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The Characterization of Carbamate Resistance in the Colorado Potato Beetle, *Leptinotarsa decemlineata* (Say), in Comparison with the House Fly, *Musca domestica* (L)

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THE CHARACTERIZATION OF CARBAMATE RESISTANCE IN THE COLORADO POTATO BEETLE, *LEPTINOTARSA DECEMLINEATA* (SAY), IN COMPARISON WITH THE HOUSE FLY, *MUSCA DOMESTICA* (L)

by

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Randy L. Rose

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ABSTRACT

The Characterization of Carbamate Resistance in the Colorado Potato Beetle, Leptinotarsa decemlineata (Say), in Comparison with the House Fly, Musca domestica (L)

by

Randy L. Rose, Master of Science

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Major Professor: Dr. William A. Brindley
Department: Biology

Resistant and susceptible populations of the Colorado potato beetle, Leptinotarsa decemlineata (Say), and the house fly, Musca domestica (L), were compared with respect to carbofuran and carbaryl toxicity in the presence and absence of the synergist, piperonyl butoxide. Resistance levels of the New Jersey population when compared with the susceptible Logan population by topical application of carbaryl and carbofuran were > 833 and 820, respectively. A resistance level of 583 was determined from carbofuran bioassays of Rutgers and NAIDM house flies. Similar levels of resistance development between these species suggests the possibility that similar resistance mechanisms may be involved.

Utilization of the synergist difference approach for evaluating synergism of these carbamates by piperonyl butoxide indicated that

the resistant strains depended to a much greater extent upon detoxication by monooxygenases than did their susceptible counterparts. While piperonyl butoxide synergism resulted in completely restoring the Rutgers strain of house flies to levels of susceptibility, New Jersey Colorado potato beetles were able to retain a significant portion of their resistance. The purpose of this study was to confirm the role that monooxygenases played in Colorado potato beetle resistance and to investigate the possibility that decreased absorption was responsible for the degree of resistance retained following piperonyl butoxide pretreatment.

Microsomal preparations from Colorado potato beetle gut and fat body were devoid of measurable monooxygenase activity as determined from O-demethylation of p-nitroanisole, in spite of attempts to clear gut contents and optimize techniques. In contrast, microsomal preparations from Rutgers and NAIDM house flies demonstrated clear differences in oxidative potential between strains.

An analysis of the distribution of NADPH-cytochrome c reductase in Colorado potato beetle microsomes revealed a loss of enzyme activity from the microsomal pellet into the soluble fraction. The solubilization of this component of the electron transport chain is suggested as a possible limiting factor for *in vitro* characterizations of the involvement of cytochrome P-450 in xenobiotic metabolism.

Determinations of NADPH oxidation from microsomal preparations from house fly abdomens and Colorado potato beetle gut and fat body did not demonstrate quantitative differences between tissue sources nor between populations of either species. In a similar manner,

NADPH-cytochrome c reductase did not vary between house fly strains nor between tissue sources of the Colorado potato beetle. There was, however, approximately a two-fold difference in NADPH-cytochrome c reductase activity between resistant and susceptible populations of Colorado potato beetle larvae.

The in vivo distribution of 1-naphthyl-N-methyl (^{14}C) carbamate in resistant and susceptible Colorado potato beetle larvae demonstrated that although significant quantitative differences did not exist between populations with respect to the rate of penetration, excretion of the radiocarbon was significantly greater in the resistant New Jersey population.

This study has been successful in establishing that monooxygenases play a chief role in Colorado potato beetle resistance to carbamate insecticides. This role was confirmed in part by an increased rate of NADPH-cytochrome c reductase activity in the resistant population, however, traditional xenobiotic metabolism could not be confirmed by other methodology examined. This may be a result of the apparent solubilization of NADPH cytochrome P-450 reductase from microsmal preparations due to the unconfirmed presence of an endogenous inhibitor. Further characterizations of resistance mechanisms need to be examined for this destructive agricultural pest.

(103 pages)

INTRODUCTION

The Colorado potato beetle, Leptinotarsa decemlineata (Say), has been reported as a serious pest of potatoes and tomatoes in several states and provinces of the Eastern United States and Canada. Because heavy reliance has been placed on the use of pesticides for control of this and other pests of potato, resistance development has recently progressed to the point where few insecticides are capable of adequate control (Hare 1980, Harris and Svec 1981). Although the potato beetle has long been known to be resistant to DDT and other chlorinated hydrocarbons, there have been few attempts to quantitate resistance levels (Hofmaster and Dunton 1961, Harris and Svec 1976, 1981, Hofmaster et al. 1967) or understand their mechanisms. Few cases of resistance to organophophates or carbamates have been reported as such, although two recent evaluations of several insecticides in Connecticut and Quebec are indicative that high resistance levels have developed against previously recommended insecticides including carbaryl, carbofuran, phosmet, methamidophos, azinphosmethyl, and malathion (Hare 1980, Harris and Svec 1981). Since the recent development of resistance to these insecticides is extremely detrimental to both potato and tomato industries it is imperative that levels of resistance be quantitated and that mechanisms and genetics of resistance be understood for this pest.

Several methods are used to quantitate resistance levels between insect populations including biological assays, in vivo and in vitro metabolism of xenobiotics, penetration of radiolabeled substrates,

and determinations of various biochemical parameters including cytochrome P-450, NADPH-cytochrome P-450 reductase, and NADPH oxidation. Although in vitro methodology provides a better understanding of the role of various body tissues and enzymes it often does not correlate well with in vivo work (Schonbrod et al. 1968, Benke and Wilkinson 1971a, Benke et al. 1972). Therefore, it becomes important when attempting to quantitate resistance or tolerance that one examines several parameters in an attempt to correlate in vitro and in vivo results.

Resistance in insects is often the result of an increased capacity for insecticide detoxication and is generally due to oxidation by monooxygenases although other metabolic routes are also implicated (Brattsten and Metcalf 1970, Oppenoorth 1971, Fukuto 1973, Plapp 1976). The use of methylenedioxyphenyl synergists such as piperonyl butoxide, known to enhance the toxicity of many organophosphate and carbamate insecticides, results in overcoming resistance mechanisms involving oxidative enzymes to the extent that the LD_{50} values of resistant populations often approach susceptible levels (Casida 1970). This characteristic response to synergists resulted in the development of the synergist ratio as a means for quantitation of in vivo monooxygenase activity (Brattsten and Metcalf 1970, 1973b, Metcalf et al. 1971). Although the synergist ratio has been widely used to demonstrate oxidative differences between insect populations and species, it has failed in some instances to explain fine differences as may occur with age related changes in oxidative ability (Lee and Brindley 1974) or

differences in the metabolism of insecticide analogs (Kiso et al. 1977, Kurihara et al. 1977). The synergist difference has been suggested as an alternative estimate which can be used to determine the extent to which a population depends upon monooxygenases for detoxication (Brindley 1977).

This study examines a known resistant and two susceptible populations of Colorado potato beetles to establish baseline mortality data for the insecticides carbaryl and carbofuran. The synergist, piperonyl butoxide, was included in the study to generate synergist differences which can be used to estimate the degree to which the potato beetles depend upon monooxygenase enzymes. Results presented here suggest that resistance development in the Colorado potato beetle is due, at least in part, to increased oxidative potential in the resistant population.

In an attempt to correlate _in vivo_ data with _in vitro_ data and clearly establish that monooxygenases are involved in the resistance mechanism for potato beetles, _p_-nitroanisole-_O_-demethylation was investigated in microsomes from both gut and fat body. When the alteration of a variety of conditions failed to result in the production of the oxidative product, _p_-nitrophenol, other biochemical parameters were investigated.

Activity of monooxygenase enzymes is dependent upon the transfer of electrons from NADPH to the terminal electron acceptor, cytochrome P-450. The principle component of the electron transfer chain is considered to be NADPH-cytochrome P-450 reductase (Goldberg 1980, Nakatsugawa and Morelli 1976, Yang 1977). The induction of cytochrome

P-450 by xenobiotics has been associated with increased levels of nearly all enzymes associated with the microsomal fraction (Goldberg 1980, Ernster and Orrenius 1973). Since resistance development is, in essence, the genetically induced state of a population, Folsom and Hodgson (1970) hypothesized that the increased oxidative metabolism of xenobiotics would be accompanied by increased levels of cytochrome P-450, oxygen uptake, and NADPH oxidation. Since increases in NADPH-cytochrome c reductase and NADPH oxidation have subsequently been associated with increased levels of cytochrome P-450 in both resistant and induced insects (Folsom and Hodgson 1970, Ahmad and Forgash 1978, Gil et al. 1974, Wilkinson and Brattsten 1973), these components were examined to demonstrate differences between the oxidative potential of resistant and susceptible Colorado potato beetle larvae.

Although resistance in insects is generally the result of increased detoxication capability, in highly resistant populations it may often be accompanied by other factors such as reduced nerve sensitivity or absorption (Plapp and Hoyer 1968, Plapp and Casida 1969, Plapp 1973, Voss 1980). Inclusion of the synergist, piperonyl butoxide, in bioassays with carbaryl or carbofuran failed to restore the resistant population of potato beetles to levels approximating the susceptible level, suggesting that factors other than detoxication may be involved. As a result, the possibility that decreased absorption was a factor in resistance was examined in vivo by following the distribution of radiolabeled carbaryl over several time periods. The effect of synergism by piperonyl butoxide was also observed.

As a result of difficulties experienced in the O-demethylation

of p-nitroanisole in Colorado potato beetles, Rutgers and NAIDM house flies were incorporated into the study to verify procedural efforts as well as to serve as reference strains to which potato beetles could be compared. Carbofuran biological assays with and without piperonyl butoxide and in vitro determinations of p-nitroanisole-O-demethylation, NADPH oxidation, and NADPH-cytochrome c reductase were all determined for both populations of house flies, and results reported here in conjunction with those of the Colorado potato beetle.

The initial goal of this study was to demonstrate that synergist differences can be used to provide reasonable hypotheses about the role of monooxygenases which could be confirmed in vivo by carbaryl metabolism and in vitro by techniques commonly used for the quantitation of monooxygenases. Difficulties encountered due to a lack of developed methodology for the in vitro determination of monooxygenase activity in the Colorado potato beetle led to the inclusion of biochemical parameters which have been shown to be involved with resistance. Results of this study provide a broad base from which further research will benefit in its attempt to identify resistance mechanisms for the Colorado potato beetle.

REVIEW OF LITERATURE

Insecticide resistance is an international problem which currently plagues entomologists not only because of increased restrictions placed upon chemical use and development, but also because of the ever increasing numbers of resistant species combined with the advanced degree of resistance development within these species. An understanding of such development in various species can be helpful in providing alternative methods of insect suppression using chemical means currently available. Part of gaining this understanding involves the determination of the mechanisms for resistance. These mechanisms can be diverse, but most commonly resistance is due to an increased ability of an insect population to withstand chemical pressure as a result of enhanced monooxygenase capability.

This literature review includes discussions of insecticide resistance, the development of resistance in the Colorado potato beetle, the use of carbamate insecticides together with synergists to estimate detoxication potential in insects, the role of monooxygenases, phsiological factors influencing the determination of insecticide resistance, and some of the problems encoutered during the *in vitro* characterization of monooxygenase activity.

Insecticide Resistance

Resistance is defined as 'the developed ability in a strain of insects to tolerate doses of toxicants which would prove lethal to

the majority of individuals in a normal population of the same species' (Oppenoorth and Welling 1976). Although resistance generally develops as a result of repeated exposure to a given chemical over a period of time, evidence exists that it may sometimes develop in the absence of chemical contact as a result of other selection pressure (Margham 1975).

The development of resistance to one chemical often results in cross resistance, defined as 'the type of protection against several compounds resulting from the discrete action of one and the same mechanism' (Georghiou and Hawley 1971). Cross resistance can also give rise to multiple resistance which is an extension of resistance to include other mechanisms of resistance.

The genetic, biological, and operational influences in the evolution of resistance have been extensively reviewed by Georghiou and Taylor (1977a, 1977b), Georghiou (1980), Plapp et al. (1979), and Davidson and Zahar (1974). The speed with which selection occurs depends upon several factors including the frequency, number, and dominance of resistant alleles, the mode of reproduction, number of offspring, generation time, the presence of refugia and immigration, the life stage(s) selected, chemical persistence and relationship to other chemicals, the level of selection pressure, and the mode of application among others (Georghiou and Taylor 1977a, 1977b). Of these factors, only the last five are considered as operational factors, or factors which can be manipulated by man. The extent to which these factors and their interactions with other factors are understood and utilized in the field for control of a given pest will determine

the rate at which resistance selection will occur.

Once resistance is attained, a reduction of selection pressure results in a leveling off of resistance to levels slightly below its maximum state where it can remain for several generations at what may be considered a heterozygous level (Keiding 1967, Devonshire and Sawicki 1979). However, in some cases, the absence of selection pressure results in complete reversions to former levels of susceptibility (McDonald 1976). Regardless of the level of resistance found in a once resistant population, the genetic background is retained and with a renewal of selection pressure former levels of resistance are rapidly restored (Keiding 1967).

The biochemical genetics of resistance have been reviewed by Oppenoorth (1965), Brown (1967), Tsukamoto et al. (1968), Plapp and Casida (1967), Plapp (1976), and Georghiou and Taylor (1977a). The inheritance of resistance is primarily the result of allelism in one principle gene although multiple resistance genes are often implicated (Brown 1967), especially in cases of high resistance levels attained in the house fly (Plapp 1976). Genes conferring resistance to DDT and pyrethroids tend to be recessive while those involved in carbamate and organophosphate resistance have always proved to be semidominant in inheritance (Plapp 1976).

Detoxication of insecticides occurs in both resistant and susceptible insects but generally occurs at a more rapid rate in resistant strains due to increased enzyme activity. Oppenoorth and Welling (1976) suggested two ways in which selection affects enzyme activity leading to resistance. The most important is the

selection for an aberrant gene resulting in an enzyme possessing different properties due to an altered amino acid sequence. In a genetic study by Terriere et al. (1971) the offspring from a resistant and a susceptible strain of house flies possessed oxidase levels of intermediate activity. These results were interpreted to mean that 'the high oxidase strain differed from the low oxidase strain in the regulation of enzyme level, possibly by possessing more genes or gene sequences for increased production of detoxifying enzymes.' In a similar study, Plapp and Casida (1969) showed that an intermediate strain of house flies possessed oxidation levels closely approximating those of the parent resistant strain yet lacked the resistance ability possessed by that parent strain, indicating that the ability to oxidize insecticides is only partially responsible for the resistance levels in the strains tested.

The second manner in which selection affects enzyme activity is the selection of regulatory factors affecting the amount of enzyme produced (Oppenoorth and Welling 1976). Fewer examples exist for this approach, one of which involves the duplication of a structural gene which occurs in the peach potato aphid, Myzus persicae, resulting in the geometrical increase of one esterase corresponding to similar geometrical increases in resistance levels (Devonshire and Sawicki 1979).

Other mechanisms for resistance include altered behavioral patterns, storage of toxins, altered target site sensitivity, and differential penetration (Margham 1975, Plapp 1976, Voss 1980). Since the primary mode of action for most carbamates and organophosphates

is the inhibition of acetylcholinesterase, altered target site sensitivity was proposed as a major resistance mechanism (Oppenoorth 1971). Although changes in acetylcholinesterase sensitivity to inhibition by carbamates and organophosphates are capable of conferring high levels of resistance (Voss 1980), demonstrating that such inhibition is a major factor in their toxic action, this mechanism is not as biologically preferred as is resistance due to metabolism and changes in the rate of absorption.

