# Study of the Absorption, Excretion, Metabolism, and Residues in Tissues in Rats Treated with Carbon-14-Labeled Pendimethalin, PROWL Herbicide

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The metabolism of the dinitroaniline herbicide pendimethalin, PROWL herbicide [*N*-(1-ethylpropyl)-3,4-dimethyl-2,6-dinitrobenzenamine], was investigated in rats by using [$^{14}$C]-4-methyl-labeled pendimethalin and [$^{14}$C]-*N*-1-ethyl-labeled pendimethalin. Rats excreted radioactivity rapidly both in urine and especially in feces at two dosage levels, 7.3 and 37 mg/kg, of [$^{14}$C]-4-methyl-labeled pendimethalin. After 96 h, the residues were less than 0.3 ppm in all tissues except fat, which had a residue of 0.9 ppm. The major metabolic routes of pendimethalin involved hydroxylation of the 4-methyl and the *N*-1-ethyl group, oxidation of these alkyl groups to carboxylic acids, nitro reduction, cyclization, and conjugation based on the identification of the 12 metabolites in urine and the tissues. Products of cyclization reactions giving methylbenzimidazolecarboxylic acids were unique to liver and kidney. All the major metabolites retained both $^{14}$C labels of pendimethalin, indicating that N-dealkylation of the isopentyl group of pendimethalin was not significant. Metabolites with one or both nitro groups of pendimethalin reduced and with one or both nitro groups of 3,4-dimethyl-2,6-dinitrobenzenamine reduced were not detected in the urine, feces, and tissues.

PROWL herbicide, pendimethalin [*N*-(1-ethylpropyl)-3,4-dimethyl-2,6-dinitrobenzenamine], is a substituted dinitroaniline herbicide that is effective against a wide spectrum of annual broadleaf weeds and grasses. It is a soil-applied herbicide manufactured by American Cyanamid Co. and is currently registered for use on a variety of agronomic crops and horticultural crops of economic importance. Pendimethalin acts by inhibiting early seedling development of susceptible species.

As part of the development effort in the evaluation of the safety of pendimethalin for food-producing crops, metabolism studies were conducted in soil, in plants, and in the goat. Metabolism studies were also conducted in the rat as a model for mammalian metabolism to aid in the assessment of safety to the consumer regarding the use of this chemical for food-producing crops.

Although the metabolism of other dinitroaniline herbicides, of which trifluralin is a prominent member, has been studied in rats (Probst and Tepe, 1969), these studies have only provided a detailed and comprehensive identification of the metabolites in the urine and feces. The object of the present paper is to elucidate the metabolism of pendimethalin in rats with special emphasis on identification of the metabolites in tissues as well as in urine. The results of the metabolism of pendimethalin in soil, plants, and the goat have not been published to date.

## MATERIALS AND METHODS

**Radiolabeled Pendimethalin.** Pendimethalin labeled with carbon-14 in the 4-methyl position was prepared from 3,4-dimethylbenzenamine [$^{14}$C]4-methyl (Amersham-Searle, Arlington Heights, IL) as described by Ziegler (1972). After purification by preparative chromatography using Quanta/Gram silica gel plates (Quantum Industries, Fairfield, NJ) developed with 1,2-dichloroethene, the [$^{14}$C]-4-methyl-labeled pendimethalin had a radiochemical purity greater than 98%, as determined by two-dimensional TLC [benzene versus heptane/triethylamine (95:5 v/v)] and specific activity of 4.3 mCi/mmol [MW = 281.3]. 3-Amino[2-$^{14}$C]pentane (ICN Chemical & Radioisotope Division, Irvine, CA) was used to prepare [$^{14}$C]-*N*-1-ethyl-labeled pendimethalin with a specific activity of 3.6 mCi/mmol and a radiochemical purity greater than 98% (Ziegler, 1974).

**Synthesis of Reference Compounds.** Table I shows the structures of the reference compounds that were prepared as potential metabolites of pendimethalin. Compounds I–XIV, XXVII–XXIX, and XXXI–XXXIII were synthesized in milligram quantities by refluxing the appropriate chloro compound with the desired amine in toluene with an acid receptor, such as triethylamine (method 1), or in 80% 1-propanol with sodium carbonate (method 2). The reaction product was isolated, purified by preparative thick-layer chromatography, and derivatized with diazomethane prior to mass spectral analysis to support the chemical structure.

Compounds XIX, XXI–XXV, and XXVII were synthesized by chemical reduction using sodium sulfide nonahydrate and sodium bicarbonate, or zinc dust and ammonium chloride or stannous chloride in ethanolic hydrogen chloride. The acetylated compounds XX and XXVI were prepared by heating the corresponding reduction product with acetic anhydride and triethylamine. The benzimidazoles XXXIV–XXXVIII were synthesized by heating the corresponding amino compound with acetic acid in the presence of 4 N hydrochloric acid. Compound XXIX, and the compounds corresponding to the monoamino and diamino derivatives of pendimethalin ($R_3$ = $NH_2$, $R_4$ = $NO_2$; $R_3$ = $NO_2$, $R_4$ = $NH_2$; or $R_3$ = $R_4$ = $NH_2$) and of 3,4-dimethyl-2,6-dinitrobenzenamine (Y = $NH_2$; $R_3$ = $NO_2$, $R_4$ = $NH_2$; or $R_3$ = $NH_2$, $R_4$ = $NO_2$, and $R_3$ = $R_4$ = $NH_2$) were prepared as described by Diehl and Trotto (1974).

The glycine conjugates XV–XVIII and XXXIX were prepared by treating the corresponding acid with tertiary butyl glycinate in tetrahydrofuran in the presence of dicyclohexylcarbodiimide followed by hydrolysis of the ester with trifluoroacetic acid.

The synthetic products were cochromatographed on TLC plates with the radiometabolites derived from the rat metabolism studies. Those compounds that were identified in urine

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**Table I. Chemical Structures of Pendimethalin Reference Compounds**[a]

| compd | structure | R1 | R2 | R3 | R4 | Y |
|---|---|---|---|---|---|---|
| pendimethalin | A | $CH_3$ | $CH_3$ | $NO_2$ | $NO_2$ | $NHCH(C_2H_5)_2$ |
| I | A | $CH_3$ | $CH_2OH$ | $NO_2$ | $NO_2$ | $NHCH(C_2H_5)_2$ |
| II | A | $CH_3$ | COOH | $NO_2$ | $NO_2$ | $NHCH(C_2H_5)_2$ |
| III | A | $CH_3$ | $CH_3$ | $NO_2$ | $NO_2$ | $NHCH(C_2H_5)CH_2CH_2OH$ |
| IV | A | $CH_3$ | $CH_3$ | $NO_2$ | $NO_2$ | $NHCH(C_2H_5)CH(OH)CH_3$ |
| V | A | $CH_3$ | $CH_3$ | $NO_2$ | $NO_2$ | $NHCH(C_2H_5)CH_2COOH$ |
| VI | A | $CH_3$ | $CH_2OH$ | $NO_2$ | $NO_2$ | $NHCH(C_2H_5)CH_2CH_2OH$ |
| VII | A | $CH_3$ | $CH_2OH$ | $NO_2$ | $NO_2$ | $NHCH(C_2H_5)CH(OH)CH_3$ |
| VIII | A | $CH_3$ | $CH_2OH$ | $NO_2$ | $NO_2$ | $NHCH(C_2H_5)CH_2COOH$ |
| IX | A | $CH_3$ | COOH | $NO_2$ | $NO_2$ | $NHCH(C_2H_5)CH_2CH_2OH$ |
| X | A | $CH_3$ | COOH | $NO_2$ | $NO_2$ | $NHCH(C_2H_5)CH(OH)CH_3$ |
| XI | A | $CH_3$ | COOH | $NO_2$ | $NO_2$ | $NHCH(C_2H_5)CH_2COOH$ |
| XII | A | $CH_3$ | $CH_3$ | $NO_2$ | $NO_2$ | $NHCH(C_2H_5)COOH$ |
| XIII | A | $CH_3$ | $CH_2OH$ | $NO_2$ | $NO_2$ | $NHCH(C_2H_5)COOH$ |
| XIV | A | $CH_3$ | COOH | $NO_2$ | $NO_2$ | $NHCH(C_2H_5)COOH$ |
| XV | A | $CH_3$ | $COHNCH_2COOH$ | $NO_2$ | $NO_2$ | $NHCH(C_2H_5)_2$ |
| XVI | A | $CH_3$ | $CONHCH_2COOH$ | $NO_2$ | $NO_2$ | $NH_2$ |
| XVII | A | $CH_3$ | $CONHCH_2COOH$ | $NO_2$ | $NO_2$ | $NHCH(C_2H_5)CH_2CH_2OH$ |
| XVIII | A | $CH_3$ | $CONHCH_2COOH$ | $NO_2$ | $NO_2$ | $NHCH(C_2H_5)CH(OH)CH_3$ |
| XIX | A | $CH_3$ | COOH | $NH_2$ | $NO_2$ | $NHCH(C_2H_5)_2$ |
| XX | A | $CH_3$ | COOH | $NHCOCH_3$ | $NO_2$ | $NHCH(C_2H_5)_2$ |
| XXI | A | $CH_3$ | $CH_2OH$ | $NH_2$ | $NO_2$ | $NHCH(C_2H_5)_2$ |
| XXII | A | $CH_3$ | COOH | $NH_2$ | $NO_2$ | $NHCH(C_2H_5)CH_2CH_2OH$ |
| XXIII | A | $CH_3$ | COOH | $NH_2$ | $NO_2$ | $NHCH(C_2H_5)CH(OH)CH_3$ |
| XXIV[b] | A | $CH_3$ | COOH | $NH_2$ | $NO_2$ | $NHCH(C_2H_5)CH_2COOH$ |
| XXV | A | $CH_3$ | COOH | $NH_2$ | $NH_2$ | $NHCH(C_2H_5)_2$ |
| XXVI | A | $CH_3$ | COOH | $NHCOCH_3$ | $NHCOCH_3$ | $NHCH(C_2H_5)_2$ |
| XXVII | A | $CH_3$ | COOH | $NO_2$ | $NH_2$ | $NHCH(C_2H_5)_2$ |
| XXVIII | A | $CH_3$ | COOH | $NO_2$ | $NO_2$ | $NH_2$ |
| XXIX | A | $CH_3$ | $CH_2OH$ | $NO_2$ | $NO_2$ | $NH_2$ |
| XXX | A | $CH_2OH$ | $CH_3$ | $NO_2$ | $NO_2$ | $NHCH(C_2H_5)_2$ |
| XXXI | A | COOH | $CH_3$ | $NO_2$ | $NO_2$ | $NHCH(C_2H_5)_2$ |
| XXXII | A | COOH | $CH_3$ | $NO_2$ | $NO_2$ | $NHCH(C_2H_5)CH(OH)CH_3$ |
| XXXIII | A | $CH_3$ | $CH_3$ | $NO_2$ | $NO_2$ | OH |
| XXXIV | B | $CH_3$ | $CH_3$ | | $NO_2$ | $CH(C_2H_5)_2$ |
| XXXV | B | $CH_3$ | $CH_2OH$ | | $NO_2$ | $CH(C_2H_5)_2$ |
| XXXVI | B | $CH_3$ | COOH | | $NO_2$ | $CH(C_2H_5)_2$ |
| XXXVII | B | $CH_3$ | COOH | | $NO_2$ | $CH(C_2H_5)CH_2CH_2OH$ |
| XXXVIII | B | $CH_3$ | COOH | | $NO_2$ | $CH(C_2H_5)CH(OH)CH_3$ |
| XXXIX | B | $CH_3$ | $CONHCH_2COOH$ | | $NO_2$ | $CH(C_2H_5)_2$ |

