Excretion Balance, Metabolic Fate, and Tissue Residues following Treatment of Rats with Thidiazuron Cotton Defoliant

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Rats rapidly metabolized and eliminated radiolabeled thidiazuron (*N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea) cotton defoliant following administration as a single oral dose and as a dietary supplement. Both urine and feces were important routes for elimination of the xenobiotic; greater than 90% of the dose was accounted for in the urine and feces by 96 h following administration to rats of a single oral dose. The parent compound was detected in the feces and to a much lesser extent in the urine of rats treated with thidiazuron as a dietary supplement but was not detected in excreta from single oral dose studies. The two major metabolites were *N*-4-hydroxyphenyl-*N'*-1,2,3-thiadiazol-5-ylurea or 4-hydroxyphenylthidiazuron and phenylurea. 4-Hydroxyphenylthidiazuron was present in the urine (free and conjugated), feces, and milk. Phenylurea was detected in urine, and its formation may represent a novel cleavage for substituted ureas. Tissue levels of radiocarbon were in the parts per billion range, and there was no evidence for the formation of persistant residues in rats.

Thidiazuron (Dropp), *N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea, is a novel cotton defoliant that promotes the formation of the leaf abscission layer and the early shedding of green leaves. It inhibits energy conservation in respiration and photosynthesis, but steric hindrance by the two large aromatic rings at both nitrogens of the urea moiety apparently abolishes effective inhibition of the photosynthetic electron flow usually observed with traditional substituted urea herbicides (Hauska et al., 1975).

Thidiazuron has low acute mammalian toxicity with an oral LD_{50} of >4000 mg/kg for rats and mice (SN 49537 Experimental Cotton Defoliant. NOR-AM Agricultural Products, Inc., Technical Information Bulletin, 1976). Toxicological studies of thidiazuron are essential to discern potential hazards to mammals that might be associated with its use as a cotton defoliant. Therefore, we investigated the excretion balance, metabolic fate, and tissue residues of thidiazuron following administration to rats as a single oral dose and as a dietary supplement.

MATERIALS AND METHODS

Compounds. Two samples of thidiazuron labeled with radiocarbon at different sites were provided by NOR-AM Agricultural Products, Inc., Woodstock, Ill. Thidiazuron-aniline-^{14}C (sp act., 14.85 mCi/mmol), designated hereinafter as thidiazuron-A-^{14}C or T-A-^{14}C, was uniformly labeled in the aniline ring. Thidiazuron-thiadiazole-^{14}C (sp act., 13.1 mCi/mmol), designated hereinafter as thidiazuron-T-^{14}C or T-T-^{14}C, was radiolabeled at the thiadiazole ring carbon adjacent to the urea nitrogen. The radiochemical purity of each sample was greater than 99% as determined by TLC and autoradiography.

NOR-AM also supplied nonradioactive samples of thidiazuron, *N*-2-hydroxyphenyl-*N'*-1,2,3-thiadiazol-5-ylurea or 2-hydroxyphenylthidiazuron, *N*-3-hydroxyphenyl-*N'*-1,2,3-thiadiazol-5-ylurea or 3-hydroxyphenylthidiazuron, *N*-4-hydroxyphenyl-*N'*-1,2,3-thiadiazol-5-ylurea or 4-hydroxyphenylthidiazuron, and 5-amino-1,2,3-thiadiazole or thiadiazole amine. Phenylurea, 2-hydroxyacetanilide, 3-hydroxyacetanilide, and 4-hydroxyacetanilide were obtained from Aldrich Chemical Co., Milwaukee, Wis.; aniline, 2-aminophenol, 3-aminophenol, 4-aminophenol, and acetanilide were purchased from Fisher Scientific Co., St. Louis, Mo.

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Table I. Chromatographic Behavior of Thidiazuron and Related Compounds[a]

Compound	Average R_f values in solvent system A	B	C
Thidiazuron	0.56	0.22	0.44
2-Hydroxyphenylthidiazuron	0.56	0.18	0.30
3-Hydroxyphenylthidiazuron	0.47	0.10	0.26
4-Hydroxyphenylthidiazuron	0.42	0.09	0.25
Acetanilide	0.66	0.48	
2-Hydroxyacetanilide	0.72	0.49	
3-Hydroxyacetanilide	0.56	0.30	
4-Hydroxyacetanilide	0.43	0.22	0.31
Aniline	0.85	0.67	
2-Aminophenol	0.83	0.56	
3-Aminophenol	0.77	0.45	
4-Aminophenol	0.62	0.30	
Phenylurea	0.40	0.15	0.38
Thiadiazoleamine	0.73	0.31	

[a] TLC was carried out on glass plates coated with a 500-μm layer of silica gel GF_{254}. The following solvent systems were used in an unsaturated chamber: A = ethyl acetate; B = chloroform-ethyl acetate (1:1); and C = chloroform-methanol (9:1).

Chromatography. TLC was used for separation of thidiazuron-^{14}C and its radiocarbon-containing metabolites. Uniplate TLC plates (20 × 20 cm, Fisher Scientific Co.), precoated with a 500-μm layer of silica gel GF_{254}, were activated for 1 h at 100 °C and stored in a dessicator until required.

The material for chromatography was spotted, and the chromatogram was developed to a height of 15 cm. The chromatographic behavior of thidiazuron and related compounds is given in Table I. After TLC the plate was placed in contact with no-screen x-ray film (Eastman Kodak Co., Rochester, N.Y.) and exposed for a minimum of 10 days.

Quantitative estimation of thidiazuron and its metabolites from TLC was accomplished by scraping the silica gel from each spot corresponding to the darkened images on the film and placing it in a scintillation vial with 10 mL of scintillation cocktail. The mixture was radioassayed.

Radioisotopic Methodology. The radiocarbon content of each sample was measured on a Beckman Model LS-100C Liquid Scintillation Spectrophotometer; the counting efficiency for radiocarbon was 94.6%. The radioactive samples were placed in glass scintillation vials, and 10 mL of appropriate counting solution was added.

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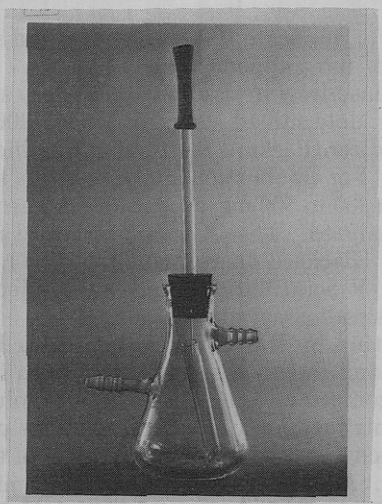

Figure 1. Apparatus used to collect milk samples from thidiazuron-treated rats.

Calculation of the amount of radioactivity in each sample was accomplished by averaging duplicate 10-min counts. All data were corrected for background, dilution, quenching, and counting efficiency.