Decreased rates of absorption have been reported for malathion resistance in the yellow fever mosquito, Aedes aegypti (L) (Matsumura and Brown 1963), for DDT (Pate and Vinson 1968), endrin (Polles and Vinson 1972), and fenitrothion (Plapp 1973) resistance in larvae of the tobacco budworm, Heliothis virescens (F); for DDT resistance of the spotted root maggot, Euresta notata (Weidemann) (Hooper 1965); and for DDT (Grigolo and Oppenoorth 1966), organotin (Plapp and Hoyer 1968), and organophosphate resistance in house flies, Musca domestica (L) (Forgash et al. 1962, Farnham et al. 1965, Hollingworth et al. 1967, Sawicki 1970, Plapp and Hoyer 1967, 1968).

Often a decreased rate of absorption does not, by itself, result in a substantial increase in resistance but when accompanied by a detoxication factor results in an increase of at least 5 to 10 times that caused by either factor alone (Sawicki 1970, Grigolo and Oppenoorth 1966, Plapp and Hoyer 1968). In addition to providing the insect with more time for detoxication, more opportunity is afforded the insect to rid itself of the toxicant by volatilization and rub-off (Forgash et al. 1962).

Plapp and Hoyer (1968) were able to demonstrate that the tin gene in the house fly confers resistance via decreased absorption to not only organotin compounds, for which its name was derived, but also to organophosphate and organochlorine insecticides. Thus decreased absorption may be a nonspecific mechanism which may readily extend to include several classes of insecticides.

Resistance Development in the Colorado Potato Beetle

The Colorado potato beetle, Leptinotarsa decemlineata (Say), is economically the most important of a complex of insect pests of potatoes which includes potato flea beetles (Epitrix cucumeris (Han)), aphids, leafhoppers, wireworms, white grubs, and cutworms (Hofmaster and Dunton 1961). If not controlled, potato beetle populations alone can completely defoliate and kill potatoes prior to tuber initiation (Moore and Hare 1979). In addition, Colorado potato beetle infestations on tomatoes have necessitated replanting in some instances and yield reductions of up to 90% in others (McClanahan 1975, Schalk and Stoner 1976a). Because heavy reliance has been placed upon pesticide use for control of this and other pests of potato and tomato, resistance development has recently progressed to the point where few insecticides provide adequate control (Hare 1980, Harris and Svec 1981).

The general pattern of resistance development in Colorado potato beetles was most recently reviewed by Harris and Svec (1981). DDT resistance was first reported in 1949 in the state of New York, only three years after its introduction, and by 1954 had spread to

several states including North Dakota, Minnesota, and Virginia (Brown 1971, Harris and Svec 1976). The subsequent spread of resistance to other chlorinated hydrocarbons was relatively rapid, resulting in their replacement by organophosphates and carbamates by the early 1960's. The principle carbamates recommended for Colorado potato beetle control have included carbaryl and carbofuran; the former for regular foilage applications and the latter for use as a systemic (Schalk and Stoner 1976b, Linduska 1978, Hofmaster et al. 1967, Hofmaster and Waterfield 1972, McClanahan 1975, McDonald 1976). In spite of constant selection pressure by these and other insecticides along with the fact that DDT resistant beetles were shown to be cross resistant to some organophophates (Hofmaster and Dunton 1961), resistance development has been relatively slow with the first documented reports appearing within the last two years (Hare 1980, Harris and Svec 1981).

The slow development of resistance to organophophates and carbamates is especially surprising in view of the fact that heavy reliance had been placed upon systemic applications of these insecticides in areas where the potato beetle is multivoltine. However, where the potato beetle is univoltine, such as in Alberta, Canada; carefully timed insecticide applications have resulted in less selection pressure and resistance development has been significantly slower as evidenced by continual use of DDT until as late as 1962 (McDonald 1976).

The reports by Hare (1980) and Harris and Svec (1981) indicate that resistance levels for previously labeled carbamates and

organophosphates including carbofuran, carbaryl, azinphosmethyl, phosmet, phorate, malathion, methamidophos, and methidathion are substantially great enough as to necessitate replacement. Insecticides currently recommended for control of these resistant populations include only aldicarb and those pyrethroids currently registered for use. However, DDT resistance in potato beetles is known to confer cross resistance to pyrethroids with the possible result being that resistance to these insecticides may be expected soon.

The evaluation of resistance development in the Colorado potato beetle has been hampered somewhat by the fact that, with the exception of the two most recent studies (Hare 1980, Harris and Svec 1981), there has been no attempt to establish susceptible levels or to use standardized methods established by the FAO for the determination of Colorado potato beetle toxicity data (Anon. 1974). It is possible that had such measures been incorporated, detection of carbamate and organophosphate resistance may have occurred as early as 1975 since several reports indicated increased difficulty in obtaining satisfactory potato beetle control, resulting in several evaluations of insecticide efficacy (McClanahan 1975, McDonald 1976, Harris and Svec 1976, Linduska 1978).

For example, the study by Harris and Svec (1976) evaluated four populations of potato beetles revealing two strains which had developed resistance to endosulfan, aldrin, endrin, and DDT. They made the observation that in a previous study by McClanahan (1975) there had been an indication of resistance development to endosulfan but it had gone undetected, presumably due to a lack of comparisons

with earlier literature. Had there been toxicity data for a susceptible strain, however, such a comparison would not have been necessary.

In addition to the problems caused by the lack of reference susceptible strains is the absence of standardization of technique. Of the four reports cited above, one used oral toxicity in combination with simulated field trials and microplot testing, two used the Potter spray tower, one in combination with systemic field trials; and the last used only systemic field trials. Although all the above testing happened to involve larvae, none used larvae of the same instar. Such conflicts in methodology make comparisons between studies difficult although each method provides useful data under given conditions.

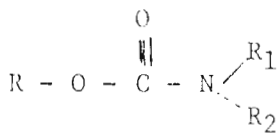
The careful quantitation of resistance for several insecticides in both susceptible and resistant populations using standardized methodology provides valuable information which can be used to detect and monitor resistance. It can also be used to provide clues for possible steps which can be taken to avoid the development of such resistance. Since naturally occurring enemies of the Colorado potato beetle are generally ineffective as control agents and resistance development to insecticides can occur in a short time, it is imperative that levels of resistance for various insecticides be quantitated and that mechanisms of resistance be understood for this pest.

Carbamate Insecticides

Carbamates are structurally related to physostigmine, the

principle alkaloid from the plant Physostigma venonosum, which was known to be an inhibitor of cholinesterase (Matsumura 1975). Chemical analogs of physostigmine were generally ineffective as insecticides due to their low lipoid solubility. However, those which were lipoid soluble were highly toxic to house flies and thrips (Kolbezen et al. 1954).

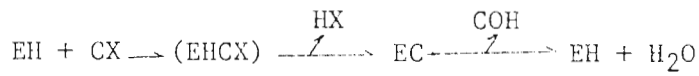
Carbamate insecticides currently used are esters of carbamic acid of the general structure



Their general characteristics, mode of action, and metabolism have been extensively reviewed by Casida (1963), O'Brien (1963, 1976), Dorough (1970), Wilkinson (1968), Matsumura (1975), Kuhr (1970), Wustner et al. (1978), and Brooks (1972).

Carbamates tend to be very selective against the cholinesterases of various species; generally having low mammalian toxicity, but not always, and possessing a large range of specificity against insect species as well (Brooks 1972).

The reversible mode of action of carbamates is described by the equation



where EH represents the esteratic site, CX represents the carbamate and leaving group, and EHCX and EC represent the complex between the esteratic site and the carbamate (Matsumura 1975). It is generally believed that the binding of the carbamate to the esteratic

site is the most important factor in determining the toxicity of a particular carbamate since the affinity for the acetylcholinesterase receptor site appears to be the most important factor in determining toxicity (O'Brien 1976, Matsumura 1975). Unlike organophosphate inhibition, the carbamylation step is relatively easy, regenerating recovered enzyme when carbamate complexing is no longer possible.

Carbamate metabolism in plants, animals, and insects has been extensively reviewed by Dorough (1970), Kuhr (1970), Fukuto (1973), and Matsumura (1975). Metabolism of carbamates was long considered to be the result of hydrolysis of the carbamate moiety and side chains resulting in the accumulation of a great deal of evidence in support of this hypothesis prior to the discovery of other metabolic pathways (Casida 1963). It is now known that metabolism of carbamates occurs primarily as a result of oxidative processes with hydrolysis and conjugation playing secondary roles. Carbamate metabolism generally results in products which are less toxic than their parent compound.

Oxidative reactions in carbamates are generally of two types; ring hydroxylation and side chain oxidation, although N-dealkylations and thioether oxidations also occur. In mammals, hydrolysis is considered a major pathway for most carbamates (Hurst and Dorough 1978) but its importance declines progressively for plants and insects. Patterns of metabolism for mammals, insects, and plants are very similar with major differences occurring in the nature of conjugation. While insect conjugates are composed of a combination of sulfate, phosphate, and sugar conjugates which are readily

eliminated, mammals are lacking in sugar conjugates while plant conjugates are predominantly composed of sugar conjugates which are stored (Kuhr 1970, Devonshire 1973).

The Use of Synergists as a Means for Estimating Detoxication Potential

A synergist is a compound which, 'when applied in combination with an insecticide is able to enhance the activity of the insecticidal component of the formulation' (Wilkinson 1966). Synergists therefore, are used to limit the ability of an organism to withstand chemical pressure by blocking the site at which detoxication of that compound occurs. Thus, more of the insecticidal component is enabled to reach the site of intoxication resulting in the enhancement of the chemical effect at that site.

The methylenedioxyphenyl compounds, such as piperonyl butoxide, sesamex, n-propylisome, and sulfoxide have long been known for their synergistic effects upon pyrethroids. The discovery by Moorefield (1958) that these compounds exerted a powerful effect upon the toxicity of methyl and dimethyl carbamates resulted in several studies in an attempt to elucidate their mode of action. Sun and Johnson (1960) first suggested that the mode of action of these synergists was through inhibition of metabolism. Several studies have since confirmed that methylenedioxyphenyl compounds exert their synergistic effect by acting as non-competitive substrates for the monooxygenase enzymes (Hodgson and Philpot 1974, Kulkarni and Hodgson 1978).

The role of synergists on metabolic transformations has been

elucidated in several insect species (Georghiou and Metcalf 1961, Shrivastava et al. 1969, Kuhr 1970, Guirguis and Brindley 1975, Ahmad et al. 1980, Osman and Brindley 1981). Georghiou and Metcalf (1961) demonstrated very early that both the absorption and excretion of carbamates in a resistant strain of house flies was significantly reduced by pretreatment with piperonyl butoxide, resulting in a seventeen-fold internal accumulation of the insecticide. The formation and excretion of insecticide metabolites has been shown to be severely inhibited by piperonyl butoxide and related synergists in house flies (Shrivastava et al. 1969, Kuhr 1970), in alfalfa leafcutting bees, Megachile pacifica (Guirguis and Brindley 1975), in the gypsy moth, Lymantria dispar (L) (Ahmad et al. 1980) and in three species of grass bugs, Labops sp. (Osman and Brindley 1981).

Since genetically acquired resistance is often associated with enhanced monooxygenase activity and synergists effectively inhibit these enzymes, synergists are often used in the characterization of resistance levels. Synergist treatments of susceptible insect populations as well as some tolerant insect species often have negligible effects upon insecticide toxicity due to low levels of monooxygenase enzymes. The same treatments applied to resistant insects, however, result in large synergistic effects, often increasing the toxicity of the selecting compound several-fold. When this occurs, the susceptibility of the resistant strain often returns to levels approaching those of the susceptible strain (Casida 1970).

Observations of these and similar relationships led to the

development of the synergist ratio, defined as the ratio of the topical LD_{50} of the insecticide alone to the LD_{50} of the insecticide plus the synergist. The synergist ratio was first suggested as a measurement for detoxication potential in the house fly by Fukuto et al. (1962), but was developed more thoroughly for several insect species as a quantitative measure of *in vivo* detoxication rates by Brattsten and Metcalf (1970, 1973b).

Although the synergist ratio has been widely used to demonstrate oxidative differences between insect populations and species, it has failed, in some instances to explain fine differences as may occur with age related changes in oxidative ability (Brattsten and Metcalf 1973a, Lee and Brindley 1974) or differences in the metabolism of insecticide analogs (Kiso et al. 1977, Kurihara et al. 1977). In addition, the low synergist ratio observed for the honey bee was interpreted by Metcalf et al. (1966) and Brattsten and Metcalf (1970) to mean that the honey bee was without the protection of a detoxifying system. A subsequent study by Gilbert and Wilkinson (1974), however, provides evidence that the honey bee possesses a monooxygenase system capable of metabolizing aldrin *in vivo* to levels nearly equivalent to those of the house fly.

Guirguis and Brindley (1975) demonstrated that in the alfalfa leafcutting bee, *Megachile pacifica*, carbaryl metabolism by 1-day-old males or 4-day-old females was greater than in 4-day-old males, yet the synergist ratio for the former two was less than for the latter. This observation led to the use of the difference between synergized and unsynergized LD_{50} doses. Similar differences were obtained from

1-day-old males and 4-day-old females corresponding to similar carbaryl persistance, while 4-day-old males had the smallest difference and the greatest internal persistance of carbaryl.