[a] Reference compounds were cochromatographed with urine, extracts of tissues, blood, fat, and feces in either TLC system, I, II, or III. Compounds identified as metabolites of pendimethalin are shown in Table II. [b] Isolated as a lactam.

and in tissues as metabolites of pendimethalin were resynthesized in amounts sufficient for toxicological testing.

The chemical structures of all standards identified as metabolites were confirmed by obtaining their infrared spectra on a Perkin-Elmer Infracord or on a Model 421 Perkin-Elmer spectrometer equipped with KRS-5 plates and a multiple internal reflectance attachment. Nuclear magnetic resonance spectra were obtained on a Varian A-60, or on a AM 500 Bruker NMR spectrometer in deuterated solvents using tetramethylsilane internal standard. Mass spectra were obtained on a Finnigan Model 1015C combined GC/MS spectrometer. Chemical ionization mass spectral analysis of the metabolites was performed on a Finnigan Model 1015C combined GC/MS spectrometer using isobutane as the reagent gas, and electron impact mass spectral analysis at 70 eV was performed on a CEC 2100B high-resolution mass spectrometer. Melting points were determined on a Thomas-Hoover melting point apparatus.

**4-[(1-Ethyl-2-hydroxypropyl)amino]-2-methyl-3,5-dinitrobenzoic Acid (X).** A mixture of 4-chloro-2-methyl-3,5-dinitrobenzoic acid (0.1 g, 0.4 mmol) and 3-amino-2-pentanol ethanedioate (0.08 g, 0.4 mmol) in 20 mL of toluene and 2 mL of triethylamine was stirred magnetically and heated to reflux overnight (method 1). The reaction mixture was cooled, diluted with 50 mL of water, acidified, and extracted with chloroform. The chloroform was removed in vacuo to afford a yellow solid. The product was separated into four yellow components by TLC on a Quanta/Gram thick-layer silica gel plate using chloroform/acetone/acetic acid (120:60:20). The yellow compound at $R_f$ 6–7 (scale of 1–10) was isolated, and after derivatization with diazomethane, it was identified as the methyl ester of X: CI/MS, $m/z$ 342, (M + H); EI/MS, $m/z$ 296 (M − 45). In the subsequent chemical synthesis of X for toxicological testing (Bullock, 1978), two diastereomeric forms were separated by fractional crystallization. One isomer was recrystallized from acetonitrile (mp 193–195 °C): $^1H$ NMR ($\delta$, DMSO-$d_6$) 0.87 (3 H, t), 1.01 (3 H, d), 1.36–1.51 (1 H, m), 1.56–1.61 (1 H, m), 2.44 (3 H, s), 3.07 (1 H, m), 3.67 (1 H, m), 4.99 (1 H, br), 8.12 (1 H, d), 8.73 (1 H, s). The mother liquor was concentrated and recrystallized from toluene to afford the second isomer (mp 136–137 °C): $^1H$ NMR ($\delta$, DMSO-$d_6$) 0.82 (3 H, t), 1.03 (3 H, d), 1.33–1.49 (1 H, m), 1.56–1.68 (1 H, m), 2.44 (3 H, s), 2.95 (1 H, m), 3.82 (1 H, m), 5.29 (1 H, br), 8.63 (1 H, d), 8.75 (1 H, s).

**4-[[1-(Carboxymethyl)propyl]amino]-2-methyl-3,5-dinitrobenzoic Acid (XI).** A mixture of 4-chloro-2-methyl-3,5-dinitrobenzoic acid (0.05 g, 0.21 mmol), 3-aminopentanoic acid (0.05 g, 0.43 mmol), and sodium carbonate (0.21 g, 2 mmol) in 20 mL of 80% 1-propanol was heated to reflux overnight (method 2). The reaction mixture was cooled, acidified, and extracted with chloroform. The chloroform extract was concentrated in vacuo to afford a yellow solid. The product was separated into three components by TLC on a Quanta/Gram thick-layer plate developed 15 cm with the solvent system benzene/dioxane/acetic acid (180:60:2). The yellow compound at $R_f$ 4.2–5.5 (scale of 1–10) was isolated, and after derivatization with diazomethane, it was identified as the methyl ester of XI: CI/MS, $m/z$ 370 (M + H); EI/MS, $m/z$ 369, 340 (M – 29), 269 (M – 73). The sample of XI prepared for toxicological testing (Trotto, 1973), after recrystallization from acetone/water, had mp 200–203 °C: $^1$H NMR (δ, DMSO-$d_6$) 0.83 (3 H, t), 1.60 (2 H, m), 2.44 (3 H, s), 2.45–2.61 (2 H, m), 3.43 (1 H, m), 7.88 (1 H, d), 8.65 (s, 1 H).

**5-Amino-4-[(1-ethylpropyl)amino]-2-methyl-3-nitrobenzoic Acid (XIX).** To compound II (18.6 g, 0.06 mol), dissolved in ethanol (300 mL) and warmed to 40 °C, was added a hot solution of sodium sulfide nonahydrate (40 g, 0.16 mol) and sodium bicarbonate (28 g, 0.33 mol) in 150 mL of water. The mixture was stirred mechanically for 30 h at 80 °C and then poured onto 500 g of crushed ice and acidified. The solid was collected by suction filtration, washed with water until the wash was neutral to litmus paper, and dried in a vacuum oven at 90 °C. The product (15.3 g, 91% yield) had mp 172–176 °C: $^1$H NMR (δ, $CDCl_3$) 0.91 (6 H, t), 1.40 (4 H, m), 2.44 (3 H, s), 3.25 (1 H, m), 4.40 (3 H, br), 7.50 (1 H, s); CI/MS, $m/z$ (rel intensity) 282 (M + H, 100), 212 (5); IR (Nujol) 3500, 1700, 1570, 1520 cm$^{-1}$.

**5-(Acetylamino)-4-[(1-ethylpropyl)amino]-2-methyl-3-nitrobenzoic Acid (XX).** To compound XIX (2.8 g, 10 mmol) was added 20 mL of acetic anhydride and 15 mL of triethylamine. The mixture was stirred at ambient temperature for 48 h. Water was added to destroy the excess acetic anhydride. Recrystallization from methanol afforded the product with mp 175–77 °C: $^1$H NMR (δ, $CDCl_3$) 0.91 (6 H, t), 1.42 (4 H, m), 2.20 (3 H, s), 2.50 (3 H, s), 2.90–3.10 (1 H, m), 4.50–5.50 (1 H, br), 7.57 (1 H, s), 8.72 (1 H, br s); CI/MS, $m/z$ (rel intensity) 352 (2), 325 (30), 324 (M + H, 100), 306 (30), 294 (2) 254 (6).

**1-(1-Ethylpropyl)-2,6-dimethyl-7-nitro-1*H*-benzimidazole-5-carboxylic Acid (XXXVI).** Compound XIX (8.4 g, 0.03 mol), 100 mL of 4 N hydrochloric acid, and 50 mL of glacial acetic acid were stirred and heated to reflux for 12 h. The solution was cooled, diluted with 200 mL of water, and extracted with ethyl acetate (3 × 150 mL). The extracts were combined, dried (anhydrous sodium sulfate), and filtered. The filtrate was removed in vacuo to afford a solid. Recrystallization from methanol gave mp 233–235 °C: $^1$H NMR (δ, $CDCl_3$) 0.84 (6 H, t), 1.97 (4 H, m), 2.70 (3 H, s), 2.93 (3 H, s), 3.92 (1 H, m), 8.75 (1 H, s), 10.0 (1 H, br); CI/MS, $m/z$ (rel intensity) 362 (<1), 344 (2), 306 (M + H, 100), 262 (2), 236 (3).

**3,5-Diamino-4-[(1-ethylpropyl)amino]-2-methylbenzoic Acid (XXV).** To compound II (9.3 g, 0.03 mol), dissolved in 120 mL of methanol, was added ammonium chloride (4.4 g, 0.084 mol) in 50 mL of water followed by zinc dust (25.6 g, 0.41 mol) in small portions. The reaction mixture was stirred mechanically and heated (70 °C) for 5 h. The reaction mixture was filtered to remove the zinc, and the filtrate was concentrated to yield a brown solid. After trituration with hot methanol, the solid had mp 214–217 °C: CI/MS, $m/z$ (rel intensity) 252 (M + H, 100), 208 (32), 206 (30), 138 (40).

