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In Vitro Transformation of Chloroacetanilide Herbicides by Rat Liver Enzymes: A Comparative Study of Metolachlor and Alachlor

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The in vitro transformation of metolachlor by rat liver cytosolic and microsomal enzymes was studied. In the presence of glutathione (GSH), the liver cytosolic enzymes catalyzed complete conversion of metolachlor to a more polar metabolite identified as the metolachlor–GSH conjugate. Liver microsomal enzymes, fortified with NADPH, catalyzed O-demethylation, benzylic hydroxylation, and N-dealkylation reactions of metolachlor. Eight oxidized metabolites were identified involving single, double, and triple hydroxylations at four different sites in the metolachlor molecule. The in vitro transformation of metolachlor by rat liver enzymes was similar to that observed with alachlor; both herbicides readily undergo conjugation with GSH and oxidation. The rate of GSH conjugation by rat liver cytosolic enzymes was significantly faster for alachlor than metolachlor. The rate of oxidation by rat liver microsomal oxygenases was similar for alachlor and metolachlor.

Metolachlor [2-chloro-*N*-(2-methoxy-1-methylethyl)-*N*-(2-ethyl-6-methylphenyl)acetamide] and alachlor [2-chloro-*N*-(methoxymethyl)-*N*-(2,6-diethylphenyl)acetamide] are the two most widely used commercial chloroacetanilide herbicides.

Alachlor Metolachlor

The metabolism of metolachlor in soil, plants, and animals was recently summarized by LeBaron et al. (1988). Although details of the studies were not presented in that report, O-demethylation and hydrolytic dechlorination of metolachlor appeared to be the major pathways of metabolism in rats. O-Demethylation has also been reported as a major pathway of metabolism for metolachlor in a soil fungus (McGahen and Tiedje, 1978) and a soil actinomycete (Krause et al., 1985).

Our laboratory has been conducting metabolism studies of chloroacetanilide herbicides in animals. Based on our experience, in vitro incubations with animal tissue fractions are very useful in predicting the in vivo metabolism and in elucidating the initial and intermediary pathways of metabolism. Alachlor is rapidly degraded in animals through a complex network of several pathways (Sharp, 1988). We have previously examined the in vitro transformation of alachlor by liver and kidney enzymes from rats, mice, and monkeys (Feng and Patanella, 1988, 1989) and have found good qualitative correlation between the results of in vivo and in vitro studies.

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The present study examines the in vitro transformation of metolachlor by rat liver enzymes. Our first objective was to compare the in vitro metabolism of alachlor with metolachlor. A second objective was to compare the results of the in vitro rat metabolism of metolachlor with those reported for the rat (LeBaron et al., 1988) and soil microorganisms (McGahen and Tiedje, 1978; Krause et al., 1985). This report describes the identification of metabolites from the in vitro transformation of metolachlor by the liver cytosolic and microsomal enzymes from male rats. The rates of transformation of metolachlor and alachlor by rat liver cytosolic and microsomal enzymes were also examined.

MATERIALS AND METHODS

Chemicals. Metolachlor (^{14}C-phenyl, UL), 15.2 mCi/mmol, was synthesized by Pathfinder Laboratories, Inc. (St. Louis, MO), and alachlor (^{14}C-phenyl, UL), 19.6 mCi/mmol, was synthesized by New England Nuclear, Inc. (Boston, MA). The chemical and radiological purities of metolachlor and alachlor were determined by HPLC analysis to be greater than 99%. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione (GSH) were obtained from Sigma Chemical Co., and *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce Chemical Co.

Liver Homogenate Preparation. Liver microsomal and cytosolic fractions (100000*g*) were prepared from the pooled organs of five naive male Long Evans rats (Charles River Laboratories, average body weight 250 g) based on a previously published procedure (Feng and Wratten, 1987). The protein concentration of the tissue fractions was measured by the method of Bradford (1976).

Liver Cytosolic Incubation. In a final volume of 1 mL, radiolabeled metolachlor or alachlor (0.05 μmol) was incubated with the liver cytosolic fraction (0.5 mg of protein) in the presence of GSH (5.0 μmol) in pH 7.4 phosphate buffer (0.1 mol) at 37 °C. For the control reaction, the cytosolic preparation was boiled for 10 min prior to incubation. The reaction mixture was sampled (100 μL) at various time intervals, and the proteins were precipitated with 100 μL of ice-cold methanol. Following centrifugation, the resulting supernatant was analyzed by HPLC/RAD (radioactivity detection).

