Metabolism of [^{14}C]Metolachlor in Bluegill Sunfish

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Bluegill sunfish were continuously exposed to [^{14}C]metolachlor at a concentration of 1 mg/L for 34 days in a flow-through system. On the 35th day, the radioactive residues were 13.9 and 220.3–250.8 ppm equivalents to [^{14}C]metolachlor in the edible and nonedible tissues, respectively. The HPLC and 2D-TLC metabolite profiles for the edible and nonedible tissues are essentially the same, indicating that common metabolites exist in both tissues. The metabolism of metolachlor in bluegill sunfish includes O-demethylation, benzylic hydroxylation of the aralkyl side chains followed by glucuronide conjugation, and reductive dechlorination with subsequent glutathione conjugation.

INTRODUCTION

Metolachlor, 2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl)acetamide, is a selective herbicide belonging to the chloroacetanilide family. It is commercially used in several crops including corn and soybeans for the control of selected annual grasses and broadleaf weeds. Heavy rainfall on a metolachlor-treated crop may result in direct discharge of the runoff water into aquatic sites and exposure of the aquatic organisms to the chemical. Since such exposure may result in residues of metolachlor and its metabolites in edible tissues of fish, it is of interest to determine the nature of these residues.

Several studies have been reported on the metabolism of metolachlor in animals, soil, and plants. The rat metabolism of metolachlor has been extensively studied by Muecke (1981) and Bissig et al. (1987). The major pathways of metolachlor metabolism in rats involved O-demethylation, hydrolytic dechlorination, and conjugation. The studies conducted on the metabolism of metolachlor in rat liver cytosolic enzymes and microsomal enzymes (Feng and Wratten, 1989) showed that glutathione conjugation and oxidative reaction were the major pathways. It has also been demonstrated in soil actinomycete (Krause et al., 1985) that O-demethylation and benzylic hydroxylation were the major metabolic pathways of metolachlor. LeBaron et al. (1988) reported that the pathway for the metabolism of metolachlor in corn and soybeans was via glutathione conjugation of the chloroacetyl side chain. However, metabolism of metolachlor in aquatic organisms is not well understood.

This paper describes the distribution, nature, and identity of the ^{14}C residues in tissues of fish following exposure to [^{14}C]metolachlor.

MATERIALS AND METHODS

Chemicals. Radiolabeled and nonradiolabeled metolachlor were obtained from the Synthesis Group, Ciba-Geigy Corp., Agricultural Division, Greensboro, NC. The [^{14}C]metolachlor was uniformly labeled on the phenyl ring with a radiochemical purity of 99.6%. The chemical purity of the nonradiolabeled metolachlor was 96.4%. The stock solution was prepared by diluting [^{14}C]metolachlor with nonlabeled metolachlor to give a final specific activity of 0.67 μCi/mg. Nonradiolabeled CGA-41638, 2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-hydroxy-1-methylethyl)acetamide, was obtained from Ciba-Geigy Synthesis Group.

Test System. A 600-L fiberglass tank was used as the exposure tank. The test solution volume was maintained with standpipe at 500 L. The details of the exposure conditions were described by Fackler (1989). A modified intermittent-flow proportional diluter (Mount and Brungs, 1967) was employed. The calibration provided a nominal concentration of [^{14}C]metolachlor equal to 1.0 mg/L in the aquarium before the addition of the bluegill sunfish. The system provided a minimum turnover rate of 6 aquarium volumes/24 h and a 90% test solution replacement time of approximately 8 h (Sprague, 1969). The dilution water used during the test was from the same source as the water which flowed into the holding tank.

This well water was routinely monitored and was characterized as having total hardness and alkalinity ranges of 27–32 mg/L $CaCO_3$ and 18–25 mg/L $CaCO_3$, respectively. The specific conductance ranged from 100 to 150 $\mu\Omega^{-1}$/cm. The dissolved oxygen concentration was measured daily and ranged from 7.8 to 9.2 mg/L. The pH was determined three times weekly and ranged from 6.8 to 7.4. The temperature was measured daily and ranged from 17 to 18 °C.

Test Organism. The bluegill sunfish (*Lepomis macrochirus*) were selected from a culture maintained at Springborn Life Sciences, Inc. The juvenile bluegill sunfish (mean wet body weights of 2.2 ± 0.8 g and mean standard lengths of 55 ± 6 mm) were acclimated in a holding tank for 2 weeks under a photoperiod of 16 h of light and 8 h of darkness prior to exposure to the chemical. All fish were fed Tetramin flakes, ad libitum, twice daily except during the 24 h prior to testing.

Treatment. The test system was allowed to equilibrate for a 2-day period, after which time a group of juvenile bluegill sunfish was transferred to the aquarium. The treated fish were continuously exposed to [^{14}C]metolachlor at 1.0 mg/L for 34 days. The temperature and photoperiod in the exposure tank were the same as in the holding tank. At test initiation, the total biomass loading was 0.93 g/L of the daily flow-through volume of the solution. Daily observations were made on the appearance and behavior of the fish.

Sample Collection. To monitor the concentration of ^{14}C residues in the exposure solution, three 5-mL water samples were collected once during the equilibration period and on days 0, 7, 14, 21, 28, and 33 during the exposure period. Each 5-mL sample was removed by pipet from the midpoint of the exposure tank and analyzed for ^{14}C content.

After 34 days of exposure, all of the fish were removed from the test system. The fish were rinsed, blotted dry, and dissected into three portions: fillet (edible tissue), viscera (nonedible tissue), and carcass (head, scales, and skeleton). All samples were frozen until analysis.

