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Microbial Hydroxylation of the Herbicide *N*-(3,4-Dichlorophenyl)methacrylamide (Dicryl)

The fungus *Rhizopus japonicus* converts the herbicide *N*-(3,4-dichlorophenyl)methacrylamide (dicryl) to *N*-(3,4-dichlorophenyl)-2-methyl-2,3-dihydroxypropionamide. This metabolite was formed by the addition of two hydroxyl groups to the ethylenic double bond of dicryl. Its structure was elucidated by nuclear magnetic resonance and mass spectrometric analysis.

Fungi of the family *Mucoraceae* are known to hydroxylate fungicidal acylanilides and related compounds. The enzyme system responsible for this reaction is nonspecific. Hydroxyl groups are introduced on aromatic rings (Wallnöfer *et al.*, 1971), methyl groups attached to the furan ring system (Wallnöfer *et al.*, 1972a), and on aliphatic side chains (Wallnöfer *et al.*, 1972b).

Since fungi of the type responsible for these reactions are commonly found in soil (Domsch, 1960), it is not unreasonable to expect hydroxylations of acylanilides to be a mode of detoxication under natural soil conditions. Because of the difficulties of isolating metabolites from a complicated matrix such as soil, we are continuing to use the fungus *R. japonicus* as a model hydroxylating organism. A number of acylanilides and related compounds are being investigated with regard to finding the "biologically labile site" towards hydroxylation.

MATERIAL AND METHODS

Chemical and Instrumentation. A sample of the herbicide *N*-(3,4-dichlorophenyl)methacrylamide (dicryl), analytical grade, was obtained from Niagara Chemical Co., Naugatuck, Conn., USA. Instruments and conditions for their operation were those described earlier (Wallnöfer *et al.*, 1971, 1972a). The 220 MHz nmr spectrum, recorded on a Varian Instrument, was obtained through the Ontario Research Foundation, Sheridan Park, Ont., Canada.

Culture Methods and Analytical Procedures. *Rhizopus japonicus* was cultured in a synthetic glucose medium (500 ml) in 1-l. Fernbach vessels on a shaker at 27° in the presence of 1.5% calcium carbonate for 1 week (Wegener *et al.*, 1967).

A standard solution of dicryl (10 mg in 1 ml of acetone–propylene glycol 1:1) was added to the culture medium to a final concentration of 20 mg/l. To observe conversion of dicryl, 10-ml portions of the culture medium were extracted with chloroform (30 ml) daily and the disappearance of the starting material was followed by uv analysis after purification by tlc (solvent A, Table I; Wallnöfer *et al.*, 1971, 1972a,b).

Isolation of the Dicryl Metabolite. The culture medium was extracted with chloroform and the extract was purified by tlc (Wallnöfer *et al.*, 1972a). The solvent system for this purification was benzene–acetic acid, 9:1. The crude material after elution from the silica was recrystallized from chloroform–carbon tetrachloride, 1:1.

RESULTS AND DISCUSSION

As observed previously with other compounds, maximum conversion of dicryl was found when the fungal growth ended. Within 1 week, 87 μmol/l. of dicryl was transformed to 19 μmol/l. of metabolite M-1; 30 μmol/l. remained unchanged in the culture medium and the rest was found unchanged in the mycelium.

Some physical data (mp, uv maxima, and R_f values) for dicryl and its metabolite M-1 are given in Table I.

The mass spectrum of the metabolite M-1 showed a

Table I. Physical Data for Dicryl and Metabolite M-1

Compound	mp, °C	uv max, nm $CHCl_3$	tlc R_f A[a]	B[b]	C[c]	D[d]
Dicryl *N*-(3,4-Dichlorophenyl)methacrylamide	105.5	264	0.76	0.76	0.99	0.83
Metabolite M-1 *N*-(3,4-Dichlorophenyl) 2-methyl-2,3-dihydroxypropionamide	101–102	252	0.03	0.31	0.72	0.25

[a] A = chloroform–benzene, 9:1. [b] B = benzene–acetic acid, 9:1. [c] C = chloroform–acetone–acetic acid, 15:2:3. [d] D = ethyl acetate–benzene, 6:4.

