Metabolism of Chloroneb by *Rhizoctonia solani* and Other Fungi

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Mycelium of *Rhizoctonia solani* incubated in a liquid medium containing chloroneb (1,4-dichloro-2,5-dimethoxybenzene) slowly converted the toxicant to a nontoxic metabolite, which was identified as 2,5-dichloro-4-methoxyphenol on the basis of infrared, nuclear magnetic resonance, and mass spectrographic analyses. About 50% of the chloroneb was converted during the first 24 hours, but no mycelial growth occurred during this period. The C_1 fragment removed from chloroneb was incorporated into both the methanol-soluble and -insoluble fractions of the mycelium. Chloroneb prevented growth of *R. solani* and *Sclerotium rolfsii* at 5 to 8 μg. per ml., but the metabolite was not toxic at 16 μg. per ml. Chloroneb was not metabolized by *S. rolfsii* or *Saccharomyces pastorianus*, but was metabolized to an unidentified product by *Neurospora crassa*.

Chloroneb (1,4-dichloro-2,5-dimethoxybenzene) is a new soil fungicide which effectively controls certain soil-borne seedling diseases of cotton (Fielding and Rhodes, 1967; Ranney and Burchfield, 1967) when the chemical is applied as a seed overcoat or an in-furrow or planter box treatment. Chloroneb enters roots and concentrates in the roots, hypocotyls, and lower stem portions of cotton (Fielding and Rhodes, 1967; Sinclair and Darrag, 1966), and in bean and cucumber seedlings (Fielding and Rhodes, 1967).

With the exception of a recent paper by Pease (1967), who reported the conversion of chloroneb to 2,5-dichloro-4-methoxyphenol in animals, little is known of the metabolism of chloroneb in higher plants or animals, or of the biological fate of chloroneb in the presence of fungi. The purpose of this investigation was to determine whether any biological alteration of the toxicant occurred as a result of fungal metabolism, and the role, if any, of the metabolites in fungitoxicity.

EXPERIMENTAL

Culture Methods. The test organisms used were *Rhizoctonia solani* Kühn; *Sclerotium rolfsii* Saccardo, ATCC 15004; *Neurospora crassa* Shear and Dodge, wild type STA-4; and *Saccharomyces pastorianus* Hansen.

R. solani and *S. rolfsii* were grown at room temperature (22° to 25° C.) for 5 to 6 days in the nutrient medium of Coursen and Sisler (1960). The medium (pH 6.4), hereafter referred to as basal medium, consisted of glucose, inorganic salts, and vitamins. A small mycelial disk of *R. solani* was placed in a 125-ml. Erlenmeyer flask containing 40 ml. of the basal medium and incubated at room temperature (Wedding and Kendrick, 1959). After 5 to 6 days the mycelial mats which developed were washed twice with sterile distilled water, removed from the flasks, and placed on filter paper pads. Disks 5 mm. in diameter were cut from the mycelial mats with a cork borer. Excess moisture was removed from the disks by blotting with filter paper prior to placing them in the test solutions. *S. rolfsii* was cultured and processed like *R. solani*.

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N. crassa was grown and conidial suspensions were prepared in sterile distilled water according to Sisler *et al.* (1967). The conidial suspensions were standardized to an absorbance of 0.5 at 600 mμ (0.3 mg. dry weight per ml.). The suspension was then centrifuged, and the distilled water was removed and replaced with a desired volume of test solution.

S. pastorianus was grown in basal medium supplemented with 2 grams of yeast extract per liter of medium at 30° C. on a water bath shaker for 18 to 24 hours. Washed cell suspensions were prepared and standardized to 0.65 mg. dry weight per ml. according to the procedure of Siegel and Sisler (1964).

Metabolism of Chloroneb-^{14}C by *R. solani*. Ten mycelial disks were incubated in 2 ml. of basal medium containing 5, 8, or 16 μg. per ml. of methyl-labeled chloroneb-^{14}C (specific activity 1.8 mc. per mmole) at 25° C. on a water bath shaker. Samples of the medium (50 to 100 μl.) were removed at 3, 6, 9, 12, and 24 hours and spotted on Adsorbosil-1 silica gel (Applied Science Laboratories) thin-layer plates (250 microns thick). Chromatograms were developed in ethyl acetate–methanol (10 to 1 v./v.). Regions of radioactivity were located on the developed plates with the aid of a Baird Atomic Model RSC-363 radiochromatogram scanner. The cochromatographed nonlabeled chloroneb standard was detected with the aid of an ultraviolet lamp.