Liver Microsomal Incubation. In a final volume of 1 mL, metolachlor or alachlor (0.05 μmol) was incubated with the liver microsomal fraction (1.5 mg of protein) in the presence of reduced NADPH (3.0 μmol) and $MgCl_2$ (5.0 μmol) in pH 7.4 phosphate buffer (0.1 mol) at 37 °C. Control incubations were conducted in the absence of NADPH and with the heat-denatured enzymes. The reaction mixture was sampled and analyzed as described above.

Metabolite Identification. The metabolites produced from the incubation of alachlor with rat liver preparations have been previously described (Feng and Patanella, 1988, 1989). For the metolachlor studies, the cytosolic incubation produced a single metabolite, which was purified by HPLC/RAD and analyzed by negative-ion fast atom bombardment (FAB) mass spectrometry. The microsomal oxidation products of metolachlor were isolated by extracting the incubation mixture with methylene chloride. The organic layer was dried with anhydrous sodium sulfate, treated with BSTFA/pyridine (4:1), and analyzed as a mixture by capillary GC/MS. In a separate experiment, the major microsomal oxidation products of metolachlor were also individually purified by HPLC/RAD prior to BSTFA derivatization and mass spectral analysis.

Instrumentation. A Waters μBondapak C-18 column (3.9 mm × 30 cm, 10 μm) was employed for the HPLC analysis of the cytosolic incubation mixtures. The gradient was programmed linearly from 20 to 100% acetonitrile in 5 mM acetate buffer (pH 4.6) over 15 min at a flow rate of 1 mL/min. The same HPLC column was employed for the analysis of the microsomal incubation mixtures. The program was maintained isocratically at 35% acetonitrile in acetate buffer (5 mM, pH 4.6) for 20 min, followed by a linear gradient to 100% acetonitrile over 5 min. The program remained at 100% acetonitrile for the duration of analysis. The HPLC effluent was sequentially detected by a UV (254 nm) monitor and by a radioactivity flow monitor (Packard RAM 7500) equipped with a solid scintillant cell. FAB mass spectral analysis was conducted on a VG ZAB instrument using glycerol as the matrix and xenon as the atom source. Capillary GC/MS analysis in the chemical ionization/isobutane (CI) mode was conducted on a Finnigan 4535 quadrupole system using a DB-5 (25 m × 0.32 mm) capillary column (J & W Scientific Inc.).

RESULTS

Metolachlor Cytosolic Incubation. Incubation of metolachlor with the rat liver cytosolic enzymes in the presence of GSH produced a single more polar metabolite. Conversion of substrate to product was complete, and formation of other products was not observed. The rate of formation of this metabolite was reduced by at least 7-fold by prior heat denaturization of the enzyme preparation, indicating that the reaction was enzyme catalyzed. After 1 h, the incubation was stopped by precipitating the proteins with methanol, and analysis by liquid scintillation counting showed that greater than 98% of the radioactivity was recovered in the supernatant. The metabolite, following isolation by HPLC/RAD, was identified by negative-ion FAB mass spectral analysis. The mass spectrum showed deprotonated molecular ions at m/z 553 (M – H) with a 10% relative intensity (RI) and deprotonated molecular ions of the sodium salt analogue (m/z 575, M + Na – 2 H, 3% RI); fragment ions of the metabolite were not observed. The molecular weight of 554 corresponded to a structure resulting from the displacement of the chlorine atom of metolachlor by the GSH-thiol (**2**; Figure 1). The transformation of metolachlor to **2** was previously observed in corn (O'Connell et al., 1988) and was also postulated to occur in rats (LeBaron et al., 1988). Among other chloroacetanilides, both alachlor (Feng and Patanella, 1988) and propachlor (Larsen and Bakke, 1983) have been shown to be metabolized via the GSH pathway in animals.

Metolachlor Microsomal Incubation. The incubation of metolachlor with the rat liver microsomal preparation in the presence of reduced NADPH led to the formation of at least eight metabolites. The formation of these metabolites was dependent on the presence of NADPH and viable enzymes. After 1 h of incubation and protein precipitation with methanol, the recovery of ^{14}C radioactivity in the supernatant was determined to be greater than 98%. A radioactivity HPLC profile of a typical incubation mixture is shown in Figure 2. For metabolite identification, the radioactivity in the deproteinated incubation mixture was extracted into methylene chloride (93% efficiency), derivatized with BSTFA, and analyzed by chemical ionization capillary GC/MS.

