Chapter 7

# Role of Carboxylesterases (ALiE) Regarding Resistance to Insecticides: Case Study of Colorado Potato Beetle (*Leptinotarsa decemlineata* Say)

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Additional information is available at the end of the chapter

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**Abstract**

Colorado potato beetle is one of the most important pests because of rapid and strongly developed resistance to insecticides. Resistant insects' populations may detoxify or degrade the toxin faster than susceptible insects, or quickly rid their bodies of the toxic molecules. Resistant populations may possess higher levels or more efficient forms of these enzymes. Insecticide metabolic destruction inside the target organism is a common defensive mechanism, decreasing the duration and intensity of the exposure of the target site, lowering the probability of a lethal outcome. Three major mechanisms of metabolic transformation of insecticides underlie the vast majority of examples of biotransformation-based resistance: (i) oxidation; (ii) ester hydrolysis; and (iii) glutathione conjugation. Pyrethrins, pyrethroids, organophosphates, carbamates and other insecticides are degraded by hydrolysis. Insecticide detoxification primarily unfolds through molecule hydrolysis on different sites, thereby splitting ester, carboxyl-ester, amide and other chemical bonds. The most important hydrolytic enzymes are phosphoric triesters and carboxylesterases (ALiE esterases). Structural mutations in mutant carboxylesterases have now been widely described showing metabolic resistance to organophosphate and pyrethroid insecticides and relatively few cases of resistance to carbamates role. Carboxylesterases role in Colorado potato beetle resistance was confirmed by many authors.

**Keywords:** insecticide resistance, metabolism, hydrolysis, carboxylesterase, ALiE esterase

## 1. Introduction

Worldwide, *Leptinotarsa decemlineata* (Say.)—Colorado potato beetle (CPB) is one of the most important pests of potatoes, an insect extremely difficult to control due to rapid and strongly developed resistance against insecticides [1–4]. None of the control techniques, during the long-lasting history, developed against this pest has provided long-term protection for potato crops. CPB still remains a major threat to potato production because of its resistance to all major groups of insecticides [5–18]. Numerous alternative control strategies for Colorado potato beetle were investigated in the last few decades [19–31].

In insects, generally, the factors that lead to resistance are (1) morphological, (2) physiological and biochemical, and (3) behavioral [32]. Regarding CPB's resistance to insecticides, the most important, investigated, and best described are the metabolic changes. Populations of resistant insects may detoxify or degrade the toxin faster than susceptible insects or quickly rid their bodies of the toxic molecules. This type of resistance is the common mechanism and often presents the greatest challenge. Resistant populations may possess higher levels or more efficient forms of these enzymes. In addition to being more efficient, these enzyme systems also may have a broad spectrum of activity. The metabolic destruction of an insecticide inside the target organism is a common defensive mechanism that leads to a decrease in the duration and intensity of the exposure of the target site and thereby lowers the probability of a lethal outcome. Three major mechanisms of metabolic transformation of insecticides underlie the vast majority of examples of biotransformation-based resistance: (i) oxidation; (ii) ester hydrolysis; and (iii) glutathione conjugation.

In the first stage, the metabolism of insecticides is manifested through many reactions, most important of which are oxidation, reduction, and hydrolysis. In the second stage, conjugates are formed, which are practically nontoxic [33]. Selective toxicity of insecticides mostly comes from the balance of the reactions included in activation and in detoxification. Pyrethrins, pyrethroids, organophosphates (OP), carbamates, and other insecticides are degraded by hydrolysis. This is the basis for the selective effect of insecticides and for insects' resistance mechanisms. Insecticide detoxification primarily unfolds through molecule hydrolysis on different sites, thereby breaking ester, carboxylester, amide, and other chemical bonds [32, 34].

The most important hydrolytic enzymes are phosphoric triesters and carboxylesterases (ALiE esterases, nonspecific, or B-esterases).

Esterase-related insect resistance is based on the following:

- Increase in the total amount of esterase—by altering regulatory genes or regulatory loci combined with structural genes, which results in change in enzyme synthesis in the organism or amplification of genes responsible for DNA methylation.
- Change in their activity—by altering structural genes that directly determine the nature of enzymes [1].

The majority of widely used insecticides are esters. This includes virtually all carbamate and OPs, most pyrethroids, and others. In almost all the cases, the hydrolysis of the ester group

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leads to a significant decrease in or elimination of toxicity. Consequently, esterase activity often plays a key role in determining the comparative responses and resistance to current insecticides [1].

In insects, the primary groups of esterases of interest hydrolyze esters of carboxylic acids, and they are, therefore, termed carboxylesterases. The topic of their nature and significance in insecticide toxicology and resistance has been reviewed by different researchers [35–38]. A useful functional method with special relevance for insecticides was developed based on the ability of the esterase to either hydrolyze OPs (type A) or to become inhibited by them (type B). There are relatively a few cases of high-level esterase-mediated metabolic resistance to carbamates. Structural mutations in mutant carboxylesterases have now been described from four insect species, showing metabolic resistance to OP insecticides [39]. The role of esterase in CPB resistance was confirmed by many authors [4, 40–44].

## 2. Hydrolytic metabolic pathways

Insecticide detoxification is primarily performed by hydrolysis of molecules at different sites. Hydrolysis means splitting of molecules by adding water. This chemical reaction splits different chemical bonds, such as ester bonds (with phosphoric, carbamine, chrysanthemum, and other acids), carboxyl ester, amide, and other bonds [32–34].

Hydrolysis of such bonds is done enzymatically and nonenzymatically. Besides hydrolases (esterases, phosphatases, carboxylesterases, and amidases), in splitting some of the mentioned chemical bonds there are also some other enzymes involved, such as mixed function oxidases (MFO) and glutathione S transferases (GST) [33]. Hydrolytically, pyrethrins and pyrethroids, organophosphates, carbamates, and some other insecticides can decompose faster or more slowly. This type of metabolism often makes a basis for a selective mode of action of insecticides and a mechanism of insect resistance to insecticides [34].

