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Mechanisms of Resistance in
Carbofuran-Resistant Colorado Potato Beetles

presented by

Joel Martin Wierenga

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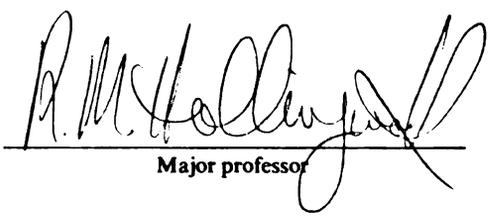

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MECHANISMS OF RESISTANCE IN CARBOFURAN-RESISTANT COLORADO POTATO BEETLES

By

Joel Martin Wierenga

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ABSTRACT

MECHANISMS OF RESISTANCE IN CARBOFURAN-RESISTANT COLORADO POTATO BEETLES

By

Joel Martin Wierenga

The Colorado potato beetle (*Leptinotarsa decemlineata* (Say)) has an increasing impact on the costs of potato production, but the biochemistry of its resistance mechanisms is largely unexamined. The aim of this research was to examine the biochemical basis for resistance to carbofuran in two strains of the Colorado potato beetle. One strain (R-mfo) was believed to be resistant due to increased mixed function oxidase activity. The other strain (R-AChE) was hypothesized to be resistant via altered acetylcholinesterase.

The rate of penetration of carbofuran was measured, and there was no difference compared with the susceptible (Susc.) strain. It is unlikely that this mechanism of resistance is important for these strains.

There were no important differences between strains for glutathione-S-transferase or arylesterase activity. However, mixed function oxidase activity was elevated 2- to 4-fold in the R-AChE strain, and 4- to 8-fold in the R-mfo strain. These differences corresponded to increased rates

of oxidative metabolism of carbofuran in vivo (120% and 400% respectively).

Acetylcholinesterase activity in each strain was assayed with acetylthiocholine following preincubation with various insecticides. The degree of inhibition varied substantially, depending on the insecticide used. The R-AChE strain was insensitive to inhibition by arylcarbamates, and the R-mfo strain was insensitive to organophosphates. All strains were insensitive to oxime carbamates compared with other species.

The concentration-log activity curves were nonlinear in most cases, indicating the presence of multiple enzyme activities. This suggests that there may be both susceptible and resistant isozymes of acetylcholinesterase present in varying proportions for each strain.

The potato glycoalkaloid α-chaconine was tested for its ability to inhibit acetylcholinesterases from the potato beetle and several other insect species. It was an effective inhibitor of all acetylcholinesterases tested except for that from the potato beetle.

It is concluded that enhanced mixed function oxidase activity is the major mechanism of resistance to carbofuran in the R-mfo strain. Altered acetylcholinesterase is the main mechanism of resistance in the R-AChE strain. More than one altered form of acetylcholinesterase is involved in resistance.

To Gramma B. and Dad Rottschafer

You taught me a lot about life

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My deepest appreciation is to God, whose faithfulness and common grace provided me with all of the elements of support necessary to achieve this goal. I finally realized how blessed I was to be able to devote my time to studying something I was interested in.

"...and I said 'I am determined to be wise'-but this was beyond me. Whatever wisdom may be, it is far off and most profound-who can discover it?"

Ecclesiastes 7: 23-24.

"The fear of the Lord is the beginning of knowledge "

Proverbs 1:7

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LIST OF ABBREVIATIONS

AChE	acetylcholinesterase
CDNB	chlorodinitrobenzene
DCNB	dichloronitrobenzene
DDT	1,1,1-trichloro-2,2-*bis*-(4'-chlorophenyl)ethane
EROD	ethoxyresorufin-O-dealkylase
HPLC	high pressure liquid chromatography
IC_{50}	concentration of inhibitor causing 50% inhibition of enzyme activity
MFO	mixed function oxidase
PROD	pentoxyresorufin-O-dealkylase
R-AChE	carbofuran-resistant Colorado potato beetle strain
R-mfo	multi-resistant Colorado potato beetle strain
R/S	resistant strain/susceptible strain ratio
SEM	standard error of the mean
Susc.	susceptible Colorado potato beetle strain
TMAA	trimethylammonium aniline

CHAPTER ONE

INTRODUCTION AND PROBLEM STATEMENT

INSECTICIDE RESISTANCE

History of Resistance

Resistance to insecticides by insect pests has been documented for over 75 years. As new pesticides have been developed, pest populations have ensured their own survival by developing resistance. When resistance occurs and the pesticide is no longer effective, replacements must be found. Resistance then develops to the new pesticide (often more quickly) and the process repeats. The cycle of resistance and introduction of new insecticides has been termed the "pesticide treadmill". The pace continues to increase and the consequences of jumping off are as catastrophic.

The first incident of insecticide resistance was reported by Melander (1914). He described populations of San Jose scale that were "...still alive under a crust of dried spray" (lime-sulfur). Melander also described resistance to lead arsenate in gypsy moth (*Lymantria dispar* L). To control the lime-sulfur resistant scales, Melander recommended using an oil spray, but with a caveat:

> "...we have noted a few individuals that have manifested a remarkable tenacity for life. Should these result in a resistant strain sometime in the future it would be necessary to use both insecticides, and then if the same individuals were doubly resistant..."

And so the first steps onto the pesticide treadmill were documented.

Between 1914 and 1946, 11 more cases of insecticide resistance were documented (e.g. scale insects, thrips, and codling moth (*Cydia pomonella* (L.)). These were all instances of resistance to inorganic pesticides (see Babers and Pratt, 1951; Forgash 1984). The development of organic insecticides (e.g. DDT) in the mid 1940s revolutionized agriculture and diminished fears of insecticide resistance. It also increased the pace of the pesticide treadmill. Weismann (1947) was the first to report resistance to DDT (in houseflies, *Musca domestica* (L.)), but others soon followed (e.g. Bohart and Murray, 1950).

Agriculture after World War II also benefitted from the introduction of organophosphorous esters which were highly effective insecticides. However, by 1950 resistance to parathion was reported in mites (*Tetranychus bimaculatus* (Harvey)) (Garman, 1950).

In the 1950s two new major classes of insecticides also became widely used: the cylcodienes and carbamates. Cyclodiene resistance was first reported in mosquitoes (*Aedes taeniorhynchus* (Weidemann)) (Gjullen and Peters 1952) and by 1954 was also found in the Colorado potato beetle (*Leptinotarsa decemlineata* (Say)) (Gauthier et al. 1981).

Carbaryl was introduced in the late 1950s, and was the first widely used carbamate insecticide (Kuhr and Dorough 1976). Within a few years, resistance was reported in the obliquebanded leafroller (*Choristoneura rosaceana*) (Smith 1963).

In the 1960s, many new members of the already known classes of insecticides were introduced. Insects which were resistant to one insecticide developed resistance to closely related compounds very quickly. Sometimes resistance to one compound gave immediate resistance to new compounds (called "cross-resistance"). The oxime carbamates were developed in the middle of the decade, but when aldicarb was registered in 1966, some pest populations were already resistant (Kuhr and Dorough 1976). The concept of "cross-resistance" became reality. It was during this decade that formamidines were also introduced (Dittrich 1966), but resistance to them was reported a few years later (Lee 1969).

The late 1960s and 1970s saw a few breakthroughs in pesticide development, the most important being the introduction of synthetic pyrethroids. These compounds were less persistent in the environment, and very low mammalian toxicity. Once pyrethroid insecticides went into widespread use, resistance developed quickly, and may have been pre-existing due to DDT cross-resistance. Resistance to resmethrin by the flour beetle (*Tribolium castaneum* (Herbst)) was reported by Champ and Campbell-Brown in 1970. It seemed that evolution was outpacing chemistry in the survival of pest populations.

Recent developments have used the products of bacteria to control pests. The use of insecticidal toxins from the bacterium *Bacillus thuringiensis* (B.t.) for insect control is the most recent major development in pest control. Although

B.t. products have been used for pest control since 1938 (Jacobs, 1950), their use was somewhat limited until recently (Feitelson et al. 1992). This was due to a lack of reliability, a narrow spectrum of activity, and readily available alternatives. However, by the mid 1980's the scene had changed. A better understanding of the toxin and its production in fermentation increased reliability. A growing number of isolates allowed control of several pests, including coleopterans, dipterans and lepidopterans. The number of available alternatives was decreasing, and the pressure to find environmentally acceptable alternatives was increasing. Probably the most important aspect of the surge of interest in B.t. was the development of molecular biology, and techniques which could manipulate the gene for the toxin (Burges, 1986). The interest in B.t. products has resulted in steady growth in its use since about 1987 (Bowen 1991).

Despite the widespread opinion that the development of resistance to B.t. would be slow and difficult, resistance to B.t. toxins was reported in the Indian meal moth *Plodia interpunctella* in 1985 (McGaughey 1985). Since then seven other pest species have shown resistance to B.t. including the tobacco budworm (Stone et al. 1989), Colorado potato beetle (Miller et al. 1990), and diamondback moth (*Plutella xylostella* (L.)) (Tabashnik et al. 1990). McGaughey and Whalon (1992) have recently reviewed the literature and examined the potential for widespread resistance to B.t.

It is evident that in the 75 years since it was discovered, resistance to insecticides has become widespread, and a fact of life for growers. Georghiou (1991) recently reviewed the magnitude of resistance, and has put the current number of resistant arthropod species at over 504. Insect pests from all of the major orders have resistance to at least one insecticide. The resistance problem is large and continues to grow. But the situation is not hopeless. There can be wide variations in susceptibility, and most pest populations can still be controlled, although at increased cost. Georghiou states "The problem is evident, the need for action compelling, and the opportunities for breakthrough substantial". The National Research Council has addressed the problems of resistance and developed strategies for the management of resistance (National Research Council, 1986), and more work on the application of resistance management tactics is being done. In the last few years the number of papers published in Journal of Economic Entomology dealing with resistance has increased (Georghiou 1990) indicating a higher degree of awareness and study of the problem.

Mechanisms of Resistance

Several different schemes have been used to classify mechanisms of resistance. Perhaps the simplest would be to classify mechanisms as either avoidance of the dose (behavioral), or tolerance of a dose that is encountered. Georghiou (1972) categorized resistance mechanisms as

behavioral, physiological, or biochemical. But there is significant overlap between those categories (physiological resistance has a biochemical component), and it doesn't seem to be a very discrete classification system.

I prefer to classify resistance based on four mechanisms: behavior, penetration, altered target site, and metabolic. This correlates the category most closely with the mechanism, and has little overlap. It also allows one to look at the biochemical, cellular, and organismal levels without confusing resistance mechanisms. Oppenorth (1984) developed useful subcategories of resistance mechanisms: qualitative and quantitative. For example, an increased detoxication capacity (metabolic resistance) could be due to the altered substrate specificity of aliesterases (Oppenoorth and Van Asperen, 1960), or to an increased amount of arylesterase which is not altered in specificity (Devonshire and Sawicki 1979).

Except for behavioral resistance, the biochemistry of these mechanisms has been investigated. The paragraphs below summarize the work which gives the current understanding of these mechanisms of resistance.

Behavioral resistance is defined as "those actions, evolved in response to the selection pressure exerted by a toxicant, that enhance the ability of the population to avoid the lethal effects of that toxicant" (Lockwood et al. 1984). Hadaway (1950) determined that mosquitoes did not light on DDT-treated surfaces, and so documented the first

instance of behavioral resistance. This mechanism of resistance has been well-documented in DDT resistant mosquitoes (see Mattingly 1962) but has also been reported in eight other orders of insects. Behavioral resistance has been documented for several classes of insecticides, including: organophosphates (Smith and Yearian 1964), carbamates (Ebeling et al. 1966), and pyrethroids (Burden 1975, Prickett and Radcliffe 1977). In their review, Lockwood et al. (1984) showed 154 cases of behavioral resistance in 45 insect species to 35 different compounds.

The relationship of behavioral resistance to other mechanisms is somewhat in dispute. Early discoveries indicated that there was an inverse relationship between behavioral and physiological mechanisms of resistance (Georghiou 1972). However, many studies have shown behavioral and physiological resistance coexisting in the same individual (e.g. Prickett and Radcliffe 1977). In about half of the cases studied, both behavioral and physiological resistance were important in resistant populations (Lockwood et al. 1984).

Altered barriers which slow the absorption of pesticides into the insect or into compartments within the insect may be present in insecticide-resistant populations. The basis for penetration resistance appears to be a change in the physicochemical properties of barriers normally present (Patil and Guthrie 1977). As a result, penetration resistance can be broad-spectrum (Sawicki and Lord 1970).

Forgash et al. (1962) were the first to describe resistance due to decreased penetration. They showed a 2- to 3-fold decrease in penetration of diazinon into a strain of resistant houseflies. In 1968, Plapp and Hoyer published an important paper in which they isolated and identified a gene (Organotin-R) responsible for penetration resistance. The presence of the mutant gene apparently resulted in a general penetration barrier with a 2-fold reduction in penetration of DDT and dieldrin. It was less effective with more polar insecticides.

Reduced uptake is a factor in some forms of resistant *Euxesta notata* (Hooper 1965), German cockroaches (*Blattella germanica* (L.)) (Ku and Bishop 1967), *Culex pipiens fatigans* (Weidemann) mosquitoes (Shrivastava 1970), *H. virescens* (Vinson and Law 1971), citrus red mites (*Panonychus citri* (McGregor)) (Hirai et al. 1973), cattle ticks (*Boophilus microplus* (Can.)) (Schnitzerling et al. 1983), and the lesser cotton armyworm (*Spodoptera exigua* (Hub.)) (Delorme et al. 1988).

The nature of the changes in the cuticle of resistant strains has only received limited study. Benezet and Forgash (1972) determined that there was no differences in the major chemical constituents of the epicuticle. Vinson and Law (1971) showed increased lipids, protein, and sclerotization in resistant tobacco budworms compared to susceptibles. Also, Patil and Guthrie (1979) showed higher levels of phospholipids in the cuticle of resistant housefly strains.

Another form of penetration resistance was characterized by Matsumura and Hayashi (1966). The nerve sheath in *Periplaneta americana* (L.) resistant to dieldrin had lower binding of dieldrin than the nerve sheath of susceptible strains. This type of penetration resistance has not been otherwise reported.

Most often, penetration resistance contributes a 2- to 3-fold degree of resistance. The importance of reduced penetration as a mechanism of resistance lies in its interaction with other resistance factors. Plapp and Hoyer (1968) recognized that fact in the title of their landmark paper, calling penetration resistance "an intensifier of resistance". Georghiou (1971) documented the synergistic relationship between penetration and other resistance mechanisms. In fact, in every case of penetration resistance discovered, the original occurrence was in combination with another resistance factor.

Increased metabolism of insecticides is the most frequently documented mechanism of resistance in insects (Matsumura 1985). This mechanism of resistance has the advantage that it can respond to a broad range of pesticides, and is highly efficient. It is also comprised of a series of enzymes which, in some cases, are induced by the presence of xenobiotics, thus reducing its energetic costs. However, metabolism of insecticides is a two-edged sword, as it can result in a more toxic compound (Aldridge and Davison 1952; Drabekand Neumann 1985).

There are four major enzyme systems important in resistance to insecticides: mixed function oxygenases, hydrolases, DDTases and glutathione-S-transferases. The earliest reported incidence of metabolic resistance was of DDT-resistant houseflies, which had elevated levels of DDTase activity (Sternburg et al. 1953).

DDTases were widely studied in the 1950s, and reviewed by Lipke and Kearns (1960). As important a factor in DDT resistance as they were, these enzymes seem to occur mostly in houseflies (with a few examples in mosquitoes) and are resistance factors relatively specific for DDT. They may be forms of glutathione-S-transferases. They are currently not of practical interest for metabolic resistance.

When examining hydrolytic mechanisms of resistance, one bumps into a forest of confusing and sometimes conflicting names for the enzymes involved. The nomenclature of enzymes allows organization and concept development, but sets artificial boundaries which can promote tunnel vision. It can be difficult to determine which group of enzymes investigators are assaying when they use non-standard substrates, or create their own nomenclature systems. Often enzymes are named based on the substrates used by the investigator. Thus one can describe the same set of enzymes as carboxylesterases, phosphatases, phosphorotriesterases, arylesterases, paraoxonases, etc. For example, in describing their "mutant aliesterase" theory, Oppenoorth and Van Asperen (1960) report an aliesterase with phosphatase

activity. It appears that in insect homogenates, there are a number of esterase activities. In some cases these activities have been named quite specifically (e.g. phosphorotriesterases) even though their narrow enzymatic activity wasn't separated from broader activities (e.g. phosphorotriesterases also hydrolyze carboxylesters). The use of "non-standard" substrates certainly can't be avoided, but including assays with a standardized set of substrates is important for comparison and it clarifies nomenclature. The nomenclature used should reflect the degree of specificity and homogeneity of the preparations, not just the substrates or inhibitors used by a particular investigation. To organize this presentation, I have generalized three groups of hydrolytic enzymes present in insects: arylesterases, aliesterases, and epoxide hydrolases.

Arylesterases, classified as A-esterases by Aldridge and Reiner (1972), include enzyme activities which have been called phosphatase, phosphorotriesterase, paraoxonase, and carboxylesterase. Arylesterases hydrolyze aromatic esters, and degrade organophosphates, although they are generally not inhibited by them. They are frequently assayed with α- or β-naphthylacetate. Resistance by this mechanism was first described by Matsumura and Brown (1961) in *Culex tarsalis* (Coquillett). This mechanism has since been widely documented in many resistant populations (see Motoyama and Dauterman 1974). Arylesterases are a diverse group of

enzymes which can show very different activities towards α- and β-naphthylacetate as well as different insecticides which they metabolize (see Oppenoorth 1985).

Aliesterases, classified by Aldridge and Reiner (1972) as B-esterases, include carboxylic ester hydrolase (EC 3.1.1.1), and some other carboxylesterases. The criteria I will use for aliesterases include inhibition by organophosphates and inability to hydrolyze acetylcholine. Aliesterases act on aliphatic substrates, and are usually assayed with methylbutyrate or malathion. This mechanism of resistance was first reported by Matsumura and Hogendijk (1964) in houseflies. Aliesterases are important resistance factors for organophosphates (Motoyama and Dauterman 1974), by metabolizing some (e.g. malathion), or by acting as a sink for others. Aliesterases also metabolize pyrethroid esters (Jao and Casida 1974), and have been documented as a mechanism of resistance in at least 10 insect species.

Epoxide hydrolases are present in insects and have been shown to metabolize cyclodienes and some juvenoids (Dauterman 1985). Some epoxide hydrolases can be inhibited by some organophosphates, however their role as factors of resistance has not been documented.

Glutathione-S-transferases are also important in metabolic resistance. These enzymes catalyze the conjugation of compounds with glutathione (γ-glutamyl-cysteinyl-glycine) which results in synthesis of mercapturic acid (Gessner and Smith 1960) or other thiol metabolites.

Shishido and Fukami (1963) showed that glutathione-S-transferases were involved in metabolism of organophosphates, and Lewis (1969) showed that they were a factor of resistance in diazinon-resistant houseflies. Several resistant insect species (e.g. tobacco budworms, houseflies, mites, and light brown apple moth (*Epiphyas postvittana*) (Suckling et. al 1990)) have been shown to have increased glutathione-S-transferase activity, although in most cases other mechanisms of resistance were also present (Oppenoorth 1985).

The mixed function oxygenases (also called MFOs, multifunction oxygenases, or polysubstrate monooxygenases) are the most important group of metabolic enzymes in the insect. This group catalyzes a broad variety of oxidation reactions including hydroxylation, dealkylation, epoxidation and desulfuration (Nakatsugawa and Morelli 1976). The mixed function oxygenase system works through a family of related b-type (P450) cytochromes.

The nomenclature for cytochrome P450 enzymes has gotten so complex that a committee was formed to standardize it (Nebert et al. 1987). Insect cytochrome P450s fall into two general groups: type I and type II. These correspond to the older, perhaps more familiar terminology of P448 (3-methylcholanthrene-induced) and P450 (phenobarbitol-induced) respectively (Nebert et al. 1987). There are several forms of these two groups present in insects, though studies have been largely limited to the Diptera. For example, Yu and

Terrier (1979) reported 6 different type II P450 activities in resistant and susceptible houseflies. More recently, a resistance-related form of P450 has been isolated from *Drosophila* and may be designated a type III P450 (Waters and Nix 1988; Sundseth et. al 1990). The role of these oxidative enzymes in metabolizing insecticides and other xenobiotics has been thoroughly reviewed by Kulkarni and Hodgson (1980).

Beside oxidative deactivation of toxicants, the mixed function oxygenase system also activates some compounds. For example, the phosphorothionate insecticides are converted to phosphates which are much more potent inhibitors of acetylcholinesterase (Aldridge and Davison 1952).

Insecticide resistance due to mixed function oxygenase activity was hypothesized as early as 1960 (Eldefrawi et. al 1960). But it was after Omura and Sato (1964) isolated and characterized cytochrome P450 that the enzymes were shown definitively to be a factor of resistance (Tsukamoto and Casida 1967). The involvement of mixed function oxygenases in resistance has been documented in vitro for several species including houseflies (Tsukamoto and Casida 1967), mosquitoes (Shrivastava et. al 1970), cabbage loopers (*Tricoplusia ni* (Hub.)) (Kuhr 1971), flour beetles (Dyte et. al 1970), and others (see Oppenoorth and Welling 1976).

In many more cases, mixed function oxygenases have been implicated in resistance because of in vivo synergism with

piperonyl butoxide, a fairly specific inhibitor of P450 (see Chang et. al 1981). The relatively small number of cases investigated in vitro is probably due in no small part to the difficulties associated with preparing insect microsomes with functional P450 (see Hodgson 1980). Whether determined in vitro or in vivo, mixed function oxygenases are the most frequently reported factor of insecticide resistance (Matsumura 1985).

Target-site interactions are second in importance to metabolism as a mechanism of resistance (Oppenoorth 1985). Examples of target-site interaction include acetylcholine receptor binding of nicotine, the effect of cyclodiene insecticides on chloride channels, and inhibition of acetylcholinesterase by organophosphate insecticides.

Although altered acetylcholinesterase has been the most studied aspect of target-site resistance, there are other examples. Cyclodiene resistance has a target-site component with defined criteria. The mechanism of resistance is not yet understood, but there may be some alteration in the binding site located on the chloride channel (see Brooks 1974, Matsumura and Ghiasuddin 1983). Similarly, knockdown resistance to DDT and pyrethroids is thought to be due to target-site insensitivity of the voltage-dependent axonal sodium channel.

Altered acetylcholinesterase as the basis for insecticide resistance was first reported by Smissaert (1964) in mites (*T. urticae* (Kock)). Since then, this mode of

resistance has been reported in several insect species (Table 1).

Housefly acetylcholinesterase has been extensively studied for several reasons. First, many cases of resistance to organophosphates and carbamates were initially reported in housefly strains. Second, houseflies are a rich source of acetylcholinesterase, and during preparation, much of the enzyme is solubilized, so 100,000 x *g* supernatants are a convenient sources of the enzyme. Third, they are easy to raise in culture, and large numbers are readily obtainable.

To avoid some of the difficulties associated with determining individual kinetic constants, the bimolecular rate constant is often used to quantify the potency of a compound. However, there is an underlying assumption that the reaction follows first order kinetics and that the reactivation rate is negligible under the experimental conditions (Main 1964). The presence of multiple isozymes can also make determining kinetic parameters difficult. In some cases where attempts were made to measure the bimolecular rate constant of acetylcholinesterase from resistant insects, the data were nonlinear (e.g. Yamamoto et. al 1983; Zhu and Brindley 1991). Acetylcholinesterase may have to be purified before kinetic characterization.

Table 1 First reported cases of resistance due to altered acetylcholinesterase.

Insect	Reference
Mite	Smissaert 1964
Tick	Lee and Botham 1966
Green Rice Leafhopper	Hama and Iwata 1971
Housefly	Tripathi and O'Brien 1973
Mosquito	Ayad and Georghiou 1975
Drosophila	Morton and Singh 1982
Brown Planthopper	Hama 1983
Lygus Bug	Zhu and Brindley 1990
Tentiform Leafminer	Pree et. al 1990
Tobacco Budworm	Brown and Bryson 1991
Colorado Potato Beetle	Ioannidis et. al 1992

RESISTANCE IN THE COLORADO POTATO BEETLE

History

The Colorado potato beetle originally fed on solanaceous weeds in the western United States, but has been a pest of potatoes since 1861 (Edgerton 1861) and was broadly distributed across the country by 1875 (Riley 1875). While many growers maintained control by hand-picking beetles, Paris Green was used as early as 1870 for Colorado potato beetle control (see Casagrande (1987) for a history of CPB management). Apparently chemical control was successful during the first part of the 20th century, because the first report of resistance in the Colorado potato beetle wasn't until the 1940s, when growers experienced control failures using arsenicals.

The advent of organic pesticides introduced DDT to widespread use. Within 8 years, Colorado potato beetle resistance to DDT was reported in several states (Gauthier et al. 1981). A few years later resistance to cyclodienes was also reported. Between 1955 and 1985 new pesticides used on Long Island, New York had an average life of 1.6 years (about 4 generations) before resistance was reported (Forgash 1985). Table 2 summarizes a history of insecticide resistance in potato beetles.

