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IN VITRO HYDROXYLATION OF BENTAZON BY MICROSOMES FROM NAPHTHALIC ANHYDRIDE-TREATED CORN SHOOTS

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In vitro metabolism of the herbicide bentazon was studied in microsomal membranes isolated from 6-day-old etiolated corn shoots. Microsomes isolated from shoots of nontreated seeds did not metabolize bentazon when assayed with NADPH or peroxides. However, microsomes isolated from shoots of seeds pretreated with naphthalic anhydride formed a single bentazon metabolite when provided with NADPH. The metabolite was identified as 6-hydroxybentazon, the major phase I metabolite produced in vivo. In vitro formation of this metabolite was strongly inhibited by carbon monoxide, nitrogen, and tetcyclacis (10 μM). The results suggest that aryl hydroxylation of bentazon in corn shoots is catalyzed by a cytochrome P-450 (E.C. 1.14.14.1) and that a seed pretreatment with naphthalic anhydride is necessary for recovery of activity in vitro.

The role that the cytochrome P-450 monooxygenase enzyme system plays in the metabolism of xenobiotics in mammals (1) and insects (2) is well established. The ability to characterize this enzyme complex in vitro has greatly advanced understanding of the types of reactions that it catalyzes.

The involvement of cytochrome P-450s in the metabolism of xenobiotics in plants has not been well characterized. There is considerable indirect evidence that cytochrome P450s play a major role in the detoxification of herbicides by plants and, hence, are important in regulating herbicide selectivity (3). However, it has proven to be difficult to demonstrate the involvement of cytochrome P-450 in herbicide metabolism with the use of in vitro assays. Presumably this is due to the low levels of cytochrome P-450s catalyzing these reactions in plants and the lability of this enzyme system during isolation (4, 5). The in vitro metabolism of herbicides by a microsomal cytochrome P-450 monooxygenase has been demonstrated in vitro in only three cases: the N-demethylation of the phenylureas (6), the aryl hydroxylation of 2,4-D (7) and the aryl hydroxylation of diclofop (8).

Abbreviations

2,4-D (2,4-dichlorophenoxy) acetic acid; PMSF, phenylmethylsulfonyl fluoride.

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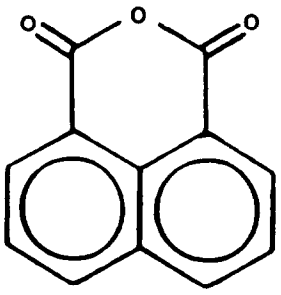

Figure 1. Structure of naphthalic anhydride.

Bentazon[1] is a herbicide that is metabolized in tolerant species, such as corn and soybean, by a two-step process involving aryl hydroxylation followed by glycosylation (9, 10). Indirect evidence suggests that the aryl hydroxylation is catalyzed by a cytochrome P-450 (3, 11). However, despite numerous attempts we, as well as others (12), have not been able to demonstrate significant rates of aryl hydroxylation of bentazon in microsomal fractions.

Herbicide safeners (also referred to as herbicide antidotes) protect some plants, primarily large-seeded grasses, against injury by selected herbicide classes (13). There is increasing evidence that safeners confer protection by causing the induction of enzymes catalyzing herbicide detoxification (14). Certain safeners have been shown to induce the synthesis of glutathione S-transferase isozymes that catalyze the metabolism of chloroacetanilide herbicides (15, 16, 17). There is also indirect evidence which suggests that pretreatment with the safener naphthalic anhydride (Fig. 1) increases the activity of monooxygenases catalyzing herbicide metabolism (18, 19).

In this paper, we demonstrate that a seed treatment with naphthalic anhydride is necessary in order to detect NADPH-dependent aryl hydroxylation of bentazon in a microsomal fraction isolated from etiolated corn shoots.

Materials and Methods

Corn seeds (Sigco Hybrid #1588) were planted in vermiculite, irrigated with water, and grown in darkness for 6 days at 26°C. Naphthalic anhydride was applied as a seed coating (approximately 0.5 g/100 g seeds) before planting.

