both the biochemical manifestations of our results using chiral, radiolabeled fluorolipids and the translation of our laboratory studies to control of termites in field situations.

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Metabolites of Diuron, Linuron, and Methazole Formed by Liver Microsomal Enzymes and Spinach Plants

Takashi Suzuki and John E. Casida*

Mouse liver microsomal oxidases convert diuron [ArNHC(O)N$(CH_3)_2$; Ar = 3,4-dichlorophenyl] to seven metabolites modified only at the dimethylamino moiety [–N$(CH_3)CH_2OH$, –N$(CH_3)CHO$, –N$(CH_2OH)_2$, –NHCH_3, –NHCH_2OH, –NHCHO, and –NH_2] and to an *N*-hydroxy derivative [ArN(OH)C(O)NHCH_3]. Linuron [ArNHC(O)N$(OCH_3)CH_3$] in this system yields the corresponding –N$(OCH_3)CH_2OH$, –NHCH_3, and –N$HOCH_3$ derivatives. Microsomal metabolism of methazole [ArNC(O)N(CH_3)C(O)] requires NADPH and gives primarily desmethylmethazole under aerobic conditions and ArNHC(O)NHCH_3 under nitrogen. Most of these metabolites are also detected in spinach leaves treated with [^{14}C]diuron and [^{14}C]methazole. Some 3-hydroxy-1-methylurea from methazole metabolism in spinach is reduced to the 1-methylurea while the remainder is conjugated as the N–O-β-D-glucoside and its 6-*O*-malonyl ester (identified by ^{1}H and ^{13}C NMR). Synthesis procedures, spectroscopic data, and potencies as Hill reaction inhibitors are given for many of the metabolites and derivatives which are useful in their identification. Methazole appears to be a proherbicide while diuron and linuron act directly and via metabolites as photosynthetic inhibitors.

Three substituted urea herbicides share a common metabolite in both plants and mammals. 3-(3,4-Dichlorophenyl)-1-methylurea (DCPMU) is formed from diuron by oxidative N-demethylation, from linuron by oxidative N-demethoxylation, and from methazole by reduction of an intermediate *N*-hydroxy metabolite (Geissbühler et al., 1975; Suzuki and Casida, 1980). DCPMU and its 3-hydroxy derivative are potent Hill reaction inhibitors, as are diuron and linuron but not methazole (Suzuki and Casida, 1980). Several of the other liver microsomal oxidase metabolites of diuron and linuron are also Hill reaction inhibitors (Suzuki and Casida, 1980).

The present investigation considers the metabolic chemistry of diuron, linuron, and methazole in microsomal enzyme systems, the applicability of these findings to plants, and the identity of two conjugated methazole metabolites in spinach.

MATERIALS AND METHODS

Spectroscopy. Proton nuclear magnetic resonance (NMR) spectra were determined with a Perkin-Elmer Model R 32-B spectrometer at 90 MHz or a Nicolet NT-180 spectrometer at 180 MHz by using $CDCl_3$ for nonpolar compounds and acetone-d_6 for polar compounds with tetramethylsilane as the internal standard (δ = 0).

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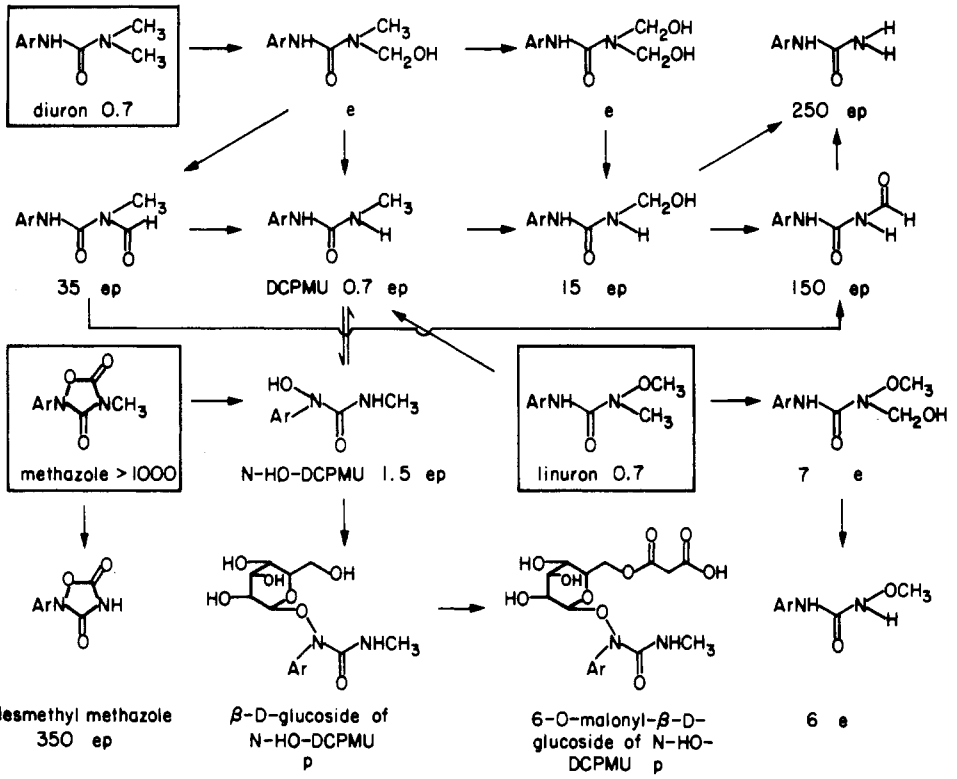


Figure 1. Metabolic pathways for diuron, linuron, and methazole. The potency of each compound as a Hill reaction inhibitor is indicated as the minimum detectable level (nanograms) on TLC. Abbreviations: Ar = 3,4-dichlorophenyl; e = enzyme metabolite detected in mouse liver microsomal systems; p = plant metabolite detected in spinach.