### 2.1. Hydrolytic enzymes

Hydrolases are widely spread in diverse plant and animal tissues and different parts of cells. In vertebrates, they can be located in blood plasma, and they are able to attack a large number of xenobiotic esters, but natural substrates of these esterases are unknown. In mammals, A-type esterases (with –SH group) can be found in the serum, and they are associated with the lipoprotein fraction. Some hydrolases, especially B-type hydrolases (with –OH group), are bound to membranes in the microsomal fraction. Up to 7% of all microsomal proteins is membrane-bound esterases. Such esterases can also be found in the serum and pancreatic fluid. Hydrolases can be present in the cellular fluid [32].

Most of these enzymes are not purified. Hence, it is often unknown if the investigated hydrolytic reactions are catalyzed by one enzyme of weak specificity (for example, one enzyme with both esterase and amidase function) or by a mixture of two or more enzymes with higher specificities. Unlike oxidases and transferases, hydrolases do not require any coenzymes; however, from time to time, they require cations for activation [32].

There are still some difficulties in naming hydrolases and their specificities for substrates. The same bonds, especially in phosphates, can be attacked not only by hydrolases but also by other enzymes (MFO and transferases). In the broadest sense, one can identify phosphatases, carboxyl esterases, carboxyl amidases, and epoxide hydrolases [41]. Cyclic phenyl saligenin phosphate, S,S,S-tributyl phosphorotrithioate (DEF), and profenofos are stated as hydrolase inhibitors [45].

### 2.2. Hydrolysis of phosphorus esters

Enzymes that catalyze a hydrolytic attack on phosphorous esters or anhydride bonds are marked as hydrolases of phosphoric triesters or phospho-triesterases. In insects, these enzymes are not purified, so they have not been adequately compared with purified mammalian hydrolases. These hydrolases do not attack phosphoro-trithioates in any scope. The hydrolase reaction is activated with 1 mM $Mn^{2+}$ and $Co^{2+}$ [46].

In several cases, the activity of these enzymes is higher in resistant insect species. Phosphate triester hydrolysis results in forming an anion metabolite that is a weak AChE inhibitor, finally resulting in detoxification of the starting compound. There are two types of phosphate-ester bonds: an anhydride bond (P—O; P—S; P—C; P—N; and others) and an alkyl-ester bond (R—O—P). There is also an alkyl–nitrogen bond (R—N—P). These bonds are split not only by hydrolases but also by MFO and G-S transferases. Enzymatic hydrolysis is found in mammals, insects, plants, and microorganisms [34].

Some of these bonds can be split nonenzymatically, for example, when it comes to chlorpyrifos oxon in trout, hydrolysis is not stimulated by $Ca^{2+}$ and EDTA does not deactivate this reaction. None of the known inhibitors has affected the hydrolysis. It is also known that trichlorfon can be nonenzymatically transformed into dichlorvos in some plant and animal species [34]. Hydrolases attack oxo forms of phosphates, such as paraoxon, diazoxon, malaoxon (**Figure 1**), and dichlorvos [46].

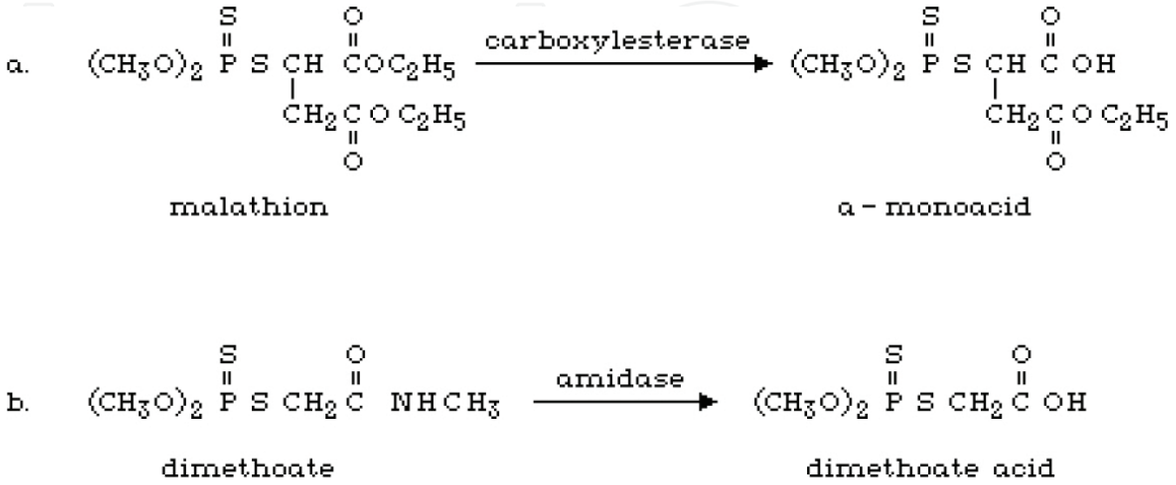


**Figure 1.** Different hydrolases and their specificities for substrates [47].

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Hydrolases (A-esterases) split not only phosphate-ester bonds such as P—O and P—S but also anhydride bonds such as P—O—P, P—F, P—C. Metabolic splitting of the P—S—aryl bond is done at P–S, hydrolytically for thiolate esters and oxidatively for thiolothionates. Fonofos is hydrolyzed after oxidative desulphurization takes place. In some mammals, trichlorfon is hydrolytically metabolized by splitting the P—C bond, and in some others (rabbits), there is a different kind of metabolic reactions. Omethoate (thiolate) is also hydrolytically metabolized in rats by splitting of the P—S bond. However, splitting of the P—S bond in the P—S alkyl structure is not hydrolytic, while splitting of the S—C bond most probably is. The former can result in a P–OH product and the latter in a P—SH product. The ester bond in malathion is split into small scale by breaking the P—S bond (about 1%) and the S—C bond (about 0.5%) by a liver homogenate. Splitting of alkyl-ester bonds in organophosphates is done not only hydrolytically but also oxidatively and through a group transfer. Hydrolysis, for example, splits the ethyl-phosphorous bond in paraoxon. This reaction is conducted in a soluble liver fraction in mammals and in vivo in insects. In rats, there is also hydrolytic O-demethylation of omethoate [34].