Radioactive Analysis. The tissues and nonextractable tissue solids were homogenized, and aliquots were oxidized in a R. J. Harvey Biological Material oxidizer OX-400 and OX-300. The resulting $^{14}CO_2$ was collected in 15 mL of Oxosol ^{14}C scintillation cocktail (National Diagnostics, Manville, NJ). Levels of radioactivity were measured by liquid scintillation counting (LSC) using either a Beckman Counter or an LKB-Wallac (Rack Beta scintillation counter). Tissue extracts and HPLC effluents were counted in ScintiVerse liquid scintillation cocktail (Fisher Scientific Co.).

Extraction and Cleanup. For isolation and identification work, homogenized tissue (20–40 g) was extracted with acetonitrile/water (8:2 v/v) or methanol/water (8:2 v/v) by stirring. The mixture was centrifuged for 10 min at 7000 rpm, and the

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Table I. ^{14}C Distribution in Edible and Nonedible Tissues of Fish Exposed to 1.0 mg of [^{14}C]Metolachlor for 34 Days

	% total ^{14}C in tissue					
tissue	hexane	$CHCl_3$/EtOAc	aqueous	protease/H_2O/MeOH extract	nonextractable	% recovery
edible	5.8	24.6	23.0	35.0	5.6	94.0
nonedible	5.8	27.3	48.1	9.4	2.9	93.5

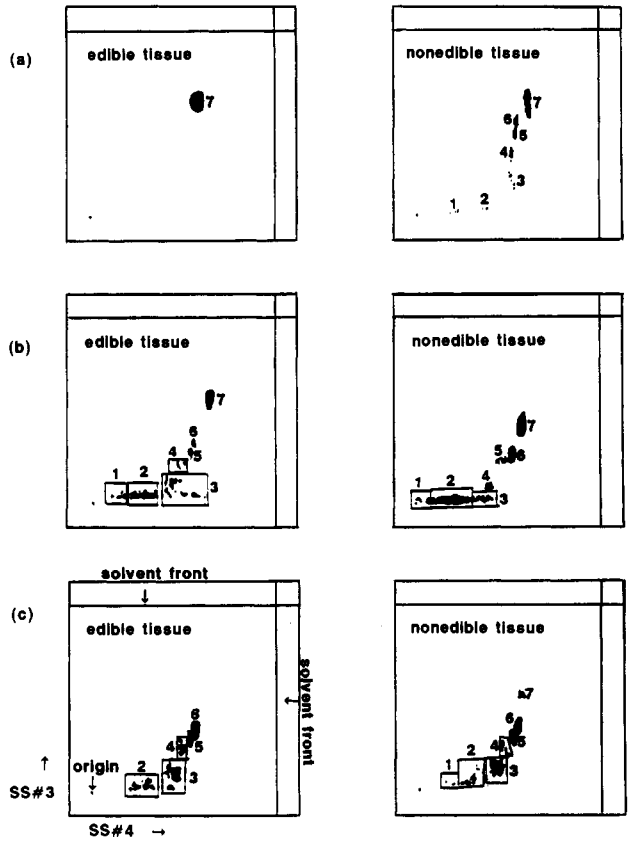

Figure 1. 2D-TLC metabolite patterns for (a) hexane fractions of edible tissue and nonedible tissue, (b) $CHCl_3$ fractions of edible tissue and nonedible tissue, and (c) EtOAc fractions after β-glucuronidase hydrolysis of aqueous fractions of edible tissue and nonedible tissue.

supernatant was decanted and filtered using glass microfiber filters (Whatman). The centrifuged pellet was reextracted twice using the same procedure. The supernatant filtrates were combined and partitioned once with hexane (1.5× volume). The methanol or acetonitrile was evaporated with a rotary evaporator under reduced pressure. The remaining aqueous solution was partitioned twice with 1.5× volume of chloroform and once with ethyl acetate. The ^{14}C content was analyzed by direct counting of the organic and aqueous fractions and by combustion of the nonextractable tissues.

The chloroform and ethyl acetate fractions were individually concentrated to dryness with a rotary evaporator. The residue was redissolved in methanol or acetonitrile for TLC and HPLC analysis.

Enzyme Hydrolyses. An aliquot of the aqueous fraction was hydrolyzed with β-glucuronidase (bovine liver, type B-1, Sigma). The aqueous sample was acidified to pH 4.6 with dilute acetic acid, and then 0.1 M sodium acetate buffer (pH 4.6) was added. The resulting solution was hydrolyzed at 37 °C overnight with the enzyme. The hydrolysate was partitioned with ethyl acetate as described above. The organic fractions were combined and concentrated for TLC and HPLC analyses.

The residue remaining after extraction (nonextractable) was treated with protease. The sample was homogenized in Tris/hydrochloric acid buffer (pH 7). The homogenate was incubated overnight at 37 °C with protease (bacterial, Sigma). The hydrolysate was centrifuged at 7000 rpm for 15 min, decanted, and filtered. The filter cake was extracted twice more by stirring with methanol/water (8:2) or acetonitrile/water (8:2) followed by centrifugation and decantation. The extracts were combined and concentrated for cleanup.

Sample Cleanup. To enhance the resolution of ^{14}C peaks, the aqueous fractions of the edible and nonedible tissue were partially purified. A portion of the aqueous fraction and the concentrate obtained from the protease-treated sample were applied to a C_{18} Bond Elut (Analytichem International) cartridge. Before sample application, each cartridge was prewashed with methanol and distilled water. The sample was applied, and the sorbent was washed with water. Radioactive metabolites were eluted with acetonitrile or methanol. Radioactive fractions were combined for HPLC analysis.

