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ARYL HYDROXYLATION OF ISOPROPYL-3-CHLOROCARBANILATE BY SOYBEAN PLANTS

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Abstract—Isopropyl-3-chlorocarbanilate-phenyl UL-^{14}C (CIPC-^{14}C) is absorbed, translocated and metabolized by soybean plants. Water-soluble metabolites in root and shoot were purified and the root major metabolite characterized. The acetylated aglucones from the β-glucosidase hydrolysis and the esters from the direct acetylation of CIPC-^{14}C polar metabolites were purified by GLC and analysed by mass spectrometry. The data showed that the phenyl ring of CIPC-^{14}C was hydroxylated by both root and shoot tissues. Isopropyl-5-chloro-2-hydroxycarbanilate (hydroxy-CIPC) was the predominant aglucone liberated by β-glucosidase from polar metabolites in root and shoot. The *o*-glucoside of hydroxy-CIPC was shown to be present, by direct acetylation and characterization. In shoot tissue the major metabolites were dechlorinated hydroxy-CIPC and were not hydrolysed by β-glucosidase. These data show that soybean root or shoot tissues hydroxylate the phenyl ring of CIPC-^{14}C but do not alter either the isopropyl alcohol moiety or the *e*arbamate bond.

INTRODUCTION

ISOPROPYL-3-CHLOROCARBANILATE (chlorpropham or CIPC) has long been used as a selective preemergence, or an early postemergence herbicide for the control of many annual grassy and broad leaf species. In spite of its many years of agricultural acceptance and much research effort, little is known about its metabolic fate in plants.

Kaufman[1] and Kearney[2,3] have demonstrated the conversion of CIPC-^{14}C to $^{14}CO_2$ in soil perfusion columns and isolated a microbial enzyme that hydrolysed CIPC to yield 3-chloroaniline. Grunow *et al.*[4] showed that the major metabolites of CIPC in rat urine were produced by *para* hydroxylation of the aromatic nucleus, hydrolysis of the carbamate bond and oxidation of the 2-propyl ester side chain; these metabolites were found in the urine as sulfate and glucuronide conjugates.

The plant metabolism of CIPC has been studied by several investigators.[5–8] James *et al*[6,7] demonstrated the formation of water soluble CIPC metabolites in several plants species. Their polar metabolites were shown to be β-glucosides of a modified CIPC molecule in which there was no cleavage of the carbamate bond. Still and Mansager[8] have shown that root-treated soybean plants absorb and translocate CIPC-^{14}C. Polar products and insoluble residues were rapidly formed in the roots. Insoluble residues were not formed in the shoots. A precursor-product relationship exists between CIPC, polar products and insoluble residues. Polar metabolites were not translocated once they are formed in either the root or shoot tissues. Analysis of the polar metabolites and the insoluble residual materials indi-

[1] D. D. KAUFMAN, *J. Agric. Food Chem.* **15**, 582 (1967).
[2] P. C. KEARNEY, *Advan. Chem. Ser.* **60**, 250 (1966).
[3] P. C. KEARNEY, *J. Agri. Food Chem.* **13**, 561 (1965).
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[5] R. H. HODGSON, Abstr. 1967 Meeting Weed Sci. Soc. Am., Washington D.C., Feb. 1967, p. 65–66.
[6] G. N. PRENDEVILLE, Y. ESHEL, C. S. JONES, G. F. WARREN and M. M. SCHREIBER, *Weed Sci.* **16**, 432 (1968).
[7] C. S. JAMES and G. N. PRENDEVILLE, *J. Agric. Food Chem.* **17**, 1257 (1969).
[8] G. G. STILL and E. R. MANSAGER *J. Agric. Food Chem.* (in press).

cated that neither the 2-propyl-alcohol moiety nor the carbamate bond of CIPC was altered by root-treated soybean plants.[8] This paper describes the isolation and characterization of a major polar metabolite found in root or shoot tissues from root-treated soybean plants.

