Phytochemistry, 1969, Vol. 8, pp. 2157 to 2169. Pergamon Press. Printed in England.

# *N*-DEMETHYLATION OF SUBSTITUTED 3-(PHENYL)-1-METHYLUREAS: ISOLATION AND CHARACTERIZATION OF A MICROSOMAL MIXED FUNCTION OXIDASE FROM COTTON*

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(*Received* 10 *June* 1969)

**Abstract**—A microsomal, mixed function oxidase that successively *N*-demethylates substituted 3-(phenyl)-1,1-dimethylurea substrates was isolated from etiolated cotton seedling hypocotyl extracts by differential and density gradient centrifugation. Active enzyme preparations were also isolated from leaves of cotton, plantain, buckwheat, wild buckwheat and broadbeans. The enzyme is located in the microsomal fraction of plant extracts and requires molecular oxygen and either NADPH or NADH as cofactors. The formation of 1 mole of formaldehyde for each mole of substrate demethylated was demonstrated with $^{14}C$ methyl-labeled substrate. The enzyme appears to be specific for substituted 3-(phenyl)-1-methylurea compounds, and the apparent $K_m$ values for three of these substrates were determined. Inhibition of the enzyme by carbon monoxide, ionic detergents, sulfhydryl reagents, chelating agents and electron acceptors, together with the demonstrated presence of a $b_5$ cytochrome and an active NADPH-cytochrome c reductase, are discussed as indirect evidence for a microsomal electron transport system in plants similar to those reported for animals. Several differences between the cotton *N*-demethylase and similar animal systems were also noted. These differences included a greater instability and a decreased sensitivity toward the insecticidal synergists 2,2-diethylaminoethyl-2,2-diphenylpentanoate (SKF 525-A) and 2-(3,4-methylenedioxyphenoxy)-3,6,9-trioxoundecane (Sesamex). The inhibition of *N*-demethylase activity by several N-methylcarbamates was investigated and the apparent $K_i$ value for the competitive inhibition by 1-naphthylmethylcarbamate was determined.

## INTRODUCTION

RECENT *in vivo* studies[1–7] have shown that substituted 3-(phenyl)-1,1-dimethylurea compounds are progressively *N*-demethylated by higher plants to less phytotoxic[7] monomethyl and demethylated derivatives.

An *in vitro* cotton leaf microsomal oxidase system which *N*-demethylates 3-(4-chlorophenyl)-1,1-dimethylurea has been recently reported by this laboratory,[8] but was not described in detail. This enzyme system was found in the microsomal fraction after differential centrifugation, and was shown to require molecular oxygen and reduced pyridine nucleotides as cofactors. In the present report, the distribution of this enzyme system in

* Use of trade names is for the purpose of identification and does not constitute endorsement by the U.S. Department of Agriculture.

[1] H. GEISSBÜHLER, C. HASELBACH, H. AEBI and L. EBNER, *Weed Res.* **3**, 277 (1963).
[2] J. W. SMITH and T. J. SHEETS, *J. Agr. Food Chem.* **15**, 577 (1967).
[3] C. R. SWANSON and H. R. SWANSON, *Weed Sci.* **16**, 137 (1968).
[4] C. R. SWANSON and H. R. SWANSON, *Weed Sci.* **16**, 481 (1968).
[5] J. H. ONLEY, G. YIP and M. H. ALDRIDGE, *J. Agr. Food Chem.* **16**, 426 (1968).
[6] R. W. FEENY and S. R. COLBY, *Weed Sci.* (Abstr.) p. 34 (1968).
[7] M. D. NEPTUNE and H. H. FUNDERBURK, JR., *Weed Sci.* (Abstr.) p. 37 (1968).
[8] D. S. FREAR, *Science* **162**, 674 (1968).

other plant species and the further characterization of the enzyme in cotton have been considered.

## RESULTS AND DISCUSSION

### *Enzyme Distribution*

The distribution of enzyme activity in the leaf tissues of several plant species is shown in Table 1. The enzyme activity in cotton, plantain, buckwheat, wild buckwheat and broadbean agrees with the active *in vivo* *N*-demethylation systems reported in these more resistant species.[1–4] The reduced enzyme activity found in the leaf preparations of the other species

TABLE 1. *N*-DEMETHYLASE ACTIVITY IN LEAF TISSUE MICROSOMAL PREPARATIONS*

| Species | Specific activity† |
|---|---|
| Cotton | 0·24 |
| Plantain | 0·24 |
| Buckwheat | 0·16 |
| Wild Buckwheat | 0·12 |
| Broadbean | 0·06 |
| Others‡ | < 0·04 |

* Reaction mixture contained from 1·5 to 2·6 mg of protein, 30 mμmoles of $^{14}C$ trifluoromethyl-labeled 3-(3-trifluoromethylphenyl)-1,1-dimethylurea, 50 μmoles of potassium phosphate, pH 7·5, 0·5 μmole of NaCN and 1 μmole of NADPH. The reaction was run for 30 min at 25°.

† Specific activity is mμmoles 3-(3-trifluoromethylphenyl)-1-methylurea formed/mg protein/30 min.

‡ Other species are okra, sorghum, corn, potato, mallow, celery and soybean.

studied may reflect a reduced ability of these more susceptible species[2, 3] to detoxify substituted 3-(phenyl)-1,1-dimethylureas by *N*-demethylation.[7]

The distribution of the enzyme activity in various cotton tissues is shown in Table 2. As a result of these studies, the most active 4- to 6-day-old etiolated cotton seedling hypocotyl tissues were used as the enzyme source in all subsequent studies.

Differential centrifugation of etiolated cotton hypocotyl extracts resulted in a distribution of enzyme activity similar to that reported in young leaf preparations[8] and corresponded to a localization of enzyme activity in the microsomal fraction (78,000–120,000 × *g* for 60–90 min).

