

Chapter 4

The Role of Plant and Microbial Hydrolytic Enzymes in Pesticide Metabolism

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Many pesticide molecules containing amide or carbamate bonds, or esters with carbonyl, phosphoryl, and thionyl linkages, are subject to enzymatic hydrolysis. Pesticide hydrolysis by esterases and amidases from plants and microorganisms can serve as a detoxification or activation mechanism that can govern pesticide selectivity or resistance, and initiate or determine the rate of pesticide biodegradation in the environment. Substrate specificity of esterases and amidases varies dramatically among species and biotypes of plants and microorganisms. The constitutive or inducible nature of these enzymes, as well as production of isozymes, is also important in the expression of these mechanisms. For example, increased aryl acylamidase activity has been reported as the mechanism of evolved resistance to the herbicide propanil in two *Echinochloa* weed species [junglerice, *E. colona* (L.) Link; barnyardgrass, *E. crus-galli* (L.) Beauv.]. Other acylamidases, such as a linuron-inducible enzyme produced by *Bacillus sphaericus*, have broad substrate specificities including action on acylanilide, phenylcarbamate, and substituted phenylurea herbicides. Many microbial hydrolytic enzymes are extracellular, thus hydrolysis can occur without uptake. Advances in molecular biology have led to an increased understanding of hydrolytic enzyme active sites, especially those conferring selective specificity. This knowledge will create opportunities for engineering novel resistance mechanisms in plants and biosynthetic and/or degradative enzymes in microorganisms.

Hydrolytic enzymes catalyze the cleavage of certain chemical bonds of a substrate by the addition of the components of water (H or OH) to each of the products. Many herbicides, fungicides and insecticides contain moieties (e.g., amide or carbamate bonds, or esters with carbonyl, phosphoryl and thionyl linkages) that are subject to enzymatic hydrolysis. A wide array of hydrolases (amidases, esterases, lipases, nitrilases, peptidases, phosphatases, etc.), with broad and narrow substrate specificities are present in animals, microorganisms and plants. Plants and microorganisms should not necessarily be expected to possess the enzymatic capacity to metabolize xenobiotic compounds. But indeed, it is the multiplicity of certain enzymes and their broad substrate specificities that make pesticide degradation possible. Thus the potential for hydrolytic cleavage of a given pesticide exists in numerous organisms.

Various levels of cellular compartmentalization for these hydrolytic enzymes exist. Some are cytosolic, while others are associated with membranes, microsomes, and other organelles. Some fungi and bacteria also excrete hydrolytic enzymes that act extracellularly on substrates, and thus pesticide detoxification and degradation may occur without microbial uptake of the compound. Certain hydrolases are constitutive, while others are inducible. The ability of an organism to hydrolyze certain pesticides can render the organism resistant to that compound. Many plants are insensitive to various classes of pesticides due to their unique hydrolytic capabilities. Furthermore, differential metabolism is an important mechanism in determining the selective toxicity of a given compound among plants and other organisms. Since molecular oxygen is not involved in hydrolytic activity, hydrolysis can occur under anaerobic and/or aerobic conditions.

In this chapter we examine the hydrolytic transformations of a variety of pesticide chemical classes by plants and microorganisms. The role of these enzymes in pesticide activation, detoxification and degradation is discussed, and opportunities for exploiting novel hydrolytic transformations of xenobiotics are examined.

Ester Hydrolysis in Plants

Esters are susceptible to hydrolysis by esterases, and to some extent by lipases and proteases. Esterases are ubiquitous in living organisms, and occur as multiple isozymes with varying substrate specificities and catalytic rates. For example, fourteen esterases have been isolated from bean (*Phaseolus vulgaris* L.) and seven from pea (*Pisum sativum* L.) (1). The biochemistry and role of plant esterases in xenobiotic metabolism has recently been reviewed (2). The physiological role of these esterases may be multifaceted, e.g., they are involved in fruit ripening, abscission, cell expansion, reproduction processes, as well as hydrolysis of ester-containing xenobiotic molecules. Varying degrees of specificity and kinetic rates are observed among plant esterases. For example, an acetyl esterase from mung bean (*Vigna radiata*) hypocotyls hydrolyzed high molecular weight pectin esters (primary physiological substrates), but could more rapidly hydrolyze low molecular substrates such as triactin, and *p*-nitrophenyl acetate (3).

Many pesticides are applied as carboxylic acid esters (Figure 1). Since the acid form is generally the active agent, esterases can play a role in pesticide activation, detoxification, and selectivity in plants (Table I.). Many herbicides have been specifically developed as esters to improve absorption into plant tissue, and to alter

Table I. Role of Plant Esterases in Herbicidal Activity in Plants

<i>Mechanism</i>	<i>Herbicide</i>	<i>Plant species</i>	<i>Citation</i>
Activation	Fenoxaprop-ethyl	Wheat, Barley, Crabgrass	(4)
	Diclofop-methyl	Wheat, Oat, Wild Oat	(5)
Detoxification	Thifensulfuron- methyl	Soybeans	(6)
	Chlorimuron-ethyl	Soybeans	(7)
Increased absorption and translocation	Quinclorac esters	Spurge	(8)
	2,4-D-Butoxyethyl ester	Bean	(9)

phytotoxic selectivity. Aliphatic derivatives of the broadleaf herbicide 2,4-D [(2,4-dichlorophenoxy)acetic acid] are widely used. The polar forms of 2,4-D are readily taken up by roots, while long-chain non-polar ester forms (butoxyethyl, and isoocetyl esters) are more readily absorbed by foliage. The ability to hydrolyze these 2,4-D esters is widely distributed in sensitive plants such as cucumber (*Cucumis sativus* L.) (10) and tolerant plants such as barley (*Hordeum vulgare* L.) (11). In a fungicide development program, ester derivatives of the herbicide 4,6-dinitro-*o*-cresol (DNOC) were assessed as fungicides, and for potential phytotoxicity on broadbean (*Vicia faba*) (12). Short-chain aliphatic esters (acetate, propionate and isobutyrate) were readily hydrolyzed and were highly phytotoxic. However, aromatic esters of DNOC (chloro- and nitrobenzoates) were hydrolyzed slowly and exhibited low phytotoxicity. The uptake of clopyralid (3,6-dichloro-2-pyridinecarboxylic acid) free acid was greater compared to that of the 1-decyl and 2-ethylhexyl esters in Canada thistle (*Cirsium arvense*) and wild buckwheat (*Polygonum convolvulus*), and in isolated cuticles of *Euonymus fortunei* (13, 14). De-esterification was essential for the 1-decyl and 2-ethylhexyl esters of this herbicide to enter the phloem and translocate to site of action.

The polycyclic alkanolic acid herbicides (PCAs), usually contain more than one ring structure (one is usually a phenyl ring) attached to an asymmetric, non-carbonyl carbon of an alkanolic acid (15). Common herbicides in this class include: diclofop-methyl {methyl ester of (\pm)-2-[4-(2,4-dichlorophenoxy)phenoxy]propanoic acid}, fenoxaprop-ethyl {ethyl ester of (\pm)-2-[4-[(6-chloro-2-benzoxazolyl)oxy]phenoxy]propanoic acid}, and fluazifop-butyl {(R)-2-[4-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenoxy]propanoic acid}. In plants, PCA-ester hydrolysis, yielding the parent

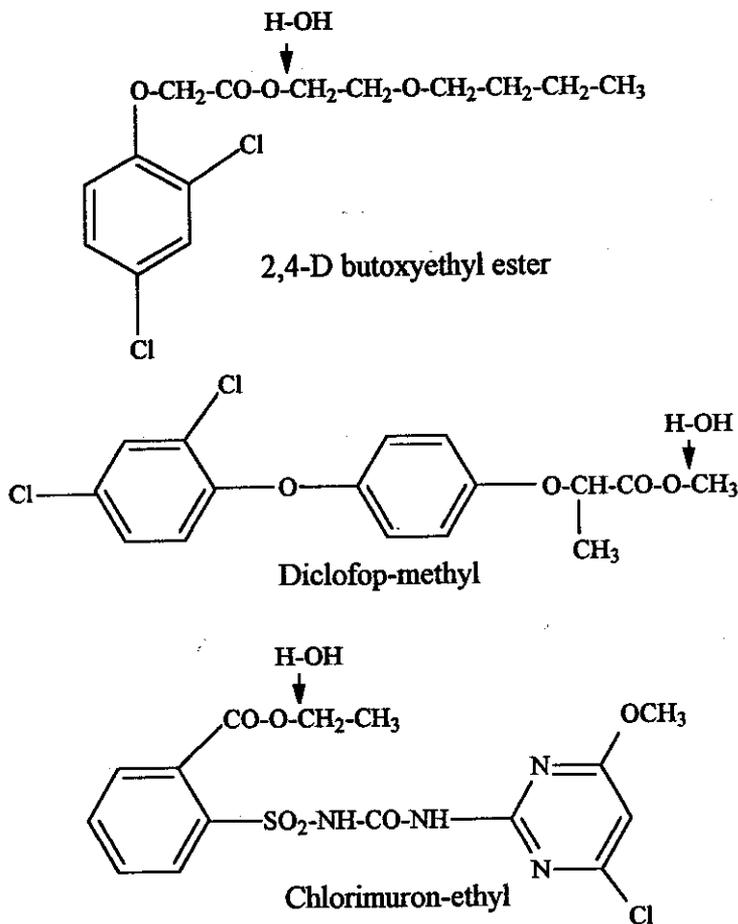


Figure 1. Selected pesticides with susceptibility to esterase-mediated hydrolysis.

