Metabolism of the Fungicide 2,6-Dichloro-4-nitroaniline in Soil

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A ^{14}C-labeled sample of 2,6-dichloro-4-nitroaniline (DCNA, Botran) was incubated in flooded soil that had been amended with D-glucose (1%, w/w). After 3 days the amount of DCNA that could be recovered from the soil decreased to 7% and to 3% after 9 days. Evolution of radioactive carbon dioxide could not be detected even after the 9-day experimental period. Much of the radioactivity (ultimately 65%) could not be removed from the soil sample by extraction with an acetone–water solution. The major metabolite that was extractable from soil was identified as 4-amino-3,5-dichloroacetanilide (ADCAA). The expected metabolic precursor of ADCAA, 2,6-dichloro-*p*-phenylenediamine (DCPD), was also isolated from the extraction solution. A third compound isolated from soil appeared to be formed by the oxidative dimerization of DCPD. This same compound was also produced by the action of horseradish peroxidase on DCPD in vitro. Six fungi sensitive to DCNA were grown on potato–dextrose agar containing 10 μg/ml of DCNA, DCPD, or ADCAA. DCPD and ADCAA did not significantly affect the growth of any of the fungi tested as compared with controls whereas DCNA completely inhibited or reduced the growth rate in all cases.

Because 2,6-dichloro-4-nitroaniline (DCNA) is widely used for control of plant disease, knowledge of its fate in soil is important for its safe use as a fungicide. Experiments on its persistence in soil have revealed that breakdown, apparently as a result of microbial action, is more rapid in soil previously treated with DCNA (Groves and Chough, 1970). In the latter case, [^{14}C]DCNA added to soil was utilized rapidly and 25 to 50% of the radioactivity associated with the chemical was recovered in CO_2 evolved from the treated soil. When [^{14}C]DCNA was added to soils not pretreated with DCNA, no $^{14}CO_2$ was evolved. However, it was not determined if DCNA had been altered without evolution of $^{14}CO_2$. Wang and Broadbent (1973) reported that only 2–4% of added DCNA was recovered after 10 days from glucose, alfalfa, and rice straw amended flooded soils as compared with 83.5% recovery from unamended flooded soils. From unamended upland soil, they reported that 99.5% of the DCNA was recovered unchanged after 4 weeks. In all the early studies, metabolic products of DCNA other than CO_2 were not identified and potential toxicity of the metabolites was not studied.

Previously we reported that a number of bacteria and fungi in nutrient media have the capacity to convert DCNA to 2,6-dichloro-*p*-phenylenediamine (DCPD) and 4-amino-3,5-dichloroacetanilide (ADCAA) (Van Alfen and Kosuge, 1974). Since certain of the organisms that were most active in DCNA metabolism are common inhabitants of soil, we determined if similar DCNA metabolites were formed in soil. The purposes of this study were: (1) to identify the principal products of DCNA metabolism in soil and (2) to determine if any of the major metabolic products of DCNA were toxic to microorganisms.

MATERIALS AND METHODS

Cultures. The cultures used in this study were obtained from the following sources in the Department of Plant Pathology, University of California, Davis: Teaching Collection, *Alternaria alternata* (Fr.) Keissler; Dr. E. E. Butler, *Rhizopus oryzae* Went et Prinsen-Geerligs (isolate number 1456), *Rhizopus stolonifer* (Fr.) Lind. (1136), and *Mucor hiemalis* Wehmer (1237); Dr. R. K. Webster, *Botrytis cinerea* Fr. (417) and Mr. D. B. Marcum, *Sclerotinia sclerotiorum* (Libert) deBary.

Chemicals. DCNA (2,6-dichloro-4-nitroaniline, Upjohn Co., Kalamazoo, Mich.) was recrystallized twice from benzene. Uniformly ^{14}C-labeled DCNA, specific activity 0.98 μCi/μmol, was a gift of Dr. Joseph Ogawa, Department of Plant Pathology, University of California, Davis. Chromatography of the [^{14}C]DCNA by silica gel thin-layer chromatography (TLC) in solvent a below showed it to be 99.7% DCNA. The remaining radioactivity (0.3%) did not move from the origin in the TLC system used. Synthesis of 2,6-dichloro-*p*-phenylenediamine (DCPD) and 4-amino-3,5-dichloroacetanilide (ADCAA) was performed as previously reported (Van Alfen and Kosuge, 1974).