### 2.3. Hydrolysis of carboxylic esters

Carboxyl esterases are also called ALiE esterases or B-esterases. The enzyme that catalyzes the splitting of the carboxyl ester bond in organophosphates is referred to as EC. 3.1.1.1. [34, 38].

It is widely spread in mammals (in the liver, kidneys, lungs, spleen, small intestine, and fluid) [34] and in insects, of both susceptible and resistant species. A purified carboxyl esterase in insects (ALiE) has the molecular weight of 16,000 Da [46].

After investigating the specificities of carboxylesterases in the liver of rats, it has been ascertained that nonphosphorous mono- and di-carboxyl esters serve as substrates. The $\alpha$-carboxyl ester bond in malathion is hydrolyzed to form malathion $\alpha$-monoacid. Similarly, the homogenate of the housefly gives $\alpha$-monoacid more than β-isomer ($\alpha/\beta$ ratio is 3.5–5.0). However, esterases taken from the horse liver and rat liver microsomes primarily produce malathion β-monoacid ($\alpha/\beta$ ratio is 0.1). Malathion di-acid in rats does not emerge in vitro, but in vivo, under the influence of an unknown factor [35, 39].

Organophosphates of different structures inhibit carboxyl esterases or the metabolism of malathion and other insecticides from this group in vivo [34]. EPN oxygen analogue and n-propyl-paraoxon inhibit these enzymes and show a strong effect ($I_{50}$ about $10^{-8}$ M) [46]. These compounds are also strong synergists for malathion and acethion in houseflies and mites. Tri-o-cresyl phosphate (TOCP), which is not an insecticide, is also tested as a carboxylesterase inhibitor. Compounds of such type are weak esterase inhibitors in vitro, but strong inhibitors of malathion metabolism in vivo. TOCP is in vivo transformed into saligenin cyclic-o-tolyl phosphate, marked as M-1, which selectively inhibits carboxyl esterases ($pI_{50}$ for the mouse plasma esterase amounts to 7.2). TOCP increases malathion toxicity four times, and M-1 100 times. Other triaryl phosphates with a 0-alkyl group can be activated metabolically in a similar way. Moreover, increased toxicity of malathion for mice is also exhibited by chlorothion (insecticide) and S,S,S-tributyl-phosphoro-trithioate (DEF) (defoliant), inhibiting its metabolism in vivo. In resistant insect species, DEF also synergizes paraoxon, azinphos-methyl,

carbamates, and DDT and with EPN it synergizes dicrotophos, dimethoate, and phorate in *Anthonomus grandis* [34].

Several cases have shown that the increased carboxylesterase activity is a mechanism of insect resistance to malathion. In general, the activity of these enzymes susceptibility to insects is low. Differences between the enzymes in susceptible and resistant insects are quantitative, and in *Tetranychus urticae*, they are also qualitative [46].

In the liver of mammals and products of several insect species, there are enzymes that hydrolyze pyrethroids. It is not excluded that carboxylesterase that hydrolyzes (+)-*trans*-resmethrin is the same one that hydrolyzes malathion. The enzymes in some insects, such as *Oncopeltus fesciatus* and *Trichoplusia ni*, degrade (+)-cis compounds, whereas isomer specificity is less pronounced in the enzymatic products from *Musca domestica* and *Blattella germanica*. Relative hydrolysis speed is much higher in mammals than in insects, which is most likely the basis of the selective toxicity of pyrethroids [34].

