# Metabolism of Metolachlor by a Microsomal Fraction Isolated from Grain Sorghum (*Sorghum bicolor*) Shoots

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A microsomal fraction isolated from the shoots of 3- to 4-day-old, dark-grown, grain sorghum (*Sorghum bicolor cv.* Funk G 522 DR) seedlings was characterized. The preparations had a cytochrome P-450 content that varied from approximately 90 to 150 pmol P-450/mg protein with cytochrome P-420 varying from 0 to 3% of the P-450 content. Type I difference spectra were formed with cinnamic acid and metolachlor, and a type II spectrum was formed with tetcyclacis. In short-term assays with [$^{14}$C]metolachlor as substrate, the preparations produced a single time-dependent product that separated on silica gel TLC plates developed in benzene/acetone (2:1, v/v). $R_F$ values for metolachlor and the metabolite were approximately 0.70 and 0.48, respectively. The microsomal reaction required NADPH and oxygen, and was inhibited by carbon monoxide, with the inhibition being partially reversed by actinic light. Compounds known to inhibit the activity of cytochrome P-450 monooxygenases (piperonyl butoxide, tetcyclacis, and tridiphane) also prevented formation of the metabolite. Identity of the metabolite was confirmed by TLC and positive ion thermospray LC/MS to be 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-hydroxy-1-methylethyl)acetamide. Hence, the reaction catalyzed by the sorghum microsomes involved O-demethylation of the methoxypropyl side chain of metolachlor.

## Introduction

In plants, as in animals, parallel pathways participate in the metabolism of pesticides. Two of the major pathways are (a) the GSH system and (b) the microsomal cytochrome P-450 monooxygenase or mfo system. For some herbicides such as metolachlor, metabolism in plants, as reviewed by LeBaron *et al.* [1], may involve the participation of both systems. The chloroacetyl side chain of metolachlor is initially considered to undergo conjugation with GSH, followed by breakdown of GSH to the cysteine moiety. Through a subsequent series of reactions that include oxidative deamination and reduction, a thiolactic acid conjugate is formed. An additional oxidation step results in the formation of the sulfoxide which serves as the terminal product [1]. For the most part, the enzymes involved have not been identified, however, conversion of thiolactic acid to the sulfoxide and other oxidative steps could be microsomally mediated. The ether of the methoxypropyl side chain of metolachlor is considered to undergo cleavage followed by conjugation of the propyl moiety with glucose [1]. The above demethylation reaction could be microsomally catalyzed, but the reaction has not been previously reported in the literature.

The role of the GSH system in the metabolism of many herbicides has been studied extensively [2, 3]. However, participation of the plant cytochrome P-450 system in the metabolism of many herbicides remains to be documented with cell-free preparations. The participation of mammalian and insect cytochrome P-450 systems in the metabolism of xenobiotics has been intensively studied

*Abbreviations:* GSH, glutathione; mfo, mixed function oxidase; metolachlor, 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)acetamide; propyl derivative of metolachlor, 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-hydroxy-1-methylethyl)acetamide; G6P, glucose-6-phosphate; diclofop, 2-[4-(2,5-dichlorophenoxy)phenoxy]propanoate; PVPP, insoluble polyvinylpyrrolidone; DTT, dithiothreitol; PBO, piperonyl butoxide; tridiphane, 2-(3,5-dichlorophenyl)-2-(2,2,2-trichloroethyl)oxirane; tetcyclacis, 5-(4-chlorophenyl)-3,4,5,9,10-pentaazatetracyclo[5,4,1,$0^{2,6}$,$0^{8,11}$]dodeca-3,9-dien.

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for over 20 years. There are a number of observations extrapolated from *in vivo* studies which suggest that plant microsomes might be involved in the oxidation of a number of herbicides. Included are the observations that metabolism is arrested by cytochrome P-450 monooxygenase inhibitors [2, 4, 5]. For herbicides, only the N-demethylation of phenylureas [6], and ring hydroxylation of 2,4-D [7] and diclofop [8] have been documented with cell-free plant preparations that have properties characteristic of cytochrome P-450 monooxygenases.

The objectives of the study reported herein were (a) to determine if a cytochrome P-450 monooxygenase preparation isolated from grain sorghum shoots [*Sorghum bicolor* (L.) Moench] catalyzed the metabolism of metolachlor, and, if so, (b) to chemically identify the product formed.