Instrumentation. The required light absorption spectra were obtained on a Perkin-Elmer Model 337 and a Beckman ACTA III spectrophotometer, respectively. The mass spectra were obtained on an Associated Electronics Industries, Ltd. MS-7 and a Varian M-66 mass spectrometer. Radioactive counting was performed as previously reported (Van Alfen and Kosuge, 1974).

Thin-Layer Chromatography. Thin-layer chromatography (TLC) was done on Eastman number 6061 silica gel plastic sheets or on Merck preparative silica gel plates in the following solvents: (a) benzene–diethyl ether (1:1, v/v); (b) benzene–petroleum ether (7:3, v/v); (c) chloroform–acetone (7:3, v/v); (d) ethyl acetate.

Time-Course Study. Dry Yolo loam soil (pH 8.6) untreated with pesticides for at least 3 years was obtained from a garden plot on the campus of the University of California, Davis. The soil was ground and mixed in a mortar and pestle. Portions (2.0 g) of the soil were placed in individual 10-ml beakers and flooded with 2.0 ml of heat sterilized 1% D-glucose solution containing 10 μg/ml of [^{14}C]DCNA. All operations were performed aseptically to avoid contamination by laboratory microorganisms. Each 10-ml beaker with soil was placed inside a 100-ml beaker which contained 15 ml of sterile 1 M KOH to trap CO_2. The 100-ml beaker was covered with filter paper and sealed with parafilm (American Can Co.). The efficiency of the system to trap CO_2 was checked by release of $^{14}CO_2$ from an acidified solution of $Na_2{}^{14}CO_3$ (100000 dpm, specific activity 5 μCi/μmol) and found to be essentially 100% effective. For controls, similar mixtures were prepared with soil that had been sterilized with propylene oxide. To

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sterilize the soil, 50 g was placed in a 1-pint jar humidified by a piece of moist filter paper, propylene oxide (2.0 ml) was added, and the jar was sealed for 6 days at 23 °C. The sterilized soil was amended with DCNA and glucose after the propylene oxide treatment. All of the samples were incubated at 25 °C in the dark.

Individual beakers of flooded soil were analyzed at regular intervals as follows: a sample of the KOH solution was examined in the liquid scintillation counter to determine if $^{14}CO_2$ had been trapped. The soil was transferred to a test tube, 4.0 ml of acetone was added, and the mixture was agitated for 1 h on a New Brunswick twist action shaker. The mixture then was filtered through Whatman No. 42 filter paper and the soil on the filter paper was washed with an additional 4.0 ml of acetone. The filtrate and the acetone washings were combined and the extracted soil was air-dried. A weighed sample of the dry, extracted soil was finely ground and placed in radioactive counting solution with 0.4 g of Cabosil (Cabot Corp.) per vial to suspend the soil particles. Ten milliliters of a toluene-based scintillation counting fluid (Van Alfen and Kosuge, 1974) was added to each vial and the radioactivity in the samples then was determined in a liquid scintillation counter. The channels' ratio method was used to correct for quenching (Bush, 1963). In a control experiment, 88% of the DCNA mixed with soil was recovered by the extraction procedure.

The acetone solution used to extract the soil sample was dried under a stream of N_2 gas. The aqueous solution which remained was extracted with 5.0 ml of benzene. The benzene fraction was dried under a stream of N_2 and the residue dissolved in 0.1 ml of acetone. The acetone solutions were chromatographed in solvent a or b. After development, the radioactive compounds on the chromatogram were located by autoradiography (Kodak No-Screen x-ray film). Portions on the chromatogram bearing radioactive compounds were cut out (plastic backed plates), individual pieces were placed in 5 ml of the toluene-based scintillation counting solution, and the samples were counted in the liquid scintillation counter.

Large-Scale Isolation of Metabolites from Soil. Yolo loam soil was amended with 100 μg of DCNA per g of soil and D-glucose (1% w/w) and then flooded with sterile water. After 5 days incubation in a 4-l. metal container, at 23 °C, the soil was extracted as described above, except that the mixture of soil–water with acetone was agitated on a rotary shaker, and the acetone–soil suspension and the benzene–aqueous solution ratios were, respectively, 1:1 and 2:1 (v/v). The benzene fraction was concentrated to 0.5 ml by evaporation, and chromatographed on Merck preparative silica gel TLC plates (2 mm thick), in solvent a. The desired metabolite was eluted from the silica gel with acetone and rechromatographed on Merck silica gel TLC plates (250 μm thick) in solvent c. The desired metabolites were eluted from the silica gel with acetone and the acetone solution evaporated to dryness. The residue was crystallized from acetone–water and the compounds analyzed by infrared spectrophotometry.

