The Toxicity, Metabolism and Distribution of Carbaryl in Three Species of Labops with and without Piperonyl Butoxide Treatment (Hemiptera: miridae)

Deifalla H. Osman
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THE TOXICITY, METABOLISM AND DISTRIBUTION OF
CARBARYL IN THREE SPECIES OF LABOPS
WITH AND WITHOUT PIPERONYL BUTOXIDE
TREATMENT (HEMIPTERA:MIRIDAE)

by

Deifalla H. Osman

A thesis submitted in partial fulfillment
of the requirements for the degree
of
MASTER OF SCIENCE
in
Toxicology

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Logan, Utah
1979
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Deifalla H. Osman
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ABSTRACT

The Toxicity, Metabolism and Distribution of Carbaryl in Three Species of Labops with and without Piperonyl Butoxide Treatment (Hemiptera:miridae)

by

Deifalla Osman, Master of Science
Utah State University, 1979

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

Carbaryl toxicity, metabolism, and distribution in adults of three species of grass bugs from the genus Labops were studied in relation to species, sex, and treatment with piperonyl butoxide. LC₅₀ values for 8 hour exposure periods ranged from 0.02-0.14, 0.03-0.3, and 0.2-0.7 µg carbaryl/vial for L. utahensis, L. hirtus, and L. hesperius respectively. The males were more susceptible to carbaryl than females. Males of L. utahensis were more susceptible than L. hirtus and L. hesperius respectively.

The synergist difference values (LC₅₀ of carbaryl alone - LC₅₀ values of carbaryl after piperonyl butoxide treatment) were measured. The percent dependency of these insects on MFOs for detoxifying carbaryl was estimated based on the theoretical synergist difference which was calculated by the equation Log LC₅₀ = 1.014 log SD - 0.009. The percent dependency values were 38-59, 25-46, and 13-33% for L. hesperius, L. hirtus, and L. utahensis, respectively. Males of L. utahensis had
the lowest percent dependency upon MFOs in detoxifying carbaryl (13%) indicating the possibility that carbaryl toxicity may be controlled by other potential defense mechanisms which are relatively ineffective themselves in view of the low tolerances of the insects to carbaryl.

Treatment with piperonyl butoxide resulted in greater enhancement of carbaryl toxicity against *L. hesperius* (synergized LC₅₀ 0.1-0.26 µg carbaryl/vial) while it showed a moderate effect on *L. hirtus* (synergized LC₅₀ 0.02-0.16 µg carbaryl/vial). Piperonyl butoxide's effect was less pronounced in the case of *L. utahensis* (synergized LC₅₀ 0.013-0.09 µg carbaryl/vial).

Unmetabolized carbaryl was the principle compound isolated from the bugs after 6 hours from treatment, being more prominent in males of *L. hirtus* (71.1% of the total metabolites) and less prominent in females hesperius (36.7% of the total metabolites).

The mechanism of detoxication appeared to include ring hydroxylation for both species and sexes. 4 and 5-hydroxycarbaryl were the only metabolites associated with the degrading of carbaryl by the bugs, since the levels of metabolites obtained were too low for accurate quantitation. Pretreatment with piperonyl butoxide prevented the appearance of both carbaryl metabolites in the organosoluble fraction and increased the accumulation of unmetabolized carbaryl. This effect was probably due to inhibition of the insect's MFO system.

Generally, this study showed a good correlation between the bioassays and the metabolic studies, thus reflecting the effectiveness of the bioassays along with synergist difference (SD) and percent dependency concepts in establishing some conclusions regarding the MFOs of *Labops*
bugs. Further application of these techniques with agricultural insects should provide a practical means of characterizing field populations for insecticide tolerance, relative levels of MFOs and their role as a defense mechanism.
INTRODUCTION

The microsomal mixed function oxidases (MFOs) are groups of enzymes which depend on NADPH as a cofactor and cytochrome P-450 as a terminal acceptor. The MFOs are distributed in different organs or tissues and play an important role in detoxication mechanisms of animals which for the most part, lead to protection of the animals. In vertebrates MFO activity in the liver is much more pronounced than in other organs such as digestive tract and lung. There is no such analogous single organ in all insects. The MFOs are associated with the endoplasmic reticulum of cells and their importance is derived from their ability to convert lipophilic substances into more polar compounds. In some cases, MFO action may "activate" a lipophilic substance by producing a metabolite which is more toxic than its parent compound.

In vitro studies of the MFO enzymes have encountered two major problems. These are selection of a proper tissue source and selection of proper methods including microsomal fraction preparations and conditions to observe maximum activity.

Carbaryl, with other carbamates, show general susceptibility to MFO detoxication. Therefore, the toxicity of a carbamate is related, in part, to how effectively and extensively the MFOs degrade it. Thus, inhibition of MFOs by a synergist, for example piperonyl butoxide, could alter the LD$_{50}$ value. Data reflecting the degree of LD$_{50}$ alteration might be used to measure the MFO activity in vivo. This has been applied by using the "synergist ratio" (Brattsten and Metcalf 1970, 1973, 1973b).
Lee and Brindley (1974) indicated that the piperonyl butoxide-carbaryl synergist ratio did not provide a good estimate of the in vivo detoxication potential as measured by EPN detoxication in alfalfa leafcutting bees. Brindley (1977) suggested that using synergist differences rather than synergist ratios may provide hypotheses that are clear and useful in estimating MFO activity in vivo.

