

Differential Metabolism of the Sulfonylurea Herbicide Prosulfuron (CGA-152005) by Plant Microsomes

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Microsomes isolated from excised shoots of 3-day-old, dark grown, grain sorghum [*Sorghum bicolor* (L.) Moench, Funk G522DR and DK 41Y] and corn seedlings [*Zea mays* (L.), Pioneer 3245] metabolized the sulfonylurea herbicide prosulfuron (CGA-152005). Corn microsomes predominantly formed a single major metabolite that resulted from hydroxylation of the phenyl ring at the C5 position. However, sorghum microsomes formed two major metabolites in an approximate 1:1 ratio. One was the 5-hydroxyphenyl metabolite, whereas the second metabolite resulted from *o*-demethylation at C4 of the triazine ring. Metabolite identity was established by mass spectrometry and co-chromatography with authentic standards. Metabolism in both corn and sorghum was greatly enhanced by pretreatment of the seed with naphthalic anhydride and by subirrigation with 2.5% ethanol 24 h prior to harvest. Metabolism required a reduced pyridine nucleotide and was affected by several cytochrome P450 monooxygenase inhibitors (carbon monoxide, tetryclacis, piperonyl butoxide, 1-aminobenzotriazole, and SKF-525A). The inhibitors differentially affected metabolism of prosulfuron. Microsomal oxidations from both untreated and inducer-treated tissue responded similarly to the inhibitors. In exploratory studies, microsomes isolated from shoots of wheat [*Triticum aestivum* L., Pioneer 2548], barley [*Hordeum vulgare* L., Boone], oats [*Avena sativa* L., Southern States 76–30-P242] and rice [*Oryza sativa* L., Gulfmont], and room ripened avocado [*Persea americana*, Mill., Hass] mesocarp tissue also primarily formed the 5-hydroxyphenyl metabolite. Titration of seven different avocado microsomal preparations with prosulfuron provided typical type I difference spectra from which an average binding constant (K_s) of $187 \pm 35 \mu\text{M}$ was obtained.

Abbreviations: 1-ABT, 1-aminobenzotriazole; alachlor, 2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide; ALS, acetolactate synthase; CGA 24704, 2-chloro-*N*-(2,6-dimethylphenyl)-*N*-(2-methoxy-1-methylethyl)acetamide; CGA-150829, 2-amino-4-methoxy-6-methyl-1,3,5-triazine; CGA-152005, prosulfuron, *N*-[[4-methoxy-6-methyl-1,3,5-triazin-2-yl] amino]carbonyl]-2-(3,3,3-trifluoropropyl)benzenesulfonamide; CGA-159902, 2-(3,3,3-trifluoropropyl)benzenesulfonamide; CGA-300406, *o*-desmethyl prosulfuron, *N*-[[4-hydroxy-6-methyl-1,3,5-triazin-2-yl]amino]carbonyl]-2-(3,3,3-trifluoropropyl)benzenesulfonamide; CGA-300408, 5-hydroxy prosulfuron, *N*-[[4-methoxy-6-methyl-1,3,5-triazin-2-yl]amino]carbonyl]-2-(3,3,3-trifluoropropyl)-5-hydroxybenzenesulfonamide; chlorsulfuron, 1-(2-chlorophenylsulfonyl)-3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)urea; *p*CMA, *p*-chloro-*N*-methylaniline; DMA, *N,N*-dimethylaniline; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; G6P, glucose-6-phosphate; HPLC, high-performance liquid chromatography; LC/ESI/MS, liquid chromatography/ electrospray ionization/mass spectrometry; metolachlor, 2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl)acetamide; NA, 1,8-naphthalic anhydride; nicosulfuron, 2-[[4,6-dimethoxypyrimidin-2-yl]aminocarbonyl]aminosulfonyl]-*N,N*-dimethyl-3-pyridinecarboxamide; PBO, piperonyl butoxide; primisulfuron, 2-[[[4,6-bis(difluoromethoxy)-2-pyrimidinyl]amino]carbonyl]amino]sulfonyl]benzoic acid; PVPP, polyvinylpyrrolidone; SKF-525A, 2-(diethylamino)ethyl-2,2-diphenylpentanoate; tetryclacis, 5-(4-chlorophenyl)-3,4,5,9,10-pentaazatetracyclo[5,4,10^{2,6},0^{8,11}] dodeca-3,9-diene; TLC, thin layer chromatography; triasulfuron, 1-(2-chloroethoxyphenylsulfonyl)-3-(6-methoxy-4-methyl-1,3,5-triazin-2-yl)urea.

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Introduction

The sulfonylurea class of herbicides, which consists of an aryl entity connected to a heterocyclic moiety by a sulfonylurea bridge, was introduced in the 1980s. These can be highly selective, broad-spectrum compounds that are used in the field at rates of a few grams/hectare, and have low mammalian toxicity. They control a wide spectrum of annual and perennial grasses, and broadleaf weeds, but give selective weed control in small grains, soybeans, rice, and other agronomic crops. Some have industrial and non-crop uses providing total vegetation control.

The mode of action of the sulfonylureas involves inhibition of ALS which plays a key role in the synthesis of the branched-chain essential amino acids leucine, isoleucine, and valine (Ray, 1989). Species selectivity among the sulfonylureas has been correlated with a rapid rate of metabolism in the tolerant crop (Beyer *et al.*, 1988). For some sulfonylureas, initial metabolism has been shown to be mediated by the cytochrome P450 monooxygenase system followed by glycosylation of the hydroxylated moieties. In cell-free studies, microsomes isolated from several-day-old, dark-grown shoots of corn have been reported to oxidize nicosulfuron, primisulfuron, prosulfuron, and triasulfuron (Moreland *et al.*, 1993; Fonné-Pfister *et al.*, 1990). Additionally, wheat microsomes oxidized chlorsulfuron and triasulfuron (Frear *et al.*, 1991; Thalacker *et al.*, 1994). The corn microsomes formed a major and a minor oxidation product from prosulfuron in an approximate 4:1 ratio (Moreland *et al.*, 1993). Wheat microsomes also produced a major metabolite from prosulfuron which resulted from hydroxylation of the phenyl ring at position C5. Identification of the metabolite was confirmed by proton NMR spectroscopy (Frear and Swanson, 1994). Two minor products were also formed by wheat microsomes, but their identities were not confirmed.

