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NEW METABOLITES OF MONURON IN EXCISED COTTON LEAVES*

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Abstract—Two new metabolites of 3-(4-chlorophenyl)-1-dimethylurea (monuron) have been isolated from cotton leaves. They have been identified as β-D-glucosides of 3-(4-chlorophenyl)-1-hydroxymethyl-1-methylurea and 3-(4-chlorophenyl)-1-hydroxymethylurea. After 24 hr, both *O*-glucosides constitute 20–25% of the methanol soluble monuron metabolites present in treated leaf tissues. The isolation and identification of these polar monuron metabolites provides direct evidence for the formation of *N*-hydroxymethyl intermediates in the oxidative *N*-demethylation of substituted phenylurea herbicides by higher plants. The significance of reactive *N*-hydroxymethyl intermediates, particularly 3-(4-chlorophenyl)-1-hydroxymethylurea, in the formation of β-D-glucosides and other polar, unknown methanol soluble and insoluble monuron residues in higher plants is discussed.

INTRODUCTION

THE OXIDATIVE *N*-demethylation of a wide range of compounds with pesticidal activity has been demonstrated in higher plants.[1–4] In many cases, stable *N*-hydroxymethyl intermediates and their *O*-glycoside conjugates have been isolated and identified as metabolities.[1–4] Phenylurea herbicides are also *N*-demethylated in plants,[5] but direct evidence for the formation of the corresponding *N*-hydroxymethyl intermediates and *O*-glycoside conjugates has not been reported.[3,5]

Indirect evidence for the enzymatic formation of an unstable *N*-hydroxymethyl intermediate during the oxidative *N*-demethylation of 3-(4-chlorophenyl)-1-methylurea has been provided by the authors.[6,7] Recent *in vivo* studies by Geissbuhler and Voss[8] have also suggested that *N*-hydroxymethyl compounds are intermediates in the metabolism of phenylurea herbicides by higher plants. These studies, together with an earlier report by Swanson and Swanson[9] on the rapid formation of unknown methanol soluble phenylurea metabolites in plantain and cotton leaf discs, suggested that phenylurea herbicides were also *N*-demethylated via *N*-hydroxymethyl intermediates which were conjugated as *O*-glycosides.

* Part II in the series "*N*-Demethylation of Substituted 3-(phenyl)-1-methylureas". For Part I see *Phytochem.* **8,** 2157 (1969).

[1] J. E. CASIDA and L. LYKKEN, *Ann. Rev. Plant Physiol.* **20,** 607 (1969).
[2] C. M. MENZIE, *Metabolism of Pesticides*, Special Scientific Report—Wildlife No. 127, 487 pp., Bureau of Fisheries and Wildlife, Washington, D.C. (1969).
[3] G. W. LUCIER and R. E. MENZER, *J. Agric. Food Chem.* **18,** 698 (1970).
[4] D. P. SCHULTZ and B. G. TWEEDY, *J. Agric. Food Chem.* **19,** 36 (1971).
[5] H. GEISSBUHLER, in *Degradation of Herbicides* (edited by P. C. KEARNEY and D. D. KAUFMAN), pp. 79–112, New York (1969).
[6] D. S. FREAR, H. R. SWANSON and F. S. TANAKA, *Beltwide Cotton Production Research Conferences, Abstr.*, Houston, Texas (1970).
[7] D. S. FREAR, H. R. SWANSON and F. S. TANAKA, in *Symposia of the Phytochemical Soc. of N. Amer.* (edited by V. C. RUNECKLES), Academic Press, New York (1971).
[8] H. GEISSBUHLER and G. VOSS, *Pure and Applied Chem.* (Suppl. Vol.) (In press) (1971).
[9] C. R. SWANSON and H. R. SWANSON, *Weed Sci.* **16,** 137 (1968).

In the present report, the isolation and characterization of phenylurea *N*-hydroxymethyl-glucosides are described. The significance of these polar conjugates, and their reactive *N*-hydroxymethyl precursors, in the metabolism of phenylurea herbicides by higher plants is discussed. A modified scheme for phenylurea herbicide metabolism in higher plants is also presented.

RESULTS AND DISCUSSION

Preliminary experiments with specifically labeled 3-(3-trifluoromethylphenyl)-1,1-dimethylurea (fluometuron), 3-(4-chlorophenyl)-1,1-dimethylurea (monuron) and monomethylmonuron were designed to test the hypothesis that the unknown polar metabolites observed by Swanson and Swanson[9] were *O*-glycoside conjugates of *N*-hydroxymethyl intermediates in the oxidative *N*-demethylation of phenylurea herbicides.

