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Detoxification of pesticides by microbial enzymes

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1. Introduction

The production and use of synthetic pesticidal chemicals increased dramatically after World War II. The need to know the fate and effect of these new chemicals in the environment has created tremendous research efforts. Early investigators were concerned with degradative processes and when it was realized that microorganisms could degrade xenobiotics, research projects were initiated to determine the basic principles of microbial metabolism. This led some scientists to be amazed at the ability of microbes to degrade chemicals, claiming that microbes were either infallible and could degrade any synthetic molecule, or, on the other hand, that certain molecules could not be metabolized and were refractory[14]. This debate led investigators toward a better understanding of the basic principles concerning the enzymology and biochemistry of pesticide-related metabolism. It also helped to establish that the persistence of xenobiotics in the environment was strongly affected by microbial activity.

In the 1970's the mood of the pesticide industry changed from being optimistic to recognizing the damages that agricultural chemicals could do to our

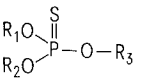
environment and public health. Pesticide usage had increased to 0.75×10^9 kg by the end of the decade[59]. Problems of chronic exposure[49], of improper waste disposal[47], environmental damage[43], and other adverse responses to pesticides required that federal laws be promulgated to govern their manufacture, distribution, and use. In developing these laws, the metabolism studies from the 1950's to 1960's helped determine which agricultural chemicals represented a danger to our chemically-dependent society. The tremendous diversity in the type of chemicals to be detoxified mandated that many different disposal techniques be considered. Physical methods of incineration, entrapment, and burial were developed, as were chemical methods involving oxidation, reduction, and hydrolysis[51]. The use of biological treatment systems persisted, and this technology is being improved. An important aspect of earlier pesticide metabolism studies helped to develop a new approach to biological pesticide waste treatment technology. Crude enzyme extracts from microorganisms were shown to hydrolyze pesticides to less toxic and less persistent compounds.

The expanding field of biotechnology has helped intensify research programs designed to detoxify pesticides that are in the environment. The 1980's have begun an area of research that is being directed towards the commercialization of many aspects of pollution control biotechnology. Among the methods being developed for large-scale use is enzyme detoxification. By the 1990's it is very likey that several hydrolase enzymes will be in use on a practical scale in order to aid in the never-ending tasks of environmental clean up.

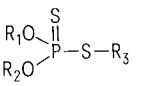
The intent of this chapter is to review the scientific literature pertaining to the treatment of various pesticides by cell-free enzyme extracts in order to show the potential of microbial enzyme systems. Our basic postulation is that some microbial enzymes can be isolated and can be effective in converting toxic or persistant pesticides to less toxic or less persistent molecules. For organophosphate, carbamate, acylanilide, phenylurea, and phenoxyacetate pesticides, hydrolases are important for initial steps of whole cell metabolism. Hydrolases are prime candidates for industrial applications because they are stable and do not require co-factors. In the table, the toxicity of some selected pesticides is listed as well as the toxicity of their major enzymatic hydrolysis product[14]. For instance, the hydrolysis of parathion to *p*-nitrophenol leads to a 122-fold decrease in overall toxicity and complete removal of parathion. For organophosphates and carbamates, hydrolysis of the parent pesticide will generally lead to a significant reduction in toxicity. For the acylanilides, phenylureas, and phenoxyacetates pesticides, hydrolysis does not generally lead to a significant reduction in toxicity; sometimes, in fact, the chemicals become more toxic. Yet the biospecificity of the pesticide molecule is destroyed, and the metabolites are not as stable in the environment as the original pesticide molecule.

2. *Organophosphates*

Organophosphate pesticides have been used as replacement chemicals for the more persistent organochlorine and mercurial compounds. Reviews have been published discussing the environmental fate of organophosphate pesticides and the role microorganisms play in their degradation[1,29,35,52,59,61]. The organophosphates are characterized as having the general formula of the type shown in figure 1. In this class, parathion (O,O-diethyl-O-p-nitrophenyl phosphorothioate) is the most extensively studied insecticide due to its intensive agricultural use. Indirect evidence of microbial participation in the degradation of parathion was shown by examining the stability of parathion in autoclaved and nonautoclaved soil and water samples[5,36].



where
R_1 and R_2 = alkyl (either ethyl or methyl) moiety
R_3 = substituted aryl or alkyl moieties



where
R_1 and R_2 = alkyl(either ethyl or methyl)moiety
R_3 = substituted alkyl or aryl moieties

Figure 1. General structure of organophosphate and dithioate pesticides.

Toxicity of selected pesticides and major metabolites

Pesticide	Major metabolite	Toxicity (mg/kg)*	Detoxification factor
Organophosphates			
Parathion	–	6	
	p-Nitrophenol	350	122
Azinphos-methyl	–	13	
	Anthranilic acid	4620	824
Carbamates			
Carbaryl	–	500	
	1-Naphthol	2590	7
Phenylureas			
Monuron	–	1480	
	4-Chloroaniline	300	− 8
Acylanilides			
Alachlor	–	1800	
	2,6-Diethylaniline	2690	2
Phenoxyacetates			
2,4-D	–	370	
	2,4-Dichlorophenol	580	2

* Toxicity reported: LD_{50} oral, rat, mg/kg, data from Christensen[14]. Dash denotes increase in toxicity.