In addition, Lee and Brindley (1974) had demonstrated that as male leafcutting bees aged, carbaryl toxicity decreased due to decreasing detoxication by monooxygenases as measured by EPN detoxication *in vitro*, while synergist ratios actually increased. When the regression line for male leafcutting bees was calculated from the synergist ratios plotted against LD_{50} values and compared to that calculated from female leafcutting bees (Figure 1) it was found that the regression lines had slopes of opposite sign (Brindley 1977). The addition of 73 other synergist ratios taken from Brattsten and Metcalf (1970, 1973b) did not establish a clear distribution of points. These observations led to a reconsideration of the physiological interpretation of synergism. Since a synergist acts by inhibiting the detoxication of an insecticide an alternative approach to the synergist ratio might be viewed as a difference. As a consequence, Brindley (1977) proposed that the synergist difference, defined as the difference between the LD_{50} of the insecticide alone and the LD_{50} of the insecticide when in combination with the synergist, be used as an alternative interpretation of insecticide-synergist toxicity data. When a regression line was calculated from synergist differences plotted against carbaryl LD_{50} values from alfalfa leafcutting bees and combined with data points taken from Brattsten and Metcalf (1970, 1973b) the points formed an orderly pattern relative to the line (Figure 2) given by the equation

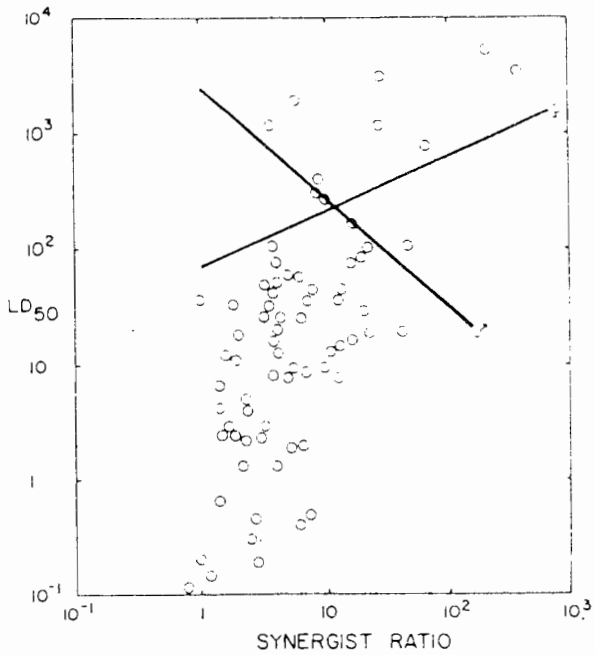


Fig. 1. Relationship of the synergist ratio to carbaryl LD_{50} values of male and female leafcutting bees and several other insect species from Brattsten and Metcalf (Brindley, 1977)

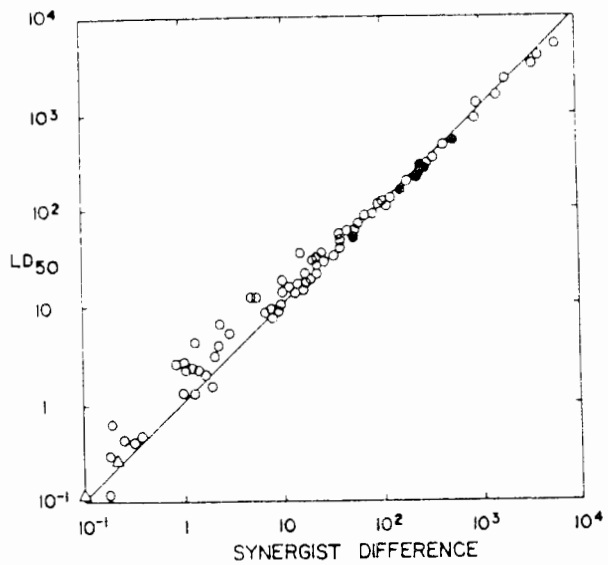


Fig. 2. Relationship of the synergist difference to carbaryl LD_{50} values of male and female leafcutting bees and several other insect species from Brattsten and Metcalf (Brindley, 1977)

(1) Log LD_{50} = 1.014 log SD + 0.01

Table 1 illustrates, using data taken from Brattsten and Metcalf (1970), the possible advantages which may be obtained by use of the synergist difference versus the synergist ratio. Two species, S. yuccae and A. eugenii, were selected from the family Curculionidae; both of which possess very different levels of tolerance yet have similar synergist ratios. Clearly, the larger synergist difference calculated for S. yuccae appears to better correlate with the tremendous tolerance of this species when compared to that of A. eugenii. Two species selected from the order Hemiptera, however, possessed very similar LD_{50} values yet very different synergist ratios. Again, the synergist difference seems to better account for differences in the metabolic capacity of these insects. Finally, A. pulicaria and R. aeneus possess very different levels of susceptibility to carbaryl, yet are synergized to an equal extent resulting in identical synergist ratios. The synergist differences for these two species demonstrate a 10-fold difference which is more indicative of the 10-fold difference in tolerance to carbaryl. These data, and other comparisons which could be taken from Table 1 poignantly illustrate the potential of the synergist difference in detecting fine differences not only between insect species and populations but also between ages, sexes, and possibly nutritional status.

Brattsten and Metcalf (1973a) examined three species of fleshflies, one blowfly, and two muscid flies for age related changes in carbaryl tolerance with and without the synergist, piperonyl

Table 1. Toxicity (μg/g) of carbaryl alone and with piperonyl butoxide to various insects: A comparison of the synergist ratio (SR) with the synergist difference (SD).

Insect Species	LD_{50}	SLD_{50}	SR	SD
Coleoptera				
Curculionidae				
Scyphophorus yuccae	2200	365	6.0	1835
Anthonomus eugenii	68	12.5	5.4	55.5
Hemiptera				
Lygaeidae				
Oncopeltus fasciatus	32.5	1.5	21.7	31
Corimelaenidae				
Allocoris pulicaria	30	9	3.3	21
Coleoptera				
Curculionidae				
Rhynchites aeneus	3	0.9	3.3	2.1

(Adapted from Brattsten and Metcalf (1970))

butoxide. Although great fluctuations in tolerance were found to occur with age within a given species, the synergized LD_{50} values for that species were found to lie within a fairly narrow range. The synergized value, therefore, may represent the 'innate' toxicity of the insecticide. Consequently, if a synergist is completely effective in inhibiting the monooxygenase enzymes, Equation 1 can be used to calculate a 'theoretical' synergist difference from the unsynergized LD_{50} value (Brindley 1977, Osman and Brindley 1981). This theoretical difference may then be compared with the actual synergist difference obtained from biological assays to determine the degree to which an insect depends upon monooxygenases for detoxication; or percent dependency.

$$(2) \quad \% \text{ Dependency} = \frac{\text{Actual Synergist Difference}}{\text{Theoretical Synergist Difference}} \times 100$$

Therefore, if the observed synergist difference is roughly comparable to the calculated synergist difference, the percent dependency should be close to 100%.

In a study of three species of grassbugs, Labops hesperius, L. hirtus, and L. utahensis (Osman and Brindley 1981), a correlation between the synergist difference and the relatve tolerances of these three species to carbaryl was observed; L. hesperius being the most tolerant (LC_{50} = 0.65 g/vial) and possessing the greatest synergist difference (SD = 0.38) and L. utahensis having the least tolerance (LC_{50} = 0.013 g/vial) and possessing the smallest synergist difference (SD = 0.004). Thus, the decrease in susceptibility between the three species was associated with an increased percent dependency

upon monooxygenases for carbaryl detoxication, with L. hesperius being highly dependent upon monooxygenases (approximately 60%) and L. utahensis having a decreased dependency upon monooxygenases (approximately 30%).

In comparison with other insects grassbugs are extremely susceptible to carbaryl as are honeybees, yet the calculated percent dependencies for both species can be as high as 60% in the former, and 65-85% in the latter (Osman and Brindley 1981). The percent dependency values, therefore, are not necessarily an indication of the susceptibility or tolerance of an insect species, but rather reflect the extent to which a species relies upon monooxygenases for insecticide detoxication. If resistance is due to increased monooxygenase activity, the calculation of percent dependency should demonstrate an increased reliance of resistant insects upon monooxygenases in comparison with their susceptible counterparts.

The Monooxygenase System

By far, the most important method of protection against xenobiotics for both mammals and insects involves the utilization of the mixed function oxidase or monooxygenase system. Monooxygenases are characterized by a dependency upon molecular oxygen and NADPH for activity, while they are inhibited by carbon monoxide and benzodioxazole synergists. Although their basic role is the metabolism of endogenous substrates including cholesterol, fatty acids, and steroids (Blumberg, 1978) their function in xenobiotic metabolism has received much greater attention.

The reactions and characteristics of the monooxygenase system

have been thoroughly reviewed by Nakatsugawa and Morelli (1976), Fukuto (1973), Kuhr (1970), Sato and Omura (1978), Brooks (1972), Wilkinson (1968), and Matsumura (1975). The reactions are very diverse including deamination, demethylation, dealkylation, aromatic hydroxylation, alkyl and N-hydroxylation, ester bond cleavage, epoxidation, oxidation of sulfides to sulfoxides and sulfones, conversion of phosphorothioates to phosphates, conversion of methylenedioxyphenyls to catechols, and oxidation of alcohols and aldehydes to acids (Matsumura 1975). The role of oxidation is the conversion of nonpolar molecules to more reactive polar molecules which can either be excreted directly or undergo conjugation in preparation for excretion. This conversion generally results in detoxication of the molecule but toxication can also occur. The principle component of the monooxygenase enzyme system is a group of ubiquitous enzymes with a characteristic carbon monoxide difference spectrum at 450 nm, known collectively as cytochrome P-450.

The role of cytochrome P-450 in xenobiotic transformations and steroid metabolism has been most recently reviewed by Blumberg (1978), Ullrich (1979), Mitani (1979), and Sato and Omura (1978). Other reviews, particularly of insect cytochrome P-450, include those by Agosin (1976), Hodgson et al. (1974), Hodgson and Tate (1976), Kulkarni and Hodgson (1976), Wilkinson and Brattsten (1973), Yu and Terriere (1979), and Terriere and Yu (1979). Cytochrome P-450 has been described as the site of oxygen activation and substrate interaction in the oxidative transformation of xenobiotic compounds (Wilkinson and Brattsten 1973). Cytochrome P-450 has been

characterized, with respect to its spectral properties, to some extent in nearly all species examined; including vertebrates, invertebrates, marine organisms, bacteria, bacteroids and yeast (Blumberg 1978, Kulkarni et al. 1976, Hodgson and Tate 1976). In all of these organisms, with the exception of bacteria, monooxygenase activity has been associated with either the endoplasmic reticulm or the inner mitochondrial membranes (Ullrich 1979).

The catalytic events involved in xenobiotic oxidation have been reviewed by Mitani (1979), Nakatsugawa and Morelli (1976), Blumberg (1978), and Ullrich (1979), and are schematically illustrated in Figure 3.

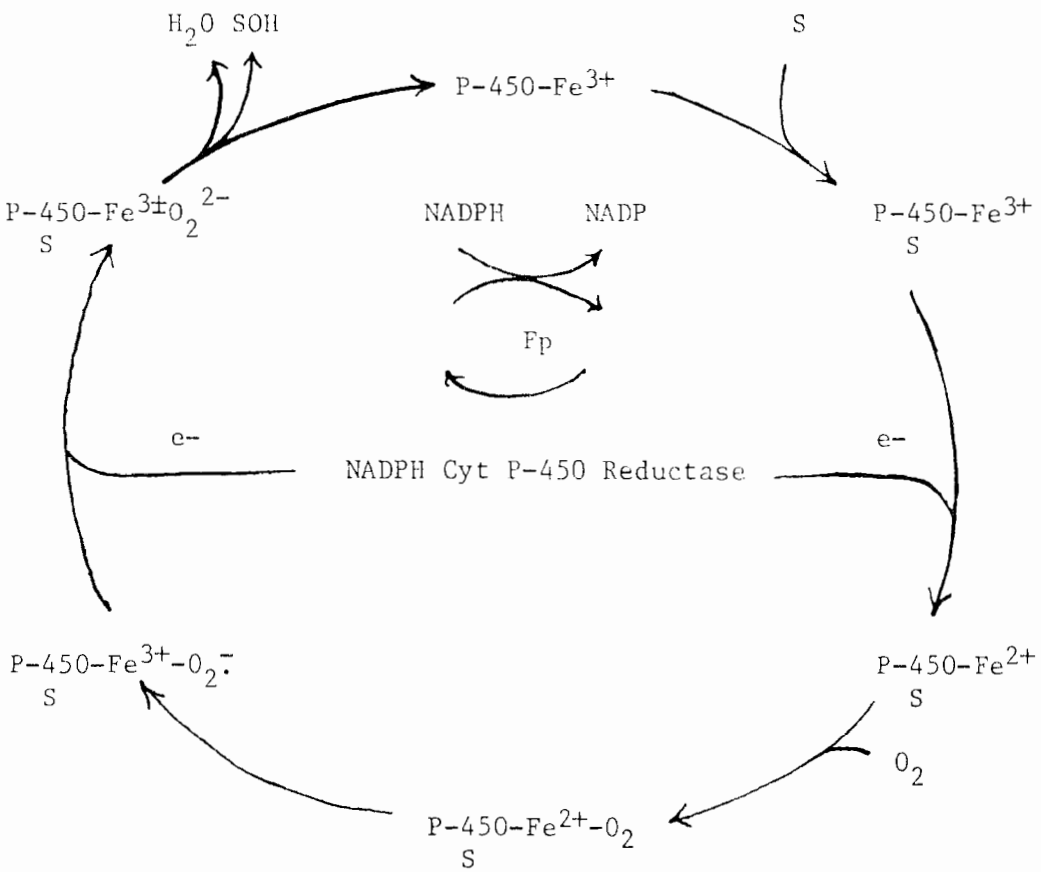


Figure 3. Hypothetical mechanism of xenobiotic metabolism by cytochrome P-450. S: substrate, Fp: flavoprotein. (Adapted from Mitani, 1979)

The initial step is the binding of the substrate to the oxidized form of cytochrome P-450. This is followed by what has been proposed as the rate limiting step of the reaction sequence (Gillette and Gram 1969, Masters et al. 1973, Ullrich 1979); the reduction of the cytochrome P-450-substrate complex by the transfer of an electron from NADPH by NADPH-cytochrome P-450 reductase in microsomes, or by NADPH-adrenodoxin reductase in mitochondria. The third step involves the addition of molecular oxygen to form an oxygenated-cytochrome P-450-substrate complex which is activated by a second electron, resulting in the rapid release of the hydroxylated product, water, and cytochrome P-450. The transfer of the second electron is thought to be mediated by NADPH-cytochrome P-450 reductase but also may involve NADH-cytochrome b_5 reductase (Nakatsugawa and Morelli 1976, Ullrich 1979).

The oxidations catalyzed by cytochrome P-450, although often broad in spectrum, can be very specific; especially those involving steroid metabolism. This observation has led to the hypothesis that several forms of cytochrome P-450 exist (Blumberg 1978), many of which possess 'slightly different, but overlapping substrate specificities' (Ullrich 1979). In insects, differences in the nature of cytochrome P-450 have been associated with induction and resistance. Thus, induced insects have been shown to have a maximum absorbance at 448 nm while the absorbance of controls occurs at 450 nm. Similarly, cytochromes P-448 and P-450 (regulated in house flies by genes on chromosomes IV and II, respectively) are involved in resistance, while cytochrome P-452 is characteristic of susceptible

strains (Agosin 1976, Hodgson et al. 1974).

The reduction of the cytochrome P-450-substrate complex by NADPH-cytochrome P-450 reductase has been demonstrated by Gillette and Gram (1969), Ernster and Orrenius (1965), and Orrenius et al. (1969). Stripp et al. (1972) demonstrated the existence of a 1:1 ratio for substrate oxidation and NADPH oxidation. Thus, the addition of cytochrome c to microsomes results in the inhibition of xenobiotic oxidation due to a loss of electron transfer to cytochrome P-450 (Gillette and Gram 1969).

A great deal of evidence indicates that the reduction of the cytochrome P-450-substrate complex is the rate limiting step for monooxygenase activity. Gillette and Gram (1969) showed that assays of NADPH-cytochrome c reductase activity were roughly proportional to N-demethylase activity in several mammalian species. Masters et al. (1973) demonstrated that antibodies inhibiting NADPH-cytochrome c reductase activity also inhibited N-demethylation of ethylmorphine to an equal extent, demonstrating that the metabolism of ethylmorphine was 'absolutely dependent upon electron transfer via NADPH-cytochrome c reductase.'