A similar analysis was made using 8 μg. per ml. of ring-labeled chloroneb-^{14}C (specific activity 0.48 mc. per mmole).

The radioactivity which remained in the mycelial disks incubated 24 hours was removed by extracting three times with 2-ml. volumes of redistilled methanol. Samples (50 to 100 μl.) of the combined methanol fractions were spotted on Adsorbosil-1 silica gel thin-layer plates and the radioactive components separated as described above.

Following extraction with methanol, the disks were placed in scintillation vials containing 10 ml. of toluene scintillation fluid—containing 4 grams of PPO (2,5-diphenyloxazole) and 0.5 gram of POPOP {*p*-bis-[2-(5-phenyloxazolyl)]-benzene} per liter—and the radioactivity was determined.

Because of the volatility of chloroneb and the major metabolite, losses of these compounds occurred when solutions were spotted and dried on silica gel plates. Therefore, quantitative measurements of the radioactivity

in chloroneb and conversion products were made using preparations chromatographed on silica gel columns. One-centimeter-diameter columns were prepared with Adsorbosil CAB silica gel 100- to 140-mesh (Applied Science Laboratories). About 10 cm. of silica gel added as an ethyl acetate slurry was packed into each column. Radioactive substances were recovered from the culture medium by extracting three times with one-half volume of ethyl acetate. This procedure removed 96 to 97% of the radioactivity from the medium. A 1-ml. sample from the combined ethyl acetate extracts was carefully layered on the silica gel column. Radioactivity was eluted with ethyl acetate–methanol (10 to 1 v./v.). One-milliliter fractions were collected and dispensed into scintillation vials containing 10 ml. of toluene-methanol (4 to 1 v./v.) scintillation fluid and counts were determined with a scintillation spectrometer.

Radioactivity in the fungal disks was removed by extracting the 10 disks from each sample three times with 2-ml. volumes of ethyl acetate. A 2-ml. sample from the combined ethyl acetate extract was layered on the column. The elution solvent and the fractionation and counting procedures were identical to those used in the analysis of the ethyl acetate extract of the culture medium.

Isolation and Identification of Chloroneb Conversion Product. Pieces of mycelium were placed in 125 ml. of basal medium in 1000-ml. flasks and the cultures were incubated at room temperature for 5 to 6 days. The basal medium was then removed and replaced with 200 ml. of sterile distilled water containing 16 μg. per ml. of chloroneb and the cultures were incubated an additional 24 hours. The culture solution was then poured through several layers of cheesecloth to remove mycelial fragments and extracted once with 50 ml. of ethyl acetate. The ethyl acetate extract was evaporated to dryness with a vacuum evaporator at 40° C. The residue was dissolved in about 2 to 3 ml. of redistilled ethyl acetate and the solution was spotted on Adsorbosil-1 silica gel thin-layer plates (250 microns thick). Chromatograms were developed with ethyl acetate–methanol (10 to 1 v./v.). Silica gel containing the ultraviolet-absorbing material corresponding in R_f to that of the metabolite was scraped from the plates and placed in 12-ml. centrifuge tubes. The silica gel was extracted three times with 2 ml. of redistilled ethyl acetate. The ethyl acetate extracts were combined and dried with a stream of nitrogen. The residue was dissolved in redistilled methanol and recrystallized three times from this solvent.

The purified metabolite in a KBr pellet was analyzed with a Perkin-Elmer 257 grating infrared spectrophotometer. A nuclear magnetic resonance (NMR) spectrum of the compound was made in acetone with a Varian 60-megahertz NMR spectrometer. The mass of the compound was determined by mass spectrographic analysis with an Associated Electrical Industries MS-9 mass spectrometer.

Metabolism of Chloroneb-^{14}C by *N. crassa*. Two milliliters of conidial suspension (0.3 or 1.5 mg. dry weight per ml.) in basal medium (supplemented with 0.01*M* citric acid and adjusted to pH 6.4 with KOH) containing 16 μg. per ml. of methyl- or ring-labeled chloroneb-^{14}C were incubated in 25-ml. flasks for 24 hours at 30° C. on a water bath shaker. The suspensions were then placed in 12-ml. centrifuge tubes and centrifuged, and the liquid phase was removed. The radioactive components in the medium and in the methanol extract of conidia were separated on thin-layer plates as previously described for *R. solani*.