For in vivo assays of bentazon metabolism, etiolated shoots (0.5 g) of 6-day-old plants were split lengthwise with a razor and placed in 2 ml of medium (25 mM MES-KOH, pH 5.0, 10 µM [^{14}C]bentazon, specific activity 2.1 mCi/mmol) contained in a 20 ml scintillation vial. Vials were incubated with shaking at room temperature for 6 h. Reactions were terminated by removing the incubation medium from the tissue with a Pasteur pipet followed by two rinses with 3 ml of distilled H_2O. Tissue was immediately frozen in liquid nitrogen and kept frozen until extraction by homogenization in 4 ml of 50% methanol using a Polytron homogenizer. Homogenates were filtered through Millipore HVLP filters and filtrates were analyzed for bentazon and metabolites as described below.

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[1]Mention of a trademark, vendor, or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Dep. Agric. or the Univ. of Minnesota and does not imply its approval to the exclusion of other products or vendors which may also be suitable.

For in vitro assays, 6-day-old etiolated seedling shoots were homogenized at 4°C with a mortar and pestle in buffer containing 0.1 M KH_2PO_4, 25 mM mercaptoethanol, and 10% glycerol (v/v) at pH 7.4. The homogenate was filtered through nylon mesh, centrifuged for 30 min at 4,000 g and the supernatant was centrifuged for 90 min at 100,000 g. The 100,000 g pellet (microsomes) was resuspended in 4 ml of buffer containing 0.1 M KH_2PO_4, 2.5 mM mercaptoethanol, and 10% glycerol (v/v). Resuspended microsomes (2.5 ml) were applied to a Sephadex G-25 column (Pharmacia PD-10) and eluted with 3.0 ml of the resuspension buffer. This fraction was used for enzyme assays and measurement of cytochrome P-450 levels.

Enzyme activity was assayed by adding 2 mM NADPH and 40 µM [^{14}C]bentazon (specific activity, 2.1 mCi/mmol) to 0.3 ml of the resuspended microsomal fraction (approximately 2 mg protein) and incubating in a shaking water bath for 2 h at 30°C. After 2 h, the reaction vials were frozen until analyzed for parent compound and metabolites as described below. Protein was measured using the method of Smith et al. (20). The requirement for oxygen was demonstrated by flushing the microsomes with N_2 for 15 min prior to assay, and for 15 min after addition of NADPH. The vial was then sealed and incubated with other samples. Carbon monoxide inhibition was demonstrated by bubbling CO through microsomes for 15 min, then flushing the vial with a mixture of 50% air/50% CO and sealing the vial. Tetcyclacis was dissolved in acetone, added to the assay vial, evaporated to dryness, and then dissolved in the assay buffer.

Procedures for the separation and identification of bentazon and metabolites were as described previously (10), with minor modification. The homogenate filtrates collected from the in vivo assays and the assay medium for the in vitro assays were passed through PrepSep C18 columns (Fisher Scientific) preconditioned with methanol and 1.0% acetic acid. Columns were washed with 2.0 ml of 1.0% acetic acid to remove water soluble compounds, and bentazon and metabolites were eluted with 2.0 ml of 80% methanol. The eluate was evaporated to dryness and redissolved in 80% acetone, filtered through a Gelman Acro LC3S 0.45 micron filter, and the filtrate was evaporated to dryness. Samples were then dissolved in 100-300 µl solvent (mobile phase) and injected onto a HPLC with UV detector and radioactivity monitor. Radiolabeled compounds were separated on a cyclobond column (Anspec) using 20%/80% acetonitrile/0.1% sodium acetate, pH 4.0, with isocratic flow at 1 ml/min. Peaks detected on the radioactivity monitor were either collected and counted by liquid scintillation spectroscopy to determine enzyme activity or were pooled and derivatized with trimethyl-silane for GC-MS. Derivatized fractions were injected directly onto the GC-MS, or in some cases were rechromatographed using reverse phase C18 HPLC chromatography (acetonitrile/H_2O, 10:90, v/v) for additional purification prior to GC-MS. GC-MS was conducted on a HP model 5980 GC coupled to a Model 5988A MS using a HP-1 crosslinked methyl silicone column (25 m x 0.32 mm). The injection port temperature was maintained at 225°C, and oven temperature was increased from 60 to 250°C at 40°C/min after injection. The MS source and interface were maintained at 225°C and 250°C, respectively. Identification of the metabolite was made by comparing HPLC retention times and GC-MS fragmentation patterns of the metabolite with those of standards provided by BASF Corp.

