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Review

Overview of Carboxylesterases and Their Role in the Metabolism of Insecticides

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Carboxylesterases hydrolyze numerous endogenous and exogenous ester-containing compounds. They play a role in the detoxification of many agrochemicals including pyrethroids, organophosphates, and carbamates. Research on these enzymes is still developing and there are several topics that should be addressed to further investigations in this area. This paper focuses on a number of these issues including enzyme nomenclature, catalytic mechanism, substrate specificity, agrochemical metabolism, role in insecticide resistance and environmental significance. It is expected that carboxylesterase research will increase with specific emphasis on isozyme and substrate identification. Future research directions are discussed and the current state of the field is evaluated.
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INTRODUCTION

Carboxylesterases (CaEs) are enzymes in the α/β hydrolase fold and catalyze the hydrolysis of carboxyl esters via the addition of water as shown in Fig. 1.[1–3] The α/β hydrolase fold also includes phosphotriesterases (such as paraoxonase),[4] cholinesterases,[5–7] and lipases[8] as well as a number of other enzymes. These enzymes are all defined by an eight-stranded mostly parallel α/β structure and a characteristic plastic nature of the protein fold that allows for large variations in domain size (Fig. 2).[3] In addition to this common structural framework, CaEs share a 2-step serine hydrolase mechanism and active site.[9]

CaEs are found in a number of tissues including liver, kidney, small intestine, heart, muscle, lung, brain, testis, nasal and respiratory tissues, adipose tissue, leukocytes, and the blood.[10] However, their expression and activity is organism dependent, with levels and activities varying widely. Polymorphisms have been reported,[11] and in particular 15 single nucleotide polymorphisms (SNPs) were identified in one CaE alone (CaE2 or CES2).[12] The esterase family is still growing as more esterases are identified and the ESTHER database currently contains 5237 nucleotide sequences for genes that encode esterases, of which 318 are CaEs.[13)]

Interest in this class of enzymes has increased due to their role in the metabolism of many agrochemicals (Fig. 3) and pharmaceuticals, as well as their role in endogenous metabolism. In particular, carboxylesterases hydrolyze pyrethroids,[14] bind stoichiometrically to carbamates[15] and organophosphates[16] as well as activate the cancer therapeutic prodrug CPT-11 through its conversion to SN-38.[17]

ESTERASE CLASSIFICATION AND NOMENCLATURE

Carboxylesterase or esterase is a common term for enzymes that hydrolyze endogenous and exogenous esters. A systematic nomenclature for the classification of these enzymes remains to be established and multiple systems are currently used. According to the International Union for

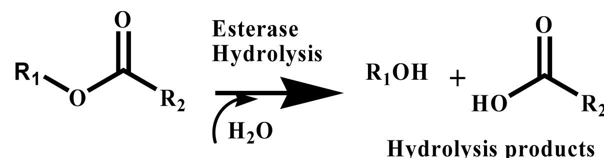

Fig. 1. Esterase hydrolysis mechanism. Esterases hydrolyze an ester via the addition of water to form the corresponding alcohol and acid.

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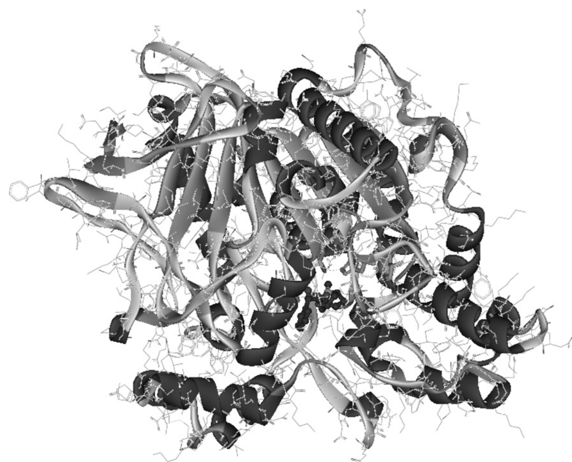

Fig. 2. Solid ribbon structure of human liver carboxylesterase 1 (hCE1) complexed with homatropine, shown as a darker ball-and-stick structure in the lower right part of the figure. The figure was generated based upon the crystal structure of Bencharit *et al*.[35] from RCSB's protein data bank (http://www.rcsb.org/pdb/cgi/explore.cgi?pdbId=1MX5). The image was created with DS ViewerPro 5.0 (Accelrys, San Diego, CA).