The distribution of the enzyme activity in the "smooth" and "rough" endoplasmic reticulum fractions of cotton microsomal preparations is shown in Table 3. The "smooth"/"rough" ratios of *N*-demethylase activity found in cotton microsomal preparations are simi-

lar to those reported for liver microsomal preparations.[9,10] The much higher "smooth"/"rough" *N*-demethylase activity ratios found in young etiolated hypocotyl preparations may reflect an increased dilution of active protein in the rough fractions with inactive ribosomal protein.[10]

TABLE 2. DISTRIBUTION OF *N*-DEMETHYLASE ACTIVITY IN COTTON TISSUES*

| Tissue | Specific activity† |
|---|---|
| Young leaves (4 weeks old) | 0·24 |
| Etiolated seedlings minus roots (7 days old) | 0·53 |
| Etiolated seedlings roots (5 days old) | 0·42 |
| Etiolated hypocotyls | |
| 4 days old | 2·33 |
| 6 days old | 2·38 |
| 8 days old | 1·25 |

* Reaction mixture contained 0·6–2·4 mg protein, 30 mμmoles of $^{14}C$ trifluoromethyl-labeled 3-(3-trifluoromethylphenyl)-1,1-dimethylurea, 50 μmoles of potassium phosphate, pH 7·5, 0·5 μmole NaCN and 1 μmole of NADPH. The reaction was run for 30 min at 25°.

† Specific activity is mμmoles 3-(3-trifluoromethylphenyl)-1-methylurea formed/mg protein/30 min.

TABLE 3. *N*-DEMETHYLASE ACTIVITY IN "SMOOTH" AND "ROUGH" COTTON MICROSOMAL FRACTIONS*

| Fraction | Specific activity† | |
|---|---|---|
| | Young leaf | Etiolated hypocotyl |
| 78,000 × *g* Pellet | 0·08 | 2·16 |
| "Smooth" | 0·11 | 2·68 |
| "Rough" | 0·04 | 0·41 |

* Reaction mixture contained from 1·3 to 2·0 mg of protein, 10 mμmoles of $^{14}C$ ring-labeled 3-(4-chlorophenyl)-1,1-dimethylurea in the case of leaf tissue or 30 mμmoles of $^{14}C$ trifluoromethyl-labeled 3-(3-trifluoromethylphenyl)-1,1-dimethylurea, in the case of hypocotyl tissue, 50 μmoles potassium phosphate, pH 7·5, 0·5 μmole NaCN, and 1 μmole of NADPH. The reaction was run for 30 min at 25°.

† Specific activity is mμmoles of either 3-(4-chlorophenyl)-1-methylurea or 3-(3-trifluoromethylphenyl)-1-methylurea formed/mg protein/30 min.

"Smooth"/"Rough" ratio of leaf tissue is 2·8, and of hypocotyl tissue, 6·5.

[9] T. E. GRAM and J. R. FOUTS, in *Enzymic Oxidations of Toxicants* (edited by E. HODGSON), p. 49, North Carolina State Univ., Raleigh, N. Carolina (1968).

[10] J. L. HOLTZMAN, T. E. GRAM, P. L. GIGON and J. R. GILLETTE, *Biochem. J.* **110**, 407 (1968).

*Reaction Products*

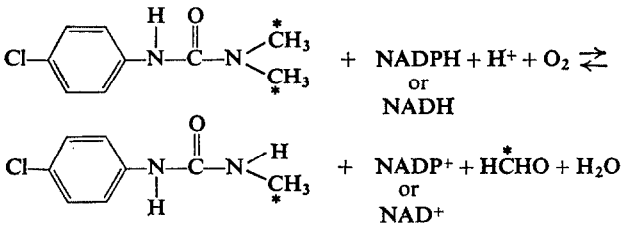
The *N*-demethylation of $^{14}C$ methyl-labeled 3-(4-chlorophenyl)-1,1-dimethylurea results in the formation of 1 molecule of formaldehyde for each molecule of 3-(4-chlorophenyl)-1-methylurea produced. Further *N*-demethylation of the 3-(4-chlorophenyl)-1-methylurea

TABLE 4. REACTION PRODUCTS OF 3-(4-CHLOROPHENYL)-1,1-DIMETHYLUREA *N*-DEMETHYLATION BY ETIOLATED COTTON HYPOCOTYL MICROSOMES*

| Treatment | Reaction products formed | | |
|---|---|---|---|
| | Formaldomethone (mμmoles) | 3-(4-Chlorophenyl)-1-methylurea (mμmoles) | Ratio of products formed |
| Plus 5,5-dimethyl-1,3-cyclohexanedione | 3·74 | 3·54 | 1·06 |
| Minus 5,5-dimethyl-1,3-cyclohexanedione | 0·07 | 3·49 | 0·02 |

* Reaction mixture contained 2·4 mg protein, 33 mμmoles of $^{14}C$ methyl-labeled 3-(4-chlorophenyl)-1,1-dimethylurea, 1 μmole of semi-carbazide, 50 μmoles of potassium phosphate, pH 7·5, 0·5 μmole of NaCN and 1 μmole of NADPH. The reaction was run for 45 min at 25° and terminated by adding 2·0 ml of 0·1 M NaOAc buffer, pH 4·5, and reacted in either the presence or absence of 0·5 ml of 0·2% aqueous 5,5-dimethyl-1,3-cyclohexanedione.

produced was not significant under the assay conditions used. However, when 3-(4-chlorophenyl)-1-methylurea was used as the substrate instead of 3-(4-chlorophenyl)-1,1-dimethylurea, *N*-demethylation to 4-chlorophenylurea was observed. This is illustrated in the results of a typical experiment shown in Table 4. The overall reaction catalyzed appears to be as follows:




*Effect of pH and Buffer Systems*

The pH optimum of 7·5 determined for etiolated cotton hypocotyl *N*-demethylase with 3-(3-trifluoromethylphenyl)-1,1-dimethylurea as the substrate corresponds to that previously reported[8] for cotton leaf *N*-demethylase with 3-(4-chlorophenyl)-1,1-dimethylurea as the substrate. The following buffer systems were all found to be equally effective for the *N*-demethylation of 3-(3-trifluoromethylphenyl)-1,1-dimethylurea by etiolated cotton hypocotyl microsomal preparations; tris (hydroxymethyl) aminoethane (TRIS), *N,N*-bis(2-hydroxyethyl)glycine (Bicine), and potassium phosphate. The use of *N*-tris (hydroxymethyl) glycine (Tricine), *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid (HEPES) and

*N*-tris (hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES) buffer systems, however, resulted in a 50–70 per cent reduction in enzyme activity under standard assay conditions. Zimmerman[11] has reported that soybean lipoxidase is inhibited in the presence of Tricine, HEPES and TES buffer systems because of reduced substrate micellization.

