# Additional observations on the chlorotoluron hydroxylase and N-demethylase activities in wheat microsomes

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# ADDITIONAL OBSERVATIONS ON THE CHLOROTOLURON HYDROXYLASE AND *N*-DEMETHYLASE ACTIVITIES IN WHEAT MICROSOMES

Short title : Oxidative reactions of chlorotoluron *in vitro*

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**Summary.** The ring-methyl hydroxylation and *N*-demethylation of chlorotoluron were studied in microsomes of wheat (*Triticum aestivum*) isolated from 5-day-old plants and cultivated cells. The two reactions were noted in both materials, but exhibited higher rates in cells than in plants. Treatement of cells with 300 µM 2,4-dichlorophénoxyacetic acid for 48 h gave mean rates of 40 and 55 pmol $min^{-1}$ $mg^{-1}$ for the ring-methyl hydroxylation and the *N*-demethylation, respectively. The reactions required NADPH as cofactor and aerobic conditions. Chlorotoluron ring-methyl hydroxylase and *N*-demethylase as well as cinnamic acid 4-hydroxylase were drastically inhibited by polyclonal antibodies raised against NADPH cytochrome c reductase. Polyclonal antibodies raised against cinnamic acid 4-hydroxylase and 7-ethoxycoumarin *O*-deethylase did not affect chlorotoluron metabolism. Cytochrome P-450 spectra of type I and type II were obtained using microsomes of wheat cells in the presence of chlorotoluron and procloraz, respectively. Among tested effectors, those known as inhibitors of cytochrome P-450 enzymes were the most efficient on ring-methyl hydroxylation and *N*-demethylation of chlorotoluron. Results are consistent with a cytochrome P-450-dependent oxidation of that herbicide in microsomes of wheat cells, which also metabolized diuron and isoproturon.

**Additional key words** - Herbicide metabolism, phenylureas, cell suspension cultures, 2,4-D, cytochrome P-450 monooxygenases.

**Abbreviations.** CA4H, cinnamic acid 4-hydroxylase ; CPUDM, chlorotoluron *N*-demethylase ; CPUH, chlorotoluron ring-methyl hydroxylase ; 2,4-D, 2,4-dichlorophenoxyacetic acid ; DMSO, dimethyl sulfoxide ; ECOD, 7-ethoxycoumarin *O*-deethylase ; SOD, superoxide dismutase.

## INTRODUCTION

Although studies of herbicide metabolism have shown that numerous herbicides undergo oxidation in plants, our knowledge of the enzymatic systems involved in these reactions is rather poor.

Frear *et al.* (1969) published the first results showing the occurrence of a cytochrome P-450 system in the *N*-demethylation of monuron in cotton (*Gossypium hirsutum*). Several years later, Makeev *et al.* (1977) showed that a similar enzymatic system was responsible for the hydroxylation of 2,4-dichlorophenoxyacetic acid (2,4-D) in cucumber (*Cucumis sp.*) leaves.

More recently, the implication of cytochrome P-450 systems in the oxidation of herbicides was established in Jerusalem artichoke (*Helianthus tuberosus*)(chlorotoluron : Fonné, 1985), corn (*Zea mays*)(chlorotoluron : Fonné-Pfister and Kreuz, 1990 ; primisulfuron : Fonné-Pfister *et al.*, 1990 ; bentazon : McFadden *et al.*, 1990), and in sorghum (*Sorghum bicolor*)(metolachlor : Moreland *et al.*, 1990). Microsomal fractions from wheat (*Triticum aestivum*) also catalyzed the oxidation of diclofop (McFadden *et al.*, 1989 ; Zimmerlin and Durst, 1990 ; Frear *et al.*, 1991) and other herbicides such as triasulfuron, chlorsulfuron and linuron (Frear *et al.*, 1991).

We previously showed that the oxidative metabolism of chlorotoluron in wheat was catalyzed by cytochrome P-450 enzymes (Mougin *et al.*, 1990, 1991). Microsomes

isolated from wheat cell cultures possessed chlorotoluron ring-methyl hydroxylase (CPUH) and chlorotoluron *N*-demethylase (CPUDM) activities by exhibiting some characteristic properties of cytochrome P-450 monooxygenases. Moreover, agrochemicals such as 2,4-D or cyometrinil, applied to cells 2 or 3 days before isolation of microsomes, were able to increase *in vitro* (a) rates of cinnamic acid 4-hydroxylase (CA4H) and lauric acid hydroxylase, two cytochrome P-450 dependent monooxygenases of higher plants, (b) levels of cytochrome P-450 contents, and (c) rates of CPUH and CPUDM activities (Mougin *et al.*, 1990). Conversely, inhibitors of P-450 decreased the CPUH and CPUDM activities when added to microsomal incubation media (Mougin *et al.*, 1991).

