# Hydroxylation and N-demethylation of chlorotoluron by wheat microsomal enzymes

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# HYDROXYLATION AND N-DEMETHYLATION OF CHLOROTOLURON BY WHEAT MICROSOMAL EN2YMES

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Abstract

1n vitro metabolism of the herbicide chlorotoluron was studied in various subcellular fractions from suspension-cultured cells of wheat (Tritrcum aestivum) treated with cyometrinil. The highest rates of degradation of herbicide were found in microsomal fractions exhibiting a NADPH-cytochrome P-450 (cytochrome c) reductase activity. Membrane fractions supported the ring-methyl hydroxylation and the first N-demethylation of chlorotoluron in the presence of molecular oxygen and NADPH. Cell treatment by cyometrinil increased the rates of these two reactions, the activity of !auric acid in-chain hydroxylase and the levels of cytochromes P-450. Ring-methyl hydroxylation and N-demethylation of chlorotoluron were drastically reduced by para-chloromercuribenzoate and by compounds draining electrons from NADPH-cytochrome P-450 (cytochrome c) reductase. Ring-methyl hydroxylase was also strongly inhibited by CO, with partial reversion by light. Combination of these data allow to postulate that ring-methyl hydroxylase of wheat belongs to the family of cytochrome P-450 monooxygenases. 1n contrast, the N-monodemethylated metabolite could also be formed in the presence of cumene hydroperoxide, and the N-demethylase activity was not affected by CO. These distinctive properties still raise some questions about the nature of the monooxygenase(s) involved in the first N-demethylation of chlorotoluron in wheat cells.

Key words: cytochrome P-450; chlorotoluron; hydroxylase; N-demethylase; cyometrinil; *Triticum aestivum*

## Introduction

Hydroxylation and N-dealkylation reactions are frequently involved in the primary metabolism of pesticides in plants. As they usually lead to less or non-toxic metabolites, they contribute to the tolerance of plants towards herbicides and often play a determining role in selectivity between crops and weeds (1,2].

Several reports suggest that hydroxylation and N-demethylation of herbicides are mediated by monooxygenases, but until now, direct experimental evidence for the involvement of cytochromes P-450 is scanty. There are indications from in vitro studies that N-demethylation of monuron (1-(4-chlorophenyl]-3,3-dimethylurea) in cotton (3], and chlorotoluron (1-[3-chloro-P-tolyl]-3,3-di- methylurea) in Jerusalem artichoke tubers [4] are supported by NAD(P)H dependent cytochrome P- 450 monooxygenases. Aryl hydroxylation of 2,4-dichlorophenoxyacetic acid (2,4-D), and of diclofop (2-(4-(2, 4-dichlorophenoxy)phenoxy]propanoate) were also reported in cucumber, and wheat micro- some preparations, respectively (5,6].

Moreover, the role of microsomal monooxygenases is suggested by the demonstration that oxidative metabolism of chlorotoluron in wheat plants and cell cultures is sensitive to various cytochrome P-450 inhibitors such as paclobutrazol, tetcyclacis and 1-aminobenzotriazole (ABT) (7,8], although ABT has been reported to also inhibit other types of monooxygenases [9]. Conversely, herbicide catabolism can be stimulated in cell cultures by treatments with compounds such as cyometrinil (a-[(cy-anomethoxy)imino]benzeneacetonitrile), dichlormid- (2,2--dichloro-N,N-di-2-propenylacetamide) and 2,4-D [8,10], although this is usually considered to be due to elevated levels of another detoxification system, glutathione-glutathione transferase [11].

This paper reports an in vitro study of monooxygenases implicated in the metabolism of chlorotoluron in wheat cell cultures treated with cyometrinil. The results indicate that the enzyme responsible for the ring-methyl hydroxylation of the herbicide probably belongs to the group of cytochrome P- 450 monooxygenases. The N-demethylase activity appears to be catalyzed, at least in part, by another P-450 monooxygenase

system.

## Materials and Methods

### Cell cultures and treatments

Achlorophyllous wheat cells (*Triticum aestivum* L., var. 'Koga II') were routinely cultivated as previously described [8]. Before the experiments, 4-8- day-old suspensions were subcultured at an inoculum density of 4 mg dry wt./ml. After 4 days of growth, the cells were put in fresh culture medium containing 400 µM cyometrinil (60 nmol/mg dry wt), except for one series of experiments (Table II) Microsomal fractions were isolated 3 days later.