In some cases, heat-labile agents of microbial origin were isolated from the soil, which helped in rapid degradation of parathion. Methyl parathion, like ethyl parathion, also disappeared rapidly in nonsterilized soils but not in sterilized soil[22]. Conclusive evidence for the involvement of microorganisms in parathion degradation was obtained when microbes were isolated from soil, and in vitro metabolism studies were subsequently performed.

The initial point of organophosphate metabolism is the hydrolysis of the aryl phosphate bond. Thus, this hydrolase (EC 3.1.3), referred to as an esterase, aryl esterase, or phosphotriesterase, is the most important enzyme in bacterial metabolism of organophosphates. An analogous enzyme has been extensively studied in both insects and mammals[19] since this reaction is a major mechanism of resistance and detoxification. In one of the earliest studies of organophosphate hydrolases, Mounter et al.[42] reported that a group of microorganisms contained enzymes capable of hydrolyzing dialkyfluorophosphates. The DFPase activity (EC 3.7.2.1) was found in 10 of 14 microbes tested and was activated by Mn^{2+} and inhibited by Ca^{2+}.

Zech and Wigand[65] isolated 2 phosphohydrolases from *E. coli* and one of the enzymes, DFPase (EC 3.8.2.1) could detoxify di-isopropyl-fluorophosphate while the other, paraoxonase (EC 3.1.1.2) hydrolyzed paraoxon. DFPase could not hydrolyze paraoxon and vice versa. Both enzymes had a very low substrate specificity ($K_m = 1.7 \times 10^{-2}$ and 5×10^{-3} mM for DFP and paraoxon, respectively), had activity at pH 8–8.5 and were unstable at room temperature.

A cell-freee preparation from *Flavobacterium* sp. ATCC 27551 described by Sethunathan and Yoshida hydrolyzed diazinon, parathion and chlorpyrifos[55]. In all 3 cases, the aromatic or heterocyclic moiety produced by hydrolysis could not be further metabolized by this bacterium. This phosphoesterase (EC 3.1.3) did not hydrolyze amino parathion or malathion, a dithioate insecticide. In another investigation with this bacterium, Brown[13] reported that the constituitive phosphotriesterase was composed of 2 protein units, one with a molecular weight of 50,000 daltons, and the other with a molecular weight of greater than 100,000 daltons.

The larger molecule was associated with an orange gum which probably contained flavoprotein. The enzyme was active with those organophosphates having an electron withdrawing aromatic or heterocylic leaving group as in parathion, paraoxon, and diazinon. The enzyme could not hydrolyze compounds with weakly electrophilic groups like 4-aminophenol. This phosphoesterase showed activity at pH values of 8–10 and was unaffected by the presence of EDTA, NaF, or NaN_3 or metal ions such as Zn^{2+}, Cu^{2+}, Co^{2+}, Mg^{2+} or Mn^{2+}. Irreversible enzyme inhibition was seen with non-ionic detergents such as triton X-100® and Tween 80®.

A crude enzyme extract obtained from a mixed bacterial culture that was grown on parathion showed exceptionally high activity for hydrolysis of at least ten other organophosphates[44]. The rate of parathion hydrolysis was 3000 nmoles/min/mg protein and was 3000 times faster than chemical hydrolysis by 0.1 N NaOH. This phosphoesterase (EC 3.1.3) was unaffected by high substrate concentration or metabolite accumulation, did not require co-factors, and was active between pH 7 and 10.5. The temperature optimum of the enzyme was 35 °C, and the crude enzyme preparation was stable at room temperature but was denatured by freezing.

The presence of various solvents at 15–450 ppm accelerated hydrolysis, but at solvent concentrations of 1000 ppm enzyme activity was inhibited. Cleavage of the arylphosphoester bond was influenced more by alkyl substituents on the phosphorous atom than by functional groups on the aromatic ring. In the case of diazinon, EPN, chlorpyrifos, and triazophos, the effect of aromatic functional groups did not prevent enzymatic hydrolysis, but a $-SCH_3$ group in the para position of fenthion inhibited hydrolysis. Both dimethyl- and diethylsubstituted dithiophosphates (ethion, azinphos-methyl, chlormephos) were not hydrolyzed by this crude enzyme preparation.

Munnecke[45] and Talbot et al.[58] covalently bound this crude enzyme extract to porous glass, silica beads or alumina and examined the properties of the immobilized enzyme for the detoxification of industrial wastewaters and concentrated pesticide solution. The immobilized enzyme exhibited approximately 50% of its soluble enzyme activity and displayed minor changes from the characteristics of free enzyme in regard to differences in pH and temperature. A reactor with immoblized enzyme had a volume of 200 ml and could hydrolyze 90% of a 10-ppm parathion solution at flow rates up to 96 l/h. Although reactors containing enzyme bound to glass showed good characteristics for the parameters examined, it was noted that the reactor containing enzyme immobilized to alumina was superior to enzyme bound to glass for high flow rates, such as those that may be encountered in industrial situations[58].

