Inhibition of Phenylamide Hydrolysis by *Bacillus sphaericus* with Methylcarbamate and Organophosphorus Insecticides

G. ENGELHARDT AND P. R. WALLNÜFER*

Bayerische Landesanstalt für Bodenkultur und Pflanzenbau, 8 Munich 19, West Germany

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The degradation of the phenylamide herbicides monolinuron, linuron, and solan by cultures of *Bacillus sphaericus* ATCC 12123 was inhibited by the methylcarbamate insecticides metmercapturon, aldicarb, propoxur, and carbaaryl and by the organophosphorus insecticides fenthion and parathion. The extent of inhibition was largest with metmercapturon and smallest with parathion. Inhibition of hydrolysis of the two phenylurea herbicides was greater than of the acylanilide compound. Tests with crude enzyme preparations of aryl acylamidase derived from *B. sphaericus* showed that the inhibition of the hydrolysis of linuron with methylcarbamates is a competitive one. The insecticides tested did not induce the enzyme, nor could they serve as its substrate.

The increased use of combinations of pesticides raises new problems relative to their potential interactions in plants and soil and their persistence properties in these materials. Numerous reports on activity of certain phenylamide herbicides as affected by carbamate or organophosphorus insecticides have been published.

Thus, the phytotoxicity of monuron [3-(p-chlorophenyl)-1,1-dimethylurea] and diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] increased after these herbicides had been applied simultaneously with the systemic insecticide phorate [O,O-diethyl S-[(ethylthio)methyl] phosphorothioate] (10). Linuron [3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea], applied in combination with phorate or methomyl [S-methyl-N-[(methylcarbamoyl)oxy]thioacetimidate], reduced seedling vigor in soybeans (12). Diuron applied with some organophosphorus insecticides was more phytotoxic to oats or corn (16, 17) than diuron by itself. Usage of chlorbromuron [3-(4-bromo-3-chlorophenyl)-1-methoxy-1-methylurea] with the insecticide carbofuran (2,3-dihydro-2,2-dimethyl-7-benzo[def]uran methylcarbamate) resulted in an increased seedling susceptibility to chlorbromuron. The basis for these phenomena appeared to be related to a reduced metabolism of chlorbromuron in corn and in barley shoots (11). Metabolism of chlorpropham (isopropyl m-chlorocarbanilate) and linuron in leaf tissues of wheat, beans, and plantains was inhibited by various organophosphorus insecticides, and the metabolism of propanil (3',4'-dichloropropianilide) in leaf tissues of tomato plants was strongly inhibited by some organophosphorus and carbamate insecticides (3). Carbaryl (naphthyl methylcarbamate) inhibited the metabolism of linuron in carrots tolerant to this herbicide (5). The tolerance of rice plants to propanil is based on the detoxication of the herbicide after its hydrolysis by an aryl acylamide (9). This enzyme has been shown to be strongly inhibited by many carbamate and organophosphate insecticides (6, 9, 15).

Organophosphorus and carbamate insecticides also inhibited the degradation of some amide, carbamate, and urea herbicides by soil microorganisms. Thus, the microbial metabolism of chlorpropham was inhibited when several methylcarbamate insecticides were present. Enzymatic studies with a purified enzyme isolated from chlorpropham-degrading soil microorganisms revealed that methylcarbamates are competitive inhibitors of the phenylcarbamate-hydrolyzing enzyme (14). Similarly, the hydrolysis of propanil by a soil fungus was strongly inhibited by p-chlorophenyl methyl carbamate and carbaryl (13).

The hydrolysis of linuron and several other herbicides and fumigicides of the phenylamide type by cultures of *Bacillus sphaericus* and by a purified aryl acylamidase derived from these organisms has been demonstrated in our laboratory (7, 8, 20–22). The purpose of this investigation was to determine if the hydrolysis of some selected phenylamides by *B. sphaericus* could be inhibited by several methylcarbamate and organophosphorus insecticides, as well as to
determine the mechanism of such an inhibition.

**MATERIALS AND METHODS**

**Chemicals.** Linuron, [14C]ureido-linuron, monuron, and monolinuron [3-(p-chlorophenyl)-1-methoxy-1-methylurea] were purchased from the Farberwerke Hoechst, Frankfurt, Germany. Solan (3-chloro-2-methyl-p-valeronitrilide) was provided by Niagara, Middleport, N.Y.; fenthiion[O,O-dimethyl-O-4-(methylthio)-m-tolylphosphorothioate] was provided by Bayer AG, Leverkusen, Germany; and diallate (S-[2,3-dichloroallyl] diisopropylthiocarbamate) was provided by BASF, Ludwigshafen, Germany. Carbaryl (naphthyl methylcarbamate) and parathion [O,0-diethyl-O-(p-nitrophenyl)phosphorothioate] were purchased from Riedel-DeHae AG, Seelze-Hannover, Germany. Propoxur (o-isopropoxyphenyl methylcarbamate), metmercapturon (4-methylthio-3,5-dimethylphenyl methylcarbamate), aldicarb (2-methyl-2-[methylthio]propionaldehyde o-[methylcarbamoyl]-oxime), and thiram [bis(dimethylthiocarbamoyl)disulfide] were purified from commercial formulations as described (20).