## Materials and Methods

### *Isolation of microsomes*

Grain sorghum seed (Funk G 522 DR) were placed in folded rolls of germination paper and placed upright in 1.0 l beakers that contained 400 ml of 0.5 strength Hoagland's solution. Seed were germinated in the dark at 28 °C for 84 to 96 h. The shoots when harvested were ground with a mortar and pestle for 30 sec with 0.3 M potassium phosphate buffer (pH 7.4) that contained 5 mM DTT and 1% insoluble PVPP (w/v). The grinding ratio of tissue: buffer was 1:6 (w/v). The homogenate was filtered through cheesecloth and centrifuged in a Sorvall RC-2 centrifuge at 4 °C for 20 min at 10,000 × *g*. The supernatant was transferred to a Beckman L 8-70 ultracentrifuge and centrifuged at 4 °C for 60 min at 100,000 × *g*. Pellets were suspended in a small volume of 0.1 M potassium phosphate buffer (pH 7.1) and stored on ice until used.

Optical difference spectra were recorded with an Aminco DW-2000 spectrophotometer with the microsomes being suspended in 0.1 M pH 7.1 potassium phosphate buffer (about 2 mg microsomal protein/ml). Dithionite-reduced carbon monoxide difference and compound-induced difference spectra were obtained following standard procedures [9, 10]. Cytochrome P-450 and P-420 were estimated from millimolar extinction coefficients of 91 and 111, respectively [11]. Microsomal protein was estimated spectrophotometrically by the method of Waddell [12] with crystalline bovine serum albumin as a standard.

### *Enzyme assays*

Microsomal incubations contained either NADH, NADPH, or an NADPH-generating system (0.25 mM $NADP^+$, 2.5 mM G6P, and 1.0 U G6P dehydrogenase), as indicated, 50 mM potassium phosphate buffer (pH 7.1), 0.1 μCi [$^{14}$C]metolachlor (8.9 μM), and microsomes (approximately 1.0 mg protein) in a total volume of 0.5 ml. Reactions were incubated at 25 °C. At the end of the incubation period, enzyme activity was terminated with 0.5 ml acetone. Precipitated protein was removed by centrifugation in a Beckman Microfuge (5 min at 10,000 × *g*). Aliquots of the supernatant were routinely spotted on silica gel (250 μ) thin layer plates and developed in benzene/acetone (2:1, v/v). The plates were radiochromatographically scanned and areas under the radioactive peaks were integrated with a Bioscan System 400 Imaging Scanner. $R_F$ values for metolachlor and the metabolite were approximately 0.70 and 0.48, respectively. Quantification of the radioactive peaks was verified by scraping of the zones from the plates followed by liquid scintillation spectrometry.

Experiments were repeated with at least three separate isolations of microsomes. Representative data are presented unless indicated otherwise. Test chemicals were dissolved in either acetone or DMSO. The final concentration of solvent was held constant at 1.0% (v/v) in all assays, including the controls.

### *Metabolite identification*

Samples of the supernatant were subjected to LC/MS separation and identification. Instrumentation included a Gilson gradient LC system, Rheodyne 7010 injector, and a DuPont Zorbax ODS column (4.6 mm × 25 cm). The mobile phase was methanol/0.1 N ammonium acetate (70:30, v/v) and the flow rate was 1.0 ml/min. Thermospray analyses were performed on a VG 12-250 mass spectrometer, equipped with a Vestec Model 701 S source and controller. The mass spectrometer was scanned over the mass range 200–300 daltons, with a 2 second scan rate. The interface was

operated in the positive ion mode, with the discharge on.

## Results and Discussion

*Microsomal spectral responses*

Shown in Fig. 1 is a typical cytochrome P-450 dithionite-reduced carbon monoxide difference spectrum obtained with the sorghum microsomes used in the study reported herein. The concentration of cytochrome P-450 in the isolations ranged from 90 to 150 pmol/mg protein. The concentration of cytochrome P-420 was typically negligible and did not exceed 3% of the P-450 content. The concentration of cytochrome P-450 in the sorghum preparations, expressed on a protein basis, is within the range reported by other investigators for crude plant preparations [8]. Reflected in Fig. 1 is a slight upwards shift in the absorption peaks of approximately 1 to 2 nm from the nominal wavelengths. Literature values for the CO absorption maximum for plant microsomes isolated from different sources vary from 448 to 452 nm [8, 13, 14].