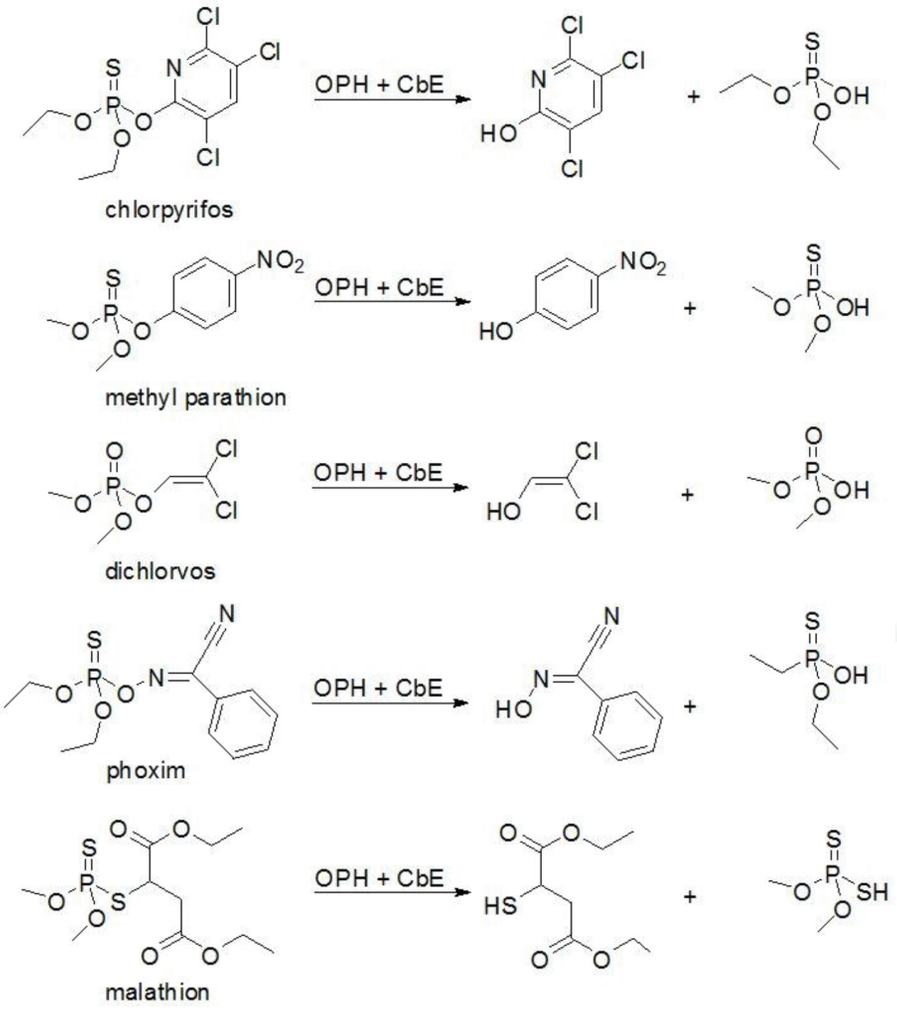


**Figure 2.** Hydrolysis of different OPs pesticides by OPH + CbE enzymes [48].

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Little is known not only about the nature of esterases that hydrolase carbamates but also about biochemistry of these processes. Enzymes in the plasma of rabbits, sheep, and pigs are more efficient in carbaryl hydrolysis than enzymes in other mammals. In insects, perhaps, it is about a nonspecific esterase, aromatic esterase, primarily responsible for carbamate hydrolysis [34].

In some organophosphates (malathion, acethion, and phenthoate), the presence of the carboxyl ester group makes these compounds susceptible to hydrolysis at that site. In malathion, hydrolysis splits one carbethoxy group, forming nontoxic monoacid (**Figure 2**). The anionic charge of the carboxyl group shifts near phosphorous so that the electrophilicity is reduced by the effect of field. Hence, malathion-$\alpha$-monoacid is inactive. The metabolism of malathion in insects and mammals is quantitatively similar, but qualitatively quite different. Due to its much faster metabolism in mammals, they are usually less susceptible to malathion than insects. Moreover, such type of malathion metabolism predominates in mammals. The presence of the carboxyl ester group in organophosphates, however, does not always lead to reduced toxicity. Mevinphos is much more toxic than it can be expected from its structure that includes the carbethoxy group. In detoxification of mevinphos, the carboxyl esterase plays no or a very little role, and this bond is hydrolyzed nonenzymatically [32, 34, 46].

Some arthropod species resistant to malathion have a much higher carboxylesterase activity than susceptible species. The carboxylesterase activity is, at least in part, responsible for resistance to organophosphorous insecticides in several insect and mite species (mosquitoes, houseflies, cicadas, and mites) [36, 40–43].

Carbamates can also be hydrolyzed by esterases (**Figure 3**). The isolated carbamic acid is immediately hydrolyzed into $CO_2$ and methyl or dimethyl-amine. The carbamate ester bond is quite stable in plants and insects, but easy to hydrolyze in the majority of animal species [32, 34].

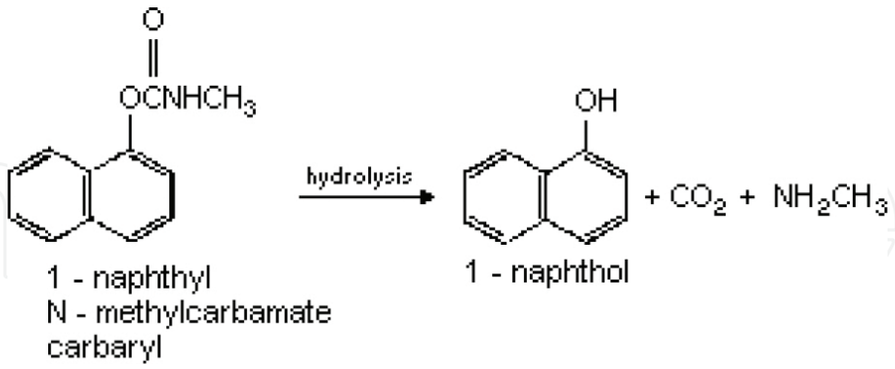


**Figure 3.** Hydrolytic pathway for carbaryl [49].

Besides, oxidative metabolites with a still intact carbamate ester bond are also subjected to hydrolysis by the same or perhaps different enzymes. In many cases, it is impossible to determine whether oxidation happens before or after hydrolysis [34]. N-substituted carbamates, the products of oxidative N-dealkylation are hydrolytically more unstable than N-methyl carbamates (about 100 times as much).

The rate of enzymatic hydrolysis depends on carbamate structure and the type of organism. Therefore, rats hydrolyze about 25% carbaryl, 33% propoxur, and 75% maxacarbate or isolan. Although most mammals hydrolyze carbamate ester bonds easily, these compounds are resistant in monkeys and pigs. On the other hand, many insects have difficulties in hydrolyzing carbamates, whereas hydrolysis is the main pathway to carbamate decomposing in *B. germanica*. The hydrolysis of carbaryl has been the most studied carbamate hydrolysis. It occurs in a large percentage in many mammals, in a small number of insect species (*B. germanica*, for example), and in a very few plants [34].