Peroxidase Studies. DCPD was tested as a substrate for horseradish peroxidase (Sigma type II), EC 1.11.1.7, by the procedure of Lieb and Still (1969). The reaction mixture was incubated for 10 min at 23 °C, and then extracted with 1 vol of chloroform. The chloroform extract was chromatographed on Eastman silica gel TLC plates in solvent a.

Toxicity of DCNA Metabolites to Certain Fungi. Difco potato–dextrose agar (PDA, 4% w/v) was amended with 10 μg/ml of DCNA, DCPD, or ADCAA. The chemicals were first dissolved in 1.0 ml of acetone and then added to 500 ml of the medium after it had cooled but not yet solidified. An equivalent amount of acetone was added to unamended medium which served as the control. The media (30 ml) were then dispensed into petri plates (100-mm diameter) which had each been marked with four radii converging at the point where the inoculum was to be placed and inoculated with plugs (4 mm diameter) from the edge of actively growing fungal colonies on PDA. The petri plates were incubated at 25 °C in the dark and daily radial growth measurements were made along these radii. Each treatment was replicated three times.

Table I. Distribution of Radioactivity into Various Fractions from [^{14}C]DCNA Treated Soil

Days	Fraction[a]			
	Soil	Benzene	Water[b]	Total
0	1 300	120 000	1 400	122 700
1	11 300	98 000	3 500	112 800
1-C[c]	1 700	102 000	1 100	104 800
2	35 000	49 000	12 700	96 700
3	50 000	39 000	15 800	104 800
3-C	2 400	123 000	1 600	127 000
4	54 000	39 000	14 500	107 500
9	57 000	26 000	9 200	92 200
9-C	6 700	107 000	1 100	114 800

[a] Values are given as total disintegrations per minute (dpm) in each fraction. [b] Total dpm in the aqueous phase after benzene extraction. [c] Distribution of the radioactivity of sterile controls after 1, 3, and 9 days is also indicated.

RESULTS

Time-Course Experiment. When the aqueous soil filtrates of the sterile controls and zero-time sample were extracted with benzene, 99% of the total extractable radioactivity as [^{14}C]DCNA was recovered in the benzene and 1% remained in the aqueous fraction. Distribution of the radioactivity in the other samples was a function of the length of the incubation period. Table I shows that the radioactivity extractable into benzene decreased rapidly with time of incubation of the soil. The 2-day benzene fraction contained less than one-half the amount of radioactivity that was present in the zero-time benzene fraction. The radioactivity bound to the soil increased rapidly and ultimately accounted for approximately 65% of the total detectable radioactivity. Throughout the 9-day experiment, no radioactivity was detected in the KOH trapping solutions, indicating that DCNA was not being metabolized to CO_2. As far as can be determined within the precision of the experiment, the data on total radioactivity recovered indicate that no major component is being lost from the extraction scheme as a function of time.

Because these procedures would not differentiate between DCNA and its metabolites in the benzene fraction, the benzene extract was further purified by chromatography by the procedures described under Materials and Methods. In addition to DCNA, the chromatogram revealed four radioactive spots with R_f values of 0.0, 0.25, 0.48, and 0.54. The spot with the R_f value of 0.54 had an intense pink fluorescence under uv light. The R_f values of two metabolites (0.25 and 0.46) were characteristic of ADCAA and DCPD, the previously reported microbial metabolites of DCNA.

To quantitate the results, the amount of radioactivity associated with each spot then was determined. The results (Figure 1) show that as DCNA is metabolized, the radioactivity appears in several major fractions. As previously noted, the soil-bound counts increase most

Table II. Growth of Fungi in Media Amended with 2,6-Dichloro-4-nitroaniline or Its Metabolites

Organism	Control[a]	DCNA[b]	DCPD	ADCAA
Botrytis cinerea	5.9 ± 0.5	0	6.4 ± 0.5	7.0
Rhizopus oryzae	37.4 ± 0.9	30.9 ± 1.2	37.4 ± 1.4	37.7 ± 1.4
R. stolonifer	40.1 ± 0.5	0	38.2 ± 1.2	35.4 ± 1.4
Mucor hiemalis	14.0 ± 0.3	3.2 ± 1.5	14.3 ± 0.4	14.8 ± 0.6
Sclerotinia sclerotiorum	18.5 ± 1.9	0	16.3 ± 2.9	17.9 ± 2.6
Alternaria alternata	4.9 ± 0.3	0	5.2 ± 0.4	5.0 ± 0.6

[a] Measurements are given in millimeters of radial growth. Each figure given is the mean plus standard deviation from 12 radii. The measurements were taken after 24 h growth at 25 °C in the dark. [b] The concentration of DCNA, DCPD, and ADCAA in the medium was 10 μg/ml.