Radiocarbon in all samples except for the $^{14}CO_2$ derived from tissues from dietary supplement studies were assayed using scintillation cocktail consisting of 1.5 L each of toluene and methyl Cellosolve, plus 2,5-diphenyloxazole (15 g) and 1,4-*bis*-2-(4-methyl-5-phenyloxazolyl)benzene (0.9 g). Permafluor V (Packard Instruments Co., Inc., Downers Grove, Ill.) scintillation cocktail was used for analysis of $^{14}CO_2$ from tissue samples of dietary supplement studies.

Experimental Animals. Male and female albino rats (Sprague-Dawley strain) were obtained from Charles River Co. (Boston, Mass.). Male rats weighing approximately 150 g each and female rats weighing approximately 260 g each (300 g when pregnant) were used in single oral dose studies of thidiazuron. Litters (7 to 14 pups) were adjusted to 10 pups/dam 1 day after birth, and pups were allowed to nurse for 10 days after birth to increase the dam's milk production to a maximum (Morag, 1970) prior to treatment with thidiazuron. Dietary supplement studies were carried out using female rats weighing approximately 200 g each. Rats were acclimated to their cages for 5 days prior to treatment.

Treating and Handling of Rats. For single oral dose studies, rats were treated orally via a stomach tube with 3 μCi of thidiazuron-^{14}C dissolved in acetone and corn oil (1:3). Three male rats were treated with thidiazuron-A-^{14}C; three male rats and four female rats were treated with thidiazuron-T-^{14}C. After treatment each rat was placed in a modified Roth metabolism chamber (one/chamber) for 96 h. Food and water were provided ad libitum.

Urine and fecal samples were collected at various time intervals after treatment and stored at −20 °C until analyzed. Tissue samples were obtained at 96-h posttreatment.

Milk samples were collected at 12-h posttreatment intervals and stored at −20 °C. To collect milk samples, female rats were injected intramuscularly with one unit of oxytocin (Pitocin, Parke-Davis) and anesthetized with an ether–air mixture blown into the metabolism cage. The rats were milked with an apparatus which was modified after Haberman (1974). The apparatus (Figure 1) consisted of a standard 125-mL suction flask that was modified by the addition of another arm. A Pasteur disposable pipet was inserted into a rubber stopper, and a 1-mL rubber bulb with the end removed served as a teat cup. A small test tube was used as the milk receptacle. A vacuum hose (water aspirator) was connected to one arm of the flask, and regulation of the pressure to the mammary gland was accomplished by application and removal of a finger to the orifice to the other arm. After collection of a 0.5–1.0-mL sample of milk, the rats were replaced in their chambers, and ten pups were allowed to feed for 1 h to complete milk extraction (Morag, 1970). Pups then were removed to separate cages.

For dietary supplement studies, the specific activity of a sample of thidiazuron-A-^{14}C and thidiazuron-T-^{14}C was adjusted to 4.0 mCi/mmol with authentic nonradioactive thidiazuron. An acetone solution of thidiazuron-^{14}C was added to standard laboratory rat chow to yield a concentration of 6.6 ppm. A Hobart mixer was used to thoroughly mix the food until radioassay of aliquots of feed demonstrated that a homogeneous mixture was attained. This thidiazuron fortified rat chow was provided ad libitum. At this concentration, animals consuming 15 g of food/day received 0.5 mg kg^{-1} day^{-1} of thidiazuron. Food cups were weighed daily to determine amounts of food consumed by the rats.

For excretion balance studies, three female rats were fed a diet containing thidiazuron-A-^{14}C and three rats were fed a diet containing thidiazuron-T-^{14}C. They were held individually in metabolism cages. After 6 days a normal nonfortified diet was resumed. Urine and feces were collected daily for 9 days after initiating treatment.

Twenty-four female rats were treated with thidiazuron-A-^{14}C and 24 rats were treated with thidiazuron-T-^{14}C as dietary supplements; they were held in groups of four/metabolism cage. Three rats from each treatment were selected at random and sacrificed every other day beginning on the first day after treatment. On day 9 the remaining rats were returned to a normal diet. Sacrificing continued every 3 days beginning on day 10. Tissue samples were removed from each rat for analysis.

Analysis of Urine. The total radioactivity in each sample was determined by radioassay of duplicate 50-μL aliquots. Urine was extracted three times with an equal volume of ethyl acetate, and the ethyl acetate extracts were combined and dried over anhydrous sodium sulfate. The ethyl acetate extract was reduced in volume to about 2 mL, and duplicate 50-μL aliquots were radioassayed. The remaining material was concentrated to 0.1 mL and subjected to TLC, autoradiography, and radioassay for quantitation of the radioactive material.

Duplicate 50-μL aliquots of the urine remaining after ethyl acetate extraction were radioassayed. Some of this material also was subjected to treatment with β-glucuronidase, aryl sulfatase, or HCl. A 0.5-mL aliquot of extracted urine was transferred to a 25-mL Erlenmeyer flask containing 4 mL of citrate–phosphate buffer (0.1 M citric acid monohydrate, 0.2 M Na_2HPO_4, pH 5.0) and 3 mg of either β-glucuronidase (15% salt form, Type B-1; bovine liver, 500 000 Fishman units/g, Sigma Chemical Co., St. Louis, Mo.) or aryl sulfatase (salt-free form, Type V: from limpets, 5–10 units/mg, Sigma Chemical Co.). To another 0.5-mL aliquot of extracted urine in buffer was added HCl to a final concentration of 9% (v/v). The control consisted of 0.5 mL of extracted urine in 4 mL of the citrate–phosphate buffer solution. After incubation in a water bath with shaking at 38 °C for 12 h the flask contents were extracted three times with equal volumes of ethyl acetate. These extracts were combined and dried over anhydrous sodium sulfate. The ethyl acetate fraction was concentrated to a volume of 2 mL and duplicate 50-μL aliquots were radioassayed. The remaining material was

concentrated to 0.1 mL and subjected to TLC, autoradiography, and radioassay. Duplicate 50-μL aliquots of the remaining water fraction also were radioassayed.

Analysis of Feces. Fecal samples from single oral dose studies were dried and ground to a powder with a mortar and pestle. The total thidiazuron-^{14}C equivalents in the feces were determined by combustion of duplicate 50-mg samples in an oxygen-purged Schöniger flask. The $^{14}CO_2$ was trapped in 6 mL of ethanolamine–methyl Cellosolve (1:2), and 3 mL of this solution were radioassayed. To gain information concerning the nature of the thidiazuron-^{14}C equivalents in the feces, some fecal samples were fractionated. The fecal powder was extracted three times with equal volumes of acetone and then three times with equal volumes of water. The acetone and water extracts were combined, and the acetone was evaporated. The remaining water extract was extracted three times with equal volumes of ethyl acetate to yield a water fraction and an ethyl acetate fraction. The water fraction was analyzed by radioassay of duplicate 50-μL aliquots. After the ethyl acetate fraction was dried over anhydrous sodium sulfate, it was reduced to a volume of 2 mL and duplicate 50-μL aliquots were radioassayed. The remaining organosoluble material was reduced to 1 mL and streaked on a TLC plate. This was subjected to chromatography, autoradiography, and radioassay. The fecal residue remaining after extraction was dried, and duplicate 50-mg samples were combusted and radioassayed.