RESULTS AND DISCUSSION

The CIPC polar metabolites from root and shoot tissues were isolated and purified as described in the Experimental. DE-52 purified polar CIPC-^{14}C metabolites from root or shoot tissue were subjected to β-glucosidase hydrolysis followed by acetylation of the aglucone. When the β-glucosidase-acetylation procedure was used with root tissue extracts, 45 per cent of the acetylated aglucone partitioned into the nonpolar solvent while 30 per cent remained in the aqueous phase. When the same procedure was used with shoot tissue extract, only 25 per cent of the radiocarbon partitioned into the nonpolar phase and 70 per cent remained in the aqueous phase. The nonpolar aglucone acetate was analysed by GLC and a number of discrete components were separated by temperature programming on OV-1 columns. Those components which contained ^{14}C label were introduced into a mass spectrometer for analysis of the aglucone acetate. Figure 1 presents the mass spectra of these compounds.

The mass spectra of isopropyl-5-chloro-2-hydroxycarbanilate is shown with its fragmentation patterns. The synthetic analog was subjected to the same acetylation and the GLC separation procedures used for the root and/or shoot of β-glucosidase aglucones. Analysis of the fragmentation patterns showed that the synthetic standard was acetylated at the 2-hydroxyl group and at the NH group of the carbanilate bond. There was a loss of two fragments of mass 42 to yield isopropyl-5-chloro-2-hydroxycarbanilate (*m/e* 229), and a loss of C_3H_6 (42) to yield *N*-(2-hydroxy-5-chlorophenyl)carbanilic acid (*m/e* 187). The fragmentation of the carbanilic acid may proceed by one of two pathways. First, a loss of CO_2 to yield 2-hydroxy-5-chloroaniline (*m/e* 143) which fragments in a characteristic fashion. The second pathway is unique for the *ortho*-hydroxy carbamates and results in the loss of water to yield the 5-chloro-2-benzoxazolinone (*m/e* 169).[9,10]

The parent ion at *m/e* 313 would be expected if the CIPC-phenyl ring was hydroxylated (isopropyl-chloro-hydroxycarbanilate) and acetylated. Two approaches were used to characterize the structure of the *m/e* 313 or *m/e* 229 ions. First, GLC elution patterns of the different acetylated hydroxy-CIPC analogs showed the two *ortho*-hydroxy analogs could be separated from each other as well as from the *meta* and *para* hydroxy analogs.[10] Second, the *ortho*-hydroxy-CIPC analogs fragmented in the mass spectrometer with 5-chloro-2-benzoxazolinone as an intermediate in the fragmentation sequence.[9] From these data, the specific GLC-retention time and the unique mass spectral fragmentation pattern, it appeared that the structure of the root and shoot aglucone was isopropyl-5-chloro-2-hydroxycarbanilate. To further substantiate this conclusion, the aglucone from β-glucosidase hydrolysis was treated with diazomethane and the methoxy derivatives analysed by GLC and mass spectrometry. From the fragmentation pattern (Fig. 2) it was clear that there was a loss of C_3H_6 to yield *N*-(methoxy-chlorophenyl)carbanilic acid. This product fragments either with the loss of CO_2 to yield methoxy-chloroaniline or with the loss of water followed by the loss of methyl to yield *m/e* 168. The fragmentation pattern of this methoxy derivative confirms the presence of the hydroxy-CIPC metabolite.

[9] G. G. STILL, *Org. Mass Spectr.* (in press).
[10] G. G. STILL and E. R. MANSAGER, *J. Chromatog.* (in press).