In Michigan, serious problems with Colorado potato beetle control began in 1987. By 1991 Colorado potato beetle populations with resistance to all groups of

Table 2. History of Resistance to Insecticides by the Colorado potato beetle.[a]

Insecticide	Year Introduced	Years Before Resistance
Arsenicals	1880	ca. 60
DDT	1945	7
Dieldrin	1954	3
Endrin	1957	1
Carbaryl	1959	4
Azinphosmethyl	1959	5
Monocrotophos	1973	0
Phosmet	1973	0
Disulfoton	1973	1
Carbofuran	1974	2
Oxamyl	1978	0
Fenvalerate	1979	2
Permethrin	1979	2
Bacillus thuringiensis[b]	1988	3

[a]Data adapted from Gauthier et al. 1981 and Forgash 1985

[b] Laboratory strain (Miller, et al. 1990), not yet observed in the field.

synthetic insecticides and several insecticide/synergist combinations were distributed throughout the major potato growing regions (Grafius et al. 1991). A recent survey by the Michigan Potato Industry Commission showed that the cost of controlling Colorado potato beetles was $15.5 million in Michigan, about 22% of the total revenues generated by potatoes (Annonymous 1992).

Colorado Potato Beetle Strains

Susc. (also known as Vestaburg). This strain was started in 1987 by collecting beetles from unsprayed volunteer potatoes in Montcalm Co., MI. Bioassay data showed that the strain was as susceptible to carbofuran and azinphosmethyl as other susceptible strains (Ioannidis et al. 1991). This strain has been maintained under laboratory conditions (see below) without selection and was used as a susceptible strain for comparison with resistant strains.

R-AChE (also known as Montcalm-C). This strain originated at the Michigan State University Research Farm at Entrican, MI. In 1987, a potato field at the research farm was sprayed with carbofuran (0.55 kg/ha). The next day, surviving larvae were collected. Adult beetles of this strain were selected by topical application with gradually increasing doses of carbofuran for three generations, then maintained at 100 µg carbofuran per adult beetle in each generation (see Ioannidis 1990). This strain is highly resistant to carbofuran (576-fold), and slightly resistant to azinphosmethyl and permethrin (Table 3). Since its

Table 3. Resistance and synergism ratios of carbofuran and azinphosmethyl in Colorado potato beetle strains.[a]

	Susceptible	R-mfo	R-AChE
Carbofuran			
Resistance Ratio[b]	1	225	576
Synergism Ratio[c]	ND[d]	15	1.3
Azinphosmethyl			
Resistance Ratio	1	445	9.1
Synergism Ratio	4.5	20	1.6

[a]adapted from Ioannidis et al. 1992

[b]LD_{50} for resistant strain/LD_{50} for susceptible strain (topical application to adults)

[c]LD_{50} for strain without/LD_{50} with piperonyl butoxide treatment (100 μg per adult for resistant strains, 50 μg per adult for the susceptible strain).

[d]not determined

resistance was not highly synergized by piperonyl butoxide, it was hypothesized that this strain was resistant primarily due to altered acetylcholinesterase.

R-mfo (also known as Long Island). This strain was collected by Dr. A.J. Forgash on Long Island in 1985 from a population which exhibited multiple resistance. It was selected with various doses of azinphosmethyl for the first 6 generations, then maintained in the laboratory under selection pressure of at least 100 µg azinphosmethyl per adult. After 1988 azinphosmethyl and carbofuran (100 µg per adult) were alternated as the selecting agents to maintain resistance to both compounds. This strain is highly resistant to both carbofuran (225-fold) and azinphosmethyl (445-fold). It is slightly resistant to permethrin. Resistance is substantially synergized by piperonyl butoxide (Ioannidis et al. 1992; Table 3). Additional data (Ahammadsahib et al. submitted) show that this strain has elevated mixed function oxidase activity.

Macomb J-P. This strain was collected from a commercial potato field in Macomb Co., MI in 1988. It is highly resistant to both carbofuran (>1000-fold) and azinphosmethyl (907-fold) (Ioannidis et al. 1991). This strain was maintained in the laboratory only briefly. Each generation was selected with 100 µg carbofuran or azinphosmethyl per adult to maintain the high level of resistance. This strain was used only in some early

characterization of acetylcholinesterase activity (see Chapter 3).

General Rearing Conditions

Colorado potato beetles were maintained at 25-28°C with a 16:8 (light/dark) light cycle. Relative humidity was not controlled. The larvae and adults were fed live potato foliage (typically variety "Atlantic") grown in clay pots in a greenhouse. Adults emerging from pupation were placed on potted potato plants. After feeding for at least 24 hours, the new adults were frozen at - 20°C, held alive on foliage at 10°C until used in experiments, or recycled into the breeding colonies. Using this method, more than 20,000 adult beetles were produced during the course of the research described here.

Before adults from the resistant strains were recycled into the colonies, they were treated with either carbofuran or azinphosmethyl. The treatment consisted of a topical application of 2 µl of insecticide (50 mg/ml in acetone) to the ventral abdomen.

STATEMENT OF THE PROBLEM

It is believed that the Colorado potato beetle is a model resistant species in many respects. Georghiou (1986) stated "Nowhere is the end of the line of effective toxicants so clearly evident as in the Colorado potato beetle...". Although many insecticides, and even household chemicals (Ghidiu 1987) have been tested against potato

beetles, the biochemical mechanisms underlying resistance have only recently begun to be investigated (Argentine 1991, Ioannidis et al. 1992).

With the R-AChE strain, bioassay data suggested that the strain had an altered acetylcholinesterase (Ioannidis 1991), but there was no direct evidence. If altered acetylcholinesterase was present in this strain, what degree of cross-resistance did it give? Although piperonyl butoxide did not highly synergize resistance, there was some synergism, and the metabolic capacity of the R-AChE strain had not been investigated.

Some aspects of resistance had been studied in the R-mfo strain, but even with synergism by piperonyl butoxide, some resistance remained, suggesting additional resistance mechanisms might be present. It could also be used as a comparison with the R-AChE strain.

To understand and compare the mechanisms of resistance operating in these strains, the biochemical and physiological resistance mechanisms would be compared with a susceptible strain. The goals of this research were as follows:

1. Measure penetration of carbofuran to determine if it is a factor of carbofuran resistance in the strains of interest.

2. Examine the activity and specificity of inhibition of acetylcholinesterase in each strain.

3. Assay glutathione-S-transferase, arylesterase, and mixed function oxidase for differences in activities.

4. Measure the rate of in vivo metabolism of carbofuran in each strain, and determine if the pattern of metabolites formed is the same in each of the strains.

CHAPTER TWO

PENETRATION RESISTANCE

INTRODUCTION

One way to attain resistance to many insecticides is by slowing or delaying absorption across the cuticle or gut. This first step in the toxicodynamics of pesticide exposure can significantly influence the toxicity of the compound. By reducing the influx of pesticides into the body, penetration barriers give mechanisms of degradative metabolism and excretion a chance to reduce the total body burden. The slower movement of toxicants into the insect could also allow for a higher proportion of the poison to be metabolized, since less would bypass saturated detoxification systems. This mechanism of resistance could confer a broad spectrum of cross-resistance to compounds having similar physical properties since it is probably based on non-specific changes in the penetration barrier.

Penetration barriers were first described as a mechanism of insecticide resistance by Forgash et al. (1962) in the housefly. They showed a 2-3 fold difference in penetration of the cuticle between diazinon-resistant and susceptible strains. This difference translated into a 5 - 9 fold level of resistance. Low levels of resistance due to cuticle penetration differences have also been shown in the German cockroach (Yu and Bishop 1967) and the mosquito (Shrivastava et al. 1970). Matsumura and Hayashi (1966) showed penetration resistance in the neural sheath surrounding the central nervous system of the German cockroach. Resistance was due to reduced binding (1- to 2-

fold) of dieldrin by proteins of the nerve cord. Penetration barriers of themselves cause fairly low levels of resistance (<10-fold), but they can have a powerful effect in synergizing other mechanisms of resistance (see Plapp 1970, Matsumura 1983).

No studies have been published showing penetration resistance in the Colorado potato beetle, but unpublished reports describe penetration resistance to permethrin (Ioannidis and Grafius 1989) and azinphosmethyl (Argentine 1991). My goal was to determine what contribution penetration barriers make to the observed resistance to carbofuran.

METHODS AND MATERIALS

To determine the rate of carbofuran uptake, 1 μg of carbofuran (1mg/ml in acetone) was applied as a single droplet to the ventral abdomen of adult beetles. After time intervals of 2 to 12 hours, dosed beetles were rinsed for 2 min in acetonitrile/water (3:1) (5 beetles per 2.5 ml). Each assay was repeated three times. The rinsates were filtered through a 0.2 micron nylon filter (13 mm Acrodisc, Gelman Sciences, Ann Arbor, MI). The filtered rinsates were injected onto a 10 cm RP-18 high pressure liquid chromatography (HPLC) column (Spheri-5, Brownlee Labs, Santa Clara, CA) through a Rheodyne 7125 injector (Rheodyne Inc., Catati, CA) fitted with a 50 μl sample loop. The HPLC system also included a Waters model 501 pump and model 490E

multiwavelength detector (Milford, MA). The mobile phase consisted of acetonitrile/water (7:3). Areas were integrated manually or with a Hewlett Packard (Houston, TX) model 3390A integrator. Recoveries at time zero were 93%, and results were quantified with external standards for peak area determination.

In later tests, 2 µl carbon-14 labeled carbofuran (0.6 µg, 50 µCi/ml in acetone, ring-labeled, provided by FMC Corp, Princeton NJ) was topically applied as above, and at various times (0 to 4 hours), beetles were rinsed and the rinsates (100 µl) applied to reverse phase TLC plates (Whatman LKC18F, Clifton NJ). Authentic standards (FMC Corp., Princeton NJ) were over-spotted on the samples. The plates were developed in acetonitrile/ 0.5 M sodium chloride (1:1). After drying, the standards were visualized by ultraviolet light, and the plates were autoradiographed. The areas of the plate showing radioactivity were scraped, and the ^{14}C-carbofuran was eluted with acetone. The samples were counted by liquid scintillation in 4 ml of Safety Solve (Research Products International Corp., Mount Prospect IL).

RESULTS AND DISCUSSION

There were no substantial differences between strains in the loss of carbofuran from the cuticle (Figure 1). Up to 8 hours after application, there were no significant differences between the resistant and susceptible strains ($p < 0.05$, Student's t-test). At 12 hours, the resistant

strains had significantly faster carbofuran absorption ($p < 0.05$). This may have been due to some mortality in the susceptible strain at 12 hours. The values obtained with HPLC analysis were confirmed (at treatment times less than 4 hours) by the carbon-14 measurements. Although Argentine (1991) reported slower penetration of azinphosmethyl in a resistant strain of Colorado potato beetle, his data are very similar to those shown in Figure 1. Argentine's results were based on a statistically significant, but small difference at a single time point (2 h) of a six hour test. The only other report of penetration resistance in the Colorado potato beetle is reduced uptake of radio-labeled permethrin in a strain of permethrin-resistant beetles (Ioannidis and Grafius 1989).

Results of penetration assays must be interpreted with caution. Either a higher or lower rate of uptake could be consistent with insecticide resistance. Obviously, lower rates of penetration tend to decrease the rate of arrival of a toxicant at the site of action. However, uptake is a complex and multicomponent diffusional process (see Welling 1977). It has been observed in the mustard beetle (*Phaedon cochleariae*) that higher rates of metabolism increased the rate of penetration of pyrethroids (Elliot et. al 1970). Presumably, the increased rate of metabolism creates a sink for the insecticide, thereby enhancing the uptake process. Thus, reductions in the permeability of the cuticle could be

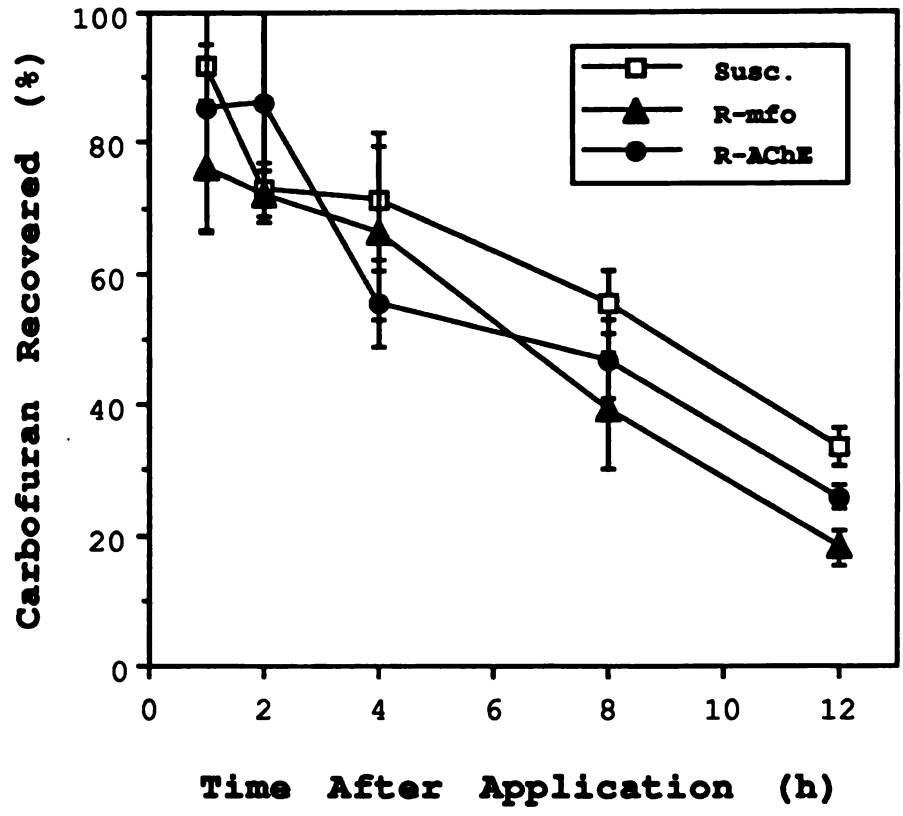


Figure 1. Recovery of carbofuran from the ventral abdominal cuticle of three strains of Colorado potato beetle as determined by HPLC.

offset by increases in the concentration gradient (force driving penetration).

Since the resistant strains did exhibit higher rates of carbofuran metabolism (see Chapter 4), the results presented here cannot rule out differences between strains in their penetration barriers. But to give the results shown here, each strain would have to compensate for the differences in metabolism with proportional differences in the characteristics of the cuticle. This would result in rates which are very similar for each strain. Overall, it is unlikely that penetrations differences play a major role in resistance of either the R-mfo or R-AChE strains.

CHAPTER THREE

TARGET SITE INTERACTIONS

INTRODUCTION

Resistance to insecticides via altered target site interactions has been documented in several insect species (Hama 1983). Almost every case of altered target site resistance has been found in an agricultural pest. This presents a serious challenge to both agriculture and the agrichemical industry. The Colorado potato beetle is a model resistant species in many respects, having developed resistance to almost every insecticide used in attempts to control it (Georghiou 1986).

Altered target-site resistance has only recently been documented in the Colorado potato beetle. Argentine (1991) observed target-site insensitivity, decreased penetration, and increased metabolism in a Colorado potato beetle strain resistant to azinphosmethyl. Altered AChE has also recently been reported in a carbofuran-resistant strain of the Colorado potato beetle (Ioannidis et al., 1992). However, neither of these reports examined this mechanism of resistance in detail nor did they discuss the general utility of altered AChE as a defense against cholinesterase inhibitors.

To develop a clearer understanding of acetylcholinesterase insensitivity as a general mechanism of insecticide resistance, in vitro assays of acetylcholinesterase from a susceptible and two carbofuran-resistant strains of Colorado potato beetle were used. The aim of this research was to define the response of

acetylcholinesterases from the resistant strains to carbofuran, and to determine if cross-resistance was conferred to other carbamate or organophosphate insecticides via altered target site resistance.

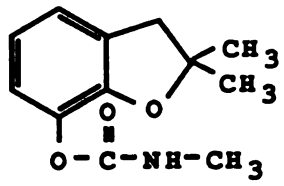
METHODS AND MATERIALS

Chemicals

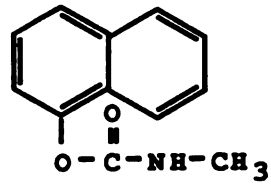
Carbofuran, 3-hydroxycarbofuran, 3-ketocarbofuran, and the N-propyl analog of carbofuran were donated by FMC Corp. (Princeton NJ); carbaryl was provided by Union Carbide Corp. (Research Triangle Park NC); azinphosmethyl oxon and propoxur were from Mobay Chemical Corp. (Kansas City MO). All other pesticides were obtained from Chem Service Inc. (West Chester PA). Figure 2 shows the structures of the insecticides used. All other chemicals were from Sigma Chemical Co. (St. Louis MO).

Acetylcholinesterase Assays

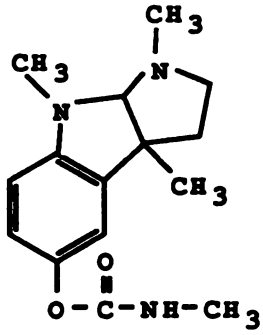
Heads and thoraces were removed from adult beetles, pooled in 0.05 M phosphate buffer (pH = 7.4) and homogenized on ice 3 x 10 s at full power with a Vertishear (Vertis Co. Gardiner NY) using the micro-fine generator. The brei was centrifuged at 3,000 x *g* for 20 min (4°C), and the supernatant was centrifuged at 100,000 x *g* for 60 min (4°C). The resulting pellet was resuspended in phosphate buffer with the Vertishear (2 x 10 s, full power) at 10 beetle equivalents per ml. This fraction was kept on ice until used in the assay. Preliminary studies showed that most



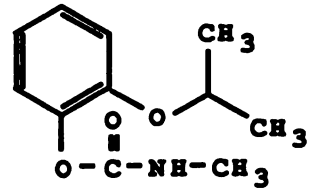
Carbofuran



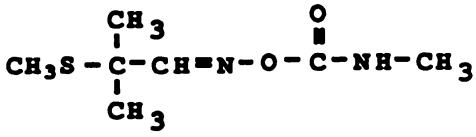
Carbaryl



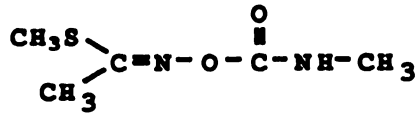
Eserine



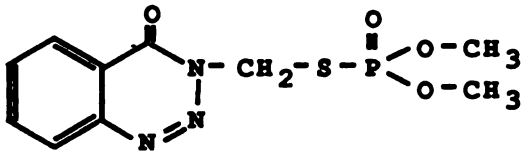
Propoxur



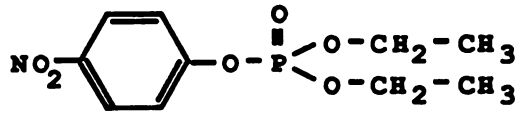
Aldicarb



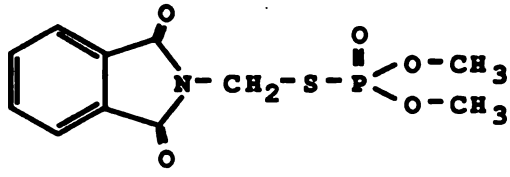
Methomyl



Azinphosmethyl oxon



Paraoxon



Phosmet oxon

Figure 2. Structures of acetylcholinesterase inhibitors used.

AChE activity was in the microsomal pellet, and head and thorax homogenates had highest specific activity.

Insecticides were dissolved in ethanol (10 mM), then diluted (100- to 500-fold) with phosphate buffer. Control assays were carried out with equivalent concentrations of ethanol alone. The assay for acetycholinesterase was after the method of Ellman et al. (1961). Briefly, 200 µl of inhibitor plus buffer was added to 300 µl of enzyme fraction and preincubated at 30°C for 10 min. After preincubation, 100 µl of 3 mM 5,5-dithio-*bis*-(2-nitrobenzoate) in phosphate buffer and 400 µl of 10 mM acetylthiocholine iodide in phosphate buffer were added. Preliminary studies showed the K_m for acetylthiocholine to be about 20 µM. To test substrate specificity, propionylthiocholine, butyrylthiocholine, or acetyl β-methylthiocholine were substituted for acetylthiocholine. Homogenates were assayed for protein by the method of Guegenrich (1984).

The reaction rate was measured at 30°C with a Shimadzu UV-265 spectrophotometer (Kyoto Japan). Absorbance readings were taken every 30 s for 10 min. Correction for the spontaneous hydrolysis of acetylthiocholine and microsomal interaction with 5,5-dithio-*bis*-(2-nitrobenzoate) were made. Data from 2 to 8 min were used to calculate the reaction rate and correlation coefficient. Results from separate experiments were pooled to calculate the mean and standard error of log percent activity remaining at each inhibitor concentration. Differences between strains at each

inhibitor concentration were tested for significance at the 95% confidence level using Student's t-test.

RESULTS AND DISCUSSION

Substrate Specificity

The hydrolysis of acetylthiocholine by Colorado potato beetle homogenates was linear for over 20 min. under the conditions described, inhibited or not. This hydrolysis was characterized as AChE activity using the criteria of Toutant (1989) as follows. The rate of hydrolysis was higher with acetylthiocholine than either propionylthiocholine or butyrylthiocholine (Table 4). The rate of hydrolysis of acetyl β-methylthiocholine was nearly equivalent to that of acetylthiocholine. And inhibition of hydrolysis by eserine (see below) was significant at 10 μM. In addition, competing reactions (e.g. carboxylesterase activity) were removed by centrifugation. These results verify that true acetylcholinesterase activity was measured.

It should be pointed out that there has been very little reported work with AChE from Colorado potato beetles. The specific activities are much lower (ca. 10-fold) than that of housefly AChE (see Appendix Two), but are only slightly lower than the specific activity reported by Argentine (1991) in Colorado potato beetle.

Inhibition by Arylcarbamates

Acetylcholinesterase from the R-AChE strain was clearly insensitive to carbofuran (Figure 3). Above 2 μM, the R-

Table 4. Rates of hydrolysis of substrates by Colorado potato beetle acetylcholinesterases.

Strain	Substrate[1]	Hydrolysis rate[2]
Susc.	ATCh	7.0 ± 0.6
	AMTCh	8.4 ± 0.8
R-mfo	ATCh	11.3 ± 0.8
R-AChE	ATCh	17.1 ± 1.7
Macomb J-P	ATCh	17.0 ± 3.4
	PTCh	10.8 ± 1.9
	BTCh	6.5 ± 1.1

[1] AMTCh: acetyl β-methylthiocholine
ATCh: acetylthiocholine
PTCh: propionylthiocholine
BTCh: butyrylthiocholine

[2] Hydrolysis rate expressed as nmole/mg protein/min. Data shown are the mean ± SEM, n = 4. Substrate concentration was 2.0 mM for AMTCh, 1.4 mM for all others (ca. 80-fold above the K_m).

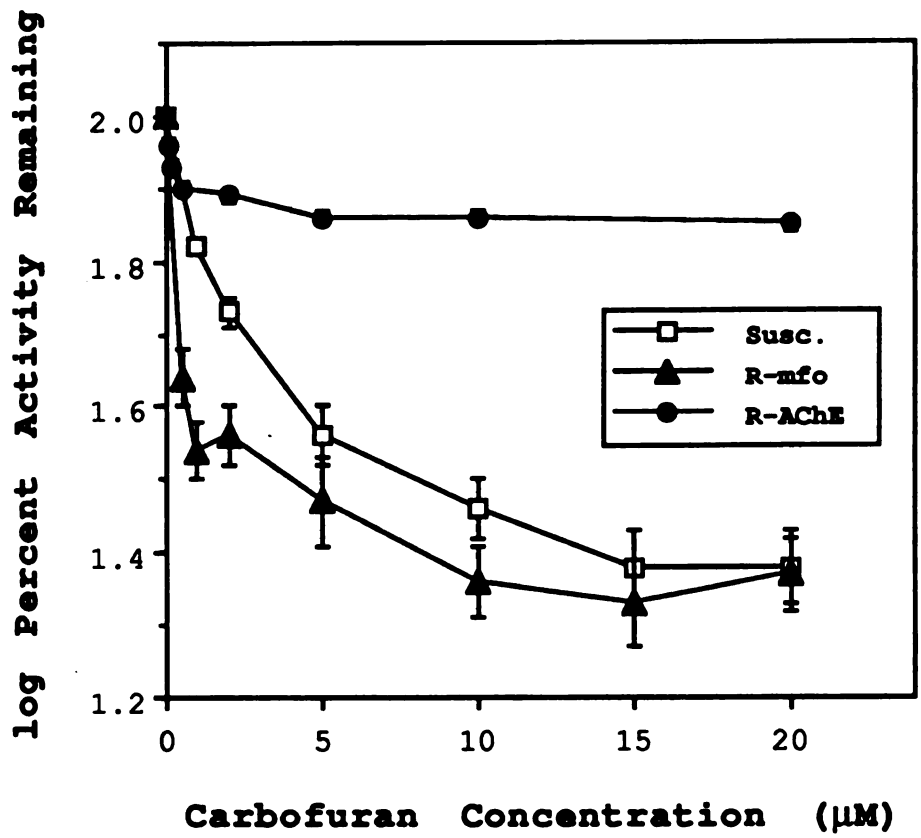


Figure 3. Inhibition of acetylcholinesterases from Colorado potato beetles by carbofuran. The enzyme was preincubated with inhibitors for 10 min. The data shown are the mean log percent activity remaining ± SEM, n = 4-6.