Fecal samples from dietary supplement studies were analyzed in a similar manner to that for total radioactivity, but a more rigorous extraction procedure was employed. Fecal samples were soaked in acetone for 15 min prior to extracting five times with equal volumes of acetone and three times with water.

Analysis of Milk. The total radioactivity in milk samples was determined by direct counting of duplicate 10-μL aliquots and by oxygen flask combustion of duplicate 50-μL aliquots with subsequent counting of the trapped $^{14}CO_2$.

After determining the total amount of thidiazuron-^{14}C equivalents, milk samples were fractionated as described by Timmerman et al. (1961), except that the amounts were reduced. The milk was mixed with an equal volume of acetonitrile in a 15-mL centrifuge tube. The milk solids were precipitated and centrifuged. The acetonitrile was decanted, and the precipitate was washed once with an equal volume of acetonitrile and twice with equal volumes of chloroform. The acetonitrile, which contained the water from the milk, was mixed thoroughly with the chloroform wash and was left to stand several minutes for phase separation. The organic solvent layer was removed, and the water fraction was extracted again with a mixture of acetonitrile and chloroform. The acetonitrile and chloroform extracts were combined and dried over anhydrous sodium sulfate. The acetonitrile–chloroform mixture was evaporatd to dryness, and 5 mL of acetonitrile were added to dissolve the residue. The acetonitrile was extracted three times with equal volumes of *n*-hexane to remove lipids. The acetonitrile and hexane fractions were concentrated to 2 mL.

Duplicate 50-μL aliquots of the acetonitrile, *n*-hexane, and water fractions were radioassayed. The milk solids were radioassayed by combustion of duplicate 50-mg samples in a Schöniger flask. The acetonitrile fraction was reduced to a volume of 0.1 mL and analyzed by TLC, autoradiography, and radioassay.

Analysis of Tissues. Total radioactivity in rat tissues from single dose studies was determined by oxygen flask combustion of duplicate 100-mg samples and subsequent radioassay of the trapped $^{14}CO_2$.

Total radioactivity in tissues from dietary supplement studies was determined using a Model 306 Tri-Carb Sample Oxidizer (Packard Instrument Co., Inc., Downers Grove, Ill.). For combustion analysis, tissue sample size ranged from 300 to 500 mg, except for fat where 50 to 100 mg were analyzed. The $^{14}CO_2$ was trapped in 7 mL of Carbo-Sorb (Packard Instrument Co., Inc.), 13 mL of Permafluor V scintillation fluid was added, and the mixture was radioassayed.

Degradation by Rat Liver Subcellular Fractions. Seven grams of freshly dissected liver from female rats were homogenized in cold 0.25 M sucrose solution. Differential centrifugation yielded a microsome plus soluble fraction which was used for in vitro studies of thidiazuron metabolism. A standard 2-mL incubation mixture contained 1 mL of 0.1 M Tris–HCl buffer (pH 7.4), 2 μmol of NADPH, 1 mL of microsome plus soluble fraction, and 200 000 dpm of thidiazuron-A-^{14}C or thidiazuron-T-^{14}C dissolved in 10 μL of acetone. Controls containing all components except the enzyme preparation were analyzed simultaneously. Samples in 25-mL Erlenmeyer flasks were incubated aerobically in a water bath at 38 °C for 1 h with constant shaking. Metabolism was terminated after the incubation period by the addition of 1 mL of ethyl acetate. The contents of the flasks were extracted three times with equal volumes of ethyl acetate. The organic extracts were combined, dried over anhydrous sodium sulfate, and evaporated to 2 mL. Duplicate 50-μL aliquots of the organic and water fractions were radioassayed. The organic fraction was reduced to 0.1 mL and subjected to TLC, autoradiography, and radioassay.

Degradation by a Model Hydroxylation System. To a 25-mL Erlenmeyer flask were added: 200 000 dpm of thidiazuron-A-^{14}C or thidiazuron-T-^{14}C, 80 μmol of EDTA, 142 μmol of ascorbic acid, 15 μmol of $FeSO_4 \cdot 7H_2O$, and 3 mL of 0.1 M phosphate buffer (pH 5.5) (Udenfriend et al., 1954). Triplicated flasks and appropriate controls were incubated in air at 38 °C for 6 h in a metabolic shaker. Another 142 μmol of ascorbic acid in 0.5 mL of buffer was added after 3 h of incubation. The experiment was terminated by the addition of 1 mL of ethyl acetate. The flask contents were extracted three times with equal volumes of ethyl acetate. The organic extracts were combined, dried over anhydrous sodium sulfate, and reduced to 2 mL. Duplicate 50-μL aliquots of the organic and water fractions were radioassayed. The organic fraction was further reduced to 0.1 mL and subjected to TLC, autoradiography, and radioassay.

RESULTS AND DISCUSSION

Excretion Balance. The cumulative percentage elimination of radioactive equivalents in urine and feces of rats treated with a single oral dose of thidiazuron-^{14}C is given in Figure 2. The total recovery of administered radiocarbon in urine and feces for the 96-h period averaged 92.7% (range 91.6 to 94.9%) for the three experiments with the vast majority of the radiocarbon (90.4% average, range 88.7 to 92.6%) being excreted during the initial 48 h. Both urine and feces were important routes for elimination of thidiazuron-^{14}C equivalents. By 96 h male rats treated with thidiazuron-A-^{14}C eliminated 49.7 and 45.2% of the dose in the urine and feces, respectively; male rats treated with thidiazuron-T-^{14}C eliminated 35.7 and 55.9% in the urine and feces; and female rats treated with thidiazuron-T-^{14}C eliminated 40.8 and 50.8% in the urine and feces. The peak level of urinary radioactivity occurred during the initial 12 h after treatment, while the peak level of fecal

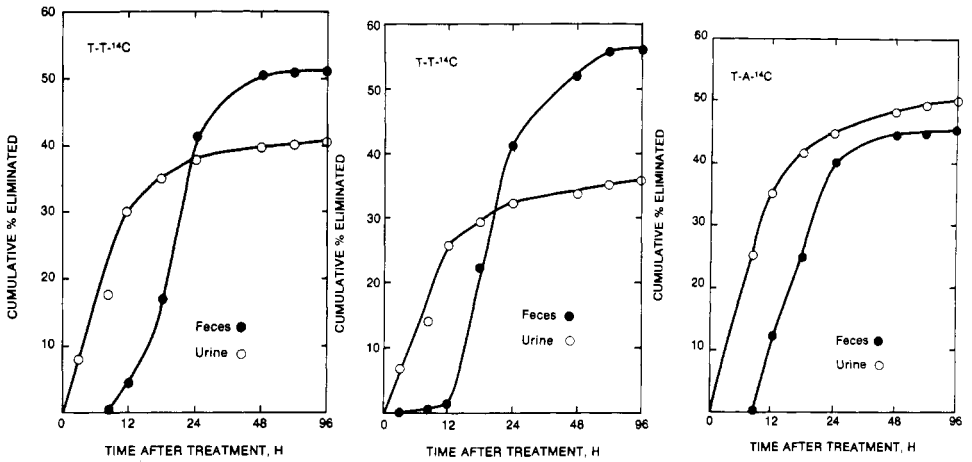

Figure 2. Cumulative percentage elimination of radioactive equivalents in urine and feces of female rats treated with a single oral dose of thidiazuron-T-^{14}C (left) and of male rats treated with a single oral dose of thidiazuron-T-^{14}C (middle) and thidiazuron-A-^{14}C (right).