Both NADPH-cytochrome P-450 reductase and cytochrome b_5 are readily solubilized by the addition of trypsin to the microsomal fraction. The addition of increasing amounts of trypsin results in a stepwise solubilization of NADPH-cytochrome c reductase (measured by observing the rate of reduction of cytochrome c, an exogenous source) which parellels the inactivation of NADPH-cytochrome P-450 reductase (assayed by measuring the rate of formation of the reduced

cytochrome P-450-CO complex at 450-457 nm following the addition of NADPH) as well as the associated NADPH-linked monooxygenase and lipid peroxidation activities (Orrenius et al. 1969). While NADPH-cytochrome P-450 reductase and cytochrome b_5 are solubilized by the addition of trypsin the major portion of cytochrome P-450 and NADH-cytochrome b_5 reductase remain in the membrane bound state. Because of this interaction Ito and Sato (1969) proposed that the former two enzymes are located on the outer layer of the microsomal vesicles. It is currently believed that when cytochrome P-450 reductase is cleaved off the microsomal membranes that it loses the hydrophobic portion of the molecule which is necessary for the interaction with cytochrome P-450 but not for cytochrome c (Ullrich 1979).

Resistance in insects has been described by Folsom et al. (1970) as a genetically determined "fully induced" state. Induction by many insecticides, phenobarbital, and other xenobiotics is preceded by an increase in mRNA and protein synthesis, resulting in an increase in the quantities of several microsomal monooxygenase enzymes (Agosin 1976, Blumberg 1978, Ernster and Orrenius 1973) including cytochrome P-450 and NADPH-cytochrome c reductase. Although induction has been shown to be 'accompanied by an increase in the level of cytochrome P-450, the corresponding increase in monooxygenase is not always proportional' (Agosin 1976, Hodgson and Tate 1976, Yu and Terriere 1979). Hence, the study by Folsom et al. (1970) demonstrated that metabolism of xenobiotics in resistant house flies was approximately 5-fold greater than in susceptible house flies while differences in measurements of cytochrome P-450,

NADPH oxidation, and oxygen consumption never exceeded 2.5-fold. As a result, Yu and Terriere (1979) suggest that qualitative differences such as the variability in the types of cytochromes and the presence of high and low spin states may be more important than the quantitative differences in cytochrome P-450 content in determining the resistance or susceptibility of a given strain of insects.

The Influence of Physiological Factors Upon Monooxygenase Activity

The influence of physiological factors upon monooxygenase activity has been reviewed by El Aziz et al. (1969), Matthews and Casida (1970), Perry and Buckner (1970), and Wilkinson and Brattsten (1973). Factors of most importance include sex, age, life stage, and nutritional status.

Females are known for their greater detoxication potential and correspondingly greater levels of enzyme activity and cytochrome P-450 than their male counterparts. Osman and Brindley (1981) demonstrated that in three species of grass bugs (*Labops sp.*), males were more susceptible to carbaryl and had less overall metabolic capacity than did females. Similar findings were reported in the American cockroach, *Periplanta americana* (Turnquist and Brindley 1975), house flies and German cockroaches, *Blatella germanica* (El-Aziz et al. 1969), and in house crickets, *Acheta domesticus* (L) (Benke and Wilkinson, 1971a). Perry and Buckner (1970) demonstrated that observed differences between sexes in monooxygenase activity and insecticide toxicity could be explained by increased levels of

cytochrome P-450 in female house flies.

Age has a profound influence upon both *in vitro* and *in vivo* experiments with insects. Benke and Wilkinson (1971a) demonstrated that in adult house crickets epoxidase activity *in vitro* increased with age to peak at two weeks corresponding to a maximal tolerance for carbaryl which was attained at three weeks. In a similar study, Turnquist and Brindley (1975) showed that in the American cockroach carbaryl toxicity was extremely age dependent; correlating well with levels of cytochrome P-450, EPN detoxication, and *p*-nitroanisole-*O*-demethylation.

Several reports deal with the influence of life stage upon monooxygenase activity in insects (Ahmad and Forgash 1975, Gould and Hodgson 1980, Benke et al. 1972, Krieger and Wilkinson 1969, Wilkinson and Brattsten 1973, El-Aziz et al. 1969). Generally, activity is lowest following ecdysis, increases to a maximum at mid-stadium, and rapidly decreases preceding ecdysis (Benke et al. 1972, Ahmad and Forgash 1975, Wilkinson and Brattsten 1973). In addition, monooxygenase activity in the final instar is often many times greater than in previous instars (Krieger and Wilkinson 1969, Ahmad and Forgash 1978, Ahmad et al. 1980). Even within a single instar monooxygenase activity has been known to increase by as much as 28-fold (Gould and Hodgson 1980).

Nutrition has long been known to be an important factor influencing monooxygenase activity but its full importance is yet to be understood. El-Aziz et al. (1969) noted that house flies given a diet of milk were more tolerant of carbamates than those fed on sucrose. Perry

and Buckner (1970) confirmed this and associated it with an increase in cytochrome P-450. Ahmad and Forgash (1975) observed that boll-fed boll weevils were more tolerant to insecticides than bloom-fed boll weevils. The same relationship was shown by them to be true of monooxygenase activity in gypsy moths fed an artificial diet instead of their regular host plant. In a similar study, Yu et al. (1979) showed that cutworm larvae fed mint leaves and mint constituents were more tolerant to carbaryl than those fed on beans and that corresponding increases in oxidase activity and cytochrome P-450 also occurred. Classic studies by Krieger et al. (1971) and Brattsten et al. (1977) demonstrate that in lepidopterans greater monooxygenase activity is associated with polyphagy and that secondary plant chemicals are capable of inducing monooxygenase levels sufficiently to provide for increased protection while feeding.

Several Considerations Relating to In Vitro Studies

The use of *in vivo* biological assays provides valuable information with respect to the ability of a given insect population to withstand insecticide selection pressure and can suggest mechanisms which may be involved in the metabolic processes. However, in order to gain an understanding of the biochemical nature and mechanism of the enzyme system or systems involved, *in vitro* studies are generally considered to be necessary. *In vitro* studies are also used to provide more precise information as to the metabolic pathways which may have been suggested by corresponding *in vivo* studies (Wilkinson 1979). Although *in vitro* studies provide for a better understanding of the role of

various body tissues and enzymes they often do not necessarily correlate with *in vivo* work (Schonbrod et al. 1968, Benke and Wilkinson 1971a, Benke et al. 1972, Gould and Hodgson 1980).

Several problems are associated with the *in vitro* quantitation of monooxygenases in insect tissues. Chief among these is the fact that insect tissue sources are severely limited, not only by their small size but also because of the numbers of insects which need to be processed at one time for analysis. The use of whole insect preparations is generally prohibitive not only due to the heterogeneous nature of such a preparation but also because of the presence of endogenous inhibitors. As a result, much *in vitro* methodology depends upon insect species which are not only large enough to be amenable for simple dissections but which can also be reared in large enough numbers to provide, within a small time frame, sufficient tissue from insects of a particular physiological condition to allow for assays.

The presence of endogenous inhibitors has been noted with many insect species. Gilbert and Wilkinson (1974) found that in the honeybee, *Apis mellifera* (L), at least 90% of the activity of the intact midgut was lost when this organ was opened by longitudinal incision. Potent inhibitors of monooxygenase activity have been associated with the gut contents of southern armyworm *Spodoptera eridania* (Cramer) (Krieger and Wilkinson 1970), cabbage loopers *Trichoplusia ni* (Hübner) (Kuhr 1971); the house cricket *Acheta domesticus* (L) (Brattsten and Wilkinson 1973), and a caddisfly larva, *Limnephilus sp.*(Brattsten and Wilkinson 1973). Other inhibitors have been found to be associated with the eye pigment

xanthommatin, in house flies, Musca domestica (L) (Schonbrod and Terriere 1971a, 1971b), fruit flies, Drosophila melamogaster, and honeybees, Apis melliifera (Brattsten and Wilkinson 1973). Tyrosinase is still another inhibitor which is found both in preparations from prepupal lepidopterans and in house fly preparations.

Several of the endogenous inhibitors associated with insect tissues have been associated with the inhibition of electron transfer from NADPH to cytochrome P-450 either by acting as an electron sink at the flavoprotein NADPH-cytochrome P-450 reductase or by its solubilization from the microsomal membranes (Wilkinson 1979). The insect eye pigment, xanthommatin, causes a marked enhancement of NADPH oxidation at concentrations as low as $4X10^{-7}M$, due to its ability to receive electrons from NADPH-cytochrome P-450 reductase thereby impeding the flow of electrons to cytochrome P-450 (Wilkinson 1979, Schonbrod and Terriere 1971b, Wilson and Hodgson 1972, Brattsten and Wilkinson 1973).

The mode of action of the tyrosinase inhibitors associated with preparations from late last-instar lepidopterous larvae and with house flies is also felt to be associated with the inhibition of electron flow to cytochrome P-450. Tyrosinase is involved with the oxidation of a variety of ortho-dihydroxy compounds which are later incorporated into the insect cuticle during the tanning process (Wilkinson 1979). Additions of 1-phenyl-2-thiourea and cyanide (both tyrosinase inhibitors) to microsomal preparations from lepidopterous larvae and house flies, respectively, are reported to have a marked stabilizing effect (Crankshaw et al. 1977, Krieger

and Wilkinson 1970).

The inhibitory properties associated with the gut contents of the southern armyworm, Spodoptera eridania (Cramer), and the house cricket, Acheta domesticus (L) have been shown to be due to the solubilization of NADPH-cytochrome c reductase (Orrenius et al. 1971, Brattsten and Wilkinson 1973). Orrenius et al.(1971) demonstrated that the presence of low concentrations of partially purified inhibitor isolated from gut contents of the southern armyworm caused a substantial inhibition of monooxygenase activity in rat liver. This was accompanied by a parallel decrease in the activity of NADPH-cytochrome P-450 reductase as NADPH-cytochrome c was solubilized. The release of less than 5% of the microsomal protein due to the action of this inhibitor resulted in 90-100% inhibition of both monooxygenase and NADPH-cytochrome P-450 reductase activity, providing further support for the assumption that this enzyme is superficially located on microsomal membranes. Solubilization of cytochrome P-450 was not found to be a factor in the inhibitory properties of these trypsin-like inhibitors although very high concentrations of trypsin do eventually result in the conversion of cytochrome P-450 to cytochrome P-420 (Orrenius et al. 1969).

Brattsten and Wilkinson (1973) also demonstrated that gut contents isolated from the house cricket were potent inhibitors of monooxygenase activity in preparations from armyworm gut and rat liver microsomes. In contrast to armyworm gut contents, those isolated from crickets exhibited substantially greater inhibitory action (40-fold) against insect monooxygenases than rat liver

monooxygenases. A decrease of NADPH-cytochrome c reductase activity in armyworm gut microsomes in the presence of partially purified gut contents inhibitor was associated with an increase in the soluble (100,000 g supernatant) fraction.

The inclusion of phenylmethanesulfonyl fluoride and Soy trypsin inhibitor in the washing medium and homogenizing medium resulted in a siginificant reduction of inhibition by house cricket gut contents. However, in the southern armyworm the inclusion of Soy trypsin inhibitor produced little effect. The addition of bovine serum albumin was shown to diminish the inhibitory effect of southern armyworm gut contents but reversed inhibition by house cricket gut contents only at extremely high concentrations. Crankshaw et al. (1979) demonstrated that in microsomes prepared from cleaned southern armyworm gut tissues the inclusion of phenylmethanesulfonyl fluoride, polyvinylpyrrolidone and EDTA increased the yield of reductase 1.7-fold.

Although adult honeybees readily metabolize aldrin in vivo to levels similar in magnitude to those reported for the house fly, homogenization of whole adult honeybees as well as various tissue sources yielded preparations completely devoid of measurable oxidase activity (Gilbert and Wilkinson 1974). Incubation of intact tissue sources, however, resulted in preparations exhibiting similar oxidative potential as have been reported for several other insect species. When it was found that simply making a longitudinal incision of the midgut resulted in a 90% reduction of monooxygenase activity the presence of an intracellular inhibitor was suspected. Subsequent studies established that this inhibitor was associated with

a ribonucleic acid moiety of a macromolecule and that inhibition should be reversed by digestion with ribonuclease (Wilkinson 1979). Other commercially available nucleic acids have since been shown to possess inhibitory properties against insect monooxygenases but had little or no effect upon mammalian tissues (Ibid).

In addition to the presence of inhibitors, other difficulties have been encountered in the preparation of insect microsomes. Benke and Wilkinson (1971b) reported that in the house cricket, Acheta domesticus (I), nearly all enzyme activity of the whole homogenate was found to sediment at 12,000 g. This resulted in the use of a sucrose density gradient to obtain microsomes, a procedure which has since been used with the Madagascar cockroach, Gromphadorhina portentosa (Benke et al. 1972), and the honeybee (Gilbert and Wilkinson 1974).

In order to obtain maximal activity from in vitro studies several procedural factors require optimization. These include homogenization techniques, homogenization media, temperature, and pH. The addition of cofactors such as bovine serum albumin and potassium cyanide should also be considered. More detailed discussions with respect to these and other factors is provided by Wilkinson and Brattsten (1973), Kulkarni and Hodgson (1975) and Wilkinson (1979).

MATERIALS AND METHODS

Chemicals

The insecticides carbaryl (1-naphthyl-N-methylcarbamate) (analytical grade, 99.9%) and carbofuran (2.3-dihydro-2.2-dimethyl-7-benyofuranyl N-methyl carbamate) (technical grade, 99%) were provided by the Union Carbide Corporation, New York, New York. The synergist, piperonyl butoxide (α-(2-(2-butoxyethoxy) ethoxy)-4.5 methylenedioxy-2-propyltoluene) (technical grade, 80%), was purchased from K and K Laboratories, Incorporated, Plain View, New York. The 1-naphthyl-N-methyl(^{14}C)-carbaryl, with a specific activity of 21mc/mmole was purchased from the California Bionuclear Corporation, San Fernando, California. Its radiochemical purity was 98%.

Nicotinomide - adenine dinucloetide phosphate NADP), reduced nicotenamide-adenine dinucleotide phosphate (NADPH), isocitric dehydrogenase, DL-isocitric acid, cytochrome c, Coomassie Brilliant Blue G-250, bovine serum albumin, and tris-(hydroxgmethyl)-aminomethane (Tris, pH 7.7) were purchased from Sigma Chemical Company, St. Louis, MO.

Insects

Colorado potato beetle adults, Leptinotarsa decemlineata (Say), which had been recently established from New Jersey and a laboratory population from Wageningen, Netherlands which has been reared continuously in the laboratory for more than 25 years were kindly provided by T. H. Hsiao (Utah State University) as resistant and susceptible populations, respectively. Adults, eggs, and first

instar larvae from the Logan, Utah area, where potatoes are not a major crop, were collected directly from the field at various times throughout the study as a second susceptible population. Larvae and adults were reared on detached potato leaves, Solanum tuberosum, obtained from greenhouse facilities or collected from an untreated field provided by the Utah State Agricultural Experiment Station. Common nightshade, Solanum dulcamara, also was used as an alternate food source. All rearing took place in a 16:8 (light: dark) photoperiod at 25 ± 1 C with 65% relative humidity in facilities separate from the laboratory. Fourth instar larvae which had reached mid-stadium (80-100 mg) were used in all procedures.