Thin layer chromatograms of methanol extracts from cotton and plantain leaf discs incubated for 4–24 hr with either trifluoromethyl-^{14}C-labeled fluometuron or phenyl-^{14}C-labeled monuron showed a rapid accumulation of polar metabolites at the origin in solvent

TABLE 1. TLC OF 3-(4-CHLOROPHENYL)-1,1-DIMETHYLUREA AND ITS METABOLITES

Compound	Solvent system* 1	2	3
Metabolites I and II	0·0	0·38	0·43
3-(4-Chlorophenyl)-1-hydroxymethylurea	0·19	0·78	0·81
4-Chlorophenylurea	0·23	0·80	0·81
3-(4-Chlorophenyl)-1-methylurea	0·47	0·83	0·89
3-(4-Chlorophenyl)-1,1-dimethylurea	0·60	0·85	0·84

* Solvent system 1 = benzene–acetone (2:1 v/v); Solvent system 2 = benzene–95% EtOH (2:1 v/v); Solvent system 3 = $CHCl_3$–MeOH–H_2O (65:25:4 v/v).

system 1 (Table 1). Rechromatography of these chromatograms in solvent system 2 (Table 1) resulted in the movement of a major portion of the polar metabolites at the origin as a single spot with an R_f value of approximately 0·50 and movement of the less polar metabolites as a group near the solvent front. Subsequent elution of the polar metabolites at R_f 0·50 with methanol and rechromatography in solvent system 1 showed that they again remained as a compact spot at the origin. Acid hydrolysis of these partially purified polar metabolites with dilute methanolic HCl (0·01 N) and chromatography in solvent system 1 indicated complete hydrolysis to approximately equal amounts of the monomethyl and the desmethyl metabolites of either fluometuron or monuron. Similar studies with carbonyl- and methyl-^{14}C-labeled monomethylmonuron resulted in the formation of apparently the same metabolites. However, acid hydrolysis of the carbonyl-^{14}C-labeled monomethylmonuron metabolites resulted in the apparent formation of only 4-chlorophenylurea. Acid hydrolysis of the methyl-^{14}C-labeled monomethylmonuron metabolites resulted in a complete loss of ^{14}C from the chromatogram.

Hydrolysis of the polar metabolites, separated by solvent system 2 (R_f 0·38) with β-glucosidase, and subsequent chromatography of the methanol soluble reaction products in solvent system 1, demonstrated the presence of a new hydrolysis product in addition to the

monomethyl and desmethyl compounds. This hydrolysis product chromatographed slightly below the corresponding desmethyl metabolites and was observed with all the specifically labeled phenylurea compounds tested, including methyl-^{14}C-labeled monomethylmonuron. In all cases, except experiments with methyl-^{14}C-labeled monuron or monomethylmonuron, a mild acid hydrolysis or a delay in the development of spotted chromatograms resulted in an apparent conversion of this labile enzyme hydrolysis product to the corresponding desmethyl metabolite. Mild acid hydrolysis or delayed chromatography of the β-glucosidase product from studies with methyl-^{14}C-labeled monomethylmonuron resulted in a complete loss of ^{14}C from the chromatogram. The behavior of this polar β-glucosidase hydrolysis product on TLC, its rapid conversion to the corresponding desmethyl compounds, and the loss of a methyl carbon after either mild acid hydrolysis or delayed chromatography suggested that it was a substituted 3-(phenyl)-1-hydroxymethylurea. Indirect evidence for the formation of such an unstable acid-labile *N*-hydroxymethylurea intermediate, by a cotton microsomal enzyme *N*-demethylase system, has been recently reported.[6,7] The highly unstable

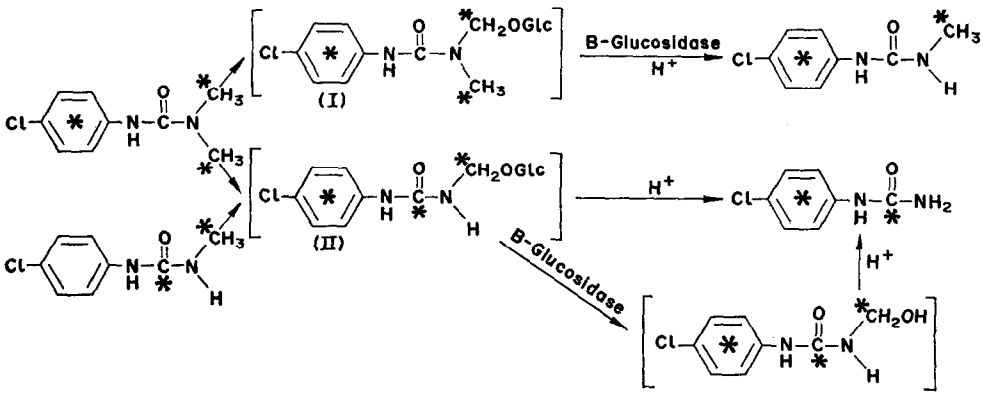

FIG. 1. CHARACTERIZATION OF METHANOL SOLUBLE METABOLITES (I AND II) WITH SPECIFICALLY LABELED 3-(4-CHLOROPHENYL)-1,1-DIMETHYLUREA AND 3-(4-CHLOROPHENYL)-1-METHYLUREA.

nature of the corresponding substituted 3-(phenyl)-1-hydroxymethyl-1-methylurea intermediates apparently precluded their detection or isolation.

A summary of preliminary experiments with specifically labeled monuron-^{14}C and monomethylmonuron-^{14}C are shown in Fig. 1. Tentative structures for two possible *N*-hydroxymethyl glycoside metabolites (I and II) and a relatively stable 3-(4-chlorophenyl)-1-hydroxymethylurea intermediate are indicated by brackets.