Thin-Layer Chromatography. Thin-layer chromatography (TLC) was performed on silica gel 60 F-254 plates, 0.25-mm thickness (Merck). Aliquots of samples and selected HPLC eluates were chromatographed with unlabeled standards. The TLC plates were developed in TLC chambers which were saturated by lining with blotter paper. Two-dimensional plates (2D-TLC) were developed 15 cm in the first solvent system (SS), dried, rotated counterclockwise 90°, and then developed 15 cm in the second solvent system. The solvent systems used were chloroform/acetonitrile (8:2 v/v) as the first system and toluene/acetic acid/water (50:50:4.5 v/v/v) as the second system. Radioactive zones were detected using either a Kodak XAR-2 X-ray film or a Berta Spark chamber (Raytest). The nonradiolabeled standards were visualized with a UV light, and photographs of the plates were taken with a Polaroid MP-3 Land camera.

HPLC Analysis. High-performance liquid chromatography (HPLC) was performed on a Perkin-Elmer 410 gradient pump system, a UV detector at a wavelength of 254 (Waters Associate Model 440), and a solid-cell radioactive detector (either Beckman 171 or Raytest Ramona-LS) Ram. The Ram and UV signals were synchronously registered on a two-channel recorder. A Beckman Ultrasphere ODS (10 mm i.d. × 25 cm, 5 μm), an Alltech Spherisorb ODS (4.6 mm i.d. × 25 cm, 5 μm), or a Du Pont Zorbax RX (4.6 mm i.d. × 25 cm, 5 μm) column was used. The mobile phases were composed of 20% methanol and 80% water containing 0.0125% trifluoroacetic acid (TFA) or 25% acetonitrile and 75% water containing 0.0125% TFA for the initial steps. The amount of methanol or acetonitrile was increased linearly up to 100%.

Nonconjugated metabolites were isolated from the organic fractions of both edible and nonedible tissues by HPLC. The organic fractions were composed of the chloroform and ethyl acetate fractions. The metabolites were effectively separated from parent metolachlor using the Beckman Ultrasphere ODS column and methanol/0.0125% TFA mobile phase.

Like the organosoluble metabolites, identification of the aqueous soluble metabolites was done with metabolites isolated from both edible and nonedible tissues. Using a Beckman Ultrasphere ODS preparative column and the same parameters used for the separation of the organosoluble metabolites, the partially purified aqueous sample was separated into five cluster peaks.

Quantification of Metabolites. The amount of metabolites in the organic fractions of the edible tissue and in the aqueous fractions from both tissues was determined by HPLC. Radioactive peaks were quantified by injecting a known amount of radioactivity into a HPLC column and collecting fractions into scintillation vials during the run. Radioactive peaks were collected individually and radioactive contents of the vials were determined by LSC. The metabolites in the organic fractions of the nonedible tissue were quantified by 2D-TLC. A known amount of radioactivity was chromatographed on a 20 cm × 20 cm silica plate and developed two dimensionally. The radioactive zones were observed as single spots and as clusters of spots. Radioactive zones were scraped into scintillation vials containing 5 mL of water. Ten milliliters of scintillation cocktail was added and mixed in a vortex. The radioactive contents of the vials were then radioassayed by LSC.

Thermospray HPLC/Mass Spectrometry (LC/MS). LC/MS spectra were obtained by a Vestec Model 201 equipped with