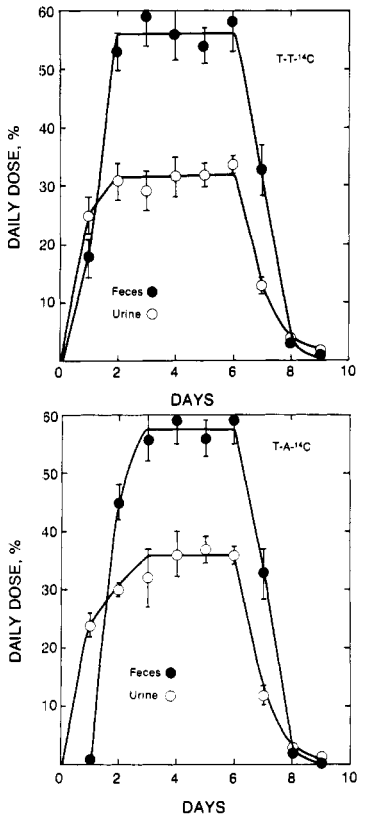

Figure 3. Elimination of radioactive equivalents in urine and feces of female rats treated with thidiazuron-T-^{14}C (upper) and thidiazuron-A-^{14}C (lower) for 6 days as a dietary supplement expressed as a percentage of the daily dose. Data are means ±SD from three rats.

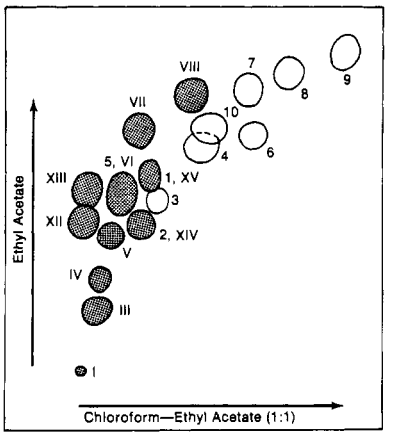

Figure 4. Diagrammatic representation of two-dimensional chromatogram obtained following TLC of organosoluble radioactive material isolated from urine of rats treated with thidiazuron-^{14}C as a dietary supplement. Roman numerals and hatched zones designate radioactive metabolites. Arabic numerals and clear zones designate authentic standards as follows: 1 = thidiazuron and 2-hydroxyphenylthidiazuron; 2 = phenylurea; 3 = 4-hydroxyacetanilide; 4 = 4-aminophenol; 5 = 3-hydroxyphenylthidiazuron and 4-hydroxyphenylthidiazuron; 6 = acetanilide; 7 = 3-aminophenol; 8 = 2-aminophenol; 9 = aniline; and 10 = thiadiazole amine. Metabolite XII was detected with thidiazuron-T-^{14}C only, and metabolite XIV was detected with thidiazuron-A-^{14}C only. Metabolites II, IX, X, and XI (Table II) were not detected in dietary supplement study.

radioactivity occurred between 12 and 24 h after treatment.

Figure 3 shows the elimination of radioactive equivalents in urine and feces of female rats treated with thidiazuron-^{14}C for 6 days as a dietary supplement. Radioactive material in both urine and feces reached a plateau between the second and third days. During the plateau stage rats eliminated about 90% of the daily dose in the urine (35%) and feces (55%). Following return of rats to untreated diet after the sixth day, thidiazuron-^{14}C equivalent in urine and feces declined rapidly. By the ninth day of the experiment or the third day on untreated food, rats treated with thidiazuron-A-^{14}C had eliminated 95.1% (37.3% urine, 57.8% feces) of the total consumed radioactive material, and rats treated with thidiazuron-T-^{14}C had eliminated 91.1% (34.5% urine, 56.6% feces) (Figure 3).

Metabolic Fate. The distribution and concentration of radioactive material in the urine of rats treated with thidiazuron-^{14}C as a single oral dose and as a dietary supplement are given in Tables II and III, respectively. For both studies the ratio of radioactive material in the organic phase to that in the aqueous phase was relatively constant at all time intervals, with 14 to 26% of the urinary radioactivity in the organic phase and 74 to 86% in the aqueous phase.

TLC of the organosoluble fraction revealed at least 15 metabolites, the majority of which obstensibly contained both ring systems, as judged by the similar chromatographic behavior of the metabolites derived from thidiazuron-T-^{14}C and thidiazuron-A-^{14}C (Tables II and III, Figure 4). The two-dimensional TLC used in the dietary supplement studies (Figure 4) indicated that several of the zones resolved by one-dimensional TLC in Table II were mixtures. For example, in solvent system A metabolite

Table II. Distribution and Concentration of Radioactive Material in the Urine of Rats Treated with a Single Oral Dose of Thidiazuron-^{14}C

Metabolite or fraction	R_f values in TLC solvent systems[a]		% urinary radioactivity at indicated sampling interval, h								
			Thidiazuron-T-^{14}C						Thidiazuron-A-^{14}C		
			Female			Male			Male		
	A	B	8-12	18-24	48-72	8-12	18-24	48-72	8-12	18-24	48-72
Organosoluble											
I	0.00	0.00	0.4	0.7	0.5	0.7	1.1	0.7	1.4	0.9	1.0
II[b]	0.10	0.02	0.2	0.1	<0.1	0.4	0.7	0.6	0.7	0.8	1.2
III	0.16		0.3	0.1	0.3						
IV[b]	0.23	0.05	0.8	1.1	1.3	3.3	2.7	2.4	2.4	2.1	2.5
V	0.37		1.8	2.0	3.9						
VI	0.47	0.08	10.4	8.8	6.6	13.4	7.2	6.7	17.0	9.8	10.1
VII	0.67	0.13	1.7	2.9	3.5	0.4	1.6	0.8	2.0	1.8	5.4
VIII	0.75	0.23	2.3	3.1	2.9	0.3	2.2	1.6	0.2	2.4	2.6
IX	0.80	0.31	0.1	<0.1	<0.1	1.3	0.2	0.5	1.6	0.8	0.7
X		0.38				<0.1	<0.1	<0.1	0.2	0.4	0.2
XI		0.16				1.2	0.6	0.3	0.9	1.1	1.7
Water soluble			82.0	81.2	81.0	79.0	83.7	86.4	73.6	79.9	74.6

[a] TLC was accomplished on glass plates coated with a 500-μm layer of silica gel GF_{254} in an unsaturated chamber; the solvents systems were ethyl acetate (A) and chloroform-ethyl acetate (1:1) (B). Solvent system A was used with female rats treated with thidiazuron-T-^{14}C. Solvent system B was used with male rats treated with thidiazuron-T-^{14}C and thidiazuron-A-^{14}C. [b] With solvent system B metabolites II and III were combined as were metabolites IV and V.