^{13}Carbon NMR spectra were also determined with the Nicolet NT-180 instrument and solvents as above. Chemical ionization–mass spectra (CI–MS) were obtained on the Finnigan 1015D mass spectrometer by using a direct introduction probe and isobutane as the reagent gas at a source pressure of ~0.3 torr. The quasi-molecular ion, $[M + 1]^+$, is reported as m/e (relative intensity) based on the ^{35}chlorine isotope. Infrared (IR) spectra were determined on a Perkin-Elmer Model 457 grating spectrophotometer using KBr disks.

Thin-Layer Chromatography (TLC). Silica gel 60 F-254 chromatoplates (E. Merck, Darmstadt, Germany) were employed with a gel thickness of 0.25 mm for analysis and 0.5 mm for preparative isolations. The solvent systems were A–C indicated in Table I or others were as specified. Resolved compounds detected in preparative work by their quenching of gel fluorescence under short-wavelength ultraviolet (UV) light were recovered by extracting the gel with acetone or dissolving the gel in water and then partitioning into ethyl acetate. ^{14}C-Labeled compounds were detected by autoradiography. Unlabeled compounds in cochromatography studies were visualized with UV. Chromogenic reagents (Stahl, 1969) were used as follows: iodine vapor; chromotropic acid for $-NCH_2OH$ compounds; anisaldehyde–H_2SO_4 for sugar derivatives. The anisaldehyde–H_2SO_4 reagent also provided good sensitivity for malonic acid.

Chemicals. Figure 1 gives the chemical structures (Ar = 3,4-dichlorophenyl) of diuron, linuron, methazole, and some of their metabolites. It also indicates designations used for a few compounds, i.e., DCPMU, N-HO-DCPMU and desmethylmethazole.

The following six ^{14}C preparations were used with specific activities of 4–16 mCi/mmol and radiochemical purities of 99.5–99.8% after purification once or twice with TLC solvent systems A and C: [*carbonyl*-^{14}C]diuron and [*carbonyl*-^{14}C]DCPMU provided by F. S. Tanaka (Metabolism and Radiation Research Laboratory, U.S. Department of Agriculture, Fargo, ND); [*phenyl*-^{14}C]-, [3-*carbonyl*-^{14}C]-, and [5-*carbonyl*-^{14}C]methazole from H. W. Dorough (University of Kentucky, Lexington, KY); [*carbonyl*-^{14}C]N-HO-DCPMU from hydrolysis of [3-*carbonyl*-^{14}C]methazole in 1 N NaOH in 80% ethanol for 10 min at 50 °C.

Syntheses. Fourteen metabolites and derivatives of diuron, linuron, and methazole (Table II) and two other metabolites [$ArNHC(O)NHCH_3$ and $ArNHC(O)NH_2$] were prepared as standards for cochromatography and for bioassay. They were generally isolated by preparative TLC (solvent system A, Table I) and recrystallization (Table II). The identity of each product was established by ^{1}H NMR and CI–MS (Table II).

$ArNHC(O)NH_2$, $ArNHC(O)NHCH_3$ (DCPMU), and $ArNHC(O)NHOCH_3$ were obtained by adding 2 equiv of the appropriate amine (NH_3, CH_3NH_2, or CH_3ONH_2·HCl plus K_2CO_3) in water to an acetonitrile solution of ArNCO. The desired products crystallized within 1 h at 20 °C. $ArNHC(O)N(CH_3)CHO$ and $ArNHC(O)NHCHO$, prepared by stirring the corresponding ureas in 88% formic acid at 110 °C for 1 h, were recovered by addition of water and ether extraction.

A mixture of $ArNHC(O)N(CH_2OH)_2$ and $ArNHC(O)NHCH_2OH$ was obtained on holding $ArNHC(O)NH_2$ in 37% formaldehyde solution in the presence of a small amount of Na_2CO_3 at 50 °C for 1.5 h. Addition of ice-cold water and extraction with ether gave $ArNHC(O)NHCH_2OH$ after purification but the bis(hydroxymethyl)urea was mostly decomposed to $ArNHC(O)NHCH_2OH$. Accordingly, $ArNHC(O)N(CH_2OH)_2$ was converted to its dimethoxy and diethoxy derivatives by treating the ether layer (50 mL) with methanol and ethanol (5 mL) and concentrated H_2SO_4 (0.5 mL) and shaking at 25 °C for 1 min. After extraction 3 times with ice-cold water, the ether phases were chromatographed to obtain $ArNHC(O)N(C-$