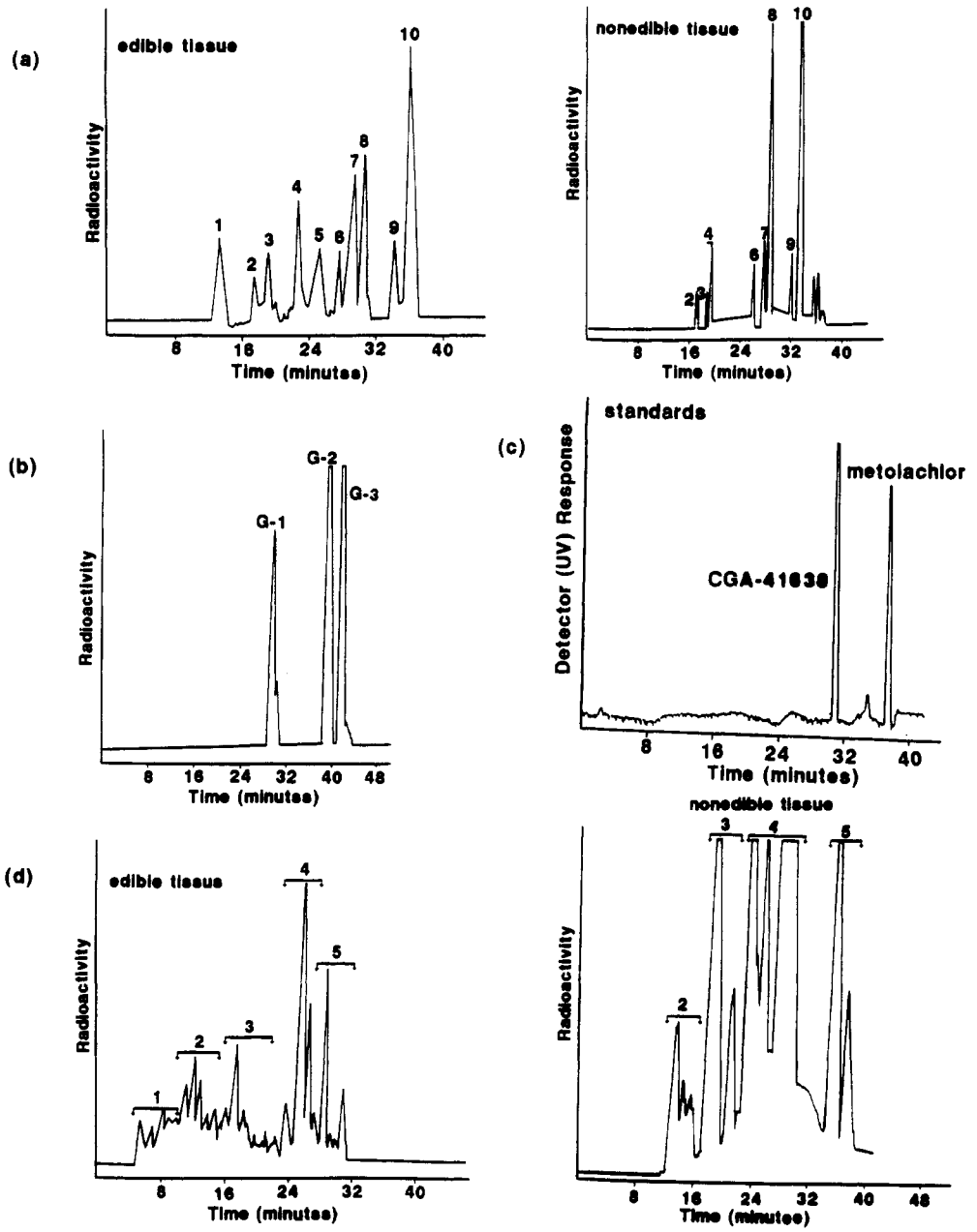

Figure 2. HPLC chromatograms of (a) organic fractions of edible tissue and nonedible tissue before enzyme hydrolysis (b) aglycons released after β-glucuronidase hydrolysis of aqueous fraction of edible tissue, (c) metolachlor and CGA-41638 standard, and (d) aqueous fractions of edible tissue and nonedible tissue.

Table II. Radioactivity Distribution of Metolachlor and Its Identified Metabolites in Fish Tissues

	% of total radioactivity in tissue	
metolachlor and metabolites	edible tissue	nonedible tissue
metolachlor (O-10)	18.0	5.7
O-4	0.6	1.5
O-7	0.4	0.4
O-8	1.5	7.7
A-1	0.1	*a*
A-2	2.9	*a*
A-3	2.2	*a*
total	25.7	15.3

[a] Not quantitated; high mass ratio of coextractants to metabolites.

a Waters Model 600MS pump and a Spherisorb ODS analytical column. An isocratic mobile phase of either 60% methanol or 70% methanol in acidified water (0.0125% TFA) was used.

Direct Insertion Probe/Mass Spectrometry (DIP/MS). Low-resolution mass spectral analysis of selected metabolites was performed on a VG-70-250-SQ Analytical instrument in the chemical ionization (CI) and electron impact (EI) mode at an electron energy of 70 eV. Samples were introduced by a heated direct insertion probe.

Fast Atom Bombardment/Mass Spectrometry (FAB/MS). Selected metabolites were analyzed by FAB/MS on a VG-70-250-SQ Analytical instrument. The spectra were acquired using an 8-kV Xe atom beam, and samples were dissolved in a glycerol/thioglycerol (10:3) matrix.

Nuclear Magnetic Resonance (NMR). The data were obtained with a 500-MHz NMR spectrometer (General Electric GN-500). Samples were dissolved in deuteriochloroform (Silano-C) into a semimicro tube with cylindrical cavity.

RESULTS

Tissue Residues. The radioactive levels in the edible and nonedible tissues of fish exposed to 1.0 mg/L [^{14}C]-metolachlor for 34 days were 13.9 and 220.3–250.8 ppm equivalents to [^{14}C]metolachlor, respectively. These radioactive residues were comparable to those obtained from the bioaccumulation study (Fackler, 1989). In this early study, the bluegill sunfish were continuously exposed to 0.10 mg/L metolachlor for 28 days in a dynamic system