RESULTS

Metabolism of Chloroneb-^{14}C by *R. solani*. A region of radioactivity (R_f 0.50) in addition to that corresponding to the parent compound (R_f 0.80) (Figure 1, *A* and *B*) was detected on chromatograms of samples of medium from *R. solani* cultures incubated 6 hours or longer with methyl- or ring-labeled chloroneb-^{14}C. The radio of radioactivity in the conversion product (region 2) to that in chloroneb (region 1) increased on extended incubation. Over 50% of the total radioactivity in the medium was in the conversion product by 24 hours (Figure 1*B*).

Methanol extracts of the hyphae also contained chloroneb-^{14}C and the ^{14}C-labeled metabolite found in the medium. Radioactivity in the metabolite increased up to 24 hours, but never exceeded that present in the chloroneb. A region of radioactivity (region 3) in addition to those corresponding to chloroneb and the metabolite was present on chromatograms of the methanol extracts of cells incubated with methyl-labeled chloroneb-^{14}C (Figure 2*B*), but was absent when ring-labeled chloroneb-^{14}C was used (Figure 2*A*).

There was no conversion of chloroneb in cultures con-

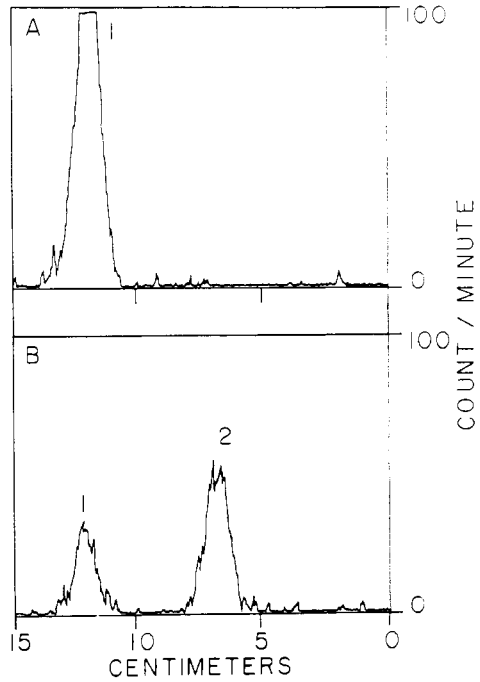

Figure 1. Radioactivity scans of thin-layer chromatograms

A. Sample of authentic chloroneb-^{14}C in basal medium
B. Sample of basal medium, *Rhizoctonia solani* mycelial disks incubated 24 hours in presence of 8 μg. per ml. of methyl-labeled chloroneb-^{14}C

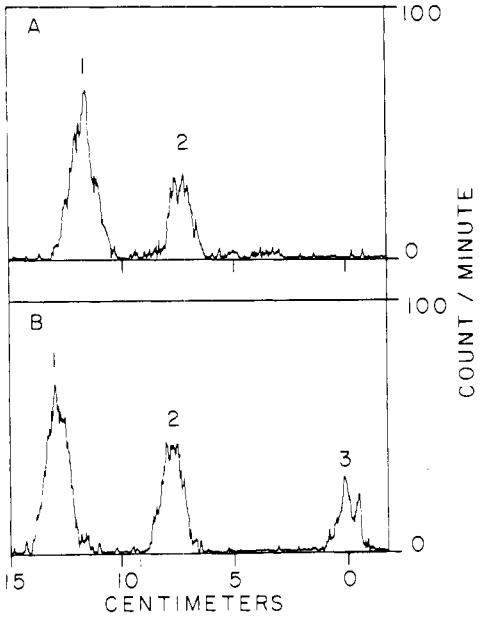

Figure 2. Radioactivity scans of thin-layer chromatograms of methanol extracts of mycelial disks of *Rhizoctonia solani* incubated 24 hours in basal medium containing chloroneb-^{14}C

A. 8 μg. per ml., ring-labeled
B. 8 μg. per ml., methyl-labeled

taining hyphal disks heated at 60° C. for 15 minutes prior to the 24-hour incubation.