Table I. Thin-Layer Chromatographic Properties and Melting Points of Diuron, Linuron, Methazole, and Some of Their Metabolites and Derivatives

compd and R substituent[i]	mp, °C	R_f values in indicated TLC solvent systems[a] A	B	C
ArNHC(O)R				
$N(CH_3)_2$[b]	158-159	0.44	0.43	0.53
$N(CH_3)CH_2OH$[c]		0.21	0.41	0.39
$N(CH_3)CH_2OCH_3$[d]	85	0.47	0.47	0.62
$N(CH_3)CH_2OC_2H_5$[d]	79	0.52	0.59	0.73
$N(CH_3)CHO$[e]	145-146	0.56	0.65	0.72
$N(CH_2OH)_2$[c]		0.08	0.39	0.40
$N(CH_2OCH_3)_2$[d]	56-57	0.54	0.47	0.83
$N(CH_2OC_2H_5)_2$[d]	liquid	0.61	0.70	0.88
$NHCH_3$[e-g]	159-160	0.31	0.52	0.27
$NHCH_2OH$[e,f,h]	165-167	0.07	0.34	0.11
$NHCH_2OCH_3$[d]	147-148	0.25	0.47	0.26
$NHCH_2OC_2H_5$[d]	146-147	0.33	0.62	0.40
NHCHO[e,h]	183-184	0.31	0.70	0.49
NH_2[e,h]	156-157	0.13	0.41	0.11
$N(OCH_3)CH_3$[b]	93-94	0.64	0.63	
$N(OCH_3)CH_2OH$[g]	136-138	0.27	0.40	
$NHOCH_3$[g]	141-143	0.40	0.59	
ArN(OH)C(O)$NHCH_3$[e,f,h]	122-123	0.27	0.62	0.69
ArNC(O)RC(O)				
NCH_3[b]	123-124	0.65	0.76	
NH[f]	152-155	0.04	0.07	

[a] A = chloroform-acetonitrile (4:1); B = diisopropyl ether-butanol (4:1); C = chloroform-pyridine (5:2). [b] Diuron, linuron, and methazole from top to bottom. [c] Chromatographed as diuron metabolite with TLC development at 0-5 °C. [d] Cochromatographs with [^{14}C]diuron metabolite after treatment with CH_3OH- or C_2H_5OH-H_2SO_4. [e] Cochromatographs with [^{14}C]diuron metabolite. [f] Cochromatographs with [^{14}C]methazole metabolite. [g] Identical chromatographic properties to linuron metabolite. [h] Cochromatographs with [^{14}C]DCPMU metabolite. [i] Ar = 3,4-dichlorophenyl.

$H_2OCH_3)_2$ and ArNHC(O)$NHCH_2OCH_3$ or the corresponding ethoxy derivatives. Analogous synthesis procedures were used for ArNHC(O)$N(CH_3)CH_2OCH_3$, ArNHC(O)$N(CH_3)CH_2OC_2H_5$, and ArNHC(O)$N(OCH_3)CH_2OH$.

ArN(OH)C(O)$NHCH_3$ (N-HO-DCPMU) was prepared by reducing $ArNO_2$ to ArNHOH by using Zn powder in 0.5% NH_4Cl aqueous solution, extraction with ether, and addition to the ether of 2-3 equiv of CH_3NCO to obtain a crystalline product recovered after 30 min at 25 °C. This product, probably ArN[OC(O)$NHCH_3$]C(O)$NHCH_3$, was shaken in 2 N NaOH in aqueous ethanol for 3 min at 25 °C, and the solution was acidified with aqueous HCl to obtain N-HO-DCPMU as a crystalline material. The *N*-hydroxyurea, ArN(OH)C(O)$NHCH_3$, is probably in equilibrium with the corresponding isourea *N*-oxide, ArN(O)=C(OH)$NHCH_3$ (Suzuki and Casida, 1980); i.e., IR (KBr) 3110 and 2880 (two hydrogen-bonded OH, probably -COH and -NOH) and 1650 cm^{-1} (C=O); CI-MS [M + 1] - O (Table II).

Desmethylmethazole was prepared via ArN(OH)C(O)-NH_2 obtained on treating an ether solution of ArNHOH (see above) with equivalent phosgene in benzene and then solvent evaporation, addition of excess NH_4OH, and ether extraction. The residue from ether evaporation was dissolved in excess 2 N NaOH solution and treated dropwise with excess ethyl chloroformate under 10 °C. Following ether extraction to remove neutral compounds, the aqueous phase was acidified and extracted with ether to recover desmethylmethazole, which was pure after recrystallization. Methazole is regenerated on treatment of desmethylmethazole with diazomethane.

The tetraacetyl derivative of ArN(O-gluc)C(O)$NHCH_3$ was synthesized by the reaction of ArN(OH)C(O)$NHCH_3$ with equivalent 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide and a slight excess of NaOH in aqueous acetone for 4 h at 20 °C (Ecke, 1973). Product isolation involved addition of water, ethyl acetate extraction, and TLC in solvent system A (R_f 0.45). The ^{13}C NMR spectra is discussed later relative to a metabolite.

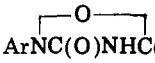
Mouse Liver Microsomal Oxidase Metabolites. The mouse liver microsomal fraction was prepared at 20% (w/v) fresh liver equivalent in 0.1 M phosphate buffer (pH 7.4). Each reaction mixture in 2.5 mL of this buffer contained the microsomal preparation (0.5 mL for diuron, linuron, or DCPMU or 1.0 mL for methazole), NADPH (0 or 2.2 μmol), and ^{14}C-labeled or unlabeled substrate (5 μg) added last in acetone (5 μL) with immediate mixing. Incubations were carried out at 37 °C for 20 min with shaking in air (or O_2, N_2, or CO when specified). Diuron, linuron, and DCPMU reaction mixtures were extracted with ether (3 mL × 4) and methazole reactions were acidified to pH 2-3 with HCl before ether extraction. The recovered ether phases were dried (anhydrous Na_2SO_4), concentrated under N_2, and analyzed by two-dimensional TLC (A × B and A × C). The ^{14}C content of each ether-extractable product, detected by radioautography and determined by direct liquid scintillation counting (LSC) of the appropriate gel region, and of the unextractable portion (aqueous phase) was related on a percentage basis to that of the original substrate.