### Preparation of microsomes

All experiments were carried out at 4°C. The cultures were filtered through a stainless steel filter and 50-60 g (fresh wt.) of cells were ground without sand in a mortar for 1 min in 200 ml of grinding buffer containing 0.1 M, potassium phosphate (pH 7.5), 20% (w/v) glycerol, 20 mM ascorbic acid, 1 mM KCN, 1 mM phenylmethyl-sulfonyl fluoride and 10% (w/w) insoluble Polyclar AT. The crude extract was filtered through two layers of cheesecloth and centrifuged at 10 000 x g (rotor Beckman JA14) for 25 min. After centrifuging the supernatant at 100 000 x g (Beckman 45Ti) for 45 min, the pellets were resuspended in 50 ml of 0.1 M phosphate buffer containing 20% (w/v) glycerol, and sedimented again. The washed pellets, resuspended in 3 or 4 ml of 0. l M phosphate buffer containing 30% (w/v) glycerol, were referred to as the miicrosomal fraction. The protein concentration m that fraction was 2-4 mg/ml. Microsomes were stored at -80°C until use, without apparent loss of activity over several weeks.

### Preparation of subcellular fractions

After grinding the cells in buffer without KCN, the filtered homogenate was submitted to three centrifugation steps: 5 min at 1000 x g, 25 min at 10000 x g and 45 min at 100000 x g The resulting pellets were resuspended in 0.1 M potassium phosphate buffer

containing 30% (w/v) glycerol, and referred to as C1, C10 and C100 pellets.

Enzymatic assays

Lauric acid m-chain-hydroxylase (IC-LAH) activity was assayed as described by Salaün et al. [12]. Activities were estimated from the sum of the 9-, 10- and 11-hydroxy laurate metabolites formed (Salaün, pers. comm.). The concentration of lauric acid m incubation medium was 100 µ.M.

Cinnamic acid 4-hydroxylase (CA4H) was deter- mined according to Salaün et al. [12], with a cinnamic acid concentration of 200 µM in the assay medium. Reaction products were analyzed by reversed phase-HPLC (Waters) with a Guard-Pack precolumn module and a µ-Bondapak C18 column (8 mm x 10 cm, 4µm). para-Coumaric and trans-cinnamic acids were separated in acetonitrile/water/acetic acid (40:60:0.2, by vol) (flow rate 1 ml/min). Radioactivities associated with the peaks were detected with a solid scintillation counter (Ramona D, Isomess), collected in 10 ml of Dynagel (Baker) and estimated by liquid scintillation counting (Beckman LS 7500).

Formation of ring-methyl hydroxylated chlorotoluron and of N-monodemethylated chlorotoluron, were followed at 25°C. The reaction mixture, adjusted to a final volume of 200 µ.1 with 0.1 M potassium phosphate buffer (pH 7.5) usually contained 16.6 kBq of [carbonyl-14C]chlorotoluron (440 kBq/mmol) with nonlabelled herbicide to a final con- centration of 500 µ.M, 1 mM NADPH, 6.7 mM glucose 6-phosphate, 2 units of glucose-6-phosphate dehydrogenase and 0.25-0.40 mg microsomal proteins The labelled herbicide was synthesized ac- cording to Gilbert and Sorma [13], then purified by TLC and HPLC. After a 2 min preincubation at 25°C, the enzymatic reaction was initiated by adding NADPH. It was stopped after 30 min with 20 µl of 4N HCl, then diluted with 5 ml water and loaded on a C18 cartridge (Sep-pak, Waters). After washing the cartridge with 5 ml water, approximately 70% of the remaining chlorotoluron was eluted with 15 ml n-hexane/chloroform (9:1, v/v). The residual herbicide and its metabolites were then eluted with 8 ml methanol. Extracts were evaporated to about 100 µl and analyzed on TLC silica plates as described [8]. After scraping the spots, radioactivity was quantified by liquid scintillation counting. Alternatively, 100-µl fractions were directly injected in reversed phase-HPLC in conditions indicated above. Controls containing the incubation

medium without microsomal proteins were also analyzed.