**Pyrethrins and pyrethroids** are quite differently hydrolyzed in living organisms. The differences are considerably due to the compound's structure and the type of organism. The hydrolysis is catalyzed by some kind of carboxylesterases. The basic metabolic pathway in pyrethroids (such as permethrin) is hydrolytic splitting of the ester bond, but oxidation is also important. Hydrolysis is, however, irrelevant and little important in the metabolism of pyrethrins (pyrethrin I, cynarin I, etc.) and related to older pyrethroids (allethrin, tralomethrin, tetramethrin, barthrin, etc.) [32, 34].

In pyrethroids with cyclopropane acid, the stereochemistry of 1,3-bond of this ring strongly affects the metabolism of these compounds. When the substituent is transposed to the carboxyl group at C-1, splitting of the ester bond is easier than when it is *cis*-positioned. The adding of a –CN group to $\alpha$-carbon of 3-phenoxybenzyl alcohol decreases the susceptibility of molecules to hydrolytic (and oxidative) decomposition. Insects normally hydrolyze pyrethroids more slowly but split the ester bond of *trans*-isomers faster than mammals. However, there is an exception. The larvae of *Chrysopa* spp., which have unexceptionally high levels of esterase, hydrolyze *cis*-isomers of permethrin and cypermethrin faster than *trans*-isomers. This level of esterase activity is undoubtedly the main factor of resistance of these species to pyrethroids [50]. Like in mammals and in insects, the main primary metabolic process of pyrethroids (permethrin, deltamethrin, cypermethrin, etc.) in plants (beans, cotton, etc.) is the splitting of the ester bond. Fenvalerate acts similarly in different plants (tomatoes, tobacco, lettuce, cabbage, etc.). In all plants, the –CN group disappears [32, 34, 50].

Permethrin, a compound without the –CN group, is hydrolyzed in mammals primarily by splitting of the ester bond, during which the *trans*-isomer is about 100 times more susceptible (rats and mice). In goats and cows, about 30% metabolites of permethrin has the preserved ester bond (both *cis*- and *trans*-isomer). Splitting of the permethrin ester bond is more difficult in fish. Insects (cockroaches, houseflies, cabbage moth, and caterpillars) tear permethrin into acid and alcohol part, among others, which form conjugates with glucose and amino acids. In all three species, *trans*-permethrin is metabolized more easily. In vivo and in vitro results are similar. The metabolism of permethrin (**Figure 4**) is, in its basis, similar in other insect species and some mites species [34, 50]. The main pathway in the metabolism of deltamethrin, cypermethrin, and cyhalothrin in mammals (mice and rats) is the splitting of the ester bond, primarily by carboxylesterases, whereby *trans*-isomers are more susceptible. These compounds are basically similarly metabolized in insects [45, 50]. Generally speaking, esterases play a main role in the metabolism of pyrethroids in caterpillars and oxidases in houseflies. In *Spodoptera littoralis* and *T. ni*, for example, prophenophos inhibits the hydrolytic decomposi-

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tion of *trans*-permethin for 65%, and *cis*-cypermethrin for more than 90%, thereby increasing the toxicity of the former four times and the latter 20 times (*T. ni*), i.e., three times for both compounds (*S. littoralis*).

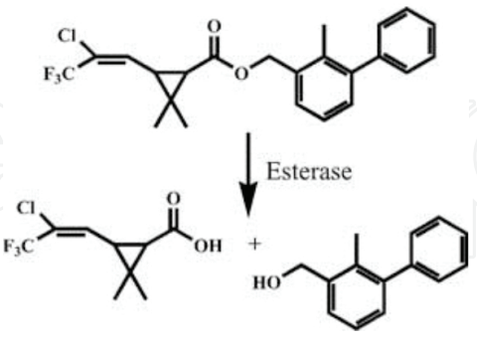


**Figure 4.** Esterase-mediated hydrolysis of the pyrethroid bifenthrin [51].

Phenyl-saligenin, a cyclic phosphate, increases the toxicity of *trans*-permethrin in *Chrysopa carnea* 68 times. Other compounds also undergo hydrolysis. For example, dinobuton (and similar compounds) is initially hydrolytically activated into dinoseb. Acaricide cycloprate is hydrolyzed into cyclopropane acid that afterward binds with carnitine, which eventually has a lethal effect [34, 50].

### 2.4. Hydrolysis of carboxamides

Amide bonds are relatively similar to ester bonds. Breaking of these bonds is catalyzed by carboxamidases. Carboxamidases are actually carboxylesterases capable of selecting amides as substrates [32]. In vertebrates, these enzymes are located only in the liver, and they are primarily related to the microsome fraction. Different divalent cations and nucleoids do not affect the enzymatic activity. The oxidative derivative of dimethoates is not hydrolyzed by carboxyamides but inhibits this enzyme. Dimethoate of amidases from different sources differs in susceptibility to EPN-induced inhibition in vivo. Hence, housefly and *Oncopeltus fasciatus* amidases are not susceptible to EPN, while mammal amidases are highly susceptible. On the whole, these enzymes are susceptible to organophosphates as inhibitors, including profenofos, TOCP, DEF, etc. [34].

Breaking the amide bond among organophosphates is primarily determined for dimethoate, dicrotophos, and vamidothion [32]. When compared to other pathways, splitting of the amide bond of dimethoates by hydrolysis amounts to 27.3% in houseflies, 2.5% in rat liver, and 0.0% of rice leaves in vitro. Splitting of the S—C bond in dimethoate is hydrolytic. Vamidothion is similarly metabolized. It has been determined that splitting of the amide bond of dimethoates and related insecticides is important for their selective toxicity. Besides, the amide bond of dimethoate is also hydrolyzed nonenzymatically on the leaf surface after oxidative desulphurization. However, the amide bond of phosphamidon is not split in plants and animals.

