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MECHANISM OF OXIDATIVE *N*-DEMETHYLATION BY COTTON MICROSOMES*

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Key Word Index—*Gossypium*; Malvaceae; oxidative *N*-demethylation in microsomes; inhibition by *N*-methylcarbamates and phenylureas.

Abstract—Inhibition studies with *N*-methylcarbamates and phenylureas were carried out to examine the mechanism for microsomal *N*-demethylation of substituted 3-(phenyl)-1-methylureas. Studies with a number of substituted *N*-methylcarbamates showed that a high electron density at the *ortho* position relative to the carbamate group was necessary for an effective inhibition of *N*-demethylation. Similar studies with several substituted phenylureas demonstrated that the proton on the aniline nitrogen atom was also necessary for effective inhibition of the microsomal *N*-demethylase system. Stable isotope studies with a methyl-d_3 and a carbonyl-^{18}O labeled 3-(phenyl)-1-methylurea were also performed; the methyl-d_3 substrate exhibited a value of $k_H/k_D = 1{\cdot}3$ for the metabolic reaction, and the carbonyl-^{18}O substrate demonstrated no isotopic exchange of labeled oxygen after *N*-dealkylation.

INTRODUCTION

OXIDATIVE *N*-demethylation of substituted 3-(phenyl)-1-methylureas[1,2] was recently demonstrated with a microsomal mixed function oxidase isolated from leaf and etiolated hypocotyl tissues of cotton plants. Metabolic products from the *N*-demethylation of 3-(4-chlorophenyl)-1,1-dimethylurea by the microsomal oxidase system were identified as 3-(4-chlorophenyl)-1-methylurea, 3-(4-chlorophenyl)-1-hydroxymethylurea, 4-chlorophenylurea, and formaldehyde. Glucose conjugates of oxidized *N*-hydroxymethyl intermediates were also isolated and characterized as major polar metabolites in excised cotton leaves treated with 3-(4-chlorophenyl)-1,1-dimethylurea or 3-(4-chlorophenyl)-1-methylurea.[1]

Inhibition studies with the cotton microsomal *N*-demethylase system were carried out to provide information on the nature of the active site and the mechanism of the reaction.[3] The *N*-demethylation of 3-(3-trifluoromethylphenyl)-1,1-dimethylurea was inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea, 3-(4-chlorophenyl)-1,1- dimethylurea and their *N*-demethylated products. These studies showed that the 3,4-dichlorophenylureas were more effective inhibitors of the *N*-demethylation reaction than their corresponding 4-chlorophenylureas. Although the demethylated products were less toxic to plants,[4,5] these compounds were more effective inhibitors of microsomal *N*-demethylation than the corresponding parent dimethyl-substituted urea. It was suggested[3] that product inhibition of this detoxication pathway might be prevented by a rapid removal of the demethylated

* Part IV in the series "*N*-Demethylation of Substituted 3-(Phenyl)-1-Methylureas". For Part III see *Phytochem.* **11**, 2701 (1972).

[1] D. S. FREAR and H. R. SWANSON, *Phytochem.* **11**, 1919 (1972).

[2] D. S. FREAR, H. R. SWANSON and F. S. TANAKA, *Phytochem.* **8**, 2157 (1969).

[3] D. S. FREAR, H. R. SWANSON and F. S. TANAKA, in *Symposia of the Phytochemical Society of North America* (edited by V. C. RUNECKLES), Academic Press, New York (1972).

[4] H. GEISSBÜHLER, C. HASELBACH, H. AEBI and L. EBNER, *Weed Res.* **3**, 277 (1963).

[5] R. B. NASHED, S. E. KATZ and R. D. ILLNICKI, *Weed Sci.* **18**, 122 (1970).

products from the active enzyme site by formation of polar metabolites and insoluble plant residues.

Several semicarbazide analogs of 3-(3,4-dichlorophenyl)-1,1-dimethylurea were investigated as inhibitors of the cotton *N*-demethylase system.[3] The behavior of the trimethyl-substituted analog was similar to the more substituted phenylureas which were less inhibitory than the corresponding dealkylated products. Studies with the mono- and dimethylsemicarbazides indicated that the insertion of an additional nitrogen atom into the side-chain did not appreciably effect the degree of inhibition.