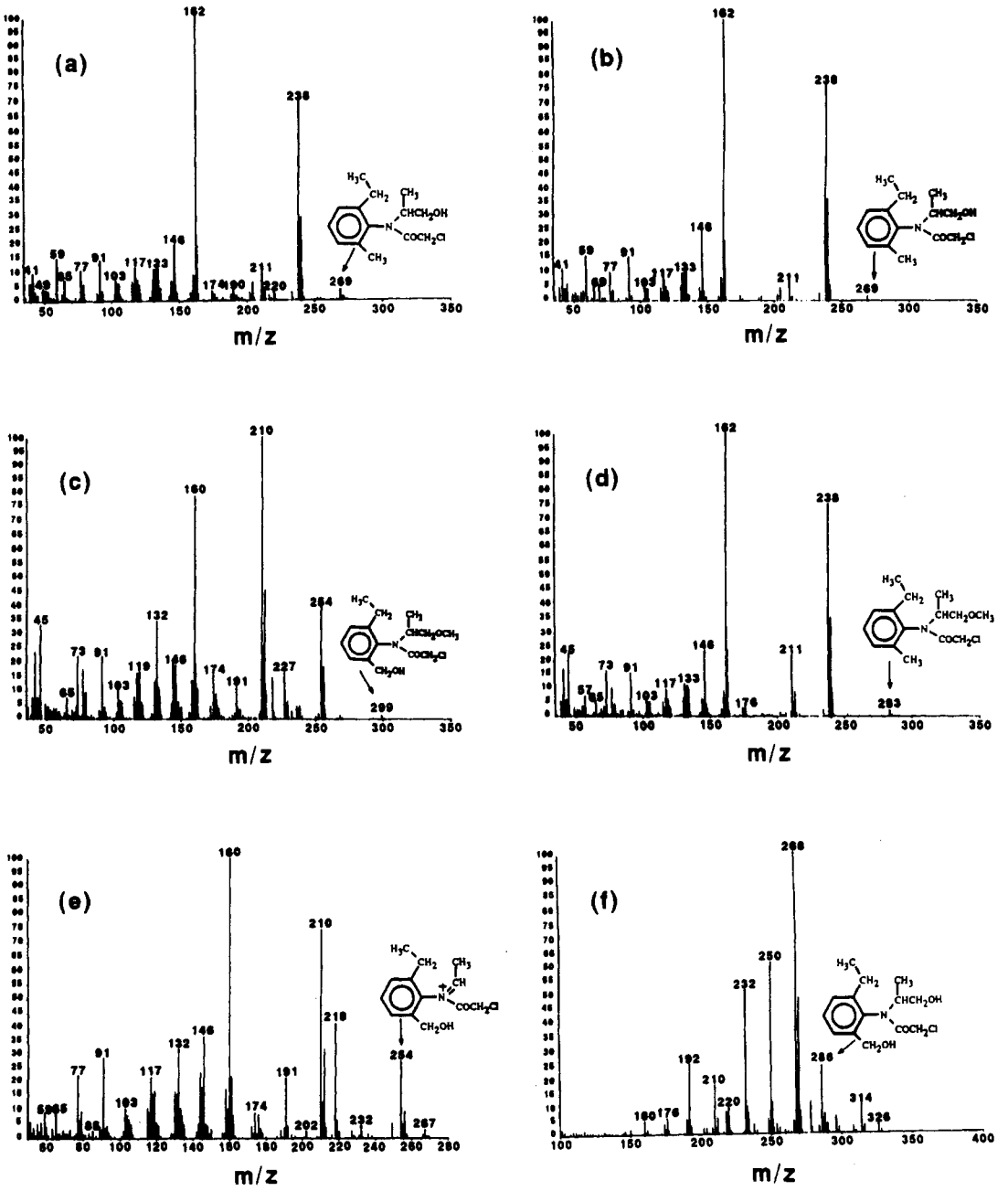

Figure 3. EI/MS spectra of (a) CGA-41638 standard, (b) metabolite O-8, (c) metabolite O-7, (d) metolachlor standard, and (e) metabolite O-4 and (f) CI/MS spectrum of metabolite O-4 or peak G-1.

followed by a 14-day depuration period. The mean equilibrium bioconcentration factors of metolachlor in bluegill sunfish were 15× in the edible tissue, 155× in the nonedible tissue, and 69× in the whole body. No effects on survival and behavior were observed during the exposure and depuration periods. The concentration of ^{14}C residues in the edible and nonedible tissues and whole fish reached steady state rapidly.

Characterization of Extractable ^{14}C Metabolites. Acetonitrile/water and methanol/water extraction resulted in 60% radioactive extractables in the edible tissue and 83.5% in the nonedible tissue. The distribution of radioactivity in the various fractions after partitioning with organic solvents is shown in Table I. Most of the radioactivity was found in the chloroform/ethyl acetate fraction and aqueous fraction for both tissues. However, the level of radioactivity in the aqueous fraction was relatively higher in the nonedible tissue, whereas the nonextractable fraction was higher in the edible tissue. These results suggest that metolachlor was extensively metabolized by the fish. The nonextractables (bound residues) in the edible tissue decreased from 40.0% to 5.6% after protease treatment followed by methanol/water extraction. This indicates that some of the radioactivity in the edible tissue was bound to proteins. Attempts to characterize the aglycons or metabolites released by protease were not successful. High mass ratio of coextractants/natural products (fats and lipids) to metabolites made characterization unattainable.

When the aqueous fraction of either the edible or nonedible tissue was subjected to β-glucuronidase, 2.2–5.8% of the total radioactivity partitioned into the ethyl acetate fraction. This indicates that either the aqueous metabolites are not all glucuronide conjugates (β-D-glucopyranosiduronic acid) or the aglycons released are too polar to partition into the organic phase. These aqueous-soluble metabolites could possibly be the other glucuronide conjugates (conjugated at N and S) and mercapturic acid pathway metabolites.

The nature of the extractable radioactivity in different fractions of both tissues was investigated by 2D-TLC and HPLC. Similar metabolite patterns were observed for

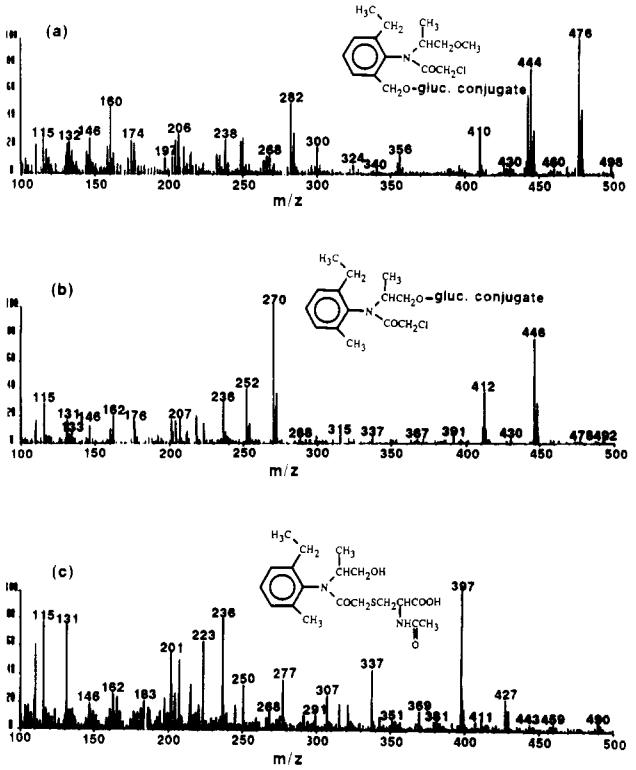

Figure 4. FAB/MS spectra of aqueous metabolites (a) A-2, (b) A-3, and (c) A-1.