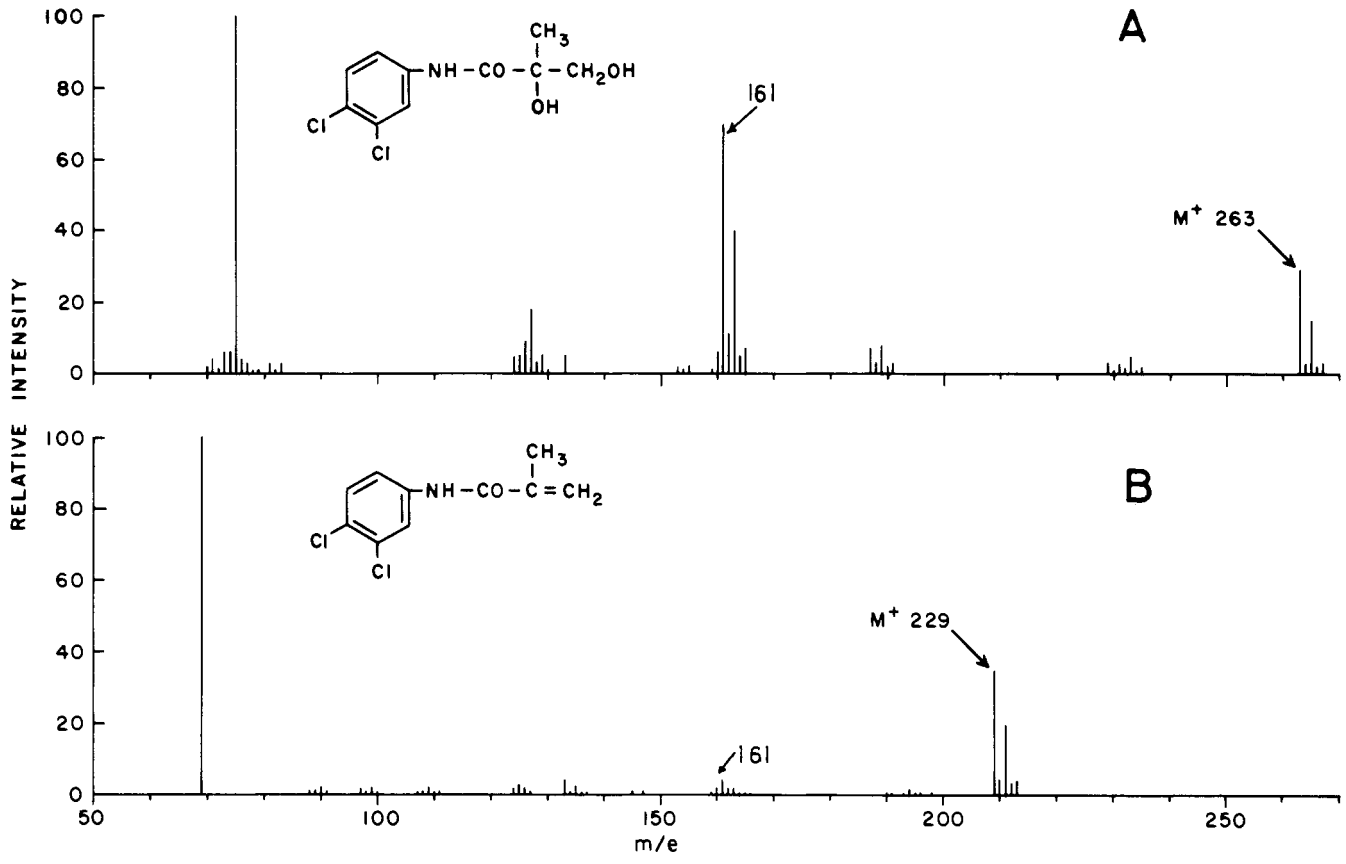

Figure 1. 70 eV mass spectra of dicryl metabolite M-1 (A) and dicryl (B)