A review of reports on low k_H/k_D isotopic effects showed that morphine was demethylated by rat liver microsomes[6-8] at a rate 1·4 times greater than *N*-trideuteromorphine. The assumption was that these data reflected a primary isotope effect and that the cleavage of a proton adjacent to nitrogen was rate limiting. Essentially the same isotopic effect of 1·3 was observed in the oxidation of trimethylamine with chlorine dioxide; however this reaction is known to proceed by electron abstraction from nitrogen and not by α-hydrogen abstraction.[9,10] Also, a model system of Sn^{2+}–HPO_4^{2-}–O_2 was used in the hydroxylation of bromobenzene-d_5 and cyclohexane-d_{12}.[11] The k_H/k_D ratios of these reactions were 1·05 and 1·28, respectively, and these data were reported as demonstrating no rate-limiting isotopic effect. It appears, therefore, that a low k_H/k_D ratio does not always reflect a primary isotopic effect.

The *N*-dealkylation of tertiary amines by an electrochemical process[12] and tertiary amine oxides by an iron catalyzed reaction[13] were studied. Both reactions suggested free radical intermediates in the dealkylation mechanism. In liver homogenates it was suggested that tertiary amines were oxidized to *N*-oxides and subsequently cleaved to secondary amines and aldehydes.[14,15] However, studies with liver microsomal fractions did not clearly establish the amine oxide group as an intermediate in the *N*-dealkylation of tertiary amines.[16] To cloud the issue further, a mechanism was proposed for the direct cleavage of tertiary amines without participation of an *N*-oxide intermediate in the dealkylation reaction.[6]

Therefore, with the identification of oxidized polar metabolic products and the examination of some inhibitors of the microsomal reaction, further attempts were made to elucidate the mechanism for the *N*-demethylation of substituted phenylureas.

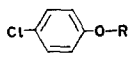
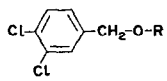
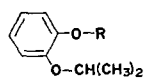
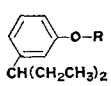
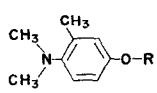
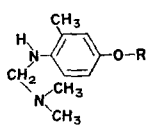
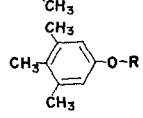
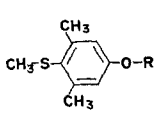
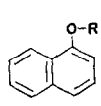
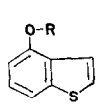
RESULTS AND DISCUSSION

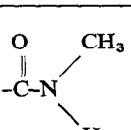
Inhibition by Substituted Carbamates

The carbamate moiety of substituted aryl–alkyl carbamates and the urea moiety of substituted phenylureas have similar structures. Inhibitor studies with several *N*-methyl-

[6] R. E. MCMAHON and N. E. EASTON, *J. Med. Pharm. Chem.* **4,** 437 (1961).
[7] R. E. MCMAHON, *J. Pharm. Sci.* **55,** 457 (1966); *Tetrahedron Letters* 2307 (1966).
[8] C. ELISON, W. H. ELLIOT, M. LOCK and H. RAPOPORT, *J. Med. Chem.* **6,** 237 (1963).
[9] D. H. ROSENBLATT, L. A. HULL, D. C. DELUCA, G. T. DAVIS, R. C. WEGLEIN and H. K. R. WILLIAMS, *J. Am. Chem. Soc.* **89,** 1158 (1967).
[10] L. A. HULL, G. T. DAVIS, D. H. ROSENBLATT, H. K. R. WILLIAMS and R. C. WEGLEIN, *J. Am. Chem. Soc.* **89,** 1163 (1967).
[11] V. ULLRICH and HJ. STAUDINGER, in *Microsomes and Drug Oxidations* (edited by J. R. GILLETTE, A. H. CONNEY, G. J. COSMIDES, J. R. FOUTS and G. J. MANNERING), p. 204, Academic Press, New York (1969).
[12] L. C. PORTIS, V. V. BHAT and C. K. MANN, *J. Org. Chem.* **35,** 2175 (1970).
[13] J. P. FERRIS, R. D. GERWE and G. R. GAPSKI, *J. Org. Chem.* **33,** 3493 (1968); *idem. J. Am. Chem. Soc.* **89,** 5270 (1967).
[14] M. S. FISH, N. M. JOHNSON, E. P. LAWRENCE and E. C. HORNING, *Biochim. Biophys. Acta* **18,** 564 (1955).
[15] C. C. SWEELEY and E. C. HORNING, *J. Am. Chem. Soc.* **79,** 2620 (1957).
[16] D. M. ZIEGLER and F. H. PETIT, *Biochem. Biophys. Res. Commun.* **15,** 188 (1964).