In preliminary studies, microsomes isolated from sorghum shoots produced two major metabolites from prosulfuron in contrast to the single major entity formed by corn and wheat microsomes. Consequently, the objectives of the studies reported herein were to (a) identify and compare the metabolites formed from prosulfuron (Fig. 1) by microsomes isolated from several species of

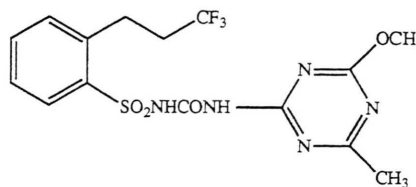


Fig. 1. Structure of prosulfuron (CGA-152005).

plants; (b) evaluate the inductive effects of seed and/or seedling treatments on the extent of prosulfuron metabolism by sorghum and corn microsomes; (c) determine the sensitivity of the oxidative reactions to cytochrome P450 monooxygenase inhibitors; and (d) explore the formation of difference spectra between prosulfuron and the microsomal preparations.

Materials and Methods

Microsomal assays

Details on the position of the radiolabel, radiochemical purity, and specific activity of the compounds studied are provided in Table I. Most of the studies were conducted with seed of the following species: grain sorghum [*Sorghum bicolor* (L.) Moench, Funk G522DR and DK 41Y] harvested in 1988; corn [*Zea mays* L., Pioneer 3245] harvested in 1990; and wheat [*Triticum aestivum* L., Pioneer 2548] harvested in 1990. All of the seed had been pretreated with captan (0.2%, w/w) and were stored under refrigeration at 4°C until used.

Seed were germinated 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. In the experiments that involved NA treatments, the seed were placed in small bags with the indicated amount of chemical and rotated for several minutes to uniformly coat the seed. Seed were maintained in the dark at approximately 28°C for 72 h. The shoots, when harvested, were ground with a chilled 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 x g. The supernatant was transferred to a Beckman

Table I. Position of the radiolabel, radiochemical purity, specific activity, and reaction mixture concentrations of the compounds studied.

Radiolabeled substrate	Radiochemical purity (%)	Specific activity [MBq/mmol]	Substrate concn. [μ M]
[phenyl- U - 14 C] Prosulfuron	97.9	677	11
[triazine- U - 14 C] Prosulfuron	98.2	548	14
[U - 14 C]phenylsulfonamide CGA-150829	95.7	141	53
[U - 14 C]triazineamine CGA-159902	99.3	437	17

L8–70 ultracentrifuge and centrifuged at 4°C for 60 min at 100,000 \times g. Pellets were suspended in a small volume of 0.1 M potassium phosphate buffer (pH 7.1) and stored on ice until used. Treatments that were used to enhance the rate and extent of prosulfuron metabolism included NA applied as a seed treatment (0.5% for sorghum and corn, and 0.2% w/w for wheat), 2.5% ethanol applied by subirrigation 24 h prior to harvest, and a combination of ethanol and NA. The subirrigation treatment involved transferring the rolls of germinating seed, after 48 h, to beakers that contained 0.5 strength Hoagland's solution and 2.5% ethanol (v/v). Dithionite-reduced, carbon monoxide difference spectra were obtained following standard procedures (Estabrook and Werringloer, 1978; Jefcoate, 1978) with an SLM/Aminco DW-2000 Spectrophotometer. Concentrations of cytochromes P450 and P420 were estimated from millimolar extinction coefficients of 91 and 111, respectively (Omura and Sato, 1964). Microsomal protein was estimated spectrophotometrically by the method of Waddell (1956) with crystalline bovine serum albumin as a standard.

The microsomal reaction mixtures placed in conical Microfuge tubes contained 0.75 mM NADPH, an NADPH-generating system (2.5 mM G6P, and 0.1 U G6P dehydrogenase), 50 mM potassium phosphate buffer (pH 7.1), 740 Bq of the radiolabeled substrate (at the molar concentration shown in Table I), and freshly isolated microsomes at an approximate protein concentration of 0.25 to 0.5 mg, in a total volume of 0.1 ml. Metabolite formation, where it occurred, was essentially linear under the conditions identified. Reactions

were incubated at 25°C for 30 min and were arrested with 0.1 ml acetone. Precipitated protein was removed by centrifugation in a Beckman Microfuge (5 min at 10,000 \times g).

Components in the supernatants obtained from the microsomal assays were routinely analyzed by one-dimensional TLC on 250 μ m silica gel plates (Whatman LK5F). The solvent system consisted of toluene/acetone/acetic acid (75:20:5, v/v). Following development, the plates were radiochromatographically scanned and areas under the radioactive peaks were integrated with a Bioscan System 400 Imaging Scanner. Concentrations of metabolites formed were estimated semiquantitatively from the chromatographic profiles and by liquid scintillation spectrometry. Corrections were made for background radioactivity and traces of radiochemical impurities by subtraction of values obtained with no-microsome control assays.

Stock solutions of the desired concentrations of inhibitors were prepared in acetone. The final concentration of acetone was held constant at 1.0% (v/v). This concentration of acetone did not interfere with the oxidative activity of the microsomes. Microsomes were incubated with the inhibitors for 5 min at 25°C in the buffered reaction mixtures before the reactions were initiated by the addition of NADPH. In the studies that involved CO, the microsomal suspension was purged with CO for 3 min prior to addition to the reaction mixture.

In vitro binding of prosulfuron to oxidized cytochrome P450 was monitored by differential spectroscopy (Jefcoate, 1978). An aliquot of the microsomal suspension was diluted with 0.1 M po-

tassium phosphate buffer (pH 7.1) to provide a protein concentration of between 1.0 and 1.5 mg/ml. The microsomal preparation (0.99 ml) was added to both the reference and sample cuvettes. After temperature equilibration, a corrected baseline was recorded between 340 and 520 nm. Aliquots (from 0.001 to 0.005 ml) of a 0.02 M stock solution of prosulfuron, dissolved in DMSO, were added directly to the sample cuvette and an identical volume of DMSO was added to the reference cuvette. One minute after mixing, absorption spectra were recorded between 340 and 520 nm. This procedure was repeated with the stepwise addition of small volumes of prosulfuron until the additions did not produce further changes in the spectrum. Results are expressed as ΔA (390–420 nm).

Metabolite identification

Microsomes isolated from shoots of NA-treated G522DR sorghum seed were used to obtain a sufficient concentration of the metabolites for structural identification. The previously described standard reaction mixture was scaled up 90-fold. The reaction was incubated and terminated with acetone as previously described. Following centrifugation to remove precipitated protein, the volume of the supernatant was reduced under a stream of N_2 and was filtered through an 0.45 μ Durapore filter (Millipore UFC3 OHV NB).