Metabolites and their derivatives were identified by two-dimensional cochromatography in all systems shown in Table I. For alkylation of the $-NCH_2OH$ compounds, the combined ether phase was concentrated to 5 mL and 0.5 mL of methanol or ethanol and 0.1 mL of concentrated H_2SO_4 were added. After the mixture was shaken 1 min at 25 °C, ice cold water (3 mL) was added and the mixture shaken. The ether phase was recovered and the aqueous phase extracted with ether (3 mL × 2). The combined ether extract was dried (Na_2SO_4) and analyzed by TLC.

Spinach Leaf Metabolites. A fresh spinach leaf (2-3 g, ~5-cm stem length) was treated with the ^{14}C-labeled compound in acetone (50 μL) by injection into the stem. The stem was placed in water, and after 48 h at 25 °C the leaf was frozen. For extraction, the frozen leaf was completely macerated with granular anhydrous Na_2SO_4 (20 g) and cold methanol-acetone (1:1, 30 mL) by using a mortar and pestle. After filtration with suction, the residue was extracted 2 additional times with methanol-acetone as above, and the residue was washed with 10 mL of the same solvent mixture to give the final unextracted portion for LSC. The combined extracts were dried (Na_2SO_4), and 10% aliquots were used for TLC as 10-cm bands on 0.5 mm thickness chromatoplates. Metabolites were tentatively identified by comparison of chromatographic properties with those of the unlabeled standards using solvent system A in all cases and additional systems as follows: B and diisopropyl ether-dioxane (1:1) for diuron, methazole, and their metabolites; chloroform-methanol-acetic acid (13:6:1) (solvent system D) for methazole, N-HO-DCPMU, and their metabolites. The glucoside of N-HO-DCPMU and its metabolites were examined in only solvent system D.

Larger amounts of two polar metabolites were obtained by holding 130 spinach leaves (390 g) for 48 h with their stems in a 200-ppm solution of N-HO-DCPMU (with tracer levels of [^{14}C]N-HO-DCPMU) during which time the leaves absorbed 750 mL which was equivalent to 150

Table II. Analytical Data for Some Metabolites and Derivatives of Diuron, Linuron, and Methazole

		NMR[b] chemical shifts, δ		CI-MS, *m/e* (rel intensity)	
no.	compd[c]	Ar, 3 H	other[a]	$[M + 1]^+$	other
1	ArNHC(O)N(CH_3)CH_2OCH_3	7.26-7.60	3.09 (s, NCH_3), 3.40 (s, OCH_3), 4.70 (s, NCH_2O)	263 (33)	231 (M - CH_3O, 100)
2	ArNHC(O)N(CH_3)$CH_2OC_2H_5$	7.26-7.60	1.28 (t, CCH_3), 3.07 (s, NCH_3), 3.61 (q, OCH_2C), 4.74 (s, NCH_2O)	277 (35)	231 (M - C_2H_5O, 100)
3	ArNHC(O)N(CH_3)CHO	7.36-7.78	3.29 (s, NCH_3), 8.55 (s, NCHO)	247 (100)	
4	ArNHC(O)N(CH_2OCH_3)$_2$	7.30-7.63	3.41 (s, $2OCH_3$), 4.80 (s, $2NCH_2O$)	293 (39)	261 (M - CH_3O, 72), 260 (M - CH_3OH, 100), 229 (M - $2CH_3O$, 39)
5	ArNHC(O)N($CH_2OC_2H_5$)$_2$	7.29-7.64	1.24 (t, $2CCH_3$), 3.62 (q, $2OCH_2C$), 4.84 (s, $2NCH_2O$)	321 (21)	275 (M - C_2H_5O, 47), 274 (M - C_2H_5OH, 100), 229 (M - $2C_2H_5O$, 32)
6	ArNHC(O)NHCH_2OH	7.38, 7.92	4.68 (m, NCH_2O)		217 ([M + 1] - H_2O, 71), 205 ([M + 1] - CH_2O, 100)
7	ArNHC(O)NHCH_2OCH_3	7.39, 7.92	3.26 (s, OCH_3), 4.63 (d, NCH_2O)	249 (21)	217 (M - CH_3O, 100)
8	ArNHC(O)N$HCH_2OC_2H_5$	7.37, 7.92	1.12 (t, CCH_3), 3.52 (q, OCH_2C), 4.68 (d, NCH_2O)	263 (13)	217 (M - C_2H_5O, 100)
9	ArNHC(O)NHCHO	7.50, 7.95	8.71 (m, NCHO)	233 (100)	
10	ArN(OH)C(O)NHCH_3	7.37-7.94	2.83 (d, NCH_3)	235 (100)	219 ([M + 1] - O, 33), 217 ([M + 1] - H_2O, 28)
11	ArNHC(O)N(OCH_3)CH_2OH	8.0, 7.39-7.70	3.78 (s, OCH_3), 5.04 (s, NCH_2O)	265 (57)	235 ([M + 1] - CH_2O, 43), 100 (100)
12	ArNHC(O)N$HOCH_3$	8.0, 7.37-7.67	3.71 (s, OCH_3)	235 (72)	160 (M - C(O)N$HOCH_3$, 100)
13		7.44-7.77		247 (21)	246 (M, 52), 159 ($Cl_2C_6H_3N$, 59), 124 (ClC_6H_3N, 100)
14	ArN(O-gluc-OAc_4)C(O)NHCH_3	7.23-7.40	1.98, 2.00, 2.03, 2.10 ($4COCH_3$), 2.86 (d, NCH_3), 4.24 (6'-CH_2 and 5'-CH), 5.16 (m, 4CH)	565 (3)	287 (12), 221 (11), 109 (100)