In this paper, the postulated involvement of cytochrome P-450 monooxygenases in chlorotoluron detoxication was studied more thoroughly by determining spectral properties of the enzymes present in microsomes, by evaluating the effects of reduced pyridine nucleotides, cofactor analogues, compounds modulating NADPH-dependent activities, polyclonal antibodies raised against plant cytochrome P-450 systems, compounds modifying hydroperoxide-dependent activities, and by examining substrate specificities of enzymes oxygenating chlorotoluron. We also investigated the ability of microsomal preparations to oxidize two other herbicidal phenylureas, namely diuron and isoproturon.

## MATERIALS AND METHODS

**Chemicals.** [Carbonyl-$^{14}$C]chlorotoluron (3-[3-chloro-p-tolyl]-1,1-dimethylurea) was synthesized according to Mougin *et al.* (1990). [Carbonyl-$^{14}$C]diuron (3-[3,4-dichloro]-1,1-dimethylurea) and isoproturon (3-[4-isopropyl]-1,1-dimethylurea) were gifts of Dr. P. Gaillardon (I.N.R.A., Dijon, France). Unlabelled chlorotoluron, diuron and isoproturon were purchased from Pestanal (Hannover, RFA). [3-$^{14}$C]*trans*-cinnamic acid was from C.E.A. (Gif/Yvette, France). Other chemicals were obtained from Sigma.

**Plant material.** Seeds of wheat (*Triticum aestivum* L., var "Etoile de Choisy") were allowed to germinate on wet paper for 5 days at 25°C in the dark. Etiolated shoots were then excised from the caryopses and stored for 1 h on ice prior to the isolation of microsomes.

**Cell cultures and treatments.** Achlorophyllous wheat cells (*Triticum aestivum* L., var. "Koga II") were routinely cultivated as previously described (Canivenc *et al.*, 1989). Treatment of cultured cells with 300 µM 2,4-D, in order to stimulate the rates of monooxygenase activity and to enhance the levels of cytochrome P-450 have also been described (Mougin *et al.*, 1991). Microsomal fractions were isolated 48 h following the beginning of 2,4-D treatment. Cell suspensions were kept for 1 h at 4°C, and then filtered through a

stainless steel filter prior to the isolation of microsomes.

**Preparation of microsomes**. Microsomes were prepared from wheat shoots (30-40 g fresh weight) or cells (55-65 g fresh weight) according to Mougin *et al.* (1991).

**Enzymatic oxidative activities using labelled substrates**. Unless otherwise stated, enzymatic activities were carried out at 25°C. Assay media for oxidative reactions contained 15% (w/v) glycerol, 0.7 mM mercaptoethanol, and 0.4-0.6 mg microsomal proteins in a 200 µl final volume of 0.1 M phosphate buffer (pH 7.5). Published methods were used to routinely assay the ring-methyl hydroxylation and the *N*-demethylation of chlorotoluron, as well as the (CA4H(Mougin *et al.*, 1991). Cumene hydroperoxide-dependent *N*-demethylation of chlorotoluron was assayed in 200 µl 0.1 M phosphate buffer (pH 7.5) plus 15% (w/v) glycerol, 0.4-0.6 mg microsomal proteins in the absence of mercaptoethanol, and 0.5 mM cumene hydroperoxide. Assays were stopped after 15 min with 100 µl absolute ethanol, in order to prevent any chemical oxidation of chlorotoluron by cumene hydroperoxide, as observed in the presence of acetonitrile under acidic conditions. After sonication and a short centrifugation (3 min) of the incubation media, substrates and metabolites of each enzymatic reaction were analyzed from two 100 µl samples by reversed phase HPLC as previously described (Mougin *et*

*al.*, 1991). Longer-time assays were run at 28°C for 90 min with chlorotoluron, diuron or isoproturon as substrates, each of them at 500 µM ([carbonyl-$^{14}$C]labelled plus unlabelled herbicide). After every 30 min of incubation, 0.5 mM NADPH and a NADPH-regenerating system were added to the medium. Incubation media consisted of 0.1 M phosphate buffer (pH 7.5), 15% (w/v) glycerol, 0.7 mM mercaptoethanol and 1 mM glutathione. Assays were stopped by addition of 1 ml *n*-hexane and sonicated. Substrates and metabolites of enzymatic reactions were successively extracted with 2 x 1 ml *n*-hexane, 2 x 1 ml diethyl-ether/*n*-hexane (1/1 , v/v) and 1 ml pure diethyl-ether. Fractions were reduced under vacuum, pooled and spotted on thin layer chromatography silica gel 60$F_{254}$ plates (Merck). Chlorotoluron (Canivenc *et al.*, 1989), diuron (Swanson and Swanson, 1968) and isoproturon (Cabanne *et al.*, 1987) extracts were analyzed as previously described.

