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Degradation of Linuron and Some Other Herbicides and Fungicides by a Linuron-Inducible Enzyme Obtained from *Bacillus sphaericus*

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Linuron [3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea] induces the formation of an enzyme (acylamidase) responsible for the degradation of a large variety of different herbicides and fungicides of the acylanilide and phenylurea type. The former type is degraded at a rate at least 10 times higher than the latter.

The microbial degradation of phenylamide herbicides and related compounds has received considerable attention in recent years. Acylanilide-, phenylcarbamic acid-, and phenylurea herbicides are degraded in soil to the corresponding anilines and various products arising from the substituents of the anilides. A number of different soil microorganisms have been isolated which are responsible for this degradation, and attempts have been made to isolate the enzymes involved to study the mechanism of degradation of these herbicides in more detail. Partially purified enzyme preparations from a strain of *Pseudomonas striata* (4) were active in degrading phenylcarbamate and acylanilide herbicides. Crude extracts from *Fusarium solani* (6) and from a *Penicillium* species (9) showed activity on acylanilides. Recently, it has been demonstrated that a partially purified enzyme preparation from *Bacillus sphaericus* ATCC 12123 is able to degrade *N'*-methoxy phenylurea herbicides, giving rise to the formation of the corresponding halogen-substituted anilines and some, as yet unidentified, degradation products (12). The present study was carried out to examine the substrate specificity of the linuron-degrading enzyme and to determine the reaction sequence by which this herbicide is degraded.

MATERIALS AND METHODS

Preparation of crude enzyme. *B. sphaericus* ATCC 12123 was grown under the conditions described previously (12). To induce the enzyme system responsible for linuron degradation, the cells were grown in the presence of 0.05 μmole of linuron per ml of medium. Cells were harvested in the late logarithmic phase of growth, washed three times with cold 0.05 M phosphate buffer (*p*H 7.5), and stored at −15 C. Cell-free extracts were obtained by grinding of cell pellets with alumina and by extraction of the resulting paste with 0.1 M phosphate buffer (*p*H 7.5).

Enzyme assays. The activity of the extracts with ureido-^{14}C-labeled linuron as substrate was determined in an assay procedure described previously (12). To determine the degradation rate of the other herbicides and fungicides used, 500 nmoles of each compound was added to 10 ml of 0.1 M phosphate buffer at *p*H 7.5 and incubated for 1 to 20 hr at 37 C after the addition of 0.1 ml of crude enzyme extract. Tests with 4-nitroacetanilide and aminoacyl-4-nitroanilides as substrates were performed at 37 C in tubes containing 1 ml of 0.1 M tris(hydroxymethyl)-aminomethane(Tris)-hydrochloride buffer at *p*H 7.5, 10^{-3} M substrate, and 0.001 to 0.1 ml of cell-free extract. Reactions were terminated after 10 min by the addition of 0.1 ml of 10% trichloroacetic acid.

Specific activity of the enzyme preparations was calculated as micromole of substrate degraded per minute (1 unit) per milligram of protein under assay conditions. Protein was determined by the method of Lowry et al. (7) by using bovine serum albumin as standard.

Analytical methods. Halogen-substituted anilines, derived from the urea, and acylanilide herbicides and aniline from the fungicides used were extracted from reaction mixtures with chloroform (11) and identified by thin-layer chromatography (TLC) on silica gel no. 150/LS 254 (Schleicher & Schuell, Dassel) by using chloroform-ethyl acetate (1:1) as solvent system. The compounds were detected on the TLC plates by spraying with a solution of 0.4% *p*-dimethylaminobenzaldehyde in acetone acidified with HCl (*p*H 2.0). 2,5-Dimethylfuran-3-carboxylic-, 2-methylbenzoic-, and 2-chlorobenzoic acid derived from the fungicides were methylated and identified by co-chromatography with known compounds by using a Varian Aerograph model 1800 equipped with a flame ionization detector. The details of this procedure were as follows. The column was a 5 foot by 2 mm inner diameter glass column packed with 1.5% XE 60 on

Kieselgur DMCS 70 to 100 mesh; temperatures were 90 C in the column, 150 C in the injector, and 160 C in the detector; gas flow rates were 30 ml/min for the carrier gas nitrogen, 30 ml/min for hydrogen, and 60 ml/min for oxygen. The amino acids formed were identified by paper co-chromatography by using an isopropanol-acetic acid-water (75:10:15) solvent system. The extent of hydrolysis of 4-nitroacetanilide and aminoacyl-4-nitroanilides was calculated from the release of 4-nitroanilide measured at 405 nm (ξ = 9,620). All other substrates were determined by quantitative residue analysis (11).