Unreacted metolachlor (**1**) accounted for 27% of the radioactivity (Figure 2). Metolachlor was not silanized, and its CI mass spectrum showed protonated molecular ions at m/z 284 (100% RI). Fragment ions were observed at m/z 252 (MH – 32, 2% RI) from the loss of methanol and indicated an intact *N*-(2-methoxy-1-methylethyl) side chain.

Eight metabolites were identified from the oxidation of metolachlor by rat liver microsomes (**3**–**10**; Figure 2), and their characteristic mass spectral ions are listed in Table I. Isotope patterns consistent with the presence of a chlorine atom were observed in the mass spectra of all these metabolites and established that the chlorine atom in metolachlor was not removed during oxidative metabolism. Metabolites **3**–**6** were primary products resulting from a single oxidation reaction at four different sites in the metolachlor molecule, and metabolites **7**–**10** were secondary products resulting from two or three multiple oxidations.

Among the initial oxidation pathways, the most abun-

Table I. Major Ions from the Capillary GC/MS Chemical Ionization Analysis of the TMS-Derivatized Products of Metolachlor Oxidation by Rat Liver Microsomal Enzymes

compd	no.	m/z (% rel intens)			
		MH	MH − 90	MH − 32	MH − 74
CH_3O, O, N, Cl, CH_3, CH_2CH_3	1	284 (100)		252 (2)	
TMSO, O, N, Cl, CH_3, CH_2CH_3	3	342 (100)			
CH_3O, O, N, Cl, $(TMSO)CH_2$, CH_2CH_3	4	372 (100)	282 (10)	340 (2)	
H, O, N, Cl, CH_3, CH_2CH_3	5	212 (100)			
CH_3O, O, N, Cl, CH_3, $CH(OTMS)CH_3$	6	372 (100)	282 (95)	340 (3)	
TMSO, O, N, Cl, CH_3, $CH(OTMS)CH_3$	7	430 (100)	340 (42)		
TMSO, O, N, Cl, $(TMSO)CH_2$, CH_2CH_3	8	430 (100)	340 (2)		
CH_3O, O, N, Cl, $(TMSO)CH_2$, $CH(OTMS)CH_3$	9	460 (100)	370 (50)	428 (18)	
TMSO, O, N, Cl, $(TMSO)CH_2$, $CH(OTMS)CH_3$	10	518 (92)	428 (15)		444 (100)

dant product (**3**, 27% of the radioactivity) resulted from O-demethylation on the *N*-(2-methoxy-1-methylethyl) side chain of metolachlor. Capillary GC/MS analysis of trimethylsilyl (TMS) derivatized **3** produced protonated molecular ions at m/z 342 (100% RI) (Table I). The molecular weight of 341 and the absence of fragment ions from the loss of methanol were consistent with a TMS-derivatized primary alcohol resulting from the loss of a methyl group on the *N*-(2-methoxy-1-methylethyl) side chain of metolachlor.

Benzylic hydroxylation at the phenylmethyl or phenylethyl positions produced the isomeric metabolites **4** and **6**, respectively. These two metabolites accounted for 31% of the radioactivity in the incubation mixture. Capillary GC/MS analysis of the TMS-derivatized metabolites produced protonated molecular ions (m/z 372) accompanied by ions from the loss of methanol (m/z 340, $MH^+ - 32$) and ions from the loss of the trimethylsilanol (m/z 282, $MH^+ - 90$). The isomeric metabolites **4** and **6** were distinguished mass spectrometrically by the intensity of the fragment ions produced from the loss of trimethylsilanol (Table I). The metabolite assigned as **6**, with the *O*-TMS function located on the phenylethyl position, would be expected to readily lose trimethylsilanol (m/z 282, 95% RI), whereas a similar loss would not be favored by the metabolite assigned as **4** with the *O*-TMS function located on the phenylmethyl group (m/z 282, 10%). Capillary GC/MS (CI) analysis of the underivatized metabolites **4** and **6** showed a similar pattern with regard to the intensity of ions resulting from the loss of water from the protonated parent ions. Among other chloroacetanilides, both alachlor (Feng and Patanella, 1989) and acetochlor (Feng, unpublished results), have also been shown to be extensively hydroxylated on the phenylethyl or phenylmethyl side chains. Fragment ions resulting from the loss of water during mass spectral analysis were very characteristic of phenylhydroxyethyl or phenylhydroxymethyl metabolites of chloroacetanilides.