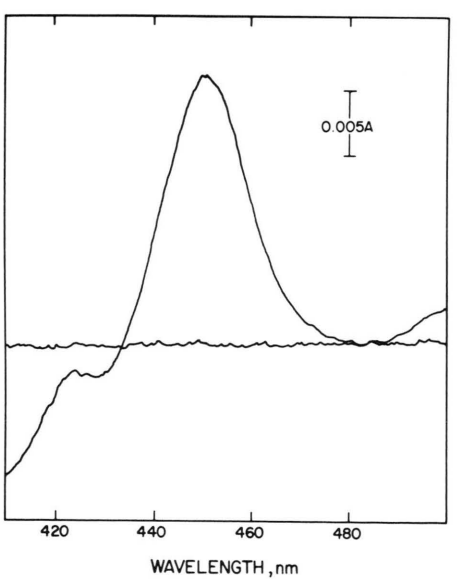


Fig. 1. Carbon monoxide difference spectrum of dithionite-reduced cytochrome P-450 obtained with a sorghum microsomal preparation. The concentrations of protein and cytochrome P-450 were 1.9 mg and 235 pmol/ml, respectively. The horizontal trace indicates the optical balance between the sample and reference cuvettes prior to treatment with dithionite and CO.

To further characterize the sorghum microsomal preparations, difference spectral responses induced by reference compounds were obtained. A large number of compounds interact with mammalian and insect microsomes to form various types of difference spectra [15, 16]. The types of difference spectra that have been described are related to binding interactions with the cytochrome P-450 heme prosthetic group [10, 17]. In contrast to the large numbers of compounds reported to form difference spectra with animal microsomes, spectral alterations of only a few compounds have been published from plant studies. Shown in Fig. 2A is a difference spectrum obtained for the sorghum microsomes with *trans*-cinnamic acid that approaches a type I spectrum. There is a peak around 395 and a trough around 425 nm. Type I ligands are considered to bind at a hydrophobic site on the P-450 protein in close proximity to the heme iron [10]. Cinnamic acid was previously reported to produce a type I spectral interaction with cauliflower and white potato microsomes [18]. However, the authors did not publish a complete spectrum, but only reported peak and trough wavelengths. In studies with animal microsomes, most type I ligands serve as substrates [10, 17]. However, some compounds that are oxidized by animal microsomes do not form type I spectra [17]. The significance of the broad secondary peak around 460 nm in Fig. 2A remains to be established.

Metolachlor also formed a difference spectrum somewhat like the one induced by *trans*-cinnamic acid (Fig. 2C). The initial peak and trough were similar, however, more distinct secondary peaks were apparent around 465 and 490 nm. The general shape of the metolachlor-induced spectrum is similar to one obtained with diclofop [8].

The sorghum microsomes formed a typical type II difference spectrum with the plant growth regulator tetcyclacis (Fig. 2B). Shown are a broad trough around 400 nm and a peak at 430 nm. Formation of a type II spectrum by tetcyclacis with plant microsomes has been reported previously [19]. Type II spectral changes are considered to result from displacement of the native sixth ligand of the heme iron by an $sp^2$ nitrogen atom of the inhibitor, *i.e.*, the inhibitor is considered to bind to the heme iron of cytochrome P-450 [10, 17]. In mammalian microsome studies, these compounds