Rosenberg and Alexander[53] obtained an inducible phosphoesterase from 2 *Pseudomonas* species grown in the presence of wither diazinon or malathion as sole phosphorous source. A crude enzyme preparation could hydrolyze aspon, monocrotophos, fensulfothion, diazinon, malathion, acephate, parathion and trithion. The enzyme preparation could not hydrolyze dimethoate, trichlorfon, methyl parathion or dichlorvos. The cleavage of the common phosphorous-oxygen bond of these pesticides was not influenced by the type of alkyl substituents since methyl, ethyl, and

propyl esters were metabolized. This enzyme was active against the dithioate insecticides malathion and carbophenothion, in contrast to the activity of the parathion hydrolase reported earlier by Munnecke[44].

All of the above organophosphate hydrolysis studies dealt with the primary hydrolysis of the parent molecule. Clearly this is an important step in the mineralization of pesticides. For example, *p*-nitrophenol, the major metabolite formed by hydrolysis of parathion, paraoxon, or methylparathion is readily metabolized by numerous microorganisms, as are other phenol metabolites from other organophosphates[6,7,25,28,48].

Dithioates constitute a major group of organophosphates. They are characterized by the general chemical formula shown in figure 1. The pesticides belonging to this group are in great demand due to their broad spectrum and high insecticidal activities. However, not much work has been reported regarding their microbial metabolism except for one compound, malathion, [O,O-dimethyl-S-(1,2-dicarbethoxyethyl)-phosphorodithioate]. Two species of the genus *Rhizobium, R. leguminosarum* and *R. trifolii* were isolated from an Egyptial soil and had powerful carboxyesterase activity (EC 3.1.1.1) toward malathion[41]. Five hydrolytic metabolites, inorganic phosphate and thiophosphate, were identified as metabolites. In a study by Mostafa et al.[40] involving 3 fungal isolates - *Aspergillus niger, Penicillium notatum* and *Rhizoctonia solani* the presence of carboxyesterase activity was indicated when malathion was degraded to different carboxylic products which constituted nearly 40% of the total metabolites. Monomethyl phosphate and thiophosphate were formed in the culture medium due to the action of 2 other enzymes, an esterase (EC 3.1.1.2) and a phosphatase (EC 3.1.3). The esterase cleaved the sulfur carbon bond of the mono- and di-acids to dimethyl phosphorothioates and dimethyl phosphorodithioates, which were ultimately demethylated through phosphatase activity. This clearly suggests the establishment of these enzymes in cultures metabolizing malathion[40].

Matsumura and Bousch[39] examined a strain of *Trichoderma viride* and a *Pseudomonas* sp. and found that malathion was rapidly metabolized by the action of a soluble carboxyesterase (EC 3.1.1.1) as evidenced by the presence of carboxylic acid derivatives in the culture medium in addition to other demethylated products and products of hydrolysis. Cell-free enzymatic hydrolysis of malathion at the phospho-sulfur bond was reported by Rosenberg and Alexander[53]. This enzyme, obtained from the *Pseudomonas* species grown on either diazinon or malathion, hydrolyzed carbophenothion and malathion, but failed to metabolize dimethoate. An alkali-extractable, heat-labile organic entity from soil, which transformed malathion to malathion monoacid, was isolated by Getzin and Rosenfield[23] from both irradiated and nonirradiated soil. This soil-free, extracellular esterase was quite stable, had a pH optimum of 6.8 and displayed normal Michaelis-Menten kinetics. The enzyme was not denatured with increased acidity (< 2.0) and was active over a wide temperature range (20–70 °C).

Most recently, Barik et al.[4] reported the enzymatic hydrolysis of malathion and other dithioate pesticides. In these studies, a constituitive, cell-free enzyme extract was obtained from 2 strains of *Arthrobacter.* The 2 enzyme extracts showed a broad pH optimum (6–9), temperature optima of 25 °C and specific activities of 1.3 and 2.0 μmoles/mg protein/min for the substrate malathion.

3. Carbamates

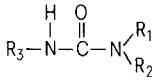
The carbamates are a structurally and physiologically heterogeneous group of compounds. Their classification is based upon the fact that they are all derivatives of carbamic acid. Carbaryl and carbofuran act as insecticides whose mode of action is that of inhibiting acetylcholinesterase, whereas chlorpropham and diallate act as herbicides and control plant growth by interfering with cell division. More is known about the microbial enzymology of phenylcarbamate herbicides than any other group of the carbamate pesticides. The best characterized carbamate hydrolyzing enzyme was obtained by Kearney and Kaufman[32] when they isolated a strain of *Pseudomonas striata* Chester from soil which could metabolize the phenylcarbamate herbicide chlorpropham (isopropyl-N-(3-chlorophenyl)carbamate, CPC) as a sole source of carbon and energy. Enzymatic cleavage of the ester and/or amide linkage of chlorpropham was quantified by the appearance of 3-chloroaniline. The esterase activity (EC 1.1.1) contained in a cell-free extract from the pseudomonad also cleaved propham, isopropyl-N-phenylcarbamate (IPC), as well as 9 other carbamates, including alpha-carbo(2,4-dichlorophenoxyethoxy) ethyl N-(3-chlorophenyl)carbamate, isopropyl-N-(3,4-dichlorophenyl)carbamate, sec-butyl-N-(3,4-dichlorophenyl)carbamate, and alpha-carboisopropoxyethyl-N-(3-chlorophenyl)carbamate.