[a] Yields as pure crystalline compounds were 35-53% for 4, 6-8, 10, and 11, 10-25% for 2, 3, 5, 9, 12, and 14, and 2-4% for 1 and 13. Yields for 10 and 13 are based on nitrobenzene. Recrystallization solvents were methanol-water for 1, 4, and 14, acetone-hexane for 2, 6, 10, 12, and 13, hexane-chloroform for 3 and 9, and acetone-water for 7, 8, and 11. [b] NMR solvents were $CDCl_3$ for 1-5 and 14 and acetone-d_6 for 6-13. [c] Ar = 3,4-dichlorophenyl.

mg of N-HO-DCPMU. The treated leaves were mixed with granular anhydrous Na_2SO_4 (1200 g) and extracted with acetone (500 mL × 3). The residue (~10 mL) after concentration at 25 °C was mixed with acetone (80 mL), water (420 mL), and a small amount of NH_4Cl and then extracted with hexane (300 mL × 3). The desired products were then recovered on extraction of the aqueous phase with ethyl acetate (200 mL × 5). After drying (Na_2SO_4) and solvent evaporation, the ethyl acetate extractives were subjected to preparative TLC developing twice with diisopropyl ether–acetone–methanol (13:6:1) to separate the two major ^{14}C metabolites, one at the origin and the second at R_f 0.20. The appropriate radioactive gel regions were extracted with acetone and the metabolites rechromatographed separately with solvent system D (R_f 0.36 and R_f 0.61). Acetone extraction gave 16 mg of the more polar and 18 mg of the less polar metabolite, each a single spot on TLC. A small portion of each metabolite was used for degradation studies. The remainder was used for ^1H and ^{13}C NMR before and after methylation (diazomethane) with subsequent acetylation (acetic anhydride–pyridine) (more polar metabolite) or acetylation only (less polar metabolite), recovering the derivatives in almost quantitative yield by preparative TLC (solvent system A).

Bioassays. The potency of the substituted ureas as inhibitors of the root and shoot growth of oat seedlings was assayed according to a reported procedure (Schuphan and Casida, 1979). They were also assayed as Hill reaction inhibitors by applying solutions of the test compound as a series of 5-mm spots on a TLC plate. Spinach leaves were macerated at 25% (w/v) in cold 0.5 M sucrose with a mortar and pestle. The filtrate from passage through four layers of gauze was kept on ice in the dark for up to 2 days. This filtrate provides results comparable to those obtained with isolated chloroplasts. TLC plates were sprayed with a 1:1 mixture of the filtrate and an 0.1% aqueous solution of 2,6-dichlorophenolindophenol sodium salt. Exposure to sunlight or a fluorescent lamp served to develop the inhibitor regions as blue-gray spots on a light green background. This technique, described earlier (Kováč and Henselová, 1977; Suzuki and Casida, 1980), is applicable to developed TLC plates providing that when solvents such as butanol and pyridine (in solvent systems B and C) are used the plates are held overnight for solvent evaporation before spraying the chloroplast preparations. Anhydrous diethyl ether contains interfering inhibitors removed by distillation and storage in the cold.

RESULTS

Mouse Microsomal Metabolites of [*carbonyl*-^{14}C]-Diuron and [*carbonyl*-^{14}C]DCPMU. Diuron yields eight microsomal metabolites (Table III) identified by TLC cochromatography (Table I) and formed only with NADPH fortification. The major metabolite is the N-deme-

Table III. Metabolites of [*carbonyl*-^{14}C] Diuron and [*carbonyl*-^{14}C] DCPMU in the Mouse Liver Microsome-NADPH-Air System

compd and R substituent[d]	radiocarbon recovery, % diuron by two methods: direct[a]	diuron by two methods: alkylation[b]	DCPMU[a]
ArNHC(O)R			
$N(CH_3)_2$	26.8 (27.6)[c]	32.5 (31.8)[c]	
$N(CH_3)CH_2OH$	3.9 (7.8)	7.5 (6.7)	
$N(CH_3)CHO$	1.8 (2.2)	1.6 (1.7)	
$N(CH_2OH)_2$	0.9 (3.6)	2.3 (3.2)	
$NHCH_3$	39.9 (37.2)	32.8 (35.0)	43.3[c]
$NHCH_2OH$	12.0 (7.0)	8.2 (7.1)	20.4
NHCHO	2.2 (3.7)	2.3 (2.4)	2.2
NH_2	1.7 (1.8)	1.6 (1.6)	4.4
$ArN(OH)C(O)NHCH_3$	0.8 (0.4)	0.3 (0.1)	4.2
trace products on TLC	0.8 (1.2)	0.5 (0.5)	2.2
unextracted	3.0 (3.5)	2.5 (2.7)	6.1
total	93.8 (96.0)	92.1 (92.8)	82.8

[a] No derivatization with TLC at 20–25 °C or, in parentheses, at 0–5 °C. [b] Conversion of $-NCH_2OH$ metabolites to $-NCH_2OCH_3$ derivatives or, in parentheses, to $-NCH_2OC_2H_5$ derivatives [except for 0.3% not alkylated with $ArNHC(O)NHCH_2OH$] prior to TLC at 20–25 °C. [c] Unmetabolized parent compound. [d] Ar = 3,4-dichlorophenyl.

thylated derivative, followed in importance by three *N*-hydroxymethyl compounds. Two formamides are relatively minor metabolites formed by further oxidation of the $-NCH_2OH$ compounds and undergoing formamidase-type cleavage to the $-NH$ derivatives. This metabolic sequence was established by demonstrating the following conversions with appropriate unlabeled standards: $ArNHC(O)NHCH_2OH$ to ArNHC(O)NHCHO and $ArNHC(O)N(CH_3)CHO$ to ArNHC(O)NHCHO, requiring both microsomes and NADPH; $ArNHC(O)N(CH_3)CHO$ to $ArNHC(O)NHCH_3$ and ArNHC(O)NHCHO to $ArNHC(O)NH_2$, requiring microsomes but no NADPH. The *N*-hydroxy derivative is relatively unstable and undergoes partial conversion to DCPMU on TLC; it is also formed on DCMPU and methazole metabolism and is considered in more detail below. The major DCPMU metabolite is $ArNHC(O)NHCH_2OH$.