NAIDM and Rutgers house flies, Musca domestica (L), were kindly provided by L. C. Terriere (Oregon State University, Corvallis, Oregon) and maintained on a CSMA diet (Ralston Purina, Kansas City, Missouri). Emerging adults were removed from holding cages at approximately 12 hour intervals and provided with a milk source, sugar, and water. In all experiments, 4 day old adults (±6 hours) were removed from the holding cages, sexed, and weighed prior to experimental use.

Biological Assays

Fourth instar Colorado potato beetle larvae were utilized in groups of 5 and 10 per dose. Each group was weighed separately in order to ensure a uniform weight distribution between groups. A minimum of 5 doses were used per assay, each of which was replicated a minimum of 3 times. Fresh insecticide solutions of carbaryl and carbofuran dissolved in technical grade acetone were prepared on a

regular basis throughout the testing period. Doses of both toxicant and the synergist, when used, were delivered topically to the dorsal abdomen by means of an electrically driven microapplicator calibrated to deliver 1 µl through a Hamilton 250-µl syringe (Hamilton Co.,Inc., Whittier California). When the applied dose exceeded the amount which could be applied in 1 µl, two simultaneous applications of 1 µl each to the dorsal abdomen and a third application to the ventral sclerites was utilized. Controls were treated in a similar manner with acetone. Treated larvae were housed in plastic containers lined with absorbant paper and were provided with fresh leaf at 24 hour intervals. Death was defined as an inability to crawl when disturbed and was determined 48 hours after treatment.

Four-day old house flies were anesthetized using a combination of carbon dioxide and cold treatment. A minimum of 10 flies were used for each of the 5 or more doses. The applied dose was delivered topically to the dorsal thorax in the same manner as described previously. Following treatment, the flies were housed in small screen cages and provided with sugar and water for 24 hours. Mortality was defined as a total absence of movement.

In order to inhibit as much monooxygenase activity as possible the maximum concentration of piperonyl butoxide resulting in no mortality to either population of each species was utilized (Brindley 1977). This dose, 10 µg piperonyl butoxide/g for potato beetles and 5 µg piperonyl butoxide/g for house flies, was applied 1 hour prior to carbamate treatment. Piperonyl butoxide-treated controls were also treated with acetone at the time of insecticide treatment.

Applications of both carbaryl and carbofuran at concentrations as low as 10 µg/µl resulted in the appearance of a barely discernible residue. At the concentration of 100 µg/µl (the maximum amount which could be dissolved in acetone) both carbamates had crystallized to the extent that they appeared as a caked powdery residue. A maximum of three applications were applied to the dorsal and ventral abdomen to avoid placing them one on top of another in the potato beetle. In the house fly, due to the lack of sufficient surface area, all three applications were applied to the same area of the dorsal thorax.

Bioassay data from several replicates were pooled and analyzed by means of computerized profit analysis (unpublished procedure). The results were plotted as percent mortality relative to the logarithms of insecticide concentration. Synergist differences and percent dependencies were also calculated for each population and insecticide.

Enzyme Preparations

The gut and fat bodies from groups of 20 to 40 potato beetle larvae were used in the preparation of potato beetle microsomes. Larvae were collectively weighed prior to dissection for gut and fat body. The posterior sclerites were clipped with a fine pair of scissors and the larvae were clipped along the dorso-longitudinal midline to expose gut and fat body. The gut was then grasped at the anterior end with forceps and the entire gut carefully withdrawn and placed in a plastic petri dish where it was covered with ice cold 0.1 M Tris buffer (pH 7.7). Fat body was next extracted by

sliding the forceps from the head to the posterior abdomen along the length of the carcass. It too was covered with ice cold buffer. Gut contents were eliminated with a gentle rolling motion of the forefinger across the gut which was immersed in buffer. The guts and fat bodies from several insects were combined, dried on filter paper, weighed, and homogenized in 2.5 mls of 0.1 M Tris buffer (pH 7.7) per g original body weight. Homogenization was performed with a motor driven Potter-Elvehjem homogenizer with a Teflon pestle for 30 seconds. The homogenate was centrifuged on a Beckman L5-65B ultracentrifuge at 15,000 g for 10 min to remove mitochondria and heavier particles. The post-mitochondrial supernatant was centrifuged at 100,000 g for 1 hour to sediment a microsomal fraction. The resulting pellet was resuspended by hand and homogenized in 1.5 ml of 0.1 M Tris buffer (pH 7.7) per g original body weight. All procedures were performed on ice.

House fly male and female adults were separately weighed prior to having their abdomens excised by a fine pair of scissors or by vigorous shaking after being frozen on dry ice. The homogenization and centrifugation scheme was similar to that of Folsom and Hodgson (1970) with some modifications. The flies were homogenized in 2.5 ml of 0.1 M phosphate buffer (pH 7.4) or 0.1 M Tris buffer (pH 7.7) per g original body weight. Homogenization was performed in a Potter-Elvehjem homogenizer with a Teflon pestle for 60 seconds. The homogenate was filtered through two layers of cheesecloth prior to centrifuging at 15,000 g for 10 min to remove the mitochrondria and heavier particles. The post-mitochondrial supernatant was centrifuged

at 100,000 g for 1 hour to sediment the microsomal fraction. For experiments involving the addition of bovine serum albumin to the microsomes for the determination of p-nitroanisole-O-demethylation, the microsomal pellet was resuspended in 0.75 M phosphate buffer (pH 7.4) per g original body weight for protein determinations. Following the determination of protein an equal volume of phosphate buffer containing 4% bovine serum albumin was added. The resulting microsomal suspension consisted of 2% bovine serum albumin in 1.5 ml 0.2 M phosphate buffer (pH 7.4) per g original body weight. For the determination of NADPH-cytochrome c reductase, NADPH oxidation, and p-nitroanisole-O-demethylation in which bovine serum albumin was not added, the microsomal pellet was resuspended in 3 times the original volume of phosphate buffer and resedimented at 90,000 g for 1 hr in order to remove a soluble diaphorase which interferes with NADPH determinations. The final microsomal pellet was resuspended in 1.5 ml of 0.1 M Tris buffer (pH 7.7) per g original body weight.

When determinations of cytochrome c oxidase and NADPH-cytochrome c reductase were necessary from the various centrifugation fractions the following procedure was utilized. After homogenization of the tissue source, 2 ml of whole homogenate were set aside for assay determinations. Following each centrifugation step, 2 ml of buffer were added to the supernatant prior to withdrawing 2 ml for the assays. The mitochondrial pellet was resuspended in 5 ml per g original body weight, while the microsomal pellet was resuspended as previously described.

Protein Determination

The determination of protein was performed as described by Bradford (1976). One hundred milligrams of Coomassie Brilliant Blue G-250 were dissolved in 50 ml of 95% ethanol. This was followed by the addition of 100 ml 85% (w/v) phosphoric acid. The resulting solution was diluted with distilled water to a final volume of 1 liter and stored for future use.

Aliquots of protein solutions were pipetted into test tubes and brought up to 0.1 ml with buffer. After filtering the reagent, 5 ml aliquots were added to each tube and the resulting solutions mixed with a vortex mixer. Absorbance was measured against a cuvette containing 0.1 ml of buffer and 5 ml of reagent at 595 nm with a slit width of 0.1 on a Zeiss PMQ-II spectrophotometer. The resulting optical densities were compared directly with a standard curve prepared with known concentrations of bovine serum albumin.

All in vitro enzyme assays with the exception of NADPH oxidation were performed in triplicate utilizing a range of protein concentrations in order to ensure linearity within replicates. Since the amount of protein in potato beetle gut and fat body microsomes was nearly always identical for a given group, amounts of protein utilized in a given assay varied from 20-250 µg depending upon the aliquot volume. The amount of protein utilized from house fly microsomes varied from 100-1200 µg per assay. In the case of NADPH oxidation the amount of protein utilized from potato beetle microsomes was the maximum amount possible from the resuspension, varying from 100-250 µg. All assays were replicated a minimum of

three times for statistical analysis unless otherwise stated.

p-Nitroanisole-O-Demethylation

The determination of p-nitroanisole-O-demethylation utilized the procedure of Kinoshita et al. (1966). A stock solution was prepared consisting of 7 mg/ml of p-nitroanisole was dissolved in a solution of 20% ethanol and 20% ethylene glycol. Each incubation consisted of a microsomal aliquot brought up to 0.5 ml with 0.1 M Tris buffer (pH 7.7) and 0.1 ml of an NADPH generating system composed of 20 µmoles $MgCl_2$, 35 µmoles sodium isocitrate, 2.5 units of sodium isocitrate dehydrogenase, and 2 µmoles NADP. Immediately prior to initiating the incubation 0.5ml of the stock solution of p-nitroanisole were made up to 5 ml with 0.1 M phosphate buffer (pH 7.8) or 0.1 M Tris buffer (pH 7.7). Initiation of the reaction was accomplished by the addition of 0.2 ml aliquots of the diluted preparation of p-nitroanisole at various timed intervals. The reaction tubes were placed in a shaking water bath at a temperature of 30° C. Termination was by the addition of 2.5 ml of cold acetone at various timed intervals. Protein was sedimented by centrifuging on a Triac Centrifuge for five minutes, after which 0.2 ml of 0.5 M glycine titrated to pH 9.4 with saturated NaOH was added. The optical density was read on a Zeiss PMQ-II spectrophotometer at 410 nm and the amount of p-nitrophenol produced was calculated from a standard curve.

NADPH Oxidation

The standard incubation procedure for NADPH oxidation was

modified slightly from that described by Folsom and Hodgson (1970). Microsomal enzyme levels varied from 0.1 to 0.2 mg protein in the case of potato beetles and from 0.1 to 1.2 mg microsomal protein in the case of house flies in a total volume of 2.5 ml of 0.1 M Tris buffer (pH 7.7). Concentrations of NADPH and cytochrome c were 0.3, and 0.114 µmoles, respectively. Rates of oxidation were measured at 340 nm on a Zeiss PMQ-II spectrophotometer and determined from the extinction coefficient (6.22×10^6 cm/mole.

Cytochrome C Oxidation

The procedure of Cooperstein and Lazarow (1951) was followed, in part, for determinations of cytochrome c oxidation. A solution of 1.7×10^{-5} M cytochrome c in 0.1 M Tris buffer (pH7.7) or 0.1 M phosphate buffer (pH 7.4), was shaken for 2 minutes after reduction with dithionite. Reaction initiation was accomplished by addition of 2 ml of reduced cytochrome c to enzyme aliquots. The rate of oxidation of cytochrome c was determined from the extinction coefficient (2.1×10^7 cm/mole) after reading the decrease in extinction at 550 nm.

NADPH-Cytochrome C Reductase

The incubation mixture for NADPH-cytochrome c reductase was modified from that described by Dallner (1963). The tissue source was brought up to 0.5 ml with 0.1 M Tris buffer (pH 7.7) or 0.1 M phosphate buffer (pH 7.4) and incubated at room temperature for 2 minutes with 0.5 ml 0.66 mM KCN. The reaction was initiated by the addition of 1 ml of cytochrome c and the NADPH generating

system making final concentrations of 0.05 and 0.1 mM, respectively. The rate of reduction of cytochrome c was determined by observing the increase in extinction at 550 nm and calculated from the extinction coefficient (2.1×10^7 cm/mole).

Data analyses for all *in vitro* methodology were performed by analysis of variance and stepwise multiple regression.

In Vivo Distribution Of Carbaryl

1-Naphthyl-*N*-methyl-^{14}*C*-carbaryl with a specific activity of 21 mCi/mmole was purchased from the California Bionuclear Corporation, San Fernando, California. Its radiochemical purity was greater than 98%. 0.5 mCi was dissolved in 1 ml benzene from which small aliquots were redissolved in acetone to give a concentration of 0.01 µg carbaryl/µl with a final specific activity of 2.1 mCi/mmole or 2770 dpm. In experiments where the concentration of ^{14}*C*-carbaryl was increased from 0.01 µg carbaryl/µl to 10 µg carbaryl/µl, the same aliquot of ^{14}*C*-carbaryl was added to the same volume of acetone containing 10 µg unlabeled carbaryl, thereby retaining the same specific activity.

Groups of 5 and 10 fourth instar larvae weighing between 90 and 100 mg were selected from both populations. Each was treated topically on the dorsal abdomen with 1 µl of labeled carbaryl in acetone delivered from a motor-driven microapplicator bearing a Hamilton 250- µl syringe (Hamilton Co., Inc., Whittier, California). In order to eliminate the possible mechanical loss of radiolabeled carbaryl the acetone solution was allowed to evaporate prior to placing the beetles into the metabolism chamber. Pretreatment

with the synergist, piperonyl butoxide, was performed 1 hr prior to treatment with radiolabeled carbaryl. The maximum non-lethal dose of piperonyl butoxide, 10 µg/g was applied to the dorsal abdomen as in the biological assays.

Ten time periods were selected for study, including 0, 1, 3, 5, 10, 20, 30, 60, 120, and 240 minutes. For each of the 7 time periods of 5 minutes duration and above, beetles were treated as quickly as possible and timing began from the median treatment time. For 0, 1, and 3 minutes, groups of 5 larvae were treated and timed individually to ensure uniformity of results but were combined as a group after the external rinse. The effect of the synergist, piperonyl butoxide, was observed only at 240 minutes.

Prior to treatment of the larvae, 5 1-µl aliquots of radiolabeled carbaryl were delivered directly into a scintillation vial for determination of the quantity of labeled carbaryl applied to the replicates. The percent radioactivity of the various samples was determined by direct comparison with the activity of this sample. A minimum of three replicates was determined for each time period examined.

Each group of treated larvae was removed from the metabolism chamber and rinsed by dipping the larvae individually through two successive 5 ml aliquots of acetone:methanol (1:1) to remove unabsorbed insecticide. The two rinses were combined and an aliquot of 1 ml placed into a scintillation vial containing 10 ml of scintillation cocktail. The rinsed beetles were transferred into a Potter-Elvehjem homogenizer and homogenized in 5 ml of an

acetone:methanol mixture (1:1) for two minutes with the variable resistor set at 60 rpm. The resulting brei was centrifuged for 3 minutes on a Triac Centrifuge and the supernatant decanted and saved. A 5 ml aliquot of the acetone:methanol mixture was added to the residue and homogenized a second time for 1 minute. The two-5 ml portions of supernatant were retained from which 0.2 ml aliquots were removed for counting into scintillation vials. Fecal extracts were obtained by the addition of two-5 ml portions of the acetone-methanol solvent to the metabolism chambers and scraping the bottom of the chambers vigorously with a spatula. Both portions were combined and aliquots of 0.5 ml retained for radio-analysis. The scintillation cocktail utilized for each aliquot was composed of 4 g PPO and 0.5 g POPOP in 1 liter of toluene in a volume of 10 ml per aliquot.

The radioactivity of the various samples was determined with a Packard Tricarb liquid scintillation spectrometer (model 2660). Quench corrections were made by using external standards ratio. The total percentage radiocarbon was calculated from the total disintegration ions per minute (dpm) for the various fractions. Statistical analyses of the various interactions between populations, time periods, and tissue sources were performed by stepwise multiple regression analysis.