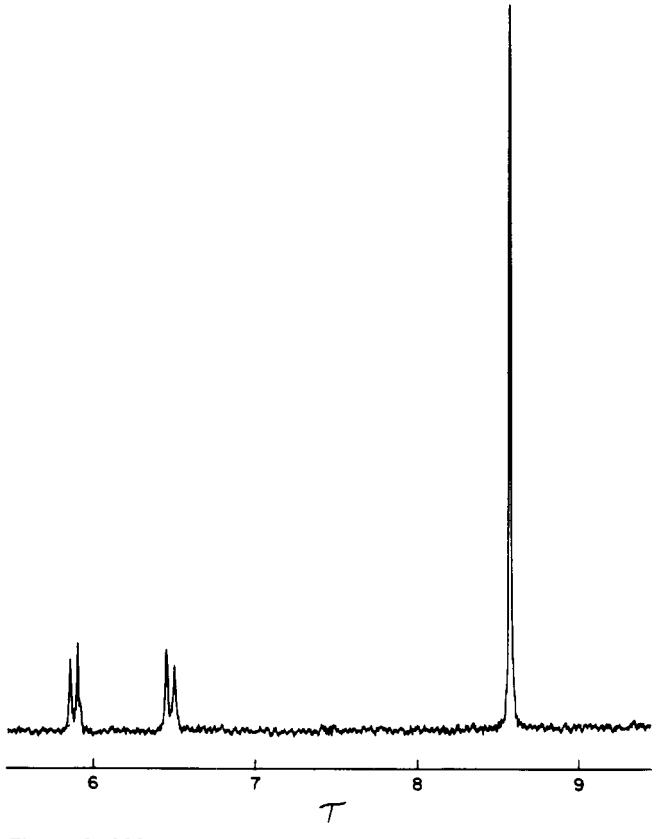

Figure 2. 220 MHz nmr spectrum of dicryl metabolite M-1 (aliphatic part); solvent: chloroform.

molecular ion at *m/e* 263 (Figure 1) corresponding to the addition of two hydroxy groups to dicryl, which shows a molecular ion at *m/e* 229. This molecular composition was confirmed by high-resolution measurement (calcd for $C_{10}H_{11}Cl_2NO_3$: 263.0116; found: 263.0115). The fragment ion at *m/e* 161 ($ArNH_2^+$; calcd for $C_6H_3Cl_2N$: 160.9801; found: 160.9797) as well as *m/e* 187 (ArN=C=O) and *m/e* 189 (ArNHCHO), (Ar = 3,4-dichlorophenyl) indicates an unchanged dichlorophenyl moiety with the two hydroxy groups in the side chain. The nmr spectrum (the aliphatic part of which is depicted in Figure 2) showed the presence of three aromatic protons (τ 2–2.8) an AB quartet (5.85, 5.90, 6.43, 6.49) corresponding to two protons and a methyl singlet at τ 8.56. These data are compatible only with M-1 being *N*-(3,4-dichlorophenyl)-2′-methyl-2′,3′-dihydroxypropionamide.

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Parathion Degradation in Submerged Rice Soils in the Philippines

The persistence of parathion (*O,O*-diethyl *O*-*p*-nitrophenyl phosphorothioate) in four Philippine rice soils was investigated in submerged and upland conditions. The parathion in the soils after incubation at 30° was extracted with a hexane–acetone mixture and the amount was determined by gas chromatography. The insecticide disappeared more rapidly from submerged soils than from upland soils. In submerged soils parathion was reduced to aminoparathion (*O,O*-diethyl *O*-*p*-aminophenyl phosphorothioate). Autoclaving of the soils increased the persistence of parathion under submerged conditions, indicating microbial participation in its degradation. Parathion degraded faster in flooded soil inoculated with parathion-hydrolyzing *Flavobacterium* sp. than in uninoculated soil.

Recent studies indicate that several chlorinated hydrocarbon insecticides persist for years in upland soils but readily break down if the soil is submerged (Castro and Yoshida, 1971; Yoshida and Castro, 1970). Likewise diazinon, an organophosphate, appears to degrade rapidly in submerged soils (Sethunathan and MacRae, 1969).

