Herbicide Transformation
I. Studies with Whole Cells of Fusarium solani

R. P. LANZILOTTA1 AND DAVID PRAMER
Department of Biochemistry and Microbiology, Rutgers-The State University,
New Brunswick, New Jersey 08903

Received for publication 19 November 1969

A strain of Fusarium solani isolated from soil by enrichment techniques used propanil (3',4'-dichloropropionanilide) as a sole source of organic carbon and energy for growth in pure culture. The primary product of the transformation of propanil by F. solani was isolated and identified as 3,4-dichloroaniline (DCA). This compound accumulated in the medium to a level (80 μg/ml) which stopped further herbicide utilization. Herbicide utilization by F. solani was influenced by various environmental and nutritional factors. It was more sensitive to acid than alkaline pH. Added glucose and yeast extract increased the rate of propanil decomposition, and the reduced aeration retarded growth of the fungus and herbicide utilization. The growth of F. solani on propionate was inhibited by added DCA.

Propanil is a relatively new acylaniline herbicide used predominantly in rice fields in which effective chemical control of weeds was nonexistent. Because of the importance of rice in world agroeconomy, the compound promises to be a significant agricultural chemical, perhaps rivaling even the auxin-derived herbicides such as 2,4-D (2,4-dichlorophenoxyacetic acid). Information regarding its fate in nature, including the identification of organisms able to transform it, will be of prime importance in its ultimate evaluation.

Earlier work from this laboratory (2) gave evidence for the oxidative alteration of this compound by microorganisms in soil. It was of interest, then, to attempt the isolation in pure culture of organisms responsible for its transformation in soil.

MATERIALS AND METHODS

Culture. The propanil-degrading fungus, Fusarium solani (Martius) Saccardo (Collin Booth, Commonwealth Mycological Institute, Surrey, England, personal communication) was an isolate obtained from Nixon Sandy loam soil by conventional enrichment techniques (Lanzilotta, Ph.D. Thesis, Rutgers University, 1968). The isolate was selected specifically for its ability to grow in mineral salts medium with propanil as sole source of organic carbon. The fungus was maintained by transfer on Czapek-Dox agar slants. Tests showed that continued subculturing on media free of propanil did not result in loss of ability to degrade the herbicide.

Media. The propanil-mineral salts medium consisted of propanil, 0.16 g; NH₄Cl, 0.2 g; MgSO₄·7H₂O, 0.1 g; KH₂PO₄, 1.19 g; K₂HPO₄, 0.216 g; NaCl, 0.1 g; CaCl₂·2H₂O, 0.01 g; Fe₃(SO₄)₂·0.0005 g; and 1,000 ml of distilled water. The final medium (pH of 6.0) could be sterilized by autoclaving (121°C) without significant hydrolysis of the herbicide.

In autoradiographic studies of propanil metabolites, the medium was modified to contain 0.10 g of propanil per liter. An absolute ethyl alcohol solution containing 4.6 mg of ring-labeled 14C-propanil per ml (specific activity, 2.18 mc/g) was prepared, and 0.1 ml (1.0 μc) of this solution was added to each 50 ml of medium before autoclaving. It was predetermined that the ratio of unlabeled to 14C-labeled propanil used here was suitable for autoradiographic identification of the herbicide and its metabolites.

In a study of the effect of 3,4-dichloroaniline (DCA) on the growth of F. solani, a medium was used which lacked NaCl, but was otherwise identical in salt composition to that described above; it contained sodium propionate at a level of 0.4 g/liter at pH 7.4.

In experiments designed to examine the effect of pH on fungal degradation of propanil in the basal salts medium, the ratio but not the molar concentration of mono- and dibasic phosphate salts was varied to obtain the desired initial pH.

The effects of glucose (0.05%), yeast extract (0.01%), Hoagland's trace element solution (1.0 ml/liter), and reduced aeration on the decomposition of propanil by F. solani were determined in a series of separate simple tests. In each case the medium was at pH 7.5. The glucose and trace elements were added to the propanil-mineral medium before autoclaving. The yeast extract was filter-sterilized and added aseptically after autoclaving. All flasks were
incubated on a rotary shaker, except for one series which remained static to reduce aeration.