The efficiency of the external, internal, and fecal procedures was verified in the following manner. Larvae which had been treated at an early time period were dipped through three consecutive 5 ml rinses of the acetone:methanol solvent system. Of the activity

recovered approximately 97% was recovered in the first two rinses. The remaining 3% recovered in the third rinse was considered negligible especially since the amount of recovered radiocarbon declined very significantly in later time periods. Larvae which had been treated in later time periods were homogenized in three consecutive 5 ml portions of the acetone:methanol solvent system. Again, recovery from the third homogenization was considered insignificant. The same procedure was repeated for the fecal extract, resulting in greater than 99% recovery from the first two rinses.

RESULTS AND DISCUSSION

Biological Assays

The population of Colorado potato beetles from New Jersey was suspected of being highly resistant to most insecticides due to intense selection pressure in the field over several generations. In contrast, the population from Wageningen, Netherlands, was considered as a reference susceptible population since it has been reared continuously in the laboratory for a period of greater than 25 years. Because potatoes are not a major crop in the state of Utah, it was suspected that a field population collected from Logan, Utah would also be susceptible to carbamates. In an effort to confirm this, topical bioassays were conducted with carbofuran following recommended procedures for the detection and measurement of resistance (Anon. 1974).

A comparison of the toxicity of carbofuran to the Wageningen and Logan populations (Table 2) reveals a small, insignificant difference between LD_{50} values obtained from these populations; confirming that the Logan population could be considered to be susceptible to carbamates. Although the Logan population does exhibit a slightly greater tolerance to carbofuran, it is interesting to note that this level of tolerance is completely obliterated by pretreatment with the synergist, piperonyl butoxide. The fact that monooxygenases may be playing a somewhat greater role in detoxication of carbofuran for the Logan population is indicated not only by a greater synergist difference but also by the calculation of a greater

Table 2. Toxicity (μg/g) of carbofuran alone and with piperonyl butoxide (p.b.) to Netherland and Utah populations of fourth instar Colorado potato beetle larvae.

Population	Insecticide	LD_{50}	95% Fiducial Limits	Slope	Percent Dependency
Netherland	Carbofuran	.47	.43 - .52	2.8	12.9
	Carbofuran + p.b.	.41	.33 - .45	3.2	
Utah	Carbofuran	.58	.50 - .64	4.1	26.3
	Carbofuran + p.b.	.43	.30 - .67	1.6	

percent dependency upon monooxygenases.

Since the Wageningen population is well adapted to laboratory conditions, thereby possessing a physiological advantage over newly established laboratory populations, it was considered to be more desirable in a comparative study to compare two field populations rather than a field versus a laboratory population. The Logan population was found to be essentially identical to the Wageningen population with respect to its susceptibility to carbofuran. As a result, all subsequent assays were performed utilizing the New Jersey and Logan populations as resistant and susceptible, respectively.

Table 3 and Figures 3 and 4 compare the toxicities of the carbamates, carbofuran and carbaryl, alone and in combination with piperonyl butoxide among resistant and susceptible populations of Colorado potato beetle larvae and four-day old adult female house flies. Both the resistant and susceptible strains of house flies were more tolerant to these carbamates than were their respective potato beetle counterparts. Levels of resistance development, as indicated by resistant factors (calculated by dividing the LD_{50} of the resistant population by the LD_{50} of the susceptible population), were exceedingly high for populations of both species, indicating the possibility of a similar resistance mechanism.

The Rutgers strain of house flies is the result of intense selection pressure by diazinon. Resistance is conferred primarily by gene(s) associated with the 5^{th} chromosome which has also been shown to be associated *in vitro* with high levels of oxidative activity (Tsukamoto et al. 1968, Perry et al. 1972). As a result,

Table 3. Toxicity (µg/g) of carbofuran and carbaryl alone and with piperonyl butoxide (p.b.) to four-day old adult female Rutgers and NAIDM house flies and to fourth instar populations of New Jersey and Logan Colorado potato beetle larvae.

Population	Insecticide	LD_{50}	95% Fiducial Limits	Slope	Percent Dependency[a]	RF[b]
M. domestica						
Rutgers	Carbofuran	6584	5194 - 8117	2.8	115	583
	Carbofuran + p.b.	13.5	9.1 - 30.4	1.8		3
NAIDM	Carbofuran	11.3	9.1 - 14.6	4.2	66	
	Carbofuran + p.b.	4.3	3.4 - 6.1	4.7		
L. decemlineata						
New Jersey	Carbofuran	492	261 - 817	1.0	93	820
	Carbofuran + p.b.	80	29 - 209	0.8		186
Logan	Carbofuran	0.58	0.50 - 0.64	4.1	26	
	Carbofuran + p.b.	0.43	0.30 - 0.67	1.6		
New Jersey	Carbaryl	>4000	undetermined	-	78[c]	>833
	Carbaryl + p.b.	1851	1598 - 2780	0.3		974
Logan	Carbaryl	4.8	3.0 - 7.0	4.1	63	
	Carbaryl + p.b.	1.9	1.0 - 3.7	4.0		

[a]Calculated by dividing the actual synergist difference by the theoretical synergist difference.
[b]RF (Resistance Factor) defined as the resistant LD_{50} divided by the susceptible LD_{50}.
[c]Determined from LD_{30} values from synergized and unsynergized carbaryl.

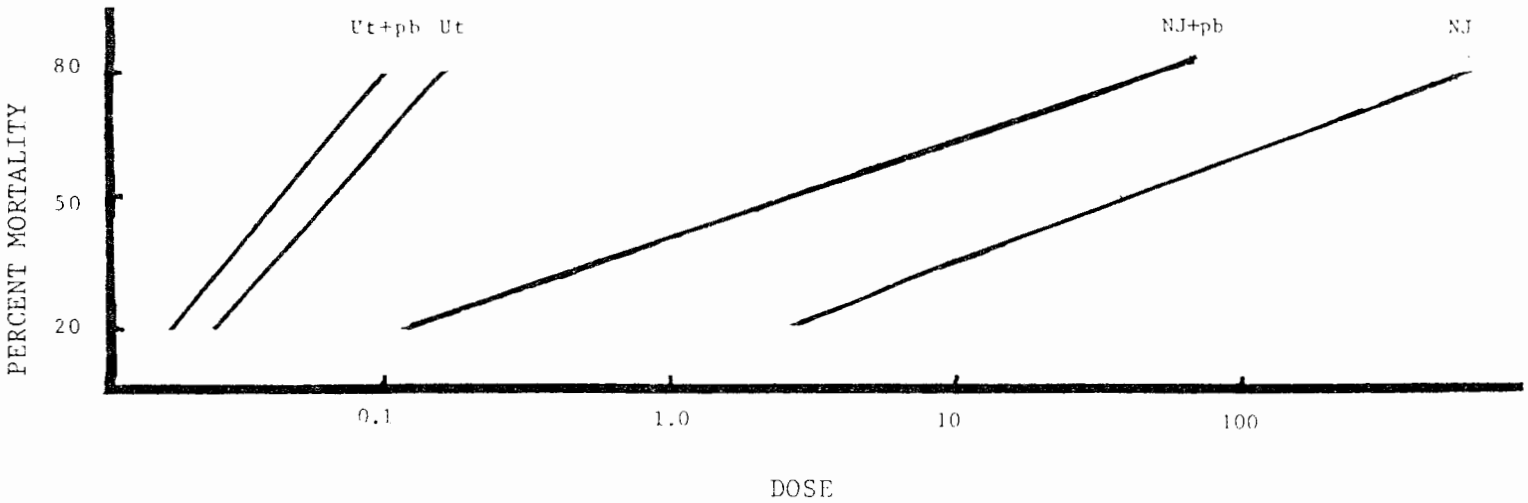


Fig. 4. Toxicity of carbofuran alone and with piperonyl butoxide (pb) to New Jersey and Logan Colorado potato beetles, µg/larva.

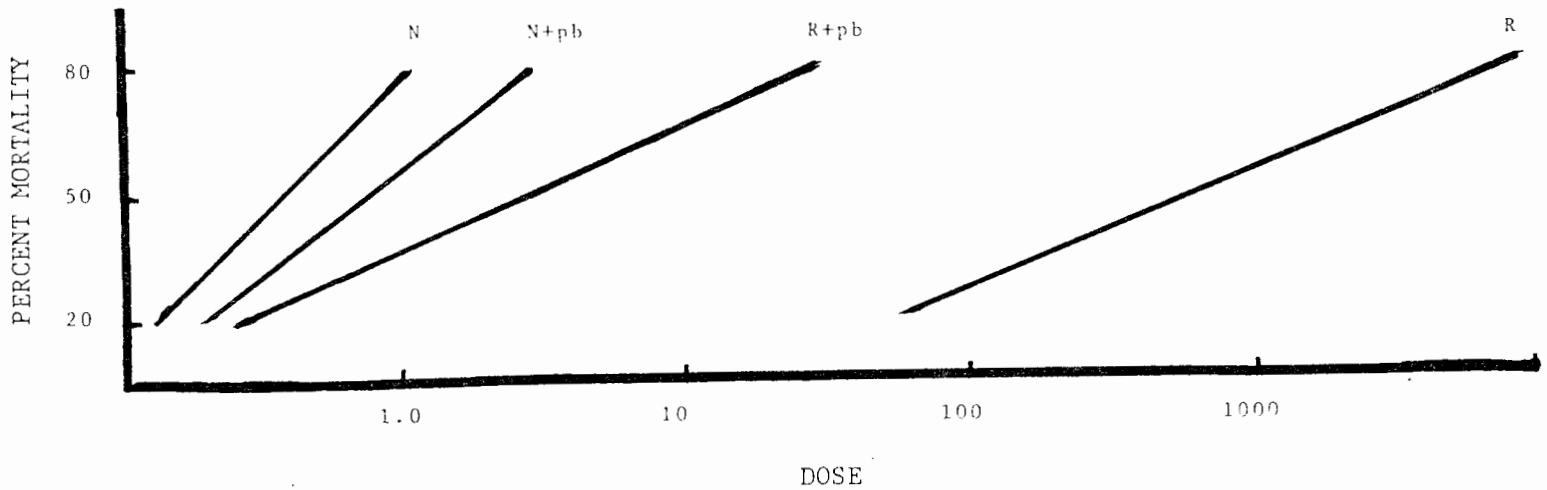


Fig. 5. Toxicity of carbofuran alone and with piperonyl butoxide to Rutgers and NAIDM four-day old house flies, µg/fly.

pretreatment with the monooxygenase inhibitor, piperonyl butoxide, has been shown to minimize the importance of the resistance factor(s) associated with the 5^{th} chromosome (Tsukamoto et al. 1968).

Concentrations of carbaryl as great as 14,000 µg/g resulted in no mortality to the Rutgers strain and in less than 40% mortality to the susceptible strain. A high degree of resistance to carbofuran was also exhibited by the Rutgers strain (LD_{50} = 6584 µg/g) relative to NAIDM house flies (LD_{50} = 11.3 µg/g). Pretreatment of the Rutgers strain with the synergist, piperonyl butoxide, resulted in a dramatic increase in carbofuran toxicity. Hence, the level of resistance was reduced from 583-fold to only 3-fold by piperonyl butoxide pretreatment. Calculation of the percent dependency upon monooxygenases is consistent with the fact that resistance in the Rutgers strain is primarily oxidative in nature.

The New Jersey population was so highly resistant to both carbofuran and carbaryl that LD_{50} values, especially for the latter, were difficult to obtain. The LD_{50} for carbofuran was 492 µg/g. However, for carbaryl the maximum concentration which could be applied (4,000 µg/g) resulted in only 30% mortality. Since carbaryl synergism was possible to some extent, this information was utilized solely for comparative purposes in order to determine an approximate percent dependency value and resistance factor for this insecticide.

In sharp contrast with the New Jersey population, Logan potato beetles were extremely susceptible to both carbofuran and carbaryl; possessing LD_{50} values of 0.58 and 4.8 µg/g, respectively. The large differences in carbamate toxicity for these populations are

reflected by resistance factors of 820 and > 833 for carbofuran and carbaryl, respectively.

Several factors are suggestive that resistance in the Colorado potato beetle may be linked with high levels of monooxygenase enzymes. Studies of resistance factors in house flies indicate that strains exhibiting the greatest resistance levels generally possess the greatest monooxygenase potential. This, coupled with the fact that carbamate metabolism in insects is primarily oxidative in nature is supported in this study by the effective reduction of carbofuran resistance to levels of susceptibility by piperonyl butoxide. Since resistance factors for the Colorado potato beetle are very similar to those of the house fly, one can hypothesize that monooxygenases are a predominant factor in carbamate resistance.

Pretreatment of the New Jersey population with piperonyl butoxide prior to treatment with both carbofuran and carbaryl resulted in large increases in their toxicity. Smaller increases were noted with the Logan population. Calculations of the percent dependency upon monooxygenases are supportive of the hypothesis that high resistance levels are attributable to high monooxygenase activity.

In contrast with the effect observed with Rutgers house flies, piperonyl butoxide pretreatment of New Jersey Colorado potato beetles did not completely eliminate resistance. Such high synergized resistance factors for carofuran and carbaryl (186 and 974, respectively) may be indicative that other resistance factors are involved. Often, the presence of two or more resistance factors provide much greater resistance potential than either factor by

itself (Sawicki 1970, Grigolo and Oppenoorth 1966, Plapp and Hoyer 1968).

Other possibilities may be invoked to explain the apparent failure of piperonyl butoxide to completely synergize carbofuran to the susceptible levels exhibited by the Logan population. It may be that the dose of piperonyl butoxide was not substantially great enough to result in the complete inhibition of the metabolism of these carbamates. However, an attempt was made in this study to eliminate this possibility by using the maximal dose of piperonyl butoxide which produced no mortality in the controls. This would hopefully ensure that the dose would be a saturating dose, requiring the full attention of monooxygenases involved in detoxication. Alternatively, it is possible that biological differences at the level of the monooxygenase P-450 receptor site exist between house flies and Colorado potato beetles, resulting in a more efficient binding of the synergist for the house flies than for the potato beetles.

Forgash (1981) also recently indicated that carbamate synergism by piperonyl butoxide was almost nonexistent. Since a two hour pretreatment time resulted in a slightly greater synergistic effect it was proposed that the lack of synergism may be due to slower penetration of the synergist than for the carbamate. Preliminary experiments conducted during the course of this study were not indicative that longer pretreatment times were necessary for greater synergism for potato beetle larvae. Further comparative work between larvae and adult Colorado potato beetles may be useful in elucidating

possible explanations for this effect.

p-Nitroanisole-O-Demethylation

Due to its relative ease of handling, p-nitroanisole-O-demethylation was selected as an in vitro means for confirmation of the hypothesis that resistance in the Colorado potato beetle was the result of oxidative metabolism. Microsomal preparations from whole larvae, isolated fat bodies, and isolated gut tissues with and without gut contents resulted in preparations which were devoid of activity. Several conditions were altered in an attempt to verify that a procedural problem did not exist. Some of these included the use of a phosphate buffer with variations in pH from 6.5 to 8.0, variations in molarity from 0.1 to 0.2 M, homogenization of tissues in a Waring blender versus the Potter-Elvehjem tissue grinder, variable centrifugation speeds, and additions of various cofactors including bovine serum albumin (BSA), 0.33 mM KCN, and 0.02 mM $MgCl_2$; all failing to result in a measureable response.

When methodology utilizing microsomal preparations were exhausted other parameters were investigated. Incubations of intact gut tissues with and without gut contents were tried without success as were whole homogenates of gut tissues and fat bodies. Had activity been present, however, it may have been precluded in these preparations since they were highly turbid, exhibiting an absorption spectrum very similar to that of the product, p-nitrophenol, likely due to the presence of carotenoids or similar products.