Cytochrome P-450 levels in the microsomal fraction were measured as described by Estabrook and Werringloer (21) using an extinction coefficient of 91 cm^{-1} mM^{-1}.

Bentazon [3-(1-methylethyl)-(1H)-2,1,3-benzothiadiazin-4(3H)-one, 2,2,dioxide], 98.5% purity, 6-hydroxybentazon, [^{14}C]bentazon (specific activity 10.5 mCi/mmol) were supplied by BASF Corp. The derivatization reagent, Tri Sil BSA, was obtained from Pierce Chemical Co. All other chemicals were obtained from Sigma.

Results and Discussion

Previous reports (3, 12) and our own research (data not shown) have indicated that corn rapidly metabolizes bentazon via aryl hydroxylation in the 6-position followed by glycosylation. However, microsomes isolated from 6-day-old etiolated corn shoots produced little or no 6-hydroxybentazon when supplied with NADPH and bentazon. The possibility that other cofactors were required or that another enzyme system

(peroxygenase, peroxidase) might be involved was considered. However, the microsomal fraction produced no metabolite of bentazon when supplied with peroxides (H_2O_2, cumene hydroperoxide) or with such cofactors as flavins or pterins. Recently, it was reported that the addition of protease inhibitors (PMSF, leupeptin) and substrate (trans-cinnamic acid) stabilized the P-450 that catalyzes the aryl hydroxylation of trans-cinnamic acid in microsomes isolated from pea (Pisum sativum L.) (4). However, the addition of protease inhibitors (0.5 mM PMSF, 1 µg/ml leupeptin) or bentazon (1 mM) to the homogenization buffer did not result in detectable aryl hydroxylation of bentazon in corn microsomes.

Experiments were also conducted to determine if bentazon hydroxylation was catalyzed by subcellular fractions other than microsomes. No activity was detected in the cytosolic fraction (post 100,000 g supernatant) when supplied with NADPH, peroxides, or cofactors required for dioxygenase activity (2-oxoglutarate, ascorbate, Fe^{2+}). As a result of the above negative results, we concluded that another approach was needed.

We examined whether pretreating corn seed with the herbicide safener, naphthalic anhydride, would increase the rate of bentazon metabolism in microsomes. Microsomes isolated from naphthalic anhydride-treated corn produced a single bentazon metabolite when supplied with NADPH in the standard assay (Fig. 2). This metabolite was identified by GC-MS as 6-hydroxybentazon, which is the major phase I metabolite produced in vivo (12). The mass spectrum of this metabolite (Fig. 3) is identical to the fragmentation pattern of a derivatized authentic standard of 6-hydroxybentazon. The structures of the major fragments are also shown.

In addition to its NADPH-dependence, other evidence suggests that the aryl hydroxylation of bentazon by corn microsomes is catalyzed by a cytochrome P-450 monooxygenase. The enzyme requires oxygen and is strongly inhibited by pretreatment