**Growth conditions and analytical methods.** Cultivation of *B. sphaericus* ATCC 12123 was performed in 25 ml of minimal medium (22) in 100-ml Erlenmeyer flasks on a shaker at 120 rpm and 30 °C. Herbicide concentration was 5 × 10^−4 M, whereas concentrations of the inhibitors varied from 10^−8 to 10^−4 M. Because of the low water solubility of most compounds, the extracts were prepared in acetone-propylenglycol (1:1), from which the specific amounts were slowly added to the culture media (usually 4 µl/ml). Growth of the bacteria was determined by measuring the optical densities of the cultures at 578 nm with an Eppendorf photometer.

For quantitative determinations of herbicide degradation the bacteria of each culture were removed by centrifugation, followed by the extraction of 10 ml of supernatant with chloroform (3 × 10 ml). Fifteen-milliliter portions of extracts, previously dried over anhydrous Na₂SO₄, were chromatographed on thin-layer chromatographic plates coated with silica gel (Schleicher and Schuell 150 G/LS 254). The developing solvent was a mixture of chloroform-benzene (9:1). The compounds thus separated were visualized with a Camag ultraviolet lamp (λ_max = 254 nm). Areas containing residual compounds were then removed from the plates, eluted with 25 ml of methanol, and subjected to ultraviolet analysis with a Beckman spectrophotometer model DB and standard solutions for comparison purposes.

**Preparation of crude extracts and enzyme assay.** The preparation of crude extracts of *B. sphaericus*, after growing in the presence of different pesticides, and assays for aryl acylamidase were performed as described (7, 21).

**RESULTS**

**Inhibition of phenoxyamide degradation in growing cultures of *B. sphaericus* ATCC 12123.** The biodegradation of linuron and monolinuron and of solan by *B. sphaericus* was inhibited by all the methylcarbamate and organophosphorus insecticides tested (Table 1). However, the growth of the bacteria was not influenced by any of the compounds. The extent of insecticide inhibition of phenoxyamide hydrolysis was different with each herbicide. The most effective or least effective inhibitor of one herbicide was also most or least effective with the other two herbicides tested.

Metmercapturon was the strongest inhibitor in each case. A total inhibition of monolinuron degradation with metmercapturon was achieved at an inhibitor concentration of 10^−7 M, of linuron degradation at 10^−6 M, and of solan degradation at 5 × 10^−8 M. The next strongest inhibitor, aldicarb, totally blocked monolinuron degradation at 10^−6 M and linuron degradation at 5 × 10^−8 M and caused a 90% reduction of solan degradation at a concentration of 10^−5 M. The insecticides parathion and fenthion were weak inhibitors of phenolamide hydrolysis by *B. sphaericus*.

The following chemicals were compared with the above insecticides: thiram, a thiocarbamate fungicide, diallate, a thiocarbamate herbicide, and monuron, a dimethyl-substituted phenylurea herbicide. This latter compound is not hydrolyzed by the *B. sphaericus* aryl acylamidase (7). Results showed that the two thiocarbamates did not influence phenolamide hydrolysis, and only a weak inhibition of linuron degradation by monuron was noticed (Table 1). Thiram inhibited growth of the bacteria at concentrations higher than 10^−4 M.

**Effect of N-methylcarbamates on linuron degradation by crude extracts of *B. sphaericus*.** Reports describing an inhibition of the degradation of several acylanilides and phenylcarbamate herbicides in plants and soil microorganisms were based on measuring the inhibition of hydrolysis of these pesticides by acylamidase enzymes. The propanil-hydrolyzing aryl acylamidase of rice appeared to be competitively inhibited by insecticidal carbamates and organophosphates (9). The chlorpropham-hydrolyzing enzyme from *Pseudomonas striata* was competitively inhibited by insecticidal carbamates and organophosphates (14). The enzymatic hydrolysis of propanil by a partially purified acylamidase from a * Fusarium* sp. was strongly inhibited by both p-chlorophenyl methylcarbamate and 1-naphthyl methylcarbamate (13). p-Chlorophenyl methylcarbamate, however, could both inhibit and induce the enzyme and was also readily metabolized by this fungus (2).