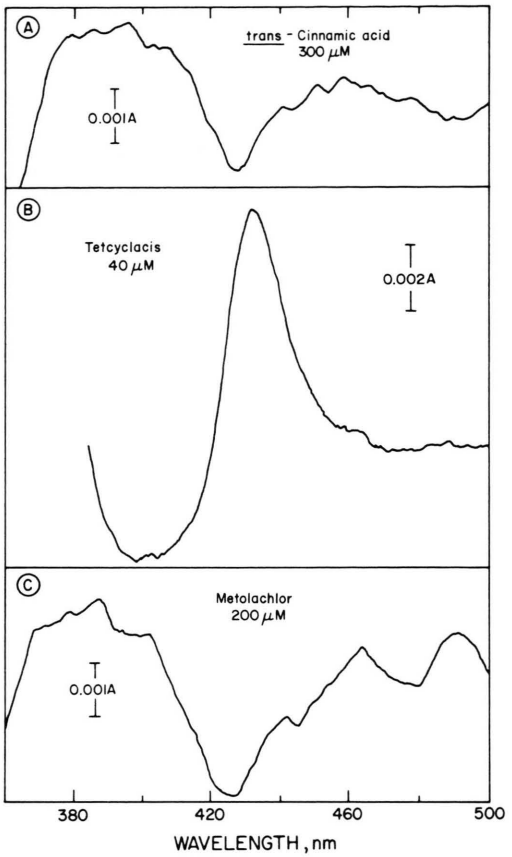


Fig. 2. Optical difference spectra obtained with sorghum shoot microsomes measured by dual wavelength spectroscopy. A. Type I-binding spectrum induced by *trans*-cinnamic acid (300 μM). The concentrations of protein and cytochrome P-450 were 1.9 mg and 235 pmol/ml, respectively. B. Type II-binding spectrum induced by tetcyclacis (40 μM). The concentrations of protein and cytochrome P-450 were 2.4 mg and 225 pmol/ml, respectively. C. Type I-binding spectrum induced by metolachlor (200 μM). Concentrations of protein and cytochrome P-450 are identified under A.

usually inhibit mfo activities. Aniline also has been shown to form a type II spectrum with microsomes [20] and responded similarly with the sorghum microsomes (data not shown).

*Metabolism of metolachlor*

The sorghum microsomes, when incubated with radiolabeled metolachlor under the conditions previously described, produced a single metabolite. As shown in Fig. 3, formation of the metabolite occurred curvilinearly with time. We have not

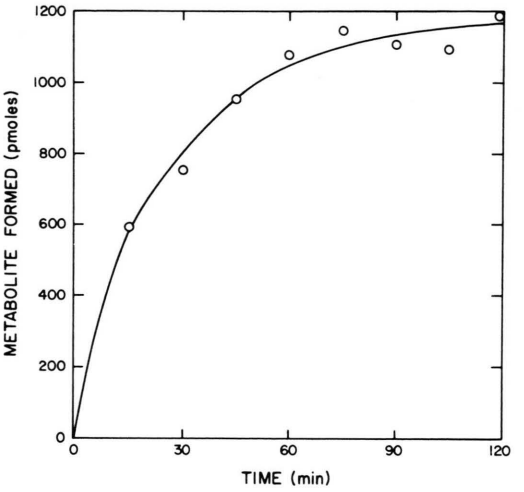


Fig. 3. Formation of the metolachlor metabolite as a function of time by sorghum shoot microsomes. The concentrations of protein and cytochrome P-450 used in each assay were 1.1 mg and 144 pmol, respectively.

identified the factor(s) responsible for the curvilinear kinetics. Substrate may have become rate-limiting, more than one P-450 isozyme may be involved, inhibitors may be present in the preparation, end-product inhibition may have occurred, or this might reflect the lability of the system.

Formation of the metabolite as a function of protein concentration by a microsomal shoot preparation is shown in Fig. 4. Under the condi-

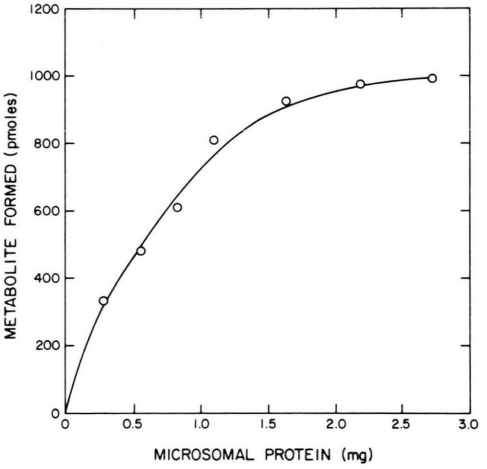


Fig. 4. Formation of the metolachlor metabolite as a function of the protein content of the sorghum shoot microsomal preparation in 60 min. In the isolation used, 132 pmol P-450 were associated with 1.0 mg protein.

tions used in the experiments, the formation of the metabolite increased curvilinearly with increasing protein concentration to about 1.5 mg and subsequently leveled off. The factors responsible for the curvilinear behavior remain to be identified.