Either 3-choroaniline, aniline, or 3,4-dichloroaniline was detected from all of the carbamates after esterase activity. Kearney later reported the purification and characterization of this enzyme[31]. With chlorpropham as the carbon source, the pseudomonad isolate was mass-cultured and the cells then sonicated. Ammonium sulfate precipitation, gel filtration and ion exchange were used to purify the enzyme. After purification the pH optimum was 8.5 and the enzyme had a broad range of activity between pH 6.0 and 9.5. With chlorpropham and propham as substrates, the esterase had specific activities of 6 and 5 nmoles/min/mg protein, respectively. The metal ions, Mg^{2+}, Mn^{2+}, Ca^{2+} and Co^{2+} were not necessary for enzyme activity. The esterase was inhibited 42% with diisopropylfluo-

rophosphate at a 10^{-6} M concentration, which implied that the hydroxyl group of serine possibly was part of the active site of enzyme-substrate interaction. The broad substrate specificity of the enzyme was demonstrated by its esterase activity against the carbamates, plus it also showed amidase activity. As an amidase (EC 3.5.1.4), the enzyme hydrolyzed 3,4-dichloroacetanilide and 3,4-dichloropropionanilide. It was proposed that large, bulky substituents in the area of the carbonyl carbon could cause steric hindrance and inhibit the hydrolysis of compounds with this property.

4. Phenylureas

The phenylureas were first developed after World War II by DuPont and approximately 25 urea herbicides are commercially available. The basic structure of the phenylurea herbicides is shown in figure 2. The microbial metabolism of urea herbicides has been investigated for many years, and the reader is referred to Geissbühler et al.[21] for a general discussion of this subject.



R_1 = methyl or methoxymethyl group
R_2 = methyl group
R_3 = substituted benzyl group

Figure 2. General chemical structure of phenylurea herbicides.

The degradation of urea herbicides by cell-free extracts of *Bacillus sphaericus* was described by Wallnöfer and Bader[64] when this organism liberated carbon dioxide from the ureido portion of the molecule and formed halogen-substituted aniline moieties[63]. Cells of *B. sphaericus* ATCC 12123, when grown in a minimal salts medium supplemented with glucose, yeast extract, and asparagine, were induced to degrade linuron [(N-3,4-dichlorophenyl)-N-methoxy-N-methylureal] in media containing 50 µmoles/l of this herbicide. Induction was not possible in minimal medium containing peptone or meat extract, however. Once cells were induced, cell-free extracts with amidase activity (EC 3.5.1.4) were capable of liberating carbon dioxide from the ureido portion of the molecule, thereby leaving the corresponding aniline moiety. In addition, the crude extract was also capable of decomposing monolinuron [N-(4-chlorophenyl)-N′-methoxy-N′-methylurea], exhibiting specific activities of 14.6 and 16.2 nmoles of hebicide degraded per min per mg protein, respectively. Monuron, a dimethylurea herbicide, was also degraded by the linuron-induced enzyme, which exhibited a specific activity of 4.1 nmoles/min/mg protein. However, fluometuron, [N-(3-trifluoromethylphenyl)-N′-N′-dimethylurea], also a dimethylurea herbicide, was not attacked by this enzyme. The enzyme expressed a specific activity of 17.8 nmoles/min/mg protein for the inducer, linuron.

The amidase (EC 3.5.1) responsible for hydrolyzing these herbicides was partially purified and Wallnöfer and Bader[63] reported a 6.6-fold purification of an enzyme having a pH optimum between 7.0 and 8.5. No enzyme activity was observed below pH 5.5, and the highest enzymatic activity was observed in the late exponential growth phase, prior to sporulation. The authors recognized the relatively low activity of the enzyme system but hypothesized that the enzyme apparently hydrolyzed N′-methoxyphenylureas at the amide linkage, which would yield phenylcarbamic acid and an unidentifiable metabolite. Phenylcarbamic acid spontaneously disintegrated to the corresponding aniline with the formation of carbon dioxide[31]. Subsequently, Engelhardt et al.[17] identified N,O-dimethyl hydroxylamine as a degradation product of linuron. The herbicide was degraded by extracts of *B. sphaericus* ATCC 12123 and the production of N,O-dimethyl hydroxylamine, as well as the products CO_2 and 3,4-dichloroaniline, allowed a mechanism of linuron degradation to be proposed.

In later enzyme work, Engelhardt et al.[16] examined the substrate specifically of the linuron-degrading enzyme and also studied the reaction sequence of linuron degradation. The investigators concluded that linuron, or a compound derived from it induced an enzyme system responsible for degrading the herbicide. The specificity of the enzyme was quite broad and the acylanilide herbicides monalide, carboxin and pyracarbolid, and several other acylanilide compounds were degraded by the linuron-induced amidase.