**Peroxidative activities.** Assay media for peroxidative reactions contained 15% (w/v) glycerol and 0.3 mg microsomal proteins in a final 1 ml volume of 0.1 M phosphate buffer (pH 7.5). Peroxygenase activity (Ishimaru and Yamasaki, 1977 a and b) was assayed with 2 mM aniline as substrate. The formation of nitrosobenzene was spectrophotometrically followed at 310 nm ($\bar{}_{310}$ = 6833 $M^{-1}$ $cm^{-1}$) according to Blée and Durst (1987) in the presence of 2 mM cumene

hydroperoxide. Controls were incubated without aniline. Peroxidase activity was determined using guaiacol as a substrate by the method of Maehly and Chance (1954). Changes in absorbance were followed at 436 nm in the presence of 2 mM $H_2O_2$ and 2 mM guaiacol. An absorbance coefficient of 25500 $M^{-1}$ $cm^{-1}$ was used. Hydrogen peroxide was omitted in controls.

**NADPH-cytochrome c reductase activity.** NADPH-cytochrome c reductase activity was measured after addition of 2 mM KCN to the reaction mixture to minimize cytochrome c reoxidation by mitochondria, according to the method of Benveniste *et al.* (1986). An extinction coefficient of 18500 $M^{-1}$ $cm^{-1}$ at 550 nm was used for activity determination.

**Other methods.** Effects of antibodies were measured by incubating microsomes with the IgG fraction of sera of rabbits immunized against several enzymes purified from Jerusalem artichoke tubers : NADPH-cytochrome P-450 reductase (Benveniste *et al.*, 1986), CA4H (Gabriac *et al.*, 1991), and 7-ethoxycoumarin O-deethylase (ECOD)(Werck-Reichhart *et al.*, 1990). Quantitative determination of hemoproteins was carried out using the method of Omura and Sato (1964). Type I and II spectra were obtained with fractions that had been enriched in endoplasmic reticulum by the following method : microsomes were layed onto a sucrose cushion (30% , w/v) and centrifuged for 90 min at 80000xg (Beckman SW

27 rotor). The fraction lying on the cushion was used for interaction spectra. For all recordings of difference spectra, the protein concentration was 1 mg $ml^{-1}$. An ethanolic solution of chlorotoluron was added to a solution of bovin serum albumin (1 mg $ml^{-1}$), the solvent was evaporated under vaccum, and the resulting solution of bovin serum albumin plus chlorotoluron was used for spectral measurements. Final concentrations of herbicide were 100 and 160 µM. Procloraz, dissolved in dimethyl sulfoxide (DMSO)15%, was administered to microsomes in the range from 100 to 400 µM. Microsomal proteins were estimated according to Bradford (1976) with bovine serum albumin as a standard. Effectors were added to the incubation media dissolved in phosphate buffer or in DMSO. In the latter case, final solvent concentration was 1% (v/v) and did not affect enzymatic activities. Controls were supplemented with the same volumes of solvent. Results are expressed as means of independent experiments (n = 2 to 4) ± SD. Enzymatic activities and cytochrome P-450 levels are expressed in pmol $min^{-1}$ $mg^{-1}$ and pmol $mg^{-1}$ of microsomal proteins, respectively.

# RESULTS

## Spectral properties of the enzymes present in microsomes of wheat

Several difference spectra of wheat microsomes were obtained (fig. 1). Reduced carbon monoxide spectra showed that microsomes isolated from wheat cells, grown for 48 h in the presence of 300 µM 2,4-D, contained about 250 pmol cytochrome P-450 per mg microsomal proteins. Low levels of P-420 were present (fig. 1 A).

The substrate binding spectrum of type I is one of the criteria often reported in favour of the involvement of a cytochrome P-450 in an enzymatic reaction. A spectrum of this type was obtained by incubating wheat cell microsomes with chlorotoluron according to the way indicated in Materials and Methods(fig. 1 B). The spectra showed a peak at 389 nm and a minimum near 410 nm for 100 and 160 µM herbicide, evidencing the binding of chlorotoluron to the catalytic site of cytochrome P-450 systems.