In fungal disks incubated with methyl-labeled chloroneb-^{14}C the radioactivity remaining after methanol extraction accounted for approximately 5% of the total radioactivity added to the cultures, compared to about 0.3% in disks incubated with ring-labeled chloroneb-^{14}C.

Quantitative analysis of the radioactive compounds recovered from *R. solani* cultures after 24-hour incubation with ring-labeled toxicant showed that 54% of the radioactivity was present in the metabolite. The percentage of metabolite in the medium was approximately 17 times greater than in the cells, whereas the percentage of chloroneb in the cells was slightly more than twice that in the medium.

No detectable alteration of methyl- or ring-labeled chloroneb-^{14}C occurred during 24 hours of incubation with mycelium (10 mycelial disks per 2 ml. of basal medium) of *S. rolfsii*.

Identification of Metabolite. Infrared spectra of chloroneb and the metabolite are shown in Figure 3. The spectrum of chloroneb (Figure 3*A*) shows several absorption bands typical of aromatic compounds (3022, 1500, and 860 cm.$^{-1}$) (Nakanishi, 1962). The strong band at 860 cm.$^{-1}$ is characteristic of 1,2,4,5-tetra-substituted aromatic compounds. Absorption bands characteristic of methoxyl groups substituted on an aromatic nucleus are present at 2968, 2939, 2905 (the three appear as a triplet), and 2845 cm^{-1}. Bands are also evident for C—H bending (1460 and 1367), asymmetrical stretching (1215), and symmetrical stretching (1026). An absorption band for C—Cl appears at 778 cm^{-1}. Specific assignments cannot be made for many bands in the region between 1225 and 950 cm.$^{-1}$ (the region of aromatic in-plane bending) because of C—C, C—O, and other single bonds which absorb in this region.

Comparable bands which appear in the spectrum of the metabolite (Figure 3*B*) are the aromatic absorptions at 3020 (weak), 1500, and 859 cm.$^{-1}$; the methoxyl absorptions at 2960, 2873, 2842, 1459, 1213, and 1031 cm.$^{-1}$; and the C—Cl band at 810 cm.$^{-1}$. The primary aromatic methoxyl band at 2842 cm.$^{-1}$ is considerably lower in intensity in the spectrum of the metabolite than in the spectrum of chloroneb. The band about 1367 cm.$^{-1}$ is reduced in intensity to a mere shoulder on the primary methyl absorption band at 1380 cm.$^{-1}$.

The major difference between the two spectra is the occurrence of a strong and broad absorption band around 3400 cm.$^{-1}$ in the spectrum of the metabolite. This is usually indicative of hydroxyl groups. The infrared data suggest that the aromatic nucleus is present in the metabolite and that the 1,2,4,5-tetra-substituted pattern of the parent compound has not been altered. However, the replacement of either a methoxyl or chloride group by a hydroxyl appears certain.