Formation of the metabolite by the microsomes had an absolute requirement for reduced pyridine nucleotide (Table I). No metabolite was formed in the absence of a reduced pyridine nucleotide. The monooxygenase reaction catalyzed by cytochrome P-450 requires the input of two electrons that are introduced one at a time at two sequential steps. Electrons can be donated by NADPH *via* the corresponding reductase or by NADH *via* the corresponding reductase and cytochrome $b_5$. NADPH was much more active than NADH. A slight synergistic response was observed when both were present. The concentration of the nucleotides were at saturating levels with respect to degradative activity.

One of the criteria for participation of a cytochrome P-450 monooxygenase in a reaction is inhibition by carbon monoxide with reversal of the inhibition by 450 nm light. Shown in Table II are the effects of gassing experiments conducted both in the dark and light on the formation of the metabolite. Reaction tubes were illuminated with actinic light (700 $\mu E/m^2/sec$) for 60 min. In the untreated controls, illumination may have had a slight stimulatory effect. Treatment by gentle bubbling (6 ml/min) of a mixture of CO and air (20/80%) for 2 min strongly inhibited formation of the metabolite. The inhibition was partially relieved by illumination. Bubbling compressed air under the same conditions, through the reaction mixture, did not alter the degradative activity of the microsomes.

Table I. Pyridine nucleotide requirement for the metabolism of metolachlor by sorghum shoot microsomal preparations.

| Pyridine nucleotide | Concn. [mM] | Activity[a] [pmol] |
|---|---|---|
| None | – | 0 |
| NADPH | 0.75 | 703 ± 61 |
| NADH | 0.75 | 176 ± 41 |
| NADH + NADPH | 0.75 + 0.75 | 826 ± 18 |

[a] Arithmetic average of picomoles of metabolite formed in 60 min by three different isolations of microsomes ± SD. The microsomal protein and cytochrome P-450 content used in the three replications averaged 1.2 mg and 100 pmol, respectively.

Table II. Effect of carbon monoxide and light on the metabolism of metolachlor by sorghum shoot microsomal preparations[a].

| Treatment | Dark | Light |
|---|---|---|
| None (control) | 405 | 427 |
| 20% CO/80% Air | 69 | 190 |
| Air | 409 | 405 |

[a] Data presented as picomoles of metabolite formed in 60 min by a preparation that contained 1.1 mg protein (120 pmol P-450). Gases were bubbled for 2 min.

Table III. Effect of cytochrome P-450 monooxygenase inhibitors on the production of a metabolite from metolachlor by sorghum shoot microsomal preparations.

| Chemical | Concn. [μM] | Inhibition[a] [%] |
|---|---|---|
| PBO | 100 | 74 ± 6 |
| Tetcyclacis | 10 | 92 ± 9 |
| Tridiphane | 40 | 70 ± 7 |

[a] Values are the arithmetic average of the inhibitions obtained with three different isolations of microsomes ± SD. The microsomal protein and cytochrome P-450 content of the preparations used averaged 1.2 mg and 130 pmol, respectively. The uninhibited controls averaged the formation of 908 ± 19 pmol of metabolite in 45 min.

Formation of the metolachlor metabolite also was prevented by compounds known to inhibit cytochrome P-450-mediated reactions (Table III). PBO is the well known insecticide synergist that has been shown to cross-react as a herbicide synergist under *in vivo* conditions. Tetcyclacis is a plant growth regulator with anti-gibberellin properties. It is an extremely potent inhibitor of plant P-450 reactions. Tridiphane, which has been shown to synergize the action of *s*-triazine, α-chloroacetamide, and thiolcarbamate herbicides under *in vivo* conditions, was more active than PBO. In a previous study, tridiphane was shown to strongly inhibit the monooxygenase activity of mouse liver cytochrome P-450 isozymes [21].

### *Metabolite identification*

Under the conditions in which the experiments were conducted, only the formation of the single metabolite was observed. The same metabolite

was also formed by mouse hepatic microsomes (data not shown). However, the mouse preparations were considerably more active than the sorghum preparations.