The metabolism of benzoyl phenyl urea (BPU) of derivatives is primarily conducted by the hydrolysis of amide bonds. In a large number of insect species (*Tribolium castaneum*, *S.*

*littoralis*, *Spodoptera exigua*, etc.), it has been recorded that these compounds (diflubenzuron, chlorfluazuron, diflubenzuron, etc.) degrade rapidly. For example, diflubenzuron is rapidly eliminated from insects (t½ about 7 h), and chlorfluazuron slowly (t½ > 100 h). Adding hydrolase inhibitors (DEF and related compounds) in food prolongs the retention time (t½ from 7 to 18 h for diflubenzuron) and increases the toxicity of diflubenzuron for *T. castaneum* and *S. littoralis*. The main metabolites in BPU hydrolysis are chloroaniline, chlorpheniramine urea, and polar metabolites. The activity of these enzymes in hydrolysis of BPU is completely inhibited by DEF or prophenophos in the concentration of about $10^{-5}$ M [32]. Therefore, DEF and prophenophos express a synergetic activity with diflubenzuron in *S. exigua* that has developed resistance to them, ranging from 3.7 (to DEF) to 5.2 times to prophenophos [34, 45].

### 2.5. Hydrolysis of epoxides

Epoxide hydrolases (or epoxide hydrases, epoxide hydratases, EH), discovered in 1968, take part in the metabolism of epoxides. These enzymes (EH) are widely spread among mammals and insects. They are located in microsomes (MEH), solution (CEH), or other parts of mammal liver cells and different insect tissues [32, 34, 44, 46]. pH optimum for the EH activity is most often in the alkaline range, and in insects, it ranges from 7.9 to 9.0 [46]. They show a pronounced specificity for substrates, and there is a great variation among the different species. The mechanism of hydration is not clear enough. Perhaps it includes a nucleophilic attack of the OH group on oxirane carbon [46]. The EH activity from liver microsomes does not depend on NADPH or $CO_2$ and this enzyme is not affected by BDO-type synergists [34]. A certain number of EH inhibitors in insects include sesamex, piperonyl butoxide, a *Cecropia* hormone, and some organophosphates, which are partially in contrast to these enzymes from the mammal liver. Nevertheless, this enzyme is inhibited for about 80% with the same concentration of $Cu^{2+}$ ions [46]. Specific EH inhibitors in mammals are phenyl-glycidoles. S,S-enantiomers are stronger inhibitors ($I_{50}$ $1.6 \times 10^{-6}$ M) than R,R-isomers. These compounds are also substrates for EH [44].

EHs catalyze the splitting of the epoxide ring of different insecticides and other compounds. Thereby, they form certain *trans*-diols that are less toxic, so these enzymes are included in epoxide detoxification processes. These processes are most studied in cyclodienes. Splitting of the epoxide ring of some cyclodienes has been shown in many insect and mammal species. In vitro, dieldrin and heptachlor epoxide are quite stable in this form of degradation. In vivo, however, especially in mammals, the main metabolite of dieldrin is *trans*-diol, which indicates the splitting of the epoxide ring [34]. Epoxides of various alkenes and arenas are enzymatically hydrolyzed, thus forming *trans*-di-hydro diols [46].

## 3. Determination of esterase activity in Colorado potato beetle

In our research, activity of Colorado potato beetle ALiE/Carboxylesterase was determined, using spectrophotometry at a wavelength of 585 μm, first described by Gomori [52]. Average enzyme was prepared out of 40 CBP fourth-instar larvae, using 40 ml of phosphate buffer

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(0.02 M, pH7). Incubation of the average enzyme of Colorado potato beetle with different inhibitors (PBO, DEF, eserine sulfate (ES)) proved that decomposition of 1-naphtyl acetate (1-NA) to 1-naphtyl (1-N) is directly related to the activity of esterase. Incubation of an average enzyme with PBO gave, in all cases, much lower reduction in activity, than in the incubation with DEF or with eserine sulfate (ES). This indicates that the formation of 1-naphtyl does not occur due to the activity of oxidase or a glutathione transferase (GST), but due to the activity of esterases.

Activity of ALiE at varying concentrations of the substrate 1-NA showed that the increasing the concentration of the substrate affects the increase in 1-N amount, and this dependence is linear. Statistical analysis has obtained high-correlation coefficient (0.9279) and a very small statistical error (SE = 0.1601), indicating a high dependence of the examined parameters. The value of the Michaelis constant (Km) was low ($6.664 \times 10^{-3}$). Km values for the activity of most enzymes typically range from $10^{-10}$ to $10^{-2}$ M $dm^{-3}$, which indicates quite high specificity of the tested enzyme for substrate.

### 3.1. Determination of the calibration line for 1-naphthol

In the experimental conditions, for the ALiE enzyme, 1-naphthylacetate (1-NA) is commonly used as a suitable substrate. Enzyme decomposes substrate 1-NA into 1 naphthol (1-N). In order to be able to determine the amount of generated 1-N, it is necessary to determine the calibration line. For these assays, it is also necessary to adjust the conditions of the experiment when examining the functioning of the enzyme. Tests were carried out in the visible region of wavelengths of light, for different concentrations of 1-N. Very low concentrations of 1-N does not give the expressed absorbance maximum. With increasing concentration, the maximum distinguishes more clearly, and most notably at higher concentrations. For the spectrophotometer device UV-VIS Perkin-Elmer 130, determined peak is at a wavelength of 585 nm and all subsequent determinations of 1-N were carried out at a wavelength of 585 nm.

| Concentration (μM) | Absorbance per replication | | | Average absorbance |
|---|---|---|---|---|
| | 1 | 2 | 3 | |
| 0.5999 | 0.205 | 0.201 | 0.233 | 0.213 |
| 1.1998 | 0.216 | 0.274 | 0.220 | 0.237 |
| 2.3995 | 0.219 | 0.234 | 0.233 | 0.229 |
| 4.7991 | 0.290 | 0.276 | 0.270 | 0.279 |
| 9.5981 | 0.323 | 0.349 | 0.358 | 0.343 |
| 19.1963 | 0.467 | 0.511 | 0.538 | 0.505 |
| 38.3925 | 0.642 | 0.643 | 0.610 | 0.632 |
| 76.7851 | 0.890 | 0.883 | 0.810 | 0.861 |
| 153.5702 | 0.822 | 0.822 | 0.815 | 0.820 |