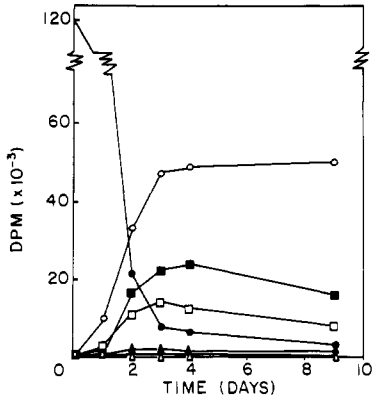

Figure 1. Distribution of radioactivity in various metabolites of [^{14}C]DCNA from soil as a function of time. The radioactivity detected on the thin-layer plates was adjusted to correct for the efficiency of detection (approximately 45%): (•) DCNA; (○) soil-bound radioactivity; (▪) ADCAA; (□) aqueous soil filtrate after benzene extraction; (▲) pink fluorescent compound; (△) DCPD.

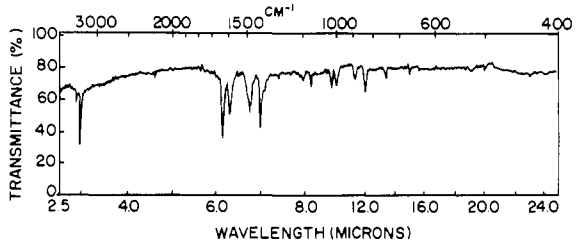

Figure 2. Infrared spectrum of pink fluorescent compound isolated from soil. KBr pellet of sample was prepared for the spectrum.

rapidly, followed by the rapid production of ADCAA appearing in the benzene fraction. The radioactivity that could not be extracted from the soil filtrate by benzene also increased significantly. At their maximum concentrations, the pink fluorescent compound and DCPD accounted for 5 and 3%, respectively, of the benzene-extractable radioactivity. Once the rate of metabolism of DCNA decreased, there was a slackening in the rate of increase of the metabolites. This was followed by a slight decrease in the amounts of all of the metabolites except the soil-bound one(s) which showed a slight net increase during this period.

Identification of Metabolites in the Benzene Fraction. To confirm that the spot with an R_f value of 0.25 was [^{14}C]ADCAA, the silica bearing the labeled material was removed from the chromatogram, and extracted with acetone. Then a portion of the acetone sample was cochromatographed with synthetic ADCAA two dimensionally, first in solvent c (R_f 0.37) and then in solvent d (R_f 0.39). The radioactive compound detected by autoradiography corresponded to the position of synthetic ADCAA on the chromatogram. A crystalline sample of the metabolite then was obtained from soil by large-scale isolation procedures as described under Materials and Methods. The infrared spectra of the soil metabolite and an authentic sample of ADCAA were identical.

DCPD could not be isolated in amounts sufficient for ir spectrophotometry. However, the soil metabolite and synthetic DCPD chromatographed identically in solvents a and b.

A pink fluorescent compound (R_f 0.54) was also detected by the chromatographic procedures. Purification from soil was achieved by the procedure described under Materials and Methods with the following variation. After elution from a TLC preparative silica gel plate developed in solvent a, the compound was adsorbed onto Bio-Rad alumina (neutral), activity grade I. The alumina sample was added to the top of a dry-packed column of the same type of alumina and the column washed with acetone. Fractions which contained the pink fluorescent metabolite were combined and then evaporated to dryness. The compound was crystallized from hexane as red needles. Since the compound decomposed above 300 °C, no melting point was determined. Mass spectrometry revealed a parent peak of m/e 346 which had P + 2 and P + 4 peaks of 138 and 68% of the parent, indicating the presence of four chlorine atoms. The exact masses of the parent and P + 2 peaks were 345.9348 and 347.9305 which correspond to a molecular formula of $C_{12}H_6N_4Cl_4$. The ir spectrum of the compound is shown in Figure 2. The uv–visible spectrum had a maximum at 290 nm and other peaks at 380 and 510 nm.