Extraction and Isolation of Metabolites

Procedures used for the extraction and isolation of metabolites I and II are described under Experimental. All chromatographic steps resulted in better than an 80% recovery of metabolites I and II. After the extraction and separation from the other ^{14}C-labeled metabolites and most of the contaminating plant materials, the metabolites were separated from each other by chromatography on Sephadex G-10. Metabolite II, metabolite I, and reference glucose were eluted with 6·2, 3·8 and 1·4 void volumes of distilled water, respectively. The effective separation of metabolites I and II was not expected on the basis of the very small differences in molecular weights. Therefore, it appeared that the substitution of a proton for

the methyl group in metabolite II resulted in a significant enchancement in the adsorption of this metabolite on Sephadex G-10. After a final TLC purification step, both metabolites chromatographed and behaved as single compounds. In some cases, both metabolites were also purified by decending paper chromatography on methanol washed Whatman 3M paper with a *n*-BuOH-EtOH-H_2O (40:11:19, v/v) solvent system. After development in this solvent system for 12 hr, both metabolites were located at an R_f value of 0·65–0·75.

Stability of Isolated Metabolites

Both metabolites (I and II) were quite stable at neutral pH in aqueous systems and could be stored for several months at −12°. Under anhydrous conditions, however, purified metabolite II was unstable in the presence of either methanol or ethanol.

Identification of Aglycone Moieties

The aglycone portion of methyl-^{14}C-labeled metabolite I was characterized by the separation and identification of the reaction products formed after acid hydrolysis. Purified metabolite I (15·1 mμmol, 114 000 dpm) was isolated from cotton leaves treated with ^{14}C methyl labeled monuron. After acid hydrolysis, any formaldehyde released was converted to the formaldomethone derivative,[10] and the reaction mixture was extracted with ether. TLC of the ether phase (solvent system 1) resulted in the separation of two ^{14}C labeled spots. One spot cochromatographed with monomethylmonuron and the other spot cochromatographed with formaldomethone. Quantitative analysis of the ^{14}C in these two spots resulted in a 98% recovery of ^{14}C as monomethylmonuron (7·4 mμmol) and the formaldomethone derivative (7·5 mμmol). These studies demonstrated that the aglycone moiety of metabolite I contained formaldehyde and monomethylmonuron in a molar ratio of 1:1. The monomethylmonuron moiety derived from the hydrolysis of metabolite I was identified by mass spectroscopy. The identification of a molecular ion at *m/e* 184 and ions resulting from the loss of methylamine (*m/e* 153) and methylisocyanate (*m/e* 127) as well as the characteristic distribution of chlorine isotopes all clearly demonstrated that monomethylmonuron was one of the two acid hydrolysis products.[10,11] Formaldomethone was identified as the derivative of the other hydrolysis product (formaldehyde) by isotope dilution and recrystallization to constant specific activity.

The aglycone portion of either methyl- or carbonyl-^{14}C-labeled metabolite II was also characterized by the separation and identification of acid hydrolysis products. Purified methyl-^{14}C-labeled metabolite II (1·41 mμmol, 6900 dpm) was hydrolyzed and any formaldehyde released was converted to the formaldomethone derivative.[10] Extraction of the reaction mixture with ether and TLC of the ether phase resulted in the isolation of a ^{14}C labeled product which cochromatographed with formaldomethone. Recoveries of over 80% of the theoretical methyl-^{14}C as formaldomethone were obtained. Further identification of formaldomethone was obtained by isotope dilution and recrystallization to constant specific activity. Acid hydrolysis of purified carbonyl-^{14}C-labeled metabolite II resulted in the recovery of over 90% of the ^{14}C as a compound which cochromatographed with 4-chlorophenylurea. Identification of this acid hydrolysis product was obtained by isotope dilution and recrystallization to constant specific activity. Additional evidence for the identification of 4-chlorophenylurea as an acid hydrolysis product of metabolite II was obtained by mass spectroscopy. The identification of a molecular ion at *m/e* 170 and a 4-chloroaniline

[10] D. S. FREAR, H. R. SWANSON and F. S. TANAKA, *Phytochem.* **8**, 2157 (1969).
[11] W. R. BENSON and J. N. DAMICO, *J. Ass. Offic. Anal. Chem.* **51**, 347 (1958).

ion at *m/e* 127 as well as the characteristic chlorine isotope distribution associated with these ions showed that the ^{14}C carbonyl portion of the aglycone moiety of metabolite II was associated with 4-chlorophenylurea. These studies demonstrated that the acid hydrolysis of metabolite II resulted in the formation of equal molar quantities of formaldehyde and 4-chlorophenylurea.