Aliquots of the supernatant were cochromatographed with selected standards by two-dimensional TLC on 250 μ m silica gel plates (Merck 60F). The solvent systems consisted of ethylacetate/isopropanol/water (130:46:24, v/v) and ethylacetate/chloroform/acetonitrile/acetic acid/water (40:40:17:1:2, v/v). Following development, distribution of the radioactive components was visualized with an AMBIS Radiosotope Detection System. Areas under the detected peaks were integrated with software provided by the vendor.

Oxidation products also were separated by reversed-phase HPLC using a Whatman Partisil 10 ODS-2 (250 mm x 9.4 mm i.d.) column. A step gradient elution was used at a flow rate of 2 ml/min with solvent A = 0.1% acetic acid in water and solvent B = acetonitrile. Step 1, 20% B for 2 min; Step 2, 20 to 40% B, 10 minute linear gradient; Step 3, 40 to 50% B, 10 minute linear gradient;

Step 4, 50% B isocratic elution for 10 minutes; Step 5, 50 to 100% B, 10 minute linear gradient; Step 6, isocratic elution for 5 minutes. The HPLC unit consisted of a solvent delivery system equipped with a pump that provided ternary low pressure mixing and a microprocessor control unit (Perkin-Elmer Series 410 LC pump), a sample injector (Rheodyne 7125), a variable wavelength UV detector (Perkin-Elmer LC-95 UV/Visible), radioisotope flow monitor (IN/US β -Ram) with a solid scintillator cell (calcium fluoride packed), and a fraction collector (ISCO Foxy II). Detector outputs were captured by a personal computer (Gateway 4SX-25) using software supplied with the radioactivity flow monitor. All solvents were HPLC grade, filtered, and degassed individually before use. Fractions were collected at 0.5 min intervals.

Fractions that corresponded to the peaks of interest were analyzed by mass spectrometry. LC/ESI/MS determinations were made with a Finnigan TSQ 7000, triple quadrupole mass spectrometer (QQQ), equipped with an Atmospheric Pressure Ionization (API) interface fitted with a pneumatically assisted electrospray head. The spray nozzle was operated at 5 kV in the positive ion mode. The 7000 was equipped with a quaternary solvent delivery system (Perkin-Elmer 410 LC Pump), a UV detector (Perkin-Elmer), a stream splitter set at 6:1 with the majority of the effluent flowing to a radioisotope flow monitor (IN/US β -RAM) and the other stream attached to the API interface. Signals were captured by a Finnigan 7000 data system. Collision-induced dissociation experiments (CID) were conducted using argon gas with collision energy in the range of 17.5–30 eV at cell pressures of approximately 0.28 Pa.

Results

Metabolite formation and identification

In exploratory studies, microsomes isolated from corn and wheat shoots formed a single major metabolite from prosulfuron, whereas microsomes from sorghum formed two major metabolites in an approximate 1:1 ratio (Fig. 2). The oxidation products were separated and collected by reversed-phase HPLC (Fig. 3) and subjected to mass spectral identification (Fig. 4). Isolate M1 had an HPLC retention time of 28 min (Fig. 3). The mass

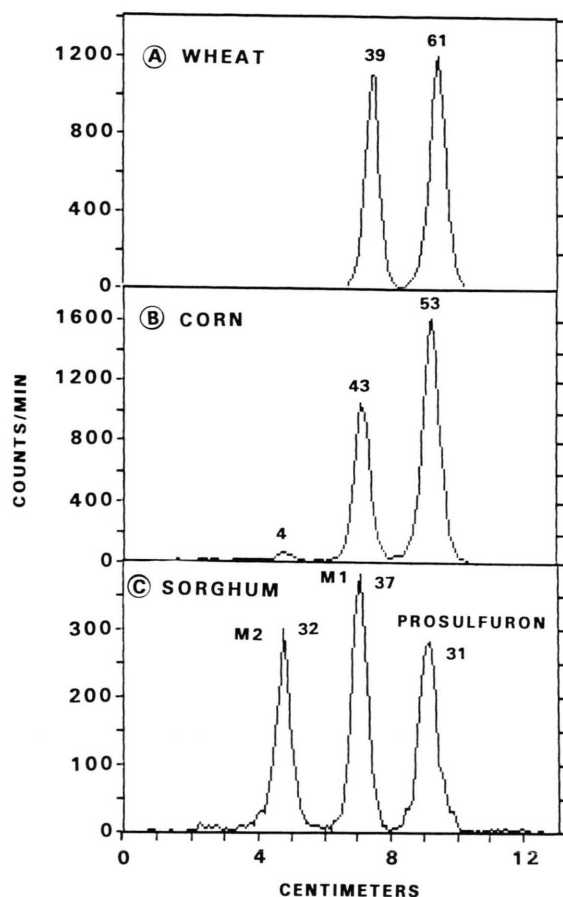


Fig. 2. Radioactivity traces of thin layer chromatographic separations of phenyl[$U-^{14}C$]prosulfuron and metabolites formed by microsomes isolated from shoots of (A) wheat, (B) corn, and (C) sorghum. Metabolite M1 is 5-hydroxyphenyl prosulfuron and M2 is *o*-desmethyl prosulfuron. The numbers above or beside the major peaks represent the % distribution of radioactivity. For all three species, microsomes were isolated from shoots of seedlings that had been seed-treated with naphthalic anhydride and subirrigated with ethanol (2.5%) 24 h prior to harvest.

spectrum showed a molecular ion of m/z 436 ($M + H$)⁺ which is 16 mass units higher than prosulfuron (not shown). The spectrum above m/z 436 also produced an ion at m/z 438 attributed to the natural abundance of sulfur isotopes and an ion at m/z 458 that corresponded to a sodium adduct. The daughter spectrum (Fig. 4A) of the m/z 436 ion showed major mass fragments at m/z 167 and 141 which is consistent with the loss of the triazine portion of the parent molecule, and suggests that the additional oxygen is on the phenyl portion of

the molecule. Isolate M1 cochromatographed in both reversed-phase HPLC (Fig. 3) and a two-dimensional TLC system (data not shown) with an authentic standard (CGA300408) in which the phenyl ring was hydroxylated at position C5. Frear *et al.* (1994) previously reported the formation of this metabolite by wheat shoot microsomes.