**Treatment of Rats.** In metabolism experiment 1, 11 male rats, Royal Hart Wistar strain (Charles River Breeding Laboratories, Wilmington, MA), were given a single oral dose of 5.5 mg (85.5 μCi) of [$^{14}$C]-4-methyl-labeled pendimethalin in corn oil equal to 37 mg/kg of body weight. The animals in this treatment group were divided into five groups of two (except group F with one animal). In experiment 2, three male rats of the same strain were given a single oral dose of 1.1 mg (17.5 μCi) of [$^{14}$C]-4-methyl-labeled pendimethalin in corn oil equal to 7.3 mg/kg of body weight to determine if there were any dose-related differences in the excretion, metabolism, and tissue residues. On the basis of published dietary-intake data for rats, the dosage rates used in experiments 1 and 2 correspond to dietary pendimethalin levels of 445 and 88 ppm, respectively.

In experiment 3, 38 Royal Hart Wistar strain male rats were given a single oral dose of 4.45 mg (20.9 μCi) of a 1:3 mixture of [$^{14}$C]-4-methyl-labeled and unlabeled pendimethalin equal to 35.6 mg/kg of body weight. In experiment 4, five male rats of the same strain were given a single oral dose of 4.0 mg (51.2 μCi) of the [$^{14}$C]-*N*-1-ethyl-labeled pendimethalin equal to 30 mg/kg of body weight. The purpose of these two experiments was to compare the metabolite profile in the urine, liver, and kidney derived from the two $^{14}$C tracers and to provide a large quantity of liver and kidney for metabolite identification.

**Collection of Samples.** In the experiments described above, the animals were housed in individual stainless steel metabolism cages (Acme Metal Products, Inc., Chicago, IL) that allowed for separate collection of urine and feces. Food (Purina Laboratory Chow, Ralston Purina Co., St. Louis, MO) and water were provided ad libitum. The urine samples from experiments 1 and 2 were collected in Dewar flasks filled with dry ice at the time intervals given in Tables III and IV, respectively. The feces samples were collected daily and refrigerated. At the conclusion of experiments 1 and 2, the cages were thoroughly rinsed with a 2:1 mixture of ethanol/water, and the rinse mixture for each experiment was assayed for radioactivity. At time of sacrifice, blood, liver, kidney, muscle, and fat were removed (Tables V and VI). The various soft tissues were rinsed with water and blotted dry with paper towels. Similar tissues within each group were pooled for analysis. In addition, in experiment 1 the gastrointestinal tract and a 3-cm × 3-cm portion of skin were removed from each rat in each group and analyzed for radioactivity. In experiments 3 and 4, the rats were sacrificed 6 h after treatment. Only the urine, liver, and kidney were collected from each animal for analysis.

**Preparation of Samples for Analysis.** The samples of tissues, feces, and gastrointestinal tract (experiments 1 and 2) were homogenized with water (5–10 mL/g) and freeze-dried. Blood was lyophilized directly. The lyophilized samples were assayed for radioactivity (Tables V and VI). The lyophilized samples from experiment 1 were extracted three times with hot ethanol. Fat was extracted for 8 h with benzene (20 mL/g). The extracts were assayed for radioactivity (Table VIII) followed by TLC. The radioactivity recovered in the benzene extract of fat was partitioned between hexane and acetonitrile followed by TLC of the acetonitrile extract.

Samples of control feces, and tissues were fortified with [$^{14}$C]-4-methyl-labeled pendimethalin, and the fortified samples were processed as described for the treated samples. The water collected from the lyophilization of the feces and tissues from experiment 1 was assayed for radioactivity.

**Radioassay Procedures.** Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instruments Inc., Downers Grove, IL) or in a Model 6880 Searle Mark III liquid scintillation system (Searle Analytic, Inc., Des Plaines, IL). Appropriate aliquots of the urine and other liquid samples were counted directly in DAM-611 scintillator solution. The lyophilized samples and postextracted solids were analyzed by combustion analysis.

Radioactive spots on thin-layer chromatograms were located by means of autoradiography using professional grade photographic X-ray film. For purposes of quantitation, each radioactive spot was scraped from the TLC plate and placed into a scintillation vial for direct counting after addition of 10.0 mL of Hydromix scintillation cocktail and 4.0 mL of water.

**Isolation of Metabolites in Urine and Liver.** Urine from rats from experiment 1 was extracted five times with 50-mL portions of chloroform followed by 50-mL portions of ethyl acetate. The extracts were dried over anhydrous magnesium sulfate and filtered. Aliquots of the organic and aqueous phases were assayed for radioactivity. Urine from the 6-h sampling was acidified to pH 2 and extracted, urine from the 12-h sampling was extracted at pH 5, and urine from the 24-h sampling was extracted at pH 10 (Table VII). The ethyl acetate extract of the 12-h urine from experiment 1 was chromatographed on silica gel plates developed with chloroform/acetone/acetic acid (180:90:30). The metabolites were isolated and rechromato-

graphed on silica gel plates developed with chloroform/methanol/water (225:66:9). Control rat urine was fortified with [$^{14}$C]-4-methyl-labeled pendimethalin and extracted as described for the urine samples from the treated rats.

Similar tissues within each treatment group (experiments 3 and 4) were pooled, homogenized with water (3–5 mL/g of tissue), lyophilized, and extracted with ethanol three times (10 mL/g). To isolate the metabolites, the rat liver extracts were concentrated in vacuo and the residual radioactivity was dissolved in 30–50 mL of water, acidified to pH 2 with 30% sulfuric acid and extracted with ethyl acetate. The extract and the aqueous phase were analyzed by liquid scintillation counting and TLC. For further cleanup, the extract was concentrated in vacuo, redissolved in 5% sodium hydroxide, and extracted with chloroform followed by ethyl acetate. The aqueous phase contained the radioactivity of interest and was acidified with 30% sulfuric acid and extracted with ethyl acetate to recover the rat liver metabolites. Metabolites from experiment 3 were derivatized with diazomethane and separated by chromatography on a silica gel column. Metabolites from experiment 4 were separated by TLC.

**Chromatographic Procedures.** Precoated silica gel 60 F-254 glass plates (EM Laboratories Inc., Elmsford, NY) were used for thin-layer chromatography. Aliquots of the radioactive extracts were chromatographed two-dimensionally on 20 cm × 20 cm plates scribed for 15-cm development in each dimension. System I [chloroform/methanol/water (225:66:9) versus chloroform/acetone/acetic acid (180:90:30)] and system II [ethyl acetate/1-propanol/water/formic acid (120:200:60:20) versus ethyl acetate/1-propanol/water/ammonium hydroxide (120:200:60:20)] were routinely used in the TLC of urine and extracts of tissues and blood. System III [xylene/chloroform/methanol (280:70:7) versus 1,2-dichloroethene/carbon tetrachloride/nitromethane (210:70:70)] was used for TLC of the extracts of fat and feces. System IV [chloroform/methanol (294:6) versus ether/methanol (270:30)] was used for TLC of the diazomethane derivatized metabolite isolates.

The diazomethane-treated sample of the rat liver metabolites from experiment 3 was chromatographed on a Lobar Size B silica gel 60 prepacked column (EM Laboratories) using 2% methanol in chloroform. Fractions were collected at 20-min intervals at a flow rate of 0.5 mL/min. The column eluate was monitored at 254 nM. A 50-μL aliquot of each fraction was assayed by liquid scintillation counting and by two-dimensional TLC in system IV followed by autoradiography. The various column fractions containing the metabolites of interest were separated and purified by TLC using chloroform/methanol (294:6) or ether/methanol (270:30). The TLC isolates were analyzed by chemical ionization mass spectrometry.

The rat liver metabolites from experiment 4 were separated by TLC using chloroform/acetone/acetic acid (180:90:30). The TLC isolates were purified by HPLC on two 4.6 mm × 25 cm stainless steel columns packed with 5-μm Whatman Partisil silica gel using 10% methanol in chloroform as the mobile phase. The column eluate was monitored for radioactivity in a flow analyzer. HPLC fractions were collected at 5-min intervals. The isolates from the HPLC were analyzed further by TLC in system I and by chemical ionization mass spectrometry. The HPLC isolates were also derivatized with diazomethane and chromatographed on silica gel plates in system IV for comparison to the sample of the diazomethane derivatized metabolites isolated from experiment 3. The radiometabolites were dissolved in a minimum amount of methanol and derivatized with diazomethane prepared from *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine.

**Analysis of Conjugates.** Urine (6 h, experiment 1) was incubated with mollusk β-glucuronidase in 0.07 M sodium acetate buffer at pH 5.0. As controls, the urine was incubated with buffer only, with buffer, enzyme, and phenophthalein glucuronide, and with buffer and phenophthalein glucuronide only. After overnight incubation at 37 °C, the samples were analyzed by TLC in system II.

**Hydrolysis of Metabolites.** The polar urinary metabolites were heated with 3 N hydrochloric acid for 3 h in an oil bath maintained at 120 °C. The sample was cooled and evaporated to dryness, and the residual radioactivity was taken up in a small amount of methanol and analyzed by TLC. In another hydrolysis experiment, designed to determine if the urinary radiometabolites could be decarboxylated, a 0.5-mL aliquot (0.22 μCi) of the rat urine from experiment 1 was heated with 5 mL of 65% sulfuric acid for 16 h in an oil bath maintained at 120 °C. The evolved gases were purged from the hydrolysis flask with compressed air into two scrubber towers containing 75 mL of ethanolamine. The ethanolamine solution was diluted to 100 mL with methanol and assayed for radioactivity. The hydrolysate was extracted with chloroform followed by ethyl acetate. Radioactivity in the extracts and in the aqueous phase was analyzed by TLC in system I. As a control, a 0.5-mL aliquot of the urine was also heated with 5 mL of distilled water as described above.