For assay of NADPH-cytochrome P-450 (cytochrome c) reductase activity, the mixture contained 50 µ,M oxidized cytochrome c, 600 µM NADPH, 2.6 mM glucose 6-phosphate, 3 units of glucose- 6-phosphate dehydrogenase, 1 mM KCN and 0.15-0.20 mg proteins. It was adjusted to a final volume of 0.6 ml with 0.1 M (pH 7.5) potassium phosphate buffer. Enzymatic activity was monitored from the increase of absorbance at 550 nm at 25°C for 2 min, using a molar extinction coefficient of 18.5/mM/cm at 550 nm for horse heart cytochrome c. Controls were not supplemented with NADPH and the regenerating system.

The assay mixture for cytochrome c oxydase contained 50 µ,M cytochrome c reduced with dithionite, 0.15-0.20 mg proteins and 0.1 M potassium phos- phate buffer to a final volume of 0. 7 ml. Controls were supplemented with 1 mM KCN. Reaction rates were estimated from the decrease of absorbance at 550 nm at 25°C for 5 min.

Inhibition studies

In order to examine the role of oxygen, reaction media were degassed under vacuum at 4°C for 15 min. The controls and assays media were then bubbled with air or nitrogen for 5 min. NADPH was added and the mixtures incubated at 25°C for a further 30 min period.

Inhibition by carbon monoxide was followed by bubbling assay media without NADPH at 4°C for 15 min in darkness with air or a CO/air (50:50, v/v) mixture. After adding NADPH, the mixtures were incubated in closed vessels for an additional 15 min period, either in darkness or under illumination with white light from a 150 W lamp at a distance of 15 cm in a water bath set at 25°C.

Effect of para-chloromercuribenzoate (p-CMB) was evaluated by incubating microsomes with the inhibitor and chlorotoluron at 4°C for 5 min. Enzymatic reactions were initiated by addition of NADPH, and the mixtures incubated for 30 min at 25°C.

A different protocol was used to study enzyme inactivation by ABT. Microsomal preparations were incubated at 25°C for 10 min with NADPH or NADPH plus ABT, in the absence of chlorotoluron. Then, the enzymatic reactions were started by addition of

the herbicide, and the mixtures incubated for a further 15 min period.

Other methods

Hemoprotein spectra were recorded at l5°C with an Uvikon 860 spectrophotometer. Quantitative de- terminations were done by the method of Omura and Sato [14], using extinction coefficients of 91/mM/cm and 185/mM/cm for cytochromes P-450 and b5, respectively. Microsomal proteins were esti- mated according to Bradford [15] with bovine serum albumin as a standard. Cyometrinil, ABT, p-CMB and cumene hydroperoxide were dissolved in dimethyl sulfoxide (DMSO). Final solvent concentrations were < 0.1 or 1% in culture and incubation media, respectively. Results are means of at least two ex- periments ±S.D. All enzymatic activities are expressed in pmol/min/mg of microsomal protein.

Results

Chlorotoluron metabolism and subcellular distribution of enzymatic activities

Aerobic incubation of microsomes with [14C]chlorotoluron and NADPH led to the formation of two labelled metabolites more polar than the herbicide. They were identified as the ring-methyl hydroxylated and the N-monodemethylated derivatives by TLC co-chromatography with authentic references (Fig. 1) and by reversed phase-HPLC.

The formation of the' e metabolites was examined in the fractions resulting from differential centrifugation of cell homogenates. As activities found in the soluble fractions were very low, even in extracts from cyometrinil treated cells, the study was done only with the C1, C10 and C100 pellets, corresponding to cell walls and nuclear debris (C1) , mitochondrial and etioplast fraction (C10), and microsomal fraction (C100).

Both the highest relative and specific activities of chlorotoluron ring-methyl hydroxylase and N-demethylase were associated with the microsomal pellet (Table I). However, these activities were also present in the C10 pellet (Table I), probably as a result of some aggregation between mitochondria and microsomes [16]. NADPH-

cytochrome P-450 (cytochrome c) reductase activity was also associated with the microsomal pellet, and in that case too, a pro- portion of this enzyme was detected in the C10 pellet. Cytochrome c oxidase, a typical marker of mitochondria, was essentially found in the C10 pellet.