acid, is the first enzymatic action on these compounds (15). For the PCAs, de-esterification is a bioactivation, not a detoxification mechanism as demonstrated for diclofop-methyl (5). The carboxyesterase responsible for de-esterification of the PCA herbicide chlorfenprop-methyl [methyl 2-chloro-3-(4-chlorophenyl)propionate] has been partially purified from oats (*Avena sativa* L.) (16) and wild oats (*Avena fatua* L.) (17). In tolerant species, the PCA-free acid is detoxified by different mechanisms, i.e., diclofop via arylhydroxylation and subsequent phenolic conjugation (18), and fenoxaprop, via glutathione conjugation (19). Rice is generally tolerant to fenoxaprop-ethyl, but under low light intensity, rice can be damaged by fenoxaprop-ethyl treatment (20). However, similar rates of *in vitro* and *in vivo* fenoxaprop-ethyl de-esterification were found in rice seedlings grown in either, normal light or low light conditions (21). Esterase activity was also measured using fluorescein diacetate (FDA) as a substrate (21). In fenoxaprop-ethyl-treated rice, FDA esterase activity was 41 % lower in shaded plants compared to unshaded plants. However, in untreated rice, FDA esterase activity was 22% lower in shaded versus unshaded plants. Hence, the phytotoxic compounds fenoxaprop-ethyl and fenoxaprop acid, persisted longer in shaded plants, which may explain this phytotoxicity to plants under low light conditions.

The herbicide chloramben (3-amino-2,5-dichlorobenzoic acid) was rapidly metabolized to an *N*-glucoside in resistant plant species, while sensitive plants formed the carboxy-glucose ester (22). The glucose ester is unstable *in vivo* due to hydrolysis by esterases, thus an equilibrium between the ester and free acid is maintained.

The selectivity of the sulfonylurea herbicides is based on several detoxification pathways, including oxidative and hydrolytic mechanisms (6). Soybean (*Glycine max* Merr.), can de-esterify thifensulfuron-methyl {methyl 3-[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]-2-thiophenecarboxylate} to the free acid (non-phytotoxic), but soybean is unable to de-esterify the herbicide analog, metsulfuron-methyl {methyl 2-[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)-amino]-carbon-yl]amino]sulfonyl]benzoate} which injures the crop plant. Soybean esterases can hydrolyze thiophene *O*-carboxymethyl esters and *O*-phenylethyl esters, but not *O*-phenyl methyl esters of certain sulfonylureas (6). Chlorimuron-ethyl {ethyl 2-[[[(4-chloro-6-methoxy-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]-benzoate} detoxification occurs *via* de-esterification of the ethyl group and dechlorination *via* homogluthathione conjugation in soybean (7). However, conjugation occurs three-fold more rapidly than de-esterification.

A recent study compared the phytotoxicity of thirteen ester derivatives (C5 to C16) of the herbicide quinclorac (3,7-dichloro-8-quinolinecarboxylic acid) for phytotoxicity against leafy spurge (*Euphorbia esula* L.) (8). Foliar application of quinclorac caused rapid death, whereas quinclorac esters applied at higher concentrations to foliage caused only phytotoxicity, not mortality. When applied to soil, quinclorac esters were metabolized by a series of and oxidations while hydrolysis was limited. This resulted in the slow release of quinclorac which increased herbicide efficacy against leafy spurge plants.

Ester Hydrolysis in Microorganisms

Studies of fenoxaprop-ethyl (23, 24) and diclofop-methyl (25) degradation in soils demonstrated a rapid hydrolysis of both compounds to their parent acids. This hydrolysis was more rapid in moist and non-sterile soils compared to dry or sterile soils, which suggested microbial degradation. The role of enzymatic hydrolysis of diclofop-methyl was reduced with certain microbial inhibitors (propylene oxide and sodium azide) (25). During hydrolysis of diclofop-methyl and fenoxaprop-ethyl in soil, enantiomeric inversion was observed (26). The S enantiomers of both compounds underwent a more rapid inversion than the R enantiomers, and rates of inversion were dependent on soil type. No inversion was observed with enantiomers of the parent compounds. Fenoxaprop acid undergoes further degradation in soil, forming metabolites such as 6-chlorobenoxazolone, 4-[(6-chloro-2-benzoxazolyl)-oxy]phenetole, and 4-[(6-chloro-2-benzoxazolyl)oxy]phenol (24). Soil pH also affects the degradation pathway of fenoxaprop-ethyl. Under acidic conditions, the rate of de-esterification was significantly lower than under neutral soil conditions, however, the benzoxazolyl-oxy-phenoxy ether linkage of fenoxaprop-ethyl was prone to non-enzymatic cleavage under acidic conditions (27). De-esterification of fenoxaprop-ethyl occurs readily in mixed microbial cultures (28) and in pure cultures and enzyme extracts of bacteria, especially fluorescent pseudomonads (27, 29). Fenoxaprop-ethyl de-esterification in bacterial enzyme preparations is pH sensitive, with the highest activity in the neutral to slightly alkaline range. The same *Pseudomonas* strains that hydrolyzed fenoxaprop-ethyl were unable to de-esterify chlorimuron-ethyl (R.M. Zablotowicz, unpublished results). Four distinct types of esterases are found in a *P. fluorescens* strain (30). These *P. fluorescens* esterases differ in substrate specificity, cellular location and structure.

Esterases have been cloned, and proteins have been sequenced from several microorganisms, e.g., two ferulic acid esterases from *Aspergillus tubingensis* (31), a cephalosporin esterase from the yeast *Rhodospiridium toruloides* (32), a chrysanthemic acid esterase from *Arthrobacter globiformis* (33), and several other esterases from *Pseudomonas fluorescens* strains (30, 34, 35). The ability of these esterases to hydrolyze ester linkages of pesticides has not been examined. Since molecular oxygen is not involved, enzymatic hydrolysis can occur under anaerobic and aerobic conditions.

The active site of prokaryotic and eukaryotic esterases contains the serine motif (Gly-X-Ser-X-Gly), originally characterized in serine hydrolase (36). This conserved peptide is part of a secondary structure of the enzyme molecule located between a β -strand and an α -helix (37). A general mechanism has been proposed for the catalytic activity of the esterase superfamily (Figure 2). This mechanism involves the formation two tetrahedral intermediates (37), with the active serine serving as a nucleophile enabling ester bond cleavage. A histidine residue in a β -strand, is also involved in formation of an ionic attachment at the carbonyl oxygen atom of the substrate during catalysis. Reaction of the second intermediate, with a molecule of water, concomitantly releases the parent acid of the substrate and regenerates the active serine of the enzyme. A similar reaction mechanism is postulated for the serine

proteases. However, a chrysanthemic acid esterase from *Arthrobacter* sp. is similar to many bacterial amidases that possess the Ser-X-X-Lys motif in the active site. This esterase may provide a unique opportunity for biotechnological synthesis, since it stereoselectively produces (+)-*t*-chrysanthemic acid, used in pyrethroid insecticide synthesis.

Certain esterases, e.g., the *R. toruloides* cephalosporin esterase, also have acetylating activity when suitable acetyl donors are present (32). The *R. toruloides* cephalosporin esterase is a glycoprotein (80 kDa glycosylated; 60-66 kDa de-glycosylated) with eight potential binding sites (Asn-X-Ser or Asn-X-Thr) for glucose. When de-glycosylated, enzyme activity was reduced about 50%, indicating an important role for glycosylation in stabilizing the native protein structure.