Isolate M2 had an HPLC retention time of 20 min (Fig. 3). The mass spectrum showed a molecular ion of m/z 406 ($M + H$)⁺. The molecular weight of 405 is 14 mass units less than prosulfuron and can be attributed to the loss of a methyl group. The daughter spectrum of the 406 ion (Fig. 4B) showed ion fragments at m/z 127 and 153 which also is consistent with a loss of the methyl group on the triazine portion of the molecule. Isolate M2 cochromatographed in both reversed-phase HPLC (Fig. 3) and a two-dimensional TLC system with an authentic standard (CGA-300406) in which the methoxy substituent at C4 of the triazine ring had been *o*-demethylated.

Microsomes isolated from shoots of NA and ethanol-treated barley [*Hordeum vulgare* L., Boone], oats [*Avena sativa* L., Southern States

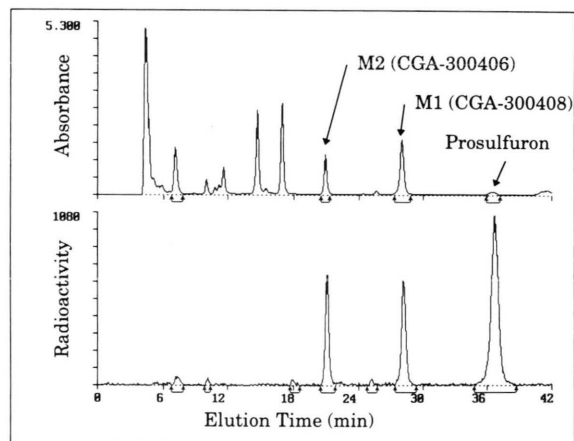


Fig. 3. Reversed phase HPLC separation of [triazine- $U-^{14}C$]prosulfuron and its metabolites formed by microsomes isolated from 3-day-old G522DR sorghum shoots that had been seed-treated with naphthalic anhydride. Prior to injection, the reaction extract was spiked with a mixture of authentic reference standards. The upper trace shows the UV absorbance spectrum of the reaction extract and the lower trace reflects the distribution of radioactivity. Radioactivity associated with the oxidation products correlated with the UV spectra of two of the standards, i.e., M1 = 5-hydroxyphenyl prosulfuron (CGA-300408) and M2 = *o*-desmethyl prosulfuron (CGA-300406).

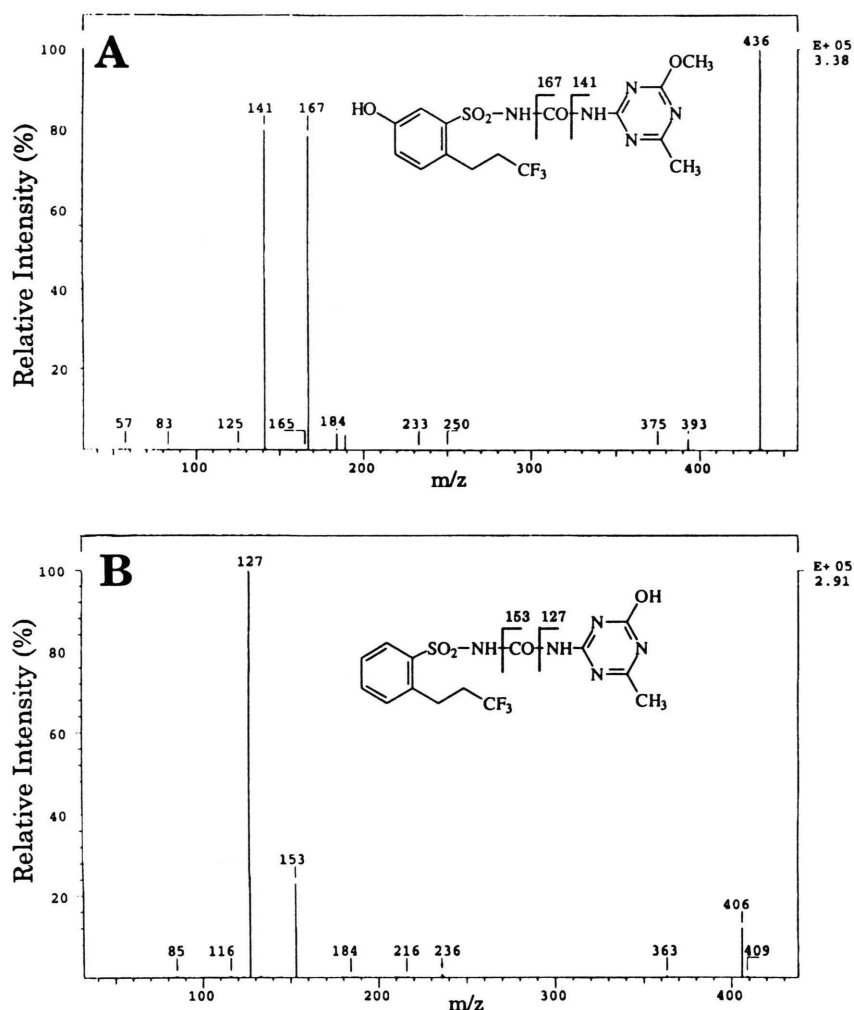


Fig. 4. Spectra obtained by LC/ESI/MS/MS of the oxidation products formed from prosulfuron by sorghum microsomes. Legend: (A) metabolite M1, 5-hydroxyphenyl prosulfuron, and (B) metabolite M2, *O*-desmethyl prosulfuron.

76-30-P242], and rice [*Oryza sativa* L., Gulfmont], and untreated room-ripened avocado [*Persea americana* Mill., Hass] mesocarp tissue also primarily formed the 5-hydroxyphenyl metabolite from prosulfuron. The rate of metabolite formation, for a minimum of two microsomal isolations, averaged 0.45 ± 0.08 , 0.59 ± 0.07 , 0.30 ± 0.06 , and 0.89 ± 0.09 nmol/mg protein/h for barley, oats, rice, and avocado, respectively.

The formation of two major metabolites by sorghum microsomes from prosulfuron in an approximate 1:1 ratio was not restricted to the G522DR cultivar which has a bronze-colored endosperm, but was also observed with microsomes isolated from shoots of the cultivar DK 41Y which has a cream-colored endosperm (Table II). As shown in

the table, the endogenous level of activity associated with the DK 41Y microsomes was approximately 1.7-fold higher than that of the G522DR microsomes with the concentrations of the two cytochromes being similar.