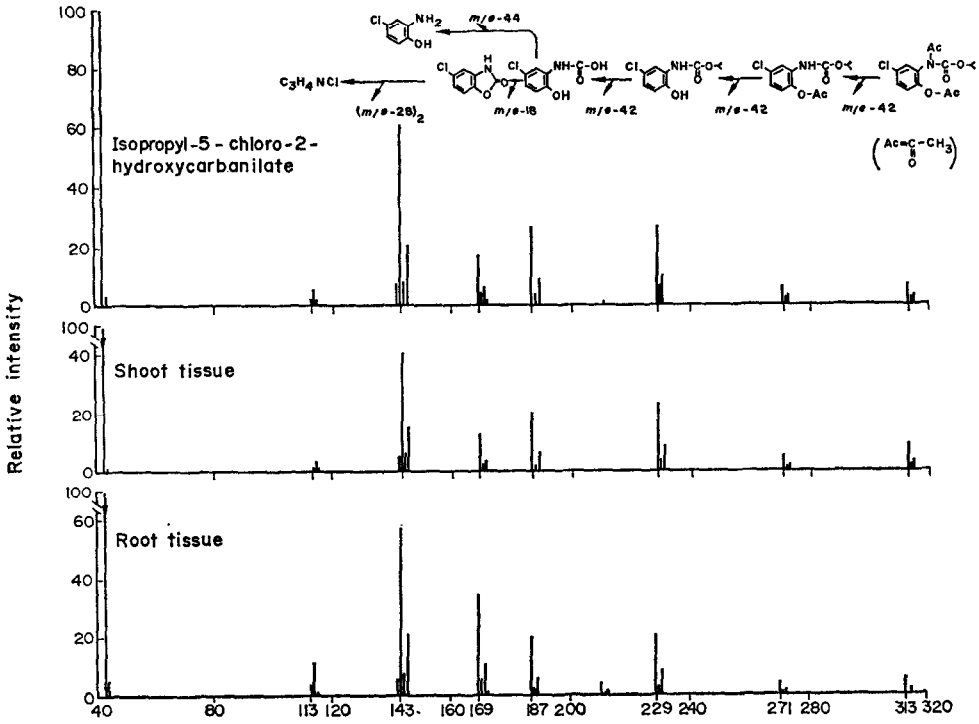

FIG. 1.

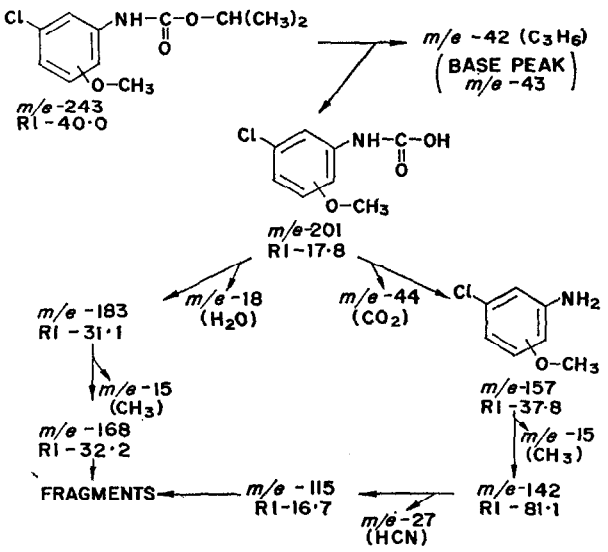

FIG. 2. SOYBEAN SHOOT CIPC-^{14}C WATER SOLUBLE METABOLITE. β-GLUCOSIDASE HYDROLYSIS AND DIAZOMETHANE DERIVATIVE OF THE AGLUCONE FOLLOWED BY GLC–MS ANALYSIS.

Mass spectral analysis of the polar fraction from root and shoot from the β-glucosidase hydrolysis-acetylation reactions (30 and 70 per cent of the total ^{14}C water-soluble CIPC-^{14}C metabolite, respectively) yielded surprising results. The root tissue showed that the same metabolite, hydroxy-CIPC, was present. However, analysis of the polar material from shoot tissue showed metabolites that appeared to be void of chlorine. Further analysis is required to confirm the character of these dechlorinated hydroxylated CIPC metabolites.