Feng *et al.* (38) transformed tobacco (*Nicotiana tabacum* L.) and tomato (*Lycopersicon esculentum* L.) with genes encoding for rabbit liver esterase 3 (RLE3). This esterase gene expressed in these plants, conferred resistance to the herbicide thiazopyr [methyl 2-(difluoromethyl)-5-(4,5-dihydro-2-thiazolyl)-4-(2-methylpropyl)-6-(trifluoromethyl)-3-pyridinecarboxylate]. These researchers proposed, that a critical assessment of microbial esterases may provide other hydrolytic enzymes that are useful in conferring pesticide (herbicide) resistance in plants. Such enzymes could also play a role in reducing the levels of pesticides, their metabolites, and other potentially harmful xenobiotics in food products.

Inhibition of Esterases in Plants and Microorganisms

The effects of two fungicides, captan [*N*-(trichloromethylthio)-4-cyclohexene-1,1-dicarboximide] and folpet [*N*-(trichloromethylthio)phthalimide], and a sulfhydryl binding inhibitor, perchloromethylmercaptan, were assessed on esterase activity of *Penicillium duponti* (39). Esterase activity using *p*-nitrophenylpropionate as substrate was inhibited 50% by all three compounds at 0.5 to 2.0 μ M. But, concentrations that totally inhibited *p*-nitrophenylpropionate esterase activity had no effect on α -naphthyl acetate esterase activity. The extreme sensitivity of certain fungal esterases to these two fungicides, suggests that part of their toxicity to fungi may be due to esterase inhibition (39).

Amide Hydrolysis in Plants

Amide and substituted amide bonds are present in several classes of pesticides, i.e., acylanilides: alachlor [2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide]; carboxin (5,6-dihydro-2-methyl-*N*-phenyl-1,4-oxathiin-3-carboxamide); diphenamid [2-chloro-*N*-[(1-methyl-2-methoxy)ethyl]-*N*-(2,4-dimethyl-thien-3-yl)]; metalaxyl [*N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)-alanine methyl ester]; metolachlor [2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl)acetamide]; and propanil, [*N*-(3,4-dichlorophenyl)propionamide], phenylureas: diuron [*N*-(3,4-dichlorophenyl)-

N,N-dimethylurea]; fluometuron [*N,N*-dimethyl-*N*-[3-(trifluoromethyl)phenyl]urea]; and linuron, [*N*-(3,4-dichlorophenyl)-*N*-methoxy-*N*-methylurea]; and carbamates: IPC (isopropyl carbanilate); and CIPC (isopropyl *m*-chlorocarbanilate). Some structural examples of these substituted amides are given for comparison (Figure 3.)

Rice (*Oryza sativa* L.) is tolerant to the acylanilide herbicide propanil, due to the presence of high levels of aryl acylamidase (EC 3.5.1.a), which hydrolyzes the amide bond to form 3,4-dichloroaniline (DCA) and propionic acid (40, 41). This enzyme activity is the biochemical basis of propanil selectivity in the control of barnyardgrass [*Echinochloa crus-galli* (L.) Beauv.] in rice. Barnyardgrass tissue was unable to detoxify absorbed propanil due to very low enzymatic activity; rice leaves contained sixty-fold higher aryl acylamidase activity than barnyardgrass leaves (40). Propanil aryl acylamidase activity is also widely distributed in other crop plants and weeds (42, 43). Plant aryl acylamidases have been isolated, and partially purified and characterized from tulip (*Tulipa gesnariana* v.c. Darwin) (44), dandelion (*Taraxacum officinale* Weber) (45), and the weed red rice (*Oryza sativa* L.) (46). Red rice is a serious conspecific weed pest in cultivated rice fields in the southern U.S. (47), and its ability to hydrolyze propanil limits the utility of this herbicide where red rice is present. Propanil hydrolysis by aryl acylamidases has also been observed in certain wild rice (*Oryza*) species (48).

With intensive use of propanil in Arkansas rice production over a thirty-five year period, barnyardgrass (initially controlled by propanil), has evolved resistance to this herbicide, and this biotype is currently a serious problem (49). Populations of propanil-resistant barnyardgrass have been verified in all southern U.S. states that use propanil in rice cultivation. A series of experiments with propanil-resistant barnyardgrass showed that the mechanism of resistance was increased propanil metabolism by aryl acylamidase activity (50, 51). Increased propanil metabolism by aryl acylamidase was also shown to be the resistance mechanism in another related weed, junglerice [*Echinochloa colona* (L.) Link] (52).

Naproanilide [2-(2-naphthylxy)-propionanilide], a herbicidal analog of propanil, was hydrolyzed by rice aryl acylamidase (53). The cleavage product, naphthoxypropionic acid is phytotoxic, however, it is hydroxylated and subsequently glucosylated as a detoxification mechanism in rice. Naproanilide hydrolysis also occurred in a susceptible plant (*Sagittaria pygmaea* Miq.), but naphthoxypropionic acid was not metabolized further in this species (53).

Initial assessment of substrate chemical structure and aryl acylamidase activity has been studied in enzyme preparations from various plants, e.g., rice (40), tulip (44), dandelion (45), and red rice (46). Nine mono- and dichloro-analogs of propanil were examined for substrate specificity of these enzyme preparations. Different profiles of hydrolytic rates were observed among species. In all species tested (not tested in red rice), little or no activity was observed with 2,6-dichloropropionanilide. In rice, greater activity was observed with 2,3-dichloropropionanilide compared to propanil; while in tulip, equal activity was observed with propanil, 2,4-dichloropropionanilide, and 4-chloropropionanilide as substrates. In a similar fashion, the effects of alkyl chain length on 3,4-dichloroanilide substrates were evaluated. Propanil was the best substrate for all four enzyme preparations. Reducing the chain length to one carbon

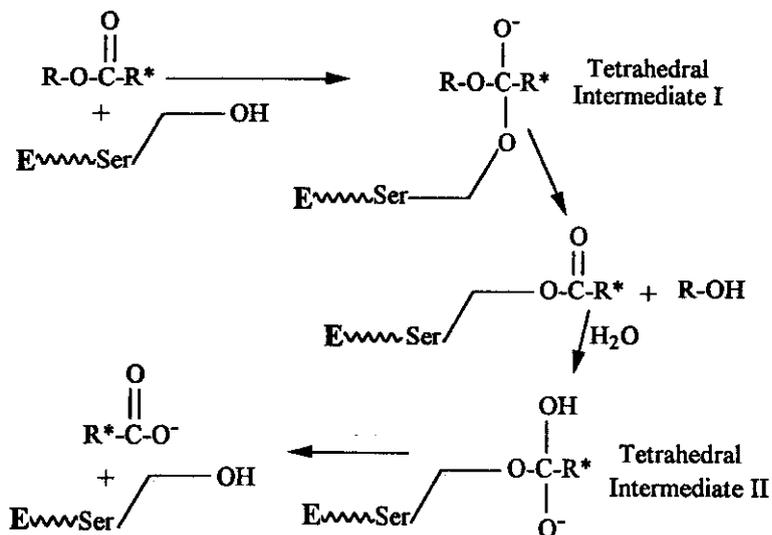


Figure 2. Schematic mechanism of esterase-mediated hydrolysis.

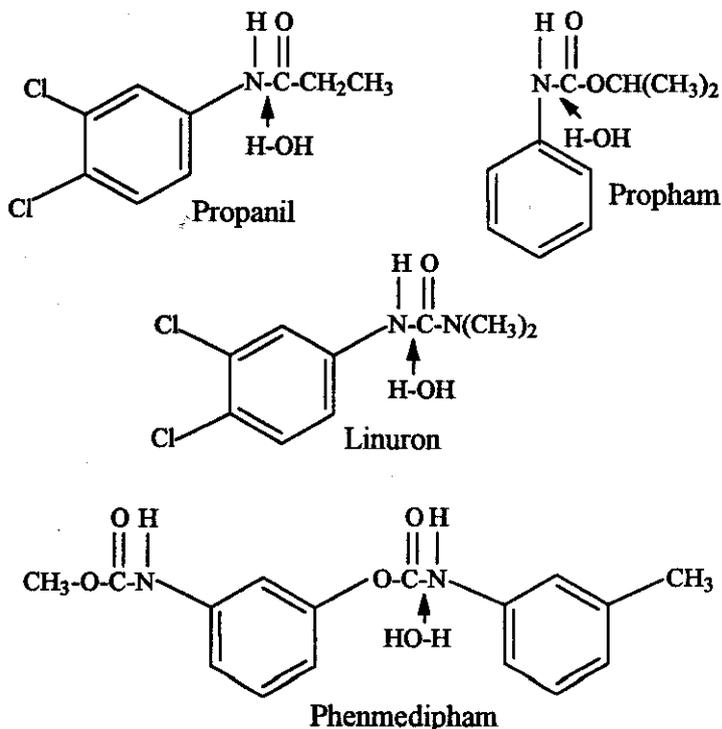


Figure 3. Selected pesticides with amide and substituted amide bonds.