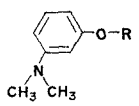
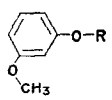
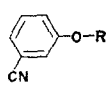
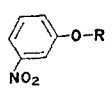
TABLE 1. INHIBITION OF MICROSOMAL *N*-DEMETHYLATION BY SOME *N*-METHYLCARBAMATES[2]

No.	Inhibitor structure*	Name	Inhibition (%)
1		4-Chlorophenyl methylcarbamate	0
2		3,4-Dichlorobenzyl methylcarbamate	0
3		2-Isopropoxyphenyl methylcarbamate	4
4		3-(3-Pentyl)-phenyl methylcarbamate	0
5		3-Methyl-4-dimethylaminophenyl methylcarbamate	0
6		4-(*N*,*N*-Dimethylamino-methyleneimino)-3-methylphenyl methylcarbamate	0
7		3,4,5-Trimethylphenyl methylcarbamate	40
8		4-(Methylthiol)-3,5-xylyl methylcarbamate	49
9		1-Naphthyl methylcarbamate	58
10		4-Benzothienyl methylcarbamate	62

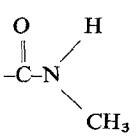
* R =  . Inhibitor concentration was 0·01 mM; Substrate was ^{14}C-trifluoromethyl-labeled 3-(3-trifluoromethylphenyl)-1,1-dimethylurea.

carbamates reveal that these compounds are effective inhibitors of microsomal *N*-demethylation (Table 1). The *N*-methylcarbamates 1–6 demonstrate virtually no inhibitory effect on the microsomal *N*-demethylase system. Even 4-chlorophenyl-*N*-methylcarbamate, which is structurally similar to 3-(4-chlorophenyl)-1-methylurea (24% inhibition) was surprisingly ineffective. Compounds 7–10, however, are effective inhibitors of the *N*-demethylation reaction. An examination of the structures of these compounds reveals that the carbamates which inhibit the microsomal *N*-demethylation reaction apparently require a high electron density at the *ortho* position relative to the carbamate group. Activation of the *ortho* position by one alkyl group is apparently inadequate to affect inhibition as evidenced by the poor inhibition with compounds 4 and 5. However, in the case of compounds 7 and 8, two alkyl groups can release their electron density to the same *ortho* position by hyperconjugation; consequently, these compounds are effective inhibitors of the *N*-demethylation reaction. Compounds 9 and 10 are also strong inhibitors of the microsomal *N*-demethylase activity. Both of these compounds are capable of mesomeric donation of electron density to the *ortho* position on the aromatic ring. From these data, it appears that a strong electron withdrawing group in the *meta* position of the *N*-methylcarbamate will prevent inhibition of the microsomal reaction, whereas a strong electron donating group in the *meta* position will enhance inhibition. The requirement for a strong electron

TABLE 2. INHIBITION OF MICROSOMAL *N*-DEMETHYLATION BY *meta*-SUBSTITUTED PHENYL *N*-METHYLCARBAMATES

No.	Inhibitor* structure†	Name	Inhibition (%)
1		3-Dimethylaminophenyl-*N*-methylcarbamate	65
2		3-Methoxyphenyl-*N*-methylcarbamate	22
3		3-Cyanophenyl-*N*-methylcarbamate	0
4		3-Nitrophenyl-*N*-methylcarbamate	0

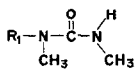
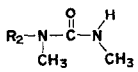
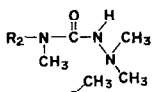
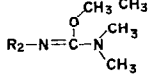
* Inhibitors illustrated in this table were synthesized in the laboratory.