*Enzyme Stability*

The effect of a modified isolation medium[12] on *N*-demethylase activity in cotton leaf preparations is shown in Table 5. The activity of microsomal preparations without any of the isolation medium components was reduced over 70 per cent when compared with the activity of the complete medium. The polyclar AT and NaCN were included in the isolation medium to reduce phenol oxidase activity and subsequent enzyme inactivation by phenol oxidation products while the isoascorbate was included as a protective reducing agent.

TABLE 5. EFFECT OF ISOLATION MEDIUM ON *N*-DEMETHYLASE ACTIVITY IN COTTON LEAF MICROSOMAL PREPARATIONS*

| Medium | Specific activity† |
|---|---|
| Complete | 0·23 |
| Minus NaCN | 0·09 |
| Minus isoascorbate | 0·08 |
| Minus polyclar | 0·08 |

* Reaction mixture contained 2·6–3·3 mg of protein, 7 mμmoles of $^{14}C$ ring-labeled 3-(4-chlorophenyl)-1,1-dimethylurea, 50 μmoles of potassium phosphate, pH 7·5, 0·5 μmole of NaCN and 1 μmole of NADPH. The reaction was run for 30 min at 25°.

† Specific activity is mμmoles of 3-(4-chlorophenyl)-1-methylurea formed/mg protein/30 min.

Washed microsomal suspensions were found to be stable for several hours at 0°, but were rapidly inactivated on freezing and thawing and on storage in the frozen state. Activity was rapidly lost at temperatures above 0° as shown in Fig. 1, and completely lost on heating at 50° for 10 min. Attempts to stabilize the enzyme activity with cysteine, dithiothreitol (DTT), 2,3-dimercaptopropanol (BAL), mercaptoethanol, and reduced glutathione were not successful. The addition of 0·5% crystalline bovine serum albumin and 25% glycerol were also unsuccessful in stabilizing the enzyme system against thermal inactivation.

*Effect of Detergents*

The effect of several detergents on enzyme activity is shown in Table 6. The anionic detergents lauryl sulfate and desoxycholate completely destroyed enzyme activity at the 0·1 per cent level while the nonionic detergents differed substantially in their ability to affect enzyme activity. Tergitol 15-S-9 appeared to be the least disruptive nonionic detergent studied in regard to its effect on enzyme activity at the 0·1 per cent level, but was found to result in almost complete inactivation of the enzyme at the 1 per cent level.

[11] D. C. ZIMMERMAN, *Plant Physiol.* **43**, 1656 (1968).
[12] W. D. LOOMIS and J. BATTAILE, *Phytochem.* **5**, 423 (1966).

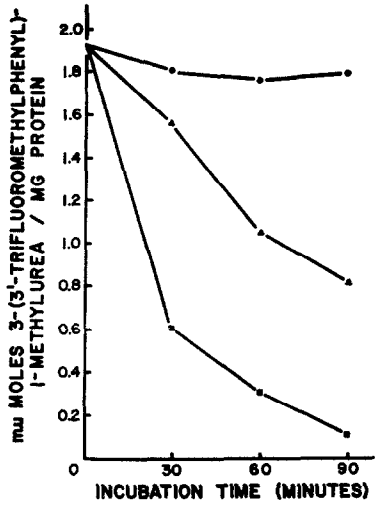



FIG. 1. THERMAL STABILITY OF COTTON HYPOCOTYL *N*-DEMETHYLASE.

The reaction mixture contained 1·2 mg of protein, 30 mμmoles of $^{14}C$ trifluoromethyl-labeled 3-(3-trifluoromethylphenyl)-1,1-dimethylurea, 50 μmoles of potassium phosphate, pH 7·5, 0·5 μmole of NaCN, 1 μmole of NADPH. The enzyme was incubated for the specified times at 0° (—●—), 15° (—▲—), and 25° (—■—). The reaction was started by adding substrate and NADPH and was run for 30 min at 25°.

TABLE 6. EFFECT OF DETERGENTS ON ETIOLATED COTTON HYPOCOTYL *N*-DEMETHYLASE ACTIVITY*

| Detergent | Concentration (% by vol.) | % of Control |
|---|---|---|
| Tergitol 15-S-9 | 0·1 | 115 |
| | 1·0 | 2 |
| Tween 20 | 0·1 | 91 |
| Tergitol NPX | 0·1 | 72 |
| TD 10 | 0·1 | 69 |
| Triton X 100 | 0·1 | 45 |
| Desoxycholate | 0·1 | 1 |
| Lauryl sulfate | 0·1 | 0 |

* Reaction mixture contained 1·4 mg protein, 30 mμmoles of $^{14}C$ trifluoromethyl-labeled 3-(3-trifluoromethyl-phenyl)-1,1-dimethyl-urea, 50 μmoles of potassium phosphate, pH 7·5, 0·5 μmole of NaCN, 1 μmole of NADPH, and detergent as indicated. The reaction was run for 30 min at 25°.

Mason *et al.*[13] have stated that detergents produce structural changes which functionally alter $P_{450}$ cytochrome and $Fe_x$ in animal microsomal systems. The sensitivity of the cotton *N*-demethylase to detergents appears to be similar to that reported for animal mixed-function oxidase systems, and may indicate the involvement of a similar microsomal electron transport system in plants and animals.

[13] H. S. MASON, J. C. NORTH and M. VANNESTE, *Fed. Proc.* **24**, 1172 (1965).