**Solvent extractions.** Primary aromatic amines were recovered from culture media as follows. The solution was brought to approximately pH 13 with NaOH and extracted twice with a half-volume of chloroform. The chloroform extracts were combined and partitioned twice with a half-volume of 1.0 N HCl. The acidic aqueous extracts were combined, brought to pH 13 by the addition of solid NaOH, and then extracted twice with a half-volume of chloroform. The chloroform extract was dried over anhydrous Na$_2$CO$_3$ and then concentrated in a flash evaporator (Buchler Instruments, Fort Lee, N.J.). A portion of solid residue was recrystallized twice from ligroin and stored.

To obtain acidic, neutral, and basic metabolites for analysis by autoradiochromatography, the following extraction procedure was employed. The medium was extracted with two half-volumes of chloroform. It was then adjusted to pH 1.0 with concentrated HCl and reextracted with chloroform as before. The acidified medium was then adjusted to pH 13.0 with solid NaOH, and the extraction procedure repeated once again. The three chloroform extracts were combined, dried over anhydrous Na$_2$CO$_3$, and concentrated to dryness in a flash evaporator. The residue was dissolved by using 10 ml of acetone for each 50 ml of medium originally extracted, and the acetone solutions were used for autoradiochromatography.

The foregoing extractions were also performed on uninoculated medium, and the products were used as controls in all analytical work.

**Thin-layer chromatography.** Thin-layer chromatography was performed by using silica gel strips cut from Eastman thin-layer chromatogram sheets with and without fluorescent indicator (Distillation Products Industries, Division of Eastman Kodak, Rochester, N.Y.). Benzene and acetone (95:5) and benzene alone were used to develop the chromatograms.

When the chromatogram sheet was impregnated with a fluorescent indicator, the spots were located by their ability to quench fluorescence under ultraviolet radiation. Primary aromatic amines, such as DCA, were also detected as bright yellow spots immediately after spraying with Ehrlich's reagent. Extremely small quantities (<0.1 µg) of DCA were detected by spraying a solution of N-(1-naphthyl) ethylene diamine dihydrochloride in absolute ethyl alcohol on chromatograms previously treated with 1.0% NaNO$_3$ in 1.0 N HCl. This process produces diazonium salts from primary aromatic amines which then couple with the ethylene derivative to form bright blue to purple spots.

In autoradiochromatography, silica gel strips were spotted with 50 µlitters of the final acetone solutions resulting from the chloroform extracts of culture media described above. They were subsequently developed with the solvent systems indicated above and then placed in contact with no-screen medical X-ray film (Eastman Kodak Co., Rochester, N.Y.). After exposures of 2 or 7 days, the films were developed and printed.

**Cultural conditions.** In most cases, media were dis-
on thin-layer chromatography. Both react with the spray reagents, develop identical spots, and melt at 72°C. Therefore, additional evidence was required for conclusive identification. Since the infrared spectrum of 4-chloroaniline is readily distinguishable from that of DCA (Standard Infrared Spectra, Samuel P. Sadtler and Sons, Inc., Philadelphia, Pa.) and since the infrared spectrum of the unknown compound matched identically to that of an authentic sample of DCA (Fig. 1), it was concluded that the lone aniline detected on thin-layer chromatographs was, in fact, DCA.

As mentioned, the solvent extraction procedure used initially was designed to recover primary aromatic amines and excluded any metabolic product not sufficiently basic to be extracted from chloroform with 1.0 N HCl, e.g., TCAB. Therefore, to obtain a more complete profile of the products of propanil metabolism in mineral medium, a second extraction procedure was applied that recovered acidic, neutral, and basic compounds of ring-labeled 14C-propanil. The autaradiochromatogram shown in Fig. 2 was developed ascendingly with benzene and acetone (95:5) and that illustrated in Fig. 3 was developed with benzene. The chromatograms were left in contact with X-ray film for 7 days, and the film was then developed and printed. The right side of both figures represents extracts of uninoculated media. Clearly, Fig. 2 and 3 show that the controls contained only one major radioactive component, propanil, and that the remaining materials were attributed to radioactive impurities reported by the manufacturer and amounted to 1.4%. The absence of major radioactive compounds other than propanil in the uninoculated control media is evidence that the herbicide was stable to both autoclaving and the chemical fractionation procedure.