† R =  . Inhibitor concentration was 0·05 mM. Substrate was ^{14}C-trifluoromethyl-labeled 3-(3-trifluoromethylphenyl)-1,1-dimethylurea.

donating group at the *meta* position of the aromatic ring appears to be specific since compounds 3, 5 and 6 all have strong electron releasing groups in positions other than *meta* to the carbamate group, and these compounds do not inhibit the *N*-demethylase reaction.

Model *N*-methylcarbamates were prepared to determine whether or not substitution of different types of groups at the *meta* position could affect *N*-demethylase inhibition. The results are given in Table 2. Compounds substituted with a strong electron donating dimethylamino or methoxy group inhibit the reaction 65 and 22%, respectively, while compounds substituted with an electron withdrawing cyano or nitro group are non-inhibitory. Since compounds 5 and 6 (Table 1) with effective electron donating groups located *para* to the carbamate group are also incapable of inhibition, it is quite apparent that *ortho* activation of the phenyl ring is necessary for inhibition. Whether enzyme attachment to the inhibitor is occurring at the *ortho* position or some other process is taking place, the exact means of participation of the position *ortho* to the carbamate group is not known.

TABLE 3. INHIBITION OF MICROSOMAL *N*-DEMETHYLATION BY SUBSTITUTED PHENYLUREAS LACKING HYDROGEN AT THE 3-POSITION OF UREA

No.	Inhibitor* structure†	Name	Inhibition (%)
1		3-(4-Chlorophenyl)-1,3-dimethylurea	0
2		3-(3,4-Dichlorophenyl)-1,3-dimethylurea	0
3		4-(3,4-Dichlorophenyl)-1,1,3-trimethyl-semicarbazide	0
4		3-(3,4-Dichlorophenyl)-1,1,2-trimethyl pseudourea	16

* Inhibitors 1, 2 and 3 were prepared in the laboratory, and 4 was supplied by E. I. DuPont DeNemours & Co., Wilmington, Del.

† R_1 = (4-chlorophenyl); R_2 = (3,4-dichlorophenyl). Inhibitor concentration was 0·01 mM. Substrate was ^{14}C-trifluoromethyl-labeled 3-(3-trifluoromethylphenyl)-1,1-dimethylurea.

Significance of the Proton Bonded to Aniline Nitrogen

For substituted phenylureas, the greater the acidity of the proton attached to the aniline nitrogen atom, the more effective the compound appears to be toward inhibition of the *N*-demethylation reaction.[3] Therefore, the apparent participation of this proton in inhibition and metabolism reactions of the substituted phenylureas has been investigated. The attachment of a methyl group at the 3-position of the side-chain of the substituted phenylureas and at the 4-position of the side-chain of the substituted phenylsemicarbazide results in complete loss of inhibition (Table 3). However, 3-(3,4-dichlorophenyl)-1,1,2-trimethyl-pseudourea, the methyl enol ether of 3-(3,4-dichlorophenyl)-1,1-dimethylurea, demonstrates one-third (16% inhibition) the inhibition observed for 3-(3,4-dichlorophenyl)-1,1-dimethyl-urea. The fact that the removal of the proton on the aniline nitrogen atom results in loss of

inhibition suggests that this proton is not only necessary for inhibition properties, but also for the metabolism of the substrate since this inhibition has been demonstrated to be competitive.[2] Furthermore, the retention of some degree of inhibition by the enol ether indicates that enolization of the carbonyl group may be involved in the mechanism for metabolism of phenylurea substrates. Studies on the inhibition of the Hill reaction by substituted phenylureas[17] have also suggested a similar requirement and involvement of the proton attached to the aniline nitrogen.