*Enzyme Inhibition*

The results of experiments on the inhibition of microsomal *N*-demethylase activity are shown in Table 7. The enzyme system was inhibited by thiol reagents, chelating reagents, electron acceptors and carbon monoxide. Most of these same reagents have been found to be effective inhibitors of the electron transport system in animal microsomal systems,[13] and provide indirect evidence to support the view that a similar electron transport system is

TABLE 7. INHIBITION OF ETIOLATED COTTON HYPOCOTYL *N*-DEMETHYLASE*

| Inhibitor | Conc. (mM) | Inhibition (%) |
|---|---|---|
| CO | 12% | 35 |
| *p*-Chloromercuribenzoate | 0·1, 0·5 | 88, 100 |
| *N*-Ethylmaleimide | 0·5, 1·0 | 22, 58 |
| $HgCl_2$ | 1·0 | 97 |
| $CuCl_2$ | 1·0 | 100 |
| $ZnCl_2$ | 1·0 | 65 |
| *o*-Phenanthroline | 1·0 | 43 |
| Bathophenanthroline | 1·0 | 36 |
| Bathocuproine | 0·1, 1·0 | 8, 33 |
| $\alpha,\alpha'$-Dipyridyl | 0·1, 1·0 | 7, 20 |
| 8-Hydroxyquinoline | 1·0 | 38 |
| *p*-Nitrobenzyl thiocyanate | 0·5 | 37 |
| Thiourea | 1·0 | 11 |
| Menadione | 0·1 | 95 |
| Riboflavin | 0·1 | 61 |
| Cytochrome c | 0·1 | 40 |

* Reaction mixture contained 1·4–1·9 mg of protein, 30 m$\mu$moles of $^{14}C$ trifluoromethyl-labeled 3-(3-trifluoromethylphenyl)-1,1-dimethylurea, 50 $\mu$moles of potassium phosphate, 0·5 $\mu$mole NaCN, 1 $\mu$mole of NADPH and inhibitor as indicated. The inhibitor and enzyme were incubated for 15 min at 25° and the reaction started by adding substrate and NADPH. CO inhibition was determined by incubating the enzyme with CO or $N_2$, 12% v/v, at 0° for 15 min. The reaction was run for 30 min at 25°.

present in cotton microsomal preparations. Direct evidence for the presence of a $P_{450}$-type cytochrome in these systems has not been obtained because of the thermal instability of the system and the presence of an interfering pigment. Estabrook[14] has recently been able to determine the presence of a $b_5$-type cytochrome system in etiolated cotton hypocotyl microsomal preparations having *N*-demethylase activity. A recent report by Moore[15] on the occurrence of a $b_5$ cytochrome in microsomal preparations from *Pisum sativum* and *Arum*

[14] R. W. ESTABROOK, private communication.
[15] C. W. D. MOORE, Ph.D. Dissertation, University of Cambridge, Cambridge, England (1967).

*maculatum* supports these findings. Moore[15] also has reported the presence of a $P_{450}$ cytochrome in plant microsomal systems.

The phenolase inhibitors, *p*-nitrobenzyl thiocyanate and thiourea[16,17] also inhibited the cotton *N*-demethylase system. Bakry[16] has proposed that these inhibitors may function by complexing through the sulfur atom with an active chelated percupryl ion intermediate in oxidations catalyzed by tyrosinase.[18] The significance of any relationship between the inhibition of *N*-demethylase activity by *p*-nitrobenzyl thiocyanate and bathocuproine and the possible direct involvement of copper in this microsomal enzyme system has not been determined. It should be noted that Mason *et al.*[13] have provided indirect evidence that copper is probably not directly involved in the animal microsomal mixed-function oxidations.

TABLE 8. INHIBITION OF ETIOLATED COTTON HYPOCOTYL *N*-DEMETHYLASE BY *N*-METHYL CARBAMATES*

| Inhibitor | Conc. (M) | Inhibition (%) |
|---|---|---|
| 4-Benzothienyl methylcarbamate | $1 \times 10^{-5}$ | 62 |
| 1-Naphthyl methylcarbamate | $1 \times 10^{-5}$ | 58 |
| 4-(Methylthiol)-3,5-xylyl methylcarbamate | $1 \times 10^{-5}$ | 49 |
| 3,4,5-Trimethylphenyl methylcarbamate | $1 \times 10^{-5}$ | 40 |
| 2,4,5-Trimethylphenyl methylcarbamate | $1 \times 10^{-5}$ | 23 |
| 2-Isopropoxyphenyl methylcarbamate | $1 \times 10^{-5}$ | 4 |
| 4-Dimethylamino-2-methylphenyl methylcarbamate | $1 \times 10^{-5}$ | 0 |
| 3,4-Dichlorobenzyl methylcarbamate | $1 \times 10^{-5}$ | 0 |
| 4-(*N*,*N*-Dimethylamino-methyleneimino)-3-methylphenyl methylcarbamate hydrochloride | $1 \times 10^{-5}$ | 0 |

* The reaction mixture contained 1·4–1·7 mg of protein, 30 mμmoles of $^{14}C$ trifluoromethyl-labeled 3-(3-trifluoromethylphenyl)-1,1-dimethylurea, 50 μmoles potassium phosphate, pH 7·5, 0·5 μmole of NaCN, 1 μmole NADPH and inhibitor as indicated. The inhibitor and enzyme were incubated at 25° for 15 min and the reaction started by adding substrate and NADPH. The reaction was run for 30 min at 25°.

Sesamex and SKF 525-A, inhibitors of microsomal mixed-function oxidases in insects and animals,[19–22] did not inhibit the etiolated cotton hypocotyl microsomal enzyme system. Sodium cyanide, sodium azide and EDTA were also not inhibitory at the 1 mM level, and product inhibition by 0·1 mM $NADP^+$ was not appreciable.