Identification of Carbohydrate Moieties

The carbohydrate moieties of purified metabolites I and II were qualitatively identified after acid hydrolysis. Thin layer chromatography of acid hydrolysates showed the presence of only one reducing sugar. This sugar cochromatographed with glucose. After acid hydrolysis and silylation, GLC showed the presence of only two peaks which cochromatographed with α- and β-D glucose. Quantitative determination of the carbohydrate moieties

TABLE 2. ENZYMATIC HYDROLYSIS OF METABOLITES I AND II

	Separated reaction products (dpm)			
	Metabolite I		Metabolite II	
Treatment	Glycoside	3-(4-Chloro-phenyl)-1-methylurea	Glycoside	4-Chloro-phenylurea
Control	4834	146	5826	224
β-D-Glucosidase	38	5240	170	6964

Aliquots (20 μl) of purified ring-^{14}C labeled metabolite I and carbonyl-^{14}C labeled metabolite II were incubated for 90 min at 30° with 100 μl of 0·01 M NaOAc buffer pH 5·25 containing 1 mg/ml β-D-glucosidase and 0·05 M KNO_3. Controls were incubated in buffer alone. Reactions were terminated by freezing. Frozen reaction mixtures were lyophilized and taken up in 50 μl of MeOH. Aliquots of the MeOH soluble reaction mixtures weres potted for TLC separation with solvent system 2 [benzene–95% EtOH (2:1, v/v)] and quantitated by liquid scintillation counting. Recoveries of ^{14}C ranged from 80 to 90%.

with glucose oxidase, which is specific for β-D-glucose, showed an average of 88% (3 determinations, 82–96%) and 82% (3 determinations, 77–86%), respectively, of the theoretical amount of glucose expected from the acid hydrolysis of purified phenyl-^{14}C-labeled metabolite I and carbonyl-^{14}C-labeled metabolite II glucosides. In addition to the specific enzyme assay, glucose was also determined with a sensitive, but relatively nonspecific colorimetric assay for reducing sugars.[12,13] This assay showed an average recovery of 123 and 129%, respectively, of the theoretical amount of glucose expected from acid hydrolysates of metabolites I and II if they were glucosides. The enzyme assays for glucose may have been somewhat low because of incomplete glucose oxidation while the colorimetric assays may have been slightly high because of small amounts of reducing or other interfering impurities present as the result of the various chromatographic and acid hydrolyses procedures used during the purification and characterization of the metabolites.

[12] J. T. PARK and M. J. JOHNSON, *J. Biol. Chem.* **181**, 149 (1949).
[13] G. ASHWELL, *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 3, p. 86, Academic Press, New York (1957).

Structures of Metabolites I *and* II

Purified methyl- and phenyl-^{14}C-labeled metabolite I was hydrolyzed in dilute acid to yield monomethylmonuron, formaldehyde and glucose in a 1:1:1 molar ratio as shown in the previous sections. Likewise, studies with either purified methyl- or carbonyl-^{14}C-labeled metabolite II have shown that acid hydrolysis of this metabolite yielded only 4-chlorophenylurea, formaldehyde and glucose in a 1:1:1 molar ratio.

The data cited above, and the hydrolysis of purified metabolites I and II by β-D-glucosidase as shown in Table 2 demonstrate that metabolite I and metabolite II are β-D-glucosides of 3-(4-chlorophenyl)-1-methyl-1-hydroxymethylurea and 3-(4-chlorophenyl)-1-hydroxymethylurea intermediates in the oxidative *N*-demethylation of monuron as indicated in Fig. 2.

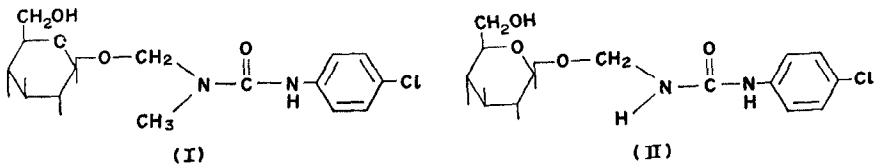

FIG. 2. STRUCTURES OF METABOLITES I AND II.

Time Studies

Time course studies were conducted to establish precursor-product relationships between the various methanol soluble monuron metabolites and to determine the significance and extend of *N*-hydroxymethyl glucoside formation. The results of a phenyl-^{14}C-labeled monuron study with excised cotton leaves and monuron are shown in Fig. 3. In this study, a rapid depletion of monuron was followed by a rapid, but transitory, accumulation of monomethylmonuron and a subsequent, almost simultaneous, formation of 3-(4-chlorophenyl)-1-hydroxymethylurea and 4-chlorophenylurea, and metabolites I and II. A slower formation of small quantities of an unknown, highly polar, methanol soluble metabolite which remained at the origin in solvent system 2 was also noted. Metabolites I and II reached a maximum of 27% of the methanol soluble metabolites after 24 hr. However, the fraction containing 4-chlorophenylurea and 3-(chlorophenyl)-1-hydroxymethylurea continued to increase during the entire 48 hr treatment. An examination of total ^{14}C recoveries in the methanol soluble and insoluble fractions indicated that the decrease in the metabolite I and II fractions after 24 hr was associated with a corresponding increase in the 3-(4-chlorophenyl)-1-hydroxymethylurea and 4-chlorophenylurea fraction. Development of chromatograms immediately after spotting and observation of autoradiograms from developed chromatograms showed that most of the ^{14}C in the R_f 0·10–0·35 zone (solvent system 1) was apparently associated with 3-(4-chlorophenyl)-1-hydroxymethylurea rather than with 4-chlorophenylurea. The decrease in the formation of metabolites I and II and in the apparent leveling off in the formation of the more polar unknown metabolites, and the continued accumulation of primarily 3-(4-chlorophenyl)-1-hydroxymethylurea may indicate a slow hydrolysis of metabolite II and a significant decrease in the ability of excised tissues to continue the biosynthesis of *N*-hydroxymethyl glucosides and other polar metabolites after 24 hr. Combustion analysis of the methanol insoluble residues resulting from

these time studies showed that approximately 20% of the ^{14}C taken up by the petiole remained in the plant tissues after 48 hr and that the accumulation of this ^{14}C labeled fraction was also slow and leveled off after 24 hr.