Samples of the metabolite were scraped and pooled from the TLC plates. It cochromatographed in three different solvent systems with a reference standard of the compound that resulted from cleavage of the ether moiety of the methoxypropyl side chain of metolachlor. The solvent systems used and $R_F$ values for the metabolite were: hexane/chloroform/ethyl acetate (3:1:1, v/v/v), $R_F$ = 0.34; benzene/acetone (2:1, v/v), $R_F$ = 0.48; and methylene chloride/acetone (95:5), $R_F$ = 0.78. Metolachlor chromatographed in the same three solvent systems with $R_F$ values of 0.67, 0.70, and 0.88, respectively.

A 0.6 ml sample of the supernatant from a 60 min reaction mixture following removal of the acetone-precipitated protein was dried, suspended in 0.15 ml methanol, and centrifuged. A 0.1 ml aliquot of the final supernatant was injected for on-line thermospray LC/MS analysis. The reconstructed ion chromatogram for $m/z$ 270 (the mass of the protonated molecular ion of the propyl derivative) from the unknown showed a small peak at the same retention time as the reference standard. Mass spectra of the unknown and the reference standard are shown in Fig. 5 A and B, respectively.

Conceivably, the methyl group is hydroxylated by the microsomes to form the unstable hydroxymethyl derivative that decayed to the propyl moiety with the release of formaldehyde. The identity suggests that the sorghum microsomes have catalyzed an O-demethylation reaction. *In vivo,* the propyl group apparently is rapidly conjugated with glucose to form a terminal product and may not accumulate in the free form.

The presence of the unconjugated propyl derivative has not been reported from plants, however, it has been identified as an early metabolite in rats and as a hydrolytic product of metolachlor under acidic conditions [1]. In plants, conjugation with glutathione seems to occur very rapidly. Consequently, the microsomal substrate, under *in vivo* conditions, may be a product derived from glutathione rather than free metolachlor.

## Conclusions

The microsomal preparation isolated from etiolated sorghum shoots and used herein, possessed properties that have been described for higher plant microsomes by other investigators. Responses elicited by the sorghum microsomes included the formation of: type I difference spectra with *trans*-cinnamic acid and metolachlor, type II difference spectra with tetcyclacis, and strong cytochrome P-450 dithionite-reduced carbon mon-

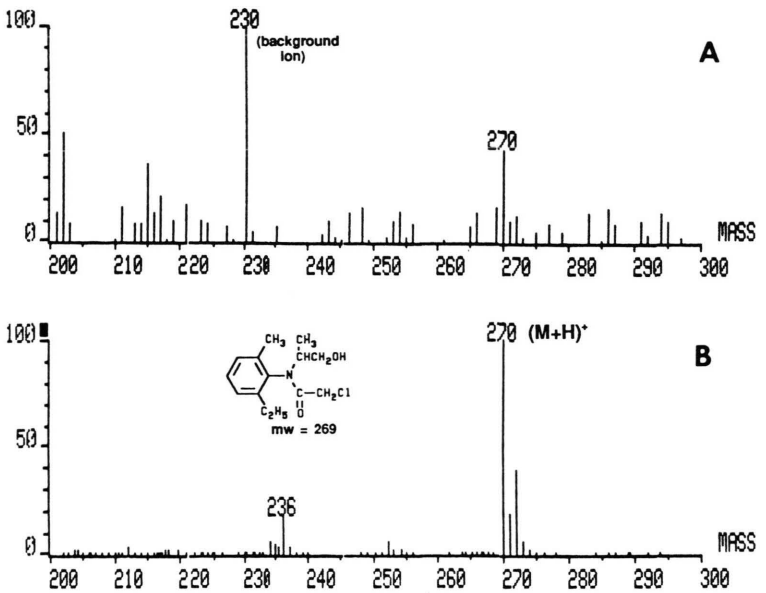


Fig. 5. On-line background-subtracted positive ion thermospray mass spectra of the unknown (A) and the reference standard (B). The protonated molecular ion of the propyl derivative of metolachlor has an $m/z$ of 270.

oxide difference spectra with only trace amounts of cytochrome P-420.

Metabolism of metolachlor catalyzed by the microsomal preparation had an absolute requirement for reduced pyridine nucleotide with NADPH being a much stronger electron donor than NADH; was inhibited by carbon monoxide with partial reversal of the inhibition by light; and was inhibited by known cytochrome P-450 monooxygenase inhibitors (PBO, tetcyclacis, and tridiphane). The microsomes O-demethylated the methoxypropyl side chain of metolachlor and the chemical identity of the product formed was confirmed by LC/MS.

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