the edible and nonedible tissue as shown in the 2D-TLC autoradiograms (Figure 1). The hexane fraction of the edible tissue contained one radioactive component, while that of the nonedible tissue contained more than one component. Similar metabolite profiles were also observed in the chloroform and ethyl acetate fractions of both tissues (Figure 1). Using solvent systems (SS) 3 and 4, seven zones were observed: a nonpolar region designated zone 7 and six regions of increasing polarity (zones 1–6). The major zone 7 and zone 6 cochromatographed with metolachlor standard and CGA-41638, respectively. The metabolites detected before enzymatic hydrolysis were also found after the β-glucuronidase hydrolysis (Figure 1). These data indicate that these metabolites are present as both free and glucuronide conjugates and that the metabolism of metolachlor is qualitatively similar in both the edible and nonedible tissues.

Like the 2D-TLC data, the HPLC profiles of the organosoluble metabolites showed several radioactive peaks, designated 1–10 (Figure 2). Peaks 1 and 5 were found only in the edible tissue, while the other peaks were found in both tissues. The average total radioactivity recovered from HPLC was 90.8% of the injected radioactivity. The distribution of radioactivity in metolachlor and its metabolites is given in Table II. Unchanged metolachlor (peak 10) was present at a relatively high concentration in the edible tissue but was present at a lower concentration in the nonedible tissue. Peaks 1–3, 5, 6, and 9 contained several minor radioactive components ranging from 0.1% to 0.5% of the total radioactivity in the tissue. These minor radioactive components exhibited chromatographic properties different from those of the nonlabeled standards. None of these minor metabolites could be isolated in sufficient quantities for definitive characterization studies. Peak 4 consisted mainly of metabolite O-4. Rechromatography of peak 7 in another mobile phase resulted in several components, with metabolite O-7 being the major peak. Metabolite O-7 was distributed at comparable levels in both edible and

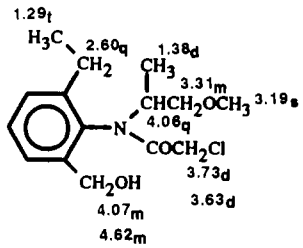

Figure 5. NMR data (values in parts per million) of metabolite O-7.

nonedible tissue. Its concentration was lower than that of the other organosoluble metabolites. Peak 8, which cochromatographed with CGA-41638 standard, was found in both tissues at relatively high concentrations compared with the other organosoluble metabolites. These metabolites were also present in the tissues as glucuronic acid conjugates.

The aglycons released by β-glucuronidase hydrolysis were separated into three major peaks, G-1, G-2, and G-3 as represented in Figure 2. Peaks G-1, G-2, and G-3 cochromatographed with metabolites O-4, O-7, and O-8, respectively. These results suggest that metabolites O-4, O-7, and O-8 are present as both free molecules and conjugates of glucuronic acid. Since the aglycons had higher radioactivity levels than their corresponding nonconjugated metabolites, they were used to isolate metabolites O-4, O-7, and O-8 for mass spectral analysis.

Using a Beckman Ultrasphere ODS preparative column and the same parameters used for the separation of the organosoluble metabolites, the partially purified aqueous sample was separated into five cluster peaks, 1–5. As presented in Figure 2, HPLC metabolite profiles for the edible and nonedible tissues are essentially the same. Cluster peaks 1–3 and 5 were further separated into several small components ranging from 0.1% to 0.6%, with >90% recovery of that injected onto HPLC column. Radioactivity associated with these four cluster peaks (1–3 and 5) accounted for 19.0% of the total radioactivity in the edible tissue. Attempts to isolate and characterize individual components using chromatographic techniques (HPLC and TLC) were unsuccessful due to the low level of radioactivity. Cluster peak 4 was also further resolved into three major peaks designated A-1, A-2, and A-3 and other minor peaks. Only a small amount of A-1 was detected in the edible tissue. The levels of radioactivity of these metabolites in the edible tissue are given in Table II. Determination of a meaningful quantitation of the levels for each metabolite in the aqueous fraction of the nonedible tissue was not possible because of the high mass ratio of coextractants (fats and lipids) to metabolites. It was apparent that there was a loss of some radioactivity during the partial purification of the aqueous fraction of the nonedible tissue. Since the level of radioactivity in the aqueous fraction was relatively higher in the nonedible tissue than in the edible tissue, it was deduced that the amounts of metabolites A-1, A-2, and A-3 would be higher in the nonedible tissue.

Spectral Analyses of Metabolites. The purified metabolites were subjected to thermospray HPLC/MS, FAB/MS, CI/MS, or EI/MS. The MS spectra of metabolites and standards are shown in Figures 3 and 4.