The vast majority of studies indicated that carbaryl may metabolize via hydrolysis, oxidations, and conjugations (Kolbezen et al. 1954; Casida and Augustinsson 1959; Casida et al. 1960; Terriere et al. 1961; Whitehurst et al. 1963; Zayed et al. 1966; Andrawes and Dorough 1967; Tsukamoto and Casida 1967a, 1967b; Kuhr 1970; Guirguis and Brindley 1975; Hurst and Dorough 1978). Hydroxylation rather than hydrolysis has been reported to be the major mode of carbaryl metabolism (Kolbezen et al. 1954; Casida and Augustinsson 1959; Casida et al. 1960; Carpenter et al. 1961; Dorrough and Casida 1964; Guirguis and Brindley 1975).

Black grass bugs are a complex of closely-related range insects (HEMIPTERA: Miridae). Data presented in this thesis show that three species of grass bug are sensitive to carbaryl insecticide but there are variations of sensitivity among and within species. These variations might be related to the level of MFO enzymes in these insect species.

The feeding injury produced by large populations of insects effectively reduced the nutritive value of the grass (Denning 1948; Todd and Kamm 1974; Higgins et al. 1977). Malecheck et al. (1977) indicated that even at low population densities, the yield and nutritional quality of intermediate wheat grass infested by black grass bugs are sharply reduced.
Since the grass bugs can become economically important pests in Utah or elsewhere, variations in MFO level would be important to understand.

Insecticide bioassays can be conducted with great precision if the physiological and environmental factors are standardized. Brindley (1975), however, found that simplified bioassay techniques could be useful if based on field populations of certain agricultural insects. This was done with a simple test kit which might indicate the early development of insect resistance to insecticides.

The experiments reported in this thesis were conducted to study the toxicity of carbaryl in three species and sexes of black grass bugs with and without piperonyl butoxide synergist. The experiments also detected the effect of species and sex upon MFOs through the interpretation of simple bioassays and confirmation by an in vivo metabolic study.

The study provides a base for further research using the bioassay methods with other insecticides potentially useful to control the bugs and other insects.
REVIEW OF LITERATURE

There is no doubt microsomal mixed function oxidases are the most important membrane bound detoxication mechanism of animals. They are distributed in several tissues or organs but may be concentrated in some tissues or organs more than others. In vertebrates their activity in the liver is much more than in other organs but there is no specific single organ in all insects.

Literature describes MFO characteristics, along with other important literature references for this study, will be discussed in the following sections.

Morphological Characteristics of MFO System

In spite of the often anomalous sedimentation behavior of insect microsomes, surprisingly little attention has been given to investigation of their ultrastructural characteristics and to determine whether indeed they have similar morphological properties to those described from mammalian liver.

The structural components of microsomal preparations from *Musca domestica* abdomens and *Prodenia eridania* gut and fat body for electron microscopy have been found to be similar to those of smooth microsomes from mammalian liver (Cassidy et al. 1969). Membraneous vesicles, broken membranes, free ribosomes, and clusters of glycogen granules have been found in *Prodenia eridania* microsomal preparations were essentially composed of materials derived from the endoplasmic reticulum.
Microsomal preparation from the fat body of American cockroaches were primarily derived from the rough surfaced endoplasmic reticulum (Brindley and Dahm 1970). Benke and Wilkinson (1971) and Benke et al. (1972) demonstrated that since the endoplasmic reticulum extends into microvilli that project into the lumen of the gut of P. eridania, it is possible that these microvilli constitute important sites of MFO activity.

From these investigations and biochemical similarity to mammals, it can be concluded that MFO activity in subcellular fractions of insect tissues is associated membrane particles derived from the endoplasmic reticulum of the cell and that these fragments and vesicles are quite similar in appearance to the microsomes from mammalian liver.

**Biochemical Characteristics of MFO System**

The microsomal enzymes and their role in insecticide toxicology have been reviewed thoroughly by Casida (1970), Wilkinson and Brattsten (1972), Agosin and Perry (1974), Ahmad (1975), Wilkinson (1976), and Walker (1978). They indicated that membranes in MFOs were composed of a great deal of lipoprotein including lipositol, phospholipid, plasmalogen and fatty acids. They also indicated that flavoprotein, NADPH-cytochrome C reductase, and cytochrome P-450, which is the terminal oxidase important for oxidation reactions, are the major components of the MFOs along with other minor components. However, there are different spectra of cytochromes and at least more than one type of P-450 in mammalian liver (Goldstein et al. 1974 and Jefcoate et al. 1969).
Cytochrome P-450 could thoroughly be inhibited by carbon monoxide resulting in blockage of MFO activity (Kuhr 1969, 1970; Benke and Wilkinson 1971; Hansen and Hodgson 1971; and Wilkinson 1976).