With sorghum and corn microsomes, the same products were formed with the radiolabel on either the phenyl ring or the triazine ring. No suggestions were obtained to indicate that the sulfonylurea bridge was cleaved by the microsomal preparations, under the experimental conditions that were used. When the radiolabeled products that would result from cleavage of the sulfonylurea bridge, i.e., phenylsulfonamide (CGA-159902) or triazineamine (CGA-150829), were tested as substrates for the corn and sorghum

Table II. Comparative metabolism of prosulfuron by microsomes isolated from shoots of 3-day-old G522DR and DK 41Y sorghum seedlings.

Cultivar	Metabolite formation ^a		Cytochrome	
	M1 (nmol/mg protein/h)	M2 (nmol/mg protein/h)	P450 (pmol/mg protein)	P420 (pmol/mg protein)
G522DR	0.68 ± 0.08	0.53 ± 0.06	94 ± 8	4.3 ± 0.9
DK 41Y	1.12 ± 0.05	0.92 ± 0.08	118 ± 18	3.7 ± 0.5

^a Data represent arithmetic averages obtained from three separate preparations of microsomes isolated from unsafened seed ± SD. Metabolite M1 is 5-hydroxy prosulfuron and M2 is *o*-desmethyl prosulfuron.

microsomal preparations, no metabolites were formed. Apparently, the entire molecule, with an intact bridge, is required for recognition by the monooxygenase(s).

Effect of seed/seedling treatments

The extent of metabolism of prosulfuron was enhanced by treatment of the sorghum seed and/

or seedlings with NA and ethanol (Fig. 5). Shown in Panel A is the extent of endogenous metabolism associated with microsomes isolated from unsafened seed with the metabolites being formed in an approximate 1:1 ratio. When the seedlings were subirrigated with 2.5% ethanol 24 h prior to harvest, there was about a 2.5-fold increase in metabolite formation (Panel B). An approximate 3-fold

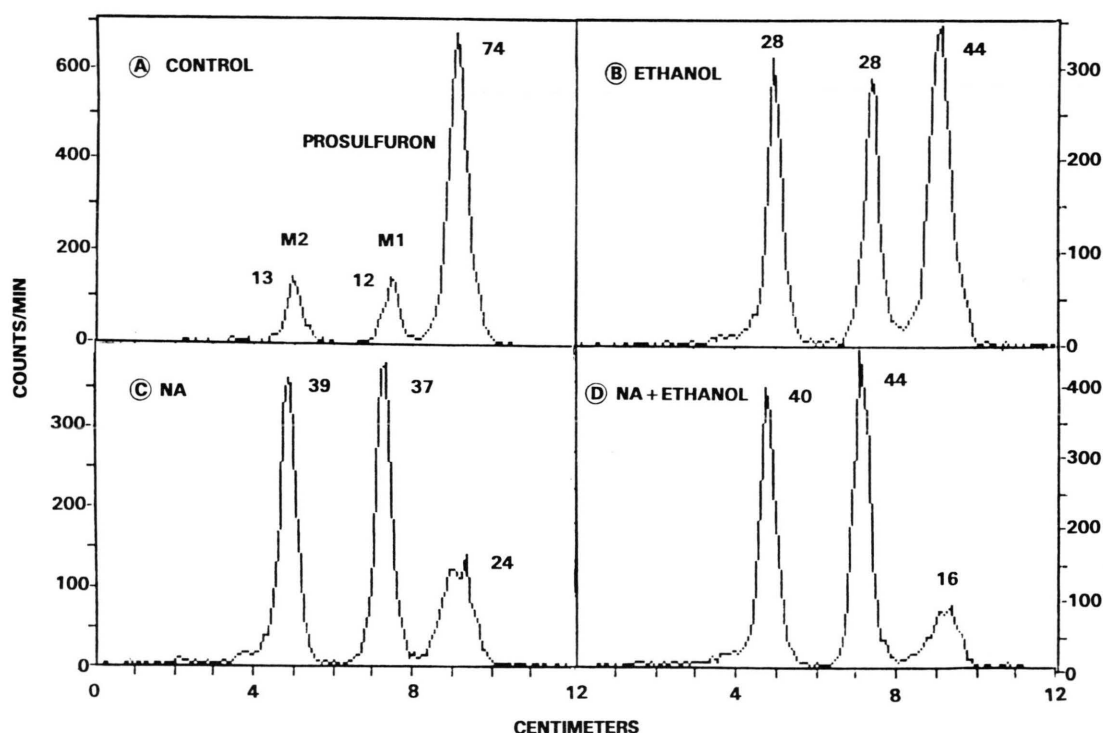


Fig. 5. Radioactivity traces of thin layer chromatographic separations of [phenyl- U - ^{14}C]prosulfuron and its metabolites formed by microsomes isolated from 3-day-old G522DR sorghum shoots. Legend: (A) untreated control, (B) subirrigated with 2.5% ethanol 24 h prior to harvest, (C) naphthalic anhydride (NA) (0.5%, w/w) applied as a seed treatment, and (D) treated with both NA and ethanol. The numbers above or beside the peaks represent the % distribution of radioactivity. Metabolites M1 and M2 are identified in the legend of Fig. 1. The P450 concentrations of the microsomal preparations in the reaction mixtures associated with Panels A, B, C, and D were 54, 55, 75, and 79 pmol/mg protein, and the protein concentrations were 0.25, 0.28, 0.23, and 0.28 mg, respectively.