The inhibition of *N*-demethylase by several *N*-methylcarbamate compounds is shown in Table 8. The combined use of substituted phenyl-*N*-methylurea herbicides and various carbamate insecticides for the control of plant pests has, in some instances, resulted in death

[16] N. BAKRY, Ph.D. Dissertation, University of California, Riverside (1966).
[17] N. BAKRY, R. L. METCALF and T. R. FUKUTO, *J. Econ. Entomol.* **61**, 1303 (1968).
[18] H. BRIGHT, B. WOOD and L. INGRAHAM, *Ann. N. Y. Acad. Sci.* **100**, 965 (1963).
[19] R. L. METCALF in *Enzymic Oxidations of Toxicants* (edited by E. HODGSON), p. 162, North Carolina State Univ., Raleigh, N. Carolina (1968).
[20] R. SCHRONBROD, D. WELLMAN and L. TERRIERE, *Bull. Entomol. Soc. Am.* **12**, 292 (1966).
[21] H. H. MOOREFIELD and N. H. WEIDEN, *J. Contrib. Boyce Thompson Inst.* **20**, 293 (1959).
[22] R. O'BRIEN, *Biochem. J.* **79**, 229 (1961).

or injury to the crop being treated.[23,24] The *in vivo* inhibition of urea *N*-demethylation in cotton leaf disks by insecticidal carbamates has been recently demonstrated by Swanson and Swanson.[4] The same carbamates that were effective inhibitors of *N*-demethylation in leaf disks were also found to be effective inhibitors of the isolated enzyme system. A particulate arylacylamidase system from rice responsible for the hydrolysis of 3,4-dichloropropionanilide has also been shown to be inhibited by insecticidal carbamates and organophosphates.[25,26] Cotton *N*-demethylase activity was not, however, inhibited by the organophosphates, *O,O*-diethyl-*O*-*p*-nitrophenyl phosphate and *O,O*-diethyl-*O*-*p*-nitrophenyl phosphorothioate at the $1 \times 10^{-5}$ M level.

*Enzyme Activities of Etiolated Cotton Hypocotyl Microsomal Fraction*

Enzyme activities associated with washed etiolated cotton hypocotyl microsomal preparations are shown in Table 9. These results compare favorably with the activities of the same enzyme systems reported in plant microsome preparations by Martin and Morton[27,28]

TABLE 9. ENZYME ACTIVITIES OF ETIOLATED COTTON HYPOCOTYL MICROSOMAL FRACTION*

| Enzyme | Specific activity† | |
|---|---|---|
| | Mean | Range |
| NADH–cytochrome c reductase | 223 | 200–248 |
| NADPH–cytochrome c reductase | 68 | 54–81 |
| NADH diaphorase | 222 | 213–231 |
| NADPH diaphorase | 65 | 60–70 |
| Succinate–cytochrome c reductase | 3 | 0–5 |

* Microsomal preparations and enzyme assays were carried out as described in Materials and Methods.

† Specific activity is mμmoles cytochrome c or 2,6-dichlorophenol-indophenol reduced/min/mg protein.

and Ragland and Hackett.[29] The presence of an active NADPH–cytochrome c reductase, and of relatively little succinic–cytochrome c reductase activity in the etiolated cotton hypocotyl preparations, again supports the possible involvement of similar electron transport systems in plant and animal microsomal systems. It also demonstrates that the amount of mitochondrial contamination is relatively low. Attempts to demonstrate the presence of NADPH and NADH oxidase activities in these preparations were unsuccessful.

*Substrate Specificity*

Etiolated cotton hypocotyl microsomal preparations are able to readily *N*-demethylate several substituted 3-(phenyl)-1-methylurea compounds as shown in Table 10. Other

[23] C. C. BOWLING and H. R. HUDGINS, *Weeds* **14**, 94 (1966).
[24] J. HACSKAYLO, J. K. WALKER, JR. and E. G. PIRES, *Weeds* **12**, 288 (1964).
[25] D. S. FREAR and G. G. STILL, *Phytochem.* **7**, 913 (1968).
[26] S. MATSUNAKA, *Science* **160**, 1360 (1968).
[27] E. M. MARTIN and R. K. MORTON, *Biochem. J.* **62**, 969 (1956).
[28] E. M. MARTIN and R. K. MORTON, *Nature* **176**, 113 (1955).
[29] T. E. RAGLAND and D. P. HACKETT, *Biochem. Biophys. Acta* **54**, 577 (1961).

substrates that have been reported to be *N*-dealkylated by *in vivo* plant systems did not function as substrates for the cotton microsomal systems. These included *N,N*-dimethyl-2,2-diphenylacetamide, which has been reported to be successively *N*-demethylated[30, 31] and 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine which has been reported to be readily *N*-dealkylated.[32]

The sulfoxidation of 2-methylmercapto-4,6-*bis*-(isopropylamino)-*s*-triazine has also been reported to occur in plants[33] and has been characterized as a microsome-NADPH-catalyzed reaction.[34] Studies with cotton *N*-demethylase preparations, however, failed to show any oxidation of $^{14}C$ ring-labeled 2-methylmercapto-4,6-*bis*-(isopropylamino)-*s*-triazine. Also, no evidence was obtained to indicate that $^{14}C$-ring-labeled 1-naphthyl methylcarbamate or 4-benzothienyl methylcarbamate were *N*-demethylated by the cotton *N*-demethylase system. Under the assay conditions used in these studies, the cotton microsomal *N*-demethylase system appears to be specific for substituted 3-(phenyl)-1-methylurea substrates.

TABLE 10. SUBSTRATE SPECIFICITY OF MICROSOMAL *N*-DEMETHYLASE FROM ETIOLATED COTTON HYPOCOTYL*

| Substrate | Specific activity† | $K_m$‡ |
|---|---|---|
| 3-(3,4-Dichlorophenyl)-1,1-dimethylurea | 4·91 | $1·5 \times 10^{-5}$ |
| 3-(3-Trifluoromethylphenyl)-1,1-dimethylurea | 4·51 | $1·3 \times 10^{-5}$ |
| 3-(4-Chlorophenyl)-1,1-dimethylurea | 3·97 | $2·9 \times 10^{-5}$ |
| 3-(4-Chlorophenyl)-1-methylurea | 2·08 | — |

* Reaction mixture contained 1·9–2·3 mg of protein, 50 mμmoles of $^{14}C$ labeled substrate for specific activity determinations, 10, 20, 40, 60 or 80 mμmoles of $^{14}C$ labeled substrate for $K_m$ determinations, 50 μmoles of potassium phosphate, 0·5 μmole of NaCN, and 1 μmole of NADPH. The reaction was run for 30 min at 25°.