AChE strain was significantly less inhibited ($p < 0.05$). In all three strains, the concentration-log activity curves were nonlinear. Inhibition by carbofuran leveled off at about 25% for the R-AChE strain. Acetylcholinesterase from the R-mfo strain responded similarly to the susceptible strain, with neither exceeding about 75% inhibition.

There was no significant difference ($p < 0.05$) between strains in response to inhibition by 3-hydroxycarbofuran (Figure 4) or 3-ketocarbofuran (Figure 5), though the R-AChE strain showed the lowest level of inhibition. In all cases, the potency of metabolites was lower (5- to 50-fold) than with carbofuran. These data are consistent with those of Metcalf et al. (1968) who showed about a 20-fold lower potency of 3 hydroxy- and 3-ketocarbofuran with housefly acetylcholinesterase.

Both the R-AChE and R-mfo strains were significantly ($p < 0.05$) insensitive to N-propylcarbofuran compared to the susceptible strain (Figure 6). In this case, the concentration-log activity curves were nearly linear. Yamamoto et al. (1977) reported altered acetylcholinesterases from the green rice leafhopper (*Nephotettix cincticeps* (Uhler)) which were insensitive to N-methylcarbamates but hypersensitive to N-propylcarbamates. The data in Figures 3 and 6 show that both resistant strains of Colorado potato beetles were insensitive to N-propylcarbofuran, and the R-AChE strain was insensitive to N-methylcarbamates.

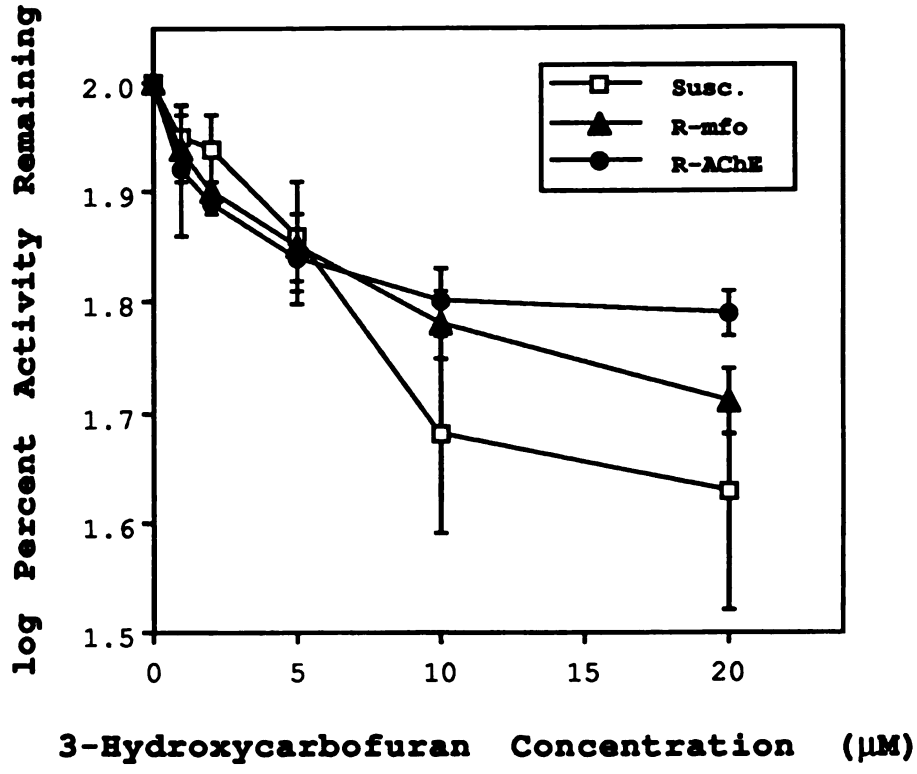


Figure 4. Inhibition of acetylcholinesterases from Colorado potato beetles by 3-hydroxycarbofuran. The enzyme was preincubated with inhibitors for 10 min. The data shown are the mean log percent activity remaining ± SEM n = 4-6.

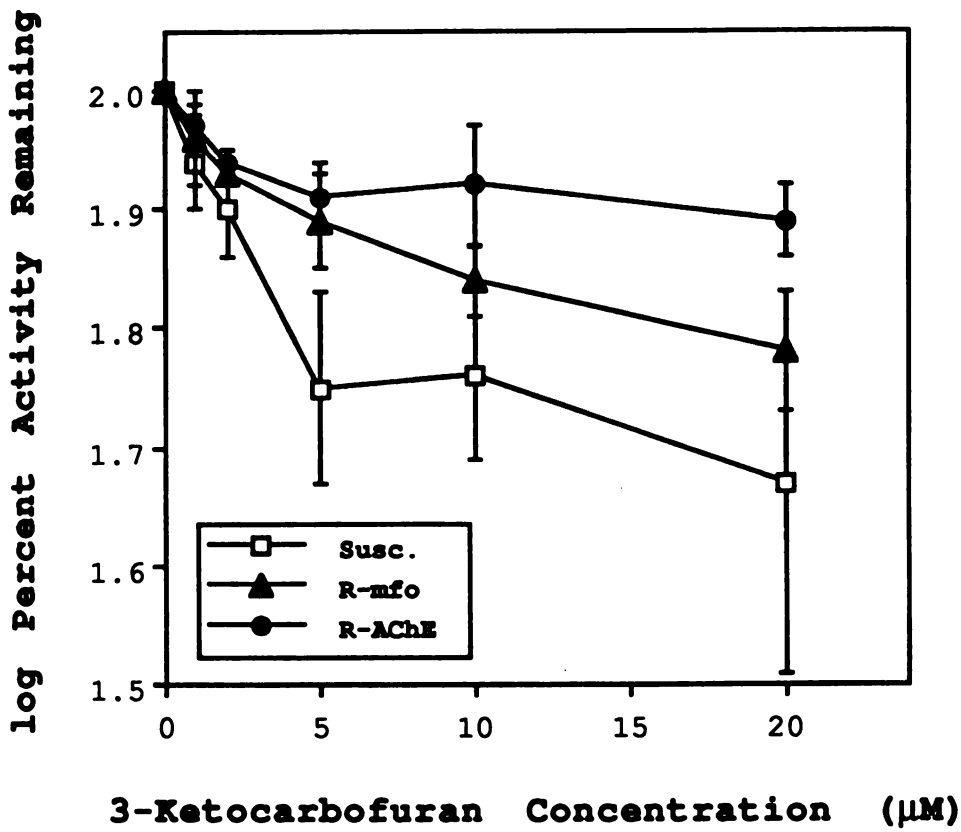


Figure 5. Inhibition of acetylcholinesterases from Colorado potato beetles by 3-ketocarbofuran. The enzyme was preincubated with inhibitors for 10 min. The data shown are the mean log percent activity remaining ± SEM, n = 4-6.

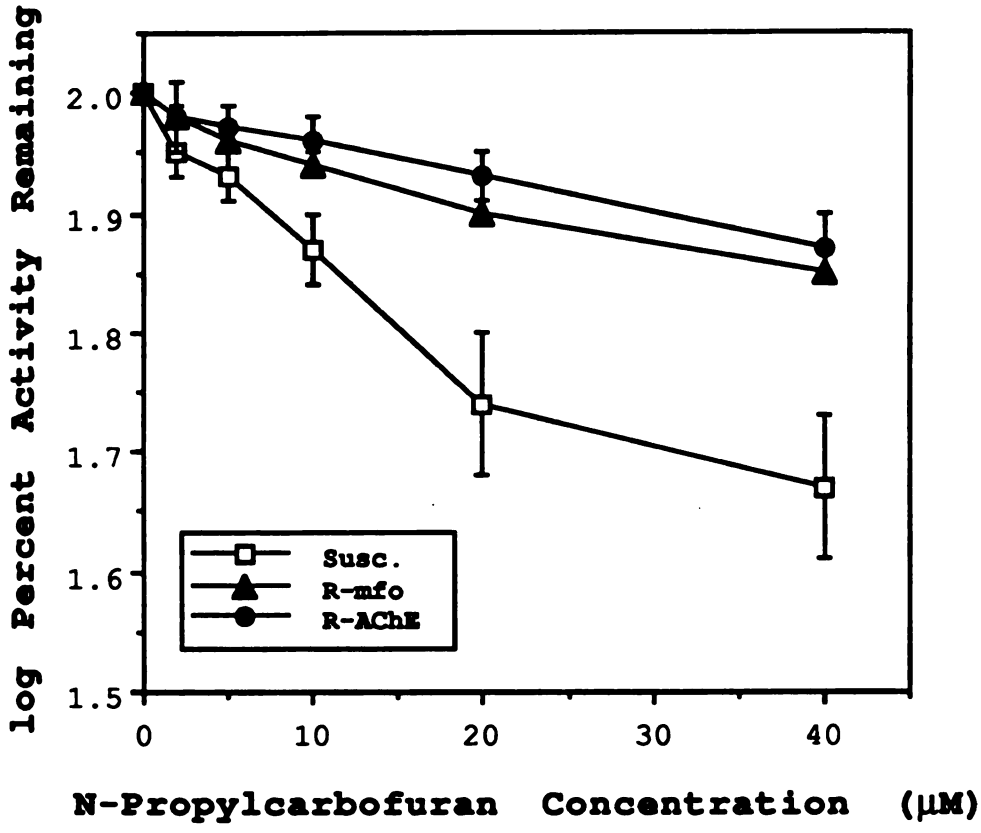


Figure 6. Inhibition of acetylcholinesterases from Colorado potato beetles by N-propylcarbofuran. The enzyme was preincubated with inhibitors for 10 min. The data shown are the mean log percent activity remaining ± SEM, n = 4-6.

The R-AChE strain showed a significantly ($p < 0.05$) lower degree of inhibition by carbaryl than the other strains at concentrations above 20 μM (Figure 7). The R-mfo strain was also less sensitive to carbaryl, but only at the highest dose (80 μM). The concentration-log activity curves were nonlinear. Inhibition of the R-AChE strain was about 35% at concentrations above 30 μM. The R-mfo strain response leveled off at about 60% inhibition. The susceptible response was nonlinear, but did not plateau. The responses of all strains showed carbaryl to be a weaker inhibitor than carbofuran by about 3-fold. This agrees with similar results of Yu et al. (1972) using the housefly and honey bee (*Apis mellifera* (L)).

The propoxur concentration-log activity curves were similar to carbofuran for all strains (Figure 8). The R-mfo and susceptible strains were sensitive to inhibition, and acetylcholinesterase activity leveled off at about 75% inhibition. Acetylcholinesterase from the R-AChE strain showed a high degree of insensitivity, with inhibition not exceeding approximately 25%. This difference was significant ($p < 0.05$) at 40 μM. The potency of propoxur was about half that of carbofuran.

The differences in the concentration-log activity curves for the three strains when eserine was used as the inhibitor (Figure 9) were not significant, but showed a trend. In this case, the R-mfo strain was the least sensitive to inhibition. The strain differences in

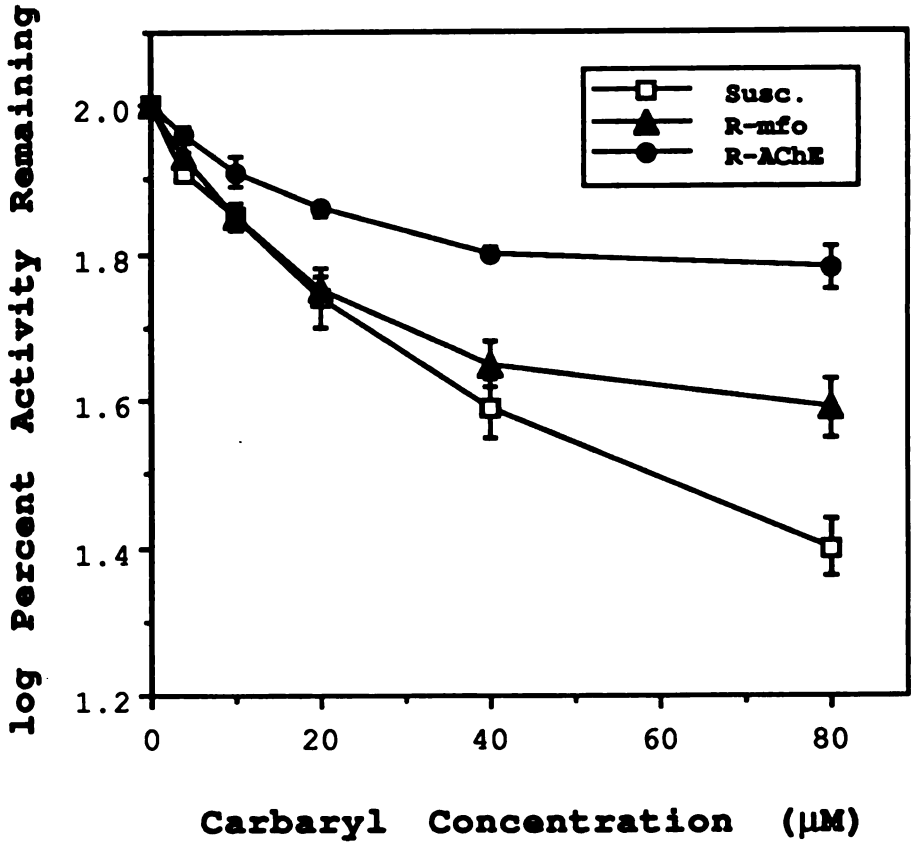


Figure 7. Inhibition of acetylcholinesterases from Colorado potato beetles by carbaryl. The enzyme was preincubated with inhibitors for 10 min. The data shown are the mean log percent activity remaining ± SEM, n = 4-6.

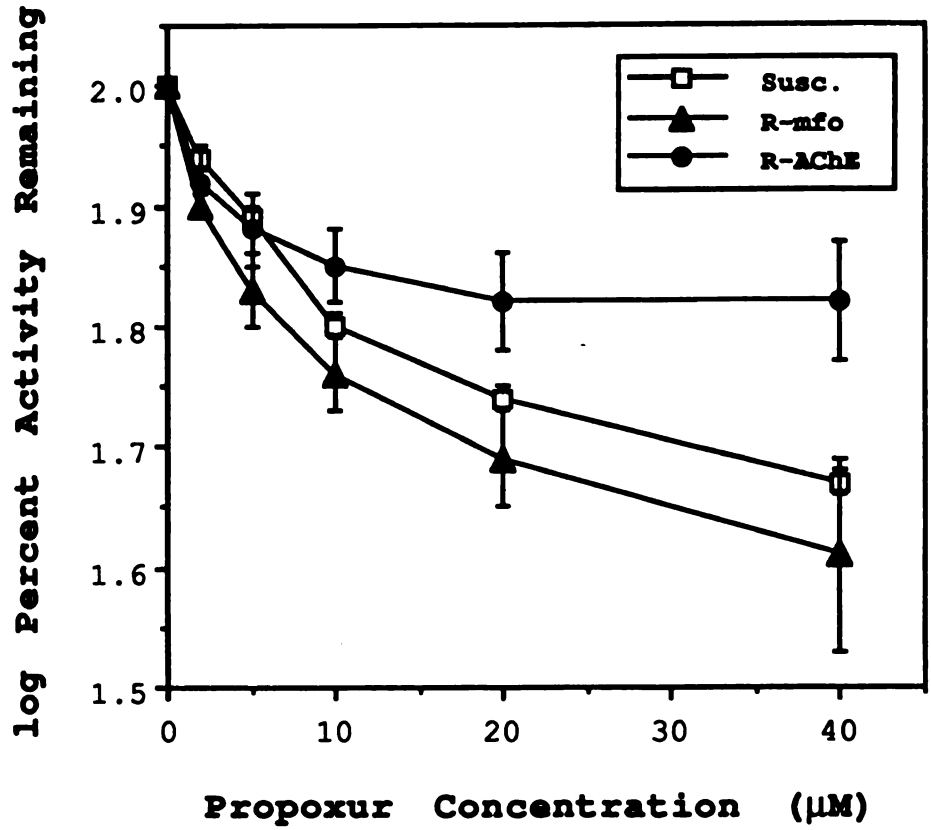


Figure 8. Inhibition of acetylcholinesterases from Colorado potato beetles by propoxur. The enzyme was preincubated with inhibitors for 10 min. The data shown are the mean log percent activity remaining ± SEM, n = 4-6.

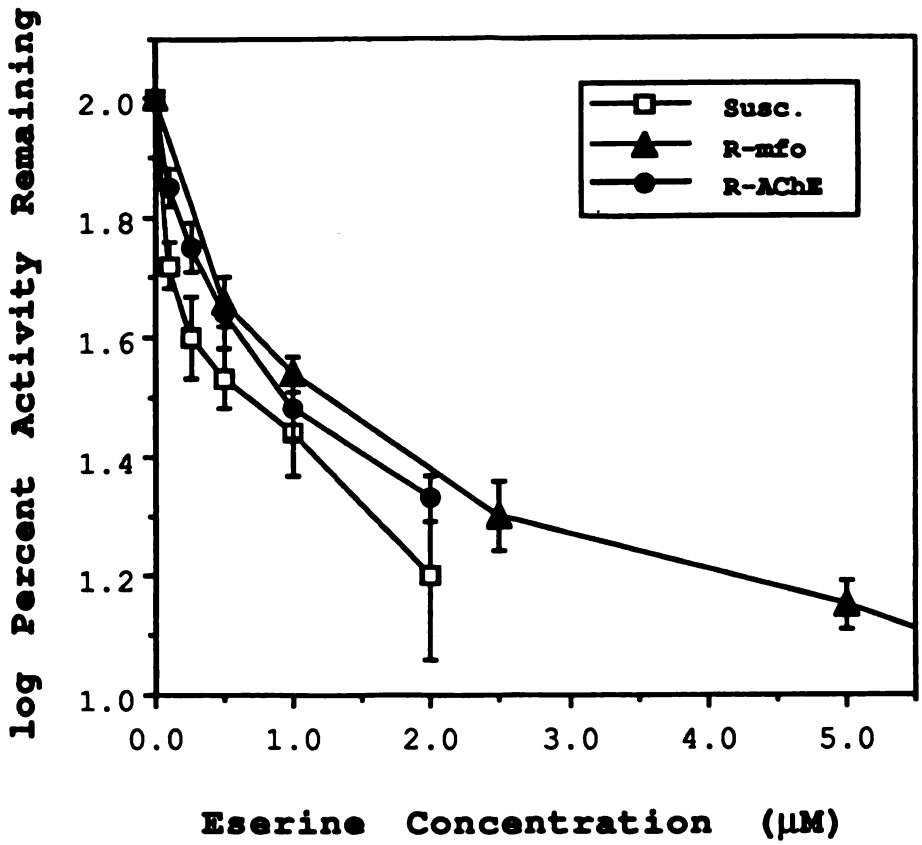


Figure 9. Inhibition of acetylcholinesterases from Colorado potato beetles by eserine. The enzyme was preincubated with inhibitors for 10 min. The data shown are the mean log percent activity remaining ± SEM, n = 4-6.

sensitivity to eserine, although not significant, are interesting considering the role that eserine plays in esterase classification. Because it is a specific acetylcholinesterase inhibitor, inhibition by eserine is a standard criterion for defining enzymatic hydrolysis of acetylthiocholine as being acetylcholinesterase activity (Toutant 1989). Eserine is often used to completely inhibit enzymatic degradation of acetylthiocholine in order to measure non-specific hydrolysis in AChE assays.

The results presented here indicate that there can be differences in sensitivity to eserine between resistant and susceptible strains. A 2- to 3-fold difference in sensitivity was reported in strains of *Drosophila* resistant to eserine (Burnell & Wilkins 1988). In addition, Hawkins and Mendel (1946) have shown significant species differences in AChE sensitivity to eserine. Therefore, caution should be used when defining the activity of altered enzymes using criteria based on sensitivity to standard inhibitors.

Inhibition by Oxime Carbamates

In addition to arylcarbamate inhibitors, two oxime carbamates were tested for their ability to inhibit Colorado potato beetle AChE. There were no significant differences ($p < 0.05$) between strains in the response to aldicarb (Figure 10). The concentration-log activity curve was nearly linear. The potency of aldicarb was substantially less than that of carbofuran, requiring about 40-fold higher concentrations for equivalent inhibition.

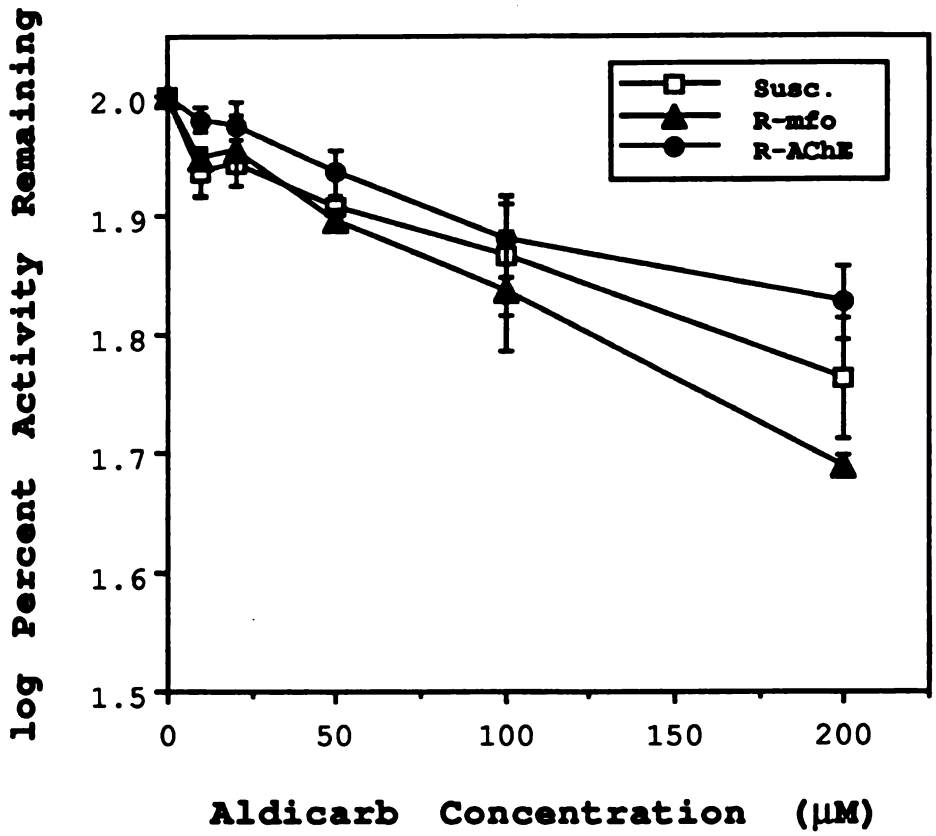


Figure 10. Inhibition of acetylcholinesterases from Colorado potato beetles by aldicarb. The enzyme was preincubated with inhibitors for 10 min. The data shown are the mean log percent activity remaining ± SEM, n = 4-6.

The R-AChE strain showed significant ($p < 0.05$) insensitivity to inhibition by methomyl at concentrations in the 50 to 200 μM range, but not at higher doses due to high variability (Figure 11). The potency of methomyl was very similar to that of aldicarb.

The concentrations of oxime carbamates required for inhibition were much higher than for arylcarbamates for all strains. Although it is difficult to assess the contribution of assay conditions, others have shown the housefly to have much lower IC_{50} (concentration causing 50% inhibition) values (typically 1 to 10 μM; see Payne et al. 1966; Weiden 1971) than found here. Aldicarb sulfoxide and aldicarb sulfone were also tested. They were approximately 35% more potent inhibitors of AChE activity, similar to their higher potency in other measurements of insect AChE.

These results were also different in that the concentration-log activity curves were linear. The fact that these strains were susceptible to aldicarb in bioassay (Ioannidis et al. 1992) indicates that in this case, aldicarb may not have its toxic effect through AChE inhibition. It has been proposed that oxime carbamates may have postsynaptic effects on acetylcholine receptors (Matsumura 1985). This may be an important action of aldicarb in these strains of Colorado potato beetle.

Inhibition by Organophosphates

It was the R-mfo strain which showed a significant ($p < 0.05$) insensitivity to azinphosmethyl oxon above 5 μM

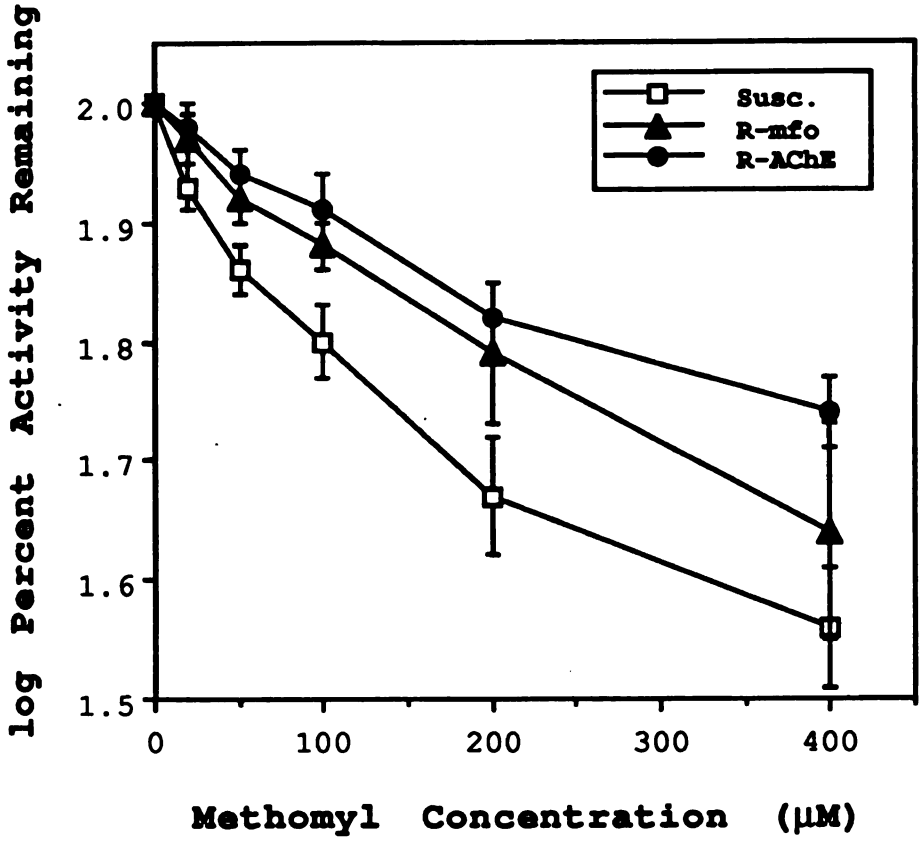


Figure 11. Inhibition of acetylcholinesterases from Colorado potato beetles by methomyl. The enzyme was preincubated with inhibitors for 10 min. The data shown are the mean log percent activity remaining ± SEM, n = 4-6.