A second approach to the analysis of the CIPC-^{14}C polar metabolites was to use a direct acetylation of samples from the DE-52 acetate columns. In this case both the root and shoot tissues yielded over 70 per cent of their total radiocarbon as acetylated derivatives. Figure 3 reports the results of GLC–MS analysis of the soybean root, CIPC-^{14}C water-soluble metabolite after direct acetylation. No parent ion peak was observed, which is in agreement with

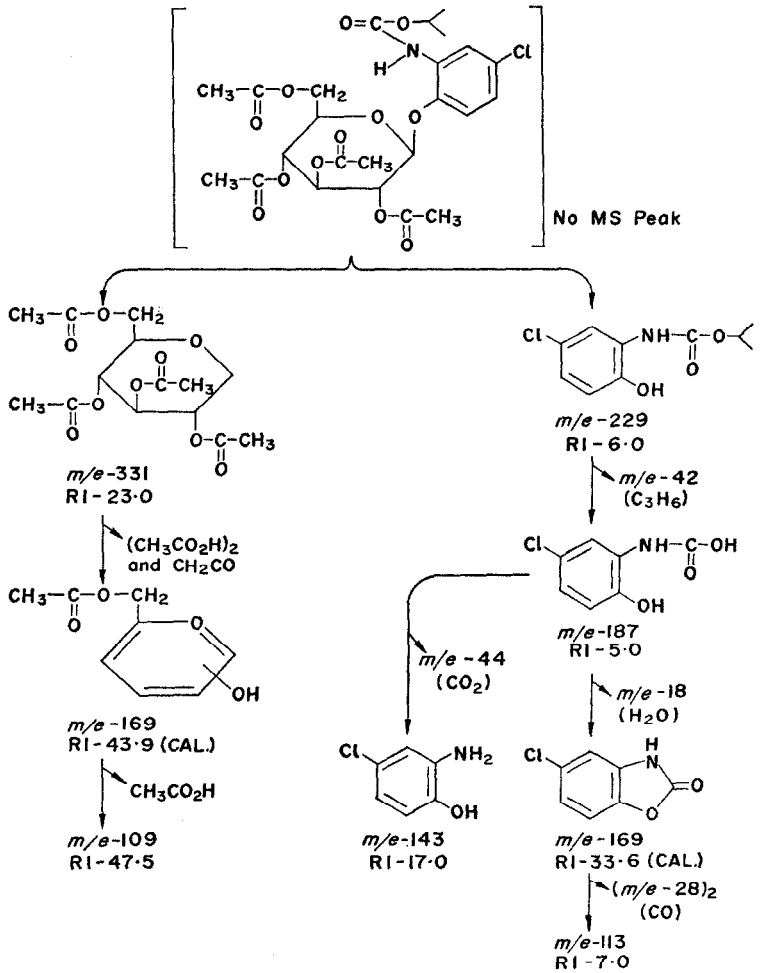

FIG. 3. SOYBEAN ROOT CIPC-^{14}C WATER SOLUBLE METABOLITE. DIRECT ACETYLATION AND GLC–MS ANALYSIS OF THE ACETYLATED PRODUCTS.

the literature,[11] as well as with the fragmentation of model compound, arbutin pentaacetate. Strong peaks at *m/e* 331, 169 and 109 are characteristic of the fragmentation of glucose pentaacetate, as reported by Biemann *et al.*[11] This pattern was also observed with arbutin pentaacetate. The aglucone yielded mass peaks at *m/e* 229, 187, 169, 143 and 113 which are characteristic of the model carbamate compound, isopropyl-5-chloro-2-hydroxy carbanilate, and the aglucone characterized from soybean shoot and root (Fig. 1). From this observation and the data from the β-glucosidase hydrolysis, it appears that the hydroxy-CIPC in soybean was conjugated as an *o*-glucoside. In shoot tissues, direct acetylation yielded only dechlorinated hydroxy-CIPC whose exact character is under investigation.