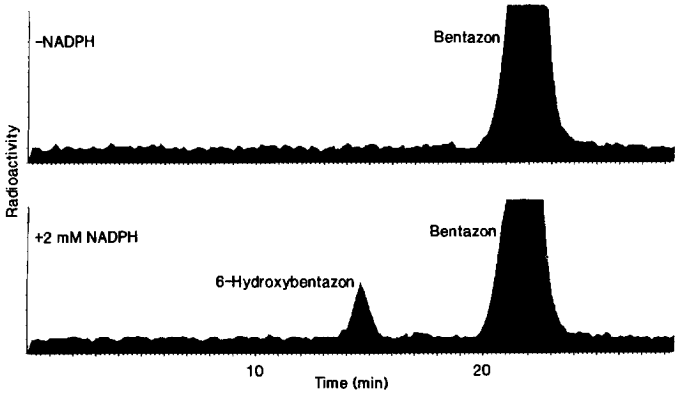

Figure 2. Radioactivity chromatograph showing the NADPH-dependence of bentazon hydroxylation measured in microsomes isolated from shoots of naphthalic anhydride-treated corn seed.

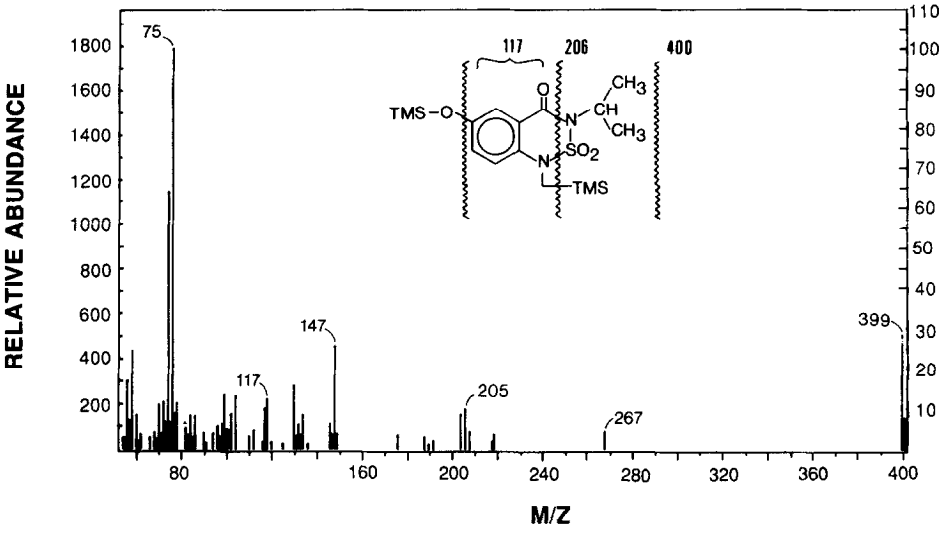

Figure 3. Mass spectrum of the trimethyl-silane-derivatized bentazon metabolite produced by a microsomal fraction isolated from shoots of naphthalic anhydride-treated corn seed.

with carbon monoxide (Table 1). The enzyme is also strongly inhibited by tetcyclacis (Table 1) which is a potent inhibitor of plant cytochrome P-450 enzymes (8). Bentazon hydroxylation was not effectively supported by NADH. Activity with 2 mM NADH was 10% of that obtained with 2 mM NADPH (data not shown).

Preliminary kinetic analysis using Lineweaver-Burke plots indicated that the enzyme catalyzing bentazon hydroxylation has an apparent K_m for bentazon of 54 μM and a V_{max} of 1.37 nmol/mg protein/h. The apparent K_m for bentazon is comparable to that reported for the cytochrome P-450 catalyzing aryl hydroxylation of diclofop in wheat (8). However, the V_{max} is lower.

It is widely known that pretreating mammalian tissues with certain xenobiotics such as phenobarbital and 3-methylcholanthrene increases cytochrome P-450 levels and the rate of metabolism of selected xenobiotics (1). These changes are attributed to the ability of various xenobiotics to cause the induction of cytochrome P-450 isozymes (1).