Peroxidase Experiments. When incubated at room temperature with horseradish peroxidase, the reaction mixture darkened rapidly and a dark-brown precipitate formed. After 10-min incubation the reaction mixture was extracted with chloroform which quantitatively removed the colored precipitate. Chromatography of the chloroform extract by TLC solvent a indicated that at least eight products were formed with the following R_f values: 0.02, 0.06, 0.24, 0.45, 0.55 (pink fluorescence under uv light), 0.64, and 0.69 (one or more compounds remained at the origin). The compounds that were visually most abundant were the pink fluorescent compound (R_f 0.55) and the R_f 0.69 compound appearing dark red-brown in daylight. Varying the concentration of the substrate (DCPD) changed the relative abundance of these two compounds. The following reaction mixture gave the maximum production of the pink fluorescent compound: 0.7 ml of 0.2 M sodium acetate buffer (pH 5.1), 0.2 ml of 0.1% H_2O_2, 1.0 ml of enzyme preparation (10 μg/ml of Sigma type II horseradish peroxidase), and 1.0 mg of DCPD added to the reaction mixture in 0.2 ml of methanol. When DCNA replaced DCPD as the substrate, no reaction occurred.

The pink fluorescent compound was extracted from the reaction mixture with 1 vol of benzene and the benzene extract was treated by the procedures described for the isolation of the pink fluorescing compound from soil. The

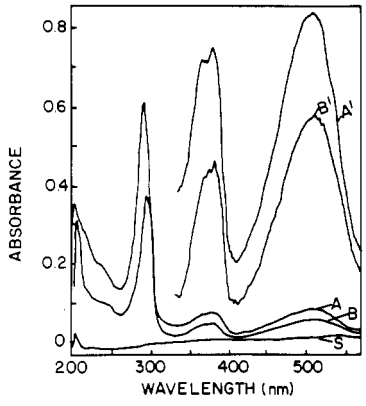

Figure 3. Comparison of uv-visible spectra of pink fluorescent compounds isolated from soil (A) and horseradish peroxidase reaction mixture (B). Methanol (S) was used as the solvent. The expanded spectra (A' and B') were run at a sensitivity of 0–0.1 as compared with 0–1.0 for the others.

absorption spectra of the pink fluorescent compound from the peroxidase reaction mixture and the DCNA metabolite from soil were identical (Figure 3) and both compounds chromatographed identically in solvents a and b.

Toxicity of DCNA Metabolites to Certain Fungi. The relative toxicity of DCNA, DCPD, and ADCAA was tested against a number of fungi. The results (Table II) show that although DCNA completely inhibited the growth of some of the fungi tested, and reduced the growth rate significantly in others, the two metabolites of DCNA did not significantly affect the growth of any of the fungi. In all cases, the radial growth of the fungi in the presence of DCPD and ADCAA after 24 h was essentially the same as the corresponding control. Daily measurements of radial growth for up to 4 days revealed no changes from the relative growth rates reported in Table II.

DISCUSSION

Experiments on the persistence of DCNA in soil have revealed that breakdown, apparently as a result of microbial action, is more rapid in soil previously treated with DCNA. Such results imply that pretreatment with DCNA causes the selection and buildup of microflora capable of metabolizing the chemical. In addition, certain soil amendments and low oxygen tension also promote utilization of DCNA by microflora. Thus, Wang and Broadbent (1973) reported that only 2–4% of added DCNA was recovered after 10 days from glucose, alfalfa, or rice-straw amended, flooded soil. Without amendment, 83.5% of the DCNA incorporated into the flooded soil was recovered. Such observations reveal that factors favoring activity of certain microflora largely determine the persistence of DCNA in soils. In our studies with glucose amended, flooded soil, DCNA is almost completely metabolized within 2 days to several compounds, two of which were not toxic to the microorganisms tested.

The finding that DCPD is a metabolite of DCNA in soil is not unexpected. Our previous work (Van Alfen and Kosuge, 1974) has shown that a large number of fungi and bacteria in culture can rapidly reduce DCNA to DCPD, especially under anaerobic conditions. While the work of Briggs and Ogilvie (1971) would suggest that an acetanilide such as ADCAA should not accumulate in soil as we found, other studies using the herbicide metobromuron confirm that an acetanilide can accumulate as a metabolite of an aniline-based pesticide (Tweedy et al., 1970). Our results (Figure 1) indicate that ADCAA accumulates to a maximum at 4 days and declines in amount thereafter.