In root fed soybean plants there was a precursor product relationship between CIPC and the polar metabolites.[8] This occurred in both root and shoot tissues. In root we have shown the polar metabolite to be the *o*-glucoside of isopropyl-5-chloro-2-hydroxy-carbanilate (hydroxy-CIPC). Root tissue also rapidly converted CIPC and hydroxy-CIPC to insoluble residues.[8] In contrast, hydroxy-CIPC is a minor component of the polar metabolites found in shoot tissue and may be the precursor of the dechlorinated hydroxy-CIPC. No insoluble residues were found in the shoots.[8]

The toxicity of isopropyl-5-chloro-2-hydroxycarbanilate has been tested in plants and shown to be of low phytotoxicity.[12] Therefore, in the case of CIPC, in soybean plants, the plant may effectively detoxify the parent molecule. No radiocarbon label from CIPC-^{14}C was found in the seed or pod tissues.[8] However, in root tissue a large portion of the hydroxy-CIPC was converted to insoluble residues.[8] The chemical character and biological significance of these insoluble metabolites is under investigation.

EXPERIMENTAL

Instrumentation. GLC was conducted by using a glass inlet and glass column of 1·8 × 0·6 cm 3% OV-1 on gas chrom Q, 80–100 mesh. Helium was used as the carrier gas with a flow rate of 60 cm^3/min; the column inlet temperature was 220° and the column oven temperature programmed from 125° to 160° with a 15 min initial hold and a 1·0 per min rate. The column effluent was split with a 10:1 stream splitter with the smallest portion passed to a flame detector. The components trapped from the GLC column were assayed for radiocarbon by liquid scintillation counting or were analysed directly in the mass spectrometer by the use of the solid sample probe. The mass spectra were measured with a Varian M-66 mass spectrometer equipped with a V-5500 console. The spectra were attained at 70 eV at a source temperature of 180° and a probe temperature of 25–50°.

Plant material. Soybeans (*Glycine max* (L.) Merr.) variety Hawkeye were germinated, grown and root-treated with CIPC-^{14}C as described by Still and Mansager[8] with one exception. Stainless-steel trays were used with each tray holding 24 soybean plants which were suspended through holes in a stainless-steel cover sheet over 4 l. of half strength Hoagland's solution. When the third trifoliate leaves were expanded, the Hoagland's solution was replaced with ½ strength Hoagland's solution which contained 1·1 × 10^{-6} M isopropyl-3-chlorocarbanilate-phenyl-^{14}C or non radiolabeled CIPC. Preparation and quantitation of these solutions are described by Still and Mansager.[8]

Extraction and purification of polar metabolites. After the plant had grown 7 days in the presence of the CIPC treating solution they were harvested by separating the roots from the shoots just below the cotyledons. Approximately 16 kilos of shoots and 6 kilos of roots were extracted by using the modified Bligh-Dyer procedure.[13] The fresh tissue (root or shoot) was ground with a Hobart mixer grinder (model A-200) by using the fine chopper plate. The ground material was collected in a mixing kettle containing methanol and $CHCl_3$ at a ratio of 2 l. methanol to 1 l. $CHCl_3$ per 1 kg tissue (fresh weight). A homogeneous extracting mixture was maintained by keeping the ratio of MeOH–$CHCl_3$–H_2O at 2:1:0·8; with the water being supplied by the plant tissues. The ground plant tissue was mixed for 20 min and filtered through several layers of cheesecloth. These solids were extracted again with $CHCl_3$ and water (1 l. $CHCl_3$ to 1 l. H_2O to 1 kg fresh weight of plant tissue) and mixed for 20 min followed by filtration. The filtrates were combined

[11] K. Biemann, D. C. DeJongh and H. K. Schones, *J. Am. Chem. Soc.* **85**, 1763 (1963).
[12] W. H. Zick (Private communication).
[13] E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Physiol.* **37**, 911 (1959).