(1) O-10. The thermospray HPLC mass spectra of metolachlor standard and O-10 both gave a protonated molecular ion at m/z 284 $(MH)^+$. These data confirm that component O-10 is unchanged metolachlor. The molecular ions at m/z 284/286 in a ratio of 3:1 indicate the presence of one chlorine atom.

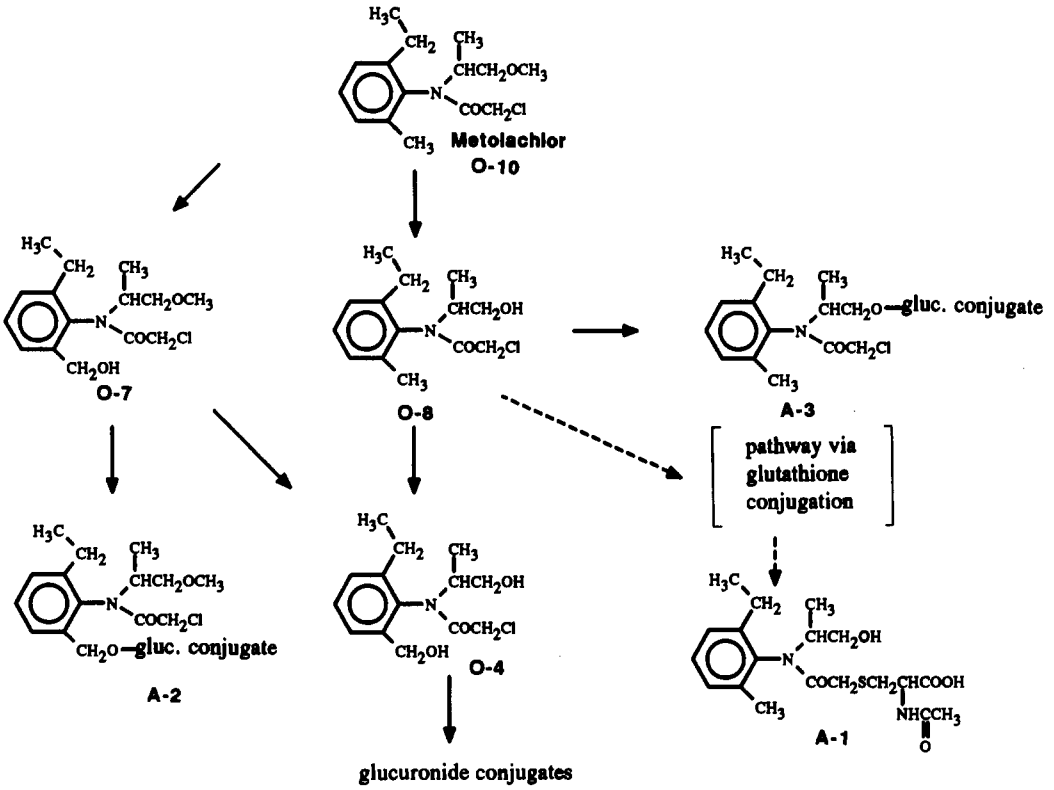

Figure 6. Proposed metabolic pathway of [^{14}C]metolachlor in bluegill sunfish.

(2) *O-8 or G-3.* The thermospray/MS spectra of synthetic CGA-41638 standard and aglycon G-3 show a protonated molecular ion at m/z 270 $(MH)^+$. As shown in Figure 3, the EI/MS spectra are identical and give molecular ion at m/z 269 and major fragments at m/z 238, 211, 162, and 146. These data indicate that aglycon G-3 is indeed CGA-41638. Metabolite O-8 cochromatographed with aglycon G-3 by both 2D-TLC and HPLC; therefore, metabolite O-8 is the nonconjugated 2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-hydroxy-1-methylethyl)acetamide. This metabolite was formed via O-demethylation on the *N*-(2-methoxy-1-methylethyl) side chain of metolachlor.

(3) *O-7 or G-2.* The thermospray/MS spectrum of the aglycon G-2 shows protonated molecular ions at m/z 300/302 in a ratio of 3:1. The EI/MS spectrum gives a very weak molecular ion at m/z 299, and it has a characteristic isotope pattern resulting from a chlorine ion (Figure 3). Comparison of the mass of G-2 to that of metolachlor standard shows the fragments of this aglycon to be 16 mass units higher than metolachlor standard, indicating the addition of an oxygen atom. The major fragments at m/z 254, 227, 210, and 160 and the rest of the spectra are consistent with the structure of a hydroxylated metolachlor. NMR data show that the benzylmethyl group rather than the benzylethyl group was hydroxylated (Figure 5). On the basis of the MS and NMR data, the structure of the aglycon G-2 is deduced as 2-chloro-*N*-[2-ethyl-6-(hydroxymethyl)phenyl]-*N*-(2-methoxy-1-methylethyl)acetamide. By both 2D-TLC and HPLC, aglycon G-2 cochromatographed with metabolite O-7; therefore, metabolite O-7 is the nonconjugated hydroxylated metolachlor.

(4) *O-4 or G-1.* The EI/MS spectrum of aglycon G-1 does not show the molecular ion but shows the major fragment peaks at m/z 254, 218, 210, and 160. The same characteristic fragment peaks were observed for the EI/MS spectrum of aglycon G-2. A CI/MS was done to obtain the molecular weight of G-1. The protonated molecular ion at m/z 286 is 16 mass units higher than the CGA-41638 standard, indicating the addition of an oxygen atom. The similarity of the fragmentation patterns of the aglycons G-1 and G-2 is indicative of hydroxylation at the benzylmethyl group. Metabolite O-4 cochromatographed with aglycon G-1. Therefore, metabolite O-4 is 2-chloro-*N*-[2-ethyl-6-(hydroxymethyl)phenyl]-*N*-(2-hydroxy-1-methylethyl)acetamide.