Table III. Distribution and Concentration of Radioactive Material in Urine and Feces of Female Rats Treated with Thidiazuron-^{14}C as a Dietary Supplement

Metabolite or fraction	% urinary or fecal radioactivity at indicated days											
	Urine						Feces					
	Thidiazuron-T-^{14}C			Thidiazuron-A-^{14}C			Thidiazuron-T-^{14}C			Thidiazuron-A-^{14}C		
	2	5	7	2	5	7	2	5	7	2	5	7
Organosoluble[a]												
I	0.6	0.3	0.6	0.3	0.5	0.4	1.7	0.9	0.9	1.2	0.8	1.0
III	0.2	0.1	0.3	0.2	0.2	0.2	1.2	0.9	1.5	0.9	1.1	.8
IV	0.8	0.7	1.0	1.2	1.2	2.5	0.8	1.1	0.8	0.8	1.6	2.2
V	0.6	0.5	0.5	0.6	0.4	0.6						
VI	17.4	16.7	19.4	11.9	13.5	11.2	35.7	17.0	19.4	35.9	19.5	19.0
VII	0.3	0.7	0.9	0.4	1.3	0.8						
VIII	0.6	1.1	1.5	1.6	2.3	2.2						
XII	0.4	<0.1	<0.1									
XIII	0.3	<0.1	<0.1	0.2	0.1	0.2						
XIV				8.4	4.7	6.0						
XV	0.4	0.2	0.7	0.2	0.2	0.2	10.4	5.3	2.9	10.9	5.2	3.2
Water soluble	78.4	79.7	75.1	75.0	75.6	75.7	8.8	7.4	8.1	10.6	6.6	6.3
Residue							41.4	67.4	66.4	39.7	65.2	67.5

[a] Organosoluble compounds were resolved two-dimensionally with TLC using solvent systems A (first direction) and B (second direction).

V was a mixture of two compounds, V and XII (thidiazuron-T-^{14}C only) and VI was a mixture of two compounds, VI and XIII. In solvent system B the origin (I) was a mixture of three compounds, I, XII (thidiazuron-T-^{14}C only), and XIII and VII was a mixture of two compounds, VII, XIV (thidiazuron-A-^{14}C only).

There were two major (>5%) organosoluble urinary metabolites. Metabolite VI clearly was the major organosoluble urinary metabolite in single oral dose (Table II) and dietary supplement (Table III) studies. Subsequent TLC experiments (e.g., Table III) showed that metabolite XIII was only a minor component (<3%) of the mixture (VI + XIII, solvent system A) in Table II. Metabolite XIV was a major metabolite in the dietary supplement study and appeared only in studies with thidiazuron-A-^{14}C (Table III, Figure 4). It likely occurred also when thidiazuron-A-^{14}C was administered to rats as a single oral dose, but its coincident R_f with metabolite VII in solvent system B made a conclusion difficult.

The majority of the radioactive compounds in the urine was water soluble, and Table IV gives the results of treatment of this material isolated from urine of rats treated with a single oral dose of thidiazuron with enzymes and acid. When compared to the control β-glucuronidase hydrolyzed about 15% and aryl sulfatase about 10% of the radioactive material resulting from either label. HCl treatment released about 50%. TLC of the organic phase from β-glucuronidase and aryl sulfatase treatments indicated the presence of several aglycons including metabolite VI, which was a major component with both enzymes and both radiolabels. Thus metabolite VI was conjugated as a glucuronide and as an ethereal sulfate.

The distribution and concentration of radioactive material in feces of rats following treatment with thidiazuron-^{14}C as a single oral dose and as a dietary supplement are given in Tables V and III, respectively. With the single oral dose the majority of the radioactive material (69 to 80%) was unextractable and remained with the fecal residue (Table V). However, the more rigorous extraction procedure employed with the dietary supplement study resulted in appreciably less unextractable material in most cases (Table III). The amount of water-soluble radioactive material in the feces was low and did not exceed 11% (Tables III and V). TLC of the organosoluble radioactive

Table IV. Hydrolysis of Water-Soluble Radioactive Material Isolated from Urine Samples of Rats Treated with a Single Oral Dose of Thidiazuron When Incubated for 12 h with Enzymes and Acid[a]

Treatment	% radioactivity					
	Organic phase			Aqueous phase		
	0-8	8-18	18-96	0-8	8-18	18-96
	Thidiazuron-T-^{14}C					
β-Glucuronidase	24.4	23.5	24.8	75.6	76.5	75.2
Aryl sulfatase	15.7	15.0	20.0	84.3	85.0	80.0
HCl	56.5	55.4	54.9	43.2	44.6	45.1
Control	6.3	7.1	10.6	93.7	92.9	89.4
	Thidiazuron-A-^{14}C					
β-Glucuronidase	28.5	20.4	24.9	71.5	79.6	75.1
Aryl sulfatase	16.5	16.8	20.3	83.5	83.2	79.7
HCl	61.1	61.8	56.6	38.9	38.2	43.4
Control	6.4	8.3	9.1	93.6	91.7	90.9

[a] Aqueous fractions from urine collected at 0-3 and 3-8 h were pooled to yield 0-8; 8-12 and 12-18 h were pooled to yield 8-18; and 18-24, 24-48, 48-72, and 72-96 h were pooled to yield 18-96. Thidiazuron-A-^{14}C data from male rats; thidiazuron-T-^{14}C data from female rats.

Table V. Distribution and Concentration of Radioactive Material from Feces of Female Rats Treated with a Single Oral Dose of Thidiazuron-T-^{14}C

Metabolite or fraction	% fecal radioactivity at indicated time interval, h		
	12-18[a]	18-24[b]	24-48[a]
Organosoluble[c]			
I	0.3	0.4	0.6
V	0.2	0.3	0.3
VI	24.6	14.0	13.9
VII	0.6	1.2	1.2
VIII	0.7	0.7	1.1
IX	0.5	0.2	0.3
Water soluble	4.5	3.0	2.6
Residue	68.6	80.2	80.0

[a] Data are means of two rats per time interval. [b] Data are means of four rats per time interval. [c] Metabolites in organic fraction were resolved by TLC with solvent system A.