Biochemistry and Molecular Biology (IUBMB) nomenclature, esterases are located within subgroup 1 of hydrolases (Enzyme Commission 3.1, EC 3.1), which is further categorized into subtypes based on the different types of ester bonds hydrolyzed. CaEs are defined as EC 3.1.1.1 in this system as they hydrolyze carboxylic esters.[18] Another common classification proposed by Aldridge groups esterases into three types (A, B and C) based on the nature of their interaction with organophosphorus insecticides such as paraoxon (see Fig. 4).[19,20] This system has been widely recognized and used by those studying esterases due to its ease of use and lack of other nomenclature systems. Enzymes that hydrolyze organophosphorus compounds (EC 3.1.1.2), including those containing an acylated cysteine in their active sites (EC 3.1.8.1), are termed A-esterases.[21,22] Esterases that are progressively inhibited by organophosphorus compounds, such as paraoxon, and have an active site serine residue (EC 3.1.1.1) are called B-esterases,[23] and those which are resistant to organophosphates but do not degrade them are C-esterases (acetylesterases or EC 3.1.1.6). While this classification system is easy to use, it is lacking in utility as it does not provide a unique classification for each esterase.

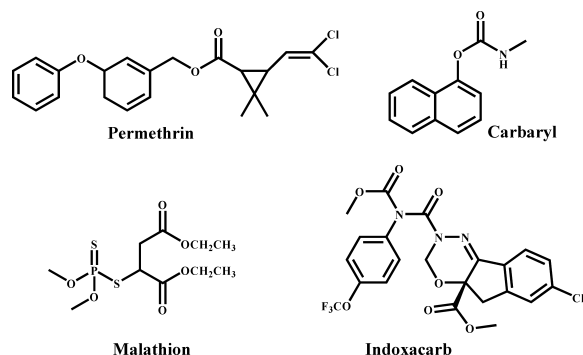

Fig. 3. Structures of common agrochemicals that bind to esterases. Permethrin is a pyrethroid, carbaryl is a carbamate, malathion is an organophosphate, and indoxacarb is an oxadiazine.

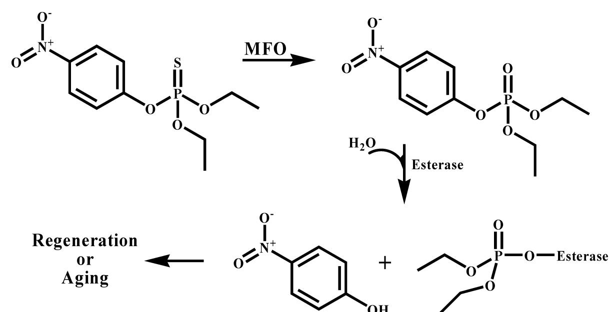

Fig. 4. Carboxylesterase inhibition mechanism for the organophosphate parathion. The organophosphate (parathion) is first activated via mixed-function oxidases (MFO) to the "active" oxon-form, which is the inhibitory structure of the compound. Paraoxon then binds to the esterase and is hydrolyzed in the process by the addition of water, releasing *p*-nitrophenol. The phosphorylated esterase can then either release the phosphate group and regain catalytic activity, or become "aged" where the phosphate remains permanently bound and the enzyme loses catalytic activity.

Apart from Aldridge's general taxonomy of esterases, different classifications have been proposed and adopted to describe the esterases in a particular species or group of closely related species. For example, Mentlein *et al*. used natural substrates to classify rat liver microsomal esterases,[24,25] while others classified esterases based on their mobility on electrophoretic gels, such as aphid esterases E1–E7,[26] and German cockroach esterases E-1 and E-2.[27] Esterases from *Culex* mosquitoes were classified using combined criteria including preference for hydrolyzing α- or β-naphthyl acetates and electrophoretic mobility.[28,29] Satoh and Hosokawa proposed the use of nucleotide homology to classify mammalian carboxylesterase isozymes into one of four families: CES1, CES2, CES3, or CES4.[10] This novel approach may provide a satisfactory systematic nomenclature, especially as additional sequence data become available.

ESTERASE HYDROLYSIS MECHANISM AND INHIBTION

The mechanism by which esterases hydrolyze their substrates has been examined using both biochemical and structural means by a number of research groups. The detailed mechanism of hydrolysis has been reviewed elsewhere[6,7,10,30–33] and is only briefly presented here. Interested readers are referred to the additional references for a more extended presentation of the hydrolysis mechanism. The exact nature of the mechanism is still open to debate, with only the current hypothesis being presented in this review.