In other experiments, designed to determine the stability of pendimethalin toward acid hydrolysis and to determine if the liver metabolites could be decarboxylated, the [$^{14}$C]-4-methyl-labeled pendimethalin and the rat liver extract (6 h, experiment 3) were heated separately with 5.5 mL of 47% sulfuric acid for 6 h in an oil bath maintained at 120 °C, and any evolved radioactivity was collected in ethanolamine. The hydrolysates were extracted with ethyl acetate, and the radioactivity in the extracts and in the aqueous phase was analyzed further by TLC. As a control, a comparable aliquot of each sample was heated with 5 mL of water and treated as described above.

**Toxicity of Pendimethalin and Its Metabolites.** Oral $LD_{50}$ values were determined in female albino mice. Pendimethalin and the metabolites listed in Table II were fed via stomach tube as 1-mL corn oil suspensions to each mouse. In the $LD_{50}$ region, five replicates were used. The mortality counts were taken 24 h after treatment and the $LD_{50}$ values are given in Table II.

## RESULTS AND DISCUSSION

**Excretion and Recovery of Administered Radioactivity.** Table III shows the excretion of the radioactivity in urine and feces following treatment of rats with [$^{14}$C]-4-methyl-labeled pendimethalin (experiment 1) at a single oral dosage level of 37 mg/kg of body weight. A high percentage of the administered dose (90.3%) was excreted within 24 h, wherein approximately 70.6% was excreted in the feces and 19.7% was excreted in the urine. The recovery of the administered dose was 97%.

Table IV shows the excretion of the radioactivity in the urine and feces following treatment with [$^{14}$C]-4-methyl-labeled pendimethalin (experiment 2) at a single oral dosage level of 7.3 mg/kg of body weight. The peak excretion in the feces was later at the higher dose. The peak excretion of the radioactivity was at 12 h in the urine from the high and low doses. The high percentage of recovery of radioactivity within 24 h in the low dose (101%) and within 96 h in the high dose (97%) indicated that metabolism of [$^{14}$C]-4-methyl-labeled pendimethalin to respiratory [$^{14}$C]carbon dioxide was insignificant.

Tables V and VI show the residual radioactivity in selected tissues, expressed as parts per million equivalents of pendimethalin, at the various times after treatment from rats dosed with 37 and 7.3 mg/kg of [$^{14}$C]-4-methyl-labeled pendimethalin, respectively. The highest residues were attained at 6 h in liver and kidney and at 12 h in fat from the high and low doses. The higher residue levels in the liver and kidney presumably result from the effectiveness of these tissues in removing pendimethalin and its closely related metabolites from blood. At the two dosage levels tested, the rats were able to rapidly eliminate the radioactivity from the liver, kidney, and muscle within 24 h. After 96 h, the $^{14}$C residues in all tissues were less than 0.3 ppm, except for fat which had a residue of 0.9 ppm (Table V). The retention of residues in the fat may be due to the lipophilic natue of pendimethalin or its metabolites. The total recovery of pendimethalin

**Table II. Relative Amounts of Pendimethalin and Metabolites in Urine and Extracts of Tissues and Blood**

| | % found in | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| compd | urine,[a] expt 1 | urine,[b] expt 3 | liver,[b] expt 1 | liver,[b] expt 3 | kidney,[b] expt 1 | muscle,[b] expt 1 | fat,[c] expt 1 | blood,[b] expt 1 | $LD_{50}$, mg/kg |
| pendimethalin | 0.4 | 0.1 | 0.3 | 1.2 | 8.8 | 28.5 | 80.9 | 2.8 | 1340 |
| I | 0.3 | 0.1 | 0.4 | 1.7 | 1.1 | 4.3 | 4.2 | 2.7 | 2140 |
| II | 2.0 | 1.0 | 0.6 | 8.9 | 5.4 | 32.2 | 5.3 | 41.0 | 1440 |
| IV | 0.3 | 5.1 | ND[d] | ND | ND | ND | 0.8 | ND | NM[d] |
| VIII | ND | 0.9 | ND | ND | ND | ND | ND | ND | NM |
| IX | 1.0 | 2.2 | ND | ND | ND | ND | ND | ND | 2330 |
| X | 30.0 | 16.5 | 5.0 | 14.9 | 6.0 | 23.5 | ND | 25.2 | 1650 |
| XI | 14.4 | 10.1 | ND | 1.1 | 1.1 | 1.2 | ND | 2.2 | >5000 |
| XX | ND | 1.4 | ND | ND | ND | ND | ND | ND | NM |
| XXVIII | 1.0 | 1.0 | ND | ND | ND | ND | ND | ND | NM |
| XXXV | ND | ND | ND | 1.3 | NM | ND | ND | ND | NM |
| XXXVI | ND | ND | NM | 16.9 | NM | ND | ND | ND | 2872 |
| XXXVIII | NM | 1.3 | NM | 18.1 | NM | ND | ND | ND | NM |
| nonmig + origin | ND | 29.4[e] | 65.8 | 91[f] | 47.8 | 4.2 | ND | 3.9 | |
| unident mig | 50.6 | 30.9[g] | 27.9 | 26.3[g] | 29.8[g] | 6.1 | ND | 22.2 | |
| total | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 91.2 | 100.0 | |

[a] Based on TLC in system II [ethyl acetate/1-propanol/formic acid/water (120:200:20:60) versus ethyl acetate/1-propanol/ammonium hydroxide/water (120:200:20:60)]. [b] Based on TLC in system I [chloroform/methanol/water (225:66:9) versus chloroform/acetone/acetic acid (180:90:30)]. [c] Based on TLC in system III [xylene/chloroform/methanol (280:70:7) versus 1,2-dichloroethene/carbon tetrachloride/nitromethane (210:70:70)]. [d] ND, not detected in urine; NM, not measured. [e] Products released by acid hydrolysis were I, II, IV, and X. [f] Products released by acid hydrolysis were XXXVI and XXXVIII. [g] No one metabolite accounted for more than 3% of the mixture; all retained both carbon-14 labels of pendimethalin, and all were derivatized with diazomethane.

**Table III. Elimination of Radiocarbon in the Urine and Feces of Rats following a Single Oral Dose of 37 mg/kg of [$^{14}$C]-4-Methyl-Labeled Pendimethalin (Experiment 1)**

| | % of administered dose | | |
|---|---|---|---|
| h after treatment | urine | feces | cum % of dose |
| 0–6 | 2.7 | 0.9 | 3.6 |
| 6–12 | 11.2 | 23.1 | 37.8 |
| 12–24 | 5.8 | 46.6 | 90.3 |
| 24–48 | 0.9 | 3.7 | 94.9 |
| 48–72 | 0.2 | 0.4 | 95.5 |
| 72–96 | 0.1 | 0.2 | 95.8 |
| recovered in urine | | | 20.9 |
| recovered in feces | | | 74.9 |
| cage rinse | | | 1.2 |
| total recovered | | | 97.0 |

**Table IV. Elimination of Radiocarbon in the Urine and Feces of Rats following a Single Oral Dose of 7.3 mg/kg of [$^{14}$C]-4-Methyl-Labeled Pendimethalin (Experiment 2)**

| | % of administered dose | | |
|---|---|---|---|
| h after treatment | urine | feces | cum % of dose |
| 0–6 | 1.1 | 9.0 | 10.1 |
| 6–12 | 12.3 | 43.0 | 65.4 |
| 12–24 | 8.4 | 26.0 | 99.8 |
| recovered in urine | | | 21.8 |
| recovered in feces | | | 78.0 |
| cage rinse | | | 1.2 |
| total recovered | | | 101 |

**Table V. Residual Radioactivity in Tissues of Rats following a Single Oral Dose of 37 mg/kg of [$^{14}$C]-4-Methyl-Labeled Pendimethalin, Expressed as Parts per Million Equivalents of Pendimethalin**

| | group (withdrawal time, h) | | | | | |
|---|---|---|---|---|---|---|
| tissue | A (6) | B (12) | C (24) | D (48) | E (72) | F (96) |
| liver | 29.8 | 11.3 | 2.6 | 1.2 | 0.8 | 0.3 |
| kidney | 16.9 | 8.0 | 1.3 | 0.8 | 0.5 | 0.3 |
| muscle | 1.3 | 1.0 | 0.2 | 0.08 | 0.07 | 0.05 |
| fat[a] | 12.2 | 12.6 | 4.9 | 2.1 | 1.6 | 0.9 |
| skin | 4.2 | 3.9 | 1.5 | 0.4 | 0.4 | 0.2 |
| blood | 5.4 | 3.9 | 0.4 | 0.3 | 0.3 | 0.01 |
| gastrointestinal tract | 206 | 111 | 4.7 | 0.6 | 0.2 | 0.1 |

[a] Includes the sum of the extractable and unextractable radioactivity.

**Table VI. Residual Radioactivity in Tissues of Rats following a Single Oral Dose of 7.3 mg/kg of [$^{14}$C]-4-Methyl-Labeled Pendimethalin, Expressed as Parts per Million Equivalents of Pendimethalin**

| | group (withdrawal time, h) | | |
|---|---|---|---|
| tissue | A (6) | B (12) | C (24) |
| liver | 4.4 | 1.6 | 0.4 |
| kidney | 5.9 | 1.7 | 0.3 |
| muscle | 0.39 | 0.87 | 0.1 |
| fat[a] | 1.1 | 1.3 | 0.8 |
| blood | 0.2 | 0.4 | 0.2 |

[a] Includes the sum of the extractable and unextractable radioactivity.

equivalents in the tissues analyzed amounted to 6.05% of the administered dose at 37 mg/kg and 5.25% at 7.3 mg/kg.

This study was designed to elucidate the nature of the metabolites in the tissues resulting from a single oral dose of pendimethalin to mammals and to define quantitatively their residues in tissues and the depletion kinetics of these residues. The measurement of the residual radioactivity in tissues of rats indicates whether any tissues serve as repositories for residues. This information can then be useful for designing residual analytical studies for the parent compound and its significant metabolites under realistic field conditions.

