence in very small quantities of a compound that may have been metabolite IX.

Methylbenzimidazoles. The methylbenzimidazoles XI and XIII are reported as in vivo trifluralin metabolites in this study for the first time. Metabolite XIII was a major metabolite, present in both the organic and the aqueous fractions of urine as well as feces. The identifications of these compounds are based on cochromatography with the synthesized reference compounds and mass spectral data and infrared spectra obtained for the isolated metabolites.

Mechanistically the methylbenzimidazoles are proposed to form from the cyclization of the in situ N-acetylated amino group. Acetylation appears to be of great significance in the excretion of amino derivatives. The conversion of the amino group on aromatic amines to an acetamido group is considered to be the major route of conjugation for this class of compounds (Williams, 1959). Clonazepam, a benzodiazepine with an aromatic nitro group, undergoes marked nitro reduction in man, rat, and dog. In man, the *N*-acetyl conjugate was found to be a significant urinary and fecal metabolite. Significant amounts of N-acetylated in vivo metabolites have been recovered after the administration of mescaline (Schreiber, 1970), glipizide (Fuccella et al., 1973; Goldaniga et al., 1973), α-(−)-methadol, an active metabolite of (+)-methadone (Sullivan et al., 1973), and hydrazine (Testa and Jenner, 1976). The major in vivo rat metabolites of nitrazepam (7-nitro-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one) and its 1-*N*-methyl derivative nimetazepam (1-methyl-7-nitro-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one) resulted from the reduction of the 7-nitro group to the corresponding amine and subsequent conversion of the amine to the *N*-acetyl derivative (Yanagi et al., 1975). A major metabolite of hydralazine (1-hydrazinophthalazine) in man and other species has been considered by several workers to be the *N*-acetyl derivative. Studies with hydralazine proved the spontaneous acetylation product to be 3-methyl-*s*-triazolo[3,4-*a*]phthalazine, a major human metabolite (Zimmer et al., 1973). This product derives from the acetylated form; the cyclization can be explained in terms of the hypothetical tautomeric form undergoing ring closure by dehydration (Hathway, 1972). Chemical evidence with 1-(aminomethyl)iso-

quinoline, an analogue of hydralazine, suggested that the cyclization was chemically rather than enzymatically mediated (the analogue produced the methylimidazole cyclization product with acetic anhydride in the presence of acid) (Zimmer et al., 1968). However, there are no previous reports of the isolation of acetamido metabolites from 2.6-dinitroaniline herbicides.

Unknowns. Unknown XV was isolated from rat urine and feces and did not cochromatograph with any available reference compound. It was more polar than the benzimidazoles; chemical tests were positive for phenolic OH. The infrared spectrum showed a strong and typical OH absorption and characteristic benzimidazole absorption bands. The mass spectrum indicated a molecular weight of 259 and the presence of an *N*-propyl group. The peak resulting from M − 29 might be either ethyl or COH loss, which is characteristic of both $ArNC_3H_7$ or phenols. From these data the structure XV is tentatively assigned. Mechanistically, it is difficult to understand how such a metabolite would be formed.

Unknown XVI was isolated from rat urine and did not cochromatograph with any of the known standards. Since a good mass spectrum could not be obtained, the metabolite should be derivatized for further mass spectral analysis. On the basis of TLC and HPLC behavior, it is quite polar and gives negative results for phenols with $FeCl_3/K_3Fe(CN)_6$ and Millon test.

Unknown XVII was isolated after acidifying the rat urine and subsequent organic solvent extraction. The methyl derivative shows a molecular ion at *m/e* 561 with *m/e* 446 and (446 + 2) ions, indicating the possible presence of sulfur. However, the compound gave negative results with the ninhydrin reagent when it was checked for possible amino acid conjugation.

Conjugation. Conjugation was analyzed by the liberation of aglycones upon incubation of the aqueous urine fraction with β-glucuronidase. TLC of the organic extract following β-glucuronidase treatment indicated the presence of metabolites III and XIII. Both metabolites were also isolated in the free form. The isolation of metabolite XIII from the aqueous fraction of rat urine as a major urinary metabolite may have resulted in part from hydrolysis of the conjugate during acid–methanol elution of the ion-exchange column. Presumably, metabolite III is conjugated through the hydroxyl group and metabolite XIII through the ring amino group. *N*-Glucuronides are not very good substrates for the β-glucuronidase used in the assay. Therefore, the incubation with that enzyme probably leads to underestimation of the quantity of the conjugate that may be present. Definitive structural characterization of the glucuronides, of course, must await analysis of the compounds themselves, rather than their aglycones.