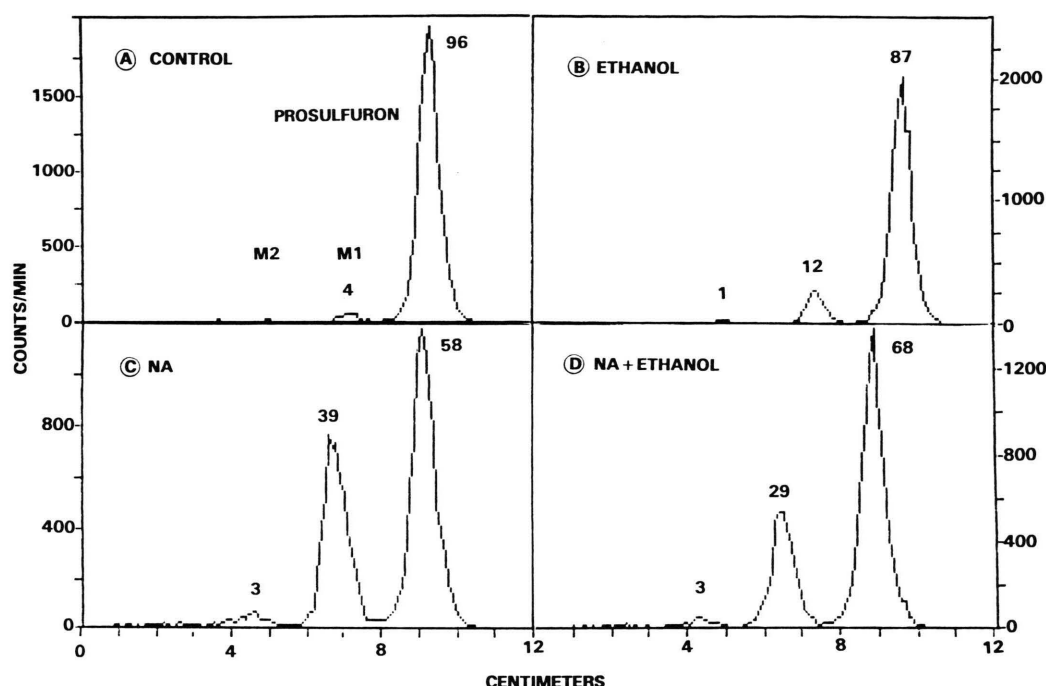


Fig. 6. Radioactivity traces of thin layer chromatographic separations of [phenyl- U - ^{14}C]prosulfuron and its metabolites formed by microsomes isolated from 3-day-old P3245 corn shoots. Legend: (A) untreated control, (B) subirrigated with 2.5% ethanol 24 h prior to harvest, (C) naphthalic anhydride (NA) (0.5%, w/w) applied as a seed treatment, and (D) treated with both NA and ethanol. The numbers above or beside the peaks represent the % distribution of radioactivity. See legend of Figure 1 for identification of M1 and M2. The P450 concentrations of the microsomal preparations in the reaction mixtures associated with Panels A, B, C, and D were 95, 106, 108, and 85 pmol/mg protein, and the protein concentrations were 0.75, 0.93, 0.74, and 0.93 mg, respectively.

increase in metabolite formation was obtained with microsomes isolated from shoots of seed that had been treated with 0.5% NA (Panel C) relative to microsomes isolated from untreated tissue (Panel A). The combination of NA and ethanol was slightly synergistic (Panel D). Availability of unmetabolized prosulfuron probably was becoming rate-limiting and product formation may not have been linear toward the end of the reaction (Panels C and D). Consequently, this may not have been a very subjective experiment. The approximate 1:1 ratio in the formation of the two metabolites by sorghum microsomes was not affected by the seed/seedling treatments.

Metabolism of prosulfuron also was enhanced by treatment of corn seed/seedlings with NA and ethanol (Fig. 6). The extent of endogenous metabolism by microsomes isolated from unsafened seed is shown in Panel A. Endogenous activity associated with corn shoot microsomes was al-

ways considerably lower than that associated with sorghum shoot microsomes (Fig. 5A). Corn microsomes primarily formed the 5-hydroxyphenyl metabolite (M1) with only a suggestion for the formation of the *o*-desmethyl metabolite (M2). Relative to the unsafened control preparations, metabolism was enhanced 3-fold by the ethanol treatment (Panel B), was enhanced 10-fold by seed-applied NA (Panel C), but antagonism occurred when the NA and ethanol treatments were combined (Panel D). However, the combination treatment did enhance metabolism 7-fold above the unsafened control preparations. NA alone and in combination with ethanol did tend to increase slightly the extent of M2 formation, but seldom accounted for more than 3% of the radioactivity in the reaction mixtures. NA provided stronger enhancement of metabolism in corn than in sorghum (Fig. 6 vs Fig. 5). At this time, we are unable to explain why the combina-

Table III. Pyridine nucleotide requirement for the metabolism of prosulfuron by microsomes isolated from 3-day-old G522DR sorghum and P3245 corn shoots.

Pyridine nucleotide	Concn. [mM]	Species and metabolites ^a		Corn M1
		Sorghum M1	M2	
None	–	0	0	0
NADPH	0.75	0.97 ± 0.00	0.88 ± 0.02	1.10 ± 0.07
NADH	0.75	0.39 ± 0.02	0.36 ± 0.01	0.11 ± 0.08
NADPH + NADH	0.75 + 0.75	1.03 ± 0.01	0.88 ± 0.02	1.18 ± 0.12

^a Metabolite formation is presented separately for the two sorghum metabolites and is quantified as nmol/mg microsomal protein/h. Data represent arithmetic averages obtained from two separate preparations of microsomes isolated from shoots of seedlings that had been treated with both naphthalic anhydride and ethanol ± SD. The concentration of cytochrome P450 in the isolations averaged 98 ± 19 and 138 ± 8, and those of cytochrome P420 averaged 5.9 ± 1.0 and 1.3 ± 0.3 for sorghum and corn, respectively. Metabolites M1 and M2 are identified in the footnote of Table II.

tion of NA and ethanol was slightly synergistic in sorghum and antagonistic in corn.

Cofactor requirement and inhibitors

Oxidation of prosulfuron by both sorghum and corn microsomes had an absolute requirement for a reduced pyridine nucleotide (Table III). The concentration of NADPH was at a saturating level with respect to degradative activity. The regenerating system was omitted from the reaction mixtures in these experiments. NADPH served as the major donor of electrons for the oxidations with NADH being slightly less than one-half as effective as NADPH with sorghum microsomes and one-tenth as effective as NADPH with corn microsomes. The combination of NADPH and NADH was not synergistic with microsomes from both species. The ability of NADH by itself to support oxidation of a substrate by sorghum microsomes contrasts with previous studies with microsomes isolated from a different collection of sorghum seed (Moreland *et al.*, 1990), but the corn response agrees with a previous report that included prosulfuron (Moreland *et al.*, 1993). The approximate 1:1 ratio in the formation of the two metabolites by sorghum microsomes was not affected by the source of reducing equivalents.