Polyacrylamide gel electrophoresis. Electrophoresis was performed at *p*H 8.3 by using 7.5% cross-linked gels in a DESAGA vertical plate electrophoresis apparatus by the method of Ornstein (8). Linuron-degrading activity was detected qualitatively and quantitatively. Incubation of the gel in 0.05 M phosphate buffer (*p*H 7.5) containing 0.1 μmole of linuron per ml for 3 hr followed by incubation of the gel for 10 min in 0.15% *p*-dimethylaminobenzaldehyde in water acidified with acetic acid to *p*H 2 showed the presence of one yellow anil band. An identical gel was sliced in 1-mm pieces (2), and individual pieces were incubated for 22 hr with ureido-^{14}C-labeled linuron under the conditions as described previously (12).

Substrates. Ureido-^{14}C-labeled 3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea (linuron), unlabeled 3-(4-chlorophenyl)-1-methoxy-1-methylurea (monolinuron), 3-(4-chlorophenyl)-1,1-dimethylurea (monuron), 3-(3,4-dichlorophenyl)-1,1-dimethylurea (diuron) were purchased from Hoechst, Frankfurt; 2-methylbenzanilide and 2-chlorobenzanilide were from BASF, Ludwigshafen. 2,5-Dimethyl-3-furancarboxanilide, 5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide (carboxin), 3-(α,α,α-trifluoro-*m*-tolyl)-1,1-dimethylurea (fluometuron), 3-(*p*-bromophenyl)-1-methoxy-1-methylurea (metobromuron), and 3-(*p*-chlorophenyl)-1-methyl-1-(1-methyl-2-propynyl) urea (buturon) were isolated from commercial formulations as described previously (11). 2-Methyl-5,6-dihydro-4-H-pyran-3-carboxanilide (pyracarbolid) was a gift from Hoechst; 3-(3-chloro-4-bromophenyl)-1-methoxy-1-methylurea (maloran) was from Ciba-Geigy, Basel; 4-chloro-α,α-dimethylvaleranilide (monalide) was from Schering, Berlin. 4-Nitroacetanilide was purchased from EGA Chemie, Steinheim, and the aminoacyl-4-nitroanilides were products of Merck, Darmstadt.

RESULTS AND DISCUSSION

Induction of linuron-degrading enzyme. Examination of cell-free extracts from *B. sphaericus* ATCC 12123 did not show any hydrolytic activity towards a variety of different herbicides, including linuron. However, considerable linuron-degrading activity was observed in extracts prepared from cells grown in the presence of this herbicide. This observation suggested that linuron induced the formation of an enzyme system in *B. sphaericus* responsible for linuron degradation. Therefore, the experiment described was performed (Table 1). As seen, simultaneous addition of chloramphenicol, a known inhibitor of protein synthesis, and linuron to growing culture prevents degradation of linuron by whole cells and no activity towards linuron was found in extracts from these cells, whereas cells grown in the presence of linuron alone degrade the herbicide as well as

TABLE 1. *Demonstration of the inducible character of the linuron-degrading enzyme*

Cultural conditions[a]	Degradation of linuron in the culture medium (%)	Specific activity of cell-free extracts as measured with linuron as substrate (units × 10^{-3}/mg of protein)
Cells grown without linuron and chloramphenicol		0.0
Cells grown in the presence of linuron[b]	100	0.12
Cells grown in the presence of linuron and chloramphenicol[c]	0.0	0.0

[a] Cell-free extracts were prepared from cells incubated under the various conditions for 2 days.
[b] Linuron, 0.05 μmoles per ml.
[c] Linuron, 0.05 μmoles per ml; chloramphenicol, 20 μg per ml.