Table VI. Concentration of Radioactive Material in Rat Milk Following Treatment with a Single Oral Dose of Thidiazuron-T-^{14}C as Determined by Direct Counting and by Combustion

Time after treatment, h	Parts per billion	
	Direct	Combustion
12	126.1 (17.4)	96.4 (10.6)
24	34.5 (4.8)	21.4 (5.7)
36	22.3 (4.3)	17.9 (3.2)
48	17.4 (3.9)	13.7 (2.6)
60	18.4 (2.1)	10.2 (1.1)
72	14.4 (2.5)	10.9 (2.2)
84	11.5 (3.6)	7.7 (1.3)
96	8.6 (1.3)	5.9 (0.8)

[a] Data based on analyses of milk samples from at least three rats. Standard deviation given in parentheses.

material isolated from the feces indicated that the major metabolite following either method of thidiazuron exposure was metablite VI (Tables III and V). In addition moderately high levels of metabolite XV were detected in the dietary supplement study Table III.

Table VI gives the concentration of radioactive material in rat milk following treatment with a single oral dose of thidiazuron-T-^{14}C. The radioactivity reached a maximum level during the initial 12 h and decreased thereafter. The results of fractionation of milk samples collected at 12-h and 24-h posttreatment are given in Table VII. The radioactivity was about equally divided among the acetonitrile and aqueous phases and in the residue. Levels

Table VII. Distribution and Concentration of Radioactive Material from Milk of Rats Treated with a Single Oral Dose of Thidiazuron-T-^{14}C

Metabolite or fraction	% radioactive material at indicated time interval, h	
	12[a]	24[b]
Acetonitrile[c]		
I	9.2	3.6
IV	5.7	3.5
VI	6.6	2.5
VII	3.0	1.1
VIII	2.6	1.0
Others (5)	8.7	4.3
Hexane	3.2	8.9
Aqueous	30.2	32.8
Residue	30.8	42.3

[a] Average of two fractionations of combined milk from four rats and three rats. [b] Fractionation of combined milk from four rats. [c] Metabolites in acetonitrile phase were resolved by TLC with solvent system A.

Table VIII. Concentration of Radioactive Material in the Tissues of Rats 96 h after Treatment with a Single Oral Dose of Thidiazuron-^{14}C[a]

Tissue	Parts per billion		
	Thidiazuron-T-^{14}C		Thidiazuron-A-^{14}C
	Male	Female	Male
Blood	16.0 (5.8)	10.3 (2.6)	10.2 (1.5)
Brain	3.0 (0.6)	5.3 (3.0)	2.0 (0.5)
Fat, omental	15.7 (4.1)	8.0 (0.8)	3.0 (0.7)
Fat, peritoneal	6.7 (0.3)	4.8 (1.3)	1.4 (1.0)
Fat, subcutaneous	5.6 (0.7)	4.3 (0.6)	3.1 (0.7)
Heart	7.9 (1.6)	5.5 (0.6)	5.1 (0.9)
Intestine	8.0 (0.3)	3.4 (1.0)	1.7 (0.3)
Kidney	9.1 (1.6)	7.5 (2.1)	8.4 (1.3)
Liver	11.7 (1.4)	12.9 (4.3)	8.0 (1.0)
Lung	9.2 (0.6)	4.6 (0.6)	5.8 (0.9)
Muscle, abdominal	7.9 (1.0)	6.5 (2.9)	2.4 (0.6)
Muscle, back	5.7 (1.2)	6.7 (0.7)	1.6 (0.5)
Muscle, leg	4.3 (1.7)	2.3 (1.3)	3.3 (1.1)
Pancreas	7.7 (1.3)	4.0 (0.6)	4.0
Spleen	7.5 (1.1)	5.1 (1.4)	2.1 (0.3)
Stomach	11.9 (0.5)	6.4 (0.7)	4.6 (0.4)
Testes	4.2 (1.7)		0.3 (0.1)
Uterus		6.1 (0.8)	
Mammary gland		5.4	

[a] Data based on analyses of tissues from three rats. Standard deviation given in parentheses.

in the hexane phase were low. Metabolite VI was a major component of the acetonitrile phase.

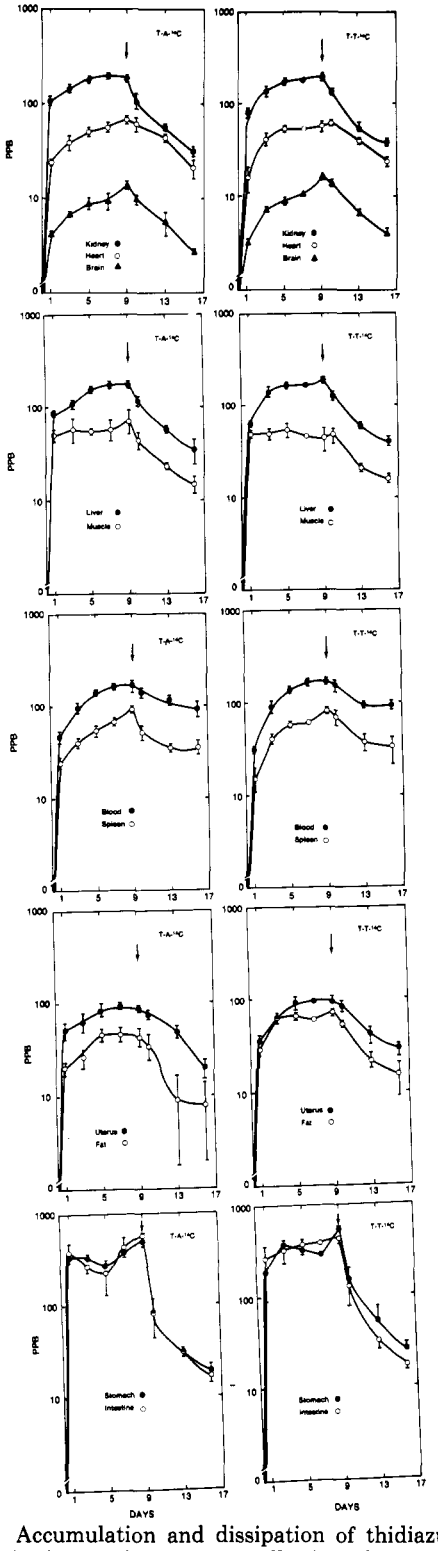

Figure 5. Accumulation and dissipation of thidiazuron-^{14}C equivalents in tissues of female rats following administration of the compound as a dietary supplement for 9 days. Arrow indicates last day on thidiazuron-treated food. Data are means ±SD from three rats at each interval except for day 7 thidiazuron-T-^{14}C study where data are from two rats.

Tissue Residues. The concentration of radioactive material in the tissues of rats 96 h after treatment with a single oral dose of thidiazuron-^{14}C is presented in Table VIII. Levels of radioactivity were low in all cases (<20 ppb); blood, kidney, and liver generally contained slightly higher levels of radioactivity than the other tissues.

Figure 5 shows the accumulation and dissipation of thidiazuron-^{14}C equivalents in tissues of female rats following administration of the compound as a dietary supplement at a concentration of 6.6 ppm for 9 days. In most tissues radiocarbon content increased during the initial days of exposure to treated food. This was followed by marked decrease in rate of accumulation and was represented by a plateau in many cases. Highest levels of radiocarbon were reached by stomach and intestine (>200 ppb); kidney, blood, and liver were intermediate (100 to 200 ppb); and heart, brain, spleen, uterus, fat and muscle were lowest (<100 ppb). By day 16 or the seventh day on untreated food greater than 50% of the maximum tissue level of radiocarbon cleared, with the exception of blood. There were no obvious differences in accumulation and dissipation of radioactive residues in rat tissues relative to the location of radiocarbon in the thidiazuron molecule (thidiazuron-T-^{14}C vs. thidiazuron-A-^{14}C).