Responses obtained with several cytochrome P450 inhibitors on the oxidation of prosulfuron by sorghum and corn microsomes is shown in Table IV. No differential inhibitory responses were associated with the seed/seedling treatments. Carbon monoxide and tetracyclacis (10 µM) inhibited,

by about 50%, prosulfuron oxidation mediated by both sorghum and corn microsomes. PBO, 1-ABT, and SKF-525A were more inhibitory of prosulfuron metabolism by corn than by sorghum microsomes. The differential sensitivity to inhibitors suggests that there could be differences in the properties of the P450 isoforms from the two species. Formation of the two metabolites by sorghum microsomes was not differentially affected by the inhibitors.

Titration of an avocado microsomal preparation with increasing concentrations (20 to 340 µM) of

Table IV. Effect of inhibitors on the metabolism of prosulfuron by microsomes isolated from 3-day-old G522DR sorghum and P3245 corn shoots.

Inhibitor	Concn. [µM]	Species and metabolites ^a		Corn M1
		Sorghum M1	M2	
		(% inhibition)		
CO	–	49	56	51
Tetracyclacis	10	42	46	64
PBO	100	13	21	72
1-ABT	250	8	4	40
SKF-525A	250	3	2	25

^a Inhibitions represent arithmetic averages of deviations from no-inhibitor controls obtained with two separate isolations of microsomes isolated from shoots of seedlings that had been treated with both naphthalic anhydride and ethanol. Production of M1 and M2 in the uninhibited controls averaged 1.07 ± 0.01 and 0.91 ± 0.02 for sorghum and 1.29 ± 0.09 nmol/mg protein/h for corn, respectively. The concentrations of cytochromes P450 and P420 in the isolations are identified in the Table III footnote. Metabolites M1 and M2 are identified in the Table II footnote.

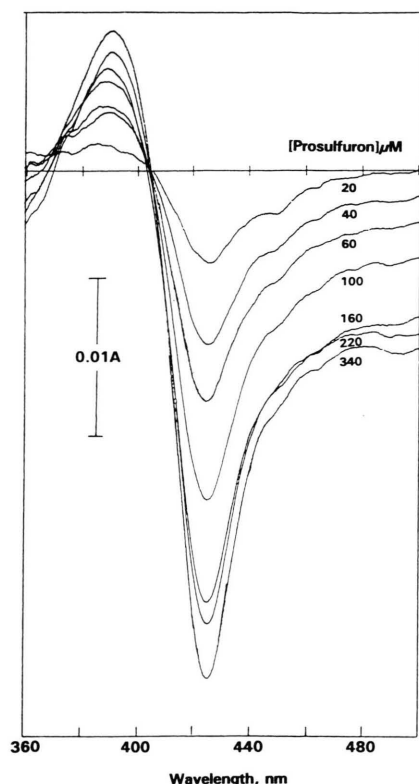


Fig. 7. Type I oxidized cytochrome P450 difference spectra obtained with an avocado mesocarp microsomal suspension that was titrated with increasing concentrations of prosulfuron (20 to 340 μM). The cytochrome P450 and protein concentrations were 670 pmol and 1.5 mg/ml, respectively.

prosulfuron provided typical type I difference spectra with peaks and troughs around 390 and 425 nm, respectively (Fig. 7). Substrate binding near the heme prosthetic group of the cytochrome P450 reaction center is a prerequisite for metabolism. Binding of a substrate is sometimes, but not always, reflected in the formation of a type I difference spectrum with oxidized cytochrome P450. Type I ligands are believed to bind at a hydrophobic site in close proximity to the heme iron (heme prosthetic group) so as to allow both perturbation of the absorption spectrum and interaction with the activated oxygen. Difference spectra have been routinely generated in studies with mammalian microsomes, but investigators have had only limited success in obtaining satisfactory difference spectra with crude plant microsomal preparations. This failure may be related to the low concentrations of cytochrome P450 in the plant preparations, to the presence of interfering pigments, or to the presence of other non-P450 heme entities. With prosulfuron, we also were unable to obtain meaningful difference spectra using the crude corn and sorghum preparations isolated in this study that possessed up to 150 pmoles of P450/mg protein. However, the preparations of microsomes isolated from ripened avocado mesocarp tissue contained up to 450 pmoles P450/mg protein and provided spectra such as shown in Fig. 7 when titrated with prosulfuron.

Absorption differences (ΔA 390–425) obtained from the spectra shown in Fig. 7 are plotted in Panel A of Fig. 8 versus the concentration (μM) of

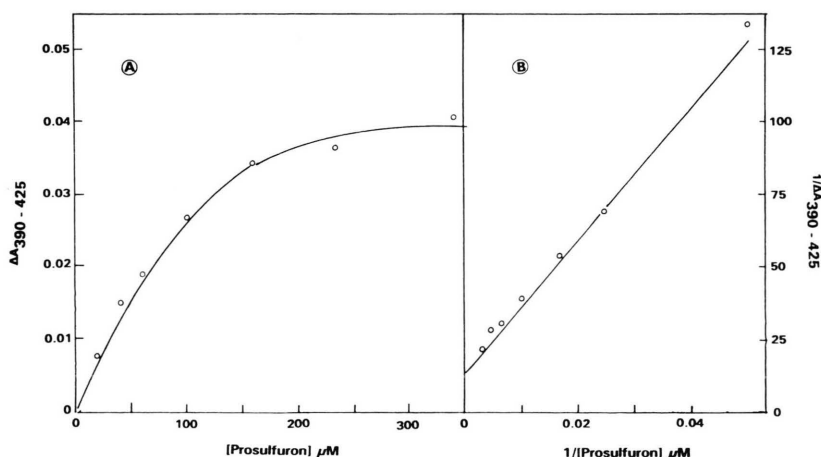


Fig. 8. Absorption differences (ΔA 390–425) were calculated from the spectra shown in Fig. 7 that were obtained with an avocado microsomal preparation. A. Spectral differences are plotted as a function of the concentration (μM) of prosulfuron. B. Double-reciprocal or Benesi-Hildebrand plot of the data shown in Panel A, i.e., $1/\Delta A$ versus $1/\text{prosulfuron}$ concentration (μM). A binding constant (K_s) of 157 μM was calculated from the linear relationship.

prosulfuron. The magnitude of the spectral difference was a function of the ligand concentration and became saturated at high concentrations of the ligand. A Lineweaver-Burk (Benesi-Hildebrand) double reciprocal plot of the data shown in Panel A, i.e., $1/\Delta A$ versus $1/\text{molar concentration}$ of prosulfuron is presented in Panel B. From this linear relationship, a binding constant, K_s , of $157 \mu\text{M}$ was calculated. A Scatchard (or Woolf) analysis in which ΔA is plotted versus $\Delta A/\text{molar concentration}$ exhibited a linear relationship and provided a K_s value of $120 \mu\text{M}$. Production of type I difference spectra with avocado microsomes was reproducible. With 7 different preparations, the K_s value averaged $187 \pm 35 \mu\text{M}$ as calculated from Lineweaver-Burk plots and $148 \pm 20 \mu\text{M}$ from Scatchard analyses. The linear relationship obtained from the two analyses suggested that prosulfuron may predominantly bind to a single P450 avocado isoform.