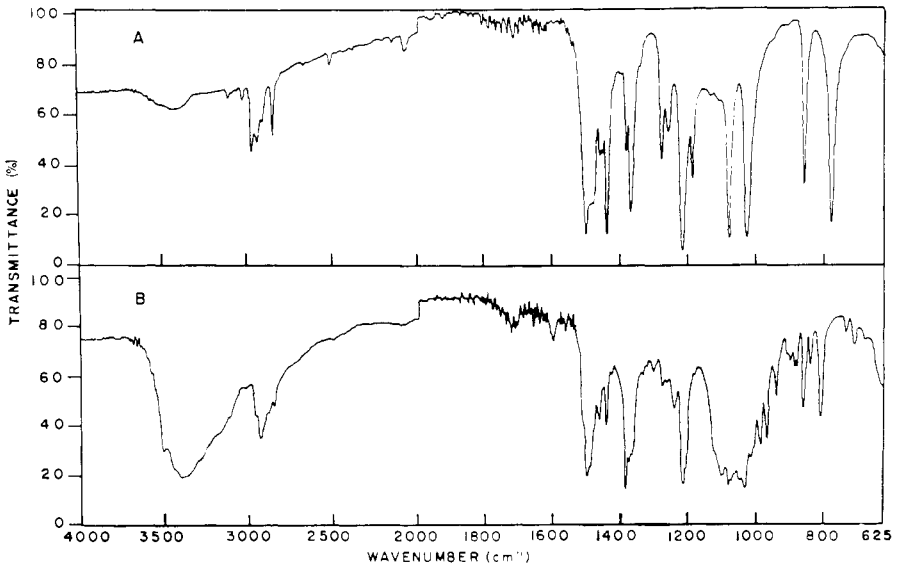

Figure 3. Infrared spectra

A. Chloroneb (1,4-dichloro-2, 5-dimethoxybenzene)
B. Chloroneb metabolite (2, 5-dichloro-4-methoxyphenol)

The NMR spectrum of chloroneb (Figure 4*A*) shows a six-proton singlet at approximately 3.84 p.p.m. (delta units) and a two-proton singlet at about 6.9 p.p.m. The six-proton absorption at 3.84 p.p.m. indicates the presence of two *O*-methyl groups and the two-proton absorption at 6.9 indicates the presence of two equivalent protons on the aromatic nucleus (Dyer, 1965).

The NMR spectrum of the metabolite (Figure 4*B*) shows a three-proton singlet at about 3.9 p.p.m. and one-proton singlets at approximately 7.18 and 7.35 p.p.m. The three-proton singlet corresponds to the protons on the *O*-methyl group and the two, one-proton singlets correspond to the now nonequivalent protons of the aromatic ring. The presence of a hydroxyl group is suggested by the one-proton singlet at about 4.25 p.p.m. and the weak absorptions at about 4.4 and 5.0 p.p.m. However, the assignment of a hydroxyl group to explain these absorptions is at best precarious. The metabolite did not dissolve readily in the acetone solvent and it slowly precipitated out of solution during the NMR analysis, which undoubtedly contributed to the poor quality of the integration curve. Nevertheless, on the basis of the infrared and NMR data the metabolite was tentatively identified as 2,5-dichloro-4-methoxyphenol.

The identity of the metabolite was substantiated by mass-spectrographic analysis. Using values of 12, 1, 16, and 35, respectively, for the mass of C, H, O, and Cl, a mass of 192 was determined for the compound. This value corresponds to that expected for the assigned structure.

Although chloroneb inhibited mycelial growth of *R. solani* and *S. rolfsii* at 5 to 8 μg. per ml., concentrations of 2,5-dichloro-5-methoxyphenol up to 16 μg. per ml. were not toxic.

Metabolism of Chloroneb-^{14}C by *N. crassa*. Two major regions of radioactivity were present on chromatograms of samples of medium in which conidia were incubated with either methyl- or ring-labeled chloroneb-^{14}C for 24 hours (Figure 5*C*). In both cases, region 1 (corresponding in R_f to chloroneb) had considerably greater activity than that of region 2 at the origin. Two major regions of radioactivity were also present on chromatograms of samples of methanol extract of conidia incubated with methyl-labeled chloroneb-^{14}C (Figure 5*B*). Region 1 again had considerably greater radioactivity than region 2. Only a trace of radioactivity other than that in the region corresponding in R_f to chloroneb was present on chromatograms of samples of methanol extracts of conidia incubated in the presence of ring-labeled chloroneb-^{14}C (Figure 5*A*). The trace of radioactivity at the origin probably corresponds to that of the metabolite found in the culture medium.