Effects of cyometrinil on the metabolism of chlorotoluron

Microsomes isolated from cells 3 days after sub- culturing catalyzed the ring-methyl hydroxylation and N-demethylation of chlorotoluron at rates ranging from 21 to 23 pmol/min/mg, and from 6 to 8 pmol/min/mg, respectively. When cells were grown for the same time in the presence of 400 µM cyometrinil, hydroxylase and N-demethylase activities were stimulated 1.6- and 3.3-fold, respectively (Table II). Cyometrinil differentially affected IC- LAH and CA4H, both known as cytochrome P-450 monooxygenases. Activity of IC-LAH was stimulated 1.4-fold, while that of CA4H was not affected (Table II). Microsomes from untreated cells contained 377 and 184 pmol/mg protein of cytochrome b5 and cytochromes P-450, respectively (Table II). These levels are comparable to those already found by others in microsomes of various plant species [17]. Cytochrome P-450 content of microsomes was increased 1.8-fold by cyometrinil treatment, whereas that of cytochrome b5 was not affected.

Cofactors and oxygen requirements

Ring-methyl hydroxylation and N-monodemethylation of chlorotoluron in microsomes required NADPH as a cofactor (Table III). No oxidations were detected in assays treated with 2% TCA just before incubation (not shown). The highest activities were obtained when the incubation media were supplemented with a NADPH-regenerating system. NADH was ineffective when given alone, as were other reductants such as sodium dithionite or ascorbic acid. Incubation in an atmosphere of mtrogen showed that molecular oxygen was necessary for the NADPH dependent activities (Table III). Conversely, cumene hydroperoxide, but not hydrogen peroxide, was also able to support a significant N- demethylase activity. Some residual metabolism could be detected in the absence of any external cofactor or under N2 conditions (Table III).

Properties of chlorotoluron ring-methyl hydroxylase and N-demethylase

The rates of microsomal ring-methyl hydroxylation and N-demethylation of chlorotoluron were almost linear up to 40 min for protein concentrations between 1.15 and 2.55 mg/ml, at pH 7.5, 25°C, and at a chlorotoluron concentration of 500 µM (not shown).

The hydroxylase activity did not follow Michaelis- Menten kinetics (not shown) in respect to chlorotoluron concentration, and a non-linear relation was obtained in Eadie-Hoffstee plots (Fig. 2). Two a parent Km values were calculated equal to 50 and 400 µM. These estimates are comparable to those prevtously found in the cases of aminopyrine and chlorotoluron N-demethylases in Jerusalem artichoke microsomes [4,16]. Numerical values of the N-demethylase activities were too variable to allow a meaningful analysis.

Effects of carbon monoxide on chlorotoluron metabolism

Activities of ring-methyl hydroxylase and N-demethylase were higher in the light than in the dark, and exhibited different sensitivities towards CO (Table IV). CO affected hydroxylase activity, its inhibition amounting to 55% in the dark and 43% in the light. The calculated partition coefficients between CO and oxygen were $K_{light}$ = 5.91 and $K_{dark}$ = 3.68. They were in the range of values found in Jerusalem artichoke tubers for delta9 unsaturated lauric acid epoxidase, another cytochrome P-450 activity [18]. The rate of light reversion was 35% for hydroxylase activity. In contrast, N-demethylase activity was not inhibited by CO.

Effects of various inhibitors on chlorotoluron hydroxylase and N-demethylase

ABT, a suicide substrate of cytochrome P-450s [19] and of methiocarb sulfoxidase, a peroxygenase-like enzyme [9], strongly inhibited chlorotoluron ring-methyl hydroxylation and N-Demethylation (Table V). N-Demethylat10n was less affected than hydroxylation, as previously observed in vivo in plants [7, 20] and cell cultures [8]. Both activities were strongly inhibited by p-CMB (Table V), an inhibitor of the NADPH-cytochrome P-450 (cytochrome c) reductase [21, 22].

Menadione, dichlorophenolindophenol (DCPIP) and cytochrome c, which behave as electron drains from the reductase, drastically inhibited chlorotoluron ring-methyl hydroxylase and N-demethylase, in accordance to the postulated involvement of the NADPH- cytochrome P-450 (cytochrome c) reductase in both oxidative reactions (Table V).

## Discussion

We previously showed that treatments of wheat cells with cyometrinil were followed by a stimulated degradation of chlorotoluron [8].·Stimulating effects of cyometrinil could also be obtained in vivo as reported above. Until recently, it was assumed that herbicide protectants such as dichlormid and cyometrinil, and herbicide synergists such as tridiphane act by stimulating (23] or inhibiting [24] glutathione- transferase, respectively. There is recent evidence, besides the evidence provided above, that these compounds might also stimulate oxidative degradation of herbicides [10, 25, 26].