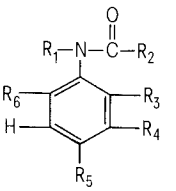
The acylanilides were degraded 10 times faster than were the methoxy-substituted phenylureas. Because the enzyme exhibited a stronger affinity for the acylanilides than the phenylurea herbicides, Engelhardt and co-workers[16] believed that the site of enzymatic hydrolysis was the peptide bond between the amino group of the aniline moiety and the carboxyl group. This led the investigators to classify the enzyme as an acylamidase (EC 3.5.1). Most recently, Engelhardt et al.[18] described the further purification and properties of the aryl acylamidase (EC 3.5.1.13) of *B. sphaericus*. This enzyme was induced by various phenylamides including acylanilide, phenylcarbamate and methoxy-substituted phenylurea pesticides. The results of this study were in contrast to previous studies by Geissbühler et al.[20] and Tweedy et al.[62] which indicated that a stepwise demethylation or demethoxylation, deamination and decarboxylation of phenylurea herbicides occurred. The enzyme investigated by Engelhardt et al.[18] resembled the enzyme from *Pseudomonas striata* Chester, studied by Kear-

ney[31], the acylanilide hydrolyzing enzymes of *Fusarium solani* reported by Lanzilotta and Pramer[34] and an enzyme from a *Penicillium* sp. described by Sharabi and Bordeleau[56]. The enzyme described by Engelhardt et al.[18] from *B. sphaericus* was produced by cells grown in media where the herbicides were present in growth media at a concentration of 0.05 µmoles/ml, with glucose, asparagine, and yeast extract serving as the primary carbon substrates. When supplied at levels of 0.05 µmoles/ml, the phenylurea herbicide, maloran [3-(3-chloro-4-bromophenyl)-1-methoxy-1-methylurea], the acylanilide herbicides, monalide (4-chloro-alpha, alpha-dimethyl-valeranilide) and propanil (3,4-dichloropropionanilide), the acylanilide fungicides, 2-chlorobenzanilide and 2,5-dimethyl-furan-3-caroxanilide, and the phehylcarbamade herbicide, propham, also induced the formation of the aryl acylamidase. However, the specific activities of the various extracts were considerably lower than those from crude extracts of linuron-induced cells. Production of the enzyme was initiated in the later exponential growth phase. A cell-free extract was prepared, and the enzyme was purified to homogeneity. The enzyme was very labile during large-scale preparation and exhibited a pH optimum between 7 and 8.5. The enzyme lacked a demonstrable metal ion requirement, was quite sensitive to inhibition by sulfhydryl reagents and some metal ions, and lost 50% of its activity when held at 40 °C for 9.5 min. The molecular weight of the enzyme was determined to be 75,000 for 9.5 min. The molecular weight of the enzyme was determined to be 75,000 daltons ±10%, and the specific activity of the purified linuron-induced enzyme was 130 nmoles/min/mg protein.

It appears that the hydrolysis of the amide bond of several phenylurea herbicides is possible by the action of acylamidase or arylacylamidase enzymes obtained from certain microorganisms. These enzymes are of particular interest for use in pesticide detoxification because they require no co-factors and exhibit relatively wide substrate specificity. Although the phenylurea herbicides have low mammalian toxicity, the hydrolysis of these compounds may be desirable for destroying the phytotoxicity of the herbicides in question.

5. *Acylanilides*

The acylanilide herbicides are effective weed killers because they provide selective toxicity at a low cost. They also exhibit relatively low mammalian toxicity. The basic structure of this class of herbicides is shown in figure 3 and they resemble, structurally, the phenylurea and phenylcarbamate herbicides. The chemistry and degradation of acylanilide herbicides was reviewed by Still and Herrett[57] and the reader is referred to this paper for a more detailed description concerning the metabolism of this group of herbicides. A review by Bartha and Pramer[8] also describes the metabolism of acylanilide herbicides. Sharabi and Bordeleau[56] described organisms that were capable of degrading karsil. Karsil could be degraded by whole cells or cell-free extracts of a *Penicillium* sp. 2-Methylvaleric acid and 3,4-dichloroaniline were produced. The karsil acylamidase (EC 3.5.1) was induced by 0.1 mM karsil and showed marked variation in enzyme activity for different substrates, including various acylanilides (including the 3 herbicides dicryl, propanil, and solan), 1 dimethylurea herbicide (diuron), and 1 phenylcarbamate herbicide (chlorpropham). After partial purification, the enzyme showed greater activity for N-(phenyl)-butyramide, when it released 5.5 nmoles of arylamine per min per mg of protein. Activity of the enzyme generally increased with increased side-chain length up to 4 carbons. Substitution or branching of the N-acyl group adversely affected enzyme activity and a 2-chloro substitution on the side chain of propanil also reduced activity. Deacylation was influenced by the nature and position of ring substituents. A 2-chloro substitution on the ring prevented hydrolysis, possibly because of a steric effect.



R_1 = an alkyl group or H
R_2 = alkyl group, CH_2Cl
R_3 = H, C_2H_5
R_4 = Cl, H
R_5 = CH_3, Cl, H
R_6 = H, C_2H_5, CH_3

Figure 3. General chemical structure of acylanilide herbicides.

As mentioned previously during the discussion of phenylurea herbicides, Kearney[31] reported that an enzyme responsible for degrading chlorpropham was isolated from *Pseudomonas striata* Chester. In this study, Kearney recognized that the phenyl carbamate-induced enzyme also showed specificity for 3,5-dichloroacetanilide and 3,4-dichloropropionanilide.