**Table 1.** Data for the calibration line for 1-naphthol.

The initial concentration contained 22.14 mg/l of 1-N. Since 1 M solution of the compound is containing 144.17 $gl^{-1}$, this concentration expressed as a molar solution amounted to 153.57 μM of the compound. Absorbance values for the nine different concentration of 1-N are shown in **Table 1** and **Chart 1**. In order to avoid the effect of these concentrations of the field in which the readings are unreliable, for the calculation of the regression line only concentrations from of 76.8 to 4.8 μM were used.

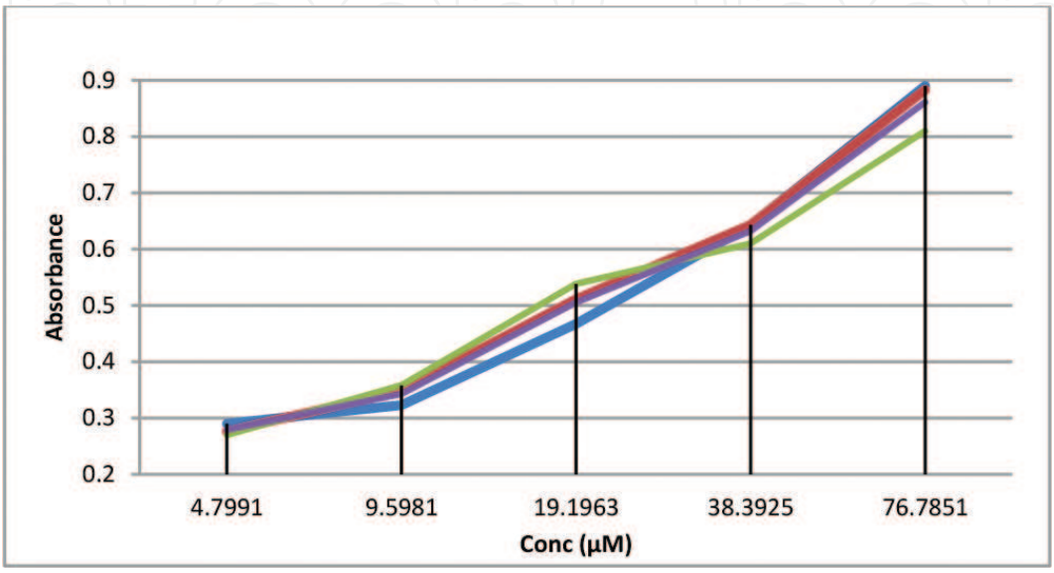


**Chart 1.** Data for the calibration line for 1 naphtol.

Statistical regression analysis calculated the following regression line for 1-N: $Y = 0.2918 + 0.0078 \times X$, as a basis for the calculation of the 1-N amounts for the appropriate absorbance. Statistical data analysis found out that the regression is linear and directly dependent. The correlation coefficient is 0.98, which indicates a high dependence of investigated parameters.

### 3.2. Determination of presence and activities of ALiE

Average enzyme was prepared out of 40 Colorado potato beetle fourth-instar larvae ($L_4$), from the locality of Dobanovci (Belgrade, Serbia), using 40 ml of phosphate buffer (0.02 M, pH7). Experimental conditions in terms of the average amount of enzyme in the required amount of the reaction mixture and the temperature were constant, but the substrate concentration was varied. It is found that the activity of Colorado potato beetle ALiE exists, since in all the examined substrate concentrations in the experimental conditions there is a degradation of the NA-1 1-N due to the enzyme (ALiE) activity (**Chart 2**).

Increasing the concentration of the substrate affects the increase in creation of 1-N, and this dependence is linear. Statistical analysis shows high correlation coefficient and a very small statistical error (as shown in **Table 2**), which indicates the high dependency of investigated parameters.

http://dx.doi.org/10.5772/66254

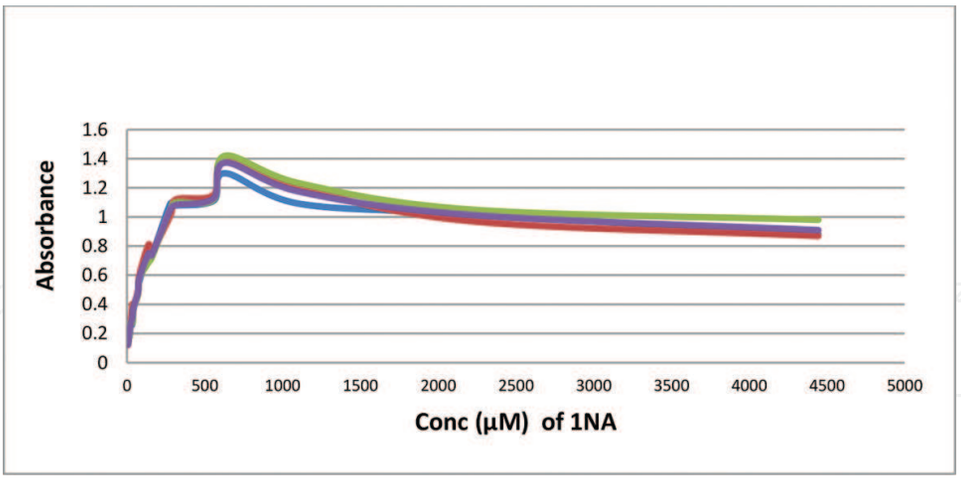


**Chart 2.** Activity of ALiE at varying concentrations of the substrate 1-NA.

The value of the Michaelis constant (Km) is small. These values of Km for most of the enzymes activity typically range from $10^{-2}$ to $10^{-10}$ M $dm^{-3}$, indicating quite high specificity of the investigated enzyme regarding substrate.