The fungicide procloraz, an inhibitor of sterol biosynthesis, combines with P-450 from bird and rat livers to give a type II spectrum (Rivière, 1983). It was found to inhibit chlorotoluron oxidation by wheat microsomes when applied at 100 µM (Mougin *et al.*, 1991). In the same concentration range (from 100 to 400 µM), procloraz induced the formation of type II spectra with a minimum at 390-400 nm and a peak at 429 nm (fig. 1 C).

**Ability of microsomes isolated from wheat shoots to metabolize chlorotoluron**

Microsomes isolated from wheat cell cultures exibited CPUH and CPUDM activities which were enhanced following subculturing of cells in the presence of 400 µM cyometrinil or 300 µM 2,4-D (Mougin *et al.*, 1990, 1991). The activities were not restricted to whole cells, microsomes isolated from wheat shoots were also able to hydroxylate and to *N*-demethylate chlorotoluron in the presence of NADPH (fig. 2 A). However, CPUH and CPUDM activities of wheat shoot microsomes were about 6-fold lower than those of microsomes of wheat cells treated with 2,4-D (fig. 2 B), amounting to 17.0 and 35.0 pmol $min^{-1}$ $mg^{-1}$ in the same conditions of incubation. No peaks corresponding to metabolites could be detected on radio-chromatograms following incubations in the absence of NADPH or microsomal proteins. In order to stimulate the CPUH and CPUDM activities of the plant microsomal preparations, we treated plants with agrochemicals such as herbicide safeners cyometrinil and naphthalic anhydride, reported to stimulate monooxygenase activities (McFadden *et al.*, 1990 ; Mougin *et al.*, 1991 ; Frear et *al.*, 1991). However, none of these treatments stimulated the microsomal metabolism of chlorotoluron. Consequently, all the following experiments were done with microsomes isolated from 2,4-D treated cell cultures.

**Effects of reduced pyridine nucleotides and cofactor analogues on chlorotoluron ring-methyl hydroxylase and *N*-demethylase activities**

CPUH and CPUDM activities were measured in the presence of cofactors applied under various conditions. Hydroxylated and N-demethylated chlorotoluron were detected after incubation with NADPH and molecular oxygen (tab. 1). The best activities were obtained with 1 mM NADPH, a 10-fold lower concentration still gave rates equal to 87-92% of maximum activities. NADH was poorly efficient when given alone. Whatever the cofactor concentrations, we did not find any synergistic interactions between NADH and NADPH (not shown). Some residual activities were detected in the absence of any external cofactor.

Effects of structural analogues of NADPH were tested at 0.5 and 2.0 mM, in the presence of 0.5 mM NADPH (tab. 2). When analogues were applied at 2 mM, they inhibited both CPUH and CPUDM. For example, 2',5'-ADP was the most efficient inhibitor, with 42-43% inhibition, whereas $NADP^+$ was less efficient (31-32% inhibitions). 2'-AMP produced little inhibition and all compounds exerted only weak inhibition at 0.5 mM.

**Effects of compounds modulating NADPH-dependent activities**

At least in theory, cytochrome P-450 monooxygenases, peroxidases, peroxygenases and flavin-containing enzymes can catalyze hydroxylation or

*N*-dealkylation reactions in plants or animals. Therefore the effects of various compounds interacting with these enzymes were evaluated on CPUH and CPUDM activities (tab. 3).

Carbon monoxide and *o*-phenanthroline are classical inhibitors of P-450 monooxygenases. When bubbled for 30 s in the incubation medium, carbon monoxide inhibited both CPUH by 54% and CPUDM by 36%. The *o*-phenanthroline which is known to chelate heme iron (Soliday and Kolattukudy, 1977) fully inhibited CPUH and CPUDM activities at 600 µM.

Mercaptoethanol, KCN, catalase, reduced glutathione and ascorbate usually inhibit peroxidase-mediated reactions (Gullner and Tyihak, 1987). In the oxidation of chlorotoluron, 2.4 mM mercaptoethanol as well as 1 mM KCN or ascorbate weakly inhibited CPUH and CPUDM, whereas catalase and reduced glutathione slightly increased the activities.