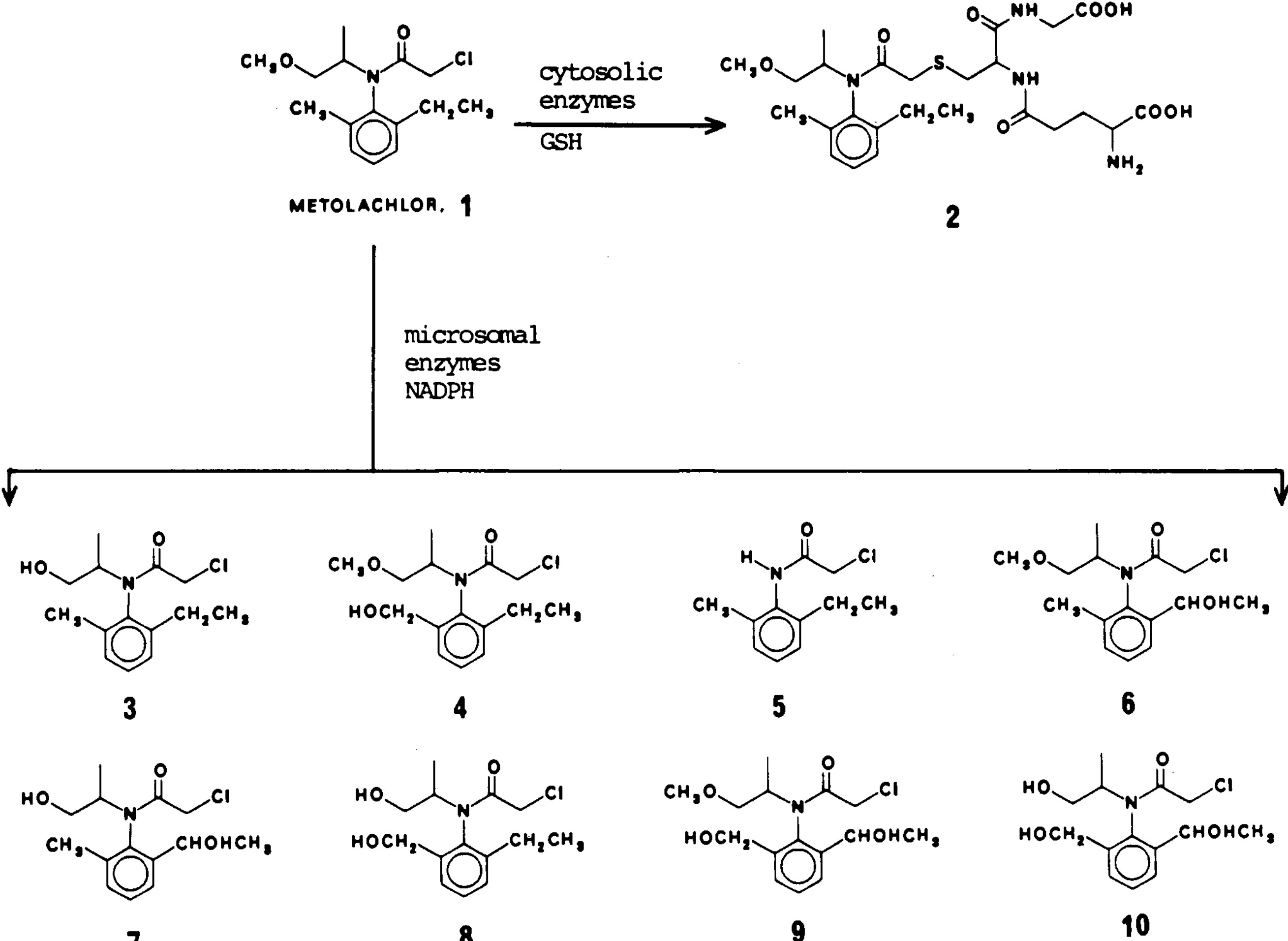

Figure 1. Summary of the in vitro metabolism of metolachlor by rat liver enzymes.