(Figure 12). The response of this strain was nonlinear. Inhibition of AChE from the R-mfo strain leveled off at about 80%. The concentration-log activity curves for the R-AChE and susceptible strains were nearly linear, showing potent inhibition by azinphosmethyl oxon.

Both the R-mfo and R-AChE strains were significantly ($p< 0.05$) less inhibited by phosmet oxon between 0.5 and 10 μM (Figure 13). The lack of significance at 10 μM was due to high standard error in the Susc. strain. With all strains, the concentration-log activity curves leveled off. For the R-mfo strain this occurred at about 50% inhibition, for the R-AChE strain at 70% inhibition, and for the susceptible strain, at 80% inhibition.

There were no significant ($p< 0.05$) differences between the acetylcholinesterase activities among the three strains when inhibited by paraoxon (Figure 14). The concentration-log activity curves flattened at about 70% inhibition. The calculated IC_{50} values for all strains (Table 5) were 60- to 90- fold higher than for paraoxon inhibition of housefly head AChE (Hollingworth et. al 1967), possibly indicating some species differences in acetylcholinesterases.

The differences seen in the sensitivity to inhibition of acetylcholinesterase from the R-AChE strain had been hypothesized, but altered acetylcholinesterase in the R-mfo strain was an unexpected result since the synergism ratios with piperonyl butoxide were high, and increased mixed function oxygenase activity had been shown (Ahammadsahib et

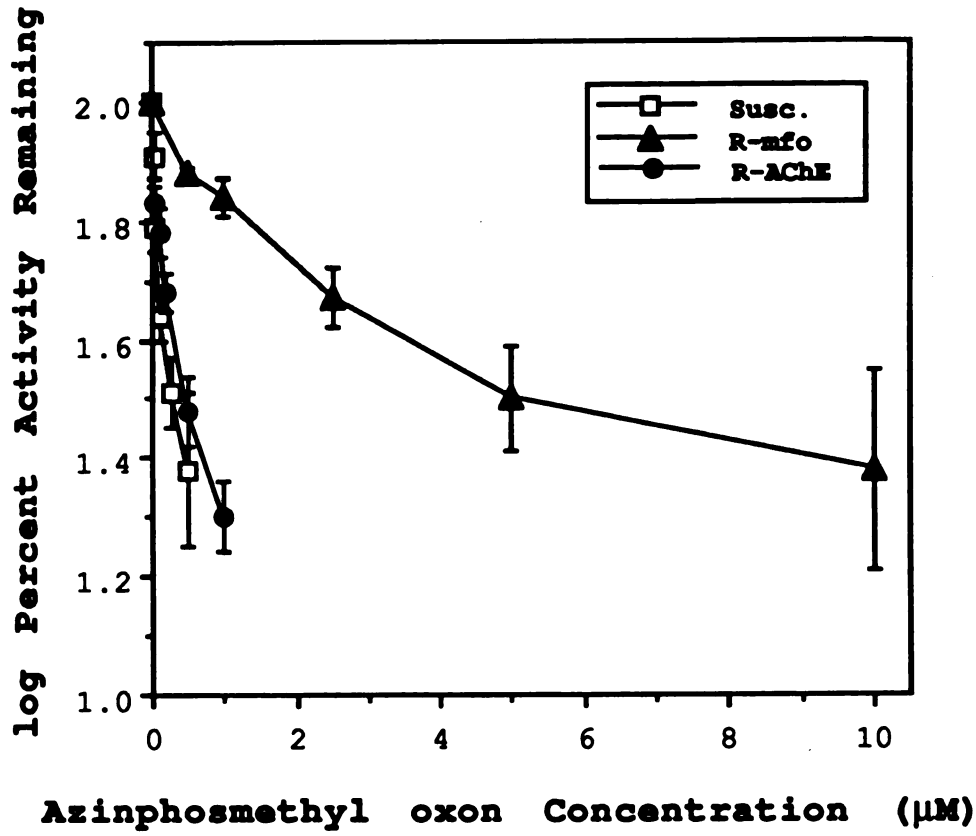


Figure 12. Inhibition of acetylcholinesterases from Colorado potato beetles by azinphosmethyl oxon. The enzyme was preincubated with inhibitors for 10 min. The data shown are the mean log percent activity remaining ± SEM, n = 4-6.

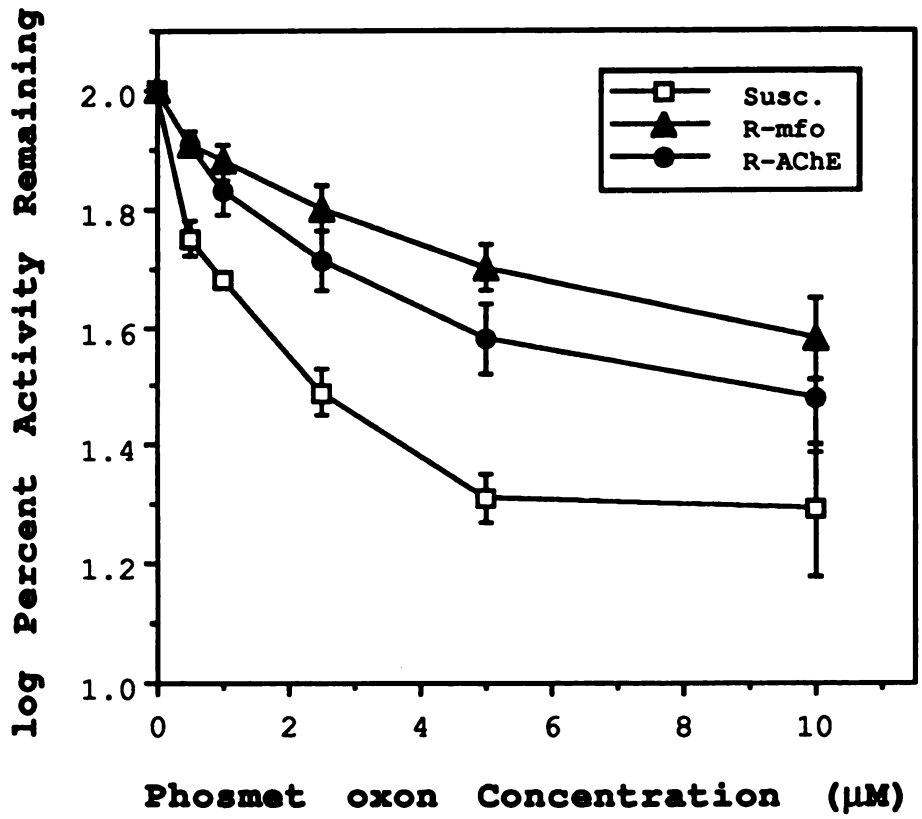


Figure 13. Inhibition of acetylcholinesterases from Colorado potato beetles by phosmet oxon. The enzyme was preincubated with inhibitors for 10 min. The data shown are the mean log percent activity remaining ± SEM, n = 4-6.

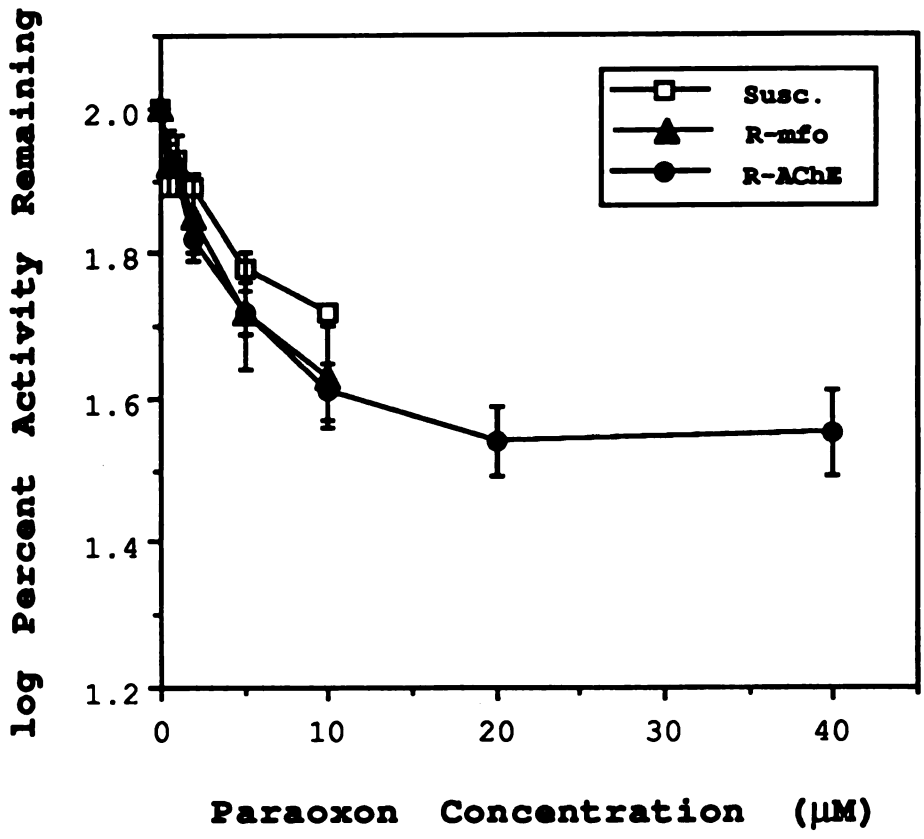


Figure 14. Inhibition of acetylcholinesterases from Colorado potato beetles by paraoxon. The enzyme was preincubated with inhibitors for 10 min. The data shown are the mean log percent activity remaining ± SEM, n = 4-6.

Table 5. IC_{50}[a] Values (μM) of several inhibitors of acetylcholinesterase from different Colorado potato beetle strains.

Inhibitor	Susc.	R-mfo	R-AChE
Arylcarbamates			
Carbofuran	2.0	0.5	>40
3-Hydroxycarbofuran	10	25	>25
3-Ketocarbofuran	18	>20	>>20
N-Propylcarbofuran	32	>>40	>>40
Carbaryl	32	32	>80
Propoxur	30	20	>>40
Eserine	0.2	0.4	2.0
Oxime Carbamates			
Aldicarb	>200	200	>200
Methomyl	180	335	>400
Organophosphates			
Azinphosmethyl oxon	0.1	2.0	0.2
Phosmet oxon	1.0	5.0	3.0
Paraoxon	9.0	6.3	6.1

[a] concentration causing 50% inhibition. Data computed from concentration-log activity curves, n = 4-6.

al. submitted). However, there was a 5-fold lower degree of inhibition by phosmet oxon, and a 20-fold decrease in sensitivity with azinphosmethyl oxon compared to the susceptible strain. Argentine (1991) also showed altered acetylcholinesterase slightly insensitive (2-fold) to azinphosmethyl oxon in a resistant strain of Colorado potato beetle. The azinphosmethyl resistant strain Argentine (1991) studied also exhibited high metabolic resistance.

Despite careful correction for background reactions (e.g. spontaneous hydrolysis of acetylthiocholine), and the lack of general esterase activities in the microsomal fractions, the AChE inhibition curves generally were nonlinear. One explanation for leveling off of the concentration-log activity curves is that there was another enzyme activity present (not acetylcholinesterase) which formed thiol groups in the incubation mixture. This enzyme activity presumably would not have been inhibited by anticholinesterase compounds. However, the amount of activity insensitive to inhibition varied greatly (<20% to 75%) depending on the inhibitor and source of AChE. Another explanation would be rapid reactivation of the carbamylated or phosphorylated enzyme. However, in all cases, the reactions were linear with time, so reactivation was not a factor. The most likely explanation for the shapes of the curves would be the presence of multiple enzyme activities capable of hydrolyzing acetylthiocholine. Efforts to purify

Colorado potato beetle AChE using affinity chromatography have so far been unsuccessful (see Appendix 3).

Without further purification, it was not considered worthwhile (and probably not possible) to derive kinetic parameters for the reactions of inhibitors or substrates with these enzymes. However, I believe that the observed patterns of inhibition can be explained by mixtures of resistant and susceptible isozymes. Very similar inhibition curves were obtained by Devonshire (1975), Yamamoto et. al (1983), Anber and Overmeer (1988), and Bull and Pryor (1990) using mixtures of acetylcholinesterases from susceptible and resistant strains (houseflies, green rice leafhoppers, mites, and houseflies respectively).

Both resistant strains showed major differences in the specificities of their acetylcholinesterases (Table 5). There were two distinct patterns. The R-AChE strain was insensitive to carbamates, and the R-mfo strain was insensitive to organophosphates. Each strain had highest insensitivity to the insecticide used to select for resistance (R-AChE: carbofuran; R-mfo: azinphosmethyl). The degree of cross-resistance provided by altered AChE in these cases appears to be limited.

Several investigators have examined the spectrum of cross-resistance with altered acetylcholinesterases. Roulston et. al (1968) showed that the ratios for inhibition of resistant and susceptible forms of AChE (R/S ratio) varied from about 0.6 to 63, depending on the inhibitor

used. In houseflies, Devonshire (1975) and Devonshire and Moores (1984) showed R/S ratios of 4 to 43, and Tripathi (1976) reported R/S ratios of 7 to 206. Similar large variations in sensitivity have been shown with *Anopheles albimanus* (Weidemann) (Ayad and Georghiou 1975) and *C. pipiens* mosquitoes (Tang et al. 1991), mites (Anber and Overmeer 1988), and H. virescens (Brown and Bryson 1991).

The spectrum of responses to inhibitors by the carbofuran resistant strains indicate that there are major differences in the alterations which give target-site insensitivity. The hypothesis that there are at least two forms of the enzyme present in each of these strains leads to two possible genetic bases for altered acetylcholinesterases: heterozygosity and gene duplication. It is possible that each of the strains is heterozygous, having one allele for altered acetylcholinesterase, and one for the normal enzyme. These alleles would have to be expressed at different levels in the different strains. Genetic studies with the R-AChE strain by Ioannidis (1990) showed that resistance was incompletely dominant with nearly perfect Mendelian ratios in crosses with susceptibles. Along with the fact that the strain was been inbred under high selection pressure for over four years, the evidence indicates that the strain is homozygous for the resistance factor.

The second possibility is gene duplication, a mechanism known to exist for esterases in aphids (Devonshire and

Sawicki 1979). Fournier (D. Fournier, INRA, Antibes, France, personal communication) recently showed that gene duplication resulted in increased AChE levels. The higher amounts of endogenous acetylcholinesterase protected *Drosophila* from mortality after exposure to cholinesterase inhibitors. Similarly, the resistant Colorado potato beetles strains tested here may have undergone a gene duplication event in which one gene subsequently produced an altered isozyme for AChE.

Several years ago, Oppenoorth (1982) suggested that the two modalities of altered acetylcholinesterase which he observed in resistant houseflies could be due to different changes in the enzyme molecule. Using site-directed mutagenesis, Mutero et al. (1992) have recently presented compelling evidence that a single amino acid change in *Drosophila* AChE causes reduced sensitivity to inhibitors, and that different amino acid substitutions resulted in different spectra of sensitivities to inhibitors. For example, replacement of tyrosine 109 by aspartate resulted in a 3-fold insensitivity to the carbamate propoxur, but no change in malaoxon inhibition or the K_m of the enzyme for acetylcholine. Other substitutions for tyrosine 109 gave up to 6-fold decreases in sensitivity, (Mutero et al., 1992). So it is possible that small alterations in the target-site (even a single amino acid) can cause large changes in sensitivity with limited cross-resistance, a pattern clearly demonstrated in this study (Table 5).

Wide variations in the specificities of altered acetylcholinesterases result in added complexity for monitoring and decision-making in resistance management programs. It is unclear how many mutant forms of AChE are viable in the Colorado potato beetle in a field environment, but clearly more than one form of acetylcholinesterase is present in the strains tested here. The observation that each of the strains developed a mutant acetylcholinesterase adapted particularly to a single selection agent raises the possibility of other mutant forms developing from different selecting agents (see Appendix 2.). The low degree of cross-resistance provided by this mechanism of insecticide resistance leaves the producer with options to control some resistant pests. However, extensive monitoring and testing will be required to determine which pesticide options will be effective.

CHAPTER 4

METABOLIC RESISTANCE

INTRODUCTION

Increased metabolic degradation of insecticides is the most frequently reported mechanism of resistance (Oppenoorth, 1985). Many aspects of metabolic resistance have been investigated, especially in houseflies. As discussed in Chapter 1, the enzymes involved in metabolic resistance include three major families: mixed function oxygenases, glutathione-S-transferases, and esterases.

While the Colorado potato beetle is notorious for its ability to develop resistance to insecticides, the mechanisms of its resistance have not been well-characterized. Argentine (1991) showed increased mixed function oxygenase and arylesterase activity in azinphosmethyl- and permethrin-resistant Colorado potato beetles. Members of the Colorado potato beetle working group at Michigan State University have shown that the R-mfo strain has increased mixed function oxygenase activity (K. Ahammadsahib, Michigan State University, personal communication).

To understand the basis for the observed resistance to carbofuran in potato beetles, it is important to determine the contribution of metabolic resistance mechanisms. The goal was to compare the activity of esterases, glutathione-S-transferases, and mixed function oxygenases in the Susc., R-mfo, and R-AChE strains. In addition, the rate of in vivo metabolism of carbofuran was compared in these strains to

determine if there were any differences in the metabolites formed.

MATERIALS AND METHODS

Chemicals

Carbofuran and carbon-14 labeled carbofuran (ring-labeled, 39.4 mCi/mmole, 0.45 mM, >98% purity) were gifts from the FMC Corporation (Princeton NJ), and azinphosmethyl was a gift of Mobay Chemical Co. (Kansas City MO). Pentoxyresorufin and ethoxyresorufin were obtained from Molecular Probes Inc. (Eugene OR). All other chemicals were obtained from Sigma Chemical Co. (St. Louis MO).

Arylesterase Assays

The heads and thoraces were removed from adult beetles, pooled in 0.05 M phosphate buffer (pH = 7.4) and homogenized on ice (3 x 10 s) at full power with a Vertishear (Vertis Co. Gardiner NY) using the micro-fine generator. The brei was centrifuged at 11,000 x *g* for 20 min (4°C), and the resulting supernatant (ca. 1 beetle equivalent per ml) was used in the assays. This fraction was kept on ice until used in the assay.

Pesticides were dissolved in ethanol (10 mM), then diluted (100- to 500-fold) with phosphate buffer. Control assays were carried out with equivalent concentrations of ethanol alone. Alpha- or beta-naphthylacetate was dissolved in acetone (6 mg/ml), then diluted 100-fold with phosphate buffer. Arylesterase activity was assayed by method of Van

Asperen (1962). Briefly, 20 µl of supernatant (25 to 40 µg protein) was added to α- or β-naphthylacetate (final concentration 65 µM). In cases where pesticides were included in the reaction mixture, 100 µl pesticide (1 mM carbofuran or 100 µM azinphosmethyl oxon) was preincubated for 10 min at 30°C before adding naphthylacetate. The reaction volume was 1 ml. Following a 10 min incubation of substrate and enzyme, 100 µl of Fast Blue BB and sodium dodecyl sulfate (3 mg/ml and 7.5 mg/ml respectively in water) was added to stop the reaction and to detect the naphthol formed. After 10 minutes, the absorbance at 600 nm (α-naphthylacetate) or 550 nm (β-naphthylacetate) was measured with a Shimadzu UV-265 spectrophotometer (Kyoto Japan). The amount of naphthol formed was determined using standard curves.

Glutathione-S-Transferase Assays

Colorado potato beetle homogenates were prepared as above. Glutathione-S-transferase activity was assayed by the method of Habig et al. (1974). Reduced glutathione (10 mM in buffer, 100 µl) was added to supernatant (100 µl, 130 to 175 µg protein). Either chlorodinitrobenzene (CDNB) or dichloronitrobenzene (DCNB) (100 µl, 10 mM in 10% ethanol) was used as the substrate. Controls were without supernatant. Buffer was added to bring the reaction volume to 1 ml, and the reaction mixture was incubated for 10 min at 30°C. The absorbance at 340 nm (CDNB) or 344 nm (DCNB) was measured. The amount of substrate conjugated was

calculated based on the extinction coefficient of 9600 $M^{-1}cm^{-1}$ (CDNB) and 8500 $M^{-1}cm^{-1}$ (DCNB) (Habig et al. 1974).

Microsomal O-Dealkylase Assays

Microsomal O-dealkylase activity was assayed using the method of Mayer et al. (1977). Abdomens from 30 adult Colorado potato beetles were ground with mortar and pestle on ice with 50 mM phosphate buffer (pH = 7.4) including bovine serum albumin (0.25 mg/ml). The brei was centrifuged at 11,000 x *g* for 10 min (4°C) and the supernatant was centrifuged at 100,000 x *g* for 1 hour (4°C). The 100,000 x *g* pellet was resuspended (30 beetle equivalents per ml) by very gentle agitation with a small paintbrush. This preparation resulted in about 60% intact P450 (40% P420) as determined by difference spectra, using extinction coefficients of 91 mM^{-1} cm^{-1} for absorbance differences between 450 and 490 nm and 110 mM^{-1} cm^{-1} for differences between 490 and 420 nm (Omura and Sato 1964).

The reaction mixture included 0.5 ml microsomes, 10 µl pentoxyresorufin or ethoxyresorufin (2 mM in ethanol), 1 µmole NADPH, and phosphate buffer in a total volume 2 ml. The reaction was measured for 10 min with a Perkin-Elmer (Norwalk CT) LS-5B fluorescence spectrometer with a 1 cm pathlength. The excitation wavelength was 530 nm and the emission wavelength was 585 nm (Burke et al. 1985). Both excitation and emission slits were set at 10 nm. The response was linear for over 10 min, and data between 2 and 8 min were used to determine the rate of O-dealkylase

activity based on a standard curve using resorufin (Mayer et al. 1977).

In Vivo Metabolism

The posterior portions of the right elytra of adult potato beetles were removed to expose the dorsal abdomen. Using a fine glass needle, 1 µg of carbon-14 labeled carbofuran (43.2 mCi/mmole, 1 mg/ml in ethanol) was injected into the dorsal abdomen. The beetles were then immobilized briefly to allow the wound to close. The injected beetles were held in 36 ml centrifuge tubes (5 per tube) at room temperature. After 2 to 8 hours, 2.5 ml acetonitrile/water (3:1) was added, and the tube vortexed for 1 min. The beetles were homogenized in the 2.5 ml acetonitrile/water with the Vertishear at power setting 60 for 30 s. The brei was centrifuged at 11,000 x *g* for 10 min (4°C). The supernatant was filtered with a 0.2 micron nylon filter (13mm Acrodisc, Gelman Sciences, Ann Arbor MI) and analyzed by HPLC. The sample was injected with a Rheodyne 7125 injector (Rheodyne Inc., Cotati CA) fitted with a 50 µl sample loop. The HPLC system also included a Waters (Milford MA) model 501 Pump, a 10 cm RP-18 column (Spheri-5, Brownlee Labs, Santa Clara CA) and a Flow One/Beta radiometric detector (Radiomatic Instrument and Chemical Co., Meriden CT). The mobile phase consisted of acetonitrile/water (1:3) delivered at 1 ml/min. The scintillation cocktail was Flow Scint III (Radiomatic Instrument and Chemical Co.) delivered at 4 ml/min.

Counting efficiency was 74%. Peak areas were integrated using Radiomatic Flow-One/Beta software. Retention times were compared against external standards for carbofuran, 3-hydroxycarbofuran, 3-ketocarbofuran, and carbofuran phenol. Recovery of injected carbofuran averaged 94% across all time points.

For all enzyme assays, protein determinations were made using a modified Lowry method (Guegenrich 1984). Results were tested for significance at the 95% level with Student's t-test.

RESULTS AND DISCUSSION

Arylesterase Assays

Both α- and β-naphthylacetate were used to assay arylesterase activities. In addition to measuring total esterase activity, true arylesterase activity was determined by measuring activity in the presence of 10 μM organophosphate (azinphosmethyl oxon). This dose would inhibit any aliesterase (aliphatic esterase) activity present (Van Asperen and Oppenoorth 1960), and allow determination of arylesterase activity only, since these A-type esterases are insensitive to organophosphates. The effect of carbofuran on enzyme activity was also tested. Arylesterase activity with α-naphthylacetate as the substrate was 337 pmole/mg protein/10 min in the susceptible strain, compared with 284 and 413 pmole/mg protein/10 min in the R-mfo and R-AChE strains respectively (Table 6).