*N*-octylamine was tested, because it is a cytochrome P-450 inhibitor and a stimulator of flavin-containing monooxygenases (Duffel *et al.*, 1981). It strongly lowered the hydroxylase and *N*-demethylase activities. Methimazole, an inhibitor of flavin-containing monooxygenases (Sabourin and Hodgson, 1984) had no effect on the tested activities, as already reported concerning the chlorotoluron *N*-demethylase of microsomes isolated from Jerusalem artichoke tubers (Fonné, 1985).

Some inhibitors of lipid peroxidation and NAD(P)H oxidases were also assayed in order to determine whether detrimental reactions reduced the P-450 activities. Methylindole, an inhibitor of lipid peroxidation (Adams *et al.*, 1987) very slightly stimulated the enzymatic activities at 100 µM (tab. 3). Superoxide dismutase (SOD) stimulated both activities, when added alone or with catalase. In the latter case, the effects corresponded to additional effects of the two compounds applied alone.

**Effects of antibodies on cinnamic acid 4-hydroxylase, chlorotoluron ring-methyl hydroxylase and *N*-demethylase activities**

CA4H, CPUH and CPUDM activities of wheat cell microsomes were evaluated in the presence of polyclonal antibodies raised against components of P-450s of higher plants (tab. 4). The three activities were drastically reduced after 10 min incubation with antibodies raised against purified NADPH-cytochrome c (P-450) reductase from Jerusalem artichoke tubers. Under the same experimental conditions, NADPH-cytochrome c reductase activity was inhibited by 75% (not shown). Wheat CA4H was drastically inactivated by antibodies raised against Jerusalem artichoke CA4H, whereas CPUH and CPUDM activities were unaffected. Antibodies raised against ECOD from Jerusalem artichoke did not inhibit activity of wheat enzymes.

**Effects of compounds modifying hydroperoxide-dependent activities**

The chlorotoluron *N*-demethylase was weakly sensitive to carbon monoxide and aminobenzotriazole, but showed a cumene hydroperoxide-dependent activity (Mougin *et al.*, 1990). That could suggest a possible participation of peroxidases or peroxygenases in the *N*-demethylation of the herbicide. Activities of two hydroperoxide-dependent enzymes, namely aniline oxidase (a peroxygenase) and guaiacol oxidase (a peroxidase) were assayed using microsomes isolated from 2,4-D treated cells. In addition, the sensitivity to mercaptoethanol of the cumene hydroperoxide-dependent CPUDM activity was evaluated because this compound is known to inhibit the peroxidative enzymes (Blée and Durst, 1987 ; Gullner and Tyihak, 1987).

No activities of the peroxygenase and peroxidase-type enzymes were detected in the absence of hydroperoxyde(s), except a basal CPUDM activity (tab. 5). However, significant activities were measured in the presence of cofactors, characteristic of CPUDM, aniline oxidase and guaiacol oxidase. CPUDM was unaffected by 1.4 mM mercaptoethanol, while peroxygenase and peroxidase activities were inhibited. Again, only the CPUDM activity was inhibited by 200 µM *p*-chloromercuribenzoate, which converts the cytochrome P-450 to inactive P-420 (Potts *et al.*, 1974). Other experiments performed with microsomes isolated from cyometrinil treated cells showed that only the

peroxidase activity was sensitive to 1 mM KCN (C. Mougin, unpublished results).

**Substrate specificities of enzymes oxygenating chlorotoluron**

Substrate specificities of cytochrome P-450 forms oxidizing chlorotoluron in wheat are not known yet. Also, the identity of the physiological substrates of these enzymes is still an enigma. A study of competition between chlorotoluron and an exogenous substrate or a known physiological substrate of P-450 was carried out to shed some light on these problems. Effects of some physiological or exogenous substrates of cytochrome P-450 systems are shown in tab. 6. Concentrations equivalent to 10 times the apparent Km were chosen for physiological substrates of P-450 cytochromes of plants. Exogenous substrates were applied at dose equivalent, in most cases, to their apparent Km values found in recent papers.

Results indicated that the addition of 300 µM cinnamic acid in the incubation medium containing 500 µM chlorotoluron slightly stimulated CPUH and CPUDM activities. By contrast, lauric acid, ferulic acid, and the exogenous substrates had some inhibiting effects toward the CPUDM activity, except the benzo[a]pyrene.

**Ability of microsomes isolated from wheat cell cultures to oxidize diuron and isoproturon**

Diuron and isoproturon are structural analogues of chlorotoluron. Diuron is *N*-dealkylated in wheat plants (Ryan *et al.*, 1981), and isoproturon undergoes a ring-isopropyl hydroxylation and a *N*-dealkylation in those plants (Cabanne *et al.*, 1987). When added to microsomes in the presence of chlorotoluron and at the same concentration as the herbicide, diuron strongly lowered both CPUH and CPUDM activities by about 70% inhibition, whereas isoproturon had no effect (Mougin *et al.*, 1991).