(5) *A-2.* The FAB/MS analysis of metabolite A-2 showed intense protonated ions at m/z 476/478 $(M + 1)^+$ and a chlorine isotopic pattern (Figure 4). This indicates that the molecular mass is 475, 176 mass units higher than that of the aglycon G-2. The fragment peak pattern of metabolite A-2 is consistent with the structure of a glucuronide conjugate. The fragment peak at m/z 300 is due to the loss of the glucuronyl moiety, indicating that the glucuronide is linked through an aliphatic hydroxyl group (Fenselau and Johnson, 1980). The isolated glucuronide was treated with β-glucuronidase, and the released aglycon was subjected to CI/DIP/MS. The mass spectral data confirm that G-2 is the aglycon of metabolite A-2. These data indicate that metabolite A-2 is the glucuronide conjugate of 2-chloro-*N*-[2-ethyl-6-(hydroxymethyl)phenyl]-*N*-(2-methoxy-1-methylethyl)acetamide.

(6) *A-3.* Similarly, FAB/MS analysis of metabolite A-3 gave protonated molecular ions at m/z 446/448 and has a chlorine isotope characteristic ion pair at m/z 270/272 (Figure 4). The loss of 176, which is the mass of a glucuronic acid moiety, produced the fragment peak at m/z 270/272. The fragment peaks are consistent with the assigned structure of a glucuronide conjugate. The spectrum of the released aglycon of A-3 was identical with that obtained for aglycon G-3. These data confirm that metabolite A-3 is the glucuronide conjugate of metabolite O-8, which is 2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-hydroxy-1-methylethyl)acetamide.

(7) *A-1.* The FAB analysis of peak 4B-2A produced a spectrum containing several fragment ions. However, the isotope cluster characteristic of a chlorine ion was not observed (Figure 4). An intense protonated molecular ion

was detected at m/z 397 $(MH)^+$. The presence of the base peak fragments at m/z 337, 268, 250, 236, 162, 146, and 131 identified A-1 as a mercapturic acid (*N*-acetylcysteine) conjugate. This metabolite was probably formed via glutathione conjugation through the displacement of the chlorine atom of the chloroacetyl side chain of metabolite O-8 and subsequent breakdown of the glutathione tripeptide to *N*-acetylcysteine.

Attempts to isolate and identify several other individual components using chromatographic techniques and mass spectrometry were unsuccessful. Low levels of radioactivity made identification impossible. However, formation of other metabolites of metolachlor such as the hydroxylated degradates found in the soil actinomycete study (Krause et al., 1985), glucuronide conjugates of these hydroxyl compounds (Feng and Patanella, 1989), and glutathione conjugates and mercapturic acid pathway metabolites (Feng and Patanella, 1988) could not be excluded.

DISCUSSION

In this study, the results showed that the pathways of the transformation of metolachlor by bluegill sunfish are very similar to those observed in animals, soil, and plants. The initial oxidation metabolites O-4, O-7, and O-8 were also the metabolites of metolachlor produced by a soil actinomycete (Krause et al., 1985) and by rat liver microsomal enzymes (Feng and Wratten, 1989). These pathways include hydroxylation of the benzylmethyl group to form metabolite O-7. In addition to a hydroxyl group at the aromatic methyl side chain, O-demethylation at the N-alkyl substituent of metolachlor occurred, yielding metabolite O-4. Metabolite O-8, resulting from O-demethylation alone, was also found as a degradation product in rats (LeBaron et al., 1988) and soil fungus (McGahen and Tiedje, 1978). These initial oxidation metabolites undergo further transformation via glucuronic acid conjugation forming metabolites A-2 and A-3. Feng and Patanella (1989) demonstrated that glucuronide conjugation of alachlor was observed using liver microsomal enzymes from rats, mice, and monkeys.

Another metabolic pathway common to chloroacetanilides is glutathione conjugation through displacement of the chlorine atom. Propachlor (Larsen and Bakke, 1981), alachlor, and metolachlor (Feng and Wratten, 1989) were shown to undergo glutathione conjugation in animals. Metolachlor was also converted to glutathione conjugates in plants (LeBaron et al., 1988). It is possible that the *N*-acetylcysteine conjugate metabolite A-1 was produced through the glutathione conjugate intermediate by bluegill sunfish liver enzyme. As in mammals, the liver is the main organ involved in the biotransformation of foreign compounds in fish (Pesonen and Andersson, 1991). Feng and Patanella (1988) demonstrated that, using cystolic fractions from rat, mouse, and monkey livers, the glutathione conjugate of alachlor was further degraded through the mercapturic acid pathway to their corresponding cysteinylglycine, cysteine, and *N*-acetylcysteine conjugates. Mercapturic acids are the most common end products of metabolism via glutathione conjugation in mammals. It appears that fish can convert glutathione conjugates to mercapturic acid in a manner similar to that of mammals.

In summary, bluegill sunfish utilized many known pathways as the detoxification mechanism in the metabolism of metolachlor. These pathways include O-demethylation and hydroxylation of the benzylmethyl group followed by glucuronide conjugation and reductive dechlorination with subsequent glutathione conjugation. The mercapturic acid conjugate was produced by the breakdown of the glutathione tripeptide to *N*-acetylcysteine. Mass spectral identification of several metabolites supports the proposed metabolic pathways (Figure 6) of [^{14}C]-metolachlor in bluegill sunfish.

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