The $-N(CH_3)CH_2OH$ and $-N(CH_2OH)_2$ metabolites are quite unstable and undergo extensive decomposition with tailing to form the $-NHCH_3$ and $-NHCH_2OH$ derivatives, respectively, on TLC at 20–25 °C. Fortunately, these $-NHCH_2OH$ compounds can be chromatographed with little or no breakdown at 0–5 °C (Table III). They are also readily alkylated with methanol or ethanol–H_2SO_4, providing an efficient and convenient derivatization procedure. Table III indicates that the $-N(CH_3)CH_2OH$, $-N(CH_2OH)_2$, and $-NHCH_2OH$ metabolites are almost quantitatively analyzed as their methylated and ethylated derivatives.

Mouse Microsomal Metabolites of [*phenyl*-^{14}C]-Methazole. Methazole undergoes slow decomposition at physiological pH to form $ArN(OH)C(O)NHCH_3$, $ArNHC(O)NHCH_3$, and unknowns with the following yields in 1.5 h at 30 °C: 3.0, 1.4, and 0.9%, respectively, in phosphate buffer (pH 7.4); 10.8, 2.4, and 1.9%, respectively, in Tris-HCl buffer (pH 8.2). DCPMU and its N–HO derivative are each formed in 2% amount on incubation of methazole with microsomes in pH 7.4 phosphate buffer for 20 min at 37 °C in the absence of NADPH (Table IV).

Methazole metabolism involves primarily oxidative N-demethylation to form desmethylmethazole under aerobic conditions and ring cleavage and reduction to form DCPMU under anaerobic conditions, in each case requiring microsomes and NADPH. The yield of desmethylmethazole is lowered greatly by 10^{-3} M SKF 525A [2-(diethylamino)ethyl 2,2-diphenylvalerate hydrochloride] or a N_2 or CO atmosphere (Table IV). DCPMU formation may require reduced cytochrome P-450 since the yield with NADPH is 93% with a N_2 and 40% with a CO atmosphere but is only 2% without NADPH. The conversion of methazole to DCPMU is greatly enhanced by iron porphyrins, i.e., 96% conversion on incubation with 10^{-3} M hematin in 0.1 M phosphate buffer (pH 7.0) for 30 min at 37 °C.

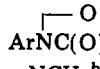
Mouse Microsomal Metabolites of Linuron. Linuron metabolism in the microsome–NADPH system yields $ArNHC(O)N(OCH_3)CH_2OH$, $ArNHC(O)NHOCH_3$, and $ArNHC(O)NHCH_3$ evident on TLC (A × B) as Hill reaction inhibitors (Suzuki and Casida, 1980).

Spinach Leaf Metabolites of [*carbonyl*-^{14}C]Diuron. The metabolites detected in the mouse microsomal system (Table III) are also evident in spinach leaves (Table V) except for $ArNHC(O)N(CH_3)CH_2OH$, $ArNHC(O)N(CH_2OH)_2$ and $ArN(OH)C(O)NHCH_3$ which may undergo partial decomposition during extraction and workup.

Spinach Leaf Metabolites of [*phenyl*-^{14}C]- and [*carbonyl*-^{14}C]Methazole. Five unconjugated metabolites are detected with [*phenyl*-^{14}C]- and [3-*carbonyl*-^{14}C]methazole but only desmethyl-methazole is detected as a metabolite of [5-*carbonyl*-^{14}C]methazole (Table V). The major metabolite is $ArNHC(O)NHCH_3$ and relatively minor metabolites are desmethylmethazole, $ArNHC(O)NHCH_2OH$, $ArNHC(O)NH_2$, and $ArN(OH)C(O)NHCH_3$.

Methazole yields two major polar metabolites with TLC R_f values of 0.36 and 0.61 in solvent system D and 0.27 and

Table IV. Metabolites of [*phenyl*-^{14}C]Methazole in the Mouse Liver Microsome System under Various Conditions

compd and R substituent[d]	radiocarbon recovery, % -NADPH[a]	+NADPH O_2	+NADPH N_2	+NADPH CO
				
NCH_3[b]	93.3	71.5 (92.4)[c]	0.2 (0.4)[c]	57.4
NH	0.0	21.8 (2.5)	1.4 (0.0)	0.1
$ArN(OH)C(O)NHCH_3$	2.0	0.2 (1.7)	0.2 (0.9)	1.0
ArNHC(O)R				
$NHCH_3$	2.0	1.7 (1.3)	93.3 (94.5)	40.4
$NHCH_2OH$	0.0	0.5 (0.1)	4.3 (3.2)	0.1
total	97.3	95.7 (98.0)	99.4 (99.0)	99.0

[a] Results are the average for buffer in an O_2 atmosphere, soluble fraction in O_2, microsome fraction in O_2, and microsome fraction in N_2 since similar results were obtained under each condition. [b] Unmetabolized parent compound. [c] Values in parentheses are for the analogous system containing 10^{-3} M SKF 525A. [d] Ar = 3,4-dichlorophenyl.