Discussion

Participation of cytochrome P450 monooxygenases in the metabolism of prosulfuron was documented by an absolute requirement for a reduced pyridine nucleotide, with NADPH being more effective than NADH (Table III), and sensitivity to P450 monooxygenase inhibitors (Table IV).

Microsomes isolated from young shoots of monocot seedlings have previously been shown to hydroxylate the aryl portion of other sulfonylureas. Specifically, such action has been documented for hydroxylation at the 5 position of the phenyl ring of chlorsulfuron, primisulfuron, and triasulfuron (Moreland *et al.*, 1993; Fonné-Pfister *et al.*, 1990; Frear *et al.*, 1991; Thalacker *et al.*, 1994), and the pyrimidine ring of nicosulfuron (Moreland *et al.*, 1993). As reported herein, the phenyl ring of prosulfuron, also is hydroxylated at the 5 position by microsomes isolated from young shoots of corn, sorghum, wheat, rice, barley, and oats, and avocado mesocarp tissue. Formation of 5-hydroxyphenyl prosulfuron has been reported previously by microsomes from wheat (Frear and Swanson, 1994) and corn (Moreland *et al.*, 1993). Sorghum microsomes, as reported herein, also formed a second major metabolite from prosulfuron that was identified by HPLC and mass spectrometry as having resulted from *o*-demethylation of the tri-

azine ring at the C4 position. The two metabolites were formed in an approximate 1:1 ratio by sorghum microsomes. Microsomes from species other than sorghum also formed trace amounts of the *o*-desmethyl metabolite, and a third metabolite that conceivably resulted from oxidation of the methyl group at C6 of the triazine ring.

The *o*-demethylation of prosulfuron by sorghum microsomes paralleled their ability to *o*-demethylate metolachlor which has been reported previously (Moreland *et al.*, 1990). Microsomes isolated from corn shoots also *o*-demethylated metolachlor (Moreland *et al.*, 1993) and microsomes from mung bean cotyledons *o*-demethylated metolachlor, CGA-24704, and alachlor (Moreland *et al.*, 1995). However, as reported herein, corn microsomes formed only small amounts of *o*-desmethyl prosulfuron, and, in exploratory studies, mung bean microsomes did not metabolize prosulfuron. We do not know if the same or different P450 sorghum *o*-demethylases are associated with the metabolism of both prosulfuron and metolachlor.

The inductive action of NA and ethanol in enhancing the rate of metabolism of many substrates has been documented extensively (Moreland *et al.*, 1993; Frear *et al.*, 1991; Thalacker *et al.*, 1994; Moreland *et al.*, 1995; and references therein). Treatment of both sorghum and corn seed/seedlings with NA and ethanol, alone and in combination, also enhanced the metabolism of prosulfuron. In sorghum, ethanol and NA were essentially equally effective, whereas in corn, NA induced the metabolism of prosulfuron about 10-fold with ethanol enhancing metabolism 3-fold. The combination of NA and ethanol seemed to be antagonistic in corn, but not in sorghum. The 1:1 ratio in the production of the two metabolites from prosulfuron by sorghum microsomes was not affected by the seed/seedling treatments. However, in the corn experiments, the safener treatments did not seem to greatly enhance the production of the minor metabolites.

It is not clear, at this time, if one or two sorghum P450 isoforms are involved in the phenylhydroxylation and *o*-demethylation of prosulfuron. No differential responses were noted relative to seed/seedling treatments, ability to accept reducing equivalents from NADPH and NADH, and sensitivity to inhibitors. In all of the above studies, the

1:1 ratio in metabolite formation was retained. However, the corn and sorghum phenylhydroxylases seemed to possess different properties as reflected in differential responses to safener treatments, ability to utilize reducing equivalents, and sensitivity to inhibitors.

Microsomes from different lots of P3245 corn seed varied somewhat in the amount of *o*-desmethyl prosulfuron that was produced. In the seed used previously (Moreland *et al.*, 1993), the ratio of M1 to M2 was approximately 4:1. However, in the seed lot used in the present study, the ratio was much lower and did not exceed 10:1, even with safener treatments (Figs. 2 and 6).

Of the plant cytochrome P450s, the avocado enzyme is the best characterized, however, the role of the P450 *in vivo* remains to be identified. O'Keefe and Leto (1989) purified a cytochrome P450 isoform from avocado mesocarp tissue that demethylated *p*CMA. Two immunologically similar polypeptides with molecular masses of 47 and 48 kD were isolated. Subsequently, a ripening-related cDNA from avocado mesocarp was sequenced that bears significant homology to mammalian cytochrome P450 sequences and codes for an *N*-terminal amino acid sequence identical to that obtained with purified avocado P450 (Bozak *et al.*, 1992). Avocado microsomes have also been reported to *N*-demethylate DMA, and to hydroxylate lauric acid (Cottrell *et al.*, 1990), nerol, and geraniol (Hallahan *et al.*, 1992).

For the most part, investigators have not been very successful in obtaining type I difference

spectra with crude preparations of monocot microsomes. However, some success has been achieved with avocado mesocarp preparations. As reported herein, we did obtain type I difference spectra for the interaction of prosulfuron with avocado microsomes and obtained an average K_s value of $187 \pm 35 \mu\text{M}$. Other investigators with avocado microsomes have published K_s values of $180 \mu\text{M}$ (O'Keefe and Leto, 1989) and $378 \mu\text{M}$ (Cottrell *et al.*, 1990) for the binding efficiency of *p*CMA; $87 \mu\text{M}$ with DMA and $339 \mu\text{M}$ with lauric acid (Cottrell *et al.*, 1990); and 7.2 and $35 \mu\text{M}$ with nerol and geraniol, respectively (Hallahan *et al.*, 1992).

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