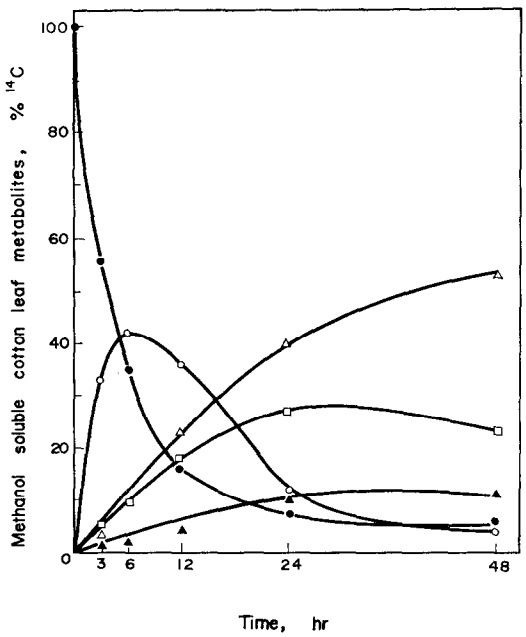

FIG. 3. TIME-COURSE STUDY OF METHANOL SOLUBLE METABOLITES FROM EXCISED COTTON LEAVES. Fully expanded greenhouse grown cotton leaves (approximately the same age and leaf surface areas) were excised with petioles. Each leaf was incubated with 1·0 ml of ring-^{14}C-labeled 3-(4-chlorophenyl)-1,1-dimethylurea ($2{\cdot}61 \times 10^{-4}$ M, 580 000 dpm). Incubations were terminated by freezing and lyophilization. Lyophilized tissues were extracted with MeOH and soluble metabolites were quantitatively determined after separation by TLC. Separated metabolites included: 3-(4-chlorophenyl)-1,1-dimethylurea (●); 3-(4-chlorophenyl)-1-methylurea (○); 3-(4-chlorophenyl)-1-hydroxymethylurea and 4-chlorophenylurea (△); metabolites I and II (□); and unknowns (▲).

Significance of N-*hydroxymethyl Intermediates in the Metabolism of Monuron*

Several reports on the metabolism of substituted phenylurea herbicides in higher plants have indicated that these herbicides are degraded to their corresponding substituted anilines.[14–16] However, recent short term *in vivo* and *in vitro* studies in this laboratory have failed to demonstrate any significant formation of substituted anilines in phenylurea herbicide metabolism. Similar findings have also been noted by Geissbuhler[5] and it appears likely that previous reports of trace quantities of substituted anilines, and, in one case, a substituted nitrobenzene,[14] may have been due to impurities in the phenylurea herbicide samples studied or to possible degradation of the herbicide (photochemical or microbial) before absorption by plant tissues.

[14] J. H. ONLEY, G. YIP and M. H. ALDRIDGE, *J. Agric. Food Chem.* **16,** 426 (1968).
[15] J. W. SMITH and T. J. SHEETS, *J. Agric. Food Chem.* **15,** 577 (1967).
[16] R. L. ROGERS and H. H. FUNDERBURK, *J. Agric. Food Chem.* **16,** 434 (1968).

Repeated short term studies (<48 hr) have shown that little 4-chlorophenylurea is present in methanol extracts of monuron or monomethylmonuron treated cotton leaves if these extracts are immediately spotted and chromatographed. Incubation of excised cotton leaves with carbonyl-^{14}C-labeled 4-chlorophenylurea for 48 hr also failed to show any significant degradation to other metabolites. Recent *in vitro* studies with a microsomal *N*-demethylase system from cotton[7] have shown that 4-chlorophenylurea is a very potent inhibitor of monuron detoxication via *N*-demethylation and have suggested that any appreciable accumulation of 4-chlorophenylurea in the plant may result in a significant enhancement of substituted phenylurea herbicide phytotoxicity. The instability of *N*-hydroxymethyl intermediates also indicates that some of the substituted phenylureas previously reported as degradation products in the plant may have been artifacts which resulted from the procedures generally used for the extraction and separation of metabolites. Therefore, it is suggested that 3-(4-chlorophenyl)-1-hydroxymethylurea may be a key intermediate in the metabolism of monuron by cotton leaves and that this highly reactive intermediate is rapidly conjugated with glucose or reacts with other endogenous compounds to prevent the formation of any appreciable concentrations of free 4-chlorophenylurea.