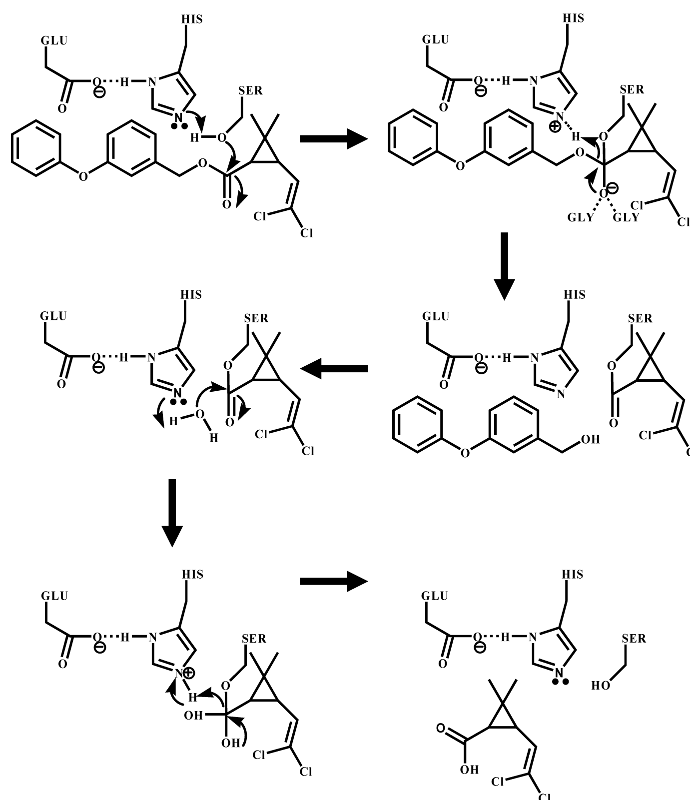

Fig. 5. Mechanism for the carboxylesterase-mediated hydrolysis of permethrin to the corresponding hydrolysis products (3-phenoxyphenyl)methanol and 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid. The mechanism is as detailed in the text. GLU is glutamate, HIS is histidine, SER is serine and GLY is glycine.

The recent publications of crystal structures of mammalian CaEs have greatly contributed to our understanding of the enzyme mechanism.[9,34,35] The general catalytic mechanism involves a catalytic triad, consisting of a Ser, His and either a Glu or Asp residue (Ser221, His468 and Glu354 for human carboxylesterase 1, hCE1[9, 35]); however recent work has discovered a potential fourth catalytic serine residue.[36] CaEs cleave esters via a 2-step process that involves the formation and degradation of an acyl-enzyme intermediate. This process is shown in Fig. 5 for the pyrethroid permethrin. A proton is transferred to the His from Ser, increasing the nucleophilicity of the Ser terminal hydroxyl group. The His is in turn stabilized by hydrogen bond formation to the Glu (or Asp). The Ser nucleophile attacks the electron deficient carbonyl moiety in the ester substrate forming a tetrahedral intermediate, which is stabilized by two Gly residues in the oxyanion hole. This intermediate collapses to form the acyl-enzyme complex, releasing the Ser and the alcohol portion of the substrate in the process. A His-activated water molecule then attacks the acyl-enzyme complex, repeating the above steps and releasing the acid portion of the substrate. The second, conserved serine residue serves to provide structural support for the spatial orientation of the Glu, thereby stabilizing the catalytic triad.[36]

The structural motif of CaEs appears to be similar to other enzymes in the α/β hydrolase-fold as discussed above. Recent crystal structures have shed new light on these enzymes and in particular have helped to explain their ability to accommodate a wide variety of substrates. The active site of human carboxylesterase 1 (hCE1) consists of a 10–15 Å deep pocket that is lined with mainly hydrophobic residues.[9] This large ($\sim$1300 Å^3) pocket is compromised of two different binding domains: the first, which is "small and rigid" and a second that is "large and flexible." The presence of these two domains may facilitate the recognition and binding of chemically divergent substrates by these enzymes.[35] Earlier studies showed the existence of a "backdoor" in the acetylcholinesterase active site that allows substrate, products, or solvent to move through the enzyme at a much higher rate than would be expected based upon the long and narrow active site gorge.[37] A similar structure was detected in CaEs, with a "side-door" separating the active site by a thin wall consisting of only four amino acids.[34] The presence of this structural feature may contribute to the promiscuity of these enzymes and enable them to hydrolyze a wide variety of substrates.