In this study the inhibition of an aryl acylam-
idase derived from *B. sphaericus* was assayed utilizing [14C](ureido)-linuron as substrate and the carbamates propoxur, carbaryl, and metmercapturon as inhibitors. To obtain some insight about the type of inhibition, linuron was used at concentrations of $10^{-5}$ to $10^{-7}$ M, propoxur and carbaryl at concentrations of $10^{-5}$ to $10^{-7}$ M, and metmercapturon at concentrations of $10^{-7}$ to $10^{-9}$ M. Enzyme assays were always started by the addition of crude extracts (usually 9 to 12 mg of protein per ml) to the incubation mixtures. The mode of inhibition of linuron hydrolysis by the aryl acylamidase was determined utilizing three graphical analyses of enzyme kinetic data: the method of Lineweaver and Burk of double reciprocal plots of enzyme activity ($v_i$) against substrate concentration ($S$); the plot of $1/v_i$ against the inhibitor concentration ($I$), developed by Dixon; and the plot of $S/v_i$ against $I$ (4, 23).

The mode of inhibition of linuron degradation in *B. sphaericus* by propoxur, carbaryl, and metmercapturon proved to be competitive. Plots of enzyme kinetic data for enzyme inhibition by metmercapturon are shown in Fig. 1.

Inhibition constants ($K_i$) for the three carbamate inhibitors were calculated from the Dixon plots. The $K_i$ value for propoxur was about $2 \times 10^{-4}$ M, for carbaryl $10^{-5}$ M, and for metmercapturon about $10^{-9}$ M. Because enzyme assays with linuron as substrate were conducted over a period of 3 h, we could not be certain how much of the depression was due to direct action of the inhibitor or to secondary alterations in the enzyme. Thus, the inhibition constants should be considered approximations.

**Phenylcarbamate and organophosphorus insecticides as substrates and/or inducers of the aryl acylamidase.** In another experiment we determined whether inhibitors could also serve as substrates or inducers of the *B. sphaericus* aryl acylamidase. Degradation of the compounds was studied over a 7-day period using the same culture conditions as described for linuron degradation.

No degradation due to microbial activity could be measured. Some hydrolysis occurred by nonbiological mechanisms, however. To investigate induction of the aryl acylamidase by selected carbamate and organophosphorus insecticides and by the nondegradable phenylurea herbicide monuron, crude extracts (protein content, 6 to 12 mg/ml) were prepared from 1-liter cultures of *B. sphaericus* grown for 48 h in the presence of each of the different pesticides ($5 \times 10^{-5}$ M). These were then assayed for aryl acylamidase activity with linuron as the substrate. No enzyme activity could be demonstrated in extracts from cells cultivated in the presence of the methylcarbamate and organo-
phosphorus insecticides. This indicated that the different insecticides tested did not act as inducers of the aryl acylamidase in *B. sphaericus*. Extracts from cells grown in the presence of a 5 x 10^{-8} M concentration of the phenylurea herbicide monuron, however, contained aryl acylamidase activity, which was about 15% of that obtained with linuron as inducer. This
result indicated that the *B. sphaericus* aryl acylamidase can also be induced by substrate analogues, which themselves do not serve as substrates of the enzyme. This phenomenon has also been described with the inducible acylamidase of *Fusarium oxysporum* (2).

**DISCUSSION**

With both whole cells and crude enzyme preparations of *B. sphaericus* metmercapturon was the strongest inhibitor of the hydrolysis of phenylamides, followed by aldicarb, carbaryl, and propoxur. The mode of action of the insecticides was based on a direct and competitive inhibition of the phenylamidase activity, similar to that observed by other investigators (2, 9, 14).

The methoxy-substituted phenylurea herbicides appear to be degraded in soil through hydrolytic processes. This is based on the finding that no significant accumulation of intermediate metabolites such as monomethyl or monomethoxy compounds, which would indicate a stepwise breakdown, were detected in soil (17, 18). These findings are similar to those with acylanilide and phenylcarbamate herbicides, which in soil are hydrolyzed to the corresponding anilines (1). If the microbial detoxication of these phenylamide herbicides in soil is based on the activity of acylamidase enzymes, the persistence of these herbicides would significantly increase when residues of the above-tested insecticides were also present in the same soil. Since these pesticide combinations are commonly used in agriculture, our laboratory studies could contribute to predicting whether pesticide combinations in soil may be hazardous because of their increased persistence. Our findings may help to explain some observations; for example, under greenhouse conditions a longer persistence of a "harmless" phenylamidine herbicide in soil was noticed (14). In these instances several pesticide applications have been performed, and interactions similar to those described above may be the reason for increased persistence.

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