**Characterization of Radioactivity in Urine.** TLC of the rat urine in system I (Figure 1) and system II (Figure 2) showed that pendimethalin was extensively metabolized by the rat. However, N-dealkylation of pendimethalin by the rat was minimal since the urinary radiometabolite profiles derived from the [$^{14}$C]-4-methyl-labeled pendimethalin and the [$^{14}$C]-*N*-1-ethyl-labeled pendimethalin, respectively, were qualitatively similar as shown in Figure 1.

A qualitative and quantitative characterization of the urinary radioactivity from experiment 1 was undertaken before the isolation and identification of the metabolites was performed. The results of the solvent partitioning of the urinary radioactivity between chloroform and water and ethyl acetate and water at pH 2, pH 5, and pH 10 are summarized in Table VII. At pH 2, 75% of the urinary

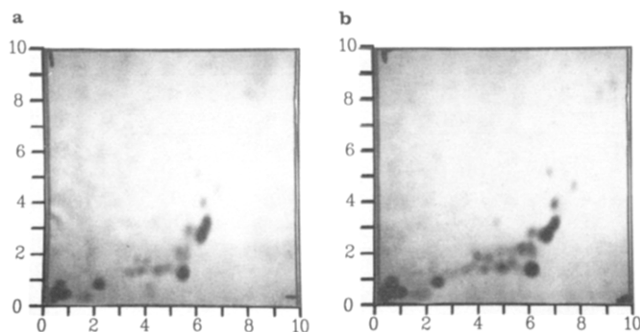


**Figure 1.** Two-dimensional TLC in system I of 6-h urine from rats receiving (a) [$^{14}$C]-4-methyl-labeled pendimethalin (experiment 3) and (b) [$^{14}$C]-*N*-1-ethyl-labeled pendimethalin (experiment 4).

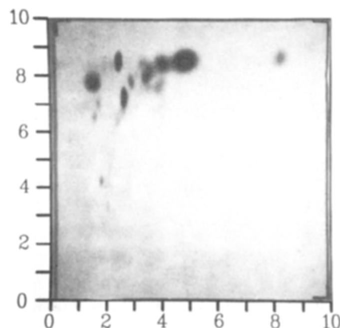


**Figure 2.** Two-dimensional TLC in system II of 6-h urine from rats receiving [$^{14}$C]-4-methyl-labeled pendimethalin (experiment 1).

**Table VII. Percentage Distribution of Radioactivity following Solvent Partitioning of Urine Samples from Rats Treated with [$^{14}$C]-4-Methyl-Labeled Pendimethalin (Experiment 1)**

| | | % of total recovered as | |
|---|---|---|---|
| h after treatment | pH | organosoluble fraction[a] | aqueous fraction |
| 6 | 2 | 75 | 25 |
| 12 | 5 | 35 | 65 |
| 24 | 10 | 6 | 94 |

[a] Represents chloroform and ethyl acetate soluble fractions.

radioactivity was extracted from the urine versus only 6% at pH 10. TLC of the various extracts revealed that pendimethalin and most of the radiometabolites were in the organosoluble fractions recovered at pH 2 or pH 5. Metabolite XI and some unidentified minor metabolites remained in the water-soluble fraction derived from the extractions of the urine at pH 2 and pH 5. Essentially all of the pendimethalin metabolites remained in the aqueous phase at pH 10, which suggested that the metabolites were probably carboxylic acids. Control rat urine was fortified with [$^{14}$C]-4-methyl-labeled pendimethalin and extracted. TLC of the extract showed that pendimethalin was stable.

The metabolites recovered in the organosoluble extract at pH 5 were converted to less polar products after derivatization with diazomethane. This confirmed the metabolites as being carboxylic acids. No significant glucuronide or sulfate conjugates were detected in 6-h urine on the basis of the results of the enzyme hydrolysis of the pendimethalin metabolites.

When the 12-h rat urine was heated (120 °C) with 65% sulfuric acid, 62% of the radioactivity was released and trapped in ethanolamine, presumably as $^{14}CO_2$. When heated with water, less than 1% of the radioactivity was trapped in ethanolamine. TLC of the organosoluble extracts of the two hydrolysis samples showed that a number of metabolites including II and X were absent from the hydrolysate with 65% sulfuric acid. This indicated that a major portion of the pendimethalin metabolites had been decarboxylated. The release of $^{14}CO_2$ suggested that the metabolites were compounds wherein the [$^{14}$C]-4-methyl group of pendimethalin had been metabolized to a carboxylic acid. No significant amount of radioactivity was released and trapped in ethanolamine when [$^{14}$C]-4-methyl-labeled pendimethalin was heated (120 °C) with 47% sulfuric acid. However, some N-dealkylation of pendimethalin to yield 3,4-dimethyl-2,6-dinitrobenzenamine had occurred.

**Thin-Layer Chromatography.** The two-dimensional TLC system I of chloroform/methanol/water (225:66:9) versus chloroform/acetone/acetic acid (180:90:30) provided a good separation of the pendimethalin reference compounds (Table I) with closely related chemical structures and the pendimethalin metabolites that were of intermediate polarity. Therefore, this TLC system was routinely used to screen the reference compounds as potential metabolites of pendimethalin by cochromatography with the urinary radioactivity, metabolite isolates, and tissue extracts.

By TLC in system I, 29.4% of the urinary radiometabolite mixture (Figure 1) either did not migrate or was poorly resolved (Table II). However, system II [ethyl acetate/1-propanol/formic acid/water (120:200:20:60) versus ethyl acetate/1-propanol/ammonium hydroxide/water (120:200:20:60)] was very effective in resolving the polar, urinary pendimethalin metabolites and left no radioactivity at or near the spotting origin of the TLC plate (Figure 2). Quantitation of the radiometabolite mixture (experiment 1) in TLC system II showed there was a minimum of 16 metabolites in the 0–6-h urine of which 3 metabolites (II, X, and XI) accounted for 46.4% of the radioactivity (Table II). There was very little pendimethalin (0.4%) in the urine.

Results from experiment 3 showed that metabolites IV, X, and XI accounted for 31.7% of the radioactivity in urine. Quantitative differences between metabolite concentrations in the urine were attributed to animal-to-animal variation in ability to metabolize pendimethalin in experiments 1 and 3.

TLC system II was not as effective as system I for separating the pendimethalin reference compounds of close chemical structure. However, TLC system III [xylene/chloroform/methanol (280:70:7) versus 1,2-dichloroethene/carbon tetrachloride/nitromethane (210:70:70)] was very effective in separating 3-amino, 5-amino, and 3,5-diamino derivatives of pendimethalin, 3-amino, 5-amino, and 3,5-diamino derivatives of the 3,4-dimethyl-2,6-dinitrobenzenamine (Diehl and Trotto, 1974), and the alcohol derivatives of pendimethalin (I, III, IV, VI, and VII). Essentially all of the urinary radioactivity remained at the spotting origin by TLC in system III.

**Isolation and Identification of Metabolites in Urine.** On the basis of the metabolism studies reported in the literature (Probst and Tepe, 1969) on other dinitroaniline herbicides, viz, trifluralin, it was anticipated that a major pathway for the metabolism of pendimethalin by the rat would be through reduction of the nitro groups and dealkylation of the *N*-alkyl group. However, the reduction products of pendimethalin or of 3,4-dimethyl-2,6-dinitrobenzenamine were not detected as terminal metabolites in the rat urine, which indicated the rat was metabolizing pendimethalin by some other major pathway.

Miyamoto (1970) has shown that 3,4-dimethylphenyl

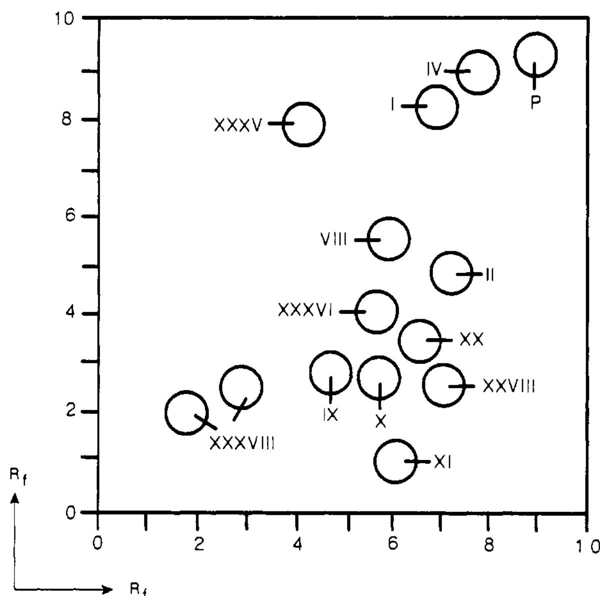


**Figure 3.** Idealized map of two-dimensional TLC $R_f$ values of pendimethalin and metabolites in [chloroform/methanol/water (225:66:9) versus chloroform/acetone/acetic acid (180:90:30)], system I.

*N*-methylcarbamate is metabolized in rats mainly through oxidation of the methyl group(s) attached to the benzene ring. Pendimethalin is an unsymmetrically substituted dinitroaniline and is different from trifluralin, which is a symmetrically substituted dinitroaniline. A number of compounds could be readily postulated as metabolites on the basis of metabolism of pendimethalin by the rat through pathways involving a combination of oxidation of the methyl group(s) and the *N*-alkyl group coupled with reduction of one or both nitro groups and conjugates thereof. Table I lists the compounds that were synthesized as potential metabolites of pendimethalin on the basis of these proposals. Of the compounds listed in Table I, 10 compounds were identified by TLC cochromatography with the urinary radiometabolite mixture as shown in Table II. Figure 3 shows a composite map of the two-dimensional TLC $R_f$ coordinates of these compounds in system I.