The hydroxyl function on the phenylethyl side chain of metabolite **6** can be located on either ethyl 1-position (benzyl) or ethyl 2-position. The transformation of metolachlor by the soil actinomycete (Krause et al., 1985) produced metabolites hydroxylated only at the benzylic position as determined by NMR. With alachlor, the hydroxylation of one or both phenylethyl side chains by animal liver microsomes also occurs exclusively at the benzylethyl position as determined by acidic hydrolysis (Feng and Patanella, 1989). On the basis of these precedents, we proposed that hydroxylation of the phenylethyl side chain of metolachlor by rat liver microsomes also occurs at the benzylic position, thereby producing metabolite **6**.

In a separate experiment, the major metabolites **3**, **4**, and **6** were individually isolated from HPLC/RAD and derivatized with BSTFA, and their structures were reconfirmed by capillary GC/MS analyses. Metabolites **3**, **4**, and **6** were also observed during the actinomycete metabolism of metolachlor and were characterized in greater detail by Krause et al. (1985).

A third type of initial oxidation reaction of metolachlor involves N-dealkylation. Since metabolite **5** was not derivatized by BSTFA, analysis by capillary GC/MS (CI) produced protonated molecular ions at m/z 212 (100% RI; Table I). Only very small amounts (1% of radioactivity) of **5** were produced from metolachlor by rat liver microsomes. Its structural identification was supported by mass spectral comparison with a synthetic standard produced by acylation of 2-ethyl-6-methylaniline with chloroacetyl chloride. Other chloroacetanilide herbicides such as propachlor (Larsen and Bakke, 1979) and alachlor (Feng and Patanella, 1989) are also N-dealkylated during animal metabolism. In contrast to metolachlor, N-dealkylation of alachlor constituted a major transformation pathway by rat liver microsomes.

Of the four additional oxidation products, metabolites **7**–**9** resulted from double oxidations and metabolite **10** from a triple oxidation. The combined radioactivity for these four metabolites accounted for 14%. These polar metabolites had retention times of less than 13 min in the reversed phase HPLC/RAD chromatogram (Figure 2). The HPLC fraction containing these metabolites was silanized and analyzed as a mixture in capillary GC/MS.

Protonated molecular ions at m/z 430 (100% RI; Table I) were observed in the mass spectra of the TMS-derivatized metabolites **7** and **8**. The molecular weight of 429 was consistent with the presence of two *O*-TMS functions and suggested that the isomeric metabolites **7** and **8** were produced by O-demethylation plus benzylic hydroxylation on either the phenylmethyl or phenylethyl side chains. The location of the *O*-TMS function on the ethyl or methyl side chains in these two isomeric metabolites could be distinguished based on the intensity of the MH − 90 ions (m/z 340) in their mass spectra (Table I), as in metabolites **4** and **6**.

Protonated molecular ions at m/z 460 (100% RI; Table I) were observed in the mass spectrum of metabolite **9** and supported the presence of *O*-TMS functions in both the phenylmethyl and phenylethyl side chains in this metabolite. Fragment ions resulting from the loss of methanol (m/z 428, 18% RI) were present, and this was consistent with an intact *N*-(2-methoxy-1-methylethyl) side chain in the metabolite structure.