Table V. Metabolites of [*carbonyl*-^{14}C]Diuron, [*carbonyl*-^{14}C]N-HO-DCPMU, and [*phenyl*-^{14}C]- and [*carbonyl*-^{14}C]Methazole 48 Hours after Treatment of Spinach Leaves

compd and R substituent[e]	radiocarbon recovery, %		
	diuron	N-HO-DCPMU	methazole[a]
ArNHC(O)R			
$N(CH_3)_2$	10.9[b]		
$N(CH_3)CHO$	4.5		
$NHCH_3$	63.9	36.5	38.8 ± 3.9
$NHCH_2OH$	8.4	4.9	2.1 ± 0.4
NHCHO	1.0		
NH	2.8		1.4 ± 0.2
$ArN(R)C(O)NHCH_3$			
OH		0.0[b]	0.2 ± 0.05
O-gluc[c]		2.0	13.1 ± 3.4
O-gluc-mal		43.2	22.1 ± 4.9
ArNC(O)RC(O)			
NCH_3			5.3 ± 1.5[b]
NH			2.5 ± 0.05
polar, unidentified[d]	1.9		
unextracted	0.8	3.0	4.0 ± 1.4
total	94.2	89.6	89.5 ± 3.9

[a] Average and standard error for two experiments with [*phenyl*-^{14}C]methazole and two with [3-*carbonyl*-^{14}C]-methazole. In comparable studies with [5-*carbonyl*-^{14}C]-methazole, only the parent compound and desmethylmethazole were detected. [b] Unmetabolized parent compound. [c] Treatment with the [3-*carbonyl*-^{14}C]-*O*-glucoside gave 10% unmetabolized glucoside and 78% [^{14}C]-*O*-malonylglucoside (total recovery, 89%). [d] Polar metabolites were relatively minor after treatment with [*carbonyl*-^{14}C]DCPMU. [e] Ar = dichlorophenyl.

0.32 in 2-propanol–25% ammonia (7:3). These metabolites are detected with the *phenyl*-^{14}C and 3-*carbonyl*-^{14}C preparations but not with 5-*carbonyl*-^{14}C starting material (Table V), indicating that in their formation the 5-carbonyl group is lost. The two polar metabolites are formed in excellent yields from N-HO-DCPMU, whereas diuron and DCPMU give little if any of these compounds (Table V). The metabolite of higher R_f is quickly metabolized to give the lower R_f compound (Table V). These observations establish an overall metabolic pathway of methazole → N-HO-DCPMU → high R_f polar metabolite → low R_f polar metabolite.

The polar metabolites were isolated from spinach treated with N-HO-DCPMU in adequate amounts and purities for spectroscopic and degradation studies. The high R_f metabolite gave a greenish blue color and the low R_f metabolite a greenish violet color with anisaldehyde–H_2SO_4, suggesting the possible presence of a sugar and modified sugar moiety, respectively. Acid hydrolysis (1 N HCl; 80 °C; 5 h) cleaved both metabolites, the less polar one to DCPMU and glucose (TLC identification) and the more polar one to DCPMU, glucose, and malonic acid (TLC in solvent system D; detection with iodine vapor and a reddish violet color with anisaldehyde–H_2SO_4). Liberation of DCPMU involves both deconjugation and possible decomposition of N-HO-DCPMU which is an acid-labile compound. Base hydrolysis (0.1 N NaOH; 50 °C; 1 h) did not modify the less polar metabolite, but it converted the more polar metabolite to the less polar metabolite and released malonic acid. Incubation with β-glucosidase (from almond; pH 5.5; 37 °C; 20 h) cleaved the less polar metabolite to N-HO-DCPMU and glucose but did not modify the more polar metabolite; malonylglucosides are not cleaved by β-glucosidase (Frear, 1976). Only the more polar metabolite was methylated with diazomethane while

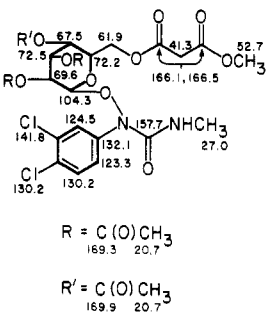


Figure 2. ^{13}C NMR data ($CDCl_3$) of 6-*O*-malonyl β-D-glucoside of 3-(3,4-dichlorophenyl)-3-hydroxy-1-methylurea, a methazole metabolite isolated from spinach, after methylation and acetylation. The signal at 130.2 ppm is probably due to two carbon atoms.

both metabolites were acetylated with acetic anhydride–pyridine. CI–MS gave $[M + 1]^+$ 565 for the acetylated derivative and 623 for the methylated and acetylated derivative as appropriate for the tetraacetylglucoside and (methylmalonyl)tetraacetylglucoside, respectively, of N-HO-DCPMU.

The conjugates were ultimately identified as the β-D-glucoside of N-HO-DCPMU and its 6-*O*-malonyl ester by examination of their NMR spectra with or without derivatization. The less polar metabolite on acetylation was completely identical with the synthetic tetraacetylglucoside, i.e., ^{1}H NMR and CI–MS (Table II) and TLC. They were also identical in ^{13}C NMR with the same assignments as shown in Figure 2 except that the 41.3-, 52.7-, 166.1-, and 166.5-ppm signals of the methylmalonyl substituent were missing and were replaced by the 20.7- and ~170-ppm signals of an additional acetyl group. The more polar metabolite prior to derivatization gave a ^{1}H NMR spectrum (acetone-d_6) with the malonyl CH_2 signal at 3.38 ppm and a characteristic doublet at 4.87 ppm (J = 7.7 Hz), confirming the β configuration of the glycosidyl linkage (van der Veen, 1963). The ^{1}H NMR spectrum ($CDCl_3$) of the more polar metabolite after methylation and acetylation differed from that of the tetraacetylglucoside (Table II) primarily by the additional methylmalonyl signals [3.45 ppm, $C(O)CH_2C(O)$; 3.80 ppm, $CH_3OC(O)$]. The ^{13}C NMR data (Figure 2) firmly established the structure of this conjugate as the 6-*O*-malonylglucoside.