The fate of the insecticide parathion (*O,O*-diethyl *O*-*p*-nitrophenyl phosphorothioate) in submerged soil is not completely understood, however. Lichtenstein and Schulz (1964) studied the persistence of parathion in upland soils. According to them, a high soil moisture content and the presence of soil microflora favored the degradation of parathion in soils. The major pathway of parathion breakdown in soils (Lichtenstein and Schultz, 1964), lake sediments (Graetz *et al.*, 1970), and microorganisms in pure culture (Mick and Dahm, 1970; Zukerman *et al.*, 1970) involves nitro reduction to its amino compound, aminoparathion (*O,O*-diethyl *O*-*p*-aminophenyl phosphorothioate).

This paper reports a study aimed at comparing the rates of decomposition of parathion in upland and in submerged soils. The effect of *Flavobacterium* sp., which had been shown earlier to hydrolyze diazinon and parathion (Sethunathan and Yoshida, 1972), on the metabolism of parathion in submerged soil was also studied.

MATERIALS AND METHODS

Persistence in Submerged and Upland Soils. We investigated the persistence of parathion in four Philippine soils: the air-dried soils Maahas clay (pH 6.6, organic matter 2.0%, total N 0.14%); Luisiana clay (pH 4.7, organic matter 3.2%, total N 0.21%); a clay loam from Pila (pH 7.6, organic matter 1.5%, total N 0.09%); and Casiguran sandy loam (pH 4.8, organic matter 4.4%, total N 0.2%). Air-dried samples of each soil were passed through a 2-mm sieve. Twenty grams of soil was placed in test tubes (25 × 180 mm) and 25 ml of distilled water was added to half of the soil samples to obtain a flooded soil condition under standing water 5-cm deep. To simulate upland conditions 4 to 6.5 ml of distilled water was added to the other samples to keep the soil moisture at field capacity. Parathion (98.76%, City Chemical Corporation, New York, N. Y., U.S.A.) dissolved in 50 μl of acetone was introduced into the soil in each tube at a concentration of 3000 ppm. The soils were incubated in an incubator at 30°.

For each treatment three replicate tubes were withdrawn for residue analysis at the start of the experiment, 7 and 14 days after incubation. To extract parathion from the soils we used the method used for diazinon (Sethunathan *et al.*, 1971). This method gave recoveries of 91 to 96%. The hexane layer was suitably diluted before being injected into a gas chromatograph equipped with a cesium bromide detector. The temperature setting was 215° for the column and the detector and was 225° for the injector port. The other gas chromatographic conditions were the same as those reported for analysis of diazinon (Sethunathan *et al.*, 1971). Under these conditions, the retention time for parathion was 3.03 min and for aminoparathion was 2.48 min.

Biodegradation of Parathion. To determine the role of microorganisms in degrading parathion in submerged soil, the rates of degradation in autoclaved and nonautoclaved lots of four soils were compared. The soils were autoclaved at 121° for 1 hr on each of three successive days to reduce microbial population. The parathion was placed in an aqueous solution (instead of dissolving the parathion in acetone) and passed through a Millipore filter (pore size, 0.45 μ). Twenty-five milliliters of the filtered solution (8 ppm) was then added to 20 g of autoclaved and nonautoclaved soils. The residues in three replicate tubes for each treatment were analyzed at the start of the experiment and at 10 days after incubation. Nonautoclaved soil undergoes rapid reduction following flooding primarily due to intense microbiological activity; however, a thin surface layer remains oxidized. These distinct zones were absent in flooded autoclaved soils, indicating reduced microbial activity.

In earlier work we found that a species of *Flavobacterium* isolated from paddy water of a field previously treated with diazinon was highly efficient in hydrolyzing diazinon and parathion (Sethunathan and Yoshida, 1972). To test the ability of this species to metabolize parathion in submerged soil, the bacterium was grown for 4 to 7 days on modified Wakimoto agar (International Rice Research Institute, 1970) and then suspended in sterile distilled water. One milliliter of the suspension was added to tubes containing 20 g of Maahas clay and the soil was flooded with 25 ml of 8-ppm parathion solution; 1 ml of sterile distilled water was added to the controls instead of the bacterial suspension. The soil samples were then incubated at 30°. Three tubes for each treatment were removed