As a result of the insurmountable difficulties associated with p-nitroanisole-O-demethylation in the Colorado potato beetles,

Rutgers and NAIDM house flies were incorporated into the study, primarily for the verification of procedural efforts. An examination of p-nitrophenol production in microsomes from the house fly demonstrated large oxidative differences between strains and sexes. The ability of both male and female Rutgers house flies to demethylate p-nitroanisole was approximately four times that of NAIDM house flies, based on both protein levels and a per fly basis (Table 4). Similar differences between these strains were also reported by Folsom et al. (1970). Although sex differences were not immediately apparent based upon protein levels, they became evident when compared on a per fly basis as others have reported (Matthews and Casida 1970). In both strains, the ability of females to demethylate p-nitroanisole was approximately 3.7-fold greater than that of males, presumably because females possess greater protein levels due to egg production.

The addition of bovine serum albumin to microsomes prepared from male and female house flies of both strains did not result in a significant enhancement of activity over that obtained from microsomes incubated in its absence. Results reported herein are the means of at least two replicates incubated with bovine serum albumin since it is normally included in determinations of *in vitro* xenobiotic house fly metabolism. There are indications, however, that its effect upon homogenates prepared from house flies of four days and older is minimal (Terriere et al. 1980).

Distribution of Cytochrome Oxidase and NADPH-Cytochrome C Reductase in Relation to the Centrifugation Scheme

In an attempt to understand why microsomal activity could not

Table 4. p-Nitroanisole-O-demethylation in four-day old adult male and female Rutgers and NAIDM house flies.

Strain	Sex	nmoles/hr/mg protein[a]	nmoles/hr/25 abdomens
Rutgers	♀	48.3 ± 11.2	57.9 ± 10.5
	♂	29.9 ± 8.5	15.5 ± 8.4
NAIDM	♀	11.6 ± 11.2	15.2 ± 10.5
	♂	5.4 ± 8.3	4.1 ± 7.1
R/S[b]	♀	4.2	3.8
	♂	5.5	3.8

[a]Results are means ± SD of at least two determinations using 2% BSA with the exception of NAIDM ♂ which is based upon 1 determination with and 2 determinations without BSA. Results of incubations wwithout BSA are, from left to right; 32.1 ± 7.9, 52.5 ± 11.6, 47.1 ± 3.8, 15.3 ± 1.9, 5.2 ± 11.2, 6.7 ± 16.5, 9.2 ± 4.7, 6.9 ± 2.3.

[b]R/S defined as the ratio of activity from the resistant Rutgers and the susceptible NAIDM strains.

be determined by means of p-nitroanisole-O-demethylation for the Colorado potato beetle the relative distribution of the enzymes cytochrome oxidase and NADPH cytochrome c reductase were examined with relation to the centrifugation procedure.

As is shown in the distribution of cytochrome oxidase (Table 5), the first centrifugation was effective in removing the bulk of the mitochondria into the mitochondrial pellet. NADPH-cytochrome c reductase, which normally sediments into the microsomal pellet as a membrane bound entity of the electron transport chain, was partially sedimented into the mitochondrial pellet. Of the remaining activity found in the mitochondrial supernatant at least 90% should have sedimented into the microsomal pellet. The microsomal centrifugation, however, sedimented less than 33% of the activity from the mitochondrial supernatant, releasing the bulk of the activity into the microsomal supernatant.

Similar centrifugation procedures examining the distributions of NADPH-cytochrome c reductase and N-demethylation in southern armyworm and house flies (Crankshaw et al. 1979, Hansen and Hodgson 1971) reveal that anywhere from 70 - 95% of the oxidative potential of the mitochondrial supernatant is sedimented into the microsomal pellet, dependant to some extent upon the homogenization media utilized. This contrasts sharply with results observed in this study for the Colorado potato beetle, since only 30% of the activity from the mitochondrial supernatant was sedimented into the microsomal pellet. The remaining 70% was lost into the microsomal supernatant. Such a loss of activity from the microsomal pellet provides a

Table 5. Distribution of cytochrome oxidase and NADPH-cytochrome c reductase in New Jersey Colorado potato beetle larvae.[a]

Fraction	Oxidase nmoles/min	%[b]	Reductase nmoles/min	%
Whole Homogenate	1245	100	767	100
Mitochondria (P-15,000)[c]	1263	101	274	36
Mitochondria (S-15,000)	16	1	392	51
Microsomes (P-100,000)	21	2	116	15
Soluble (S-100,000)	0	0	277	36

[a]Results are the means of at least five seperate replications.
[b]Percent of total activity observed in the whole homogenates.
[c]Definitions: P = pellet, S = supernatant.

possible clue for the obvious lack of microsomal monooxygenase activity from gut and fat body preparations of the Colorado potato beetle.

The tremendous loss of NADPH-cytochrome c reductase from the microsomal pellet indicates that at some point during the preparation procedure, solubilization of NADPH-cytochrome c reductase may be occurring. This results in a loss of the ability to pass electrons on to cytochrome P-450, which is necessary for the oxidative process to occur. Although some reductase activity remains in the microsomal pellet, it is most likely not interacting with cytochrome P-450 since it is suspected that the hydrophobic portion of the enzyme, necessary for interaction with cytochrome P-450, is not necessary for interaction with cytochrome c (Ullrich 1979).

Several endogenous inhibitors associated with insect tissues have been linked with the solubilization of NADPH-cytochrome c reductase from microsomal membranes (Wilkinson 1979). Inhibition is primarily associated with gut contents and has been found in southern armyworm, cabbage loopers, house crickets, and caddisfly larvae (Wilkinson 1979). The fact that in the cabbage looper, the addition of 1/10 of the contents from one gut results in 60 - 90% inhibition of both gut and fat body homogenates (Kuhr 1971) indicates the potential power of such inhibitors. During the isolation of gut and fat bodies from as many as 20 - 30 potato beetle larvae, contamination by at least this much of the gut contents is unavoidable.

In one single, unreplicated experiment, an attempt was made to

demonstrate the possible existence of an endogenous inhibitor associated with potato beetle microsomes. The addition of a small aliquot of potato beetle microsomes obtained from the gut to house fly microsomes resulted in a slight increase in the O-demethylation of p-nitroanisole. This enhancement of activity, however, was reduced by the addition of larger aliquots. Perhaps the increase in house fly monooxygenase activity following the addition of potato beetle microsomes is due to greater access of potato beetle microsomes to house fly NADPH-cytochrome P-450 reductase enzymes or vice versa. The fact that enhancement was greater by smaller aliquots than by larger may be indicative of either a better integration of smaller aliquots into house fly microsomes or an indication of the presence of some inhibitory property of potato beetle microsomes.

NADPH-Cytochrome C Reductase and NADPH Oxidation

NADPH-cytochrome P-450 reductase is closely associated with cytochrome P-450 and is thought to be involved in the reduction of the cytochrome P-450 substrate complex. Folsom and Hodgson (1970) and Ahmad and Forgash (1973) demonstrated that the oxidation of NADPH can be inhibited by CO and sulfhydryl inhibitors, indicating that electron flow to cytochrome P-450 is through NADPH-cytochrome P-450 reductase. Ernster and Orrenius (1965) showed that induction of monooxygenases generally results in the associated induction of NADPH-cytochrome P-450 reductase. While measurements of NADPH-cytochrome P-450 reductase have been shown to roughly coincide

with differences between resistant and susceptible monooxygenase activity, NADPH-cytochrome c reductase determinations do not correlate to the same extent, although their activity is enhanced (Gillette and Gram 1969). Similarly, Folsom et al. (1970) and Ahmad and Forgash (1978) demonstrated that NADPH oxidation and NADPH-cytochrome c reductase activity was increased significantly in resistant house flies and in induced gypsy moth larvae, respectively. However, these increases did not reflect the total levels of resistance and induction which could be demonstrated by xenobiotic metabolism.

Rather than pursue other xenobiotic routes of metabolism in the Colorado potato beetle, an examination of NADPH oxidation and NADPH-cytochrome c reduction was considered as an alternate route to demonstrate quantitative differences between populations of Colorado potato beetles. The activities of these components of the electron transport chain were also briefly examined in Rutgers and NAIDM house flies.

NADPH oxidation and NADPH-cytochrome c reductase activities were examined in both the gut and fat body of both populations of Colorado potato beetle larvae (Table 6). Neither component of the electron transport chain was found to differ significantly between tissue sources on the basis of protein determinations. Since both weight and protein were approximately equal in both tissues, the specific activities reported probably reflect relative differences between tissues.

The endogenous rate of NADPH oxidation was extremely low and sometimes difficult to measure in potato beetle microsomes when

Table 6. NADPH oxidation and NADPH-cytochrome c reductase activities (nmoles/min/mg protein ± SE) of microsomal preparations from New Jersey and Logan populations of the Colorado potato beetle.[a]

Assay	New Jersey		Logan		R/S[b]
	Gut	Fat Body	Gut	Fat Body	
NADPH Oxidation					
-cytochrome c	5.7 ± 7.2	8.2 ± 7.1	5.4 ± 4.2	6.8 ± 2.9	1.1
+cytochrome c	57.8 ± 30.2	44.6 ± 21.5	39.2 ± 12.3	47.6 ± 20.4	1.2
NADPH-Cytochrome C Reductase	61.5 ± 31.5	60.1 ± 30.6	32.6 ± 11.7	35.1 ± 3.1	1.8

[a]Results are means of at least four replications.
[b]R/S defined as the ratio of activity from the resistant Rutgers and the susceptible NAIDM strains.

compared with that obtained from house fly preparations (Tables 6 and 7). The addition of cytochrome c to the incubation as an electron acceptor, however, significantly enhanced activity of NADPH oxidation (approximately 7.5-fold in potato beetles and 4.4-fold in house flies). Although the New Jersey population had slightly greater activity in most cases, population differences were not statistically different due to high variability between groups of larvae. Especially surprising was the result obtained from NADPH oxidation in the house flies, since it too was not found to vary significantly between strains. This was in sharp disagreement with results obtained by Folsom et al. (1970) who demonstrated 2-fold differences in NADPH oxidation, oxygen consumption, and cytochrome P-450 content in Rutgers and NAIDM house flies using similar techniques.

NADPH-cytochrome c reductase was found to vary significantly between New Jersey and Utah populations of Colorado potato beetle larvae to levels similar to those reported for NADPH oxidation and NADPH-cytochrome c reductase in insect and liver microsomes which are either genetically or chemically induced (Folsom et al. 1970, Ahmad and Forgash 1973, Gillette and Gram 1969). In contrast, differences in NADPH-cytochrome c reductase between resistant and susceptible house flies failed to be demonstrated.

Results reported here for resistant and susceptible Rutgers and NAIDM house flies contradict those reported for the same strains utilizing approximately the same techniques for NADPH oxidation (Folsom et al. 1970). Although determinations of NADPH-cytochrome

Table 7. NADPH oxidation and NADPH-cytochrome c reductase activities (nmoles/min/mg protein ± SE) of microsomal preparations from Rutgers and NAIDM house flies.

Assay	Rutgers	NAIDM	R/S[a]
NADPH Oxidation[b]			
-cytochrome c	14.6 ± 2.7	16.7 ± 3.0	.87
+cytochrome c	58.5 ± 13.6	78.8 ± 15.2	.74
NADPH-Cytochrome C Reductase[c]	65.1 ± 5.8	64.7	1.00

[a]R/S defined as the mean ratio obtained from gut and fat body activities from the resistant Rutgers and susceptible NAIDM strains of house flies.
[b]Results are means of at least four replications.
[c]Results are means of two and one replicates for Rutgers and NAIDM, respectively.

c reductase were not replicated sufficiently in this study for statistical analysis, replicates of NADPH oxidation demonstrate no quantitative differences between strains. Discrepencies between these studies may be due to simple variability which occurs between laboratories but is more likely to be the result of differences in sexing and aging of house flies since in this study only four-day old females ± 8 hours were used while in the previous study both males and females of between five and eight days of age were used. The relative endogenous rates for both strains in this study are very similar to the endogenous rate reported for the Rutgers strain, but the addition of cytochrome c resulted in the elevation of activity by more than 3-fold above that reported by Folsom et al. (1970).

The results of *in vitro* methodology suggest that resistance in Colorado potato beetle larvae is associated with a general increase in activity of at least one component of the electron transport chain, NADPH-cytochrome P-450 reductase. The interaction of this component with cytochrome P-450 presumably results in the *in vivo* detoxication of carbamate insecticides but this cannot be confirmed at present by the *in vitro* determination of *p*-nitroanisole-*O*-demethylation. Since NADPH-cytochrome c reductase is known to be lost during the process of homogenization and/or centrifugation from the microsomes into the microsomal supernatant it is possible that the lack of xenobiotic metabolism as measured by *O*-demethylation is due to its lack of interaction with cytochrome P-450. Other xenobiotic oxidations need to be examined to further characterize resistance in the Colorado potato beetle *in vitro*.

In Vivo Distribution of
Radiolabeled Carbaryl

Pretreatment of the New Jersey population of Colorado potato beetle larvae with the monooxygenase synergist, piperonyl butoxide, resulted in a significant increase in the toxicity of carbofuran. This implicated a high degree of dependence upon monooxygenase enzymes for carbamate detoxication, however, since piperonyl butoxide pretreatment did not restore the New Jersey population to levels approximating the susceptible Logan population it was suggested that more than one factor may be involved in the resistance mechanism of the New Jersey population. Since decreased absorption is often a preferred mechanism of resitance in insects, especially for DDT and other chlorinated hydrocarbons to which the New Jersey population is most likely resistant, an examination of possible differences between the two populations in rates of penetration and excretion was performed utilizing radiolabeled carbaryl.

The results of the in vivo distribution of radiolabeled carbaryl over ten time periods and at two dose levels with and without piperonyl butoxide are shown in Table 8.