resulting in a MeOH–$CHCl_3$–H_2O ratio of 2:2:1·8 and filtered through Whatman No. 1 in a basket centrifuge. The filtrate was collected in a single carboy and allowed to separate into two phases. Sometimes the sharpness of the interface was enhanced by the addition of small amounts of methanol or $CHCl_3$. The $CHCl_3$ which has been shown to contain only unaltered CIPC was discarded.[8] The polar layer was concentrated *in vacuo* at 30° to approximately 1–2 l. and then lyophilized.

The freeze-dried polar extract was solvated in water at 4° and centrifuged at 1×10^5 g for 2 hr. The precipitate was found free of radiocarbon and discarded. The aqueous supernatant was adjusted with HCl to a concentration of 0·01 N and extracted six times with *n*-BuOH. The aqueous acidic solution was discarded and the *n*-BuOH was removed *in vacuo* leaving an aqueous solution. This solution was adjusted to pH 3·4 in preparation for absorption of the impurities on basic aluminum oxide (Woelm) as described by Crout.[14] The solution and basic aluminum oxide were vigorously stirred while the pH was maintained at 8·5. After the pH was stabilized the mixture was filtered through paper (Whatman No. 4) and the basic aluminum oxide washed with water. All the filtrates were adjusted to pH 3·5 with HCl as soon as they were separated from the basic aluminum oxide. The pooled filtrates were concentrated by lyophilization. The concentrated aqueous solution was adjusted to 0·01 N with HCl and extracted with *n*-BuOH (× 4) and the acid solution discarded. The *n*-BuOH solution was washed twice with a cold 0·1 N NaOH and twice with water. Both the basic and aqueous solutions were discarded and the remaining solution was mixed with hexane and water to a concentration of *n*-BuOH-hexane-H_2O (1:10:1).[15] The *n*-BuOH-hexane phase was washed 3 times with water which removed all the radiolabeled material from the organic phase. This aqueous phase contained the polar metabolites which were concentrated by lyophilization for DE-52 acetate column chromatography.

DEAE cellulose, anion exchange columns (Whatman DE-52) were converted to the acetate form and two gradient elution patterns were used for purification of the CIPC-polar metabolites. The first elution used a nine chamber gradient with 0·4 N HOAc in chambers 3, 6, 7, 8 and 9 with water in chambers 1, 2, 4 and 5. The ^{14}C labeled material eluted between 0·08 N and 0·15 N acetate. This sample was re-chromatographed on DE-52 acetate eluting with a linear concentration gradient of acetic acid from zero to 0·15 N acetate. Two poorly separated peaks eluted between 0·06 N and 0·1 N acetate. These two peaks were combined and used for the characterization studies.

All β-glucosidase reactions were run in pH 4·8 NaOAc buffer at 37° for 4 hr. The acetylation of the aglucone and the direct acetylation reactions were by use of the procedure described by Paulson and Portnoy.[16] The methoxy derivatives of isopropyl-5-chloro-2-hydroxycarbanilate were prepared according to the procedure of Schlenk and Gellerman.[17]

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[14] J. R. CROUT, in *Standard Methods of Clinical Chemistry* (edited by DAVID SELIGSON), Vol. 3, p. 62, Academic Press, New York (1961).
[15] P. A. SHORE and J. S. OLIN, *J. Pharmicol. Exptl. Therap.* **122**, 295 (1958).
[16] G. D. PAULSON and C. E. PORTNOY, *J. Agric. Food Chem.* **18**, 180 (1970).
[17] H. SCHLENK and J. L. GELLERMAN, *Anal. Chem.* **32**, 1412 (1960).

Key Word Index—Glycine max; Leguminosae; herbicide; isopropyl-3-chlorocarbanilate; CIPC; aryl hydroxylation; detoxification.