Several different types of CaE inhibitors have been reported in the literature. The main structural motifs include trifluoromethyl ketone (TFK)-containing inhibitors, organophosphate derivatives, carbamates and sulfonamides.

Each of these compound classes has been used to study CaE biochemistry and function. TFK-containing inhibitors are based upon the inclusion of an electron deficient carbonyl moiety in the molecule, which covalently binds to the enzyme.[38,39] These compounds are slow tight-binding inhibitors, but the covalent bond is reversible and the enzyme is reactivated on the order of days to weeks.[40] The oxon-forms of OP insecticides, such as paraoxon (*O,O*-diethyl *p*-nitrophenyl phosphate), can be very potent CaE inhibitors ($k_i=1.4\times10^6\ M^{-1}\ min^{-1}$ for rat serum CaE[41]), and potentially lead to aging of the enzyme following phosphorylation.[42] The phosphorylated enzyme can either release the OP substrate (where it undergoes hydrolysis similar to an acyl group, but at a much slower rate) or it can undergo aging, where the enzyme is essentially catalytically dead (*i.e.*, the OP acts as a suicide substrate). These processes are shown in detail in Fig. 4. A similar reaction can occur with carbamates; however, the methylcarbamoylated enzyme is less stable than the phosphorylated enzyme, thus accounting for the decreased toxicity of some carbamates relative to OPs.[16] The reversible nature of carbamate binding makes them useful for kinetic studies.[36] Other commonly used CaE inhibitors include 2-(*o*-cresyl)-4*H*-1,2,3-benzodioxa-phosphorin-2-oxide (CBDP), tetraisopropylpyrophosphoramide (iso-OMPA), *S,S,S*-tri-*n*-butyl phosphorotrithioate (DEF), and the fluorophosphorous derivative diisopropylphosphorofluoridate (DFP).[43] Recent reports of the identification of CaE selective inhibitors containing a sulfonamide moiety are interesting in that they are the first isoform-selective inhibitors reported for CaEs.[44] The mechanism of inhibition is still unclear, but it is hypothesized that the oxygen atoms of the sulfone moiety form hydrogen bonds with residues in the active site, thereby preventing the substrate from accessing the catalytic residues. The identification of additional isoform-selective inhibitors will be crucial for the future study and identification of CaEs, because they will enable researchers to selectively remove CaE isoform-specific activity in tissue preparations. The use of CaE inhibitors for use as pesticide synergists has been proposed. While at first pass an interesting idea, the resulting increase in mammalian toxicity would most likely be unacceptable. However, there is a precedent as shown by the co-application of piperonyl butoxide (PBO, a mixed-function oxidase inhibitor) with pyrethroids to synergize insect selective toxicity.[45]