The isolation and characterization of the unknown methanol soluble metabolites which remained at the origin of thin layer chromatograms developed in solvent system 2 has not been successful. This fraction accounts for 11 % of the methanol soluble phenyl-^{14}C-labeled monuron metabolites after 24 hr (Fig. 3). Chromatography of this unknown methanol soluble fraction on DEAE-cellulose and Sephadex G-10 has resulted in the separation of several minor unidentified fractions, but the majority of the ^{14}C activity associated with this fraction either remained tightly associated with the chromatographic support materials or was eluted in a very broad, tailing band. Hydrolysis of this polar phenyl-^{14}C-labeled metabolite fraction with 1·0 N HCl for 16 hr resulted in almost a complete recovery of ^{14}C as 4-chlorophenylurea and a small amount of monomethylmonuron. When excised cotton leaves were incubated for 24 hr with methyl- and carbonyl-^{14}C-labeled monomethylmonuron, 13–19 % of the ^{14}C methanol soluble metabolites remained at the origin in solvent system 2. Hydrolysis of the carbonyl-^{14}C-labeled metabolites for 24 hr with 0·5 N methanolic HCl and chromatography in solvent system 2 indicated that 90 % of the ^{14}C in these metabolites was released as carbonyl-^{14}C-labeled 4-chlorophenylurea. These studies, together with the indirect evidence provided in the time studies (Fig. 3) suggest that either 3-(4-chlorophenyl)-1-hydroxymethylurea or its β-D-glucoside (metabolite II) may be the principal precursors of the unknown methanol soluble metabolites. When excised cotton leaves were incubated for 24 hr with pruified carbonyl-^{14}C-labeled metabolite II, less than 2 % of the absorbed ^{14}C was associated with either the unknown methanol soluble metabolites or with the methanol insoluble residues. In these studies, over 86 % of the absorbed ^{14}C was recovered in the methanol soluble fraction. The only compounds detected in this fraction were unreacted metabolite II and a small amount of degradation products, 4-chlorophenylurea and/or 3-(4-chlorophenyl)-1-hydroxymethylurea. These results indicated that the subsequent metabolism of metabolite II was very limited in excised cotton leaves. This may be due to poor penetration of metabolite II into leaf cells. However, these studies suggest that the precursor to both unknown methanol soluble and insoluble metabolites may be the highly reactive 3-(4-chlorophenyl)-1-hydroxymethylurea intermediate and not its β-D-glucoside (metabolite II).

Attempts were made to characterize the nature of the methanol insoluble residues in excised leaves. Excised cotton leaves were incubated for 24 hr with 5×10^{-4} M (1·0

$\mu c/\mu mol$) methyl-^{14}C-labeled monuron and monomethylmonuron, phenyl-^{14}C-labeled monuron and carbonyl-^{14}C-labeled monomethylmonuron. In each case, approximately 20% of the ^{14}C was found in the methanol insoluble residues after a 24-hr incubation. Hydrolysis of the insoluble residues from these various treatments with 1·0 N HCl for 16 hr and extraction of the hydrolysates with methanol resulted in a recovery of 80–90% of the ^{14}C from the carbonyl- and phenyl-labeled residues, but only 15–23% of the ^{14}C from the methyl-labeled residues. The major portion of the ^{14}C recovered from the carbonyl- and phenyl-^{14}C-labeled residues was 4-chlorophenylurea. Only unknown, polar compounds were released by acid hydrolysis of methyl-^{14}C-labeled residues. The results of these studies demonstrated that almost all of the ^{14}C in the carbonyl- and phenyl-labeled residues was recovered as 4-chlorophenylurea, while most of the ^{14}C in the methyl-labeled residues remained insoluble. The recovery data presented above suggest that the methyl groups associated with the insoluble residues may be covalently linked through the reactive *N*-hydroxymethyl group in a manner similar to the *O*-glucosides. However, all attempts to demonstrate the release of any significant amounts of ^{14}C labeled formaldehyde were negative. Digestion of carbonyl-^{14}C-labeled methanol insoluble residues with trypsin, ficin, pepsin, protease and cellulase also failed to release any significant amounts of ^{14}C labeled methanol or water soluble products. Hydrolysis of methyl-^{14}C-labeled insoluble residue from monomethylmonuron treatments with 6 N HCl in a sealed tube under N_2 at 110° for 64 hr also failed to release any significant quantities of methanol soluble ^{14}C. The failure of enzyme digestions to release carbonyl-^{14}C-labeled products and strong acid hydrolysis to release methyl-^{14}C-labeled products suggests that the methanol insoluble