† Specific activity is the amount of 3-(3,4-dichlorophenyl)-1-methylurea, 3-(3-trifluoromethylphenyl)-1-methylurea, 3-(4-chlorophenyl)-1-methylurea or 3-(4-chlorophenyl) urea formed/mg protein/30 min.

‡ $K_m$ values were obtained from Lineweaver-Burk plots.

*Kinetic Studies*

The apparent $K_m$ constants for 3-(4-chlorophenyl)-1,1-dimethylurea, 3-(3,4-dichlorophenyl)-1,1-dimethylurea and 3-(3-trifluoromethylphenyl)-1,1-dimethylurea were calculated from Lineweaver–Burk plots (Table 10). The apparent $K_m$ for 3-(4-chlorophenyl)-1-methylurea was not determined because of the formation of a significant quantity of an unknown product in addition to 4-chlorophenylurea. This product had an $R_f$ slightly lower than 4-chlorophenylurea in the benzene–acetone (2:1) solvent system and required NADPH for its formation.

The competitive inhibition of 3-(3-trifluoromethylphenyl)-1,1-dimethylurea *N*-demethylation by 1-naphthyl methylcarbamate is shown in Fig. 2. The competitive nature of the observed inhibition is supported by the structural similarities between the inhibitor and the

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[31] T. GOLAB, R. J. HERBERG, S. J. PARKA and J. B. TEPE, *J. Agr. Food Chem.* **14**, 592 (1966).
[32] R. H. SHIMABUKURO, *J. Agr. Food Chem.* **15**, 557 (1967).
[33] E. EBERT and P. W. MÜLLER, *Experientia* **24**, 1 (1968).
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substrate, and by the reported carbamoylation of liver microsomal proteins by several *N*-methylcarbamates.[34] The apparent $K_i$ for 1-naphthyl methylcarbamate was found to be $1{\cdot}5 \times 10^{-6}$ M. Kinetic studies for determining the nature of 1-napthyl methylcarbamate inhibition were carried out by incubating the inhibitor with the enzyme for 15 min at 0° before the addition of substrate and NADPH to initiate the reaction. This was done to reduce the rate of the "nonenzymatic" hydrolysis of the inhibitor, and to eliminate the thermal inactivation of the enzyme during the incubation period.

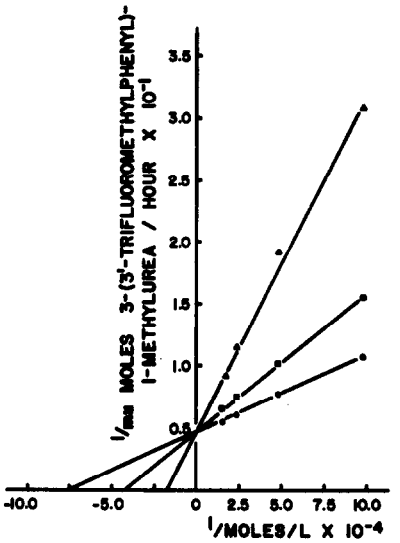


FIG. 2. LINEWEAVER–BURK PLOT OF COTTON HYPOCOTYL *N*-DEMETHYLASE ACTIVITY WITH 1-NAPHTHYL-METHYLCARBAMATE AS THE INHIBITOR.

The reaction mixture contained 2·2 mg protein, 10, 20, 40 or 60 mμmoles of $^{14}$C trifluoromethyl-labeled 3-(3-trifluoromethylphenyl)-1,1-dimethylurea, 50 μmoles of potassium phosphate, pH 7·5, 0·5 μmole of NaCN, 1 μmole of NADPH, and 0 (—●—), $5 \times 10^{-6}$ M (—▲—), or $1 \times 10^{-6}$ M (—■—) 1-naphthyl methylcarbamate. The enzyme and inhibitor were incubated at 0° for 15 min and the reaction started by addition of substrate and NADPH. The reaction was run for 30 min at 25°.

## MATERIALS AND METHODS

### *Materials*

Polyclar AT (research powder grade) was purchased from General Aniline and Film Corporation, Melrose Park, Illinois. Isoascorbic acid (D-araboascorbic acid) was purchased from Eastman Organic Chemicals, Rochester, New York. $^{14}$C labeled formaldehyde, $^{14}$C carbonyl labeled 4-chlorobenzoic acid, and $^{14}$C labeled dimethylamine hydrochloride were purchased from New England Nuclear, Boston, Massachusetts. All other reagents used were obtained commercially and were of the highest purity available.

### *Synthesis of $^{14}$C-Carbonyl-Labeled 3-(4-Chlorophenyl)-1-Methylurea*

For the preparation of $^{14}$C-carbonyl-labeled 3-(4-chlorophenyl)-1-methylurea, 22·8 mg of $^{14}$C carbonyl-labeled 4-chlorobenzoic acid was refluxed with 2 ml $SOCl_2$ for 2 hr on an oil bath. After removal of excess $SOCl_2$, the 4-chlorobenzoyl chloride, in 0·75 ml of dry acetone was treated, with stirring, at 20–25°, with a solution of 0·2 g $NaN_3$ in 1 ml water. After 30 min, the 4-chlorobenzoyl azide was extracted with $CHCl_3$, the extract taken to dryness and stored in a desiccator.

To effect the Curtius rearrangement, the 4-chlorobenzoyl azide was dissolved in 5 ml dry toluene and heated at 100–105° until $N_2$ evolution ceased (after 2 hr). The solution of 4-chlorophenylisocyanate in toluene was cooled in an ice bath, and 5 ml $CHCl_3$, saturated with anhydrous $MeNH_2$, was added rapidly with stirring. The mixture was stirred for 15 min, and the solvent and excess $MeNH_2$ removed by rotary vacuum evaporation (yield 78 per cent).