Our results provide the first demonstration of the in vitro oxidative metabolism of chlorotoluron by wheat microsomes. The subcellular fraction catalyzes two concurrent reactions, leading to the formation of the ring-methyl hydroxylated and the N-monodemethylated herbicide. These derivatives are thus identical to the primary metabolites formed in whole plants. Ring-methyl hydroxylation and N- demethylation proceed at comparable rates in vitro, while ring-methyl hydroxylation is the major metabolic pathway in plants.

The reactions responsible for the production of the two metabolites are enzymatic. In wheat microsomal fractions, they were associated with sizeable levels of cytochrome P-450s, and with various enzymes known as markers of endoplasmic reticulum, such as NADPH-cytochrome P-450 (cytochrome c) reductase, CA4H and IC-LAH. These last two are cytochrome P-450 monooxygenases. The 10000 x g pellets, essentially enriched in mitochondrial and etioplasts, exhibited low rates of ring- methyl hydroxylase and N-demethylase activities, probably originating from contaminating microsomal membranes.

The activity of the ring-methyl hydroxylase exclusively required molecular oxygen and NADPH. It was clearly inhibited by CO and this inhibition was partly reverted by light. It is also very sensitive to p-CMB, which blocks thiol groups of the NADPH-cytochrome P-450 (cytochrome c) reductase, and to alternate electron acceptors such as menadione, DCPIP and cytochrome c. Taken together, these data strongly suggest (but do not prove) that ring- methyl hydroxylase belongs to the family of cyto- chrome P-450 monooxygenases. At that point, it would be important to also demonstrate type I binding difference spectra in wheat microsome preparations incubated with chlorotoluron. However, until now, the spectra we have obtained were not fully typical, as reported by others concerning several substrates of plant cytochrome P-450 monooxygenases, such as lauric acid incubated with microsomes of Jerusalem artichoke [21], or diclofop acid with microsomes of wheat (Zimmerlin and Durst, pers. comm.).

It is likely that the N-demethylase activity of wheat microsomes supplemented with chlorotoluron is also of cytochrome P-450 type. Supportive evidence for this hypothesis is provided by the requirement of the reaction for oxygen and NADPH, and the inhibitory effects of p-CMB, menadione, DCPIP and cytochrome c. Although inhibition of N-demethylase by ABT does not provide a totally definitive argument, it is worthy to observe that the level of inhibition is of the same range as that found for CA4H, i.e., much higher than that reported for the sulfoxidase of methiocarb [9]. Insensitivity of chlorotoluron N-demethylase to CO does not inevitably disprove the P-450 hypothesis, because cytochrome P-450s of plants have various degrees of sensitivity to CO, as reported about w- and midchain hydroxylation of palmitic acid by bean microsomes [27]. Moreover, a complete insensitivity of a cytochrome P-450 to CO has been given in the case of aromatization of androstenedione [28]. The fact that cumene hydroperoxide is able to replace molecular oxygen may indicate that a part of the N-demethylase activity could depend on a peroxygenase-type enzyme, similar to that described from pea [29]. Further studies are needed to examine this possibility. How- ever, it has been shown that cytochrome P-450 monooxygenases such as p-chloro-N-methylaniline demethylase from castor bean [30] and avocado [31] can use a hydroperoxide as oxygen donor, so that the question remains open.

In brief, it appears that two microsomal mono-oxygenases of wheat, exhibiting a drastic

difference to CO, are involved in ring-methyl hydroxylat10n and N-demethylation of chlorotoluron. It will be of interest to establish if other herbicides can also be substrates of similar enzymatic systems.

Acknowledgments

The authors thank Drs. F. Durst and J.P. Salaün for helpful discussions and identification of metabolites of laurate hydroxylase. They are also grateful to Dr. E. Ebert (Ciba-Ge1gy, Basle) for, the generous gift of cyometrinil and metabolite standards.