Conclusion. In the present study, at least five different types of reactions, hydroxylation, N-dealkylation, nitro reduction, cyclization, and conjugation, are involved in the metabolism of trifluralin in rats. The possible metabolic pathways are depicted in Figure 1. Hypothetical intermediate metabolites are shown in brackets to indicate the most likely routes leading to terminal metabolites. While the routes leading to most metabolites can be explained in terms of known enzymatic reactions, the place of metabolite XV is unknown.

Further work with trifluralin metabolism should involve a bile cannulation experiment to characterize the glucuronide conjugates more completely and establish the contribution of enterohepatic circulation. Hanasono et al. (1978) have reported excretion of 41% of the dose in the bile within 48 h after administration of ethalfluralin to rats. Emmersion and Anderson (1966) have shown that approximately 12% of the administered dose was excreted in the bile within 24 h. Further work should also elucidate the structures of the unknowns XVI and XVII reported in this study.

This study has shown that the metabolism of trifluralin in rats is much more complicated than reported in the earlier studies of Emmerson and Anderson. Work on the in vitro metabolism of trifluralin in rat liver microsomal preparations and in soil has shown the presence of the several major pathways of metabolism leading to hydroxylated, dealkylated, reduced, and cyclized products such as those reported here. This study confirms and corroborates in an in vivo situation these results from other systems and extends the results of Emmerson and Anderson to show that trifluralin metabolism in mammals will yield a similar pattern of metabolism as in these other biological systems.

ACKNOWLEDGMENT

Our thanks to Judd O. Nelson for supplying certain synthetic trifluralin analogues and for helpful discussions. Part of a dissertation presented by F.Ü.E. to the Graduate School, University of Maryland, in partial fulfillment of the requirements for the Ph.D. degree. A contribution to USDA regional research project NE-115, The Metabolism and Fate of Pesticides and Their Residues in or on Agricultural Commodities. Scientific Article No. A-4136, Contribution No. 7121, of the Maryland Agricultural Experiment Station, Department of Entomology.

Registry No. I, 2077-99-8; II, 62421-58-3; III, 62421-59-4; IV, 445-66-9; V, 2078-04-8; VI, 2078-02-6; VII, 2078-01-5; VIII, 2078-06-0; IX, 55702-44-8; X, 51026-15-4; XI, 98858-39-0; XII, 98858-40-3; XIII, 98858-41-4; XIV, 53901-40-9; XV, 98858-43-6; Pr_2NH, 142-84-7; [*trifluoromethyl*-^{14}C]trifluralin, 95768-52-8; α,α,α-trifluoro-2,6-dinitro-1-chloro-*p*-toluidine [*trifluoromethyl*-^{14}C], 98858-42-5; trifluralin, 1582-09-8.

LITERATURE CITED

Brimfield, A. A.; Street, J. C.; Futrell, J.; Chatfield, D. A. *Pestic. Biochem. Physiol.* **1978**, *9*, 84.

Emmerson, J. L.; Anderson, R. C.; *Toxicol. Appl. Pharmacol.* **1966**, *9*, 84.

Fales, H. M.; Jaouni, T. M.; Babashak, F. F. *Anal. Chem.* **1973**, *45*, 2302.

Fisher, D. L.; St. John, L. E., Jr.; Gutenmann, W.; Wagner, D.; Lisk, D. *J. Dairy Sci.* **1965**, *48*, 1711.

Fuccella, L. M.; Tamassia, V.; Valzelli, G. *J. Clin. Pharmacol.* **1973**, *13*, 68.

Golab, T.; Althaus, W. A.; Wooten, H. L. *J. Agric. Food Chem.* **1979**, *27*, 163.

Golab, T.; Herberg, R. J.; Day, E. W.; Raun, A. P.; Holzer, F. J.; Probst, G. W. *J. Agric. Food Chem.* **1969**, *17*, 576.