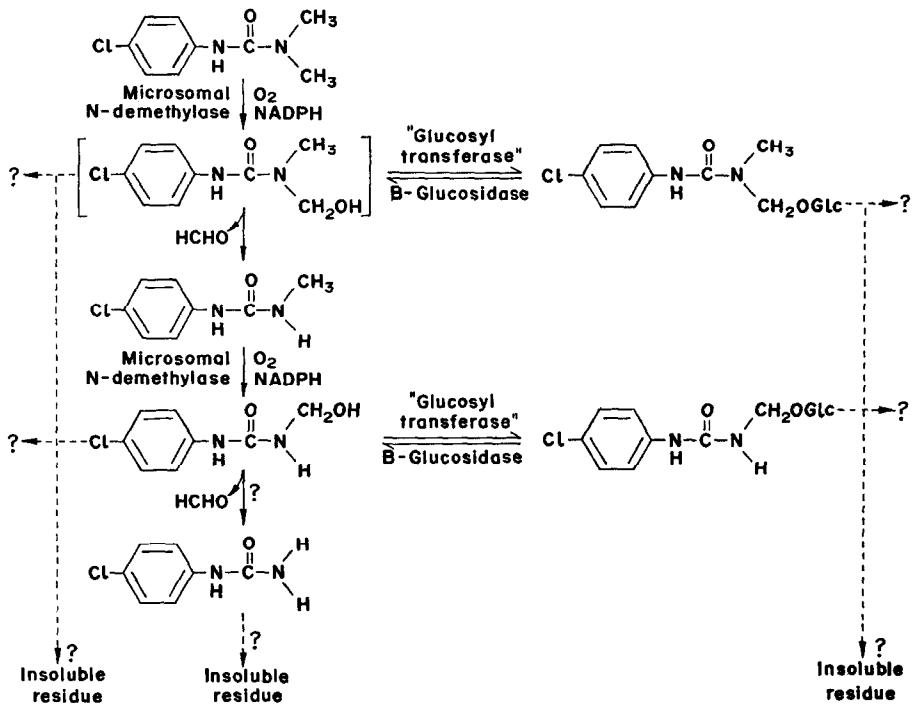

FIG. 4. METABOLISM OF 3-(4-CHLOROPHENYL)-1,1-DIMETHYLUREA IN EXCISED COTTON LEAVES.

residues may be associated with the lignin fraction of the plant. A recent study by Neptune[17] has provided evidence that insoluble fluometuron residues may also be associated with the lignin fraction.

A proposed scheme for the metabolism of monuron is shown in Fig. 4. This scheme was developed primarily on the basis of previous *in vitro* studies[6,7,10] and on the results and discussion presented in the present report. It suggests a key role for *N*-hydroxymethyl intermediates, particularly the 3-(4-chlorophenyl)-1-hydroxymethylurea intermediate, in the formation of β-D-glucosides (metabolites I and II) and other polar, unknown methanol soluble metabolites and insoluble residues of monuron in higher plants.

EXPERIMENTAL

Radiocarbon labeled compounds. Carbonyl-^{14}C 3-(4-chlorophenyl)-1-methylurea (5·85 μCi/μmol) was synthesized by F. S. Tanaka.[10] Ring (1·0 μCi/μmol) and methyl (3·4 μCi/μmol)-^{14}C 3-(4-chlorophenyl)-1,1-dimethylurea were also synthesized by F. S. Tanaka.[18] Methyl-^{14}C 3-(4-chlorophenyl)-1-methylurea (2·2 μCi/μmol) was synthesized in 74% yield with 250 μCi of methylamine-^{14}C-hydrochloride (6·55 μCi/μmol) as described previously.[18] Carbonyl-^{14}C 4-chlorophenylurea (5·85 μCi/μmol) was isolated and purified from cotton microsomal preparations[10] treated with carbonyl-^{14}C 3-(4-chlorophenyl)-1-methylurea. Trifluoromethyl-^{14}C 3-(3-trifluoromethylphenyl)-1,1-dimethylurea (2·39 μCi/μmol) was provided by CIBA Corporation, Vero Beach, Florida. D-Glucose-^{14}C (U) (8·0 μCi/μmol) was purchased from Schwarz-Mann, Orangeburg, N.Y.

Instrumentation. MS were obtained at 70 eV by the direct insertion of solid samples into a Varian M-66 spectrometer with the ion source at 180°. Quantitative measurements of ^{14}C were made with a Packard 3375 liquid scintillation spectrometer. Thin layer and paper chromatograms were monitored for ^{14}C with a Packard 7201 radiochromatogram scanner and eluates from the column chromatography were monitored for ^{14}C with a Picker Nuclear Scinti/Flow system. Chromatography of TMS carbohydrate derivatives was carried out on a Microtek MT 220 gas chromatograph. Beckman DU and DB spectrophotometers were used for visual and ultraviolet spectral analyses.

Determination of ^{14}C. MeOH soluble ^{14}C-ureas, their metabolites and hydrolysis products were quantitatively determined by liquid scintillation counting after extraction or chromatographic serparations.[19,20] The ^{14}C remaining in leaf tissue residues after MeOH extraction were quantitatively determined as $^{14}CO_2$ by the oxygen flask combustion method of Oliverio *et al.*[21] HCHO-^{14}C released by acid hydrolysis with 1·0 N HCl at 35° for 2 hr was trapped as the formaldomethone derivative, separated by ether extraction and TLC (solvent system 1), and determined by liquid scintillation counting.[10]

Plant materials and treatment. Fully expanded, greenhouse grown[10] cotton (*Gossypium hirsutum* L.) leaves, including petioles, were excised under distilled water. Each petiole was placed in a 15 × 75 mm test tube containing 1·0 ml of aqueous ^{14}C-labeled substrate. In most preparative experiments, 1–2 × 10^6 dpm of the appropriate ^{14}C labeled phenylurea compound was supplied to each of several leaves at concentrations approaching 5 × 10^{-4} M. The rapid uptake of the ^{14}C labeled treating solution was followed by an additional 1·0 ml of distilled water. After the uptake of the distilled water, the test tubes were filled with distilled water and maintained at 70°F and 60% R.H. with a 12 hr photoperiod until harvest. More than 90% of the ^{14}C label was absorbed by the excised leaves under these conditions. Metabolism was terminated either by freezing and lyophilization or by direct extraction with methanol.