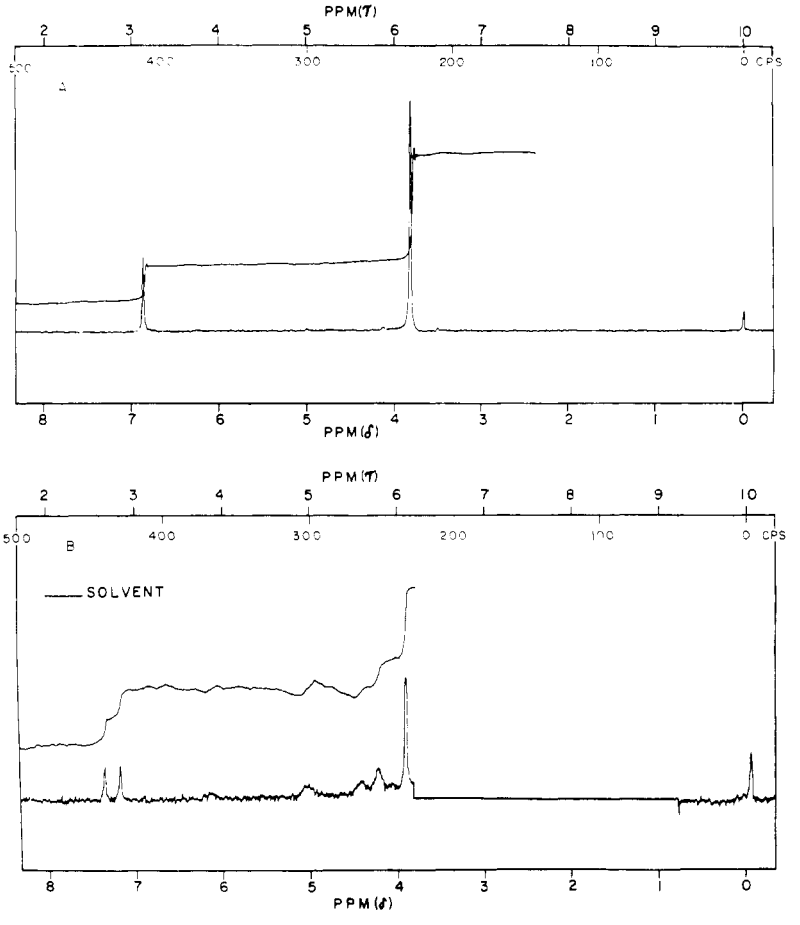

Figure 4. Nuclear magnetic resonance spectra

A. Chloroneb (1,4-dichloro-2,5-dimethoxybenzene) in CCl_4
B. Chloroneb metabolite (2,5-dichloro-4-methoxyphenol) in acetone

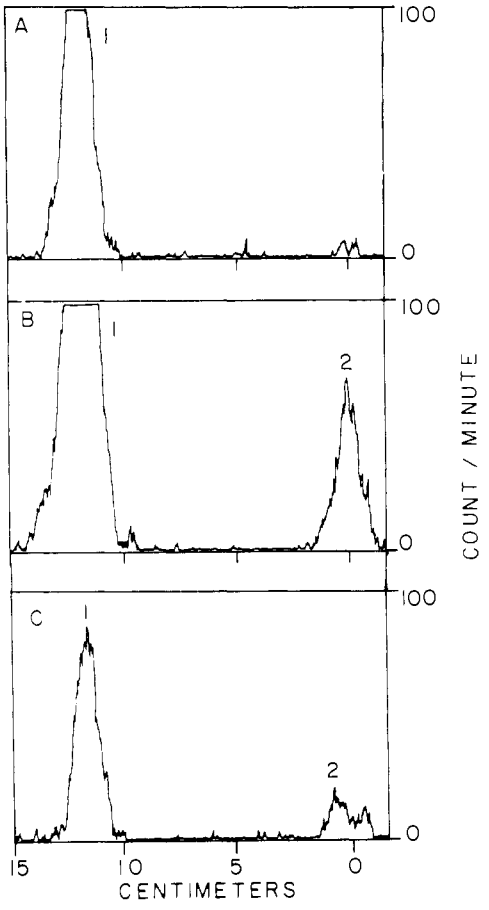

Figure 5. Radioactivity scans of thin-layer chromatograms of methanol extracts of *Neurospora crassa* conidia and of culture medium after incubation for 24 hours with 16 μg. per ml. of chloroneb -^{14}C

A. Methanol extract of conidia incubated with ring-labeled-^{14}C
B. Methanol extract of conidia incubated with methyl-labeled chloroneb-^{14}C
C. Medium from cultures incubated with ring-labeled chloroneb-^{14}C

The radioactivity which remained after methanol extraction in conidia incubated with methyl-labeled chloroneb-^{14}C accounted for approximately 4.5% of the total radioactivity added to the cultures. On the other hand, radioactivity remaining in the conidia incubated with ring-labeled toxicant was less than 0.8% of that added to the culture.

Saccharomyces pastorianus cells (about 5 mg. of dry weight per ml.) cultured in basal medium containing chloroneb-^{14}C did not metabolize the toxicant. All the radioactivity added to the cultures remained in the parent compound after 24 hours.