Since the pink fluorescent compound isolated from soil was the same as the one isolated from the DCPD–peroxidase reaction mixture, a similar oxidation of DCPD may account for its formation in soil. Wang and Broadbent (1973) also detected a pink fluorescent compound in flooded Columbia fine, sandy loam soil that had been amended with glucose and DCNA. The low amount of pink fluorescent compound relative to the amount of ADCAA in this experiment may perhaps be explained by the fact that oxidation would not be favored under the anaerobic conditions of flooded soil. Briggs and Ogilvie (1971) observed that soil slurries amended with 3-chloro-4-methoxyaniline contained a pink compound which they isolated and identified as 3-chlorobenzoquinone–4-(3'-chloro-4'-methoxy)anil. They also found that the latter compound was formed by a peroxidase-catalyzed oxidation of peroxidase 3-chloro-4-methoxyaniline. In addition, peroxidase catalyzes the formation of a tetrachloroazobenzene from 3,4-dichloroaniline, a soil metabolite of the herbicide Propanil (Bartha and Pramer, 1970).

Although spectroscopic data are not sufficient to identify the structure of the pink fluorescent metabolite of DCNA, the accurate mass of the parent peak (345.9347) suggests a molecular formula of $C_{12}H_6Cl_4N_4$ (345.9348). Since it is known that this compound is formed by oxidation of DCPD, it is undoubtedly a dimer of DCPD. The lack of typical aromatic bands in the 2000–1660- or 900–700-cm^{-1} regions together with the lack of the typical double absorption band of primary amines at 3500–3200 cm^{-1} and C–N stretching of aromatic amines at 1350–1250 cm^{-1} support the evidence from the molecular formula that this compound is highly oxidized. The structure could be a double quinone diimine coupled by either an azine or anil. The intense band in the uv spectrum at 290 nm and the compound's fluorescence would support such a structure.

Although several other oxidative products of DCPD were formed in the peroxidase reaction mixture, none of these products was visually detected among the DCNA metabolites that were extracted from DCNA-amended soil. The composition of the microflora of the soil may be important in determining which metabolites of DCNA are formed and their relative abundance. The soil preparation procedure used in these experiments may also have affected the relative abundance of the metabolites since it has been observed that peroxidase activity is decreased by air drying of soil (Bartha, 1971).

Experiments show that after prolonged incubation, a substantial portion of DCNA is converted to compounds that could not be extracted from the soil by our procedures. Although the compounds have not been identified, they may represent metabolites bound to soil particles (Hsu and Bartha, 1974). The radioactivity not extractable from soil was obviously not DCNA, since DCNA was readily extracted from all of the sterile controls as well as the zero time sample.

ADCAA and DCPD appear to be detoxification products of DCNA because they failed to inhibit growth of all the fungi and bacteria that were tested. In high nutrient soils that are subject to uncontrolled flooding, DCNA may be a poor choice for a fungicide since it is metabolized rapidly under those conditions.

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Synthesis and Isolation of 1-Hydroxy-2-propyl 3-Chlorocarbanilate from Soybean Plants Treated with Isopropyl 3-Chlorocarbanilate

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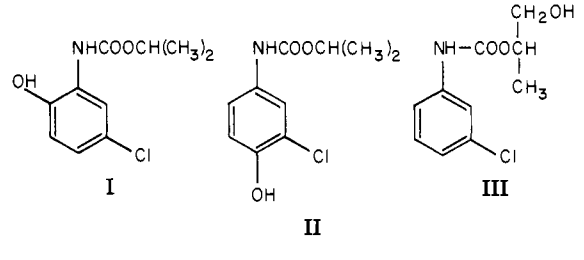
The metabolism of isopropyl 3-chlorocarbanilate (CIPC) to form an alkyl hydroxylated metabolite in soybean shoots was indicated by alkaline hydrolysis–distillation of 3-chloroaniline from polar plant extracts. The presence of 1-hydroxy-2-propyl 3-chlorocarbanilate (III) in hydroponically grown plants was confirmed by autoradiograms of two-dimensional TLC plates, both of III and the acetate derivative of III, t_R of III on HPLC and t_R of the heptafluorobutyrate derivative of III on GLC. The presence of III in soil-grown plants was confirmed by the alkaline hydrolysis–distillation of 3-chloroaniline from polar extracts, t_R of the acetate of III on HPLC and GLC, and the mass spectral analysis of the acetate of III. Soil grown soybean shoots produce a higher percentage of III when compared to the aryl hydroxylated metabolites which are the predominant metabolites found in hydroponically grown plants. Synthetic III (mp 88.5–91.0 °C, 68%) was prepared by HCl cleavage of 1-triphenylmethoxy-2-propyl 3-chlorocarbanilate (V) (mp 138–139 °C, 82%) which was prepared from 1-triphenylmethoxy-2-propanol (IV) and 3-chlorophenyl isocyanate.