In plants, treatment with selected xenobiotics increases microsomal cytochrome P-450 content and, in some cases, the metabolism of xenobiotics such as herbicides. Treating Jerusalem artichoke with herbicides (monuron, dichlobenil, 2,4-D) and phenobarbital increased microsomal cytochrome P-450 content (22, 23). Treating corn leaves with naphthalic anhydride increased the rate of metabolism of the herbicide chlorsulfuron, which is thought to be metabolized by a cytochrome P-450 monooxygenase (18).

We examined the effect of naphthalic anhydride on cytochrome P-450 levels in corn shoots. Cytochrome P-450 levels in microsomal fractions isolated from shoots of nontreated seed ranged from 0.16 to 0.23 nmol/mg protein which is in close agreement with values previously reported for corn shoots (24). P-450 levels in microsomes isolated from naphthalic anhydride-treated seed exhibited a wider range, from 0.18 to

Table 1
Effect of cytochrome P-450 inhibitors on bentazon hydroxylation in vitro

Treatment	% Inhibition
Control	--
N_2	82% ± 9
CO	70% ± 13
10 μM tetcyclacis	92% ± 2

Rate of formation of 6-hydroxybentazon in the control was 0.93 nmol/mg protein/h. Results are expressed as the mean ± standard error (n=4).

0.32 nmol/mg protein. Because of the larger variation in cytochrome P-450 levels in naphthalic anhydride-treated shoots, the average P-450 content (0.22 ± 0.05 nmol/mg protein) was not significantly different from nontreated shoots (0.19 ± 0.03 nmol/mg protein). Spectrophotometry measures total P-450, however, and the induction of a specific isozyme of P-450 that catalyzes aryl hydroxylation of bentazon may not be detectable.

We therefore examined the effect of naphthalic anhydride treatment on the in vivo rate of bentazon metabolism in corn shoots. In preliminary experiments with apical segments of corn coleptiles, there were no significant differences in the rate of bentazon metabolism in control and naphthalic anhydride-treated corn shoots. However, we considered the possibility that the uptake of the herbicide by intact coleoptiles may limit the rate of in vivo metabolism and prevent the expression of enhanced metabolism due to naphthalic anhydride treatment. To promote the absorption of bentazon, the coleoptiles were split lengthwise with a razor which eliminated the restriction on uptake imposed by the cuticle. In addition, the pH of the incubation medium was lowered from pH 6.5 to 5.0. Lower pH increases the amount of the undissociated form of bentazon and hence promotes uptake (25). When these changes were made, bentazon metabolism was 70% higher in the naphthalic anhydride-treated tissue; 1.16 ± 0.10 nmol/h/g fresh wt in naphthalic anhydride-treated tissue versus 0.68 ± 0.03 nmol/h/g fresh wt in nontreated tissue. Naphthalic anhydride-treatment did not alter the pattern of herbicide metabolism. In both nontreated and treated shoots, the metabolite formed was the glycosyl conjugate of 6-hydroxybentazon.

The fact that there was a significant increase in metabolism of bentazon in naphthalic anhydride-treated tissue with only a small increase in total P-450 content suggests that naphthalic anhydride may act by increasing the level of a specific isozyme(s) in corn shoots responsible for bentazon metabolism. However, we can not rule out the possibility that naphthalic anhydride may also have other effects in vivo which serve to stabilize enzyme activity during isolation.

Advances in the characterization of cytochrome P-450s involved in herbicide metabolism have been hampered by the difficulty in obtaining microsomal fractions that

exhibit activity in vitro (3, 5). Our work shows that pretreating corn with naphthalic anhydride allows for the in vitro study of the cytochrome P-450 catalyzing bentazon hydroxylation. This effect of naphthalic anhydride pretreatment is not limited to bentazon. Pretreating wheat shoots with naphthalic anhydride caused a 30-fold increase in the in vitro activity of the P-450 catalyzing the aryl hydroxylation of the herbicide diclofop (D.S. Frear, USDA-ARS, Bioscience Research Lab, Fargo, ND, personal communication). Naphthalic anhydride may prove to be a useful tool that will allow for the in vitro characterization of P-450s that catalyze herbicide metabolism in plants.

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