(i.e., the acetamide analog), decreased activity by 18 to 50%, compared to propanil. Increasing the alkyl chain length or inserting alkyl branching, reduced activity by 60 to 100%.

Aryl acylamidases from several plant species have been purified to homogeneity (54). The enzymes from orchardgrass and rice are quite similar. Both have molecular weights of about 150 kDa, and are membrane bound. These enzymes share a common pH optimum of 7.0, similar K_m 's for propanil, and are both inhibited by the insecticide carbaryl. A gene for aryl acylamidase has been cloned from Monterey pine (*Pinus radiata*) male pine cones (55). This protein, comprised of 319 amino acids, is similar to esterases containing a serine hydrolase motif in the active site.

Amide Hydrolysis in Microorganisms

As we have reviewed (56), aryl acylamidases have been characterized in diverse species of algae, bacteria and fungi, and propanil has been the most studied pesticide substrate. The distribution of aryl acylamidases and related hydrolytic enzymes produced by selected microorganisms are summarized (Table II). Differences in substrate specificity and inducibility of these enzymes are found among genera and species of microbes. For example, aryl acylamidases produced by *P. fluorescens* strains RA2 and RB4 had a substrate range limited to certain acylanilides (propanil, nitroacetanilide, and acetanilide), and were ineffective on several herbicides containing substituted amide bonds: phenylureas (linuron and diuron), a phenylcarbamate (CIPC), chloracetamides [alachlor and the *N*-dealkylated metabolite 2-chloro-*N*-(2',6'-diethylacetanilide)], and a benzamide {pronamide [3,5-dichloro(*N*-1,1-dimethyl-2-propynyl)benzamide]} (66). Similar substrate specificities are found in other bacterial strains (Table III). Other microbial acylamidases also have activity restricted to certain acylanilides, e.g., *Fusarium oxysporum* (69).

Some acylamidases, such as a linuron-inducible enzyme produced by *Bacillus sphaericus* (59), and an extracellular coryneform aryl acylamidase (60), have wide substrate specificities including hydrolytic action on acylanilide, phenylcarbamate, and substituted phenylurea pesticides. In a *Fusarium oxysporum* strain, there are two distinct aryl acylamidases: one induced by propanil, the second induced by *p*-chlorophenyl methyl carbamate (69). One study has compared the metabolism of several phenylurea herbicides by bacteria and fungi (70). Nine fungal species were more effective in degrading linuron or isotroturon [3-(4-isopropylphenyl)-1,1-dimethylurea] compared to fenuron (1,1-dimethyl-*N*-phenylurea), but *N* or *O*-dealkylation (not hydrolysis) was the major degradation mechanism. Only one of five bacterial isolates (a pseudomonad) metabolized linuron with the formation of 3,4-dichloroaniline, indicating hydrolytic cleavage of the urea bond. The hydrolytic mechanism for phenylurea herbicides by a coryneform-like bacteria has also been shown with an organism capable of hydrolyzing linuron > diuron > monolinuron [*N*-(3-chlorophenyl)-*N*-methoxy-*N*-methylurea] >> metoxuron [*N*-(3-chloro-4-methoxyphenyl)-*N,N*-dimethylurea] >>> isotroturon (71).

Table II. Selected Microbial Species that Produce Aryl Acylamidases and Related Hydrolytic Enzymes

<i>Organism</i>	<i>Substrate</i>	<i>Inducer</i>	<i>Citation</i>
<i>Anacystis nidulans</i>	CIPC, IPC	nt*	(57)
<i>Aspergillus nidulans</i>	Propanil, (propionanilide**)	Constitutive	(58)
<i>Bacillus sphaericus</i>	Linuron, carboxin, IPC	Linuron	(59)
Coryneform-like, strain A-1	CIPC, linuron, naproanilide, propanil	(Acetanilide)	(60)
<i>Fusarium oxysporum</i>	Propanil, CIPC, (acetanilide)	Propanil and phenylureas	(61)
<i>Fusarium solani</i>	Propanil, (acetanilide)	Propanil and (acetanilide)	(62)
<i>Nostoc entophyllum</i>	Propanil	nt	(63)
<i>Penicillium</i> sp.	Karsil, propanil, (acetanilide)	Karsil	(64)
<i>Pseudomonas fluorescens</i>	(Acetanilide), (<i>p</i> -Nitroacetanilide, <i>p</i> -Hydroxyacetanilide)	(Acetanilide)	(65)
<i>P. fluorescens</i>	Propanil, (acetanilide, nitroacetanilide),	Constitutive	(66)
<i>P. picketti</i>	Propanil	Constitutive	(67)
<i>P. striata</i>	CIPC, IPC, Propanil	CIPC	(68)

NOTE: *nt = not tested; ** compounds in parenthesis are not used as pesticides.

Propanil hydrolysis yielding DCA (72,73) is the major mechanism for dissipation of this compound in soil. Many microorganisms hydrolyze propanil to DCA, and enzymes from several species have been isolated, partially purified, and characterized. For example, ninety seven bacterial isolates were collected from soil and flood water of a Mississippi Delta rice field over a two year period following propanil application (Table IV). Overall, 37% of the soil and water isolates exhibited propanil hydrolytic activity. Although activity was observed in both gram-positive and gram-negative isolates, there was a greater frequency of propanil-hydrolysis among gram-negative bacteria. The hydrolytic activity of cell-free extracts of several of these isolates, and other rhizobacterial cultures on several substrates, was assessed using methods described elsewhere (66). Only acylanilides were hydrolyzed, with no detectable hydrolysis of carbamate and substituted urea herbicides (Table V). All isolates, except AMMD and UA5-40, were isolated from soil or water that had been previously

Table III. Aryl Acylamidase Activity on Five Substrates in Cell-Free Extracts from Ten Bacterial Strains

Genera	Strain	Source	Product formed (nmol mg ⁻¹ protein h ⁻¹)			CIPC
			Propanil	2-NAA	Acetanilide	
<i>Bacillus</i> sp.	S92B2	S	18.1	4.6	16.4	nd
<i>Flavobacterium</i> sp.	W92B14	W	59.2	38.7	28.2	nd
<i>P. cepacia</i>	AMMD	R	13.9	0.6	5.1	nd
<i>P. fluorescens</i>	RA-2	R	14,310	5,540	15,940	nd
<i>P. fluorescens</i>	RB-3	R	14.0	254	49	nd
<i>P. fluorescens</i>	RB-4	R	35,218	11,017	30,510	nd
<i>P. fluorescens</i>	UA5-40	R	0.3	1.4	1.4	nd
<i>P. fluorescens</i>	W92B12	W	14.5	25.8	17.0	nd
<i>Rhodococcus</i> sp.	S92A1	S	15.9	10.2	3.5	nd
<i>Rhodococcus</i> sp.	S93A2	S	144	18.7	191	nd

NOTE: S = soil isolate; W = water isolate; R = rhizosphere isolate. Cell-free extracts (CFE, 1 to 8 mg protein mL) were prepared as described elsewhere (66). The assay mixture contained 800 nmol substrate and 0.2 mL CFE in a final volume of 1.0 mL potassium phosphate buffer (pH 8.0, 50 mM), incubated at 30°C for 15 min to 4 h. Acetanilide hydrolysis determined by diazotization; 2-NAA (2-nitroacetanilide) hydrolysis determined spectrophotometrically at 410 nm (66); Propanil, CIPC, and linuron hydrolysis determined by HPLC (66).

Table IV. Recovery of Propanil-Hydrolyzing Isolates from a Mississippi Delta Rice Soil and Flood Water

Year	Source	Gram-stain reaction	Propanil-hydrolyzing isolates	Total Isolates tested
1982	Soil	Negative	9	13
		Positive	2	19
	Water	Negative	4	8
		Positive	6	15
1983	Soil	Negative	5	9
		Positive	3	11
	Water	Negative	3	8
		Positive	4	14
Total soil			19	52
Total, water			17	45
Total gram-positive			15	59
Total gram-negative			21	38

NOTE: Bacteria were isolated on tryptic soy agar (10%), from soil and water following propanil application. Individual colonies were subcultured, ascertained for purity and tested for Gram stain reaction. Propanil hydrolysis assessed in cell suspensions (log 11.0 cells ml; 50 mM potassium phosphate, pH 8.0; 800 μ M propanil) after 24 h incubation. Propanil and metabolites were determined by HPLC as described elsewhere (66).

exposed to propanil. *P. fluorescens* strains RA-2 and RB-4 exhibited aryl acylamidase activity several orders of magnitude higher than any other organism isolated in our studies (56, 66).