Lanzilotta and Pramer[33] used propanil (3′,4′-dichloropropionanilide) at a concentration of 0.16 g/l of basal salts medium and isolated a strain of *Fusarium solani* from soil that could utilize the herbicide as a sole source of carbon and energy. The proposed pathway for the metabolism of propanil is shiown in figure 4. The primary metabolite 3,4-dichloroaniline, accumulated in the medium up to a level of 80 µg/ml. This level of 3,4-dichloroaniline prohibited further utiliza-

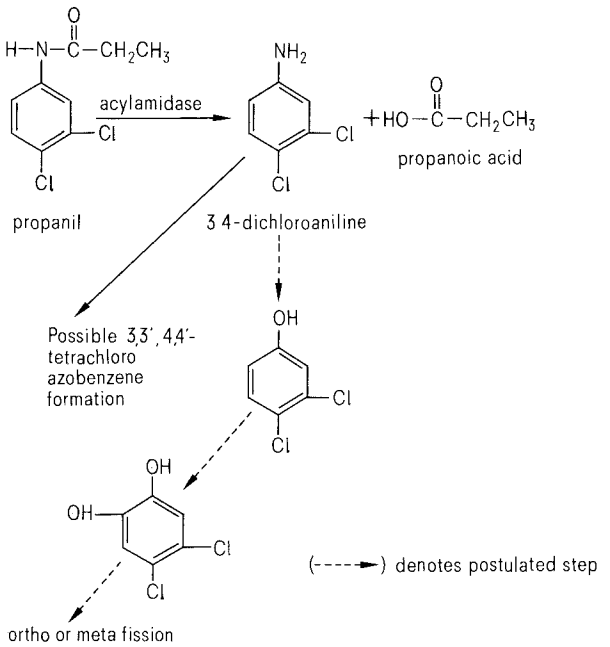


Figure 4. Proposed pathway for the metabolism of propanil.

tion of the herbicide and allowed only minimal growth. When glucose (0.05%) and yeast extract (0.01%) were added to propanil-containing basal salts medium, the rate of propanil degradation increased, wheras reduced aeration retarded fungal growth and herbicide metabolism. The increased rate of propanil degradation due to the addition of glucose or yeast extract was assumed to be a result of increased cell population at the onset of propanil utilization. The investigators believed that the acylamidase involved probably functioned during usual metabolic activities of the fungus, acting on substrates more 'normal' than propanil.

In further studies with the acylamidase of *F. solani*, Lanzilotta and Pramer[34] characterized the fungal aryl acylamidase (EC 3.5.1). Replacement cultures, previously grown on Czapek-Dox broth, were used to produce the enzyme when they were placed in 0.05 M phosphate buffer (pH 7.4) to which had been added either 150 ppm propanil or 270 ppm acetanilide. A crude enzyme extract was obtained from the acetanilide-containing medium and the enzyme was partially purified and concentrated by ammonium sulfate precipitation. The enzyme was active over a pH range of 7.5–9.0, was inactivated in 10 min at 50 °C and showed maximum enzyme activity at 38 °C. The enzyme exhibited a K_m of 0.195 mM for acetanilide. Propachlor was a competitive inhibitor of acetanilide hydrolysis. Lanzilotta and Pramer[34] also investigated the influence of chemical configuration on the rate of hydrolysis of various structurally related acyanilides. The effect of para-substitution generally decreased the rate of hydrolysis. Acetanilide was also hydrolyzed 42 times faster than propionanilide, and when a chlorine atom was present on the acyl chain of acetanilide, the rate of hydrolysis decreased approximately 67%. The phenylurea herbicides monuron and fenuron, and the phenylcarbamate herbicide, propham, were not hydrolyzed by this enzyme preparation.

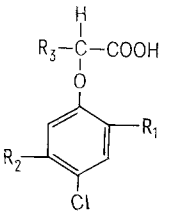
Blake and Kaufman[12] also characterized acylanilide-hydrolyzing enzymes from *F. oxysporum* Schlecht. The enzymes were induced by propanil and PPG-124 (p-chlorophenylmethylcarbamate), and both enzymes were separated into fractions greater than 100,000 daltons or less than 50,000 daltons. The greater than 100,000 daltons fraction exhibited 95% of the total amidase activity. The investigators were unable to demonstrate whether the amidases were similar, but reported that the two enzymes possibly were different. The enzymes were produced by cells cultured in a basal salts medium containing 0.2% sucrose and 0.1% yeast extract. Cells were grown for 3 days at 25 °C in the presence of either 50 ppm propanil or 10 ppm PPG-124, then harvested by filtration and immediately frozen and lyophilized. Active enzyme preparations were produced at 5 °C by grinding the lyophilized cells and precipitating the proteins with ammonium sulfate. The proteins were then purified by ultrafiltration. Enzyme activities were determined by assaying the rate of chloroaniline formation. With the propanil-induced enzyme, the greatest specific activity of the enzyme was observed for the inducer, and was 9.25 nmoles/min/mg protein. The PPG-124-induced enzyme expressed a specific activity of 66.66 nmoles/min/mg protein.