We tested the *in vitro* oxidation of these two herbicides by microsomes isolated from 2,4-D treated cells. Microsomes were able to metabolize diuron (fig. 3 A) and isoproturon (fig. 3 B) probably to *N*-dealkylated metabolites, in the presence of NADPH. The rates of reactions were in the same range for the two herbicides, corresponding to 3-5 pmol $min^{-1}$ $mg^{-1}$ of each metabolite. These rates were lower than those obtained for the ring-methyl hydroxylation and *N*-demethylation of chlorotoluron. No transformation could be detected in the absence of NADPH cofactor.

## DISCUSSION

Wheat plants actively metabolize the herbicide chlorotoluron *in vivo* (Gross *et al.*, 1979 ; Cabanne *et al.*, 1987). The plant enzymatic systems metabolizing chlorotoluron are constitutive, but their activities need to be stimulated for *in vitro* studies. Using wheat cell cultures, Canivenc *et al.* (1989) reported the marked effects of 2,4-D and cyometrinil on chlorotoluron metabolism. The stimulations noted *in vivo* were found in microsomal preparations, allowing to show the involvement of cytochrome P-450 monooxygenases in chlorotoluron metabolism (Mougin *et al.*, 1990, 1991).

This paper provides additional observations involving cytochrome P-450 enzymes in chlorotoluron metabolism in microsomes isolated from 2,4-D treated cells.

NADPH was the preferred electron donor for chlorotoluron oxidation, although NADH was able to support appreciable reaction rates. The involvement of NADPH cytochrome P-450 reductase was also evidenced by the effects of structural analogues of NADPH that compete with the coenzyme at the level of the flavoprotein (Salaun *et al.*, 1980). Interestingly, antibodies raised against NADPH cytochrome c reductase from Jerusalem artichoke drastically inhibited both CPUH and CPUDM activities of wheat cells. Because antibodies raised against CA4H did not interact with

enzymes responsible for chlorotoluron metabolism, the cytochrome P-450 forms responsible for the CA4H, CPUH and CPUDM activities are probably different.

Type I spectra of P-450 were obtained with wheat cell microsomes in the presence of chlorotoluron, demonstrating the binding of the herbicide in a catalytic site of P-450. Carbon monoxide, which is the most classical inhibitor of P-450, inhibits both CPUH and CPUDM, although this latter enzyme seems to be less sensitive. Procloraz induced a type II binding spectra when added to microsomes, suggesting the binding of the fungicide to the heme iron of P-450, in agreement with the inhibition of chlorotoluron oxygenation already described (Mougin *et al.*, 1991). The cytochrome P-450 nature of enzymes oxidizing chlorotoluron is strengthened by the effects of *o*-phenanthroline and *n*-octylamine on both CPUH and CPUDM activities.

No strong interactions were observed between CPUH and CPUDM activities and physiological or exogenous substrates of P-450. Again, chlorotoluron metabolism is probably not mediated in wheat by CA4H, laurate in-chain hydroxylase, or ferulic acid 5-hydroxylase.

The various inhibitors of peroxidases, peroxygenases and flavin-containing monooxygenases never exerted significant inhibiting effects on CPUH and CPUDM. Incubations done in the presence of cumene hydroperoxide and inhibitors of peroxidase and peroxygenase-type enzymes showed that the properties of

the chlorotoluron *N*-demethylase are distinct from those of these enzymes.

Taken together, these new results agree with those previously obtained *in vivo* and *in vitro*, and are consistent with the hypothesis that cytochrome P-450 enzymes catalyze the ring-methyl hydroxylation and the *N*-demethylation of chlorotoluron in microsomal preparations isolated from wheat cell cultures.

The phenylureas diuron and isoproturon were also found to be oxidized by microsomes isolated from wheat cells. This result is worth comparing with previous data showing that diuron acted as a good competitor of chlorotoluron metabolism *in vitro*, whereas isoproturon had no effect (Mougin *et al.*, 1991). Identities of the enzymes involved in these oxidations need to be confirmed by additional experiments.