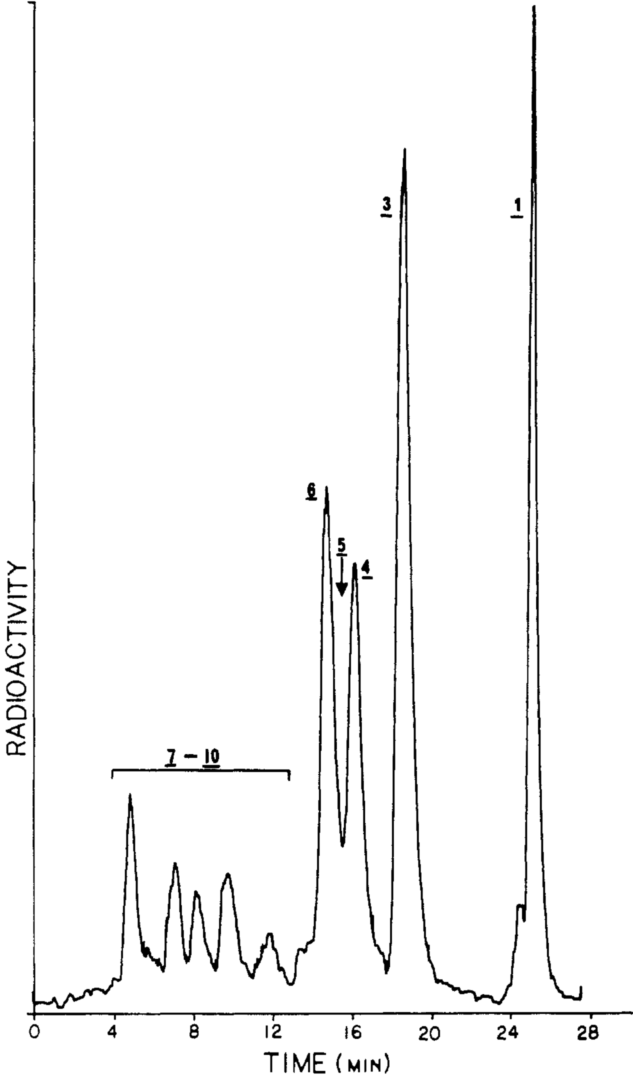

Figure 2. [14]C radioactivity chromatogram produced by HPLC separation of a 1-h incubation mixture of metolachlor with rat liver microsomal enzymes.

The mass spectral analysis of TMS-derivatized metabolite **10** showed protonated molecular ions at m/z 518 (92% RI; Table I). This molecular weight was consistent with the presence of three O-TMS functions and suggested a structure resulting from O-demethylation and benzylic hydroxylations at both the phenylmethyl and phenylethyl side chains of metolachlor. The mass spectrum was characterized by the absence of fragment ions resulting from the loss of methanol and by the presence of fragment ions at m/z 444, which appeared to have resulted from the loss of trimethylsilane (MH − 74).

Comparative Metabolism of Metolachlor and Alachlor. On the basis of results from the present studies with metolachlor and from the previous studies with alachlor (Feng and Patanella, 1988, 1989), it is apparent that both chloroacetanilides are readily transformed by the liver enzymes of rats.

Conjugation of metolachlor or alachlor with GSH presumably takes place via catalysis by liver cytosolic GST (Jakoby and Habig, 1980). With identical incubation parameters, the rates of GSH conjugation by rat liver cytosol were examined for both metolachlor and alachlor (Figure 3). A comparison of the initial velocities of the reactions showed that the rate of formation of the GSH conjugate was about 20 times faster for alachlor than metolachlor. On the basis of similar incubations with heat-denatured enzymes, the contribution from the non-

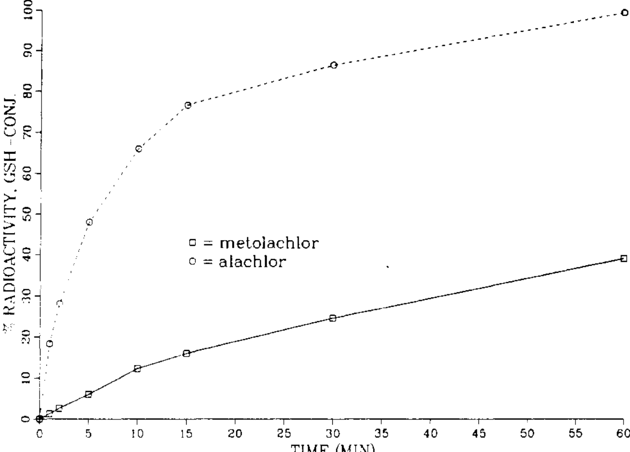

Figure 3. Rate of formation of the GSH conjugates of metolachlor and alachlor catalyzed by GSH-fortified rat liver cytosolic enzymes.

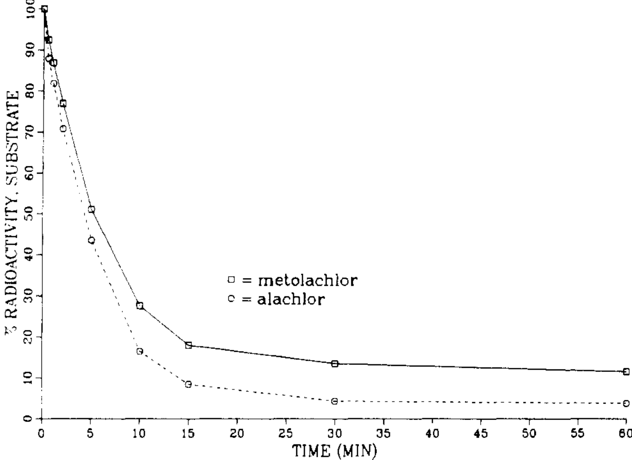

Figure 4. Rate of oxidation of metolachlor or alachlor catalyzed by NADPH-fortified rat liver microsomal enzymes.

enzymatic conjugation of metolachlor and alachlor with GSH was judged to be insignificant.