Hodgson (1967) reported two water-soluble metabolites of isopropyl 3-chlorocarbanilate in barley, cucumber, and soybean. James and Prendeville (1969) reported the isolation of suspected β-glycosidic conjugates and postulated that the isopropyl moiety of CIPC was oxidized by smartweed, tomato, and parsnip plants. Extensive studies of hydroponically grown soybeans by Still and Mansager (1971–1973) showed the presence of the aryl hydroxylated metabolites, isopropyl 5-chloro-2-hydroxycarbanilate (I) and isopropyl 3-chloro-4-hydroxycarbanilate (II), but provided no indication of alkyl hydroxylation. Unpublished work at PPG Industries (1973) on soybean plants confirmed the presence of I and II but also indicated the presence of a third metabolite. This paper reports the joint investigation resulting in the identification of this third metabolite, 1-hydroxy-2-propyl 3-chlorocarbanilate (III).

EXPERIMENTAL SECTION

Synthesis of Metabolites. Compounds I and II were prepared by the method of Grunow et al. (1970). Preparation of the compounds by a different route has been described by Bobik et al. (1972).



Barberton Technical Center, Chemical Division, PPG Industries, Inc., Barberton, Ohio 44203 (J.L.W., G.G.E.) and Agricultural Research Service, U.S. Department of Agriculture, Metabolism and Radiation Research Laboratory, Fargo, North Dakota 58102 (G.G.S.).

In the following preparations, care was taken to use anhydrous solvents and reagents and to exclude atmospheric moisture from the reaction vessel.

Compound IV (Figure 1) was prepared from chlorotriphenylmethane and 1,2-propanediol in the presence of pyridine by the method of Havbrandt et al. (1969); 86% yield of IV; mp 99–100 °C (lit. mp 96–97 °C). The use of purified chlorotriphenylmethane (Renfrow and Hauser (1943)) was found essential for obtaining high purity product.

To a refluxing solution of 202.0 g (0.633 mol) of IV in 1000 ml of benzene was added 95.4 g (0.621 mol) of 3-chlorophenyl isocyanate over a 25-min period. After refluxing for 14 h, unreacted isocyanate was destroyed by adding 50 ml of isopropyl alcohol and refluxing for an additional 15 min. The product, 1-triphenylmethoxy-2-propyl 3-chlorocarbanilate, crystallized after the addition of 1500 ml of hexane. The product was removed by filtration, washed with 200 ml of hexane, and vacuum dried to yield 241.1 g (82%) of white crystals: mp 138–139 °C; ir (mull) 3310 and 1695 cm^{-1}; NMR ($CDCl_3$) δ 6.9–7.6 (m, 19, ArH), 6.72 (br s, 1, NH), 5.17 (m, 1, CH), 3.20 (d, J = 5 Hz, 2, CH_2), 1.24 (d, J = 6.5 Hz, 3, CH_3). Anal. Calcd for $C_{29}H_{26}ClNO_3$: C, 73.79; H, 5.55. Found: C, 73.52; H, 5.12.

Hydrogen chloride was passed through a sintered glass bubbler into a solution of 240.4 g (0.510 mol) of V in 1200 ml of chloroform. The temperature rose from 24 to 28 °C over a 24-min period and then dropped to 24 °C during the subsequent 115 min of the addition period. About 20 g of hydrogen chloride was added during the first 50 min and only an additional 4 g during the remaining 89 min. After sparging the solution with nitrogen for 70 min to remove excess hydrogen chloride, the solvent was removed under vacuum using a 25–35 °C water bath. The 262 g of solid residue was dissolved in 450 ml of carbon tetrachloride and 93 g of crude 1-hydroxy-2-propyl 3-chlorocarbanilate was obtained upon cooling. The product was