*Synthesis of $^{14}C$ Methyl-Labeled 3-(4-Chlorophenyl)-1,1-Dimethylurea and 3-(3,4-Dichlorophenyl)-1,1-Dimethylurea*

For the preparation of $^{14}C$ methyl-labeled 3-(4-chlorophenyl)-1,1-dimethylurea, 16·3 mg $^{14}C$-dimethylamine hydrochloride was placed in a vacuum system and converted to the free amine by the addition of 0·5 ml of 2·5 M aqu. NaOH. In order to trap volatilized water, an ascarite drying tube was placed in the vacuum system between the sample and the cold trap. The dimethylamine was transferred by a vacuum transfer technique into a vessel containing 2 ml toluene, 2 ml $CHCl_3$, and either 33·8 mg 4-chlorophenylisocyanate or 41·4 mg 3,4-dichlorophenylisocyanate held in a cold bath of dry ice-2-propanol. After transfer, the sample was removed from the cold bath and allowed to stand at room temp. overnight in a sealed system [3-(4-chlorophenyl)-1,1-dimethylurea, yield 94 per cent, 3-(3,4-dichlorophenyl-1,1-dimethylurea, yield 92 per cent].

*Plant Materials*

Leaf tissues were excised from young plants grown in the greenhouse with one-half strength Hoagland's nutrient solution and with a 12-hr photoperiod. The species studied included: cotton (*Gossypium hirsutum* L.), plantain (*Plantago major* L.), wild buckwheat (*Polygonum convolvulus* L.), buckwheat (*Fagopyrum esculentum*), broadbean (*Vicia faba*), corn (*Zea mays* L.), sorghum (*Sorghum vulgare*), mallow (*Malva rotundifolia* L.), soybean (*Glycine max* cv. Merrill), okra (*Hibiscus esculentus* L.), potato (*Solanum tuberosum* cv. cobbler), and celery (*Apium graveolens*). Etiolated Stoneville 213 cotton seedlings were grown at 32° and 70 per cent relative humidity in subirrigated vermiculite.

*Preparation of Microsomal Fractions*

Excised leaf and etiolated seedling tissues were rinsed with distilled water, blotted dry, and ground to a fine powder with liquid $N_2$ in either a mortar and pestle or in a vented stainless-steel Waring Blendor assembly. The frozen tissue powders were slurried with 4 vol. of 0·3 M potassium phosphate buffer, pH 7·5, containing 0·25 M sodium isoascorbate, 0·001 M NaCN and one-half weight of Polyclar AT (insoluble polyvinylpyrolidione). After standing 15 min, the slurry was squeezed through four layers of cheesecloth and differentially centrifuged. The microsomal fraction was sedimented at $78,000 \times g$ for 80 min after removal of larger particulate matter by centrifugation at $1500 \times g$ for 10 min and at $17,500 \times g$ for 20 min.[8]

Unless stated otherwise, the microsomal preparations from 50–150 g of fresh tissue were washed by resuspension in 30–90 ml of 0·1 M potassium phosphate buffer containing 0·001 M NaCN followed by sedimentation again at $78,000 \times g$ for 80 min. Washed microsomal pellets were resuspended in a small volume (1–5 ml) of the same buffer to give approximately 4·0 mg of protein/ml for enzyme assay. Microsomal preparations used in the pH and buffer studies were washed and resuspended in distilled water before enzyme assay with appropriate buffer systems.

The separation of "smooth" and "rough" microsomal fractions was carried out with density gradient centrifugation according to the procedure of Dallner[35] as modified by M. J. Chrispeels *et al.*[36] All enzyme extraction and centrifugation procedures were carried out at 0–4°.

*Enzyme Assays*

Microsomal *N*-demethylase activity was determined by following the rate of demethylated product formation. The standard reaction mixture contained 5–30 m$\mu$moles of $^{14}C$ labeled substrate, 0·05 $\mu$mole of NaCN, 50 $\mu$moles of $K_3PO_4$, pH 7·5, 1·0 $\mu$mole of NADPH and 1–3 mg of washed microsomal protein in a final volume of 1·0 ml. The reaction was initiated by the addition of NADPH and incubated for 30 min at 25°. Controls were run without NADPH. Enzyme activity was linear for 40 min and was proportional to enzyme concentration up to 4 mg of protein. The reaction was terminated by rapid freezing in a dry ice-acetone bath followed by lyophilization. The lyophilized reaction mixture was extracted with 500–600 $\mu$l. of methanol and the demethylated reaction product was separated from the substrate by TLC[3] for quantitative determination by liquid scintillation counting.[8, 37]

Succinate–cytochrome c reductase, NADPH and NADH oxidase, NADH and NADPH cytochrome c reductase, and NADH and NADPH diaphorase activities were determined according to the procedure reported by Ragland and Hackett.[29] Protein concentration was determined by the method of Lowry *et al.*[38] with crystalline bovine serum albumin as the standard. Inhibition studies were conducted by incubation of the enzyme and inhibitor for 15 min, followed by the addition of substrate and NADPH to initiate the reaction.

*Identification and Quantitative Determination of Reaction Products*

Formaldehyde was identified as the 5,5-dimethyl-1,3-cyclohexanedione derivative (formaldomethone) by cochromatography in two TLC systems, autoradiography, and mass spectroscopy. Formaldomethone had an $R_f$ of 0·95 in benzene–acetone (2:1 v/v) and of 0·33 in a benzene–ethyl acetate (95:5 v/v). The substrate, 3-(4-chlorophenyl)-1,1-dimethylurea and other reaction product, 3-(4-chlorophenyl)-1-methylurea remained

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[37] F. SNYDER and N. STEPHEN, *Anal. Biochem.* **4**, 128 (1962).
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at the origin in the latter solvent and had $R_f$s of 0·39 and 0·57, respectively, in the former solvent.[3] After separation and purification by TLC, the isolated formaldomethone was further identified by autoradiography and cochromatography with known formaldomethone. The mass spectrum of the methone derivative isolated from the reaction corresponded with the mass spectrum of known formaldomethone.