The ethyl acetate extract of the urine at pH 2 was resolved into 16 discrete radioactive bands by TLC in chloroform/acetone/acetic acid (180:90:30). Metabolite X, a major urinary metabolite, appeared as a yellow compound on the silica plate. The yellow color indicated that the nitro groups of the metabolite were not reduced. The metabolite was isolated and separated into two components by TLC using chloroform/methanol/water (225:66:9). The two components were isolated and derivatized with diazomethane. Chemical ionization mass spectral analysis of both derivatives gave a (M + H) ion at $m/z$ 342 and a secondary ion at 255. Since the mass spectral analysis of the methyl ester derivative of II gave an (M + H) ion at $m/z$ 326 and a secondary ion at 255 and both derivatives of metabolite X gave a (M + H) ion that was 16 amu higher than the parent ion for the methyl ester of II, it is suggested that the two components of metabolite X were hydroxylated forms of II. The hydroxyl group in these components of metabolite X resided in the *N*-(1-ethylpropyl) group on the basis of the observation that both isolates and II gave secondary ions at 255 which correspond to the ion from fragmentation and loss of the *N*-(1-ethylpropyl) group.

Compound IX and compound X, which differ only in the location of the hydroxyl group in the *N*-(1-ethylpropyl) group, were synthesized for TLC comparison with the metabolite isolates. In the synthesis of X, a mixture of two compounds was obtained. The compounds were identical by TLC to metabolite X, which sometimes, but not always, could be partially resolved by TLC in system I. The components of the synthetic mixture were separated by TLC and purified. Chemical ionization mass spectral analysis of each synthetic component, after derivatization with diazomethane, gave a (M + H) ion at $m/z$ 342. The data indicated that the *dl*-threo and *dl*-erythro isomer of X had been synthesized and separated. The two components of metabolite X, the major metabolite of pendimethalin in rat urine, were diastereomers of X. This constitutes the first report of the isolation and identification of diastereomeric metabolites from the metabolism of a dinitroaniline compound in rats. Compound IX was subsequently identified as a minor metabolite by TLC.

In the subsequent resynthesis of X for toxicological testing (Bullock, 1978), the two diastereomers were separated by fractional crystallization. One diastereomer had mp 193–195 °C, and the other diastereomer had mp 136–137 °C. The mixture of the two diastereomers of X was used for toxicological testing. Although no assignment of structure could be made for the diastereomers of X based on proton NMR analysis, it was observed that the isomer with the higher melting point had the greater $R_f$ on a silica gel plate developed with chloroform/acetone/acetic acid (180:90:30). Palamareva and Kurtev (1977) reported the TLC $R_f$ values for a series of 1,2-disubstituted 1,2-diarylethanes and showed that $R_f$ erythro > $R_f$ threo. Therefore, it is suggested that the isomer with mp 193–195 °C and greater $R_f$ might be the erythro isomer of X. However, further analyses are needed to unequivocally determine if this proposal is correct.

Chemical ionization mass spectral analysis of a minor metabolite, as the methyl ester derivative, gave a (M + H) ion at $m/z$ 256. This metabolite was identified as XXVIII. By TLC, XI was identified as a major metabolite, and II, IV, VIII, XX, and XXXVIII were identified as minor metabolites (Table II) in rat urine. These results showed that pendimethalin was metabolized in rat mainly through hydroxylation and oxidation of the methyl group(s) and the *N*-alkyl group. There were very few N-dealkylated metabolites on the basis of TLC comparison of the metabolites derived from the two $^{14}C$-labeled versions of pendimethalin. Reduction of II with zinc dust in the presence of ammonium chloride yielded the diamino compound XXV. Compound XXV was not a urinary metabolite of pendimethalin. Acetylation of XXV yielded the diacetyl compound XXVI, which was very similar by TLC, but not exactly coincident, with one of the polar unidentified urinary metabolites. The 3-amino compound XXVII, obtained from the reduction of II with stannous chloride in ethanolic hydrogen chloride, the corresponding acetylated derivative, and the 5-amino compound XIX were not detected as a metabolites in the urine. The 5-(acetylamino) compound XX was a minor urinary metabolite (1.4%).

Another polar urinary metabolite was stable toward acid hydrolysis. The metabolite was isolated and purified by HPLC using the mobile phase of chloroform/methanol/acetic acid (900:80:20). Chemical ionization mass spectral analysis was unsuccessful because of the low volatility of the metabolite. Although the metabolite could be derivatized with diazomethane, mass spectral data of the derivatized metabolite were inconclusive. This metabolite

**Table VIII. Extraction of [$^{14}$C]Pendimethalin Derived Radioactivity from Tissues, Blood, and Feces of Rats following a Single Oral Dose of 37 mg/kg of [$^{14}$C]-4-Methyl-Labeled Pendimethalin**

| sample | interval, h | $^{14}$C residue | % of radioactivity extracted[a] | % of radioactivity unextracted |
|---|---|---|---|---|
| muscle | 6 | 1.3 | 92.6 | 7.4 |
| muscle | 12 | 1.0 | 91.6 | 8.4 |
| liver | 6 | 29.8 | 78.6 | 21.4 |
| liver | 12 | 11.3 | 65.1 | 34.9 |
| kidney | 6 | 16.9 | 87.0 | 13.0 |
| kidney | 12 | 8.0 | 71.1 | 28.9 |
| fat | 6 | 12.2 | 88.0 | 12.0 |
| fat | 12 | 12.6 | 93.0 | 7.0 |
| fat | 24 | 4.9 | 89.0 | 11.0 |
| blood | 6 | 5.4 | 85.8 | 14.2 |
| blood | 12 | 3.9 | 77.6 | 22.4 |
| feces | 6 | | 89.7 | 10.3 |
| feces | 12 | | 78.9 | 21.1 |

[a] Fat was extracted with benzene. All other samples were extracted with ethanol.

accounted for 2% of the radiometabolite mixture and was not identified.

**Identification of Metabolites in Feces, Tissues, and Blood.** Major portions of the $^{14}$C residues in rat feces, tissues, and blood samples from experiment 1 were extractable (Table VIII). Of the radioactivity excreted by rats in experiment 1, 90% was recovered in the feces within 24 h after treatment. Of the radioactivity excreted in the 12-h sample, 78.9% was extracted with ethanol. The major component (74%) in the extract of the 12-h feces was isolated by TLC [xylene/chloroform/methanol (280:70:7)] and identified as pendimethalin by chemical ionization mass spectral analysis. A minor fecal metabolite from the TLC was also isolated and purified by TLC. Mass spectral analysis of the metabolite isolate gave an (M + H) ion at $m/z$ 280, which was 2 amu less than pendimethalin. The metabolite was postulated to be a derivative of pendimethalin resulting from the loss of water from III or IV. The chemical structure of this minor metabolite was not verified through chemical synthesis. Additional minor fecal metabolites that were identified by TLC included II, IV, and X. These minor metabolites may have resulted from the cross contamination of the feces and urine samples during collection. The reduction products of pendimethalin and 3,4-dimethyl-2,6-dinitrobenzenamine were not detected as terminal metabolites in the extracts of the feces.

The high percentage of radioactivity excreted in the feces and identified as pendimethalin indicated that pendimethalin was incompletely absorbed from the gastrointestinal tract and passed through the rat essentially unchanged. Analysis of control feces fortified with [$^{14}$C]-4-methyl-labeled pendimethalin showed that pendimethalin was stable under the conditions used to analyze the feces from the treated rats. Less than 0.1% of the radioactivity was recovered in the water from the lyophilization of the feces samples from experiments 1 and 2.

TLC analysis of the 6- and 12-h extracts of the liver, kidney, muscle, and blood in systems I and II showed that many of the metabolites in the urine were common to those found in the tissues (Figures 4 and 5). This established the relevance of the urinary metabolites to those found in the tissues. Pendimethalin was also shown to be stable under the conditions used to analyze the treated tissues on the basis of the analysis of the control tissues fortified with [$^{14}$C]-4-methyl-labeled pendimethalin. For the tissue extracts from experiment 1, the compounds that were identified included pendimethalin, I, II, X, and XI (Table II). In 6-h muscle, pendimethalin, II, and X were identified as the major components, and these accounted for 84.2% of the $^{14}$C residue. Minor metabolites in the muscle were identified as I and XI. In 6-h blood, II and X were identified as the major components, and these accounted for 66.2% of the $^{14}$C residue. Minor compounds in the blood were identified as pendimethalin, I, and XI. The major component (80.9%) of the $^{14}$C residue in 6-h fat was identified as pendimethalin, and minor compounds were identified as I, II, and IV (Table II). The analysis of the

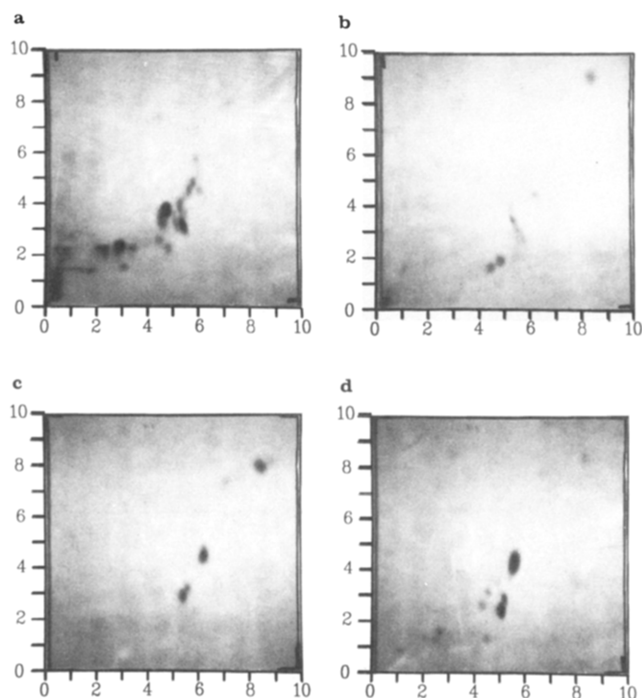


**Figure 4.** Two-dimensional TLC in system I of extracts of (a) 6-h liver, (b) 6-h kidney, (c) 6-h muscle, and (d) 6-h blood from rats receiving [$^{14}$C]-4-methyl-labeled pendimethalin (experiment 1).