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**Table 1.** *Effects of reduced pyridine nucleotides on chlorotoluron ring-methyl hydroxylase (CPUH) and N-demethylase (CPUDM) activities.*

| Incubation conditions | | Relative activities (%) | |
|---|---|---|---|
| | | CPUH | CPUDM |
| NADPH | 1.0 mM | 100.0[a] ± 2.6 | 100.0[b] ± 4.9 |
| NADPH | 0.5 mM | 92.5 ± 3.9 | 97.2 ± 2.9 |
| NADPH | 0.1 mM | 87.4 ± 5.4 | 92.2 ± 2.6 |
| NADPH | 0.0 mM | 18.7 ± 1.7 | 9.0 ± 0.5 |
| NADH | 10.0 mM | 85.7 ± 8.2 | 48.2 ± 4.2 |
| NADH | 1.0 mM | 53.5 ± 3.5 | 38.0 ± 2.8 |

Activities are expressed as % of maximal activities : [a]34.0 and [b]42.5 pmol $min^{-1}$ $mg^{-1}$. Incubations were conducted for 25 min in the presence of NAD(P)H regenerating system.

**Table 2.** *Effects of pyridine nucleotide analogues on chlorotoluron ring-methyl hydroxylase (CPUH) and N-demethylase (CPUDM) activities.* Activities were assayed during 15 min runs in the absence of a cofactor regenerating system.

| Incubation conditions | | Relative activities (%) | |
|---|---|---|---|
| | | CPUH | CPUDM |
| NADPH | 0.5 mM | 100.0[a] ± 1.1 | 100.0[b] ± 2.0 |
| + 2'-AMP | 0.5 mM | 104.5 ± 2.0 | 97.4 ± 2.9 |
| + 2'-AMP | 2.0 mM | 95.1 ± 7.1 | 91.3 ± 5.7 |
| + 2',5'-ADP | 0.5 mM | 82.4 ± 2.1 | 89.2 ± 5.9 |
| + 2',5'-ADP | 2.0 mM | 57.1 ± 4.2 | 58.1 ± 5.3 |
| + $NADP^+$ | 0.5 mM | 90.9 ± 7.9 | 102.5 ± 6.0 |
| + $NADP^+$ | 2.0 mM | 68.0 ± 5.5 | 69.0 ± 3.6 |

Activities are expressed as % of control values : [a]42.5 and [b]55.8 pmol $min^{-1}$ $mg^{-1}$.

**Table 3.** *Effects of various compounds on NADPH-dependent chlorotoluron ring-methyl hydroxylase (CPUH) and N-demethylase (CPUDM) activities.*

| Incubation conditions | | Relative activities (%) CPUH | Relative activities (%) CPUDM |
|---|---|---|---|
| Control | | 100.0[a] ± 3.5 | 100.0[b] ± 3.7 |
| Carbon monoxide | | 46.0 ± 0.6 | 63.7 ± 8.3 |
| Control | | 100.0[c] ± 4.1 | 100.0[d] ± 2.3 |
| o-Phenanthroline | 600 µM | 54.4 ± 5.8 | 56.6 ± 2.6 |
| Mercaptoethanol | 2.4 mM | 91.5 ± 3.2 | 89.8 ± 1.8 |
| KCN | 1.0 mM | 85.0 ± 9.6 | 84.5 ± 6.8 |
| Catalase | 1,67 µkat | 115.4 ± 9.3 | 110.2 ± 6.8 |
| Reduced glutathione | 1.0 mM | 116.5 ± 5.0 | 123.2 ± 8.5 |
| Ascorbate | 1.0 mM | 90.6 ± 8.4 | 84.3 ± 6.3 |
| *n*-Octylamine | 3.0 mM | 29.8 ± 2.7 | 30.5 ± 3.8 |
| Methimazole | 200 µM | 103.8 ± 7.0 | 97.3 ± 1.3 |
| Methylindole | 100 µM | 106.2 ± 7.8 | 119.6 ± 12.0 |
| SOD | 1,67 µkat | 123.1 ± 6.3 | 122.2 ± 8.0 |
| SOD + catalase | 1,67 + 1,67 µkat | 131.3 ± 15.2 | 129.7 ± 1.4 |

Activities are expressed as % of control activities : [a]40.2, [b]51.2, [c]37.2 and [d]42.6 pmol $min^{-1}$ $mg^{-1}$.