METHODS FOR ESTERASE STUDY

One of the major obstacles in the biochemical identification and characterization of CaEs is the choice of substrate to measure activity in the presence of multiple isozymes. Many studies report general esterase activity using colorimetric substrates such as *p*-nitrophenyl acetate (PNPA) or α-naphthyl acetate.[10] These substrates are convenient to use due to their low cost, availability, and relatively cheap optics required for data acquisition (colorimetric assays). However, it is likely that some CaE isozymes do not hydrolyze these substrates efficiently, if at all. It is therefore not appropriate to use a single substrate to examine the esterase activity of crude tissue homogenate. Given the likelihood that multiple esterase isoforms are present in the preparation, it is necessary to have a battery of reporters for full characterization of enzyme activity. Correlation analyses performed on PNPA and pyrethroid hydrolysis activity in human liver microsomes showed very little correlation between the hydrolytic profiles ($r^2=0.29$ for a fenvalerate surrogate), suggesting that different enzymes are involved in the hydrolysis of the two substrates.[46] Therefore, monitoring of PNPA activity, or that of other general substrates, may not provide an accurate account of pyrethroid hydrolysis. Similarly, Stok *et al*. reported that the portion of pyrethroid hydrolysis activity in mouse liver microsomes was only 0.5% that of total esterase activity (as measured by PNPA).[14] Attempts to develop more selective reporters of esterase-mediated hydrolysis of pyrethroid insecticides have been described. For example, Riddles *et al*. prepared substrates by coupling a colored leaving group (*p*-nitrophenol) to pyrethroid acids.[47] However, these surrogates still vary greatly in the alcohol portion from commercial pyrethroids. The substrates synthesized by Stok *et al*. represent an additional improvement in that the alcohol portion is more structurally similar to pyrethroids. This enhancement was reflected in the high correlation between the rates of synthetic substrates hydrolysis ($r^2=0.92$) with that of the pyrethroid insecticides.[14] Huang *et al*. have further developed these substrates by synthesizing optically pure analogs.[48] However, ultimately the best measure of hydrolysis activity is to test the actual pesticide or substrate. The importance of substrate choice for monitoring esterase activity was further demonstrated by Wheelock *et al*. who reported that the IC_{50} of diazinon-oxon or chlorpyrifos-oxon varied with the substrate used in the assay.[49] Diazinon-oxon was a potent inhibitor (nM IC_{50}) when assays were conducted with PNPA, however no inhibition was observed when α-cyano-2-naphthylmethyl acetate was used as a reporter. Both substrates have identical acid moieties, yet provide widely divergent results. These data strongly suggest that a battery of substrates should be employed when measuring CaE activty to ensure that an accurate indication of enzyme activity is obtained.

AGROCHEMICAL METABOLISM AND ROLE IN INSECTICIDE RESISTANCE

Three different classes of agrochemicals, pyrethroids, OPs, and carbamates, interact with CaEs (structures are shown in Fig. 3).[15,42,50,51] Major pathways for agrochemical metabolism involve a number of different enzyme systems that are beyond the scope of this review, including P450 monooxygenases (P450 MOs), glutathione *S*-transferases (GSTs), phosphotriesterases, as well as CaEs. It is well known that variability in CaE levels[46,52,53] as well as relative isozyme

abundance[14,54] contribute to the selective toxicity of ester-containing insecticides in both mammals and insects.[55] However, stereochemistry is also extremely important in esterase-associated metabolism. Many agrochemicals, including pyrethroids and some OPs, contain chiral centers that greatly affect their subsequent metabolism. Since varying stereoisomers of both OPs and pyrethroids exhibit differential toxic effects,[50,56–59] stereospecific hydrolysis is essential in the determination of their toxic properties. In studies on the metabolism of pyrethroids such as allethrin,[50] resmethrin,[60,61] phenothrin,[62] permethrin,[63,64] and cypermethrin,[64] it has been demonstrated that the *trans*-isomers are more rapidly hydrolyzed than the corresponding *cis*-isomers in both mammals and insects. These findings support the generally observed lower toxicity of *trans*-isomers.[65,66] A recent study with a recombinant pyrethroid-hydrolyzing CaE originally isolated from mouse liver found that *trans*-permethrin and *trans*-cypermethrin were hydrolyzed 22-fold and 4-fold faster than their *cis*-isomers, respectively.[14] In addition, assays conducted with four fenvalerate isomers showed that the two less toxic enantiomers (αR, 2*R*)- and (αS, 2*R*)-fenvalerate were hydrolyzed ~50- and 5-fold faster than the (αR, 2*S*) enantiomer. The most toxic fenvalerate enantiomer (αS, 2*S*)-fenvalerate (or esfenvalerate) exhibited almost no CaE-based hydrolysis, which supports the observations from previous studies with crude mouse liver microsomes.[67,68] Further testing on recombinant pyrethroid specific esterases (BAC36707 and NM_133960[14]) using pyrethroid-surrogate substrates, found that the esterase-specific stereospecificity varied significantly among 8 cypermethrin enantiomers, the least toxic (1*S*, *trans*, αS)-stereoisomer was hydrolyzed ~300-fold faster than the two most potent isomers (1*R*, *cis*, αR)-and (1*R*, *cis*, αS)-cypermethrin.[48] These findings indicate that the study of esterase stereospecificity may have a significant impact on the design of more efficient and selective insecticides.