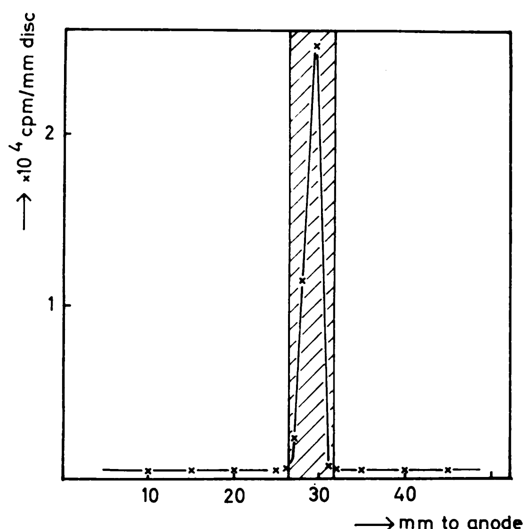

FIG. 1. *Polyacrylamide gel electrophoresis of the linuron-degrading enzyme. The shaded area represents activity staining. The crosses denote the activity of gel slices as determined with radioactive linuron.*

TABLE 2. *Chemical names, degradation products, and degradation rates of various phenylamides*

Chemical name of compound (trade name)	Degradation products		Specific activity of cell-free extract (units × 10^{-3}/mg of protein)
	Anilines	Acids	
Herbicides			
3-(4-Chlorophenyl)-1,1-dimethylurea (monuron)	Nil	Nil	0.0
3-(3,4-Dichlorophenyl)-1,1-dimethylurea (diuron)	Nil	Nil	0.0
3-α,α,α-Trifluoro-*m*-tolyl)-1,1-dimethylurea (fluometuron)	Nil	Nil	0.0
3-(*p*-Chlorophenyl)-1-methyl-1-(1-methyl-2-propynyl)urea (buturon)	Nil	Nil	0.0
3-(4-Chlorophenyl)-1-methoxy-1-methylurea (monolinuron)	4-Chloroaniline[a]	CO_2 + unknown	0.20
3-(3,4-Dichlorophenyl)-1-methoxy-1-methylurea (linuron)	3,4-Dichloroaniline[a]	CO_2 + unknown	0.20
3-(*p*-Bromophenyl)-1-methoxy-1-methylurea (metobromuron)	4-Bromoaniline[a]	Not determined	0.18
3-(3-Chloro-4-bromophenyl)-1-methoxy-1-methylurea (maloran)	3-Chloro-4-bromoaniline[a]	Not determined	0.11
4-Chloro-α,α-dimethyl-valeranilide (monalide)	4-Chloroaniline[a]	Not determined	2.38
Fungicides			
3,5-Dimethyl-furan-3-carboxanilide	Aniline[a]	2,5-Dimethyl-furan-3-carboxylic acid[b]	28.1
2-Methyl-5-hydroxymethyl-furan-3-carboxanilide	Aniline (trace)	Not determined	Tr
5,6-Dihydro-2-methyl-1,4-oxathiin-3-carboxanilide (carboxin)	Aniline	Not determined	2.52
2-Methyl-5,6-dihydro-4-H-pyran-3-carboxanilide (pyracarbolid)	Aniline	Not determined	0.35
2-Methyl-benzanilide	Aniline	2-Methylbenzoic acid[b]	3.22
2-Chloro-benzanilide	Aniline	2-Chlorobenzoic acid[b]	18.7
Other acylanilides			
4-Nitroacetanilide	4-Nitroaniline[c]	Not determined	90.0
L-Alanine-4-nitroanilide	4-Nitroaniline[c]	Ala[d]	309
N-Acetyl-L-alanine-4-nitroanilide	4-Nitroaniline[c]	Not determined	5.00
Glycin-4-nitroanilide	4-Nitroaniline[c]	Gly[d]	106
L-Leucine-4-nitroanilide	4-Nitroaniline[c]	Leu[d]	55.8
L-Phenylalanine-4-nitroanilide	4-Nitroaniline[c]	Phe[d]	7.63

[a] Thin-layer chromatography.
[b] Gas-liquid chromatography.
[c] Spectrophotometry.
[d] Paper chromatography.

extracts prepared from them. Furthermore, chloramphenicol itself did not inhibit linuron degradation when added to a cell-free extract prepared from induced cells. Therefore, it is concluded that linuron or a compound derived from it induces the formation of an enzyme system responsible for the degradation of the herbicide.

Gel electrophoresis of cell-free extract. Geissbühler (3) and Tweedy (10) obtained evidence for enzymatic degradation of phenylurea herbicides by stepwise demethylation and demethoxylation. If such a mechanism would be operative in *B. sphaericus*, the induction of the linuron-degrading enzyme system might involve the formation of several enzymes.