The cumulative rates of penetration, calculated from the data presented in Table 8, were used to transform the data into the rate of penetration per minute (Table 9) according to methods of calculation published by Sun (1968). The rate of penetration per minute was calculated from the observed values according to $(P_2-P_1)/(t_2-t_1)$ where P_1 is equal to the percent penetrated at time t_1. Graphical presentations of this data revealed the presence of three rates of penetration, the first of which was exceedingly rapid,

Table 8. *In vivo* distribution of radiolabeled carbaryl alone and with piperonyl butoxide (p.b.) in the New Jersey and Logan populations of Colorado potato beetles at various time intervals and dose levels.[a]

Dose Level	Time After Treatment (min)	% Penetrated		% Excreted		% Internal	
		New Jersey	Logan	New Jersey	Logan	New Jersey	Logan
0.01 µg/larva							
	0	3.7 ± 8.9	15.9 ± 46.7	-	-	13.1 ± 8.6	10.8 ± 11.4
	3	82.9 ± 6.8	93.7 ± 2.2	32.1 ± 5.4	29.3 ± 15.8	60.5 ± 9.9	65.8 ± 20.2
	5	90.4 ± 4.6	95.1 ± 0.2	27.3 ± 19.0	13.2 ± 6.9	61.4 ± 12.3	72.8 ± 9.7
	10	94.8 ± 2.9	98.5 ± 1.1	31.8 ± 13.4	14.9 ± 4.2	65.5 ± 16.5	70.6 ± 12.3
	20	97.2 ± 2.0	98.9 ± 0.5	25.8 ± 13.2	8.9 ± 5.6	68.2 ± 14.5	76.6 ± 5.1
	30	97.3 ± 1.8	96.2 ± 3.7	29.4 ± 9.2	11.3 ± 2.3	58.9 ± 14.0	75.0 ± 5.8
	60	97.5 ± 3.0	99.1 ± 0.1	39.0 ± 7.5	27.0 ± 7.6	46.4 ± 0.9	59.6 ± 14.8
	120	97.5 ± 3.6	98.8 ± 0.4	42.9 ± 2.6	24.8 ± 7.4	42.4 ± 0.9	58.6 ± 28.9
	240	98.7 ± 1.1	99.2 ± 0.2	42.8 ± 11.1	31.8 ± 7.0	27.6 ± 6.3	35.2 ± 4.2
	240[b]	99.1 ± 0.1	98.6 ± 1.2	41.0 ± 3.6	27.5 ± 6.3	29.9 ± 10.6	34.8 ± 11.3
10 µg/larvae							
	240	76.3 ± 10.5	-	54.4 ± 5.0	-	24.2 ± 2.1	-
	240[b]	69.6 ± 4.1	-	54.4 ± 5.0	-	24.2 ± 2.1	-

[a]Results are means ± SE of at least three replications.
[b]Larvae were pretreated with 10 µg/g piperonyl butoxide 1 hour before treatment with radiolabeled carbaryl.

Table 9. Percentage of radiolabeled carbaryl penetrated, and the penetration rate per minute, in Colorado potato beetle populations at various time periods following topical treatment at 0.01 µg/beetle.

Time after Treatment (min)	New Jersey		Logan	
	penetrated %	rate/min[a] %	penetrated %	rate/min %
0	3.7	-	15.9	-
1	65.0	61.3	80.9	65.0
3	82.9	9.0	93.7	6.4
5	90.4	3.8	95.1	6.4
10	94.8	0.9	98.5	0.7
20	97.2	0.2	98.9	0.8
30	97.3	0.0	96.2	0.0
60	97.5	0.0	99.1	0.0
120	97.5	0.0	98.8	0.0
240	98.7	0.0	99.2	0.0

[a] Rate of penetration = $\frac{P_2 - P_1}{t_2 - t_1}$ where P_1 = percent penetrated at time t_1 (Sun 1968).

occurring within the first 5 minutes after application, resulting in approximately 90% of the applied dose being absorbed by both populations. This was followed by a second phase of penetration which also was very rapid up until 20 minutes had elapsed. A third period followed in which a slow steady state of penetration continued for the duration of the experiment.

In nearly all time periods examined, the Logan population had absorbed a greater percentage of the radiolabeled carbaryl than had the New Jersey population. Statistical analysis, however, did not reveal significant differences in penetration between populations.

Excretion of radiolabeled carbaryl and metabolites began almost immediately but was relatively stable during the first 30 minutes (Table 8, Figure 6). This was followed by a rapid phase of excretion occurring in both populations up until 1 hour, after which it remained at relatively stable levels throughout the remaining testing period. Excretion of the New Jersey population was consistently at levels approximately twice those of the Logan population.

The amount of internal carbaryl and metabolites peaked at 20 minutes for both populations and began a rapid decline up to 1 hour. This was followed by a more gradual decline throughout the remaining time periods. Increases in fecal excretion corresponded very closely with decreases in internal activity for both populations,producing a mirror image effect (Figure 6).

Statistical analyses of external, fecal, and internal fractions for the ten time periods did not demonstrate an overall statistical difference between the Logan and New Jersey populations. However,

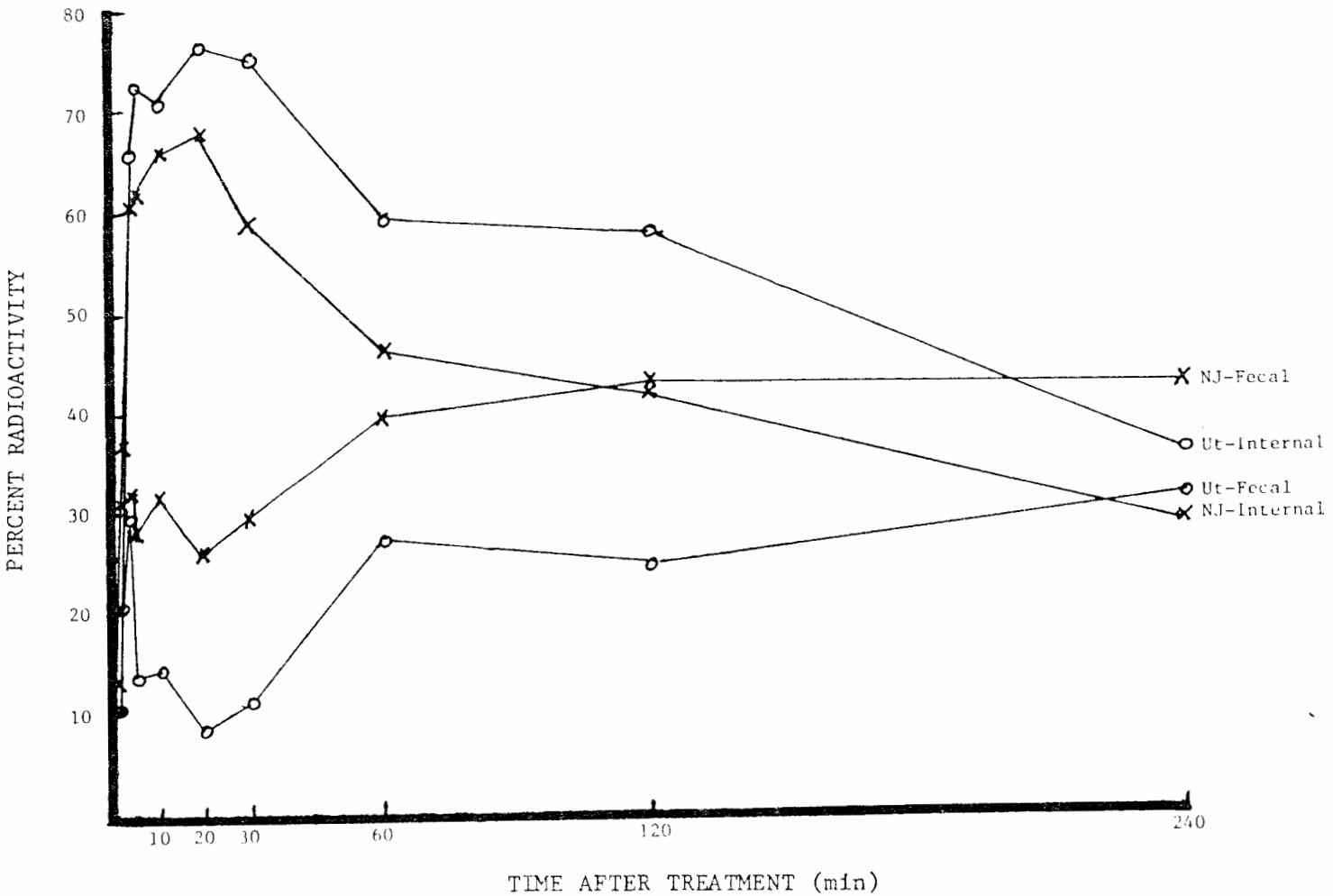


Fig. 6. In vivo distribution of 1-naphthyl N-methyl (^{14}C) carbamate in fecal and external extracts from fourth instar New Jersey and Logan populations of the Colorado potato beetle.

time periods, sources, and interactions between time and sources, and populations and sources were all significant at the 99% level. Of most importance is the significant interaction between populations and sources since this is indicative of the variability seen between the populations and the levels of excretion and internal accumulation within the beetles.

Addition of the synergist, piperonyl butoxide, appeared to show no effect on the amount of radioactivity recovered from any tissue after 240 minutes. Because the dose of radiolabeled carbaryl used in this study was very low (0.01 µg/larva) to avoid the possibility of killing the Logan population when synergized, it was felt that although the synergist may have been effectively blocking the monooxygenases, that the dose of toxicant may have not been sufficient to challenge the site. Therefore, application of the toxicant at 10 µg/larva to the New Jersey population was examined with and without piperonyl butoxide pretreatment. This dose generally resulted in a 20% mortality of the New Jersey population. However, even at this dose, piperonyl butoxide could not be shown to have an effect on penetration, excretion, or internal accumulation; even though greater than 20% of the applied dose remained on the cuticle due to the residual effect of the high concentration applied (Table 9).

In resistant and susceptible house flies, pretreatment with a synergist such as piperonyl butoxide generally does not alter the rate of penetration for most carbamates but does 'reduce the amount of metabolite excretion by resistant strains' (Kuhr 1970). This reduction in metabolism results in the accumulation of the parent

compound to levels high enough that death results. In both the bollworm, Heliothis zea, and the tobacco budworm, Heliothis virescens, the majority of material excreted was found to be unchanged carbaryl (66 and 54%, respectively) (Plapp 1973). Similar results have been reported for the gypsy moth, Lymantria dispar (L) (Ahmad et al. 1980). Metabolism of carbaryl by resistant and susceptible strains of the cabbage looper, Trichoplusia ni (Hubner), was significantly reduced by piperonyl butoxide, with the reduction in the resistant strain closely paralleling the magnitude of the increased metabolic capacity of that strain (Kuhr 1971).

The puzzling results of pretreatment with piperonyl butoxide in resistant Colorado potato beetle larvae are difficult to interpret without additional information. The dose level at which the synergist was applied (0.1 µg/g) had been determined by bioassays to be nontoxic for both populations. Dose levels as high as 100 µg/g only occasionally produced mortality, with no obvious difference occurring between populations. Perhaps for this portion of the study it would have been wise to have used this higher dose to ensure maximum inhibition, however, biological assays utilizing the lesser dose level had been very effective in blocking the site of detoxication of both carbamates to a certain extent. Increasing the dose level of radiolabeled carbaryl also did not seem to have any effect. An examination of the metabolic products in the resistant New Jersey population would have been useful in interpreting these results since piperonyl butoxide may have resulted in blocking metabolism of carbaryl while exerting no effect upon its excretion.

Although differences in the rate of penetration are present, they alone are not sufficient to account for differences in toxicity observed between resistant and susceptible Colorado potato beetles. The rate of excretion in the resistant New Jersey population, however, is nearly twice that of the Logan population, accounting for a significant reduction of radiolabeled carbaryl and its metabolites from the internal fraction of the New Jersey beetles. This, combined with a slightly decreased absorption rate may account for a great deal of the difference observed between populations. A likely resistance mechanism for carbaryl may simply be an increased capacity for excretion of toxic chemicals as suggested by the fact that piperonyl butoxide did not seem to exert a visible effect upon levels of radiolabeled materials in the internal fraction. Much more work is needed to elucidate fully mechanisms of resistance in this pest.

SUMMARY

Resistant and susceptible populations of the Colorado potato beetle, Leptinotarsa decemlineata (Say), and the house fly, Musca domestica (L), were compared with respect to carbofuran and carbaryl toxicity in the presence and absence of the synergist, piperonyl butoxide. Resistance levels of the New Jersey population when compared with the susceptible Logan population by topical application of carbaryl and carbofuran were > 833 and 820, respectively. A resistance level of 583 was determined from carbofuran bioassays of Rutgers and NAIDM house flies. Similar levels of resistance development between these species suggested the possibility that similar resistance mechanisms may be involved.

Applications of the synergist, piperonyl butoxide, resulted in dramatic increases in carbamate toxicity for both resistant strains, while lesser increases were observed in the susceptible strains. Utilization of the synergist difference approach for evaluating synergist data indicated that the resistant strains depended to a much greater extent upon detoxication by monooxygenases than did their susceptible counterparts. This was also reflected by calculations of percent dependency upon monooxygenases as given by the equation, Log LD_{50} = 1.014 log SD - 0.01. The New Jersey population of potato beetles, like the Rutgers strain of house flies showed a high degree of dependency upon monooxygenases (93 and 115% dependency, respectively) for carbofuran detoxication. Logan potato beetles and

NAIDM house flies showed a lesser dependency upon monooxygenases for detoxication (26 and 66% dependency, respectively) as is evidenced by their greater susceptibility. Synergism by piperonyl butoxide resulted in the restoration of the Rutgers strain of house flies to levels of susceptibility but failed to increase carbamate toxicity to such an extent for the New Jersey population of potato beetles. This resulted in the formulation of the hypothesis that for the New Jersey population, resistance mechanisms other than monooxygenase detoxication may be involved.

An attempt to conclusively demonstrate that resistance in Colorado potato beetles was associated with increased monooxygenase activity involved the use of *in vitro* p-nitroanisole-O-demethylation assays. Microsomal preparations from whole larvae, isolated fat body, and isolated gut tissues with and without gut contents resulted in preparations which were devoid of activity. In contrast, microsomal preparations from Rutgers and NAIDM house flies demonstrated a four-fold oxidative difference between strains.

Determinations of the distribution of NADPH-cytochrome c reductase between fractions obtained during centrifugation revealed that less than 15% of the total activity of this enzyme was sedimented into the microsomal pellet with the majority of activity occurring in the microsomal supernatant. Since the solubilization of NADPH-cytochrome c reductase has been associated with specific inhibitors isolated from insect preparations it is likely that this was also occurring in Colorado potato beetles due to contamination by gut contents.

NADPH oxidation and NADPH-cytochrome c reductase were examined for quantitative differences which may be associated with increased levels of cytochrome P-450 in resistant potato beetles and house flies. NADPH oxidation in gut and fat body from Colorado potato beetles and from abdomens of house flies was not found to differ significantly between resistant and susceptible populations of a given species nor between tissue sources. This result was contradictory to a previous study performed with house flies, perhaps due to age and sex differences between the studies. In a similar manner, NADPH-cytochrome c reductase did not vary between house fly strains nor between tissue sources from the Colorado potato beetle. There was, however, nearly a two-fold difference observed between populations of the Colorado potato beetle. This is consistent with the idea that resistance of the New Jersey population is associated with cytochrome P-450.

The in vivo distribution of 1-naphthyl-N-methyl-(^{14}C)-carbamate was examined in an attempt to explain why biological assays involving piperonyl butoxide did not restore resistant beetles to levels approximating those of the susceptible population. Results indicated that decreased penetration in resistant potato beetles is probably not a significant factor. However, the excretion of radioactivity in the New Jersey population was approximately twice that of the Logan population, confirming that quantitative differences do exist between these populations.

This study has been successful in establishing that monooxygenases play a chief role in Colorado potato beetle resistance to carbamate insecticides. However, the in vitro characterization of

resistance in this insect is difficult, possibly due to the presence of an inhibitor which solubilizes NADPH-cytochrome c reductase and is likely to be associated with the gut contents. The results of biological studies indicate that either piperonyl butoxide is not as effective in inhibiting monooxygenases in potato beetles as in house flies or that another mechanism of resistance may be involved which could not be confirmed by this study. Further work with this insect needs to be done to confirm the presence of monooxygenases further and to demonstrate the existence of a monooxygenase inhibitor more conclusively.

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