The pH optimum of the greater than 100,000 dalton fraction of the propanil-induced enzyme was 10, whereas the pH optimum of the greater than 100,000 daltons fraction of the PPG-124-induced enzyme was 9. When the substrate specificities of both enzymes were investigated, it was noted that the acyl chain length definitely affected the rate of hydrolysis. The effect of number and position of chloro-substituents was also determined, and para-substitution appeared to increase the rate of hydrolysis, whereas ortho-substitution reduced the rate of hydrolysis. Both enzymes were capable of hydrolyzing acylanilides but were incapable of hydrolyzing the phenylurea herbicides linuron or diuron and the propanil-induced enzyme could not hydrolyze PPG-124 or chlorpropham.

6. Phenoxyacetates

The phenoxyacetate herbicides, which include 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and 4-chlroro-2-methylphenoxyacetic acid (MCPA) have been used extensively for the control of weeds since their introduction

in the mid 1940's. The basic structure of the phenoxyacetates is shown in figure 5.



R_1 = Cl or CH_3
R_2 = H or Cl
R_3 = H or CH_3

Figure 5. General chemical structure of phenoxyacetate herbicides.

The recent concern over the possible mutagenicity of phenoxyacetate herbicides because of TCDD (2,4,7,8-tetrachlorodibenzo-p-dioxin) contamination of 2,4,5-T has resulted in restriction on their use and increased interest in their biochemistry and metabolism.

Loos[37] reviewed the chemistry, degradation and mode of action of phenoxyacetate herbicides and the microbial metabolism and degradation of these compounds has also been reviewed[2,30]. In general, 2,4-D disappears more rapidly than other phenoxyacetates after application to soil, usually within 2–3 weeks. MCPA and 2,4,5-T persist for a more variable period of time after application, ranging from 6 weeks or longer for MCPA and between 6 and 38 weeks for 2,4,5-T[37]. Biodegradation of phenoxyacetates in aquatic environments occurs more slowly than in soil, possibly because of lower cell densities in these environments[26]. A cell-free extract obtained by Loos et al.[38] from an *Arthrobacter* sp. was capable of catalyzing the degradation and dehalogenation of 2,4-D, MCPA and 4-chlorophenoxyacetic acid. First, the side chain of 2,4-D, attached by ether linkage to the aromatic ring, was detached without prior hydroxylation of the ring to form 2,4-dichlorophenol. In 3 h, 92% of the organic chlorine in 2,4-D was released. This information can be used to calculate a specific activity of 0.3 nmoles/min/mg protein for the overall dehalogenation reactions. Data reported by Tiedje and Alexander[60] can be used to estimate a specific activity of 0.1 nmoles/min/mg protein for their enzyme preparation [EC 1.14.99 (monoxygenase) or possibly EC 3.3.2 (hydrolase)] when it catalyzed the conversion of 2,4-D to 2,4-dichlorophenol.

Bollag et al.[10] continued this work and found the enzymes from the *Arthrobacter* sp. hydroxylated 2,4-dichlorophenol to form 3,5-dichlorocatechol. From their data a specific activity of 6.2 nmoles/min/mg protein can be calculated. NADPH and oxygen were required for the reaction, indicating the involvement of a mixed function oxidase (EC 1.14.13.7). The 3,5-dichlorocatechol was subsequently converted to an UV-absorbing compound (2-chloro-4-carboxymethylene-but-2-enolide) and free chloride[9]. Oxygen was required for this reaction, but no pyridine nucleotides or iron were needed. The specific activity of the enzyme preparation of this reaction can be estimated as 4.4 nmoles/min/mg protein. The lyophilized, cell-free enzyme extract retained its activity toward chlorocatechol for several months.

Bollag et al.[11] also obtained a strain of *Flavobacterium peregrinum* from a soil enrichment which was capable of degrading MCPA. The enzymatic activity of this organism and the *Arthrobacter* sp. isolated by Loos et al.[37] toward MCPA was then compared. Both cell-free extracts catalyzed the conversion of MCPA to 4-chloro-2-methylphenol. The specific enzyme activity for dehalogenation can be estimated to be 0.1 nmoles/min/mg protein for the *Arthrobacter* extract and 0.01 nmoles/min/mg protein for the extract from the *Flavobacterium* sp. The extract from the *Arthrobacter* sp. also catalyzed the specific dehalogenations of 4-chloro-2-methylphenol and 4-chlorocatechol. The *Flavobacterium* sp. extract did not release chloride from the phenol. Subsequently, the *Arthrobacter* extract was fractionated by gel filtration. Active fractions were not capable of hydroylating 4-chloro-2-methylphenol to a catechol unless NADPH was added. Dechlorination occurred only after the catechol was formed.