Table 6. Esterase activity (nmole/mg protein/10 min)[a] in Colorado potato beetle homogenates using α-naphthylacetate as the substrate .

	Susceptible	R-mfo	R-AChE
Untreated	611 ± 56	519 ± 47	863 ± 89[b]
Control	479 ± 42	476 ± 22	686 ± 86
Carbofuran[c]	535 ± 69	374 ± 33[d]	676 ± 90
Azinphosmethyl oxon[c]	337 ± 28[d]	284 ± 38[d]	413 ± 78[d]

[a]data are the mean ± SEM, duplicate assays, n = 5-6; control assays included 0.2% acetone.

[b]significantly different from susceptible ($p < 0.05$)

[c]Carbofuran at 100 μM; azinphosmethyl oxon at 10 μM.

[d]significantly different from control ($p < 0.05$)

Carbofuran (100 μM) caused a decrease in activity in the R-mfo strain which could be due to competition with the substrate α-naphthylacetate.

The R-AChE strain had slightly (ca. 25%) higher total esterase activity. Arylesterase activity was nearly the same with β-naphthylacetate as the substrate (Table 7). In the presence of carbofuran, both the susceptible and R-mfo strains showed a significant decrease in activity, perhaps due to competitive inhibition. As both tables show, there are few differences between strains in these hydrolytic enzymes. Although the R-AChE strain may have slightly higher total esterase activity (ca. 25%), the relevance to the observed levels of resistance is questionable.

The esterase assay results were about 50% lower than those of Argentine (1991), who used abdominal homogenates. In a single test, using abdominal homogenates, arylesterase activity was almost identical with that reported by Argentine (1991). Argentine showed no difference in arylesterase activity between his susceptible and azinphosmethyl-resistant strain, but showed 25 to 50% higher arylesterase activity in permethrin- and abamectin-resistant strains.

It is sometimes difficult to compare esterase assay results with other reports because the nomenclature is somewhat confusing, and investigators often include inhibitors to isolate various esterase activities. Thus it can be difficult to distinguish between aliesterase,

Table 7. Esterase activity (nmole/mg protein/10 min)[a] in Colorado potato beetle homogenates using β-naphthylacetate as the substrate .

	Susceptible	R-mfo	R-AChE
Untreated	593 ± 26	666 ± 67	721 ± 99
Control	646 ± 41	629 ± 53	847 ± 29[b]
Carbofuran[c]	443 ± 71[d]	434 ± 35[d]	712 ± 99
Azinphosmethyl oxon[c]	388 ± 49[d]	266 ± 20[d]	591 ± 91[d]

[a]data are the mean ± SEM, duplicate assays, n = 5-6; control assays included 0.2% acetone.

[b]significantly different from susceptible ($p < 0.05$)

[c]carbofuran at 100 μM; azinphosmethyl oxon at 10 μM.

[d]significantly different from control ($p < 0.05$)

arylesterase, carboxylesterase and general esterase activities. Indeed, it may not even be correct to attempt to isolate these activities as it is unclear that they are due to separate enzymes. Had there been significant differences in total esterase activity, there would have been more impetus to investigate the various modalities of esterase activity in each strain further.

Glutathione-S-Transferase Assays

Activity was assayed using two substrates which distinguish the two major classes of glutathione-S-transferases (Clark et al. 1984). With CDNB as the substrate, glutathione-S-transferase activity was about 350 nmole/mg protein/10 min in all three strains (Table 8). Using DCNB, activity was about 10 nmole/mg protein/10 min. These values are about 50% lower than the values reported by Argentine (1991) with abdominal homogenates. The values are similar to those found in *Spodoptera frugiperda* (Yu 1984). In a single test with abdominal homogenates, there was no difference in activity with CDNB compared to head and thorax homogenates. While there was about an 8-fold increase in activity with DCNB compared to head and thorax homogenates. As shown in Table 8, neither carbofuran nor azinphosmethyl had any effect on glutathione-S-transferase activity using chlorodinitrobenzene as the substrate.

Mixed Function Oxygenase Assays

The preparation of viable microsomes from Colorado potato beetle abdomens proved to be rather difficult.

Table 8. Glutathione-S-transferase activity (nmole/mg protein/10 min)[a] in Colorado potato beetle homogenates.

Substrate	Susc.	R-mfo	R-AChE
Chlorodinitrobenzene	361 ± 28	367 ± 40	343 ± 35
+ Carbofuran[b]	357 ± 20	355 ± 11	343 ± 15
+ Azinphosmethyl oxon[b]	353 ± 23	366 ± 25	294 ± 42
Dichloronitrobenzene	14 ± 1	10 ± 2	6 ± 2

[a]data are the mean ± SEM, duplicate assays, n = 5-6; control assays included 0.2% acetone and substrate.

[b]carbofuran at 100 μM; azinphosmethyl oxon at 10 μM.

[c]significantly different from susceptible ($p < 0.05$)

[d]significantly different from control ($p < 0.05$)

Because others also had difficulties with the preparation (Rose and Brindley 1985, J. Argentine, University of California, Irvine, personal communication) several different procedures were attempted, based on published techniques (e.g. Hung and Sun 1989; Yu 1991; Argentine 1991). Crude homogenates, isolated tissue homogenates, and a variety of homogenization buffers and techniques were used without success. However, by using a mortar and pestle to homogenize the abdomens (K. Ahammadsahib, Michigan State University, personal communication), viable microsomes were produced, showing mixed function oxygenase activity. It is likely that the microsomes are unusually sensitive to mechanical stress. For example, any microsome preparations which were vortexed had no mixed function oxygenase activity.

Determinations of P450 levels in each strain showed the R-mfo strain to have a significantly lower concentration of P450 ($p < 0.05$) (Table 9.). Figure 15 shows a representative difference spectrum. Normally about 60% of the cytochrome P450 was viable, as has been reported by Argentine (1991) for his preparations. The relatively low survival of P450 in these preparations introduced some uncertainty into the enzyme measurements. p-Nitroanisole-O-demethylase assays (Kinoshita et al. 1966) were used to assess the viability of initial microsomal preparations. The resulting rates of 24 nmole/mg protein/min were very close to those of

Table 9. Concentrations of cytochrome P450 and P420 (μmole/mg protein) from different strains of Colorado potato beetles.

Strain	P420	P450	% P450
Susc.	1.93 ± 0.26	2.56 ± 0.51	57
R-mfo	0.60 ± 0.30[a]	1.28 ± 0.54[a]	68
R-AChE	0.84 ± 0.20[a]	2.09 ± 0.97	71

[a]significantly different from Susc. ($p < 0.05$).

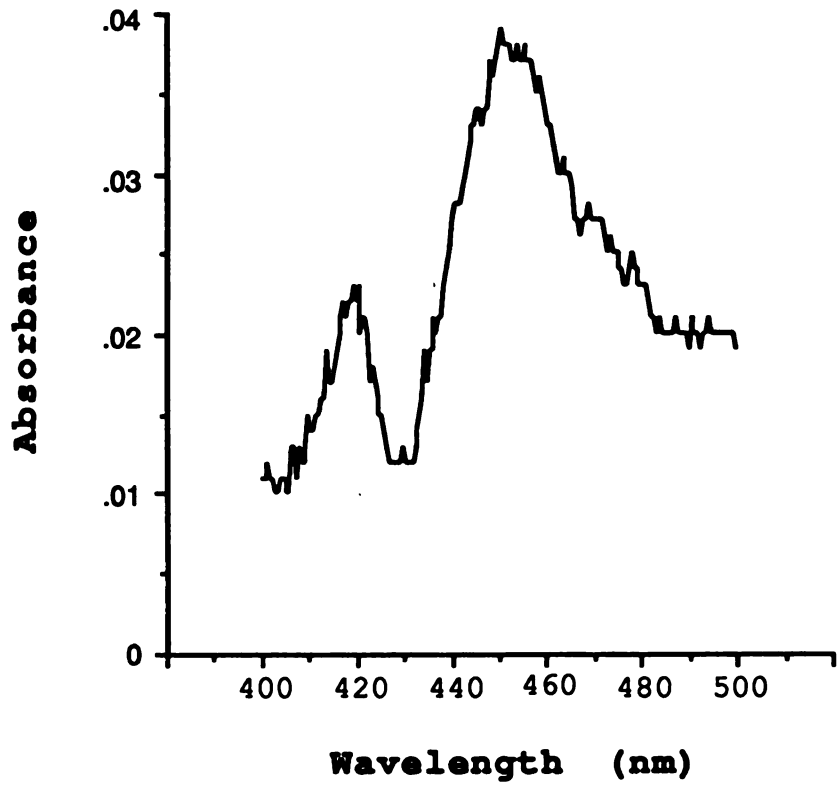


Figure 15. Representative P450 difference spectrum from the R-mfo strain.

others (25 nmole/mg protein/min; K. Ahammadsahib, Michigan State University, personal communication). In turn, this is reasonably typical of rates seen with microsomal preparations from other insects (Ahammadsahib et al. submitted).

Because the P450 enzymes assayed were not purified and studied extensively, I will refer to what is now known generally as type I and type II cytochrome P450s. As mentioned earlier, these correspond to P448 (3-methylcholanthrene-induced) and P450 (phenobarbitol-induced) respectively (Nebert et al. 1987). The two assays used to measure O-dealkylase activity distinguish between type I and type II in vertebrates. Pentoxyresorufin-O-dealkylase (PROD) activity is indicative of type II, and ethoxyresorufin-O-dealkylase (EROD) activity indicative of type I MFO activity (Burke et al. 1985).

Both resistant strains had higher mixed function oxidase activity. The results show that the R-mfo strain has about 2-fold higher EROD and PROD activities on a protein basis, and about 4-fold higher levels when expressed on a cytochrome basis (Figure 16). The R-AChE strain had elevated EROD of about 1.5-fold. Values for the Susc. strain were 0.153 ± 0.006 pmole/mg protein/min and 0.60 ± 0.002 pmole/μmole P450/min for EROD, and 0.405 ± 0.020 pmole/mg protein/min and 0.158 ± 0.008 pmole/μmole P450/min for PROD.

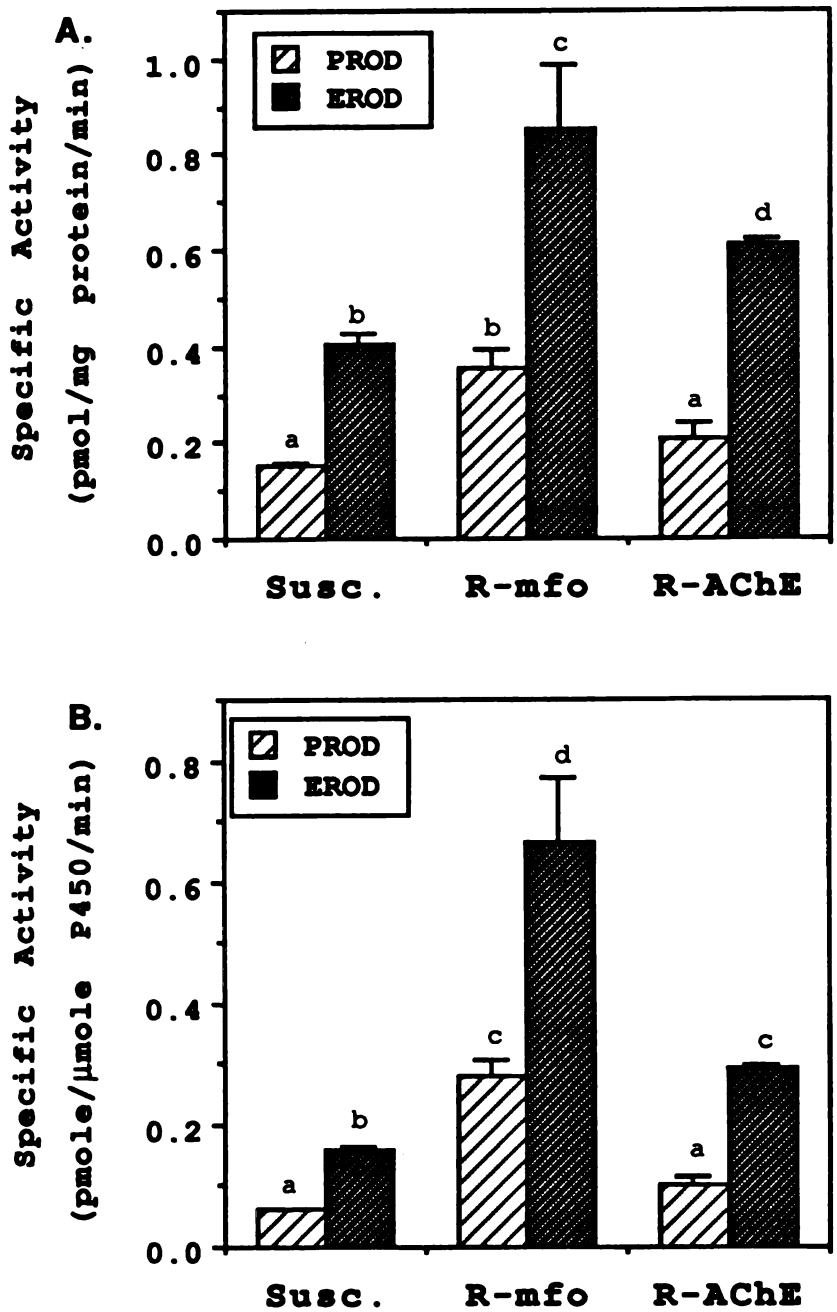


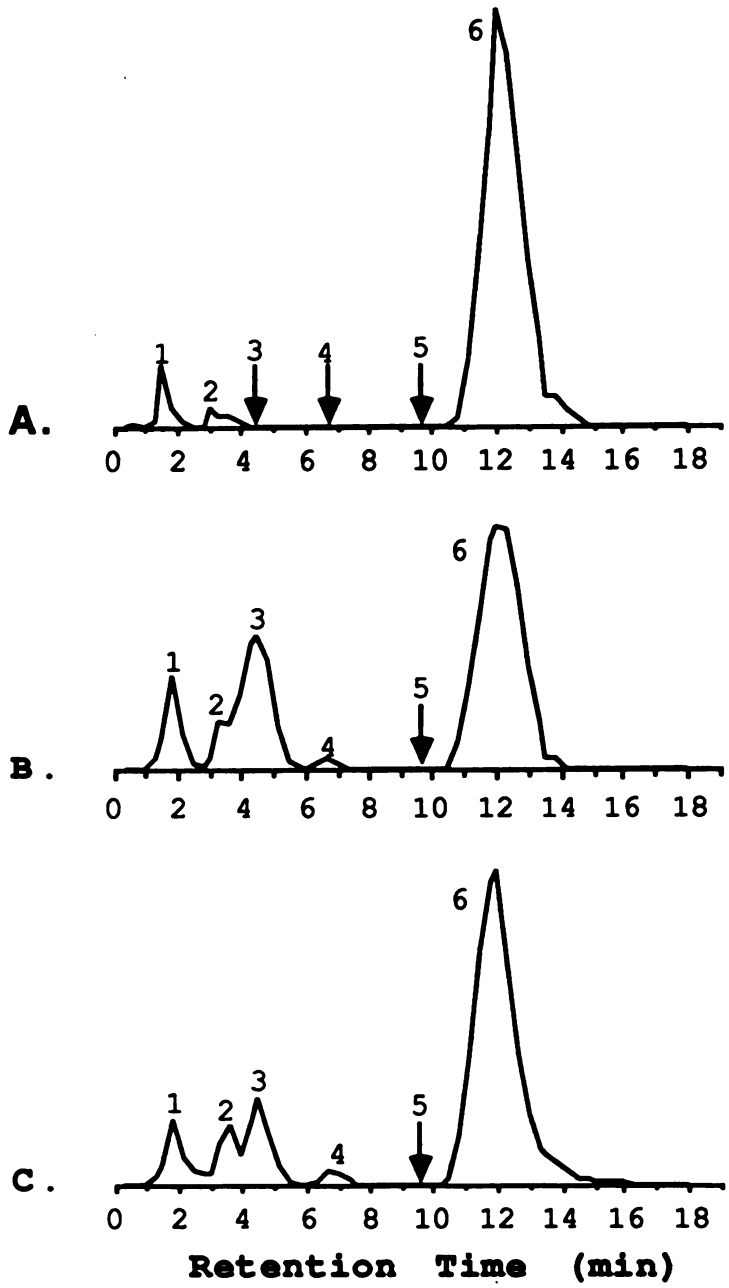
Figure 16. Ethoxyresorufin-O-dealkylase (EROD) and pentoxyresorufin-O-dealkylase (PROD) activity in Colorado potato beetle microsomes expressed on a protein (A) or cytochrome P450 (B) basis. Data shown are the mean ± SEM, n = 3-4. Values with different letters are significantly different ($p < 0.05$).

It is interesting that the R-mfo strain had lower concentrations of P450 in the microsomes, but higher EROD and PROD activity. This indicates that there is a qualitative change in these enzymes which may be responsible for resistance.

These results confirmed earlier unpublished work with the R-mfo strain, and the hypothesis that the R-mfo strain had increased mixed function oxygenase activity. The elevated levels of EROD in the R-AChE strain may relate to the slight degree of synergism provided by piperonyl butoxide in this strain (synergism ratios were 1.3 to 1.6). Increased metabolism can significantly enhance the protection afforded by altered target-sites, and the two mechanisms are often associated (Oppenoorth 1985).

In Vivo Metabolism of Carbofuran

To assess the overall rate of metabolism, and to get a picture of the type of metabolites formed, metabolism of carbon-14 labeled carbofuran in vivo was examined by HPLC. The conversion of carbofuran to more polar metabolites was measured to determine the rate of metabolism (Figure 17). Three of the five major peaks from the in vivo assays corresponded to standard compounds. The chromatograms showed peaks that co-chromatographed with 3-hydroxycarbofuran, 3-ketocarbofuran, and carbofuran. Carbofuran phenol was not detected. One peak was more polar than 3-hydroxycarbofuran and could have been a conjugate of carbofuran or its metabolites. The metabolites seen in vivo



Peak 1 = unknown (conjugate?)
Peak 2 = 3-ketocarbofuran
Peak 3 = 3-hydroxycarbofuran
Peak 4 = unknown
Peak 5 = carbofuran phenol
Peak 6 = carbofuran

Figure 17. Chromatograms from in vivo metabolism of carbofuran in different strains of Colorado potato beetle (4 hours post-injection). A. Susc., B. R-mfo, and C. R-AChE.

correspond with the oxidative degradation of carbofuran by the pathway described by Metcalf et al. (1968) in houseflies and depicted in Figure 18.

The relative rates of metabolism in each strain were consistent with the results of the in vitro mixed function oxidase assays. The rate of carbofuran metabolism in vivo in the susceptible strain was 19 ng/h. The rate for the R-AChE strain was 1.2-fold higher (Figure 19), comparable with its 1.5- to 2-fold increase in EROD activity in vitro. The R-mfo strain exhibited a 4-fold increased rate of carbofuran metabolism in vivo. This corresponded to the 2- to 4-fold increases in both EROD and PROD. Based on Figure 19, it is likely that the overall rate of metabolism is nonlinear in the R-AChE and Susc. strains, especially during time periods less than two hours after injection. However, after two hours, the process appears to be fairly linear with good correlations to straight lines (r^2 = 0.80 to 0.98).

There was little or no difference between strains in the non-oxidative metabolic enzymes measured. The relationships between in vivo carbofuran metabolism and in vitro O-dealkylase activity are strong indications that mixed function oxygenases are important in carbofuran resistance, especially for the R-mfo strain.

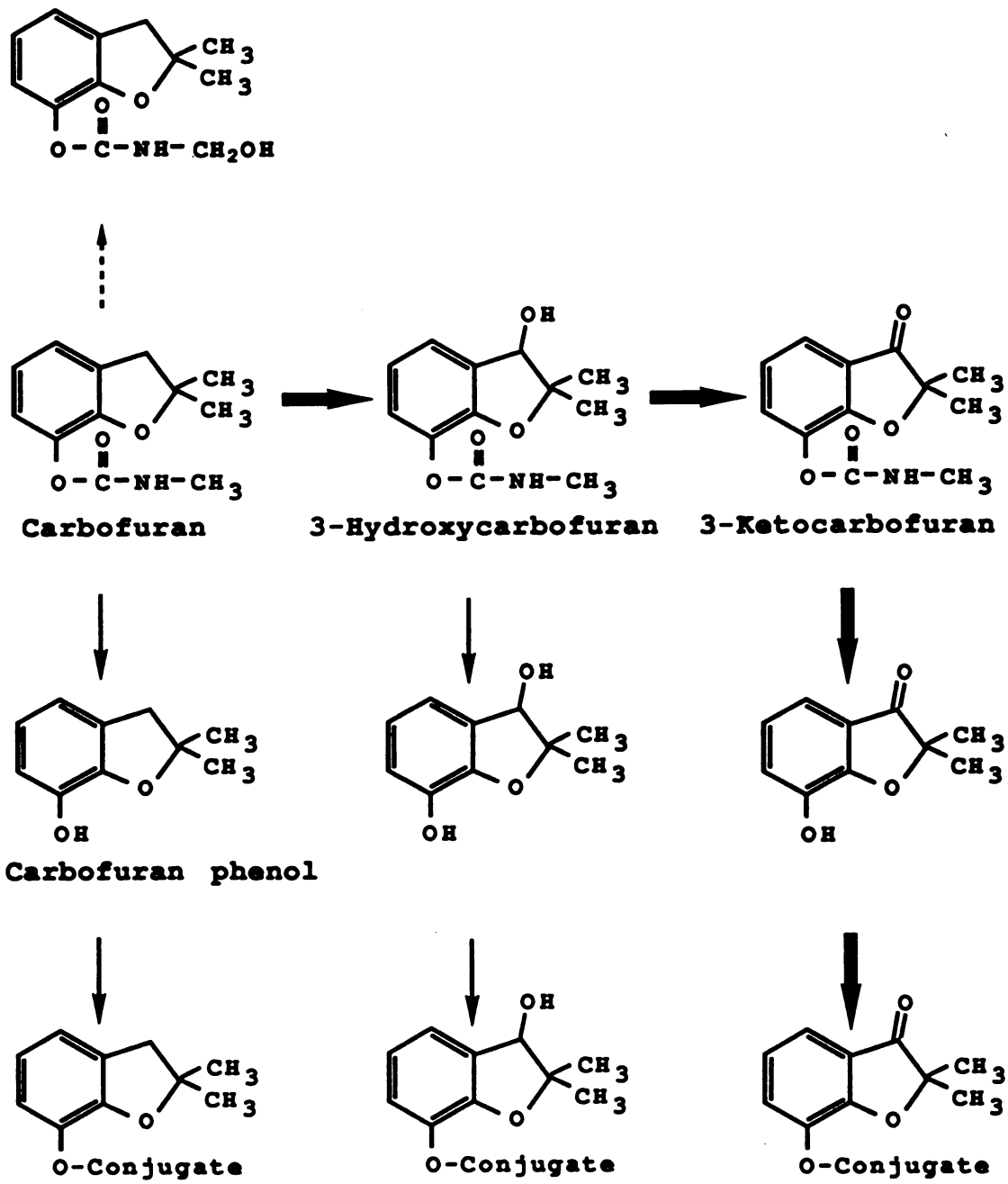


Figure 18. Metabolic pathway of carbofuran. Heavy lines indicate major route of metabolism in insects (from Metcalf et al. 1968).

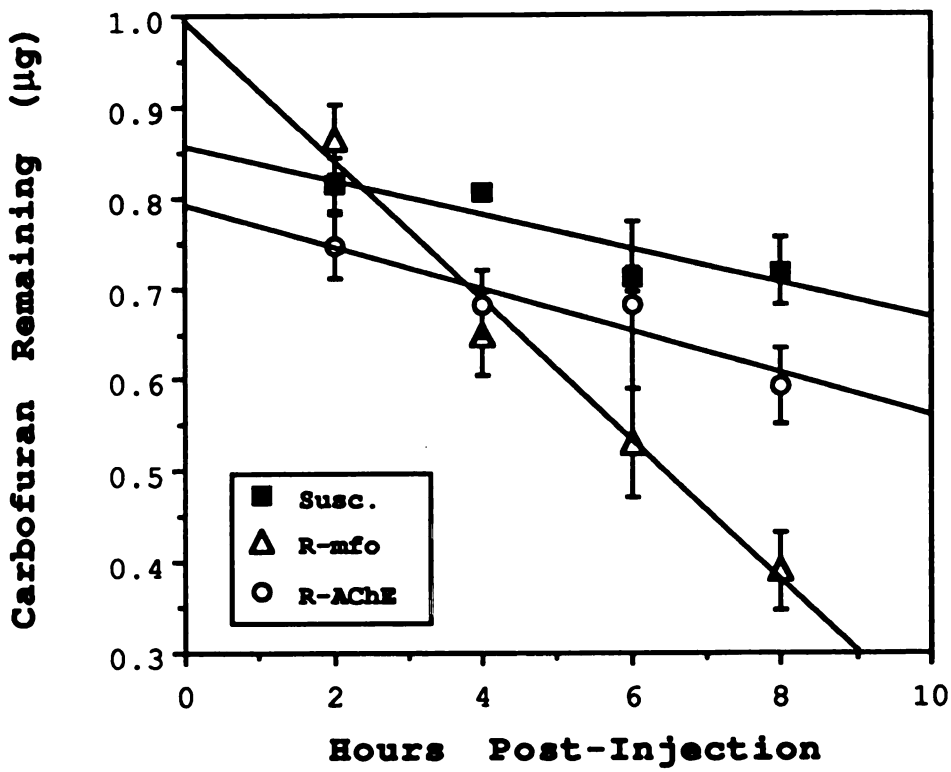


Figure 19. Metabolism of carbofuran in vivo. Lines were fit to the data by the least squares method. Rates were 19.0 ng/h (Susc.), 23.1 ng/h (R-AChE), and 76.9 ng/h (R-mfo); r^2 values were 0.80 to 0.98.