**Table 4.** *Effects of polyclonal antibodies on cinnamic acid 4-hydroxylase (CA4H), chlorotoluron ring-methyl hydroxylase (CPUH) and N-demethylase (CPUDM) activities.* Microsomes and antibodies were pre-incubated for 10 min at 25°C. Enzymatic assays were started by adding the other components to the reaction media, and incubated for 25 min. Controls were supplemented with pre-immune sera.

| Incubation conditions | Relative activities (%) | | |
|---|---|---|---|
| | CA4H | CPUH | CPUDM |
| Control | 100.0[a] ± 8.0 | 100.0[b] ± 0.9 | 100.0[c] ± 8.7 |
| anti-reductase IgG | 3.9 ± 0.4 | 0.0 | 0.0 |
| Control | 100.0[d] ± 3.1 | 100.0[e] ± 4.4 | 100.0[f] ± 3.2 |
| anti-CA4H IgG | 5.8 ± 0.6 | 100.5 ± 4.0 | 106.3 ± 0.9 |
| anti-ECOD IgG | 107.8 ± 9.4 | 107.2 ± 3.2 | 107.8 ± 1.7 |

Activities are expressed as % of control activities : [a]5397, [b]46.3, [c]92.6, [d]4719, [e]49.2 and [f]64.8 pmol $min^{-1}$ $mg^{-1}$.

**Table 5.** *Effects of various compounds on (hydro)peroxide-dependent activities.* Cofactors were cumene hydroperoxide for chlorotoluron N-demethylase (CPUDM) and aniline oxidase activities, and hydrogen peroxide for guaiacol oxidase. MSH : mercaptoethanol.

| Incubation conditions | Enzymatic activities | | |
|---|---|---|---|
| | CPUDM (pmol $min^{-1}$ $mg^{-1}$) | aniline ox. (nmol $min^{-1}$ $mg^{-1}$) | guaiacol ox. (nmol $min^{-1}$ $mg^{-1}$) |
| without cofactor | 3.8 ± 0.4 | 0.0 | 0.0 |
| with cofactor | 46.1 ± 1.2 | 8.0 ± 0.8 | 1164.2 ± 31.0 |
| +MSH 1.4 mM | 45.4 ± 1.5 | 0.0 | 18.5 ± 2.2 |

**Table 6.** *Effects of various physiological and exogenous substrates of cytochrome P-450 systems on chlorotoluron ring-methyl hydroxylase (CPUH) and N-demethylase (CPUDM) activities.*

| Incubation conditions | | Relative activities (%) | |
|---|---|---|---|
| | | CPUH | CPUDM |
| Control | | 100.0[a] ± 4.7 | 100.0[b] ± 2.8 |
| Cinnamic acid | 300 µM | 127.0 ± 12.2 | 111.1 ± 5.8 |
| Lauric acid | 200 µM | 97.9 ± 7.3 | 79.1 ± 1.6 |
| Ferulic acid | 300 µM | 67.3 ± 1.4 | 63.5 ± 1.4 |
| 7-Ethoxycoumarin | 500 µM | 70.4 ± 3.7 | 75.0 ± 11.3 |
| p-Chloro-N-methylaniline | 500 µM | 60.8 ± 7.9 | 65.5 ± 3.6 |
| Tolbutamide | 500 µM | 67.8 ± 7.1 | 67.8 ± 4.6 |
| Benzo[a]pyrene | 500 µM | 82.4 ± 0.5 | 107.1 ± 8.5 |

Activities are expressed as % of control activities : [a]40.8 and [b]51.9 pmol $min^{-1}$ $mg^{-1}$.

## LEGENDS TO FIGURES

**Figure 1.** *Difference spectra of wheat cell microsomes.* A, reduced carbon monoxide spectrum ; B, chlorotoluron-enzyme type I binding spectrum. Herbicide concentrations were 0, 100 and 160 µM ; C, procloraz-enzyme type II binding spectrum. Final concentrations of fungicide ranged from 100 to 400 µM.

**Figure 2.** *Metabolism of chlorotoluron by microsomes isolated from wheat shoots (A) and cultured cells (B).* Radioactive peaks on thin layer chromatography elution profiles co-chromatographied with hydroxymethyl-chlorotoluron (a), *N*-monodemethylated-chlorotoluron (b) and chlorotoluron (c). Chlorotoluron concentration in assay media were 500 µM. Incubations were run for 90 min with 0.5 mg microsomal protein, under aerobic conditions and in the presence of NADPH.

**Figure 3.** *Thin layer chromatography elution profiles of 90 min incubations with microsomes isolated from wheat cells in the presence of diuron (A) and isoproturon (B).* Incubations with diuron (d) and isoproturon (i) produced polar metabolites. Herbicide concentration in assay media were 500 µM. Incubations were run for 90 min with 0.5 mg microsomal protein, under aerobic conditions and in the presence of NADPH.