Table IX. Concentration of Thidiazuron-^{14}C Equivalents When Incubated for 1 h with Microsomal + Soluble Rat Liver Fraction

Metabolite or fraction	% radioactivity: Thidiazuron-T-^{14}C	% radioactivity: Thidiazuron-A-^{14}C
Organosoluble[a]		
Thidiazuron	18.8	23.5
I	13.2	8.6
II	2.5	1.2
III	3.3	1.4
IV	2.6	4.5
V	2.0	5.4
VI	26.3	31.8
VII	3.8	3.1
VIII	3.6	4.4
IX	2.7	0.8
Others	6.1	3.8
Water soluble	15.5	11.5

[a] Metabolites in organic fraction were resolved by TLC with solvent system A.

Identification of Radiocarbon. The identification of major metabolites VI, XIV, and XV was undertaken. Metabolite VI, which was present in urine (free and conjugated), feces and milk, was detected in studies using thidiazuron-A-^{14}C and thidiazuron-T-^{14}C. It also was formed in vitro by rat liver microsomes plus soluble fraction (Table IX) and by the model hydroxylating system. Thus it seemed probable that both aromatic rings were present and that the urea bridge was intact. When aliquots of metabolite VI, isolated and purified from rat urine, were spotted on filter papers and sprayed with chromogenic reagents, such as *p*-nitrobenzenediazoniumfluoborate, Gibbs's reagent, and ferric chloride–potassium ferricyanide (Krishna et al., 1962), a positive result was obtained, suggesting the presence of at least one phenolic hydroxyl group. Low-resolution mass spectrometry (Figure 6) indicated a molecular ion peak at m/e 236, 16 mass units higher than that of the parent thidiazuron. Measurement of metabolite VI parent ion yielded a mass of 236.03673 (calculated for $C_9H_8O_2N_4S^+$ 236.03679); measurement of m/e 208 yielded a mass of 208.02887 (calculated for $C_9H_8O_2N_2S^+$ 208.03064). Thus metabolite VI contained one hydroxyl group probably on the phenyl moiety. This location was confirmed by measurement of the base peak (m/e 135) which yielded a mass of 135.03146 corresponding to the fragment $C_7H_5O_2N^+$ (calculated mass 135.03202). There are obviously three sites for hydroxyl attachment to the phenyl moiety with the 4 position being favored based on typical electrophilic substitution. TLC in solvent system C (Table I) of authentic samples of 2-hydroxyphenylthidiazuron,

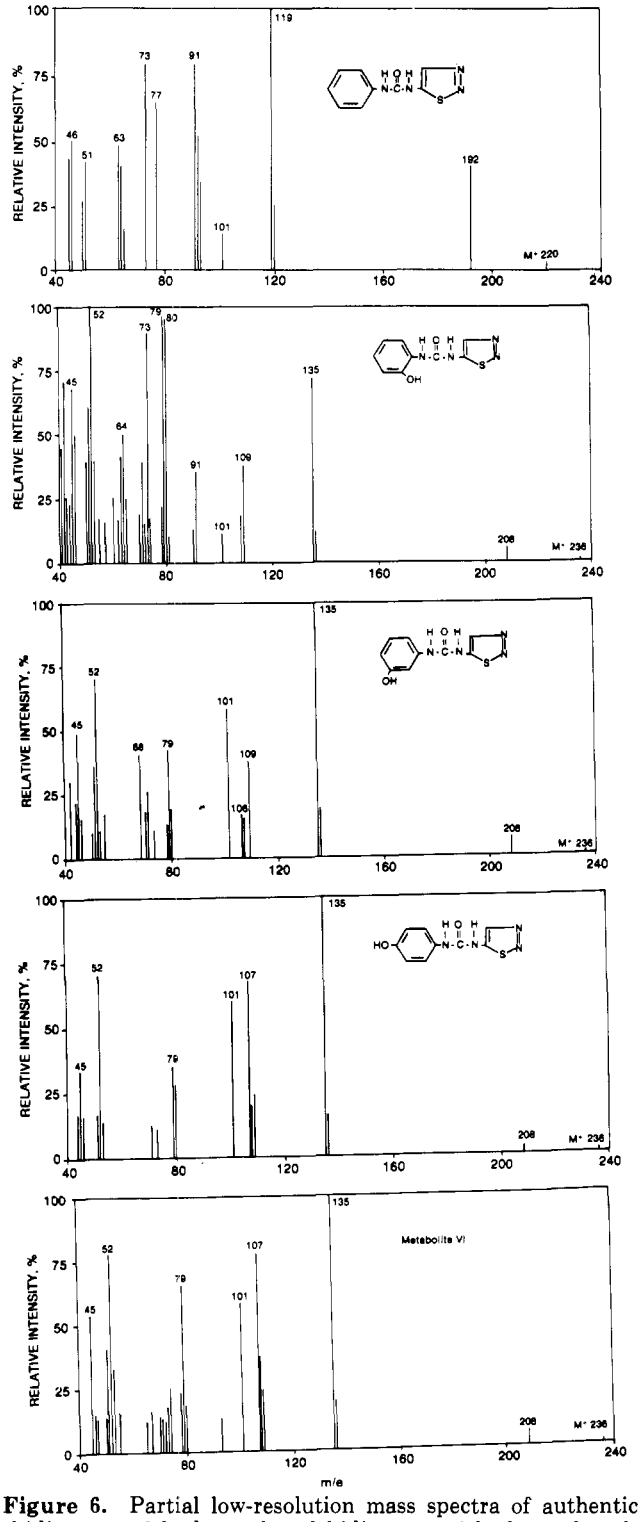

Figure 6. Partial low-resolution mass spectra of authentic thidiazuron, 2-hydroxyphenylthidiazuron, 3-hydroxyphenylthidiazuron, 4-hydroxyphenylthidiazuron, and of metabolite VI isolated from urine of thidiazuron-treated rats.