The ability to hydrolyze propanil was studied in fifty-four isolates of fluorescent pseudomonads collected from three Mississippi Delta lakes (74). Overall, about 60% of the isolates hydrolyzed propanil, and all the propanil-hydrolyzing bacteria were identified as *P. fluorescens* biotype II. Most propanil-hydrolyzing *P. fluorescens* isolates transformed DCA to 3,4-dichloroacetanilide. The potential for acetyl transferase activity by these *P. fluorescens* aryl acylamidases should not be overlooked, since the enzyme isolated from *Nocardia globerula* (75) and *Pseudomonas acidovarans* (76) possesses this activity. The amidase from *Rhodococcus* sp. strain R312 has both amidase and acyl transferase activity. This enzyme was expressed in *Escherichia coli* and utilized in kinetic studies on acyl transferase activity (77). This purified enzyme catalyzed acyl transfer from amides and hydroxamic acids to only water or hydroxylamine. Further aspects of acetyl transferase activity will be addressed in the later presentation on bialaphos and phosphinothricin detoxification/resistance.

A variety of methods (radiological, spectrophotometric, and HPLC) are available to measure enzymatic hydrolysis of amides. A colorimetric method for measuring aryl acylamidase activity in soil using 2-nitroacetanilide (2-NAA) as substrate was recently developed (78). Acylamidase (2-NAA) activity was several fold lower than other

Table V. ^{14}C -Propanil Metabolism by Cell Suspensions of Soil, Water and Rhizosphere Bacteria

Genera	Strain	Source	% ^{14}C Recovered in methanolic extracts			
			Propanil	DCA	3,4-DCAA	Origin
<i>Bacillus</i> sp.	S92B2	S	nd	71.1	10.5	18.5
<i>Flavobacterium</i> sp.	W92B14	W	nd	88.6	4.5	6.9
<i>P. cepacia</i>	AMMD	R	74.5	6.9	18.6	Nd
<i>P. fluorescens</i>	RA-2	R	nd	100	Nd	Nd
<i>P. fluorescens</i>	RB-3	R	2.5	91.0	5.0	1.5
<i>P. fluorescens</i>	RB-4	R	nd	100	Nd	Nd
<i>P. fluorescens</i>	UA5-40	R	100	nd	Nd	Nd
<i>P. fluorescens</i>	W92B12	W	70.3	9.2	4.2	16.3
<i>Rhodococcus</i> sp.	S92A1	S	15.9	10.2	3.5	Nd
<i>Rhodococcus</i> sp.	S93A2	S	nd	96.1	3.9	Nd

NOTE: DCA = 3,4-dichloroaniline; 3,4-DCAA = 3,4-dichloroacetanilide; Origin = highly polar metabolites (immobile in benzene: acetone solvent); S = soil isolate; W = water isolate; R = rhizosphere isolate; nd = none detected. Cell suspensions [48 h tryptic soy broth cultures, log $11.0 \text{ cells mL}^{-1}$; potassium phosphate buffer (50 mM, pH 8.0)] were treated with propanil (800 μM , 8.0 kBq mL^{-1}) and incubated at 28°C , 150 rpm, 24 h. Propanil and metabolites identified by TLC and radiological scanning as described elsewhere (56).

hydrolytic enzymes such as alkaline phosphatase (3 to 5%), and aryl sulfatase (5 to 13%). Aryl acylamidase activity was 1.3- to 2.5-fold higher in surface no-till soils compared to conventional-tilled soils. This would be expected due to the greater microbial populations and diversity associated with the accumulation of soil organic matter in the surface of no-till soils. Maximal aryl acylamidase activity was observed at pH 7.0 to 8.0, and activity was reduced at assay temperatures above 31°C . Thermal inactivation has also been reported for a purified aryl acylamidase from *P. fluorescens* (65).

With respect to chloroacetamide herbicides, there is only limited evidence in the literature for cleavage of the substituted amide bond. The fungus *Chaetomium globosum* was shown to hydrolyze substituted amide bonds of alachlor (79) and metolachlor (80). A unique cleavage of propachlor [2-chloro-*N*-(1-methylethyl)-*N*-phenylacetamide] at the benzyl C-N bond, by a *Moraxella* isolate, has been demonstrated (81). However, little is known about this mechanism, or its distribution among other species. Recently, two bacteria (*Pseudomonas* and *Acientobacter*) capable of metabolizing propachlor were described (82). Both strains initially dehalogenated propachlor to *N*-isopropylacetanilide. The *Acientobacter* strain then hydrolyzed the amide bond, before the release of isopropylamine, and prior to ring cleavage. The *Pseudomonas* strain initially transformed propachlor via *N*-

dealkylation. This metabolite was then cleaved at the benzyl C-N bond as in the *Moraxella* isolate described above (81).

Aryl acylamidases have been purified from several bacterial species including, *B. sphaericus* (59), a coryneform-like bacterium (60), *Nocardia globerula* (75), *P. aeuriginosa* (83), *P. fluorescens* (65), and *P. pickettii* (67). These various enzymes have been shown to be quite diverse. Among these four genera, the aryl acylamidases ranged in size from 52.5 kDa for the *P. fluorescens* enzyme, to about 127 kDa for the coryneform and *N. globerula* aryl acylamidases. The *P. pickettii* enzyme is a homodimer, while the other enzymes are monomers. A novel amidase from *P. putida*, specific for hydrolyzing *N*-acetyl arylalkylamines, was purified to homogeneity (84). This protein (MW =150 kDa) is a tetramer of four identical subunits. This enzyme hydrolyzed various *N*-acetyl arylalkylamines containing a benzene or indole ring, and acetic acid arylalkyl esters, but not acetanilide derivatives. To our knowledge, no genes for specific pesticide-hydrolyzing aryl acylamidases have been cloned. A multiple alignment and cluster analysis has been performed on amino acid sequences of 21 amidases or amidohydrolases (85). A hydrophobic conserved motif [Gly-Gly-Ser-Ser (amidase signature)] has been identified which may be important in binding and catalysis. Amidases from prokaryotic organisms also have a conserved C-terminal end, not found in eukaryotes. These studies also indicate similarities of amino acid sequences among amidases, nitrilases and ureases.

Metals, i.e., Hg^{++} , Cu^{++} , Cd^{++} and Ag^{+} , that affect sulfhydryl groups differentially inhibited bacterial aryl acylamidases (59, 60, 65, 83). EDTA did not inhibit the *B. sphaericus* (59) and *P. fluorescens* (65) enzymes, but 2.5 mM EDTA inhibited the coryneform-like aryl acylamidase by about 90% (60). An aryl acylamidase from *P. aeuriginosa* required Mn^{++} or Mg^{++} (83), but these cations were not required by other enzymes mentioned above.

Initial studies of the interaction of chemical structure and microbial (*Penicillium* sp.) aryl acylamidase activity were evaluated in an enzyme inducible by karsil [*N*-(3,4-dichlorophenyl)-2-methylpentanamide] (64). Activity was greater with longer alkyl amide substitution, i.e., activity was 4, 262 and 1000 units for acetanilide, propionanilide, and butyranilide, respectively. Of the six herbicides evaluated, propanil had the highest activity (520 units), compared to karsil (70 units), solan [*N*-(3-chloro-4-methylphenyl)-2-methylpentanamide] (55 units), dicryl [*N*-(3,4-dichlorophenyl)-2-methyl-2-propenamide] (40 units), and no activity was detected with diuron and CIPC. The hydrolysis of seven *para*-substituted acetanilides by three bacterial species (*Arthrobacter* sp., *Bacillus* sp., and *Pseudomonas* sp.) was compared to rate constants for alkaline-mediated hydrolysis (86). In these studies, *para* substitution did not affect acetanilide hydrolysis by resting cells of these bacteria, however alkaline-mediated hydrolysis was highly affected. These studies suggest different intermediates and mechanisms for biotic versus abiotic transformations.