CHAPTER FIVE

CONCLUSIONS

SPECIFIC CONCLUSIONS

Each of the goals outlined in Chapter One were accomplished by investigating the biochemical basis of resistance in two carbofuran-resistant strains of the Colorado potato beetle. It is apparent from the results presented in Chapter Two that altered rates of penetration can in no way account for the observed levels of resistance. In fact, in these strains, it is unlikely that altered penetration is even a component of resistance.

Enhanced metabolism of insecticides is the most common mechanism of resistance, and was observed in both the R-mfo and R-AChE strains. It is likely that neither the hydrolytic enzymes nor the glutathione-S-transferases contribute significantly to resistance to carbofuran in either of these strains. In fact, it is interesting that the R-mfo strain, which descended from a multiply resistant strain, did not show elevated levels of either of these enzyme systems. In both resistant strains, the mixed function oxygenase system was elevated. In the case of the R-AChE strain, one of the major subclasses of oxidative enzymes (type I) was enhanced. In the other resistant strain, both subclasses of enzyme (type I and type II) were enhanced. The enhanced levels of mixed function oxygenases corresponded to increased rates of metabolism of carbofuran in vivo. The metabolism appeared to follow the established metabolic pathway for carbofuran.

The contribution of altered acetylcholinesterase to resistance was critical for the R-AChE strain. The acetylcholinesterases from this strain were highly insensitive to inhibition by many arylcarbamates. The R-mfo strain showed insensitivity to inhibition by organophosphates, but not arylcarbamates. These results indicate that changes in acetylcholinesterase can cause very diverse changes in specificity of the enzyme, a phenomenon recently reported in other systems. The other important conclusion from the acetylcholinesterase data is that there are multiple forms of the enzyme present in all of the strains, and it is likely that different isozymes have different sensitivities to inhibition by insecticides. This has distinct consequences for populations with altered acetylcholinesterases. It is unclear how many mutant forms of AChE are viable in the Colorado potato beetle in a field environment, but clearly more than one form is present in the strains tested. The observation that each of the strains developed a mutant acetylcholinesterase adapted particularly to a single selection agent raises the possibility of other mutant forms developing from different selecting agents. Some alterations will probably result in a low degree of cross-resistance (as seen with these strains), but other alterations could provided a broader range of protection. This means that extensive monitoring and testing will be required to determine which pesticide options will be effective.

When the various resistance mechanisms are compared to the levels of resistance in each strain, generally the degree of resistance is accounted for by the amount of protection conferred by the resistance mechanisms (Table 10). For example, the level of resistance for the R-mfo strain to azinphosmethyl (445-fold) can be explained by 20-fold synergism (contribution of metabolism by MFO) and about 20-fold insensitivity of acetylcholinesterase. However, the correlations break down when considering resistance to carbofuran by the R-mfo strain. While it is 225-fold resistant to carbofuran, metabolism contributes about 15-fold resistance, and altered AChE only about 1.5-fold. This leaves about a 10-fold level of resistance unaccounted for. It is difficult to offer any explanation for this outcome.

GENERAL CONCLUSIONS

In conducting the research described here, I have come to some general conclusions about insecticide resistance that may seem obvious, but were not always so, during their synthesis.

From a systematic, scientific approach to the study of resistance, it is often useful to isolate mechanisms of resistance for study. There are several dangers to this approach. First, it is easy to lose sight of the importance of interactions between mechanisms, and the likelihood that multiple mechanisms of resistance are at work. For example, in studying altered acetylcholinesterase, I was surprised

Table 10. Comparison of resistance levels and resistance mechanisms in the resistant strains of Colorado potato beetle.

	R-AChE	R-mfo
Carbofuran		
Resistance ratio	576	225
Synergism ratio	1.3	15
AChE ratio	>>20	1.5
metabolism ratio	1.2	4.0
penetration ratio	1	1
Azinphosmethyl		
Resistance ratio	9.1	445
Synergism ratio	1.6	20
AChE ratio	2	20
metabolism ratio	1.5	4.0
penetration ratio	1	1

that the R-mfo strain showed insensitivity to organophosphates. This was because I was focused on the metabolic mechanisms of resistance that we felt were important in that strain. On reflection, it should have been somewhat obvious that continuous selection with organophosphate might select for at least some degree of change in acetylcholinesterase.

Second, I believe that it is important to correlate in vitro mechanistic assays with whole animal studies. It is a leap of logic to assume that inhibition of an enzyme in a homogenate preparation corresponds to in vivo inhibition. I was able to correlate enhanced in vitro O-dealkylase activity with enhanced oxidative metabolism in vivo. But there were some technical barriers to doing the same with acetylcholinesterase inhibition. As good as those data are, comparable results from whole animal studies would make the case for AChE insensitivity as the major source of resistance in the R-AChE strain truly compelling. To really understand the basis of resistance, one must engage in a complete analysis of the impact of a compound and its metabolic fate in vivo.

Another tendency towards compartmentalization is with enzymes. The nomenclature of enzymes allows organization and concept development, but can be confusing if the nomenclature is not used accurately. The nomenclature used should reflect the degree of specificity and homogeneity of the preparations, not just the substrates or inhibitors used

in a particular investigation. Standard substrates should always be used for comparison and for a frame of reference.

The interactions between resistance mechanisms is important when examining the basis for resistance. Early on it had been suggested by Plapp and Hoyer (1968) that there was a relationship between penetration and metabolic mechanisms of resistance. The implications of this relationship for interpreting penetration data was discussed in Chapter Two. Oppenoorth (1985) and many others mention that increased metabolism often occurs in cases of altered AChE. Altered target-site mechanisms usually need a "helper" to attenuate the concentration of toxicants at the site of action, because the mutations in the enzyme are often not striking enough to render it completely insensitive to inhibition. It is even likely that multiple resistance mechanisms act synergistically. While it is not always easy to detect and identify interactions between resistance mechanisms, it is important to assess the toxicodynamics of an insecticide to understand its mode of action, and find points at which it is vulnerable to resistance development.

The picture of resistance in the Colorado potato beetle developed in this research is based on a few inbred strains which have been selected for several years with one or two insecticides. The resulting patterns of target-site insensitivities are reasonably clear. However, the relevance of these patterns to field populations is unknown.

Typically, a very diverse selection pressure is put on heterogeneous populations, so the alterations in AChE in a field environment may be of a more general nature. This points out an area deserving further study. A survey of potato beetle populations using a fairly large, standard set of inhibitors is necessary to discern any patterns of altered AChE development in the field.

I have come to a belief in the power and inevitability of insecticide resistance. The focus of pest control research should be towards beating the evolutionary clock. This can be done in two ways: first, by speeding the development and of new compounds, modes of action, and resistant plant varieties. This is the strategy that has been used since 1914, frequently called the pesticide treadmill. The second tactic is to extend the usefulness of current technology. This is accomplished by efficient, managed use of the technology, and by conserving, or even increasing the resource of susceptibility. This needs to be done on an ecosystem basis. Once a gene for resistance is fixed in a population, it is very difficult to remove it. I do not believe that either one of these approaches, of themselves, is sufficient. Both approaches must be applied to the problem to ensure safe, effective, and cost-efficient protection of food and fiber production.

FUTURE RESEARCH

The low potency of the oxime carbamates in inhibiting acetylcholinesterases from all of the Colorado potato beetle strains is quite interesting. Although interactions of oxime carbamates with acetylcholine receptors has been suggested as a possible mechanism of action of these compounds, it has not really been documented. It would be interesting to determine if the lack of acetylcholinesterase inhibition by oxime carbamates is a general feature of Colorado potato beetles, or a unique feature of the strains tested here.

The data presented here are a strong indication that organophosphates and carbamates bind to acetylcholinesterase in different ways. Although most certainly the active site of the enzyme is the same for both classes of compounds, and there are many structure-activity relationship studies reported, the nature of the binding sites hasn't been fully investigated. In the early 1970s, O'Brien et. al (1974) conducted a preliminary investigation to develop a model of inhibitor binding for acetylcholinesterase. The resulting model included three new binding sites: hydrophobic, indophenyl, and charge transfer complex. In the intervening 20 years, little has been done to follow up on that work, but since that time, the three-dimensional structure of the enzyme has been elucidated. With the sophisticated molecular modeling programs now available, it should be

possible to gain more insight into the binding of inhibitors to acetylcholinesterase.

The hypothesis that there are multiple forms of acetylcholinesterase extant in each strain leads to the obvious need for more effort to purify and evaluate cholinesterase isozymes. The work I did in that arena was largely unsuccessful, and is presented in Appendix Three. However, the issue still remains, and is important. The possibility of producing several acetylcholinesterases which adequately perform the normal functions of the enzyme, but offer several modalities of protection from exogenous inhibitors is a complex, and serious potential problem. It has implications for strategies to control resistant populations, pesticide use (mixtures and rotations), and study of the genetics of resistance. As biotechnology advances, it may be quicker and easier to purify the isozymes by first cloning the gene(s), then express the proteins through translation systems.

The first work that I did with the potato glycoalkaloid, α-chaconine (see Appendix 2) was done to assess the impact of resistance to conventional insecticides on tolerance of host plant resistance factors that may be related to acetylcholinesterase inhibition. It was interesting to find that acetylcholinesterase from one of the resistant strains was hypersensitive to α-chaconine compared with the susceptible strain. The very high levels of chaconine required for inhibition led to a comparative

investigation with other species. The Colorado potato beetle is highly insensitive to chaconine, even when comparing a closely related species. This then brings up some questions. Are Colorado potato beetle cholinesterases generally insensitive to chaconine, and if so, is chaconine really a host plant resistance factor? Secondly, how do conventional insecticides and host plant resistance factors interact if they exploit the same target? How much does exposure to one agent increase the likelihood of resistance to the other?

Finally, it would be interesting to investigate further the effects of selection on the ultimate mechanisms of resistance expressed. For example, in the beetle strains I investigated, the resistance was the result of selections with only one or two compounds. The R-mfo strain came from a multi-resistant stock, and exhibited more than one mechanism of resistance, depending most on metabolism. I expect that the cholinesterase inhibitors used to select for resistance would affect the changes in acetylcholinesterases, and hence their specificities. The influence of selecting agents on the mechanism(s) of resistance which ultimately provide protection for the pest populations may be a complex question. So I believe that it is very important to conduct a large scale survey of Colorado potato beetle acetylcholinesterases. Only by this means can we come to understand the alterations in the enzyme which occur in the field. It is possible that

acetylcholinesterases develop a "general" alteration when confronted with selection from diverse inhibitors. If this happens, it is possible that further study of the enzyme could yield inhibitors which preferentially inhibit resistant isozymes (similar to the hypersensitivity of resistant green rice leafhoppers to N-propylcarbamates (Hama, 1983)). Although this would be a fairly large undertaking, it is one which deserves study. The answers could have a tremendous impact on extending the life of current and future pest control agents.

CONCLUSION

The goals of the research described here were to examine various mechanisms of resistance to determine which were important to the enhanced survival of resistant strains when challenged with the pesticide carbofuran. The preceding paragraphs and chapters can be succinctly condensed. The R-mfo strain depends primarily on increased mixed function oxygenase activity to protect it from carbofuran and other insecticides. It also has an altered acetylcholinesterase which provides additional protection from organophosphates. The R-AChE strain depends primarily on altered acetylcholinesterase for resistance to carbofuran. It also has some increased mixed function oxygenase to supplement the protection of altered acetylcholinesterase.

CHAPTER SIX

LITERATURE CITED

Ahammadsahib K.I., R.M. Hollingworth, M.E. Whalon, P.M. Ioannidis and E.J. Grafius. Polysubstrate monooxygenases and other xenobiotic-metabolizing enzymes in susceptible and resistant Colorado potato beetle. Submitted to Pesticide Biochemistry and Phsyiology.

Aldridge W.N. and A.N. Davison. 1952. The inhibition of erythrocyte cholinesterases by tri-esters of phosphoric acid. Biochemical Journal 52: 663-671.

Aldridge W.N. and E. Reiner. 1972. Enzyme inhibitors as substrates. *In* A.Neurenberger and E.L. Tatum [eds.], Frontiers of Biology Vol. 26. American Elsevier, New York.

Anber H.A.I. and W.P.J. Overmeer. 1988. Resistance to organophosphates and carbamates in the predacious mite *Amblyseius potentillae* (Garman) due to insensitive acetylcholinesterase. Pesticide Biochemistry and Physiology 31: 91-98.

Annonymous. 1992. December 1991 Colorado potato beetle survey results. Potato Growers of Michigan Inc. and The Michigan Potato Industry Commission. DeWitt, MI.

Argentine J.A., 1991, Biochemistry and genetics of insecticide resistance in the Colorado potato beetle. Ph.D. dissertation, University of Massachusetts.

Argentine J.A., J.M. Clark and D.N. Ferro. 1989. Genetics and synergism of resistance to azinphosmethyl and permethrin in the Colorado potato beetle (Coleoptera: Chrysomelidae). Journal of Economic Entomology 82: 698-705.

Ayad H. and Georghiou G.P. 1975. Resistance to organophosphates and carbamates in *Anopheles albimanus* based on reduced sensitivity of acetylcholinesterase. Journal of Economic Entomology 68: 295-297.

Babers F.H. and J.P. Pratt. 1951. Development of insect resistance to insecticdes II. A critical review of the literature up to 1951. U.S. Department of Agriculture/Agricultural Research Administration Bureau of Entomology and Plant Quarantine Report E-818.

Benezet H.J. and A.J. Forgash. 1972. Reduction of malathion penetration in houseflies pretreated with silicic acid. Journal of Economic Entomology 65: 895-896.

Bohart R.M. and W.D. Murray. 1950. DDT resistance in *Aedes nigromaculatus* larvae. Proceedings of the California Mosquito Control Association 18: 22-23.

Bowen N. 1991. World Agrochemical Markets. P.J.B. Publications, Richmond, UK.

Brooks G.T. 1974. Clorinated insecticides. Vol. II. Biological and environmental aspects. CRC Press, Cleveland.

Brown T.M. and P.K. Bryson. 1991. Characteristics of methyl paraoxon-resistant acetylcholinesterase of *Heliothis virescens*. *In* Seventh International Congress of Pesticide Chemistry Abstracts, International Union of Pure and Applied Chemistry, Hamburg, Germany.

Bull D.L. and N.W. Pryor. 1990. Characteristics of resistance in house flies subjected to long-term concurrent selection with malthion and permethrin. Pesticide Biochemistry and Physiology 37: 101-115.

Burden G.S. 1975. Repellency of selected insecticides. Pest Control 43: 16-18.

Burges H.D. 1986. Impact of *Bacillus thuringiensis* on pest control with emphasis on genetic manipulation. MIRCEN Journal 2: 101-120.

Burke M.D., S. Thompson, C.R. Elcombe, J. Halpert, T. Haaparanta and R.T. Mayer. 1985. Ethoxy-, pentoxy-, and benzyloxyphenoxazones and homologues: A series of substrates to distinguish between different induced cytochromes P-450. Biochemical Pharmacology 34: 3337-3345.

Burnell A.M. and N.P. Wilkins. 1988. An investigation of the *in vitro* inhibition of acetylcholinesterase by the carbamate inhibitor eserine sulphate in eserine resistant strains of *Drosophila melanogaster*. Comparative Biochemistry and Physiology 90C: 215-220.

Casagrande R.A. 1987. The Colorado potato beetle: 125 years of mismanagement. Bulletin of the ESA 33: 142-150.

Champ B.R. and M.J. Campbell-Brown. 1990. Insecticide resistance in Australian *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). II. Malathion resistance in eastern Australia. Journal of Stored-Product Research 6: 111-131.

Chang K.M., C.F. Wilkinson and K. Hetnarski. 1981. Spectral and inhibitory interactions of methylenedioxyphenyl compounds with southern armyworm (*Spodoptera eridania*) midgut microsomes. Pesticide Biochemistry and Phsyiology 15: 32-42.

Clark A.G., N.A. Shamaan, W.C. Dauterman and T. Hayaoka. 1984. Characterization of multiple glutathione transferases from the house fly *Musca domestica* (L.). Pesticide Biochemistry and Physiology 22: 51-59.

Dauterman W.C. 1985. Insect metabolism: extramicrosomal. *In* G.A. Kerkut and L.I. Gilbert [eds.], Comprehensive insect biochemistry physiology and pharmacology 12: 713-730. Pergamon, New York.

Delorme R., D. Fournier, J. Chaufaux, A. Cuany, J.M. Bride, D. Auge and J.B. Berge. 1988. Esterase metabolism and reduced penetration are causes of resistance to deltamethrin in *Spodoptera exigua* HUB (Noctuidea: Lepidoptera). Pesticide Biochemistry and Physiology 32: 240-246.

Devonshire A.L. 1975. Studies of the acetylcholinesterase from houseflies (*Musca domestica* L.) resistant and susceptible to organophosphorus insecticides. Biochemical Journal 149: 463-489.

Devonshire A.L. and G.D. Moores. 1984. Different forms of insensitive acetylcholinesterase in insecticide-resistant house flies (*Musca domestica*). Pesticide Biochemistry and Physiology 21: 336-340.

Devonshire A.L. and R.M. Sawicki. 1979. Insecticide-resistant *Myzus persicae* as an example of evolution by gene duplication. Nature 280: 140-141.

Dittrich V. 1966. N-(2-methyl-4-chlorphenyl)-N',N'-dimethylformamidine (C-8514/Schering 36268) evaluated as an acaricide. Journal of Economic Entomology 59: 889-893.

Drabek J. and R. Neumann. 1985. Proinsecticides, pp. 35-85. *In* D.H. Hutson and T.R. Roberts [eds.], Insecticides. Wiley & Sons, New York.

Dyte C.E., D.G. Rowlands, J.A. Daly and D.G. Blackman. 1970. *In* Pest infestation research 1969. Agriculture Research Council, Report of the Pest Infestation Laboratory, Slough England, pp 41-42.

Ebeling W., D.A. Rierson and R.E. Wagner. 1968. Influence of repellency on the efficacy of blatticides. III. Field experiments with German cockroaches with notes on three other species. Journal of Economic Entomology 61: 751-766.

Edgerton, J. 1861. Potato Insects. Praire Farmer 8: 116-117.

Eldefrawi M.E., R. Miskus and V. Sutcher. 1960. Methylenedioxyphenyl derivatives as synergists for carbamate insecticides on susceptible, DDT-, and parathion-resistant houseflies. Journal of Economic Entomology 53: 231-234.

Elliot M., M.G. Ford and N.F. Janes. 1970. Insecticidal activity of the pyrethrins and related compounds III. Penetration of pyrethroid insecticides into mustard beetles (*Phaedon cochleariae*). Pesticide Science 1: 220-223.

Ellman G.L., K.D. Courtney, V. Andres and R.M. Featherstone. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 7: 88-95.

Feitelson J.S., J. Payne and L. Kim. 1992. *Bacillus thuringiensis*: Insects and beyond. Biotechnology 10: 271-275.

Forgash A.J. 1984. History, evolution and consequence of insecticide resistance. Pesticide Biochemistry and Physiology 22: 178-186.

Forgash A.J. 1985. Insecticide resistance in the Colorado potato beetle. Massachusetts Agricultural Experiment Station Research Bulletin 704: 33-52.

Forgash A.J., B.J. Cook and R. C. Riley. 1962. Mechanisms of resistance in diazinon-selected multi-resistant *Musca domestica*. Journal of Economic Entomology 55: 544-551.

Garman P. 1950. Parathion resistant red spiders. Journal of Economic Entomology 43: 53-56.

Gauthier N.L., R.N. Hofmaster and M. Semel. 1981. History of Colorado potato beetle control, pp. 13-33. *In* J.H. Laschomb and R.A. Casagrande [eds.], Advances in potato pest management. Hutchinson Ross, Stroudsburg PA.

Georghiou G.P. 1971. Isolation, characterization and resynthesis of insecticide resistance factors in the housefly, Musca domestica. *In* Proceedings, 2nd International IUPAC congress on pesticide chemistry (A.S. Tahori, Ed), Vol 2: 77-94, Gordon and Breach, New York.

Georghiou G.P. 1972. The evolution of resistance to pesticides. Annual Review of Ecology and Systematics 3: 133-168.

Georghiou G.P. 1986. The magnitude of the resistance problem, pp. 14-43. *In* Pesticide Resistance: Strategies and tactics for management. National Academy Press, Washington, DC.

Georghiou G.P. 1990. Overview of Insecticide Resistance, pp. 18-41. *In* M.B Green, H.M. LeBaron, and W.K. Moberg, [eds.], Managing resistance to agrochemicals. ACS Symposium Series 421. American Chemical Society, Washington DC.

Georghiou G.P and A. Legunes-Tejeda. 1991. The occurrence of resistance to pesticides in arthropods. Food and Agriculture Organization of the United Nations, Rome.

Gessner, T. and J.N. Smith. 1960. Comparative detoxication VII. The metabolism of chlorobenzene in the locust. Excretion of unchanged chlorobenzene and cysteine conjugates. Biochemical Journal 75: 165-172.

Ghidiu G.M. 1987. Laboratory tests of household materials for Colorado potato beetle toxicity. New Jersey Grower 10: 3-4.

Gjullen C.M. and R.F. Peters. 1952. Recent studies of mosquitoe resistance to insecticides in the San Joaquin valley in California. Mosquito News 12: 1-7.

Grafius E.J., B. Bishop, W. Boylan-Pett, J. Sirota and K. Brix. Colorado potato beetle management 1991 potato research report. Michigan Potato Research Report 23: 65-76.

Guegenrich F.P. 1984. Microsomal enzymes in toxicology-Analysis and separation, pp. 605-634. *In* A.W. Hayes [ed.], Principles and Methods of Toxicology. Raven Press, New York.

Habig W.H., M.J. Pabst and W.B. Jakoby. 1974. Glutathione-S-transferase: The first enzymatic step in mercapturic acid formation. Journal of Biological Chemistry 249: 7130-7139.

Hadaway, A.B. 1950. Observations on mosquito behavior in native huts. Bulletin of Entomological Research 41: 63-78.

Hama H. 1983. Resistance to insecticides due to reduced sensitivity of acetylcholinesterase, pp. 299-332. *In* G.P. Georghiou and T. Saito [eds.], Pest resistance to pesticides. Plenum, New York.

Hama H. and T. Iwata. 1983. Resistance to carbamate insecticides and its mechanism in green rice leafhopper, *Nephotettix cincticeps* Uhler. Japanese Journal of Applied Entomology and Zoology 17: 154-159.

Harris C.R. and H.J. Svec. 1981. Colorado potato beetle resistance to carbofuran and several other insecticides in Quebec. Journal of Economic Entomology 74: 421-424.

Hawkins R.D. and B. Mendel. 1946. True cholinesterases with pronounced resistance to eserine. Journal of Cellular and Comparative Physiology 27: 69-85.

Hirai K., T. Miyata and T. Saito. 1973. Penetration of ^{32}P-dimethoate into organophosphate resistant and susceptible citrus red mite *Panonychus citri* McGregor (Acarina: Tetranychidae). Applied Entomology and Zoology 8: 183-190.

Hodgson E. 1980. Characterization of cytochrome P-450 in studies of insecticide resistance, pp. 207-228. *In* G.P. Georghiou and T. Saito [eds.], Pest resistance to pesticides. Plenum, New York.

Hollingworth R.M., R.L. Metcalf and T.R. Fukuto. 1967. The selectivity of sumithion compared with methyl parathion: Metabolism in susceptible and resistant house flies. Journal of Agriculture and Food Chemistry 15: 250-255.

Hooper G.H.S. 1965. The mechanism of DDT resistance in spotted root maggot *Euxesta notata*. Journal of Economic Entomology 58: 601-611.

Hung C-F and C-N Sun. 1989. Microsomal monooxygenases in diamondback moth larvae resistant to fenvalerate and piperonyl butoxide. Pesticide Biochemistry and Physiology 33: 168-175.

Ioannidis P.M. 1990. Characterization, synergism and inheritance of resistance to azinphosmethyl, carbofuran and permethrin insecticides in the Colorado potato beetle (Coleoptera: Chrysomelidae). Ph.D. dissertation, Michigan State University, E. Lansing.

Ioannidis P.M. and E.J. Grafius. 1989. Insecticide resistance in the Colorado potato beetle *Leptinotarsa decemlineata* in Michigan. Pesticide Resistance Newsletter 1 (2): 20-22.

Ioannidis P.M., E.J. Grafius and M.E. Whalon. 1991. Patterns of insecticide resistance to azinphosmethyl, carbofuran and permethrin the Colorado potato beetle (Coleoptera: Chrysomelidae). Journal of Economic Entomology 84: 1417-1423.

Ioannidis P.M., E.J. Grafius, J.M. Wierenga, M.E. Whalon and R.M. Hollingworth. 1992. Selection, inheritance, and characterization of carbofuran resistance in the Colorado potato beetle (Coleoptera: Chrysomelidae). Pesticide Science 34: (in press).