Stable Isotope Studies

The identification of 3-(4-chlorophenyl)-1-hydroxymethylurea as an unstable intermediate involved in the *N*-demethylation of 3-(4-chlorophenyl)-1-methylurea shows that substrate oxidation occurs at the methyl position. Examination of this oxidation reaction using 3-(4-chlorophenyl)-1-methylurea methyl-d_3 demonstrates an isotopic effect in the *N*-demethylation reaction. A value of $k_H/k_D = 1{\cdot}3$ is obtained by a comparison of the quantity of demethylated product from the methyl-H_3 with methyl-d_3. The low value for k_H/k_D suggests a secondary rather than a primary isotope effect with the rate-limiting step being an electron abstraction or possibly an *N*-oxide rearrangement.

In consideration of an *N*-oxide intermediate and the requirement of a labile proton on the aniline nitrogen atom, the possibility for the participation of a nitrone rather than an *N*-oxide intermediate in the metabolism of substituted phenylureas might be considered if enolization of the carbonyl group occurred prior to oxidation of the aniline nitrogen. Under the influence of light, nitrones can form oxaziridine compounds.[18] A substituted oxaziridino[2,3-a]pyrrolidine[18] has been suggested to photodecompose via a zwitterion intermediate to yield rearrangement products. In this regard substituted phenylureas may be metabolized in a manner similar to the light induced reactions since many of the photolytic products[19] of 3-(4-chlorophenyl)-1,1-dimethylurea are identical to the biological oxidation products. If oxaziridine and zwitterion intermediates are involved in the metabolic reaction, the carbonyl oxygen would participate in an equilibration step with the oxygen required in this reaction. This would lead to an oxygen isotopic exchange if the carbonyl group was labeled with ^{18}O.

To examine the degree of participation of the carbonyl group in the *N*-demethylation reaction, ^{18}O-carbonyl labeled 3-(4-chlorophenyl)-1,1-dimethylurea has been prepared with an initial ^{18}O isotopic abundance of 23·2 atom %. In the dealkylated metabolite, however, the ^{18}O isotopic abundance has also been estimated as 23 atom % which verifies that isotopic oxygen exchange does not occur. Unfortunately, the lack of carbonyl oxygen exchange demonstrates that the proposed equilibration step involving a zwitterion intermediate is not involved. Perhaps a nitrone intermediate[20] abstracts the proton from the methyl group in the oxidation reaction. At this time, however, a method has not been developed to demonstrate this mechanism. On the other hand, a mechanism similar to that elucidated for the enzyme acetoacetate decarboxylase[21] can also be discounted since isotopic exchange is not observed. In this reaction, Hamilton and Westheimer[22] were able to demonstrate that the

[17] D. E. MORELAND, *Ann. Rev. Plant Physiol.* **18,** 365 (1967).
[18] G. G. SPENCE, E. C. TAYLOR and O. BUCHARDT, *Chem. Rev.* **70,** 231 (1970).
[19] D. G. CROSBY and C. S. TANG, *J. Agric. Food Chem.* **17,** 1041 (1969).
[20] H. K. KIM and P. M. WEINTRAUB, *J. Org. Chem.* **35,** 4282 (1970).
[21] F. H. WESTHEIMER, *Search* **1,** 34 (1970).
[22] G. A. HAMILTON and F. H. WESTHEIMER, *J. Am. Chem. Soc.* **81,** 6332 (1959).

^{18}O isotopic enrichment of carbonyl-^{18}O acetoacetic acid is lost from the final decarboxylation product. In the mechanism for acetoacetate decarboxylation the β-keto group is attacked by the ϵ-amino group of a lysine residue to form an imine bond between substrate and enzyme. After decarboxylation the imine bond is hydrolyzed to regenerate the carbonyl group. For substituted phenylureas, there is apparently no binding of the carbonyl with the enzyme to form an imine bond since all the isotopic oxygen is retained after demethylation of the substrate.