No residual propanil was detected by chromatography of extracts of inoculated media (left sides of Fig. 2 and 3). The major radioactive metabolite had the Rf value of DCA in both solvent systems and responded to spray reagents as would DCA. In addition, several minor radioactive components were detected in extracts of the inoculated media in both solvent systems.
Although the identity of these substances was not established, none corresponded to 3,3',4,4'-tetrachloroazobenzene in $R_f$ value, and none was a primary aromatic amine. Actually, there is some doubt that they were fungal metabolites of propanil since their appearance coincided with the formation of a reddish-brown substance in the medium which was subsequently linked with a photochemical alteration of DCA. In fact, when DCA was added to medium that was sterilized but not inoculated and stored in the light, the reddish-brown material appeared after 2 to 3 days. When this medium was extracted and chromatographed, the material was resolved into three to five components, depending on the time of incubation before extraction, the type and intensity of light source, and the solvent system used. The $R_f$ values of some of these compounds matched those of the radioactive unknowns. Furthermore, like the unknowns, none of the photochemically produced substances were primary aromatic amines.

Growth of *F. solani* on propanil as the only carbon source was limited by other than the supply of herbicide. To examine this phenomenon more fully, an experiment was designed to correlate growth with propanil degradation. In this study, the decomposition of propanil was conveniently followed by analysis of DCA released in the medium during fungal development (Fig. 4). It can be seen that DCA production reached a maximum in 7 days after inoculation or 5 days after the onset of propanil degradation, and, at that time, 70% of the propanil present initially in the medium had been decomposed. The course of DCA formation was essentially sigmoidal. Growth paralleled propanil decomposition and was maximum after 6 days. The decrease in mycelial mass noted from the sixth through the eighth day of incubation suggested cell leakage or lysis induced by the accumulation of a toxic level of DCA. Filaments harvested on days 8, 9 and 10 were highly vacuolated and frequently devoid of cytoplasm when examined under the microscope. It appeared that at a concentration of approximately 4 mg/50 ml, DCA interferes with metabolic activity and with the osmotic integrity of cells. When 70% (5.6 mg) of the added herbicide was transformed, sufficient DCA had accumulated in the medium to inhibit further transformation.

The slight decrease in DCA concentration recorded between days 7 and 8 coincided with the formation of a reddish-brown material in the medium and therefore was attributed, at least in part, to the photochemical reactions.
TABLE 1. Effect of 3,4-dichloroaniline (DCA) concentration on growth of Fusarium solani

| DCA mg | Days of growth * | 2 | 3 | 4 |
|--------|----------------|---|---|---|
| 0      |                | 3.0 | 14.4 | 15.6 |
| 2      |                | 0.5 | 4.7  | 16.9 |
| 3      |                | 0.6 | 1.6  | 10.7 |
| 4      |                | 0   | 0.2  | 1.0  |
| 5      |                | 0   | 0    | 0    |

* Expressed as milligrams (dry weight) per 50 ml.

To test the supposition that DCA inhibits fungal metabolism, an experiment was performed to measure the effect of various concentrations of DCA on the growth of F. solani in propionate. (Propionate and DCA are produced in equimolar amounts by the hydrolysis of propanil, and it is the only substrate available to the fungus as it develops on the herbicide.) The amount of propionate supplied was approximately 10 times greater than that which the fungus obtains by complete hydrolysis of the amount of propanil (8 mg/ml) usually supplied in the medium. Nevertheless, as can be seen from the data listed in Table 1, DCA is toxic. Inhibition of growth of F. solani on propionate increased with increasing DCA concentration. The rate but not the extent of growth was reduced by 2 mg of DCA. Toxicity was more marked at 3 mg, and, at 4 mg, DCA reduced growth in 4 days by 90%. Five milligrams of DCA/50 ml of medium was completely inhibitory, and there appears to be support for the suggestion that accumulation of DCA to toxic levels inhibits the continued hydrolysis of propanil by F. solani.

Results of a study of the influence of pH on the decomposition of propanil showed that the onset of decomposition was more rapid at increased pH (4.0 to 7.5). After 7 days, however, there was no significant difference in the extent of DCA formation in media of pH 5.0 to 7.5. No optimum was discernable, but there was no growth at pH 4.0.

The effects of various nutritional factors and aeration on the utilization of propanil by F. solani are summarized in Table 2. No treatment decreased the 3-day lag that consistently preceded herbicide utilization by the fungus. Both glucose and yeast extract increased the subsequent rate of propanil decomposition. Only glucose increased the extent of decomposition which reached a maximum of 81% in 5 days, after which an apparent loss of DCA was observed.

Propanil decomposition was favored by increased aeration. Static cultures had degraded only 47% of the added herbicide after 7 days, whereas 70% was degraded in the shaken cultures.