A structure-activity study evaluated acylanilide herbicide chemical structure using model substrates and four bacterial strains [two *Arthrobacter* spp. (BCL and MAB2), a *Corynebacterium* sp. (DAK12), and an *Acinetobacter* sp. (DV1)] capable of growth on acetanilide (87). When the nitrogen of acetanilide was alkylated (methyl or ethyl), the compound was an unsuitable substrate for all four strains. When a *para*-

methyl group was present on the acetanilide ring, activity occurred in all strains, but was lower than acetanilide activity in DV1 and one of the *Arthrobacter* strains, (MAB2). When the methyl group was in the *ortho* or *meta* position, activity similar to that on acetanilide occurred in two strains (DAK12 and BCL), but was only about 30% of the acetanilide rate in MAB2, and undetectable in DV1. Little or no activity in all four strains was found with dimethyl substitution in the 2 and 6 positions. Consequently, alachlor and metolachlor (with 2 and 6 alkyl substituents on the ring) are more persistent in soil compared to propachlor with an unsubstituted aniline ring (88).

The fungicide ipridione [3-(3,5-dichlorophenyl)-*N*-isopropyl-2,4-dioximidazolidine-1-carboxamide] undergoes several potential amide hydrolytic reactions. Ipridione degradation by an *Arthrobacter*-like strain (89) and three pseudomonads (*P. fluorescens*, *P. paucimobilis*, and *Pseudomonas* sp.) has been reported (90). Initial cleavage of ipridione forms *N*-(3,5-dichlorophenyl)-2,4-dioximidazoline and isopropylamine. The imidazolidine ring is cleaved, forming (3,5-dichlorophenylurea)acetic acid, which can be further hydrolyzed to 3,5-dichloroaniline. 3,5-Dichloroaniline is a major metabolite observed in soils where microflora have adapted the ability for enhanced ipridione degradation (91).

Inhibition of Plant and Microbial Amidases

Propanil hydrolysis is inhibited by various carbamate and organophosphate insecticides in plants (40, 92) and microorganisms (93). Competitive inhibition of aryl acylamidase activity by these compounds was the basis for increased (synergistic) injury to rice, caused when insecticides were applied to rice in close proximity to propanil application (94). Synergistic effects of propanil with several agrochemicals: carbaryl (1-naphthyl *N*-methylcarbamate); anilofos {*S*-[2-[(4-chlorophenyl)(1-methyl-ethyl)-amino]-2-oxoethyl] *O,O*-dimethyl-phosphoro-dithioate}; pendimethalin [*N*-(1-ethylpropyl)-3,-dimethyl-2,6-dinitrobenzenamine]; and piperophos {*S*-[2-(2-methyl-1-piperidinyl)-2-oxoethyl]*O,O*-dipropyl phosphorodithioate} in propanil-resistant barnyardgrass were recently detected using a chlorophyll fluorescence technique (95). Carbaryl's synergism is due to its competitive inhibition of aryl acylamidase (40), but the exact mechanism of the other synergists in propanil-resistant barnyardgrass is presently unknown.

Interactions of insecticides and soil aryl acylamidase activity have also been reported (93). When soil was treated with *p*-chlorophenyl methyl carbamate, propanil hydrolysis was substantially inhibited, and subsequent formation of tetrachlorodiazobenzene was reduced 10- to 100-fold. Carbaryl at 100 μ M inhibited propanil aryl acylamidase activity by 10 to 70% in several bacterial strains (66, 96). The *Fusarium solani* propanil-aryl acylamidases were insensitive to high concentrations of carbaryl and parathion (*O,O*-diethyl-*O*-4-nitrophenyl phosphorothioate), however the chloroacetanilide, herbicide ramrod (*N*-isopropyl-2-chloroacetanilide), competitively inhibited acetanilide hydrolysis.

Carbamate Hydrolysis in Plants

Carbamates have a broad spectrum of pesticidal activity and include commonly used insecticides, herbicides, nematocides (aldicarb {2-methyl-2-(methylthio)propanal *O*-[(methylamino)carbonyl]oxime} and fungicides. There are three major classes of carbamates: methyl carbamates: aldicarb, carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranol methylcarbamate), and carbaryl; phenylcarbamates: CIPC, IPC, and phenmedipham; and thiocarbamates: EPTC (*S*-ethyl dipropyl carbamothioate), butylate [*S*-ethyl bis(2-methylpropyl)carbamothioate], and vernolate (*S*-propyl dipropylcarbamothioate). The behavior of insecticidal carbamates has been extensively reviewed (97, 98). Studies on IPC (99) and CIPC (100, 101) in plants indicate that the major metabolic route is aryl hydroxylation and conjugation. Plants do not cleave the carbamate bond of the phenylcarbamate herbicides, which is distinct from the initial metabolism in either microorganisms or animals. The thiocarbamates such as EPTC are metabolized in tolerant species such as corn, cotton (*Gossypium hirsutum*), and soybeans via initial oxidation to the sulfoxide followed by glutathione conjugation (102, 103). The pathway for EPTC metabolism in plants is similar to that found in mouse liver microsomes (104).

Carbamate Hydrolysis in Microorganisms

Microbial degradation of carbamates occurs readily in soil. Accelerated degradation of certain carbamate insecticides has led to ineffectiveness of these compounds, e.g., control of phyloxera in vineyards by carbofuran (105). Bacterial isolates from several genera (*Arthrobacter*, *Achromobacter*, *Azospirillum*, *Bacillus*, and *Pseudomonas*) can hydrolyze various insecticidal carbamates (106). Hydrolysis is the major pathway for the initial breakdown of carbofuran, but little is known about the fate of the metabolites formed by this mechanism (106). Mineralization of the carbonyl group of carbofuran occurs more extensively compared to mineralization of the ring structure (107). The toxicity of aldicarb is greatly reduced when it is hydrolyzed to the oxime and nitrile derivatives (108), but oxidation of aldicarb to the sulfone or sulfoxide yields compounds with similar or greater toxicity. Organophosphorus compounds: paraoxon (phosphoric acid diethyl-4-nitrophenyl ester), chlorfenvinphos [phosphoric acid 2-chloro-1-(2,4-dichlorophenyl)ethyl diethyl ester], and disulfoton {phosphorodithioic acid *O,O*-diethyl *S*-[2-(ethylthio)ethyl]ester} which are esterase inhibitors, suppressed carbofuran hydrolysis in soil for 3 to 21 days (109). The persistence of carbofuran was increased when these esterase inhibitors were combined with the cytochrome P-450 inhibitor, piperonyl butoxide. This synergism is due to the inhibition of both major mechanisms of carbofuran degradation, i.e., hydrolysis and oxidation.

Some microbial aryl acylamidases [*P. striata* (110), *B. sphaericus* (59) and a coryneform-like isolate (60)] can hydrolyze certain phenylcarbamates, e.g., CIPC and IPC, as summarized in Table II. However, the *P. striata* aryl acylamidase is unable to hydrolyze methylcarbamates such as carbaryl. Carbamate hydrolases such as the

Arthrobacter phenmedipham-hydrolase, is specific for phenylcarbamates (111), whereas the *Achromobacter* carbofuran-hydrolase is specific for methylcarbamates (112).

A cytosolic carbamate-hydrolase has been purified from a *Pseudomonas* sp. (113). This enzyme is composed of two identical dimers with a molecular weight of 85 kDa each, and is active on carbaryl, carbofuran and aldicarb as substrates (113). The *Achromobacter* carbofuran-hydrolase has been purified to homogeneity and has a molecular weight of 150 kDa. This enzyme is either cytoplasmic or occurs in the periplasmic space, and requires Mn^{++} as an activator (114). It has no urease activity and does not hydrolyze benzamide. The genes for the *Achromobacter* carbofuran-hydrolase have been cloned, however they were poorly expressed in many gram-negative bacteria such as *E. coli*, *Alcaligenes eutrophus*, and *P. putida*. This indicates that secondary processing is required to produce a functional enzyme. The genes for phenmedipham-hydrolase (*pcd*) have also been cloned and the protein purified to homogeneity (111). The phenmedipham-hydrolase is a monomer with a molecular weight of 55 kDa, and contains the esterase motif (Gly-X-Ser-X-Gly). The phenmedipham-hydrolase also has hydrolytic activity on the traditional esterase substrate, *p*-nitrophenylbutyrate. The *pcd* gene was expressed in tobacco, and conferred resistance to phenmedipham at rates 10-fold higher than normal field application rates (115). Although bacteria have been isolated that possess diverse hydrolytic degradation mechanisms for carbaryl, few organisms are capable of complete mineralization of the entire molecule. When a bacterial consortium (two *Pseudomonas* spp.) was constructed, both hydrolysis and aromatic mineralization of carbaryl occurred (116).