Jacobs S.E. 1950. Bacteriological control of the flour moth *Ephestia kuehniella* Z. Proceedings of the Society for Applied Bacteriology 13: 83-91.

Jao L.T. and J.E. Casida. 1974. Insect pyrethroid-hydrolyzing esterases. Pesticide Biochemistry and Physiology 4: 465-472.

Kinoshita F.K., J.P. Frawley and K.P. Dubois. 1966. Quantitative measurement of induction of hepatic microsomal enzymes by various dietary levels of DDT and toxaphene in rats. Toxicology and Applied Pharmacology 9: 505-513.

Kring J.B. 1958. Feeding behavior and DDT resistance of *Epitrix cucumeris*. Journal of Economic Entomology 51: 823-828.

Ku T-Y. and J.L. Bishop. 1967. Penetration, excretion and metabolism of carbaryl in susceptible and resistant German cockroaches. Journal of Economic Entomology 60: 1328-1332.

Kuhr R.J. 1971. Comparative metabolism of carbaryl by resistant and susceptible strains of the cabbage looper. Journal of Economic Entomology 64: 1373-1378.

Kuhr R.J. and H.W. Dorough. 1976. Carbamate insecticides: Chemistry, biochemistry and toxicology. CRC Press, Cleveland.

Kulkarni A.P. and E. Hodgson. 1980. Metabolism of insecticides by mixed function oxidase systems. Pharmacology and Therapeutics 8: 379-475.

Lee S.C. 1969. Resistance to parathion and kelthane of mites in Korea. Korean Society of Plant Protection 12: 91-96.

Lewis J.B. 1969. Detoxication of diazinon by subcellular fractions of diazinon resistant and susceptible houseflies. Nature 224: 917-918.

Lipke H. and C.W. Kearns. 1960. DDT-dehydrochlorinase. Advances in Pest Control Research 3: 253-287.

Lockwood J.A., T.C. Sparks and R.N. Story. 1984. Evolution of insect resistance to insecticides: a reevaluation of the roles of physiology and behavior. Bulletin of the ESA 30: 41-51.

Main A.R. 1964. Affinity and phosphorylation constant for the inhibition of esterase by organophosphates. Science 144: 992-995.

Matsumura F., 1983. Penetration, binding and target insensitivity as causes of resistance to chlorinated hydrocarbon insecticides, pp. 367-386. *In* G.P. Georghiou and T. Saito [eds.], Pest resistance to pesticides. Plenum, New York.

Matsumura F. 1985. Toxicology of insecticides. Plenum, New York.

Matsumura F. and A.W.A Brown 1961. Biochemistry of malathion resistance in *Culex tarsalis*. Journal of Economic Entomology 54: 1176-1185.

Matsumura F. and S.M. Ghiasuddin 1983. Evidence for similarities between cyclodiene type insecticides and picrotoxinin in their mechanisms. Journal of Environmental Science and Health B 18: 1-14.

Matsumura F. and M. Hayashi 1966. Dieldrin: Interaction with nerve components of cockraoches. Science 153: 757-759.

Matsumura F. and C.J. Hogendijk. 1964. The enzymatic degradation of parathion in organophosphate-susceptible and resistant houseflies. Journal of Agriculture and Food Chemistry 12: 447-453.

Mattingly P.F. 1962. Mosquito behavior in relation to disease eradication programmes. Annual Review of Entomology 7: 419-436.

Mayer R.T., J.W. Jermyn, M.D. Burke and R.A. Prough. 1977. Methoxyresorufin as a substrate for the fluorometric assay of insect microsomal O-dealkylases. Pesticide Biochemistry and Physiology 7: 349-354.

McGaughey W.H. 1985. Insect resistance to the biological insecticide *Bacillus thuringiensis*. Science 229: 193-195.

McGaughey W.H. and M.E. Whalon. 1992. Potential for insect resistance to *Bacillus thuringiensis*. ARS/USDA *Bacillus thuringiensis* Resistance Scientific Conference. Washington DC.

Melander A.L. 1914. Can insects become resistant to sprays? Journal of Economic Entomology 7: 167-173.

Metcalf R.L., T.R. Fukuto, C. Collins, K. Borck, S. Abd El-Aziz, R. Munoz and C.C. Cassil. 1968. Metabolism of 2,2-dimethyl-2,3-dihydrobenzofuranyl-7 N-methylcarbamate (furadan) in plants, insect, and mammals. Journal of Agriculture and Food Chemistry 16: 300-311.

Miller D.L., U. Rahardja and M.E. Whalon. 1990. Development of a strain of Colorado potato beetles resistant to the delta-endotoxin of B.t. Pest Resistance Management Newsletter 2 (2): 25.

Motoyama N. and W.C. Dauterman. 1975. Interstrain comparison of glutathione-dependent reactions in susceptible and resistant houseflies. Pesticide Biochemistry and Physiology 5: 489-495.

Mutero A., M. Pralavorio, V. Simeon and D. Fournier. 1992. Catalytic properties of cholinesterases: importance of tryosine 109 in *Drosophila* protein. NeuroReport 3: 39-42.

Nakatsugawa T. and M.A. Morelli. 1976. Microsomal oxidation and insecticide metabolism, pp. 61-114. *In* C.F. Wilkinson [ed.], Insecticide biochemistry and physiology. Plenum, New York.

Nebert D.W., M. Adesnik, M.J. Coon, R.W. Estabrook, F.J. Gonzales, F.P. Guengerich, I.C. Gunsalus, E.F. Johnson, B. Kemper, W. Levin, I.R. Phillips, R. Sato and M.R. Waterman. 1987. The P450 gene superfamily: Recommended nomenclature. DNA 6: 1-11.

O'Brien R.D., B. Hetnarski, R.K. Tripathi and G.J. Hart. 1974. Recent studies on acetylcholinesterase inhibition, pp. 1-13. *In* G.K. Kohn [ed.], Mechanism of pesticide action. ACS Symposium Series vol 2, American Chemical Society, Washington DC.

Omura T. and R. Sato. 1964. The carbon monoxide-binding pigment of liver microsomes. Journal of Biological Chemistry 239: 2370-2378.

Oppenoorth F.J. 1982. Two different paraoxon-resistant acetylcholinesterase mutant in the house fly. Pesticide Biochemistry and Phsyiology 18: 26-27.

Oppenoorth F.J. 1984. Biochemistry of insecticide resistance. Pesticide Biochemistry and Physiology 22: 187-193.

Oppenoorth F.J. 1985. Biochemistry and genetics of insecticide resistance. *In* G.A. Kerkut and L.I. Gilbert [eds.], Comprehensive insect physiology biochemistry and pharmacology Vol 12: 731-773. Pergammon Press, New York.

Oppenoorth F.J. and K. Van Asperen. 1960. Allelic genes in the housefly producing modified enzymes that casue organophosphate resistance. Science 132: 298-299.

Oppenoorth F.J. and W. Welling. 1976. Biochemistry and physiology of resistance, pp. 507-551. *In* C.F. Wilkinson [ed.], Insecticide biochemistry and physiology. Plenum, New York.

Patil V.L. and F.E. Guthrie. 1979. Cuticular lipids of two resistant and a susceptible strain of houseflies. Pesticide Science 10: 399-406.

Payne L.K., H.A. Stansbury and M.H.J. Weiden. 1966. The synthesis and insecticidal properties of some cholinergic trisubstituted acetaldehyde O-(methylcarbamoyl) oximes. J. Agric. Food Chem. 14: 356-365.

Plapp FW, 1970, On the molecular biology of insecticide resistance,pp 179-192. *In* R.D. O'Brien and I. Yamamoto [eds.], Biochemical toxicology of insecticides. Academic, New York.

Plapp F.W. and R.F. Hoyer. 1968. Insecticide resistance in the house fly: Decreased rate of absorbtion as the mechanism of action of a gene that acts as an intensifier of resistance. Journal of Economic Entomology 61: 1298-1303.

Pluthero F.G. and Singh R.S. 1984. Insect behavioral responses to toxins: practical and evolutionary considerations. The Canadian Entomologist 116: 57-68.

Pree D.J., D.E. Archibald and K.J. Cole. 1990. Insecticides resistance in spotted tentiform leafminer (Lepidoptera: Gracillariidae): Mechanisms and management. Journal of Economic Entomology 83: 678-685.

Prickett A.J. amd C.A. Ratcliffe. 1977. The behavior of *Tribolium castenaeum* (Herbst) and *Sitophilus granarius* (L.) in the presence of insecticide-treated surfaces. Journal of Stored-Products Research 13: 145-148.

Riley C.V. 1875. Seventh annual report on the noxiuous, beneficial, and other inscts of the state of Missouri. Regan and Carter, Jefferson City, MO.

Rose R.L. and W.A. Brindley. 1985. An evaluation of the role of oxidative enzymes in Colorado potato beetle resistance to carbamate insecticides. Pesticide Biochemistry and Phsyiology 23: 74-84.

Roulston W.J., H.J. Scnitzerling and A.C. Schunter. 1968. Acetylcholinesterase insensitivity in the Biarra strain of cattle tick *Boophilus microplus* (Can.), as a cause of resistance to organophosphorus and carbamate acaricides. Australian Journal of Biological Science 21: 759-767.

Sawicki R.M. and A.W. Farnham. 1968. Examination of the isolated autosomes of the SKA strain of houseflies (*Musca domestica* L.) for resistance to several insecticides with and without pretreatment with sesamex and TBTP. Bulletin of Entomological Research 59: 409-421.

Sawicki R.M. and K.A. Lord. 1970. Some properties of a mechanism of delaying penetration of insecticides into houseflies. Pesticide Science 1: 213-217.

Sawicki R.M. and A.D. Rice. 1978. Response of susceptible and resistance aphids *Myzus persicae* (Sulz.) to insecticies in leaf-dip bioassays. Pesticide Science 9: 513-516.

Schnitzerling H.J., J. Nolan and S. Hughes. 1983. Toxicology and metabolism of some synthetic pyrethroids in larvae of susceptible and resistant strains of the cattle tick *Boophilus microplus* (Can.). Pesticide Science 14: 64-72.

Shishido T. and J. Fukami. 1963. Studies on the selective toxicity organic phosphorus insecticides. II. The degradation of ethyl parathion, methyl parathion, methyl paraoxon and sumithion in mammal, insect and plant. Botyu-Kaguku 28: 69-76.

Shrivastava S.P., G.P. Georghiou, R.L. Metcalf and T.R. Fukuto. 1970. Carbamate Resistance in Mosquitos. Bulletin of the WHO 42: 931-942.

Smissaert H.R. 1964. Cholinesterase inhibition of spider mites susceptible and resistant to organophosphate. Science 143: 129-131.

Smith A.G. 1963. Control of apple leaf rollers in Nelson. Orchardist New Zeeland 34: 315-318.

Smith W.W. and W.C. Yearian. 1964. Studies of behavioristic insecticide resistance in houseflies. Journal of the Kansas Entomological Society 37: 63-77.

Sternburg J., E.B. Vinson and C.W. Kearns. 1953. Enzymatic dehydrochlorination of DDT by resistant flies. Journal of Economic Entomology 46: 513-515.

Stone T.B., S.R. Sims and P.G. Marrone. 1989. Selection of tobacco budworm for resistance to a genetically engineered *Psuedomonas fluorescens* containing the δ-endotoxin of *Bacillus thuringiensis* subsp. *kurstaki*. Journal of Invertebrate Pathology 53: 228-234.

Suckling D.M., K.F. Armstron and J.G.I. Khoo. 1990. Selection with azinphosmethyl influences glutathione-S-transferase activity in the light brown apple moth, *Epiphyas postvittana* (Lepidoptera: Tortricidae). Pesticide Biochemistry and Physiology 38: 9-17.

Sundseth S.S., C.E. Nix and L.C. Waters. 1990. Isolation of insecticide resistance-related forms of cytochrome P-450 from *Drosophila melanogaster*. Biochemical Journal 265: 213-217.

Tabashnik B.E., N.L. Cushing, N. Finson and M.W. Johnson. 1990. Field development of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). Journal of Economic Entomology 83: 1671-1676.

Tang Z.-H., R.J. Wood and S.L. Cammack. 1990. Acetylcholinesterase activity in organophosphorus and carbamate resistant and susceptible strains of the *Culex pipiens* complex. Pesticide Biochemistry and Physiology 37:192-199.

Toutant J.-P. 1989. Insect acetylcholinesterase: catalytic properties, tissue distribution and molecular forms. Progress in Neurobiology 32: 423-446.

Tripathi R.K. 1976. Relation of acetylcholinesterase sensitivity to cross-resistance of a resistant housefly strain to organophosphates and carbamates. Pesticide Biochemistry and Physiology 6: 30-34.

Tsukamoto M. and J.E. Casida. 1967. Metabolism of methylcarbamate insecticides by the $NADPH_2$ requiring enzyme system from houseflies. Nature 213: 49-51.

Van Asperen K. 1962. A study of housefly esterases by means of a sensitivie colorimetric method. Journal of Insect Physiology 8: 401-416.

Van Asperen K. and F.J. Oppenoorth. 1960. The interaction between organophosporus insecticides and esterases in homogenates of organophosphate susceptible and resistant houseflies. Entomologia Experimentalis et Applicata 3: 68-83.

Vinson S.B. and P.K. Law. 1971. Cuticular composition and DDT resistance in the tobacco budworm. Journal of Economic Entomology 64: 1387-1390.

Waters L.C. and C.E. Nix. 1988. Regulation of insecticide resistance-related cytochrome P450 in *Drosophila melanogaster*. Pesticide Biochemistry and Physiology 30: 214-227.

Weiden M.H.J. 1971. Toxicity of carbamates to insects. Bull. W.H.O. 44: 203-213.

Weismann R.von. 1947. Untersuchungen uber das physiologiesche Verhalten von *Musca domestica* L. verschiedener Provenienzen [Differences of susceptibility to DDT of house flies from Sweden and Switzerland]. Schweizerische Entomologische Gesellschaft (Mitteilungen) 20: 484-504.

Welling W. 1977. Dynamic aspects of insecticide interactions. Annual Review of Entomology 22: 53-78.

Yamamoto I., Y. Takahashi and N. Kyomura. 1983. Suppression of altered acetylcholinesterase of the green rice leafhopper by N-propyl and N-methyl carbamate combinations, pp. 579-594. *In* G.P. Georghiou and T. Saito [eds.], Pest resistance to pesticides. Plenum, New York.

Yu C-C., R.L. Metcalf and G.M. Booth. 1972. Inhibitors of acetylcholinesterase from mammals and insects by carbofuran and its related compounds and their toxicities towards these animals. Journal of Agriculture and Food Chemistry 20: 923-926.

Yu C-C. and L.C. Terriere. 1979. Cytochrome P450 in insects 1. Differences in the forms present in insecticide-resistant and susceptible house flies. Pesticide Biochemistry and Physiology 12: 239-248.

Yu S.J. 1984. Interactions of allelochemicals with detoxication enzymes of insecticide-susceptible and resistant fall armyworms. Pesticide Biochemistry and Physiology 22: 60-68.

Yu S.J. 1991. Insecticide resistance in the fall armyworm *Spodoptera frugiperda* (J.E. Smith). Pesticide Biochemistry and Physiology 39: 84-91.

Zhu K.Y. and W.A. Brindley. 1990. Acetylcholinesterase and its reduced sensitivity to inhibition by paraoxon in organophosphate-resistant *Lygus hesperus* Knight (Hemiptera: Miridae). Pesticide Biochemistry and Physiology 36: 22-28.

APPENDICES

APPENDIX ONE

APPENDIX 1

Record of Deposition of Voucher Specimens*

The specimens listed on the following sheet(s) have been deposited in the named museum(s) as samples of those species or other taxa which were used in this research. Voucher recognition labels bearing the Voucher No. have been attached or included in fluid-preserved specimens.

Voucher No.: 1992-02

Title of thesis or dissertation (or other research projects):

Mechanisms of Resistance in Carbofuran-Resistant Colorado Potato Beetles

Museum(s) where deposited and abbreviations for table on following sheets:

Entomology Museum, Michigan State University (MSU)

Other Museums: None

Investigator's Name (s) (typed)
Joel M. Wierenga

Date June 11, 1992

*Reference: Yoshimoto, C. M. 1978. Voucher Specimens for Entomology in North America. Bull. Entomol. Soc. Amer. 24:141-42.

Deposit as follows:

Original: Include as Appendix 1 in ribbon copy of thesis or dissertation.
Copies: Included as Appendix 1 in copies of thesis or dissertation.
Museum(s) files.
Research project files.

This form is available from and the Voucher No. is assigned by the Curator, Michigan State University Entomology Museum.

APPENDIX 1.1

Voucher Specimen Data

Page 1 of 1 Pages

Species or other taxon	Label data for specimens collected or used and deposited	Number of: Eggs	Larvae	Nymphs	Pupae	Adults ♀	Adults ♂	Other	Museum where deposited
Leptinotarsa decemlineata (Say) Colorado Potato Beetle									
Strains: Vestaburg susceptible	MI Montcalm Co. nr. Vestaburg Aug. 1987					3	3		
Montcalm-C carbofuran-resistant	MI Montcalm Co. MSU Potato Res. Farm nr. Entrican Jul. 1987					3	3		
Long Island multiple resistant	MI Ingham Co. MSU Entomology Lab. Apr. 1992					3	3		
Note: these specimens are from MSU laboratory colonies started in 1985 and 1987 (see voucher number 1990-02).									

(Use additional sheets if necessary)

Investigator's Name(s) (typed)

Joel M. Wierenga

Date June 11, 1992

Voucher No. 1992-02

Received the above listed specimens for deposit in the Michigan State University Entomology Museum

Curator 11 June 1992 Date

APPENDIX TWO

APPENDIX TWO

INHIBITION OF INSECT ACETYLCHOLINESTERASES BY THE POTATO GLYCOALKALOID α-CHACONINE

Introduction

The major glycoalkaloid components of potatoes are α-solanine and α-chaconine, alkaloids differing only in their sugar moieties (Figure 20). These molecules have been implicated in instances of food poisoning (e.g. MacMillan and Thompson 1979), and several toxic effects in mammals have been identified. Among the effects described are membrane disruption (Roddick et al. 1988), teratogenicity, and acetylcholinesterase inhibition (see review by Jadhav et al. 1981). Entomologists and potato breeders have investigated the role of glycoalkaloids in host plant resistance to insect pests, especially the Colorado potato beetle (*Leptinotarsa decemlineata* (Say)). These studies have examined the effects on insect behavior (Harrison and Mitchell 1988), growth and development (Sinden et al. 1986) and crop damage (Sanford et al. 1990). Potato breeders have exploited high alkaloid levels to confer host plant resistance to the Colorado potato beetle, although one variety (Lenape) was withdrawn from the market due to high levels of glycoalkaloids in the tuber (Nishie et al. 1971).

In the present study, we evaluate the ability of α-chaconine to inhibit acetylcholinesterase from several

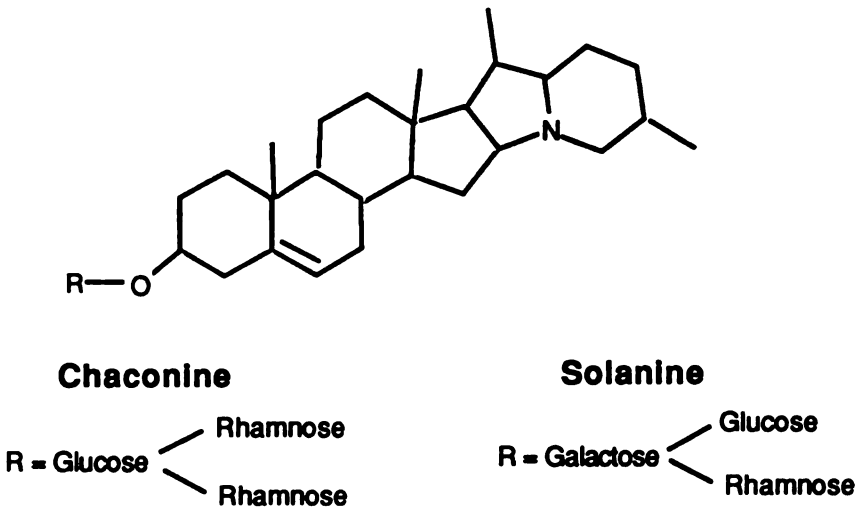


Figure 20. The structures of chaconine and solanine

insect species, including Colorado potato beetles susceptible and resistant to conventional insecticides.

Materials and Methods

All insects were maintained at 28°C and 16: 8 light/dark. Relative humidity was not controlled. A susceptible and two insecticide resistant Colorado potato beetle strains were fed fresh potato foliage. Susceptible German cockroaches, *Blattella germanica* (L.), were fed dog chow and water ad lib. Adult *Aedes aegypti* (L.) mosquitoes from the laboratory of Dr. A. Raikhel (Michigan State University) and houseflies, *Musca domestica* (L.), purchased from Carolina Biological Supply (Burlington NC) were fed sugar water ad lib. Cottonwood leaf beetles, *Chrysomela scripta* (F.), were from the laboratory of Dr. L. Bauer (USDA, E. Lansing MI) and raised on cottonwood foliage. All chemicals used, including α-chaconine, were purchased from Sigma Chemical Co. (St. Louis, MO).

Adult insects (2 to 10 days old) were used in all of the in vitro assays for AChE activity. Heads and thoraces (Colorado potato beetle and German cockroach) or whole insects were used to produce tissue homogenates. Insects were homogenized with a Vertishear (Gardiner, NY) microfine homogenizer (2x 10 s setting 80) in 50 mM phosphate buffer (pH 7.4). The homogenate was centrifuged at 5,000 x *g* for 20 min (4°C), and the supernatant centrifuged at 100,000 x *g* (4°C) for 1 h. The resulting pellet was resuspended to give

0.1 - 5 mg/ml protein with approximately 7.3 units of AChE activity per ml, and assayed for AChE activity (Ellman et al. 1961). Briefly, phosphate buffer including 0.0625N HCl (0 to 200 µl), and 0 to 200 µl α-chaconine (2.5 mM in 0.0625N HCl) were added to 300 µl homogenate (20 - 500 µg protein). Following preincubation for 10 min at 30°C, 100 µl 5,5-dithio-*bis*-(2-nitrobenzoate) (3 mM) and 400 µl acetylthiocholine iodide (10 mM) were added for a total volume of 1 ml.

The reaction rate at 30°C was measured using a Shimadzu UV-265 spectrophotometer (Kyoto, Japan). Absorbance readings (at 412 nm) were taken every 30 s for 10 min. The reaction was linear for over 10 minutes. Data points between 2 and 8 min were used to calculate the rates and correlation coefficients. The reaction rates at various chaconine concentrations were used to calculate the mean percent inhibition and construct concentration-inhibition curves. The Lowry method as modified by Guegenrich (1984) was used for protein determination. For housefly and cottonwood leaf beetle homogenates, the 100,000 x *g* supernatant (100 - 2000 µg protein) was assayed for AChE activity using the procedure above.

In vivo toxicity studies were carried out with Colorado potato beetles. For feeding studies, 25 mg of α-chaconine was applied to potato leaves (petioles with 5 leaflets each, ca. 225 $\mu g/cm^2$). Young second instars (12 per petri dish) were placed on the leaves (2 petioles per dish), and

mortality was assessed daily for five days. For injection studies, adult beetles (2 to 10 days old) were injected ventrally in the abdomen with 40 μg α-chaconine in 2 μl 0.25N HCl. Controls were injected with 0.25N HCl only. In some cases adults were pretreated with piperonyl butoxide 2 h before injection with chaconine. The piperonyl butoxide was applied topically in 2 μl (12.5 mg/ml acetone) to the ventral abdomen. The beetles were immobilized on their backs for 5 to 10 min. Each treatment group consisted of 10 or 15 beetles, the experiments were repeated once. Mortality was assessed daily for three days.

Results and Discussion

Both beetle species had the lowest AChE activity (ca. 7 nmole/mg protein/min) (Table 11), while the housefly had the highest specific activity, similar to that seen by others (e.g. Lenoir-Rousseaux 1985). The soluble enzyme (100,000 x *g* supernatant) had 5- to 18-fold lower activities compared with the microsomal fraction, indicating that most of the enzyme activity was membrane-bound.

Initial AChE assays showed that at 200 μM, α-chaconine was a slightly better AChE inhibitor than solanine (25% and 20% inhibition respectively) in the susceptible strain. This is consistent with the findings of others in numerous species (see Roddick et al. 1988). For most of the insects assayed, the concentration causing 50% inhibition (IC_{50}) was in the 5 to 40 μM range. This is similar to the sensitivity

Table 11. Inhibition of Insect Acetylcholinesterases by α-Chaconine.

Insect	Specific Activity[a] (nmole/mg prot./min)	IC_{50}[b] (μM)
German Cockroach (M)[c]	56.4 ± 4.1	9.7
Mosquito (M)	32.7 ± 2.9	8.8
Housefly (M)	155.5 ± 2.7	34
Housefly (S)	8.4 ± 1.8	36
Cottonwood leaf beetle (M)	7.5 ± 0.4	6.7
Cottonwood leaf beetle (S)	1.4 ± 0.1	>>40
Colorado potato beetle (M)		
Susceptible	7.0 ± 0.6[d]	979
Carbofuran-resistant	17.1 ± 1.7[d]	968
Multi-resistant	11.3 ± 0.8[d]	272

[a] data are the mean ± SE, n = 4-6
[b] data computed from dose response curves, n = 4-6
[c] M = membrane-bound; S = soluble
[d] data from Ioannidis et al. 1992; Vestaburg, Montcalm-C, and Long Island strains respectively.

of mammalian AChE to α-chaconine. Alozie et al. (1978) report IC_{50} values of 4 and 30 μM for bovine erythrocyte AChE and horse serum butyrylcholinesterase respectively.