3-hydroxyphenylthidiazuron, and 4-hydroxyphenylthidiazuron and of metabolite VI excluded the 2-hydroxyphenyl derivative. Metabolite VI and 4-hydroxyphenylthidiazuron yielded pink spots in chromogenic tests with *p*-nitrobenzenediazoniumfluoborate, while 3-hydroxyphenylthidiazuron yielded a yellow-orange spot. Further, the low-resolution mass spectrum of metabolite VI was similar to that of authentic 4-hydroxyphenylthidiazuron and differed from that of authentic 3-hydroxyphenylthidiazuron in the relative abundance of some significant fragments (Figure 6). For example, *m/e*

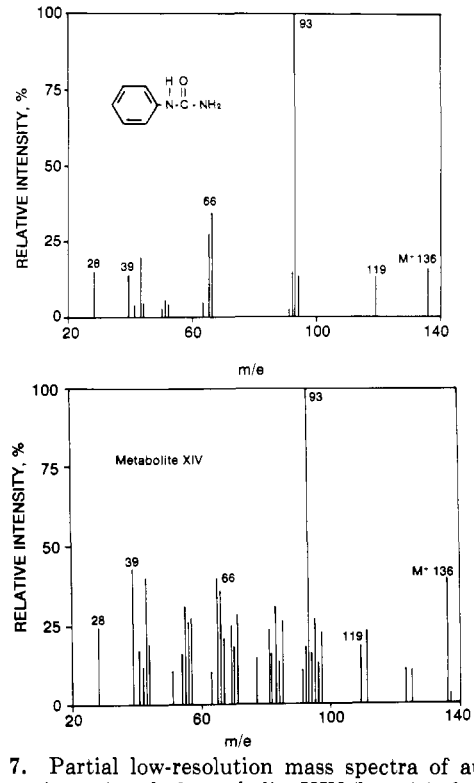

Figure 7. Partial low-resolution mass spectra of authentic phenylurea (upper) and of metabolite XIV (lower) isolated from urine of thidiazuron-treated rats.

107 ($C_6H_5ON^+$) was 75% of the base peak for metabolite VI, 68%, of the base peak for authentic 4-hydroxyphenylthidiazuron, and only 15% of the base peak for authentic 3-hydroxyphenylthidiazuron. Thus metabolite VI was identified as 4-hydroxyphenylthidiazuron.

Metabolite XIV was a major metabolite in the urine of rats fed thidiazuron-^{14}C as a dietary supplement and probably also was present in urine of rats treated with a single oral dose. It was detected only in studies using thidiazuron-A-^{14}C. Metabolite XIV did not yield a positive result when tested with the chromogenic reagents mentioned above; thus, it was concluded that a hydroxyl moiety was not present. Metabolite XIV cochromatographed with authentic phenylurea in TLC solvent systems A, B, and C (one-dimensional) and in the two-dimensional system using A and B (Table I, Figure 4). Examination of low-resolution mass spectra of metabolite XIV and authentic phenylurea (Figure 7) supported the identification of metabolite XIV as phenylurea.

Metabolite XV was a major component of the feces and a minor component of the urine of rats given thidiazuron-^{14}C as a dietary supplement. Further it was present when both radiolabeled thidiazuron samples were used. Metabolite XV cochromatographed with authentic 2-hydroxyphenylthidiazuron and with thidiazuron itself. When metabolite XV was spotted on filter paper and sprayed with the chromogenic reagents listed above a negative result was obtained, suggesting the absence of a phenolic hydroxyl moiety. Thus metabolite XV was probably the parent compound thidiazuron.

Figure 8 summarizes the major paths for thidiazuron metabolism by rats. The most important reaction was hydroxylation of the 4 position of the aniline moiety to yield 4-hydroxyphenylthidiazuron. Hydroxylation of the aromatic nucleus of substituted urea herbicides in vivo by mammals has been previously reported and is a fairly common reaction for these compounds (Geissbühler et al., 1975). Phenylurea, which was first isolated and identified

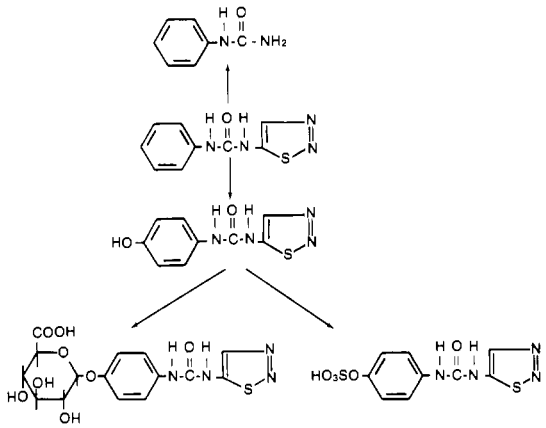

Figure 8. Major paths for thidiazuron metabolism by rats.

as a thidiazuron metabolite from goat urine in our laboratory (Benezet et al., 1978), apparently formed by cleavage of the N–C bond between the thiadiazole ring and the urea bridge. Many substituted urea herbicides, such as diuron and fluometuron, form phenylureas by oxidative N-dealkylation (Geissbühler et al., 1975). Phenylurea obviously cannot be formed from thidiazuron by dealkylation; hence, its formation in goats and rats probably represents a novel cleavage for substituted urea herbicides. We currently are studying the mechanism for its formation. There was little, if any, cleavage by rats of thidiazuron at the urea bridge since aniline, thiadiazole amine, and aminophenols were not detected.

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Measurement of Available Lysine in Processed Beef Muscle by Various Laboratory Procedures

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Three procedures were compared for determining available lysine in beef muscle damaged by heat and glucose or by heat alone (Carpenter, Silcock, and an enzymic method using pronase). The pronase procedure was the most sensitive to lysine damage in beef muscle protein caused by early Maillard, advanced Maillard, and protein–protein type reactions. All amino acids released by pronase from the treated materials except tryptophan were determined. Beef muscle meals processed in the presence of glucose had a greatly reduced release of lysine, histidine, arginine, cystine plus cysteine, glutamic acid, and methionine. Although a fall of over 50% of methionine released by pronase was found, there was no evidence of a release of methionine sulfoxide or methionine sulfone from the meat meals. Beef muscle prepared in the absence of glucose showed a low release of lysine, aspartic acid, glutamic acid, serine, and cystine plus cysteine. This indicated that cross-linkage reactions between lysine and asparagine or glutamine had probably occurred.

The nutritive value of a protein depends not only on its content of essential amino acids but also on the availability of these amino acids for absorption and utilization by the monogastric animal. A loss of availability of amino acids can occur by different mechanisms depending on the conditions of processing of the protein.

Heating proteins in the presence of reducing sugars under mild conditions can cause reactions of the sugar with free NH_2 groups of the protein (early Maillard reactions). Heating protein under severe conditions in the presence of reducing sugars causes more advanced Maillard reactions with destruction of some lysine units. Furthermore, according to Carpenter and Booth (1973), inter and intra peptide linkages can occur during advanced Maillard reactions causing a drop in the availability of amino acids and the general digestibility of the protein. In the absence of sugars, heating of proteins may produce many cross-linking reactions, particularly between lysine and glutamine or lysine and asparagine (Hurrell et al., 1976). According to Carpenter and Booth (1973) such cross-linkages in heated protein very likely hinder digestion in the animal.

Methods using pronase to predict damage of amino acids in processed foodstuffs have been described by Provansal et al. (1975) and Rayner and Fox (1976). Pronase released lysine has been shown to have excellent potential in measuring available lysine in rapeseed meal by its comparison with the Silcock procedure (Rayner and Fox, 1976). However, Hurrell and Carpenter (1974) have shown that the Silcock procedure does not measure lysine availability

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