Discussion

Several enzymes capable of degrading a wide variety of pesticides have been described in this chapter. None of these enzymes is practical for immediate routine industrial use, but the potential importance of their development for pesticide disposal has been indicated. Enzymemediated pesticide disposal can be applied to many situations where removal of a hazardous chemical is desired. In agricultural practices, methods for detoxifying pesticides are nonexistent, yet unwanted pesticides are often generated by miscalculations in mixing or spraying, or by residues remaining in containers. Soil spills are not uncommon on farms either, and safe methods for detoxifying spilled pesticides are needed. In any of these situations a suitable pesticide detoxifying enzyme preparation could be used by the consumer to allow safe and efficient disposal of unwanted chemicals. The enzymatic removal of parathion from pesticide containers has already been demonstrated[46] when it was observed that 85 g of residual parathion in a 200-l drum were hydrolyzed after parathion hydrolase was added. Within 16 h, 90% of the parathion present in the drums as a 48% emulsifiable concentrate was hydrolyzed. Methyl parathion, diazinon, and dursban at concentrations up to 1% technical compound could also be enzymatically hydrolyzed.

The feasibility of using enzyme preparations to clean up soil spills has also been demonstrated. Studies by Domsch et al.[15] and by Barik and Munnecke[3] indicated that when parathion hydrolase was added to soils, it efficiently hydrolyzed 1% parathion or diazinon within 24 h to less toxic and unstable products.

Enzymes capable of degrading pesticides could also be used in agriculture to prevent carry-over of crop protection chemicals. Spray tanks and other equipment containing phenoxyacetate, phenylurea, acylanilide, or other herbicides could be treated with an appropriate enzyme to remove residual pesticide. This would prevent carry-over and accidental phytotoxicity during subsequent spraying procedures.

Detoxification by enzymes rather than by whole microbial cells is particularly beneficial because enzymes sometimes can tolerate environmental extremes better than whole microbial cells. Extremes of pH and temperature, as well as high salt and solvent concentrations are often encountered in pesticide production wastewaters. The enzyme parathion hydrolase, for example, could tolerate salt concentrations up to 10%, solvent concentrations of 1% and temperatures up to 50 °C. These 3 parameters would have prevented growth of the *Pseudomonas* sp. that produced the hydrolase[46]. The use of enzymes is also attractive because the transport of pesticides into whole microbial cells can be problematic. Such membrane transport problems could be avoided when soluble enzymes are employed in disposal processes.

The use of immoblized enzymes in a continuous flow column for detoxification of pesticide production wastewaters has also been described[11,58]. This system was examined to determine its ability to hydrolyze residual parathion in process wastewaters before discharge into waste treatment systems. The enzyme column was effective in reducing parathion levels from 10 ppm to 500 ppb in approximately 1 min and the enzyme remained active under continuous use conditions for over 70 days. Enzymes that have the greatest importance for pesticide detoxification are those that can function without co-factors or co-enzymes and can detoxify a pesticide molecule by a hydrolytic or other simple enzymatic reaction. Presently, there are some enzymes that would be useful for detoxification reactions but are not likely cadidates for immediate development because they require co-factors or co-enzymes. For example, oxygenase enzymes (EC 1.14.12 and EC 1.14.13) require molecular oxygen and either NADPH or NADH to initiate aromatic ring fission. The development of these enzymes for large-scale industrial applications will be prohibited until economical methods for co-factor regeneration are discovered. Carboxy-lyase enzymes (EC 4.1.1) could also be important degradative enzymes for industrial pesticide detoxification use, but these require thiamin-diphosphate or pyridoxal-phosphate, and until an inexpensive source of these co-factors is available, carboxy-lyase enzymes cannot be developed for treatment systems.

Some limitations of pesticide detoxification by enzyme technology should be considered here. It is a fact that some compounds are not detoxified by enzymatic hydrolysis, but instead, the metabolites have increased toxicity (table). In such instances, initial enzymatic treatment of a parent compound may cause the molecule to subsequently be degraded more rapidly by whole microbial cells. Some pesticides do not pose toxicity problems, but because they are used in such large quantities there is a need for proper disposal technology. Because enzyme mediated disposal can occur under mild conditions, we feel that the enzymatic treatment of low toxicity compounds is highly desirable.

7. Future directions and goals

It was estimated that the demand for industrial enzymes would average a growth rate of 8% over the 6-year period between 1979–1985[50]. Although the use of enzymes for detoxifying pesticides has been limited on the industrial scale, large scale production and use is quite feasible, especially since a wide variety of enzymes have already been described and studied. Much interest has been expressed by chemical companies to develop and expand this enzyme technology further. Several enzymes exhibit a variety of different functions and it is most likely that other enzymes that have yet to be characterized in depth may also serve to detoxify pesticides and other forms of hazardous wastes. One such group of enzymes is the halidohydrolases. It would be desirable if enzyme systems could be developed to dehalogenate aromatic compounds since the basic nucleus of many pesticides is a halogenated aromatic ring. Two halidohydrolases have been described that were induced by chloroacetate and dichloroacetate. These enzymes used a hydroxyl group from water to replace the halogen in the substrate[24]. Dechlorination of aromatic compounds has been described by Janke and Fritsche[27].

Investigators are searching for new detoxification enzymes in several laboratories. These enzymes are being sought in hopes of detoxifying several forms of toxic compounds. At his time, the enzymes that catalyze simple hydrolytic reactions are best for immediate development for large scale use. There is particular interest in developing enzymes for detoxifying a wide variety of wastes because such treatment is gentle and safe. Once the existing detoxification enzymes have been developed, produced and used on a large scale, then the search for and development of other new enzymes, including those that dehalogenate or require co-factors, may occur.

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