Thiocarbamates have been developed as herbicides (butylate, EPTC and vernolate) and fungicides. Repeated application of these compounds to soil led to the development of microbial populations with accelerated thiocarbamate degradation capability. Soils adapted to EPTC degradation also rapidly degraded a related thiocarbamate, vernolate (117). However, soils adapted to butylate did not rapidly degrade EPTC and vernolate (117). EPTC metabolism occurs in many genera of bacteria (*Arthrobacter*, *Bacillus*, *Flavobacterium*, *Pseudomonas*, and *Rhodococcus*) and fungi (*Fusarium*, *Paecilomyces*, *Penicillium*) (118). Both hydrolytic and oxidative mechanisms have been proposed for EPTC degradation due to the fact that dipropylamine was found in the media of *Arthrobacter* and *Rhodococcus* strains TE1 (119) and BE1 (120). Evidence from another *Rhodococcus* strain JE1, indicates initial hydroxylation of the propyl group of EPTC, followed by *N*-dealkylation, forming propionaldehyde and *N*-depropyl-EPTC (121). Cytochrome P-450s, responsible for EPTC *N*-dealkylation, have been cloned from several *Rhodococcus* strains (122-124).

Hydrolysis of Organophosphate Insecticides by Plants

Organophosphate insecticides {parathion, malathion [*S*-1,2-bis(carboethoxy)ethyl-*O*-*O*-dimethyl dithiophosphate], and coumaphos (*O*-3-chloro-4-methyl-2-oxo-2H-

chromen-7-yl *O,O*-diethyl phosphorothioate)) have replaced many of the chlorinated insecticides, because they are more effective and less persistent. Wheat (*Triticum aestivum* L.) and sorghum (*Sorghum vulgare* L.) rapidly degrade dimethoate [*O,O*-deimethyl-*S*-(*N*-methylcarbamoylmethyl)phosphorothiolothionate] to products, which suggests hydrolytic metabolism (125). Crude enzyme extracts from wheat germ were able to hydrolyze malathion to dimethylphosphorothionate and dimethylphosphorothiolthionate (126). Metabolism of *O*-ethyl *O*-(4-methylthio)phenyl *S*-propylphosphorodithioate (sulprofos) was studied in cotton (127). The major metabolites were the sulfoxide and sulfone derivatives, indicating oxidation as a major initial transformation. Formation of the phenol and gluconoside conjugates of sulprofos indicates hydrolysis of the phospho-phenol bond, but enzymatic hydrolysis has not been confirmed. However, other enzymes, such as mixed-function oxidases and glutathione *S*-transferases (GST), may be equally important in the detoxification of organophosphorus insecticides in plants (128).

Hydrolysis of Organophosphate Insecticides by Microorganisms

The degradation of organophosphate insecticides has been studied extensively in several gram-negative bacterial strains, especially *Pseudomonas diminuta* and in *Flavobacterium* ATCC 27551 (129). Hydrolysis of the organophosphorus insecticides occurs via nucleophilic addition of water across the acid anhydride bond; thus the enzymes named parathion-hydrolases and phosphotriesterases are actually organophosphorus acid anhydrases (130). The parathion-hydrolase can be either cytosolic or membrane-bound, depending upon the bacterial species. The *Flavobacterium* enzyme is membrane-bound, and is a single unit of 35 kDa, whereas the enzyme from strain SC (gram-negative, oxidase-negative aerobic, non-motile, rod shaped bacterium) is composed of four identical subunits, each with a molecular weight of 67 kDa (129). This enzyme is also a membrane-bound protein (129). A coumaphos-degrading *Nocardia* isolate, B-1 (131) produces a cytoplasmic parathion hydrolase composed of a single 43 kDa subunit (129). These results indicate that enzymes possessing very diverse characteristics can have the same catalytic function. The aryldiphosphatase gene from *Nocardia* strain B-1 (*adp*-gene) has nothing in common with *opd* genes from other sources, and has most likely undergone independent evolution (132).

The bacterial parathion-hydrolase genes (*opd*) have been cloned from *P. diminuta* (133, 134) and *Flavobacterium* ATCC 27551 (135). The nucleotide sequence for the *Flavobacterium* and *P. diminuta* *opd* genes are identical (134, 135). The *opd* genes were poorly expressed in *E. coli*, and the *Flavobacterium* hydrolase was a much larger protein when expressed in *E. coli* compared to the native *Flavobacterium* hydrolase. When the hydrolase was expressed in *Streptomyces lividans*, it was of similar size to that produced in *Flavobacterium*, but was synthesized in larger quantities and was secreted extracellularly (136). Production of an extracellular hydrolase is ideal for remediation processes because detoxification does not require bacterial uptake.

Nitrile Hydrolysis in Plants

Nitrile groups are essential moieties in the phytotoxicology of the herbicides, bromoxynil (3,5-dibromo-4-hydroxybenzoxynitrile), cyanazine {2-[[4-chloro-6-(ethylamino)-1,3,5-triazin-2-yl]amino]-2-methylpropanenitrile}, and dichlobenil (2,6-dichlorobenzoxynitrile), and the fungicide chlorothalonil (2,4,5,6-tetrachloro-1,3-benzenedicarbonitrile). Initial enzymatic hydrolysis of the nitrile group produces an amide. The amide is subsequently converted to the carboxylic acid, which may be decarboxylated. This metabolic pathway occurs for bromoxynil in wheat (137) and for cyanazine in wheat, potato (*Solanum tuberosum*) and maize (*Zea mays* L.) (138, 139).

Nitrile Hydrolysis in Microorganisms

In bacteria, the cyano group of bromoxynil can also be hydroxylated to the respective carboxylate by several species: *Fexibacterium* sp. (140) and *Klebsiella pneumoniae* (141). The *K. pneumoniae* utilizes bromoxynil as a nitrogen source, rather than a carbon source, with 3,5-dibromo-4-hydroxybenzoate accumulating as an end-product. Alternatively, an oxidative pathway, mediated by pentachlorophenol-hydroxylase (flavin monooxygenase) from *Flavobacterium* sp. ATCC 39723 (currently classified as a *Sphingomonas*), directly liberates cyanide, forming dibromohydroquinone (142). Formation of the hydroquinone derivative, rather than the hydroxybenzoate derivative, renders bromoxynil more prone to complete mineralization. *Klebsiella* bromoxynil-nitrilase genes (*bxn*) have been cloned, sequenced, and the protein purified (143). The *bxn* genes have been expressed in plants, resulting in bromoxynil-tolerant plants (144). Commercial application of this technology is currently being used in cotton and potatoes to produce herbicide-resistant crops.

Role of Phosphatases and Sulfatases in Pesticide Degradation

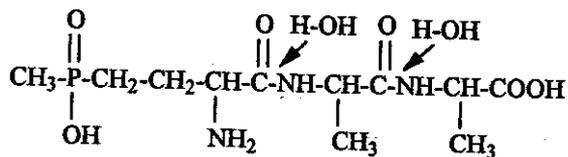
There is limited literature available on the role of phosphatases and sulfatases in pesticide metabolism. The insecticide endosulfan [1,2,3,4,7,7-hexachlorobicyclo [2.2.1]-2-heptene-5,6-bisoxymethylene sulfite] is metabolized *via* both oxidative and hydrolytic mechanisms *in vitro* by the white rot fungus, *Phenerochaete chrysosporium* (145). Under both nutrient-rich and nutrient-limiting conditions, endosulfan is metabolized to endosulfan diol. This indicates a different metabolic route, catalyzed by a sulfatase rather than a lignin peroxidase. Other studies have shown that endosulfan diol is also formed by hydrolytic cleavage of endosulfan by static cultures of the fungus *Trichoderma* sp. (146). These observations also suggest a role for sulfatase in fungal metabolism of endosulfan.

Genetic Engineering of Crops for Bialaphos/Glufosinate Resistance

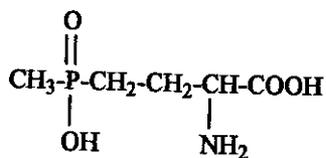
Numerous phytotoxic metabolites produced by microorganisms have been isolated, identified and tested for their potential as herbicides. Of these, bialaphos and phosphinothricin (PPT) are the most successful. Glufosinate (ammonium salt of phosphinothricin) and bialaphos have been developed as major commercial herbicides (see Figure 4). These compounds are included in this discussion because bialaphos is hydrolyzed by plant and microbial enzymes to yield the active herbicide (PPT), and also because PPT can be enzymatically acetylated at the primary amine moiety to yield the *N*-acetyl compound (non-phytotoxic) which may be acted on by hydrolases and/or transferases to yield the active phytotoxin (Figure 4). Furthermore, metabolism of the peptidyl compound bialaphos, and the resistance of transformed plants to phosphinothricin, are based on the presence or absence of transaminase and/or hydrolytic enzyme activity.