It is of interest that N-HO-DCPMU is quickly and almost completely conjugated in spinach while no evidence was obtained for glucosides or malonylglucosides of $ArNHC(O)N(CH_3)CH_2OH$ and $ArNHC(O)NHCH_2OH$.

Biological Activity of Diuron, Linuron, Methazole, and Their Metabolites and Derivatives. Diuron and linuron are potent Hill reaction inhibitors and are converted metabolically to additional Hill reaction inhibitors (Figure 1). Demethylation does not alter the activity of diuron but reduces the potency of linuron by 10-fold and of DCPMU by 357-fold. The $-NCH_2OH$ and $-NCHO$ derivatives are relatively low in activity. Methazole is inactive per se and on demethylation, so it must act via N-HO-DCPMU or more likely DCPMU if its herbicidal activity is reflected by its ability to inhibit the Hill reaction (Figure 1).

Several ureas are moderately effective inhibitors of the growth of oat seedlings, affecting root growth without marked effect on shoot growth at levels of 100 ppm or below. The ppm concentrations to inhibit root growth by 50% were as follows: >100 for methazole; 50–100 for desmethylmethazole and N-HO-DCPMU; 20–30 for diuron, linuron, and the $-NHCHO$ and $-NHOCH_3$ derivatives; 5–10 for DCPMU and the $-N(OCH_3)CH_2OH$, -N-

$(CH_3)CHO$, $-NHCH_2OH$, $-NHCH_2OCH_3$, and $-NH_2$ derivatives.

DISCUSSION

Metabolic reactions of diuron, linuron, and methazole in liver microsomal enzyme systems and spinach leaves are given in Figure 1. Major pathways involve oxidation of $-NCH_3$ substituents ($-NCH_3 \rightarrow -NCH_2OH \rightarrow -NCHO$) and presumably $-OCH_3$ substituents ($-OCH_3 \rightarrow -OCH_2OH$) with spontaneous loss of formaldehyde ($-NCH_2OH \rightarrow -NH$; $-OCH_2OH \rightarrow -OH$) or formamidase liberation of formic acid ($-NCHO \rightarrow -NH$). *N*-Hydroxy metabolites play an important role with each herbicide even though the reduction reaction ($-NOH \rightarrow -NH$) is strongly favored over N-hydroxylation under the conditions examined. The reactions observed are notable for the absence of hydroxylation or other modifications of the 3,4-dichlorophenyl substituent or extensive cleavage of the urea moiety.

Some of the oxidized metabolites appear to be observed for the first time in this study, i.e., desmethylmethazole, N–HO–DCPMU, the $-N(CH_2OH)_2$, $-N(CH_3)CHO$, and $-NHCHO$ derivatives of diuron (Suzuki and Casida, 1980), and the $-N(OCH_3)CH_2OH$ metabolite of linuron (analogous metabolite of monolinuron reported by Schuphan and Ebing, 1978). The $-N(CH_3)CHO$ or $-NHCHO$ analogues of diuron and/or monuron are known to form in the Fe^{2+}–ascorbic acid oxidizing system (Tanaka and Wien, 1979) and photochemically (Crosby and Tang, 1969; Tanaka et al., 1977). Several metabolites are detected in low yields, possibly due to their instability. The *N*-hydroxyureas are suggested to undergo rapid metabolic reduction to the corresponding ureas (Baskakov, 1973), yet N-hydroxylation is still evident by the formation of N–HO–DCPMU from DCPMU and diuron. The $-NCH_2OH$ metabolites are sufficiently unstable in free and conjugated form to justify special procedures for their analysis. This is conveniently achieved by conversion to their alkoxy derivatives, e.g., $ArNHC(O)NHCH_2OCH_3$ (Dorough et al., 1973; Ivie et al., 1973; Tanaka et al., 1972).

Studies with liver microsomes detected all of the unconjugated diuron and methazole metabolites found in plants (Figure 1). Accordingly, the mechanisms of diuron and methazole metabolism revealed by the microsomal enzyme investigations are probably applicable to the plant systems. The liver microsomal system metabolizes methazole by a sequence involving NADPH-dependent reductive cleavage of the oxadiazolidinedione ring, probably at the N–O bond, decarboxylation, and reduction of N–HO–DCPMU to DCPMU. Plant metabolism of methazole yields DCPMU, $ArNHC(O)NH_2$, and polar metabolites which may be relevant to the differential tolerances of various species although these polar products are not identified (Butts and Foy, 1974; Dorough et al., 1973; Jones and Foy, 1972; Keeley and Thullen, 1979). In spinach the polar metabolites are the N-*O*-glucoside of N–HO–DCPMU and its 6-*O*-malonyl ester.

Diuron, linuron, and several of their metabolites are potent Hill reaction inhibitors. In contrast, methazole appears to be a proherbicide. The selectivity of methazole may be related in part to the relative rates of the bioactivation pathway involving reductive cleavage of methazole via N–HO–DCPMU to DCPMU as opposed to the detoxification processes of oxidative N-demethylation and conjugation of N–HO–DCPMU.

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