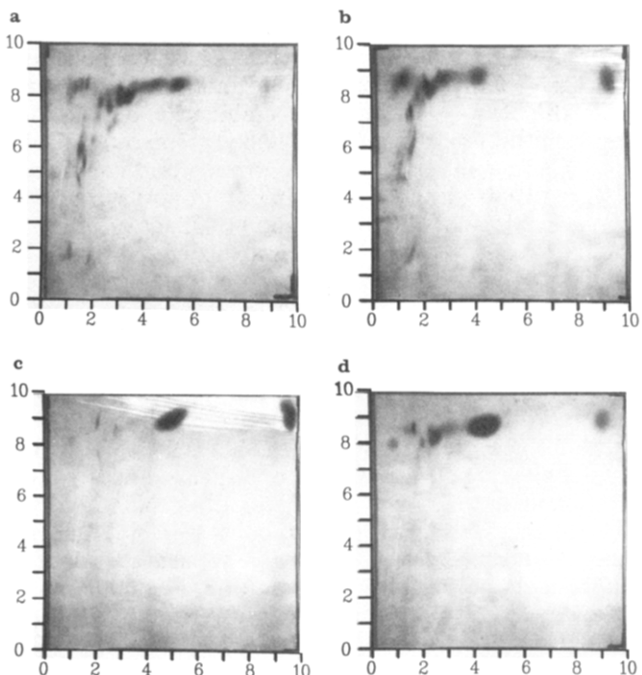


**Figure 5.** Two-dimensional TLC in system II of extracts of (a) 6-h liver, (b) 6-h kidney, (c) 6-h muscle, and (d) 6-h blood from rats receiving [$^{14}$C]-4-methyl-labeled pendimethalin (experiment 1).

12- and 24-h samples of fat showed that the amount of I decreased from 4.2% to 1.9%, while the amount of II increased from 5.3% to 9.3%.

As shown in Table VIII, 21.4–34.9% of the $^{14}C$ residue in the liver and 13.0–28.9% of the $^{14}C$ residue in kidney in the 6- and 12-h samples, respectively, was not extractable. Although the amount of unextracted residue in the liver and kidney was higher at 12 h, the total $^{14}C$ residue in these tissues was less. In 6-h liver (experiment 1), only 6.3% of the extract of the $^{14}C$ residue could be identified as pendimethalin, I, II, and X (Table II). In 6-h kidney, only 22.4% of the extractable $^{14}C$ residue could be identified as pendimethalin, I, II, X, and XI. In experiment 1, 65.8% of the extractable radioactivity from liver and 47.8% of the extractable radioactivity from kidney did not migrate from the TLC spotting origin when system I was used. For liver, another 30% of the extractable radioactivity did migrate (Figure 4a,b) and contained two additional major unidentified metabolites (XXXVI and XXXVIII) that were not quantitatively significant in urine (Figure 1a). TLC of the liver and kidney extracts in system II (Figure 5a,b) revealed the extracts contained an extensive number of metabolites.

**Isolation and Identification of Major Metabolites in Liver.** Efforts to produce the major unidentified liver metabolites XXXVI and XXXVIII by incubation of [$^{14}C$]-4-methyl-labeled pendimethalin with rat liver using the in vitro maintenance technique of Sullivan (1972) or incubation with rat liver homogenates were unsuccessful. These in vitro experiments only produced the pendimethalin metabolites identified as I–IV, VI–VIII, XXVIII, and XXIX (Table I). The formation of these metabolites in the in vitro experiment served to elucidate the oxidative pathway of pendimethalin metabolism in the rat liver. The major unidentified liver metabolites XXXVI and XXXVIII may be terminal products of in vitro metabolism of pendimethalin in the rat liver since they were not detected in the in vitro experiments nor were they observed in the urine.

In experiment 3, the large-scale in vivo rat metabolism experiment with [$^{14}C$]-4-methyl-labeled pendimethalin, the $^{14}C$ levels were 18 ppm in liver and 19.2 ppm in kidney. In experiment 4, involving [$^{14}C$]-*N*-1-ethyl-labeled pendimethalin, the average $^{14}C$ levels were 21.8 ppm in liver and 12.9 ppm in the kidney. The residue levels in the 6-h liver and 6-h kidney from experiments 3 and 4 were comparable to those found in experiment 1 (Table V). Extraction of the tissues with ethanol recovered 53% of the $^{14}C$ liver residue from experiment 3 and 85% of the $^{14}C$ liver residue from experiment 4. For kidney, 41% and 59% of the $^{14}C$ residue was extracted, respectively. TLC of the extracts showed that the metabolite profiles were comparable to those shown in Figure 3a,b. Comparison of the profiles of the metabolites in liver and kidney derived from the separate $^{14}C$ versions of pendimethalin showed that the metabolites were not fragments of pendimethalin but were simply modifications of pendimethalin analogous to the metabolites detected in urine.

The initial solvent partitioning of the extracts of the liver from experiments 3 and 4 between ethyl acetate and water at pH 2 recovered 75.9% and 69% of the radioactivity in the ethyl acetate extracts, respectively. The ethanol extracts of the kidney from experiments 3 and 4 were partitioned between chloroform and water at pH 10 followed by ethyl acetate and water at pH 3. For the kidney extract from experiment 3, 46.6% of the $^{14}C$ residue was extracted with chloroform and 26.6% was extracted with ethyl acetate. For experiment 4, 21.4% of the $^{14}C$ residue was extracted with chloroform and 40.4% was extracted with ethyl acetate. Metabolites XXXVI and XXXVIII were recovered in the ethyl acetate extracts and were characterized as carboxylic acids on the basis of their reactivity to diazomethane. When the $^{14}C$ residue in liver was heated (120 °C) with 47% sulfuric acid, only 3.6% of the radioactivity was evolved and trapped in ethanolamine versus 4.5% when heated with water. This suggested that the metabolites in the liver extract were not readily decarboxylated.

TLC in system I of the ethyl acetate extracts of the liver and the kidney showed a radiometabolite profile similar to that of the ethanol extracts of the respective tissues. Therefore, it was concluded that the metabolites in the liver and kidney extracts were not formed in the workup procedure involving extraction with ethyl acetate. The ethyl acetate extract of the metabolite mixture derived from the [$^{14}C$]-4-methyl-labeled pendimethalin (experiment 3) was repartitioned between chloroform and water at pH 10 followed by ethyl acetate and water at pH 2 for further cleanup. The ethyl acetate extract was derivatized with diazomethane and chromatographed on a silica gel column using 2% methanol in chloroform to afford several fractions of radioactivity. The major fraction of radioactivity collected between 100 and 150 mL of eluant (fraction 2) contained the methyl ester derivatives of metabolite X, the unidentified liver metabolite XXXVI, and a number of other metabolites on the basis of TLC in system IV. Metabolite XXXVI, as the methyl ester, was separated from the mixture by TLC and isolated. Chemical ionization mass spectral analysis of the isolate, as the methyl ester, gave an (M + H) ion at $m/z$ 310 and an (M + H) ion at $m/z$ 320. By high-resolution mass spectral analysis of the metabolite isolate, ions at 319.1517 ($C_{16}H_{21}N_3O_4$), 290.1098 ($C_{14}H_{16}N_3O_4$), and 232.0711 ($C_{11}H_{10}N_3O_3$) were observed. These data suggested a 2-methylbenzimidazole-5-carboxylic acid structure for metabolite XXXVI, which could form an acetylated amino precursor, either biologically, chemically on workup or upon treatment with diazomethane, or chemically during vaporization in the mass spectrometer.

Since it was possible that some secondary chemical reaction could occur due to diazomethane derivatization to alter the basic structure of the metabolite isolate, mass spectral data were sought on liver metabolite XXXVI that avoided derivatization with diazomethane to support the structure of the metabolite. The metabolite derived from the experiment with [$^{14}C$]-*N*-1-ethyl-labeled pendimethalin was isolated. The metabolite appeared radiochemically pure by TLC analysis; however, by HPLC on a Partisil silica gel column, the metabolite isolate was separated into two components (A and B) by using 10% methanol in chloroform as the mobile phase. Chemical ionization mass analysis of isolate A gave an (M + H) ion at $m/z$ 306, 14 amu less than the methylated metabolite, and an (M + H) ion at $m/z$ 324. Mass spectral analysis of isolate B gave only an (M + H) ion at $m/z$ 306. Diazomethane derivatization of A and B followed by TLC in system III showed that these were identical with the metabolite in fraction 2 from the silica gel column chromatography.

On the basis of the mass spectral data, 1-(1-ethylpropyl)-2,6-dimethyl-7-nitro-1*H*-benzimidazole-5-carboxylic acid (compound XXXVI, Table I) was proposed as the metabolite in fraction B. Compounds XX and XXXVI were proposed as the components in fraction A. However, the spectral data would also be consistent for a metabolite wherein the 3-nitro group instead of the 5-nitro group of

II was reduced by the rat to yield XXVII, followed by acetylation and cyclization.

Compound II was reduced with sodium sulfide nonahydrate and sodium carbonate to yield XIX followed by heating XIX with glacial acetic acid and 4 N hydrochloric acid to yield XXXVI. Spectral data (NMR, IR, and MS) for XXXVI confirmed the chemical structure. Mass spectral analysis of the synthetic compound gave an (M + H) ion at $m/z$ 306, identical with the metabolite in fraction B. Derivatization of XXXVI with diazomethane followed by mass spectral analysis gave an (M + H) ion at $m/z$ 320 which was identical with the spectral data for the metabolite isolate in fraction 2. Compound XXXVI was identical by TLC in system I with the major metabolite in liver. This metabolite accounted for 16.9% of the radiometabolite mixture in the extract from liver (Table III, experiment 3) and 7.1% of the radiometabolite mixture in the extract from kidney (experiment 1).