Bialaphos is a tripeptide comprised of a unique amino acid, L-2-amino-4-[hydroxy(methyl)phosphinyl]butyric acid (PPT) linked to two L-alanyl moieties. The compound was isolated from cultures of *Streptomyces viridochromogenes* (147), and *Streptomyces hygroscopicus* (148). The natural form of PPT is the L-isomer (L-PPT), and it was the first reported naturally-occurring amino acid containing a phosphinic group. Bialaphos was initially found to have some antifungal (*Botrytis cinerea*) and antibacterial activity (147, 149), thought to be attributed to L-PPT. Glutamine reversed growth inhibition caused by bialaphos in *Bacillus subtilis* cultures (147). PPT also strongly inhibited glutamine synthetase [E.C. 6.3.1.2; GS] activity in *E. coli* (147). Later examination of L-PPT for phytotoxicity by Hoechst AG showed that it possessed strong phytotoxicity, and this compound was patented as a herbicide (150). Synthesis of the DL-PPT ammonium salt resulted in the commercial herbicidal formulation of this active ingredient. Bialaphos is rapidly degraded by microorganisms in soil to PPT (151), which is also rapidly degraded, with half-life of 4 to 7 days in soils (152, 153). In a test of 300 bacterial isolates from soil, all strains degraded L-PPT to the 2-oxo analog of PPT via transamination (154). Glufosinate (PPT) is a non-selective, postemergence herbicide used for weed control in orchards and vineyards, in chemical fallow situations, as a preharvest desiccant, as a burn-down herbicide of cover crops and/or weeds prior to no-till planting (155), and for weed control in transgenic crops resistant to the herbicide (156).

Bialaphos is absorbed through plant leaves, and some translocation (of bialaphos or its metabolites) occurs (151). Bialaphos is metabolized in plants soon after absorption to yield PPT. Bialaphos does not inhibit GS, but is rapidly metabolized by peptidases in plant tissues yielding PPT (157, 158). The D-isomer of PPT is not a GS inhibitor (159), has no herbicidal activity (156), and is not acetylated in transgenic plants, which have been transformed with resistance to L-PPT (160). There is also a lack of degradative metabolism of L-PPT in non-transformed plants, but there is rapid conversion to the acetylated product in plants genetically altered for PPT resistance.



Bialaphos



Phosphinothricin

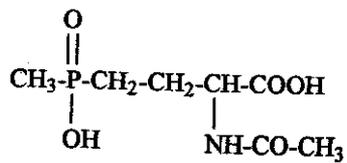
*N*-Acetyl-phosphinothricin

Figure 4. Chemical structures of bialaphos, phosphinothricin and *N*-acetylated phosphinothricin.

Biotechnological approaches have been utilized in studies on the biochemistry of PPT in microorganisms and plants. The biosynthetic pathway of bialaphos has been completely elucidated using various techniques (161). Beginning with precursors containing three carbon atoms, bialaphos is produced in a complex series of over a dozen steps (161). One step involves an acetyl CoA-dependent reaction that modifies either demethyl-PPT or PPT. The *bar* gene is responsible for resistance to bialaphos in *S. hygroscopicus*, and encodes for the acetyl transferase, which converts PPT to an acetylated non-phytotoxic metabolite (162). Although this acetyl transferase is not classified as a hydrolytic enzyme *per se*, it does form an amide bond that could therefore be susceptible to hydrolytic and/or transferase activity. As pointed out previously, deacetylases have been studied in plants, microorganisms, as well as in mammalian systems, and nitroacetanilide substrates have been utilized to facilitate assaying their activities (163). Such plant or microbial enzymes could act on *N*-acetyl-PPT to release the phytotoxic compound, PPT.

Over the past several years, many vegetable and cereal crop species have been transformed with genes imparting resistance to PPT. Cloning of a PPT-resistant gene (*bar*) from *S. hygroscopicus* (164) and the transformation of PPT-resistant plants has been accomplished (165). A similar gene (*pat*) from *S. viridochromogenes* Tü 494, with the same function, was simultaneously isolated and has also been introduced into various plant species (160, 166, 167). Presently, more than 20 crop plant species have been transformed for resistance to PPT in this manner. Some of these genetically altered plants are resistant to PPT at rates as high as 4 kg ha⁻¹ (ca. 10 times the lowest normal field application rate) (164). This indicates a high degree of incorporation of acetyl transferase expression.

The previously known anti-fungal activity of bialaphos and glufosinate was recently assessed on three pathogens (*Rhizoctonia solani*, *Sclerotinia homoeocarpa*, and *Pythium aphanidermatum*) *in vitro* and *in vivo* on PPT-resistant transgenic creeping bentgrass (*Agrostis palustris*), an important turfgrass (168). Results indicated that bialaphos can simultaneously control weeds and fungal pathogens in this transgenic grass. Furthermore, bialaphos has antibiotic activity against *R. solani* Kühn that causes rice sheath blight (169), and *Magnaporthe grisea* (Herbert) Barr (148) that causes rice blast disease. Substantial suppression of sheath blight symptoms was reported when bialaphos was applied to transgenic plants which had been infected with *R. solani* prior to herbicide treatment (170). Inoculated transgenic rice plants [bialaphos-resistant (*bar*) gene] had reduced lesions and other symptoms of rice blast disease after bialaphos treatment (171). It is assumed that these pathogens are controlled by bialaphos and PPT, because the microbes lack the ability to rapidly metabolize the compounds to non-fungitoxic products. Thus, it appears possible to control some serious diseases by using *bar*-transgenic rice cultivars and bialaphos for weed and disease control. Glufosinate has also been successfully used to control the weed red rice (a conspecific weed of cultivated rice) in *bar*-transformed rice (172).

Bialaphos and PPT are unique among commercial herbicides in that they have both potent antibiotic and herbicidal properties. This dual strategy will no doubt be utilized more widely with the increasing availability of PPT-resistant crops.

Summary and Conclusions

Generally, our understanding of microbial hydrolytic enzymes has been greatly increased during the past decade, but information on plant hydrolytic enzymes is not as advanced. Although advances have been made, most of the information on microbial hydrolytic enzymes, has not been focused directly on pesticide metabolism. Many hydrolytic enzymes have been reported to have multiple activities (amidase, esterase, transferase), but most have not been examined for multiplicity, especially with regard to the metabolism of pesticides. Also the knowledge about the precise physiological role of these hydrolytic enzymes is insufficient. Moreover, information is needed on enzyme mechanisms and regulation of enzyme activity. Many enzyme active sites or receptor sites recognize only one stereochemical geometry. Thus, understanding enzyme multiplicity, physiological role, mechanism, and regulation, may lead to the development of more specific regulators (e.g., inhibitors, activators), so that more specific and efficacious pesticidal compounds can be developed using a biorational design. The use of techniques such as protein engineering may provide additional insight on the relationship of protein structure and substrate specificities of hydrolytic enzymes in plants and microorganisms.

Many industrial synthetic processes produce racemic mixtures, in which only one enantiomer is biologically active. Hydrolytic enzymes have high potential value in the development of bioprocesses for production of compounds useful to agriculture and other industries. Enzymes have the unique ability to facilitate stereospecific transformations and thus, biosynthetic approaches may be more effective in some industrial syntheses. The cloning of an *Arthrobacter* esterase gene that stereospecifically produces (+) *t*-chrysanthemic acid, utilized in the synthesis of pyrethroid insecticides, is one example demonstrating this biotechnological strategy. This enzyme occurs in low amounts in this bacterium, however cloning and over-expression could permit industrial-scale preparation. Certain hydrolytic enzymes are also being considered for remediation of contaminants, e.g., nitrilases for solvents such as acetonitrile (173), amidases for acrylamides (174), and atrazine [6-chloro-*N*-ethyl-*N*-(1-methylethyl)-1,3,5-triazine-2,4-diamine] chlorohydrolyase, to degrade atrazine (see chapter by Sadowsky and Wackett in this volume).

As we have discussed, crop engineering for resistance to herbicides, based upon microbial hydrolytic enzymes, is a commercial success for bialaphos and phosphinothricin. Future herbicide technologies may utilize other unique microbial hydrolytic enzymes that can be developed for engineering crop resistance to other herbicides. Other novel pesticides (fungicides, insecticides and herbicides) may also be designed as potent inhibitors of hydrolytic enzymes, or that would be activated or detoxified by specific plant or microbial hydrolytic enzymes.

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