DISCUSSION

The metabolic conversion of chloroneb to 2,5-dichloro-4-methoxyphenol which occurs in cultures of *R. solani* also occurs in animals (Pease, 1967). Demethylation to the corresponding hydroxy derivatives frequently occurs in the metabolism of methoxylated aromatic compounds (Axelrod, 1956; Bray *et al.*, 1955; Henderson, 1957; Woodcock, 1964, 1967; Woodings, 1960). Henderson (1957) found that *Haplographium*, *Hormodendrum*, and *Penicillium* species all decompose monomethoxybenzoic acids by demethylation to the corresponding hydroxybenzoic acids. In the case of veratric acid (3,4-dimethoxybenzoic acid), *Hormodendrum* and *Penicillium* species selectively demethylate only one of the methoxyl groups to form vanillic acid (3-methoxy-4-hydroxybenzoic acid) (Henderson, 1957). In bacterial dissimilation of veratric acid by *Pseudomonas* species there is a sequential demethylation first to vanillic or isovanillic acid (3-hydroxy-4-methoxybenzoic acid) and then to protocathechuic acid (3,4-dihydroxybenzoic acid) (Woodings, 1960).

Dissimilation of aromatic methyl ethers yields the corresponding hydroxy derivatives and formaldehyde (Axelrod, 1956; Woodings, 1960). Chloroneb is probably oxidatively metabolized by *R. solani* to 2,5-dichloro-4-methoxyphenol and formaldehyde; however, labeled formaldehyde could not be identified in the culture medium resulting from the metabolic dissimilation of methyl-labeled toxicant (Menzer and Casida, 1965). This does not eliminate the possibility that the C_1 fragment is liberated as formaldehyde. Since chloroneb is slowly converted to the metabolite, the C_1 fragment may be utilized in cellular metabolism as rapidly as it is produced. Studies using methyl- and ring-labeled chloroneb-^{14}C indicate that part or all of the methoxyl groups which are removed are subsequently incorporated into cellular constituents. When methyl-labeled chloroneb-^{14}C was used in metabolism studies with *R. solani*, three regions of radioactivity were detected on chromatograms of methanol extracts of the hyphae. In addition to the regions corresponding to chloroneb-^{14}C and 2,5-dichloro-4-methoxyphenol-^{14}C, a third region or radioactivity occurred at the origin on the thin-layer chromatograms. The radioactivity of the compound(s) in this region undoubtedly originated from the methoxyl group of chloroneb, because radioactivity was not present at this location when ring-labeled chloroneb was used. Similarly, the appreciable amount of radioactivity which could not be extracted from the hyphae with methanol after exposure to methyl-labeled chloroneb originated from the methoxyl group of the toxicant. Almost all the radioactivity could be removed by methanol when ring-labeled toxicant was used. The labeled C_1 fragment is apparently transferred to tetrahydrofolic acid and utilized as a methyl source in the synthesis of methionine, thymine, and other methyl-requiring cellular constituents.

N. crassa also dissimilated a small fraction of methyl- and ring-labeled chloroneb-^{14}C. The unidentified product in the culture medium which remained at the origin of chromatograms may be a glycoside of the aglycone 2,5-dichloro-4-methoxyphenol. The metabolism must involve removal of at least one methyl group of chloroneb, since the radioactivity at the origin of chromatograms of methanol extracts of conidia incubated with methyl-labeled chloroneb-^{14}C was present in only trace amounts when ring-labeled toxicant was used. As with *R. solani*, there was greater incorporation of radioactivity into the hyphae with methyl-labeled than with ring-labeled toxicant.

With regard to the four fungal species used in this study, the ability or lack of ability to metabolize chloroneb is not the primary factor determining sensitivity to the toxicant. *S. pastorianus* did not metabolize chloroneb, but it was the most resistant of the four species tested (Hock and Sisler, 1969). *N. crassa* was relatively insensitive, but metabolized the toxicant more slowly than *R. solani*, which was highly sensitive. Chloroneb was not metabolized by *S. rolfsii*, an organism which was as sensitive to the toxicant as *R. solani*. However, the former organism never overcame the effect of the toxicant as did the latter. Ability to metabolize chloroneb, therefore, was probably the factor responsible for *R. solani* ultimately overcoming the effect of the toxicant. Although *R. solani* detoxified chloroneb, the process was rather slow. The effects of 5 to 8 μg. of chloroneb per ml. were overcome only when the amount of hyphae per milliliter of solution was rather large.

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