The *N*-demethylated reaction products, 3-(4-chlorophenyl)-1,methylurea, 3-(3,4-dichlorophenyl)-1-methylurea and 3-(3-trifluoromethylphenyl)-1-methylurea were identified by direct comparison with known compounds, with techniques of cochromatography, autoradiography and mass spectroscopy. The structures of the *N*-demethylated products were clearly established by their mass spectra because of the presence of the respective molecular ions, and major peaks resulting from the cleavage of methylisocyanate ions[39] as well as the characteristic distribution of chlorine isotopes and a characteristic loss of one F ion observed with known 3-(3-trifluoromethylphenyl)-1-methylurea samples.

Formaldehyde produced during the *N*-demethylation of $^{14}C$ methyl-labeled 3-(4-chlorophenyl)-1,1-dimethylurea was quantitatively determined by trapping as the semicarbazide derivative followed by conversion to formaldomethone and separation by TLC. The procedures used for the isolation of the formaldehyde produced were modifications of the reported methods of Johnson and Mackenzie[40] and Mackenzie.[41] The standard reaction mixture with $^{14}C$ methyl-labeled 3-(4-chlorophenyl)-1,1-dimethylurea was used, except that 1 $\mu$mole of semicarbazide was included to trap any radioactive formaldehyde formed during the reaction. Addition of 1 $\mu$mole of semicarbazide to the assay medium did not affect enzyme activity. The reaction was terminated after 45 min at 25° by the addition of 2·0 ml of 0·1 M NaOAc buffer, pH 4·5. One-half ml of 0·2% aqueous 5,5-dimethyl-1,3-cyclohexanedione was added, the volume adjusted to 4·0 ml with distilled water, and the reaction mixture kept at room temp. for 20 hr. The formaldomethone formed and the 3-(4-chlorophenyl)-1-methylurea produced were quantitatively extracted with ether (5 × 4 ml). The combined ether extracts were evaporated to dryness and dissolved in 1·0 ml of methanol for TLC separation. 300 $\mu$l of the methanol extract was spotted on 250 $\mu$ HF silica gel plates together with known nonradioactive formaldomethone, 3-(4-chlorophenyl)-1,1-dimethylurea and 3-(4-chlorophenyl)-1-methylurea standards. The plates were developed in benzene–acetone (2:1 v/v) for 15 cm, visualized under u.v. light, and the radioactive formaldomethone and 3-(4-chlorophenyl)-1-methylurea quantitatively determined.[37]

*Acknowledgements*—Analytical grade carbamates used for inhibition studies were generously provided as follows: 4-benzothienyl methylcarbamate from Mobil Chemical Company, Metuchen, New Jersey; 1-naphthyl methylcarbamate, 3,4-dichlorobenzyl methylcarbamate, and 4-(*N*,*N*-dimethylaminomethylene-imino)-3-methylphenyl methylcarbamate hydrochloride from Union Carbide Company, New York; 2,4,5-trimethylphenyl methylcarbamate and 3,4,5-trimethylphenyl methylcarbamate from Shell Chemical Company, New York; and 4-(methylthiol)-3,5-xylyl methylcarbamate, 2-isopropoxyphenyl methylcarbamate and 4-dimethylamino-2-methylphenyl methylcarbamate from Chemagro Corporation, Kansas City, Missouri. Analytical grade organophosphates were provided as follows: *O*,*O*-diethyl-*O*-*p*-nitrophenyl phosphate by American Cyanamide Company, Princeton, New Jersey, and *O*,*O*-diethyl-*O*-*p*-nitrophenyl phosphorothioate by Monsanto Company, St. Louis, Missouri. Insecticide synergists SKF 525-A (2,2-diethylaminoethyl-2,2-diphenylpentanoate) and Sesamex [2-(3,4-methylenedioxyphenoxy)-3,6,9-trioxoundecane] were supplied by Smith, Kline, and French Laboratories, Philadelphia, Pennsylvania, and by Shulton, Clifton, New Jersey. Surfactant TD10 was provided by Hodag Chemical Company, Skokie, Illinois, while surfactants Tergitol NPX and Tergitol 15-S-9 were provided by Union Carbide Company, New York, New York. 3-(4-Chlorophenyl)-1,1-dimethylurea, 3-(4-chlorophenyl)-1-methylurea, 3-(4-chlorophenyl)-urea, 3-(3,4-dichlorophenyl)-1,1-dimethylurea, 3-(3,4-dichlorophenyl)-1-methylurea and 3-(3,4-dichlorophenyl)-urea were supplied by E. I. duPont deNemours and Company, Wilmington, Delaware. CIBA Corporation, Vero Beach, Florida, provided 3-(3-trifluoromethylphenyl)-1,1-dimethylurea, 3-(3-trifluoromethylphenyl)-1-methylurea and 3-(3-trifluoromethylphenyl)-urea. *N*,*N*-Dimethyl-2,2-diphenylacetamide, *N*-methyl-2,3-diphenylacetamide and 2,2-diphenylacetamide were supplied by Eli Lilly and Company, Greenfield, Indiana. $^{14}C$ Carbonyl-labeled 3-(3,4-dichlorophenyl)-1,1-dimethylurea were obtained from E. I. duPont deNemours and Company, Wilmington, Delaware; $^{14}C$ carbonyl-labeled *N*,*N*-dimethyl-2,2-diphenylacetamide from Eli Lilly and Company, Greenfield, Indiana; $^{14}C$ ring-labeled 4-benzothienyl methylcarbamate from Mobil Chemical Company, Metuchen, New Jersey; $^{14}C$ trifluoromethyl-labeled 3-(3-trifluoromethylphenyl)-1,1-dimethylurea from CIBA Corporation, Vero Beach, Florida; $^{14}C$ ring-labeled 2-methylmercapto-4,6-*bis*-(isopropylamino)-*s*-triazine, and $^{14}C$ ring-labeled 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine from Geigy Chemical Corporation, Yonkers, New York; $^{14}C$ ring-labeled 3-(4-chlorophenyl)-1,1-dimethylurea from Dr. R. E. Kadunce of this laboratory; and $^{14}C$ ring-labeled 1-naphthylmethylcarbamate from Mr. V. J. Feil, Animal Husbandry Division USDA, Metabolism and Radiation Research Laboratory, Fargo, North Dakota.

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