Golab, T.; Herberg, R. J.; Gramlich, J. V.; Raun, A. P.; Probst, G. W. *J. Agric. Food Chem.* **1970**, *18*, 838.

Goldaniga, G. C.; Maraone, C.; Pianezzola, E.; Valzelli, G.; Ambrogi, V. *Arzneim.-Forsch.* **1973**, *23*, 242.

Gutenmann, W. H.; Lisk, D. J. *J. Dairy Sci.* **1970**, *53*, 1289.

Hall, R. C.; Giam, C.-S. *J. Agric. Food Chem.* **1972**, *20*, 546.

Hanasono, G. K.; Moss, C. L. *Toxicol. Appl. Pharmacol.* **1979**, *48*, A7.

Hanasono, G. K.; Occolowitz, J. L.; Wolen, R. L. *Toxicol. Appl. Pharmacol.* **1978**, *45*, 321 (Abstract).

Hathway, D. E. In "Foreign Compound Metabolism in Mammals"; Hathway, D. E., Senior Reporter; The Chemical Society: London, 1972; Vol. 2, p 179.

Heck, H. A.; Dyer, R. L.; Scott, A. C.; Anbar, M. *J. Agric. Food Chem.* **1977**, *25*, 901.

Kearney, P. C.; Plimmer, J. R.; Wheeler, W. B.; Kontson, A. *Pestic. Biochem. Physiol.* **1976**, *6*, 229.

Kennedy, G. L., Jr.; Keplinger, M. L.; Fancher, O. E.; Stone, J. D.; Calandra, J. C. *Toxicol. Appl. Pharmacol.* **1975**, *33*, 341.

Leitis, E.; Crosby, D. G. *J. Agric. Food Chem.* **1974**, *22*, 842.
Lyle, R. E.; LaMattina, J. L. *J. Org. Chem.* **1975**, *40*, 438.
Nelson, J. O.; Kearney, P. C.; Plimmer, J. R.; Menzer, R. E. *Pestic. Biochem. Physiol.* **1977**, *7*, 73.
Opdycke. J. C.; Miller, R. W.; Menzer, R. E. *J. Agric. Food Chem.* **1982**, *30*, 1223.
O'Sullivan, D. G. *J. Chem. Soc.* **1960**, 3278.
Poole, C. F. In "Handbook of Derivatives for Chromatography"; Blau, K., King, G. S., Eds.; Heyden and Son: London, 1977; p 152.
Schreiber, E. C. *Annu. Rev. Pharamcol.* **1970**, *10*, 77.
Sullivan, H. R.; Due, S. L.; McMahon, R. E. *Res. Commun. Chem. Pathol. Pharmacol.* **1973**, *6*, 1072.
Testa, B.; Jenner, P. "Drug Metabolism: Chemical and Biochemical Aspects"; Marcel Dekker: New York, 1976; p 315.
Waring, R. H.; Pheasant, A. E. *Xenobiotica* **1976**, *6*, 262.
Williams, R. T. "Detoxification Mechanisms", 2nd ed.; Wiley: New York, 1959; p 428.
Williams, P. P.; Feil, V. J. *J. Agric. Food Chem.* **1971**, *19*, 1198.
Yanagi, Y.; Haga, F.; Endo, M.; Kitagawa, S. *Xenobiotica* **1975**, *5*, 245.
Zimmer, H.; Glasgow, D. G.; McClanahan, M.; Novinson, T. *Tetrahedron Lett.* **1968**, 2805.
Zimmer, H.; Kokosa, J.; Garteiz, D. A. *Arzneim.-Forsch.* **1973**, *23*, 1028.

Received for review April 8, 1985. Accepted August 27, 1985. Contents of this publication do not necessarily reflect the views and policies of the University of Maryland, nor does mention of trade names or commercial products constitute their endorsement or recommendation for use.