EXPERIMENTAL

Inhibition studies. The plant material, preparation of the microsomal fractions, and the handling of the enzyme assays were carried out as previously described.[2] For the inhibition studies, the reaction mixture contained 1·4–1·7 mg of protein, 30 nmol of ^{14}C-trifluoromethyl labeled 3-(3-trifluoromethylphenyl)-1,1-dimethylurea, 50 μmol K_3PO_4, pH 7·5, 0·5 μmol NaCN, 1 μmol NADPH and 5–10 nmol of inhibitor. The enzyme and inhibitor were incubated at 0° for 15 min and the reaction was started by addition of substrate and NADPH. The reaction was allowed to proceed for 30 min at 25°.

Synthesis of isotopically labeled substrates. The synthesis of 3-(4-chlorophenyl)-1-methylurea methyl-d_3 was carried out using the method described for preparation of methyl-^{14}C substituted phenylurea compounds.[23] Methyl-d_3-amine hydrochloride (98% minimum isotopic purity) was purchased from Diaprep Incorporated, Atlanta, Georgia. The reaction mixture contained 2 g of $CD_3NH_2.HCl$ (28·4 mM) and 4·4 g (28·4 mM) of 4-chlorophenylisocyanate. The product was recrystallized from benzene–acetone, and the deuterium content was verified by NMR and MS. Synthesis of ^{18}O labeled 4-chlorobenzoic acid was achieved by carbonation of a Grignard reagent according to the method of Van Bruggen *et al.*[24] $BaCO_3{}^{18}O$ was purchased from the Miles Laboratories, Kankakee, Illinois. The 4-chlorophenylmagnesium bromide was prepared from 4-bromochlorobenzene by standard methods. The method described for the preparation of carbonyl-^{14}C substituted phenylureas[23] was used for the synthesis of carbonyl-^{18}O labeled 3-(4-chlorophenyl)-1,1-dimethylurea. Starting with 100 mg of $BaCO_3$–^{18}O, the overall yield for the synthesis was 23·7%. The product was purified by preparative TLC. A considerable quantity of ^{18}O was lost through isotopic exchange during the multiple step synthesis. The initial isotopic abundance of ^{18}O in the $BaCO_3$ was 93·1 atom %, and that of the final product was 23·2 atom % as estimated by MS.

Deuterium isotope effect. Experimental conditions were identical to those used for the inhibition studies, except the incubation period was eliminated. The reaction was followed by the addition of 3-(4-chlorophenyl)-1-methylurea carbonyl-^{14}C (specific activity of $1{\cdot}12 \times 10^6$ dpm/16 μg). To 16 μg of radioactive material was added 144 μg of 3-(4-chlorophenyl)-1-methylurea methyl-d_3 as carrier. After the addition of the radioactive material, the final isotopic content at the methyl position of the substrate was 11·9% hydrogen isotope and 88·1% deuterium label. The *N*-demethylase reaction product, 4-chlorophenylurea, was isolated by TLC and estimated by liquid scintillation counting. The 4-chlorophenylurea yield for the control reaction without deuterium isotope was 5·23 nmol, and the yield of product with deuterium present was 4·60 nmol. The deuterium isotope effect was calculated as follows: $k_H/k_D = 5{\cdot}23/4{\cdot}60 \times 0{\cdot}881 = 1{\cdot}3$.

Oxygen exchange study. Experimental conditions were identical to those utilized in the deuterium isotope study. Substrate for this study was ^{18}O-carbonyl labeled 3-(4-chlorophenyl)-1,1-dimethylurea. The dealkylated product, 3-(4-chlorophenyl)-1-methylurea, was isolated from several microsomal reactions by TLC, and the combined sample was examined for ^{18}O content by MS. The ^{18}O isotopic abundance in the dealkylated metabolite was approx. 23 atom %.

[23] F. S. Tanaka, *J. Agric. Food Chem.* **18**, 213 (1970).
[24] J. T. Van Bruggen, C. K. Claycomb and T. T. Hutchens, *Nucleonics* **7**, 45 (1950).