Isolation and purification of metabolites I *and* II. Excised cotton leaves treated with ^{14}C-labeled substrates were frozen with dry ice, lyophilized and homogenized in an Omnimixer with 5 parts of MeOH (v/fr. wt). The homogenate was filtered on a 10-μ Teflon filter and washed with MeOH until negligible ^{14}C was detected in the filtrate. The combined filtrates were concentrated to a small volume on a rotary evaporator at room temp. Metabolites I and II were separated from other ^{14}C-labeled metabolites in the methanol extract by preparative TLC on 500 μ silica gel HF plates with benzene–95% EtOH (2:1). Metabolites I and II (R_f 0·38) were scraped off the TLC plates, eluted with MeOH, taken almost to dryness on a rotary evaporator and dissolved in a minimum volume of distilled water (1–5 ml). The concentrated solution of metabolites was then placed on a 2·5 × 25 cm column of DEAE-cellulose which had been previously equilibrated with 2 M Tris-HCl buffer pH 8·0 and exhaustively washed with distilled water. Metabolites I and II were eluted from the DEAE column as one unresolved peak in less than two void volumes with distilled water at a flow

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rate of 1 ml/min. Peak fractions were pooled, lyophilized, taken up in a minimum volume of distilled water (1–3 ml) and placed on a 1·5 × 90 cm column of Sephadex G-10. Metabolites I and II were separated on the G-10 column by elution with distilled water at a flow rate of 1 ml/min. The eluted fractions for each peak were pooled, lyophilized and taken up in a small volume of MeOH, Me_2CO or distilled water. Final chromatographic purification was achieved by TLC on 250 μ HF plates with $CHCl_3$–MeOH–H_2O (65:25:4). The purified metabolites were scraped from the thin-layer chromatograms, eluted from the silica gel HF with MeOH or Me_2CO, and stored at −12° in MeOH, Me_2CO or distilled water.

Hydrolysis of Metabolites I *and* II. Metabolites I and II were acid hydrolyzed at 22–40° for 12 hr with 0·01–0·1 N HCl. Both metabolites were also hydrolyzed with β-D-glucosidase (1 mg/ml) in 0·1–0·2 ml of 0·01 M NaOAc buffer containing 0·05 M KNO_3 (pH 5·25) for 90 min at 30°.

Glucose determinations. A modified TLC procedure of Gal[22] was used for the qualitative determination of glucose. Samples of purified metabolites I and II (10–20 μg) were hydrolyzed for 12 hr at room temp. with 10–25 μl. of 0·01 N HCl. The hydrolysates were directly spotted on 250 μ silica gel HF plates, developed three times with an ascending solvent system [MeOAc–*iso*PrOH–H_2O (18:1:1)] and visualized with a *p*-aminobenzoic acid spray reagent.

Glucose was also qualitatively determined by GLC. Purified metabolites I and II (15–20 μg) were hydrolyzed with 100 μl of aq. 0·1 N HCl for 12 hr at room temp. Residual HCl and water were removed with a stream of air and three 20-μl methanol rinses. The air dried hydrolysis products were dissolved in 4–6 μl of Tri-Sil Z, allowed to react for 1 hr at room temp. and injected (1–2 μl aliquots) directly into the gas chromato graph. Chromatographic separation of the TMS derivatives was achieved on a 180 cm × 4 mm glass column packed with 3% OV-1 on 60–80 mesh Gas-Chrom Q. The oven was maintained at 175°, the injector was 225°, the flame ionization detector block was 225° and the N_2 carrier gas flow was 70 ml/min.

Microdeterminations for glucose were made with β-D-glucose: oxidoreductase (E.C. 1.1.3.4). The Glucostat reagent (Worthington Biochemicals Corporation) was made up to 50 ml with a 0·1 M potassium phosphate buffer pH 7·0. Purified metabolites I and II (10–25 μg) were hydrolyzed in capped tubes (15 × 75 mm) at 40° for 12 hr with 0·40 ml of 0·1 N HCl. The hydrolysate was neutralized (pH 7·0) with NaOH and 1·0 ml of Glucostat reagent was added. The enzyme reaction was allowed to go to virtual completion at 25° for 1 hr and terminated with 0·05 ml of 5 N HCl. After 10 min, absorbance was determined at 400 nm. Absorbance was linear from 0–25 μg of glucose with a reagent blank set at zero. Acid hydrolysates of purified metabolites I and II (10–20 μg) were also analyzed for glucose by the ferricyanide method of Park and Johnson[11] as described by Ashwell.[12]

Determination of formaldomethone and 4-chlorophenylurea. Formaldomethone was quantitatively determined at 258 nm in EtOH with a molar extinction coefficient of $2{\cdot}46 \times 10^4$ l. mol^{-1} cm^{-1}. EtOH solutions of 4-chlorophenylurea were quantitatively determined at 244 nm with a molar extinction coefficient of $2{\cdot}25 \times 10^4$ l. mol^{-1} cm^{-1}.

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Key Word Index—Gossypium hirsutum; Malvaceae; herbicide metabolism; monuron; 3-(4-chlorophenyl)-1-dimethylurea; oxidative *N*-demethylation; *N*-hydroxymethyl-β-D-glucoside conjugates.