To explore this, extracts from *B. sphaericus* cells

grown in the presence of linuron were subjected to polyacrylamide gel electrophoresis. Activity staining of gels with unlabeled linuron and examinations of discs for activity towards ureido-^{14}C-labeled linuron made it very likely that a single protein is responsible for linuron degradation since degradation of linuron could not be prevented by protein separation (Fig. 1).

Site of enzymatic attack and substrate specificity. The hydrolysis of linuron by the enzyme could follow two possible mechanisms. Attack at the linkage between the carboxy group and the 1-methyl, 1-methoxy-substituted nitrogen would yield the corresponding phenylcarbamic acid. This compound is unstable and disintegrates spontaneously to the halogen-substituted aniline and CO_2 as suggested previously (12). Attack at the amide linkage between the aniline and the carboxy group would give rise to the immediate formation of the halogen-substituted aniline and *N*-methyl-*N*-methoxy-carbamic acid. Because of the instability of the latter, *N,o*-dimethylhydroxylamine and CO_2 were also formed as degradation products.

To decide which of the two possibilities is realized, a study was made to determine whether various other acylanilides could also be degraded by the enzyme and which degradation products are formed. The results are summarized in Table 2.

It should first be emphasized that only enzyme preparations obtained from cells grown in the presence of linuron showed activity towards this large variety of different compounds. This means that the linuron-induced enzyme displays a rather low specificity. An investigation was then made as to whether some of the tested compounds, besides being substrates, could also serve as inducers of this enzyme. Preliminary experiments revealed that, in addition to linuron, 2,5-dimethyl-furan-3-carboxanilide was also effective as inducer at one-fourth of the induction rate of linuron.

These results indicate that the cell-free extract from *B. sphaericus*, grown in the presence of linuron, hydrolyzed various acylanilides and methoxy-substituted phenylureas to anilines and the corresponding acids (Table 2). The acid moiety formed during the decomposition of the methoxy-substituted phenylureas, however, could not be determined because of the rapid dissociation of this metabolite as described above. Therefore, the substrate specificity of the enzyme strongly suggests that the peptide bond between the amino group of the aniline moiety and the carboxy group is the site of enzyme attack. The pathway shown in Fig. 2 is proposed.

The degradation rate of different acylanilides was compared with that of several substituted urea compounds (Table 2). It is apparent that all

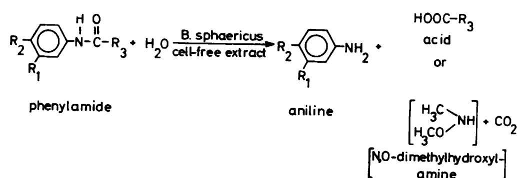

FIG. 2. *Proposed mechanism of degradation of phenylamide compounds. R_1:-H, -Cl; R_2:-H, -Cl, -Br, -NO_2; R_3:acyl-, aminoacylresidue, alkylaminoresidue (for details, see Table 2).*

acylanilides are degraded at a rate at least 10 times higher than that of the methoxy-substituted phenylureas. The highest specific activity of the enzyme was observed with aminoacylanilides as substrates, which would classify the enzyme as an acylamidase (EC group 3.5.1.). Furthermore, it is noteworthy that the enzyme was not only effective on L-alanyl-4-nitroanilide but also on *N*-acetyl-L-alanyl-4-nitroanalide, thus showing a rather unlimited substrate specificity, which is also reflected in its ability as an acylamidase to degrade methoxy-substituted phenylurea compounds. However, it is difficult to explain why the decomposition of urea herbicides appears to be specific for the methoxy-substituted phenylureas. Generally, it appears that the activity of cell-free extract of *B. sphaericus* increased with hydrophilic character of the substrates. This might be in line with the inability of the enzyme to degrade 1,1-dimethyl phenylureas, which exhibit the least hydrophilic character of all compounds tested.

A preliminary test with isopropyl carbanilate indicated that the cell-free extract was even active on this compound, forming aniline as a major product.

In their studies on phenylamides, Kearney et al. (4) and Lanzilotta et al. (6) did not find any activity towards 1,1-dimethyl phenylureas and concluded that the enzymes from *Pseudomonas striata* (4) and *Fusarium solani* (6) were not able to degrade phenylureas in general. These results agree with our observation that the enzyme of *B. sphaericus* did not decompose the 1,1-dimethyl phenylurea herbicides. These authors did not use the methoxy-substituted phenylurea herbicides in their investigations, and it might be possible that the enzymes they described were also active to some extent towards these compounds.

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