As outlined above, the catalysis of CaE substrates involves binding, formation of a tetrahedral intermediate, and then hydrolysis to regenerate free enzyme. In the case of some organophosphate substrates, this last step (dephosphorylation) is extremely slow, and the esterase (a B-esterase according to the classification of Aldridge) is rendered catalytically inactive (Fig. 4). In the case of most other insecticidal esters, hydrolysis proceeds to completion and the insecticide is split into two biologically inactive products (Fig. 5). Notable exceptions include malathion (an organophosphate) and indoxacarb (an oxadiazine) (see Fig. 3). In both cases, differences in hydrolytic potential between target (insect) and non-target (mammalian) species are believed to be responsible for the selective toxicity of these compounds. Malathion is a proinsecticide that may either be activated by oxidative desulfuration or inactivated by hydrolysis at one of two carboxyl esters. Insecticide-susceptible insects are predisposed toward activation of malathion rather than detoxification, presumably because their titer of a carboxylesterase is lower than that in mammals.[69] In contrast, indoxacarb is also a relatively insect-selective proinsecticide but, in susceptible insects, is activated by hydrolysis of a carboxyl ester, a reaction that occurs more readily in insects than mammals.[70]

Selection of insect populations by repeated administration of insecticides may result in an enhanced capability for insecticide metabolism, and reports of insecticide resistance associated with CaE activity are widespread. Early reports documented enhanced activities of CaEs in malathion-resistant house flies, *Musca domestica*,[71] and mosquitoes.[72] Pyrethroids also are susceptible to hydrolysis by CaEs,[73–75] and CaE-based, pyrethroid resistance has been reported in a wide array of pest insects.[76–78]

There are three general mechanisms by which carboxylesterases are associated with resistance to insecticides. First, resistance may arise in populations through selection for insects that possess and express multiple copies of a gene for a carboxylesterase (gene amplification). Overproduction of these enzymes has been demonstrated in the green peach aphid, *Myzus persicae*,[78–80] in Culicine mosquitoes[29,77,81,82] and the brown planthopper, *Nilaparvata lugens*.[83] Resistance is due, not to an overall increase in catalytic efficiency, but to the high titer of carboxylesterases present that serves as an "insecticide sink" and delays or prevents interaction between toxin and target site. Resistance due to this mechanism (sequestration) may be further heightened by the co-expression of target sites with reduced sensitivity to insecticides.[78]

Metabolic resistance may also occur through selection for and expression of mutant carboxylesterases. Point mutations within structural genes (as opposed to regulatory genes) result in enzymes with an enhanced capability for insecticide metabolism. This phenotype was initially described over 40 years ago in the house fly,[84] and has been well studied in the Australian sheep blow fly, *Lucilia cuprina*.[85] In early studies, resistance to parathion in laboratory strains of the house fly was associated with increased hydrolysis of organophosphorus esters by phosphotriesterases and simultaneously, a decreased hydrolysis of aliphatic carboxylesters.[84] Oppenoorth and van Asperen's hypothesis, which came to be known as the "mutant aliesterase theory," was that both decreased carboxylesterase activity and increased phosphotriesterase activity in these strains were due to a single mutation in an esterase gene. Years later, a similar phenomenon was described in *L. cuprina*,[86] and in recent studies, a gene (*Lc*α–*E7*) encoding an esterase (E3) from diazinon-resistant strains of *L. cuprina* was isolated, sequenced, and expressed.[85] The *Lc*α–*E7* gene from resistant flies was found to contain a single point mutation that resulted in the loss of aliesterase activity and the gain of low (but measurable) activity toward organophosphorus insecticides. A recently described, alternate hypothesis is that the point mutation in the aliesterase gene may derepress the transcription of non-hydrolytic enzymes (*e.g.*, P450 MOs) that catalyze ester cleavage and im-

part resistance.[87)]

Finally, metabolic resistance may also be conferred through enhanced transcription of non-amplified, structural genes for insecticide detoxifying enzymes. In this case, mutations in regulatory genes result in the over-expression of these enzymes, and the capacity for insecticide metabolism is enhanced. This mechanism has been demonstrated for other classes of enzymes associated with resistance (GSTs and P450 MOs[88–93)]) but, as yet, it has not been shown to occur in carboxylesterase-mediated metabolic resistance.