Metabolism of Isouron [3-(5-*tert*-Butyl-3-isoxazolyl)-1,1-dimethylurea]: Rapid Conversion of the Herbicide to Less Phytotoxic Metabolites in a Resistant Plant, Sugarcane

Masakatsu Ishizuka,* Yutaka Kondo, Takenao Nagare, and Yasuyoshi Takeuchi

The metabolism of isouron [3-(5-*tert*-butyl-3-isoxazolyl)-1,1-dimethylurea] in a resistant plant, sugarcane, and inhibitory activities of isouron and its metabolites on photosynthetic O_2 evolution in *Chlorella* were investigated. [^{14}C]Isouron was applied in a 10^{-4} M solution to the plants through the roots for 4 h. After a 168-h metabolic period unchanged isouron comprised 22.7% of the total radioactivity found in the plants, and the following metabolites were formed by N-demethylation and hydroxylation of *tert*-butyl group: monomethylurea derivative [3-(5-*tert*-butyl-3-isoxazolyl)-1-methylurea] (6.4%); (hydroxymethyl)urea derivative [3-(5-*tert*-butyl-3-isoxazolyl)-1-(hydroxymethyl)urea] (2.4%); urea derivative [3-(5-*tert*-butyl-3-isoxazolyl)urea] (2.0%); (hydroxy-*tert*-butyl)dimethylurea derivative [3-[5-(1,1-dimethyl-2-hydroxyethyl)-3-isoxazolyl]-1,1-dimethylurea] (0.7%); (hydroxy-*tert*-butyl)monomethylurea derivative [3-[5-(1,1-dimethyl-2-hydroxyethyl)-3-isoxazolyl]-1-methylurea] (1.5%); (hydroxy-*tert*-butyl)urea derivative [3-[5-(1,1-dimethyl-2-hydroxyethyl)-3-isoxazolyl]urea] (14.3%). Monomethylurea derivative had an inhibitory activity about half that of isouron on photosynthetic O_2 evolution, but the other metabolites had no significant activities. Furthermore, isouron was converted to more polar metabolites, which were tentatively identified as O-glucosides of 3-(5-*tert*-butyl-3-isoxazolyl)-1-(hydroxymethyl)-1-methylurea (10.5%) and (hydroxymethyl)urea (9.1%) and (hydroxy-*tert*-butyl)urea (4.2%) derivatives.

INTRODUCTION

Isouron is an isoxazolylurea herbicide for controlling total vegetation in noncroplands (Ito et al., 1979) and for selective weed control in sugarcane fields (Yukinaga et al., 1979a). As isouron inhibits the Hill reaction in isolated spinach chloroplasts, the primary site of its action has been suggested to be in the photosynthetic electron-transport system (Yukinaga et al., 1979b). We previously reported that the major metabolic pathways of the herbicide in a susceptible plant, bean, were N-demethylation and hydroxylation of the *tert*-butyl group, followed by glucose conjugation (Ishizuka et al., 1982a). It was found that, in bean plants, the rate of isouron metabolism was slow; much of the isouron absorbed by the plant remained unchanged, and the major metabolite was the first demethylation product, the monomethylurea derivative. Since in many cases differences in the rate of metabolism of a herbicide among different plant species cause a selective action of the chemical (Jensen, 1982; Jacobson and Shimabukuro, 1984), a relative insensitivity of sugarcane plant to isouron may be derived from its rapid conversion in the plant to metabolites that are less active on photosynthetic electron transport.

The purpose of this study, therefore, is to establish the pathway and rate of isouron metabolism in a resistant plant, sugarcane, and to determine the inhibitory activities of isouron and its metabolites on photosynthetic O_2 evolution in *Chlorella*. A preliminary account of this work has been given earlier (Ishizuka et al., 1982b).

MATERIALS AND METHODS

Chemicals. Isouron and its related compounds were synthesized at Shionogi Research Laboratories, Fukushima-ku, Osaka, Japan. [^{14}C]Isouron labeled at the 5-position of the isoxazole ring was also prepared at the laboratories and had a specific activity of 8.28 mCi/mmol. Its radiochemical purity as determined by thin-layer chromatography (TLC) was greater than 98.5%.

Plant Materials. Vegetative stalk propagule pieces of sugarcane (*Saccharum officinarum* L. cv. NCo-310) were surface sterilized with a 0.1% (w/v) sodium hypochlorite solution for 15 min, washed with tap water, and planted in moist sand. The seedlings sprouted were grown for 3

Aburahi Laboratories, Shionogi Research Laboratories, Shionogi & Company, Limited, Koka-cho, Shiga 520-34, Japan.

0021-8561/85/1433-1070$01.50/0