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Table I. Subcellular distributions of chlorotoluron ring-methyl hydroxylase and N-demethylase, NADPH-cytochrome P-450 (cytochrome c) reductase and cytochrome c oxidase Relative activities are expressed as % of total activities found in pellets C1 +C10 + C100. Specific activities are given in pmol/min/mg for ring-methyl hydroxylase and N-demethylase of chlorotoluron and nmol/min/mg for NADPH- cytochrome P-450 (cytochrome c) reductase and cytochrome c oxidase.

| | Relative activities | | | Specific activities | | |
|---|---|---|---|---|---|---|
| | $C_1$ | $C_{10}$ | $C_{100}$ | $C_1$ | $C_{10}$ | $C_{100}$ |
| Ring-methyl hydroxylase | 2.9 ± 0.2 | 20.2 ± 0.5 | 76.9 ± 1.1 | 2 4 ± 0 3 | 2.0 ± 0.1 | 23.9 ± 0.6 |
| *N*-Demethylase | 0.1 ± 0.1 | 30 5 ± 1.4 | 69.4 ± 1.3 | 0.1 ± 0 1 | 4.4 ± 0.4 | 30 6 ± 0.6 |
| NADPH-cyt. *c* reductase | 1.6 ± 0.1 | 32.8 ± 1.9 | 65.6 ± 1.9 | 5.8 ± 0 3 | 14.7 ± 1.2 | 90.0 ± 0.7 |
| Cytochrome *c* oxidase | 7.2 ± 1.1 | 91.9 ± 1.4 | 0.9 ± 0.2 | 88.0 ± 20.1 | 138.6 ± 1.3 | 4 0 ± 1.3 |

Table II. Effects of a cyometrinil treatment on enzymatic NADPH-dependent activities and cytochrome contents of wheat cell microsomes. CPUH, chlorotoluron ring-methyl hydroxylase; CPUDM, chlorotoluron N-demethylase.

| | Enzymatic activities(pmol/min/mg) | | | | Cytochromes (pmol/mg) | |
|---|---|---|---|---|---|---|
| | CPUH | CPUDM | IC-IAH | CA4H | $b_5$ | *P*-450 |
| Controls | 22.0 ± 0.7<br>(100) | 6.6 ± 0.9<br>(100) | 382.0 ± 22.0<br>(100) | 1540.0 ± 41.0<br>(100) | 377.0 ± 12.5<br>(100) | 184.0 ± 37.5<br>(100) |
| Cyometrinil | 33.8 ± 1.8<br>(155) | 22.2 ± 0.6<br>(329) | 545.0 ± 13.0<br>(143) | 1460.0 ± 98.0<br>(95) | 388.0 ± 10.5<br>(103) | 329.0 ± 11.0<br>(179) |

Table III. Cofactors and oxidant forms requirements for chlorotoluron ring-methyl hydroxylase and N-demethylase activities. Activities are expressed as % of maximal activities of controls. The control values were: *32.8, **28.1, •30.8, "'36.3, *25.2, *27.9 pmol/mm/mg.

| Incubation conditions | Conc. | % of maximal activities | |
|---|---|---|---|
| | | Hydroxylase | *N*-Demethylase |
| Complete medium (NADPH 1 0 mM) | | 100.0* ± 0.5 | 100.0** ± 0 5 |
| NADPH | 1.0 mM[a] | 60.7 ± 0.1 | 77.1 ± 0.0 |
| NADPH | 0.5 mM | 62.6 ± 0.3 | 54.4 ± 0.1 |
| NADPH | 0.1 mM | 44.2 ± 0.1 | 48.8 ± 1.2 |
| no NADPH | | 12.8 ± 0.1 | 11.5 ± 0.1 |
| NADH | 0.5 mM[a] | 7.4 ± 0.1 | 0 3 ± 0.1 |
| Dithionite | 1.0 mM | 9.7 ± 0.5 | 13.0 ± 0.7 |
| Ascorbate | 1.0 mM | 11.1 ± 0.3 | 22.2 ± 1.7 |
| Complete medium (NADPH 1 0 mM) | | 100.0† ± 2.9 | 100.0‴ ± 3.6 |
| Cumene hydroperoxide | 1.0 mM | 30.7 ± 2.9 | 110.2 ± 1.1 |
| Hydrogen peroxide | 1.0 mM | 34.9 ± 1.4 | 0.0 |
| Complete medium (NADPH 1.0 mM) | | 100.0‡ ± 11.8 | 100.0§ ± 8.7 |
| $N_2$ | | 24.1 ± 2.2 | 16.3 ± 0.1 |

[a]Without cofactor regenerating system.