**DISCUSSION**

The results of this study showed that F. solani hydrolyzed propanil and formed DCA and propionic acid. The propionate served as a source of carbon and energy for growth of the fungus, whereas DCA accumulated as the major metabolic product. No evidence could be obtained to indicate that the fungus could further transform the DCA, although such reactions as dehalogenation (8) and conversion to azo-benzenes (3, 4) of chloroanilines are reported to occur in soil. In addition, no phenolic products were evident which may result from the ring hydroxylation that often precedes ring rupture in the microbial dissimilation of chloroaryls (6, 7, 10). In contrast, the relative recalcitrance of chloroanilines is supported in work by Alexander and Lustigman (1) who demonstrated that aniline disappeared from a dilute soil suspension in 4 days, but o-, m-, and p-chloroanilines persisted for more than 64 days.

The apparently nonmetabolic, photochemical products formed from DCA in mineral medium may be complex polymers of DCA in which the primary amino groups are linked covalently and, therefore, are unable to react with reagents used to detect and identify anilines.

The ability of DCA to accumulate in the mineral medium and inhibit further metabolic activity of the fungus is not understood. Possibly, DCA may repress further synthesis of the acyl-amidase that hydrolyzes propanil or otherwise inhibits its action. This inhibition may cause suppression of growth even when the need for propanil hydrolysis is obviated by supplying the products of hydrolysis directly to the medium. This is reasonable in light of the fact that the
acylamidase involved most likely functions in the usual metabolic activity of the fungus, acting on some "normal" substrate other than propanil. In other words, bypassing the need to hydrolyze propanil for growth may not bypass the need for the acylamidase involved in that hydrolysis.

Subsequent kinetic studies (Lanzilotta and Pramer, Appl. Microbiol., submitted for publication) have showed, however, that the acylamidase of F. solani does not exhibit product inhibition, at least when acetanilide is used as substrate.

The increased rate of decomposition of propanil in mineral media supplemented with glucose and yeast extract was apparently a reflection of an increased cell population at the onset of propanil utilization rather than to increased activity.

The inhibition of propanil decomposition by the addition of Hoagland's trace element solution may have been the result of using too high a level of one or more specific metals or to a synergistic effect resulting from a combination of metals that are not toxic individually.

ACKNOWLEDGMENTS

This investigation, published as part of the Journal Series of the New Jersey Agricultural Experiment Station, was supported by Public Health Service grant ES-16 from the Division of Environmental Health Sciences.

We are grateful to Rohm & Haas Co., Philadelphia, Pa., for generously supplying propanil and 14C-propanil.

LITERATURE CITED

1. Alexander, M., and B. K. Lustigman. 1966. Effect of chemical structure on microbial degradation of substituted benzenes. J. Agr. Food Chem. 14:410-413.
2. Bartha, R., R. P. Lanzilotta, and D. Pramer. 1967. Stability and effects of some pesticides in soil. Appl. Microbiol. 15:67-75.
3. Bartha, R., H. A. B. Linke, and D. Pramer. 1968. Pesticide transformation: production of chloroaazobenzenes from chloroanilines. Science 161:582-583.
4. Bartha, R., and D. Pramer. 1967. Pesticide transformation to aniline and azo compounds in soil. Science 156:1617-1618.
5. Bratton, A. C., E. K. Marshall, Jr., D. Babbitt, and A. R. Hendrickson. 1939. A new coupling component for sulfanilamide determination. J. Biol. Chem. 128:537-550.
6. Byrde, R. J. W., and D. Woodcock. 1957. Fungal detoxification. 2. The metabolism of some phenoxy-N-alkyl carboxylic acids by Aspergillus niger. Biochem. J. 65:682-686.
7. Gaunt, J. K., and W. C. Evans. 1961. Metabolism of 4-chloro-2-methyl-phenoxycetic acid by a soil microorganism. Biochem. J. (Proc. Biochem. Soc.) 79:25P-26P.
8. Kaufman, D. D., and P. C. Kearney. 1965. Microbial degradation of isopropyl-N-3-chlorophenylcarbamate and 2-chloroethyl-N-3-chlorophenylcarbamate. Appl. Microbiol. 13:443-446.
9. Lu, K. C., J. E. Dawson, and M. Alexander. 1959. A microchemical method for detecting antifungal substances. Arch. Mikrobiol. 33:182-183.
10. MacRae, I. C., M. Alexander, and A. D. Rovira. 1963. A decomposition of 4-(2,4-dichlorophenoxy) butyric acid by Flavobacterium sp. J. Gen. Microbiol. 32:69-76.
11. Pese, H. L. 1962. Separation and colorimetric determination of monuron and diuron residues. J. Agr. Food Chem. 10:279-281.