Compound XX was prepared from the acetylation of XIX. The mass spectral analysis of XX gave (M + H) ions at $m/z$ 306 and 324 which were identical with the spectral data for the HPLC isolate from fraction A. The (M + H) ion at $m/z$ 306 was assigned to XXXVI. The presence of the (M + H) ion at $m/z$ 306 was attributed to cyclization of XX when the analyte was being vaporized in the sample probe during the mass spectral analysis. Compound XX could not be converted chemically to XXXVI by treatment with diazomethane. Heating XX in xylene in the presence of $p$-toluenesulfonic acid or heating in benzene with thionyl chloride did not result in cyclization to XXXVI. The 5-(acetylamino) compound was a minor metabolite in the kidney (0.5%). The 3-amino compound XIX and the 5-amino compound XXVII were not detected in the extracts of the tissues.

In an attempt to prepare the isomer of XXXVI, the 3-amino compound XXVII was heated with acetic acid in the presence of 4 N hydrochloric acid. By TLC, the reaction product did not coincide with any of the metabolites in the liver; however, the identity of the product as the isomer of XXXVI was not fully verified.

In the metabolism of trifluralin in the rat, Erkog and Menzer (1985) reported that the mono- and diamino metabolites of trifluralin were not acetylated during ethyl acetate elution of silica gel plates or by TLC employing a solvent system containing acetic acid, nor could they detect any formation of methylbenzimidazole compounds from the mono- and diamino compounds. In the metabolism study with pendimethalin, although XIX was not detected as a metabolite of pendimethalin, the possibility that XIX could be converted to either XX or XXXVI under the conditions used to isolate the metabolites was not investigated. However, by TLC in the solvent system employing acetic acid (system I), the 5-amino compound XIX was not converted to the 5-(acetylamino) compound XX or to the cyclization product XXXVI. Although there was evidence to show that XXXVI could form from XX during chemical ionization mass spectral analysis, the formation of the cyclization product XXXVI in the metabolism of pendimethalin is most likely an in vivo process rather than an in vitro process analogous to the formation of the 2-methylbenzimidazole compounds reported in the metabolism of trifluralin. The reduction products of pendimethalin and of 3,4-dimethyl-2,6-dinitrobenzenamine were not detected as metabolites in the extracts of liver and kidney.

Fraction 3 from the column chromatography of the diazomethane derivatized liver metabolite mixture eluted between 150 and 200 mL and contained a derivatized metabolite which by chemical ionization mass spectral gave an (M + H) ion at $m/z$ 264. Fraction 4 eluted between 200 and 250 mL, and fraction 5 eluted between 250 and 300 mL. Mass spectral analysis of fractions 4 and 5 combined gave (M + H) ions at $m/z$ 280, 282, and 264. Of these ions, the $m/z$ 264 ion was the most abundant and the others were relatively small. A 2,2-diethyl-2,3-dihydro-1$H$-benzimidazole or a dehydro derivative of the methyl ester of XXVI was postulated for the $m/z$ 264 ion. A chemical synthesis of these compounds was not pursued since these were relatively minor metabolites.

Fraction 6 from the column chromatography of the derivatized liver mixture eluted between 390 and 470 mL and contained two derivatized metabolites. One of the two derivatized metabolites corresponded to the second major unidentified liver metabolite. The two derivatized metabolites were separated by TLC and isolated. By chemical ionization mass spectral analysis, one isolate gave an (M + H) ion at $m/z$ 336. The other isolate gave an (M + H) ion at $m/z$ 322. The isolate with $m/z$ 336 was 16 amu higher than the methyl ester derivative of the XXXVI. These data suggested a methyl ester derivative of a metabolite with the hydroxyl group in the $N$-alkyl side chain.

A mixture of compounds was obtained in the synthesis of XXXVIII. The mixture was separated by TLC, and two products were isolated. The spectral data for two isolates gave an (M + H) ion at $m/z$ 322, as the free carboxylic acid, and an (M + H) ion at $m/z$ 336 after derivatization of the isolates with diazomethane. The spectral data were consistent for the $dl$-threo and $dl$-erythro isomers of XXXVIII and the metabolite. Among other postulates for the $m/z$ 336 ion for the derivative of the metabolite would be the methyl ester of the benzimidazolecarboxylic acid isomer derived from reduction of the 3-nitro group instead of the 5-nitro group of X, followed by acetylation and cyclization. The 2,3-dihydro derivative of the methyl ester of XXXVI was a postulate for the metabolite isolate in fraction 6 with an (M + H) ion at $m/z$ 322. These postulated structures were not verified through chemical synthesis. The benzimidazolecarboxylic acid (XXXVII) prepared from compound IX was not a metabolite in the liver. On the bais of the spectral data, the second major metabolite in liver was characterized as a benzimidazole-5-carboxylic acid with a hydroxyl group and is most probably XXXVIII.

On the basis of the chemical synthesis and spectral data for the cyclized metabolites, 65% of the extractable liver residue and 45% of the extractable kidney residue from experiments 1 and 3 (Table II) were identified or characterized.

**Identification of Polar, Water-Soluble Radioactivity in Urine and Liver.** TLC in system I of the urinary radiometabolite mixture (experiment 3) showed 29% of the radioactivity was extremely polar and remained at or near the TLC origin. The radioactivity was isolated and hydrolyzed with 3 N hydrochloric acid to release several metabolites (I, II, IV, and X) to show there were conjugates in the urine. The glycine conjugates (Table I) were not detected as urinary metabolites. On the basis of identifications made in experiments 1 and 3, 69% of the urinary radioametabolite mixture was attributed to the compounds shown in Table II. The unidentified, migrating urinary metabolites, accounting for 30.9% of the radiometabolite mixture, were converted to less polar products after derivatization with diazomethane, which showed these unidentified metabolites were carboxylic acids. These metabolites were of minor importance since no one

**Figure 6.** Proposed metabolism scheme for pendimethalin in rats (compounds shown in brackets are probable intermediates but were not detected).

metabolite in this fraction individually accounted for more than 3% of the mixture on the basis of TLC in system II (Figure 2).

Approximately 30% of the radiometabolite mixture derived from the liver was water-soluble. This radioactivity remained at the spotting origin after TLC in system I. Hydrolysis of the water-soluble radioactivity released the two benzimidazolecarboxylic acid metabolites, XXXVI and XXXVIII, which suggested that these compounds might be present as conjugates in the liver. Hydrolysis of the polar, origin-bound radioactivity amounting to 47.8% of the extractable residue from the kidney was not attempted. It is conjectured that the radioactivity could in part also be due to conjugates. The intractable $^{14}C$ residue in the liver and kidney was not identified.

**Conclusion.** The results of the metabolism study show that pendimethalin was metabolized by the rat as depicted in Figure 6. The primary sites for metabolism of pendimethalin involve the hydroxylation and the oxidation of the *N*-alkyl group and the 4-methyl group of the aromatic ring, reduction of the 6-nitro group followed by acetylation, and ring closure to the 2-methylbenzimidazole-5-carboxylic acid compounds and conjugates thereof. Metabolites derived from the oxidation of the 4-methyl and *N*-alkyl group were predominant in urine.

The mono- and diamino reduction products of pendimethalin and 3,4-dimethyl-2,6-dinitrobenzenamine were not detected as terminal metabolites in the rat urine, feces, and extracts of tissues. By comparison, Probst and Tepe (1969) and Erkog and Menzer (1985) report that the primary metabolic route for metabolism of trifluralin in the rat was reduction and N-dealkylation of one or both alkyl groups on the basis of metabolites identified in the urine and feces. Ethylbenzimidazoles arising from cyclization reactions have been reported as in vitro metabolites of trifluralin from rat liver microsomal

incubations (Nelson et al., 1977). The 2-methylbenzimidazole-5-carboxylic acid metabolite of pendimethalin was first reported as an in vitro liver metabolite in rats (Zulalian et al., 1976). The ethyl and 2-methylbenzimidazoles have also been reported as in vivo metabolites of trifluralin in the rat (Erkog and Menzer, 1985). It was not unequivocally determined whether pendimethalin metabolites derived from the reduction of the 3-amino group followed by acetylation and cyclization to benzimidazolecarboxylic acids were in urine, liver, and kidney. If present in urine, these would constitute minor metabolites.

There was no evidence of any terminal metabolites in the urine derived from hydroxylation or oxidation of the 3-methyl group of pendimethalin in the rat to XXX or XXXI, respectively. Metabolites derived from these compounds could not be ruled out. In addition, metabolites resulting from a combination of hydroxylation and or oxidation of the *N*-alkyl groups and reduction of the nitro groups are also possible. However, if any of these metabolites were present, they would constitute minor metabolites. Since the major metabolic pathway for metabolism of pendimethalin by the rat was elucidated, the chemical synthesis of these additional compounds was not warranted, particularly when it was shown that none of the remaining unidentified metabolites of pendimethalin individually accounted for more than 3% of the radiometabolite mixture in the urine.

The relevance of utilizing the excretory products of an animal as a source of metabolites for isolation and identification depends upon assessment of the metabolite composition residing in the tissues as noted in the metabolism of robenidine in the rat (Zulalian and Gatterdam, 1973) and especially in the chicken (Zulalian et al., 1975), wherein a unique metabolite was found in the chicken liver but not in the chicken excreta. In the rat study with pendimethalin, the finding of the 2-methylbenzimidazole-5-carboxylic acid metabolites as unique metabolites in the liver and kidney, but not in the urine, again emphasizes the importance of evaluating the residual tissue metabolites in metabolism studies. This metabolism study extends the knowledge on the fate of dinitroanilines in the rat and shows that pendimethalin is metabolized by several major pathways leading to hydroxylated, oxidized, and cyclized products. It also showed the formation of diastereomeric metabolites. The metabolites of PROWL herbicide, pendimethalin, which were tested orally in female mice were less toxic than pendimethalin (Table II).

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