ENVIRONMENTAL ROLE OF ESTERASES

An organism's sensitivity to pyrethroid, OP, or carbamate exposure may be influenced by its endogenous CaE activity. Therefore, measurement of CaE activity may be useful in predicting the effects of agrochemical exposure upon ecosystem health.[94)] However, there are not currently enough data available in the literature to fully examine this issue. CaE activity is most likely important for pyrethroid detoxification in some species of fish, but work by Glickman and coworkers showed that the most important factor in rainbow trout sensitivity to permethrin was sensitivity of the target site, the sodium channel.[95)] It is still possible that in some cases, different species will have greater levels of esterase activity, which could affect the ability to detoxify agrochemicals. Glickman *et al*. showed that carp have higher levels of esterase activity and a greater ability to hydrolyze permethrin than rainbow trout,[96)] which could account for observed interspecies differences in pyrethroid toxicity. This issue should be examined in more detail following the acquisition of additional data on CaE levels in multiple species, especially aquatic invertebrates.

The use of acetylcholinesterase (AChE) activity as a biomarker of organism exposure to agrochemicals (OPs and/or carbamates) is well documented in the literature.[97–100)] However, recent studies have suggested that AChE activity alone is not an appropriate biomarker[100)] because some OPs have increased affinity for CaE over AChE. The preferential inhibition of CaE over AChE following exposure to OPs and potentially carbamates suggests that CaE activity will provide a more sensitive endpoint.[15,101–103)] However, there is also the issue of the endogenous titers of the respective enzymes, which could greatly affect use in environmental monitoring. It may be appropriate to begin collecting data on CaE activity in environmental samples. One difficulty with this approach, versus that of using AChE, is the choice of substrate employed for monitoring purposes. As discussed above, the use of routine substrates such as PNPA, while representing the largest amount of data available in the literature, may not be directly relevant to CaEs that will interact with agrochemicals. Further work will be required in the development of universal and appropriate assays for evaluating the use of CaE activity as a biomarker.

CONCLUSIONS

The interest in CaEs is evident by the increasing numbers of citations available in searchable databases. The importance of these enzymes in the metabolism of agrochemicals and insect resistance is well established. The role of CaEs in pharmaceutical metabolism, while beyond the scope of this review, will strongly drive the development of this field. Interested readers are directed to recent reviews on the use of esterases in designing soft- and prodrugs for the pharmaceutical market[104–106)] as well as the use of esterase activity in the development of the anti-tumor agent CPT-11.[107)] Esterase activity has been shown to be important in a number of commercial drugs including lovastatin[108)] and β-blockers.[11)] It is likely that pharmaceutical companies will invest heavily in creating a library of cloned and expressed CaEs, similar to that which is currently performed with P450 MOs. If these tools became commercially available, they could be very useful to the agricultural chemistry research community. Apotential avenue of research is the study of agrochemical:drug interactions, either through CaE inhibition or substrate competition.

Whereas much of the CaE research in the future is likely to be driven by pharmaceutical rather than agricultural applications, the development of agrochemicals remains an important research area that would benefit from a more comprehensive understanding of CaEs. For example, exploitation of both qualitative and quantitative differences in CaE profiles between mammals and insects have been shown to provide the basis for pharmacokinetic selectivity of insecticides. In addition, a number of ecological questions regarding CaEs in herbivorous insects remain to be explored. For example, little is known about the role of CaEs in allelochemical metabolism or in host-plant acceptance. Finally, compared with other detoxifying enzymes, there have been few studies to determine how activities of CaEs are regulated, and the extent to which activities are induced in response to xenobiotics.

Given the importance of CaEs in the detoxification of antipersonnel agents such as sarin, soman and VX, there is a great deal of interest in these enzymes from the U.S. government. It has been hypothesized that CaE activity could be formatted to serve as a prophylactic to protect soldiers from exposure to chemical weapons.[41)] This area will mostly likely be a source of increased funding given the current emphasis on terrorism-related research. Other interesting applications of CaE activity include the treatment of cocaine overdose patients.[35)]

The major needs of the research community include information on the endogenous roles of CaEs and development of a universally accepted nomenclature system. The field is beginning to examine isozyme abundance and distribution in multiple organisms and the expected increase in identified isozymes could become complicated. The next wave of necessary tools to advance the field includes the production and

distribution of cloned CaEs, identification of isozyme-selective inhibitors and new substrates. In particular, substrates should be isozyme-selective, cheap, and readily available. It would be desirable to have a battery of substrates that mimicked agrochemicals and pharmaceuticals of importance, similar to that which has been done for pyrethroids. The creation of CaE knockout mouse would be of particular interest in elucidating the endogenous role of CaEs. The study of CaEs is still wide open for researchers to make their contribution into understanding the roles and functions of this interesting family of enzymes.

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