Colorado potato beetle AChE was highly insensitive to α-chaconine (IC_{50} ca. 300 to 900 μM) compared with the other species tested, even the cottonwood leaf beetle, a closely related herbivorous species (Table 11).

In comparing soluble and membrane-bound acetylcholinesterases, we found a puzzling difference. In houseflies, both forms of AChE responded similarly to α-chaconine, but there was a significant ($p < 0.05$; 15-fold) difference in the inhibition of soluble enzyme from cottonwood leaf beetles. Membrane disruption, an established property of chaconine, or other, unknown factors might be a species-dependent factor of inhibition.

In the bioassays on Colorado potato beetles, even very high levels of α-chaconine on the leaves (225 $\mu g/cm^2$), did not reduce feeding or development rate (time to molts) in either the susceptible or multiresistant strains. These results correspond with those of Harrison and Mitchell (1988) and Sinden et al. (1986). Likewise, injection of 40 μg α-chaconine did not cause mortality in either strain. To discount the possibility that oxidative metabolism of these alkaloids was preventing toxicity, beetles were pretreated with the synergist piperonyl butoxide at a dose that inhibits mixed function oxygenase activity (Ioannidis et al. 1992), but again, there was no mortality. There is evidence

from other feeding studies that some species (e.g. potato leafhopper) are relatively sensitive to solanidine glycoalkaloids (Sinden et al. 1986, Sanford et al. 1990).

The Colorado potato beetle is highly adapted to these glycoalkaloids (Kogan 1976, Harrison and Mitchell 1988). One aspect of this adaptation could be altered AChE in response to glycoalkaloid exposure from continuous feeding on potatoes, although α-chaconine is poorly absorbed by the gut of mammals (Nishie et al. 1971).

Acetylcholinesterase from the multiresistant strain of Colorado potato beetle was more sensitive to α-chaconine than the susceptible strain by about 3-fold. The multiresistant strain has an altered AChE which is insensitive to organophosphate but not carbamate insecticides, and appears to have both susceptible and resistant isozymes. The IC_{50} values for carbofuran was 2 μM (susceptible), 0.5 μM (R-mfo), and ca. 60 μM (R-AChE) (Chapter 3). These differences in sensitivity to carbofuran may be correlated to difference in the binding of α-chaconine to the enzyme.

The results presented here show good evidence that insect targets other than metabolic systems can respond to host plant resistance factors, so development and breeding programs for host plant resistance factors should consider the target. Although it is possible that selection pressure by either plant resistance factors or conventional insecticides could make the target more susceptible to the

other, utilization of a target already under selection pressure may be risky. For example, the success of Bacillus thuringiensis (B.t.) transgenic plants currently under development may be affected by the use of B.t. as a commercial spray during the period before transgenic plants are released.

Conclusion

Alpha-chaconine was an effective inhibitor of all insect acetylcholinesterases tested except for the Colorado potato beetle. The relative insensitivity of AChE from that pest may represent an adaptation to feeding on potato foliage. If insecticide resistance leads to reduced responses to natural toxins, host plant resistance may not be an effective strategy of pest management. Exposure to plant alkaloids could enhance the survival of some pests to insecticide exposure. Further studies are required to determine in vivo toxicity to apparently sensitive species, but as a naturally derived AChE inhibitor, α-chaconine may be useful for controlling some species.

References Cited

Alozie S.O., S.P. Sharma and D.K. Salunkha. 1978. Inhibition of rat cholinesterase isozymes in vitro and in vivo by the potato alkaloid alpha-chaconine. Journal of Food Biochemistry 2: 259-276.

Ellman G.L., K.D. Courtney, V. Andres and R.M. Featherstone. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 7: 88-95.

Guegenrich F.P. 1984. Microsomal enzymes involved in toxicology-analysis and separation, pp. 609-634. In Principles and Methods of Toxicology. Raven PRess, New York.

Harrison G.D. and B.K. Mitchell. 1988. Host-plant acceptance by geographic populations of the Colorado potato beetle *Leptinotarsa decemlineata*. Journal of Chemical Ecology 14: 777-788.

Ioannidis P.M., E.J. Grafius, J.M. Wierenga, M.E. Whalon and R.M. Hollingworth. 1992. Selection, inheritance and characterization of carbofuran resistance in the Colorado potato beetle (Coleptera: Chrysomelidae). Pestic Sci 34: in press.

Jadhav S.J., R.P. Sharma and D.K. Salunkha. 1981. Naturally occurring toxic alkaloids in foods. Critical Reviews in Toxicology 9: 21-104.

Kogan M. 1976. The role of chemical factors in insect/plant relationships. In Proceedings of the XV International Congress of Entomology, College Park, Entomological Society of America, pp. 211-227.

Lenoir-Rousseaux J-J. 1985. Les Arthropodes possèdent une même forme moléculaire majoritaire d'acetylcholinesterase. Comptes Rendus des Seances de la Societe de Biologie et de ses Filiales 179: 741-747.

MacMillan M. and J.C. Thompson. 1979. An outbreak of suspected solanine poisoning in schoolboys: examination of criteria of solanine poisoning. Quarterly Journal of Medicine 48: 227-243.

Nishie K., M.R. Gumbmann and A.C. Keyl. 1971. Pharmacology of Solanine. Toxicology and Applied Pharmacology 19: 81-92.

Roddick J.R., A.L. Rijnenberg and S.F. Osman. 1988. Synergistic interaction between potato glycoalkaloids α-solanine and α-chaconine in relation to destabilization of cell membranes: Ecological implications. Journal of Chemical Ecology 14: 889-902.

Sanford L.L., K.L. Deahl, S.L. Sinden and T.L. Ladd Jr. 1990. Foliar solanidine glycoside levels in *Solanum tuberosum* populations selected for potato leafhopper resistance. American Potato Journal 67: 461-466.

Sinden S.L., L.L. Sanford, W.W. Cantelo and K.L. Deahl. 1986. Leptine glycoalkaloids and resistance to the Colorado potato beetle (Coleoptera: Chrysomelidae) in *Solanum chacoense*. Environmental Entomology 15: 1057-1062.

APPENDIX THREE

APPENDIX THREE

ACETYLCHOLINESTERASE PURIFICATION

Introduction

In the early stages of this research project, the acetylcholinesterase assay method was developed. This involved the initial characterization of AChE from a field strain of Colorado potato beetles. Subcellular fractionation showed highest specific activity in the microsomal fraction, although there was some activity in the supernatant as well. The addition of detergent (Triton X-100) solubilized a significant amount of the enzyme, but the specific activity was lower then the microsomal fraction without detergent. The pH and temperature range was broad, indicating a stable enzyme. The optimum activity was obtained at 35°C, and pH 7.4. The temperature was reduced to 30°C for running the assays to reduce the rate of spontaneous hydrolysis of acetylthiocholine. The K_m was for acetylthiocholine was approximately 20 μM. The V_{max} was approximately 12 nmole/mg protein/min, and occurred at a substrate concentration of 75 μM.

The striking differences in specificity between AChE from the three CPB strains led to the conclusion that there would be differences in either the binding constant, carbamylation/phosphorylation constant, or possibly the reactivation rate of the enzyme. To determine the altered

interaction with inhibitors, it was necessary to purify the enzyme to near homogeneity. Two techniques have been used to purify insect AChE. Most early efforts consisted of ammonium sulfate precipitation followed by size exclusion and ion exchange chromatographies (Lee et al. 1974).

Affinity chromatography was first used to purify insect acetylcholinesterase by Steele and Smallman (1976). This technique usually results in high purification (> 1,000 fold), often with good recovery. Only a few investigators have reported affinity purification of insect acetylcholinesterase (Table 12). Although the acridinium resin used by Gnagey et al. (1987) appeared to be a good choice as a ligand, the multi-step synthesis was complicated and I felt it was beyond my capability. My initial choice was to use trimethylammonium aniline. This ligand gave both good purification and recovery of housefly AChE (Table 12). In this project, good recovery was especially important due to limitations in the number of beetles the colonies could produce, and the low amount of enzyme per beetle.

Trimethylammonium Aniline Column

Trimethylammonium aniline was synthesized by the methods of Traylor and Singer (1967) and Berman and Young (1971). Approximately 3 g of N,N-dimtheyl-1,3-phenylenediamine dihydrochloride was added to excess (15-fold) acetic anhydride. A few drops of sulfuric acid (ca. 1 drop/5ml acetic anhydride) were added. The solution was

Table 12. Affinity purification of insect acetylcholinesterases with different ligands.

Ligand	Purification	Recovery(%)	Insect	Reference
trimethyl ammonium aniline	900	51	Housefly	Steele & Smallman 1976
	1,200	43	" "	Tripathi & O'Brien 1977
acridinium	4,300	23	Drosophila	Gnagey et al. 1987
procainamide	7.5	44	Mosquito	Bonning et al. 1989
3-(carboxyphenyl) ethyldimethyl ammonium	1,400	3	Lygus bug	Zhu et al. 1991

heated until it turned orange, then let cool completely, whereupon it turned reddish-brown in color. Four volumes of water were added slowly. The resulting yellow solution was cooled to 4°C, then poured into excess (4-fold) cold 5N sodium hydroxide. This solution was filtered to obtain gold crystals (yield ca. 60%). The crystals were washed in water, then filtered and dried. These were added to excess (10-fold) methyl iodide and refluxed in acetone for 30 min. After cooling at 4°C, the solution was filtered. The resulting light orange crystals had a melting point of 219-221°C (yield ca. 32%).

The orange crystals were dissolved in a mixture of ethanol and hydrochloric acid (1:1), then refluxed for 2 h. After cooling, five volumes acetone were added to precipitate the product. The result was a pink powder with a melting point of 179°C (yield ca. 25%) this was stored under nitrogen at 4°C. Mass spectrum analysis (Michigan State University Mass Spectrum Laboratory) showed high relative abundance of the molecular ion at 151 m/z, consistent with the structure of trimethylammonium aniline.

Trimethylammonium aniline was coupled directly to Affi-Gel 202 (Bio-Rad, Richmond CA) using 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (equivalent to Bio-Rad EDAC reagent). The gel was washed alternately with high and low pH salt solutions, then rinsed with distilled water. It was then diluted 1:1 with distilled water, and Trimethylammonium aniline (50 μmole/ml of gel) was added.

With gentle stirring, the carbodiimide (1mg/ml of gel) was added, and the pH adjusted to 4.7-5.0 with 1N HCl. The mixture was stirred overnight. The gel was washed with 50 mM phosphate buffer (pH 8.0), then slurried into a 1 x 10 cm column. The resulting column had a nominal flow rate of 8 ml/h and a void volume of 2.0 ml.

Column capacity was tested using electric eel AChE (type V-S, Sigma Chemical Co. St. Louis, MOO). Fractions (1 ml) were collected with an ISCO Retriever II (ISCO Inc., Lincoln, NE) fraction collector. Four void volumes of running buffer were collected, then eluting buffer (10 mM hexamethoniun bromide) was added. Four additional void volumes were collected. All fractions were assayed for AChE activity as described earlier. The results showed the gel to have a capacity of 0.09 units (Figure 21). This capacity approximated the amount of AChE activity in 180 Colorado potato beetles. The column was regenerated with 10 volumes of 6 M guanidine HCl (Tripathi and O'Brien, 1977) for future use.

Beetle heads and thoraces were homogenized in phosphate buffer (pH 7.4) with 0.1% Triton X-100. The brei was centrifuged at 20,000 x *g* for 30 min and the supernatant was loaded onto the column (ca. 0.007 units, or 15 beetle equivalents). Phosphate buffer was run through the column, and 16 (1 ml) fractions were collected. Elution buffer (as above) was run and 15 additional fractions collected. Each fraction was assayed for AChE activity. Almost all pre-

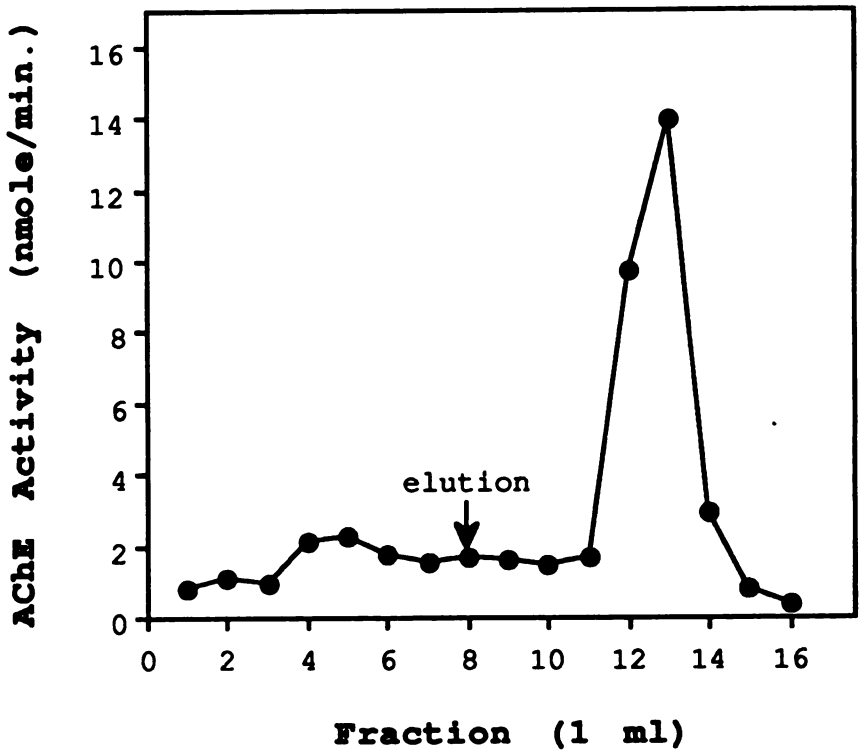


Figure 21. Affinity chromatography of electric eel acetylcholinesterase. The ligand used in the column was trimethylammonium aniline. The first 8 fractions were collected with running buffer. Fractions 9 to 16 were collected with 10 mM hexamethonium bromide.

elution fractions showed AChE activity, indicating that Colorado potato beetle AChE was not being retained by the column (Figure 22). This result was confirmed in another trial with even less AChE injected (ca. 0.003 units), again with no retention by the column.

Procainamide Column

A procainamide column was made based on the method of Bonning et al. (1988). Sepharose 4B gel (2 g) was swelled in 0.5 M sodium chloride, then washed and rinsed alternately with distilled water and 0.5 M NaCl. Procainamide (Sigma Chemical Co., St. Louis MO) in water (100 mg/15ml) was added to the washed gel in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (480 mg) and the pH was adjusted to 4.5 to 6.0. This mixture was stirred slowly at room temperature for 24 h. The solution was decanted from the gel, and the gel was washed alternately with 1 M NaCl buffered with 50 mM monobasic potassium phosphate and 1 M sodium chloride buffered with 50 mM dibasic sodium phosphate. Finally, the gel was washed with distilled water and slurried into a 2 x 10 cm column. The nominal flow rate was ca. 20 ml per h with a void volume of 4.0 ml.

The column capacity and function was tested with mosquito acetylcholinesterase. Homogenates of adult mosquitoes (*Aedes aegypti* (L.)) were made in phosphate buffer (pH 7.4) with 1 mg/ml Triton X-100. The 100,000 x *g*

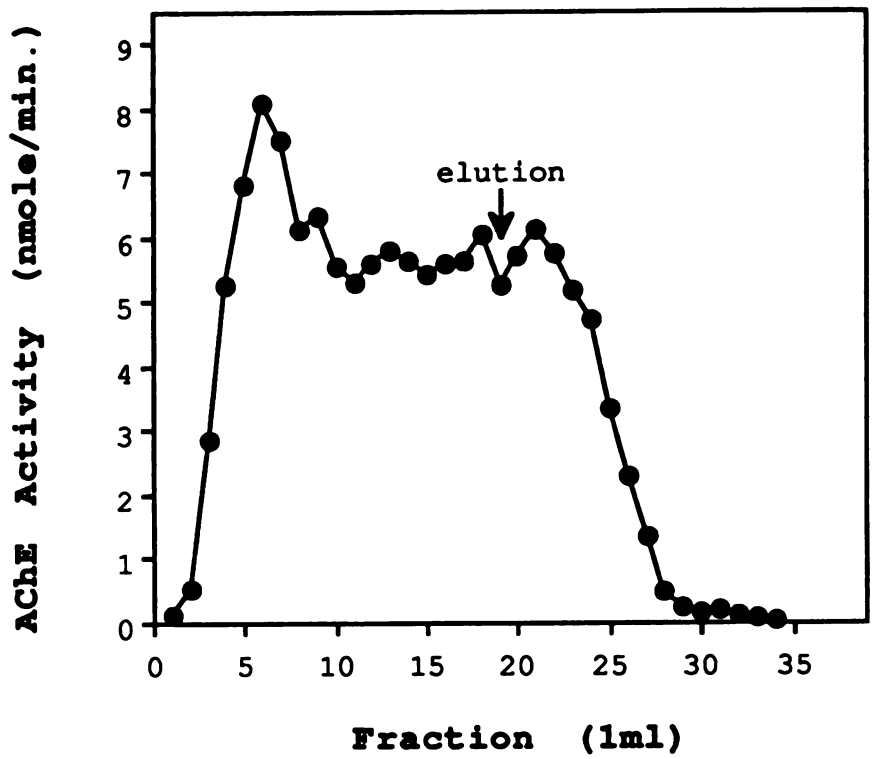


Figure 22. Affinity chromatography of Colorado potato beetle acetylcholinesterase. The ligand used in the column was trimethylammonium aniline. The first 20 fractions were collected with running buffer. Fractions 21 to 34 were collected with 10 mM hexamethonium bromide.

supernatant (3 ml) was loaded onto the column. There was a single peak of AChE activity which eluted with running buffer. The retained AChE activity was eluted with 50 mM decamthonium bromide. Three peaks of protein were eluted (Figure 23). These peaks were assayed for acetylcholinesterase activity using the methods described earlier (Chapter 3). Peak 1 showed a 10-fold purification, and peak 2 a 2-fold purification. There was no acetylcholinesterase activity in peak 3. Peaks 1 and 2 represented a total of 40% recovery of the total added activity (compared to 44% recovery and 7.5-fold purification obtained by Bonning et al. (1988)). Having established suitable conditions with mosquito acetylcholinesterase, the same technique was applied to Colorado potato beetle AChE. Homogenates were made as described for mosquitoes, although in some cases proteins were first concentrated by precipitation with ammonium sulfate. The 20 to 40% cut was centrifuged, then resuspended in phosphate buffer. At least 0.01 units of acetylcholinesterase (20 beetle equivalents) were loaded onto the column. A single, large peak was eluted with running buffer (Figure 24). There was no significant acetylcholinesterase activity retained by the column.

The poor performance of the two affinity columns, made it unprofitable to continue the purification. However it is possible that the acridinium resins of Gnagey et al. would give better results.

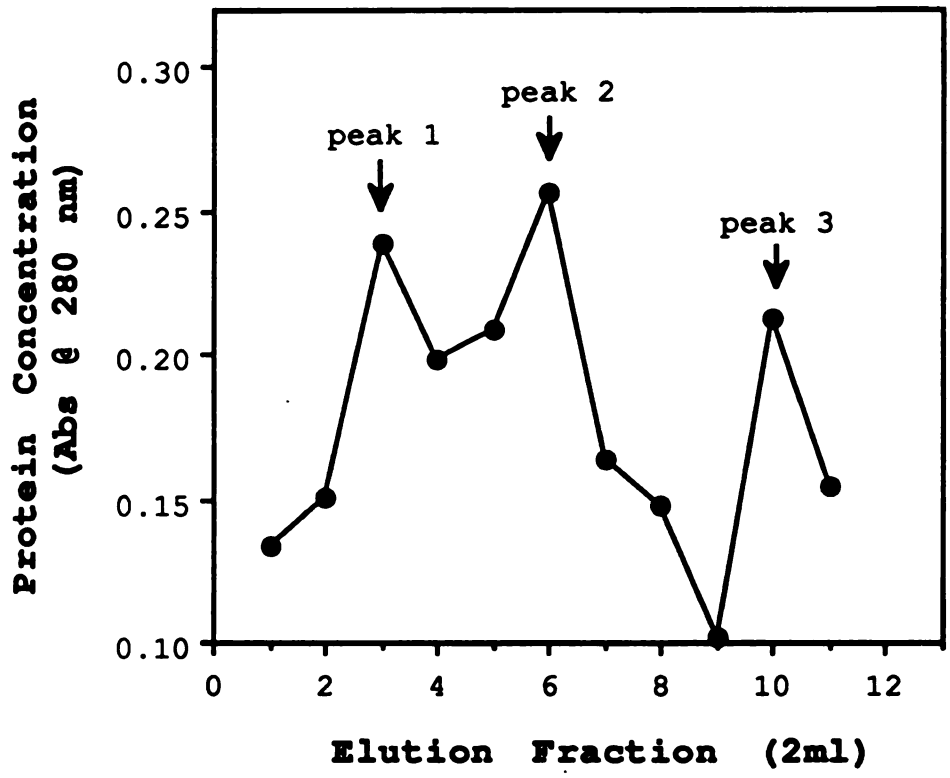


Figure 23. Procainamide affinity chromatography of mosquito acetylcholinesterase. Fractions were collected using 50 mM decamethonium bromide.

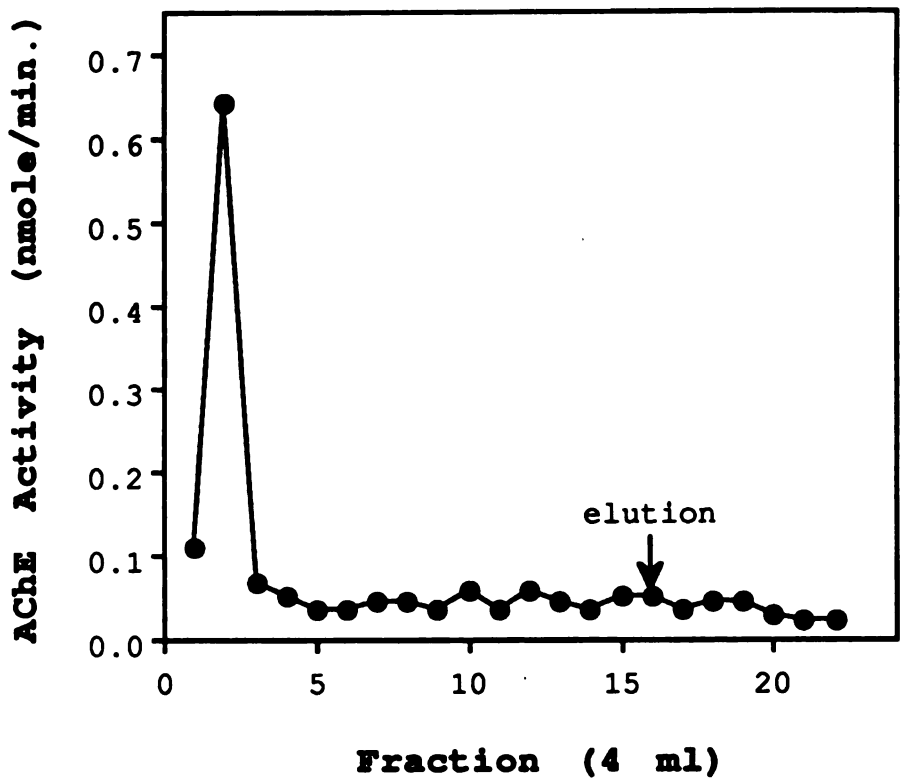


Figure 24. Procainamide affinity chromatography of Colorado potato beetle acetylcholinesterase. The first 16 fractions were collected with running buffer. Fractions 17 to 22 were collected with 50 mM decamethonium bromide.

References Cited

Berman J.D. and M. Young. 1971. Rapid and complete purification of acetylcholinesterases of electric eel and erythrocyte by affinity chromatography. Proceedings of the National Academy of Sciences 68: 395-398.

Bonning B.C., C.A. Malcom and J. Hemingway. 1988. Purification and characterisation of acetylcholinesterase from *Culex pipiens* Say. Pesticide Science 24: 278-280.

Gnagey A.L., M. Forte and T.L. Rosenberry. 1987. Isolation and characterization of acetylcholinesterase from *Drosophila*. Journal of Biological Chemistry 262: 13290-13298.

Lee A-H., R.L. Metcalf, and C.W. Kearns. 1974. Purification and some properties of house cricket (*Acheta domesticus*) acetylcholinesterase. Insect Biochemistry 4: 267-280.

Steele R.W. and B.N. Smallman. 1976. Acetylcholinesterase of the house-fly head affinity purification and subunit composition. Biochimica Biophysica Acta 447: 147-157.

Traylor P.S. and J.S. Singer. 1967. The preparation and properties of some tritiated diazonium salts and related compounds. Biochemistry 6: 881-887.

Tripathi R.K. and R.D. O'Brien. 1977. Purification of acetylcholinesterase from housefly brain by affinity chromatography. Biochimica Biophysica Acta 480: 382-389.

Zhu K.Y., W.A. Brindley and T.H. Hsiao. 1991. Isolation and purification of acetylcholinesterase from *Lygus hesperus* (Hemipter: Meridae). Journal of Economic Entomology 84: 790-794.