The rates of oxidation of metolachlor and alachlor by NADPH-fortified rat liver microsomal enzymes are shown in Figure 4. On the basis of initial velocities of substrate disappearance, alachlor was oxidized at a slightly faster rate than metolachlor.

DISCUSSION

The in vitro metabolism of metolachlor by rat liver enzymes is summarized in Figure 1. The GST enzymes in the liver cytosol catalyzed the formation of metolachlor–GSH conjugate **2**. Further degradation of **2** by liver cytosolic enzymes from rats was not observed. The liver microsomal enzymes catalyzed multiple-oxidation reactions of metolachlor, including O-demethylation, N-dealkylation, and benzylic hydroxylations. These reactions required the presence of NADPH and are typical of reactions catalyzed by the microsomal cytochrome P-450 mixed-function oxygenases (Wislocki et al., 1980).

The results from the present study showed that the pathways of in vitro transformation of metolachlor by rat liver enzymes are very similar to those observed with alachlor (Feng and Patanella, 1988, 1989). Both herbicides are readily conjugated with GSH via catalysis by GST and are oxidized at multiple sites via catalysis by cytochrome P450 mixed-function oxygenases. One major difference observed between metolachlor and alachlor in the present study was the 20-fold faster rate of GSH conjugation with alachlor than metolachlor. On the basis of these results,

we predict that although the rates of oxidation of alachlor and metolachlor are expected to be comparable, the rates of their detoxication in rats via GSH conjugation are expected to be faster for alachlor than metolachlor.

In a comparative plant metabolism study, O'Connell et al. (1988) observed a 2- to 3-fold faster rate of GSH conjugation for alachlor than metolachlor in corn. It is apparent that, in both plants and animals, alachlor can be expected to be metabolized at a faster rate than metolachlor by GST enzymes. It is possible that the longer persistence of metolachlor than alachlor in soil (Walker and Brown, 1985) is also influenced by the reactivity of these chloroacetanilides with the GST enzyme in soil microorganisms.

A good correlation has been observed between the in vitro (Feng and Patanella 1988, 1989) and in vivo (Sharp, 1988) metabolism of alachlor in rats. By analogy, our in vitro results suggest GSH conjugation, O-demethylation, and benzylic hydroxylation as the major pathways of metolachlor metabolism in rats. LeBaron et al. (1988) postulated GSH conjugation as a pathway of metolachlor metabolism in rats; our identification of the metolachlor–GSH conjugate during in vitro metabolism provides direct evidence for this pathway. LeBaron et al. (1988) also reported O-demethylation and hydrolytic dechlorination as major pathways and benzylic hydroxylation as a minor pathway in the metabolism of metolachlor in rats. With the exception of hydrolytic dechlorination, O-demethylation and benzylic hydroxylation of metolachlor have been demonstrated during our in vitro metabolism studies. It is noteworthy that the in vitro oxidation of metolachlor by rat liver microsomes produced metabolites that were almost identical with those observed with the soil actinomycete (Kraus et al., 1985). In comparison, metabolite **3** resulting from O-demethylation of metolachlor was the only common product of degradation in the soil actinomycete (Kraus et al., 1985) and fungus (McGahen and Tiedje, 1978). Conjugation of metolachlor with GSH by soil microorganisms was not detected.

In summary, the in vitro metabolism of metolachlor with rat liver preparations demonstrated pathways of GSH conjugation, O-demethylation, benzylic hydroxylation, and N-dealkylation, all of which were either postulated or observed in rat metabolism studies (LeBaron et al., 1988). Our work supports the utility of in vitro methods in predicting and rationalizing the metabolism of chloroacetanilides in animals.

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Registry No. **1**, 51218-45-2; **2**, 106133-39-5; **3**, 65513-61-3; **4**, 51218-45-2; **5**, 32428-71-0; **6**, 121073-75-4; **7**, 96394-95-5; **8**, 96411-89-1; **9**, 121073-76-5; **10**, 121073-77-6; alachlor, 15972-60-8.

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