| Correlation coefficient | Statistical error | Regression line | Km ($Mdm^{-3}$) |
|---|---|---|---|
| 0.9279 | 0.1601 | $Y = 0.2849 + 0.00189 \times X$ | $6.664 \times 10^{-3}$ |

**Table 2.** ALiE activity at varying substrate (1-NA) concentrations—statistical parameters.

### 3.3. Determining the type of enzyme activity using inhibitors

For experiments with inhibitors, we used the average enzyme from Colorado potato beetle of the fourth-grade larvae (L4), population Dobanovci, which contains not only a complex of enzymes but also a variety of other compounds that can react with inhibitors. PBO is a typical oxidase inhibitor and therefore is often used as an insecticide synergist, which are subject to oxidative detoxification. DEF is a specific inhibitor of ALiE esterases, while the ES is specific cholinesterase inhibitor.

Pre-incubation of the enzyme with PBO showed minimal reduction in activity in the degradation of 1-NA. Statistical analysis showed that there are still significant differences in the results for the variant, which applies only to the enzyme and variant combinations of enzymes and piperonyl butoxide.

Pre-incubation of the enzyme with DEF resulted in significantly decreased activity of the enzyme. Differences in the enzyme activity without inhibitor and pre-incubation with DEF are very significant. There are very significant differences between variants, such as the enzyme pre-incubated with the PBO and variants when pre-incubated with DEF.

The greatest reduction in the enzyme activity was obtained when the enzyme is pre-incubated with eserine sulphate (ES). Statistical analysis showed that there are very significant differences

between the basic enzyme activity and its activity after pre-incubation with ES. Duncan's test proved that all the variants belong to different groups.

Different concentrations of the CPB average enzyme (ALiE) from population Dobanovci, depending on the increase in the concentration of the enzyme, produce increasing amounts of 1 N, at a constant amount of substrate (1 NA). Similar instances happened with variable concentrations of substrates in the presence of a constant amount of average enzyme. Since the reactions are specific to this group of enzymes, these results indicate their distinguished activity in potato beetle. Incubating the average enzyme with different inhibitors has been proven in the case of Colorado potato beetle that degradation of 1-NA into 1-N comes under the influence of esterase. Incubation of average enzyme with PBO gave in all cases much lower lower reduction in activity, than the impairment of enzyme activities incubated with DEF or eserine sulphate (ES), which indicates that the creation of 1-N does not come due to the effect of oxidase or glutathione transferase (GST), but due to the effect of esterase (**Table 3**).

| Treatment | Absorbance per replication | | | | | Average | Median | Variance | Standard deviation | Duncan test |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | | | | | |
| Enzyme | 0.82 | 0.88 | 0.82 | 0.82 | 0.90 | 0.848 | 0.82 | $1.52^{-3}$ | 0.039 | a |
| Enzyme + PBO | 0.80 | 0.80 | 0.78 | 0.80 | 0.84 | 0.804 | 0.80 | $4.80^{-4}$ | 0.022 | b |
| Enzyme + DEF | 0.72 | 0.70 | 0.70 | 0.75 | 0.72 | 0.718 | 0.72 | $4.20^{-4}$ | 0.020 | c |
| Enzyme + ES | 0.66 | 0.65 | 0.69 | 0.66 | 0.67 | 0.665 | 0.66 | $1.75^{-4}$ | 0.013 | d |

$LSD_{0.05}$ = 0.06.
$LSD_{0.01}$ = 0.08.

**Table 3.** Statistical parameters of the inhibitor effect on the activity of CPB larvae ALiE.

## 4. Conclusion

Resistant insect populations may detoxify or degrade the toxin faster than susceptible insects, or quickly rid their bodies of the toxic molecules. Resistant populations may possess higher levels or more efficient forms of these enzymes. The metabolic destruction of an insecticide inside the target organism is a common defensive mechanism. Three major mechanisms of metabolic transformation of insecticides underlie the vast majority of examples of biotransformation-based resistance: (i) oxidation; (ii) ester hydrolysis; and (iii) glutathione conjugation. Pyrethrins, pyrethroids, organophosphates, carbamates, and other insecticides are degraded by hydrolysis. The most important hydrolytic enzymes are phosphoric triesters and carboxylesterases (ALiE esterases, nonspecific, or B-esterases). Structural mutations in mutant carboxylesterases have now been widely described showing metabolic resistance to organophosphate and pyrethroid insecticides. There are relatively few cases of high-level esterase-mediated metabolic resistance to carbamates. The role of carboxylesterases in Colorado potato beetle's resistance to insecticides was confirmed by many authors. CPB is resistant to all major

groups of insecticides, including organophosphates and carbamates. Insecticide resistance presence and level are measurable [53]. ALiE's role in the emergence of resistance to organophosphorus and other insecticides in insects, especially in Colorado potato beetle, is investigated. In most of the insect species in which the testing was performed, the dependence of the increase in the activity of the enzyme matched with the increase of the insecticide resistance. Probably, the primary role of this enzyme is its importance for the absorption of organophosphorus insecticides, which becomes nontoxic, and then to gradually decompose to nontoxic components. Increased concentrations of the average CPB enzymes produced increasing amounts of 1-naphthol (1-N) at a constant amount of substrate (1-NA). Similar results happened with variable concentrations of substrate (1-NA) in the presence of a constant amount of average enzyme. Since the reactions are specific to this group of enzymes, these results indicate their distinguished activity in Colorado potato beetle.

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