Table IV. Effects of light and carbon monoxide on chlorotoluron ring-methyl hydroxylase and N-demethylase activities.

| | Enzymatic activies (pmol/mn/mg) | |
|---|---|---|
| | Hydroxylase | *N*-Demethylase |
| Dark | 21 5 ± 1 2 | 20.2 ± 1.4 |
| Dark + CO(% inhibition) | 9 6 ± 0.9(55 2) | 26.3 ± 2 1 |
| Light | 29.0 ± 0.2 | 25 2 ± 0 5 |
| Light + CO(% inhibition) | 16.4 ± 0.1(43 1) | 26 0 ± 2 4 |
| Reversion (%) | (35 1) | |

Table V. Effects of inhibitors on chlorotoluron ring-methyl hydroxylase and N-demethylase activities. % of inhibition are expressed with respect to control values which were: *20.9 ± 2.4, **15.4 ± 0.4, t31,5 ± 2.6, '"33.1 ± 2.0 pmol/min/mg.

| Inhibitors | Conc. | % of inhibition | |
|---|---|---|---|
| | | Hydroxylase | *N*-Demethylase |
| ABT | 100 μM | 73.8 ± 4.6* | 56.6 ± 2 0** |
| *p*-CMB | 500 μM | 91.3 ± 1.3† | 66.0 ± 5 0‡ |
| Menadione | 100 μM | 99.6 ± 0.1 | 100.0 ± 0 0 |
| DCPIP | 100 μM | 100.0 ± 0.0 | 100.0 ± 0 0 |
| Cytochrome *c* | 20 μM | 86.6 ± 3 4 | 93.7 ± 1.3 |

Fig. 1. Thin-layer radiochromatogram of metabolites (A,B) formed after 1-h incubation of wheat microsomes plus chlorotoluron (C) Experimental conditions are described in Materials and Methods. Arrows refer to the chromatographic mobility of standards: I, ring- methyl hydroxylated chlorotoluron; II, N-monodemethylated chlorotoluron; III, chlorotoluron.

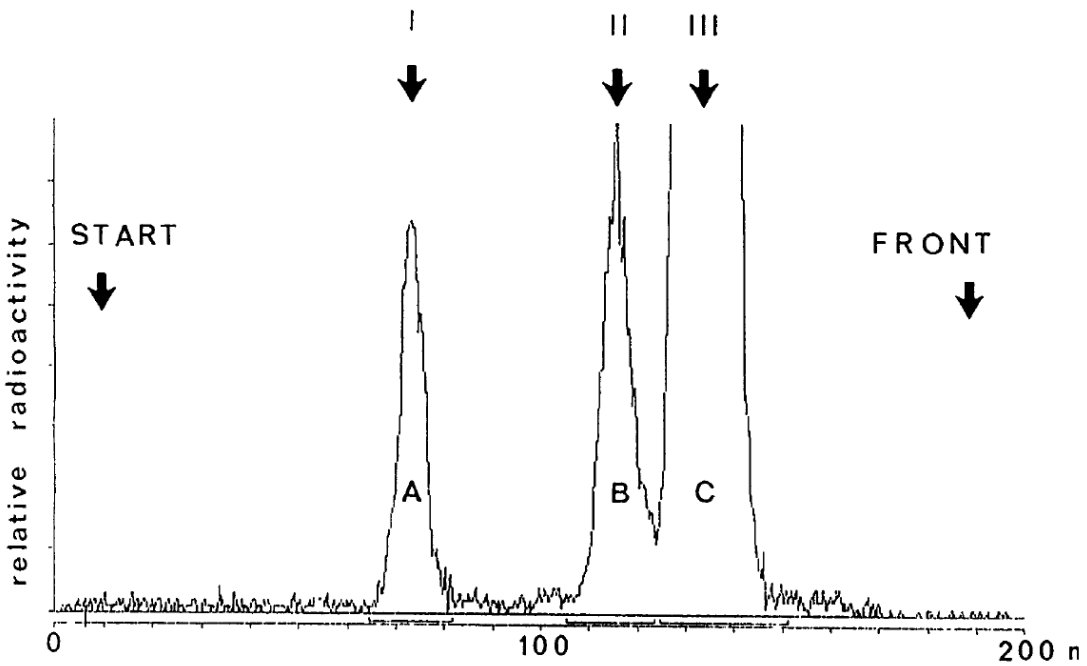

Fig.2. Eadie-Hoffstee plot of chlorotoluron ring-methyl hydroxylase activity. Chlorotoluron concentrations ranged from 12 to 1000µ.M.