Many investigators have been able to demonstrate the importance of insect MFOs in insecticide metabolism. In the presence of molecular oxygen (O₂) and HADPH, MFOs have been found to catalyze insecticide oxidative reactions through a number of pathways (Wilkinson 1968; Casida 1970; Brooks 1972; Wilkinson 1976; Paine 1978). The importance of the insect MFOs in insecticide metabolism was first indicated by Sun and Johnson in 1960. Nakatsugawa and Morelli (1976) have listed the MFO-mediated reactions including the hydroxylation of aromatic and alicyclic rings, the hydroxylation of aliphatic side chains, the dealkylation of aromatic and cyclic ethers, the dealkylation of substituted amines, the epoxidation of double bonds, the oxidation of thioethers, and the desulfuration of phosphorothionates to phosphates. In addition to these oxidative reactions, insect MFOs also catalyze the reduction of aromatic nitro and azo compounds and affect the hydration of epoxide rings. For the most part, these react with insecticidal compounds and constitute detoxication reactions, but in some cases, such as in desulfuration of phosphorothionates, the reaction is responsible for activating the compound to one that is highly toxic (Wilkinson and Brattsten 1972). Wilkinson (1976) indicated that the oxidation reaction(s) insecticides may undergo depends largely on their chemical structures, which may possess more than one site at which the MFOs can attack.

Figure 1 illustrates the most important sequences in the microsomal oxidation of foreign compounds in mammalian liver that results
Fig. 1. Interaction of electrons, oxygen and substrate in the microsomal oxidase system. (Nakatsugawa and Morelli 1976)

in transfer of electrons or reducing equivalents through a series of redox components from NADPH to cytochrome P-450. In this scheme, the substrate(s) first forms a complex with the oxidized form of cytochrome P-450, which is then reduced by the flow of electrons from NADPH. The reduced cytochrome P-450 substrate complex subsequently combines with molecular oxygen and the resulting complex rapidly breaks up to give the oxidized substrate and water, at the same time regenerating oxidized cytochrome P-450 for further substrate binding. Some inhibitors of MFOs may act by complexing with cytochrome P-450 but failing to dissociate from it. A flavoprotein, which may \textit{in vitro} pass electrons to cytochrome C and is, therefore, called NADPH-cytochrome C-reductase, is the major intermediate between NADPH and cytochrome P-450. \textit{In vivo}, however, cytochrome C would not be involved. Strimatter and Velick (1956) reported that another redox pathway that transfers electrons from NADH to cytochrome b$_5$ via a flavoprotein and NADH-cytochrome b$_5$ reductase. A recent discussion of these pathways has
been offered by Hodgson and Tate (1976). The overall reaction may be formulated as follows, where $A$ is the oxidized form and $AH_2$ is reduced form of cytochrome P-450: (Goldstein et al. 1974)

$$NADPH + A + H^+ \rightarrow AH_2 + NADP^+$$

$$AH_2 + O_2 \rightarrow \text{"active oxygen complex"}$$

$$\text{active oxygen complex} + \text{substrate} \rightarrow \text{oxidized substrate} + A + H_2O$$

$$\text{HADPH} + O_2 + \text{substrate} + H^+ \rightarrow NADP^+ + \text{oxidized substrate} + H_2O$$

Information that is available on substrate binding to cytochrome P-450 in insect microsomes indicated that substrate binding spectra in insect preparations are similar to those reported for hepatic microsomes. (Remmer et al. 1969; Wilkinson and Brattsten 1972) Different spectra may be formed as a result of substrate binding to cytochrome P-450. Hodgson and Tate (1976) indicated that different spectra may be formed by methylendioxyphenyl compounds and reduced cytochrome P-450 from insecticide-susceptible and resistant strains of houseflies which reveals that, although some variations occur, essentially the same types of spectra are formed.

**Resistance**

Oppenoorth (1965) has defined resistance as the ability of a strain of insects to tolerate doses of toxicant which could prove lethal to the majority of individuals in a normal population of the same species. Insect resistance has been suggested to be inherited or acquired (Plapp 1976; Oppenoorth and Welling 1976; Oppenoorth et al. 1977).
Oppenoorth and Welling (1976) indicated that resistance due to induction of a greater detoxication capacity has never shown that such induced alteration is inherited. Since the resistance in many instances was found to be at least partially due to high levels of MFOs, discussion will emphasize the contribution of MFOs to the insect's resistance.

Several genetic studies have confirmed the fact that microsomal oxidation is controlled by genes on different chromosomes. Oppenoorth (1967); Schonb~o:d et al. (1968); Tsukamoto et al. (1968) demonstrated some oxidative resistance contributions of both chromosomes 2 and 5 in different resistant housefly populations. They also indicated that only oxidative genes confer metabolic resistance to carbamate insecticides in housefly which both oxidative and nonoxidative metabolic resistance genes are known to be important. Plapp (1970) indicated that insects with multiple series of genes are capable of producing larger amounts of detoxifying enzymes. Georghiou (1971), found that epoxidation of aldrin to dieldrin in a resistant housefly strain was higher than in any of its substrains carrying one chromosome only. Different strains of the same species and different species have different cross-resistance spectra suggesting that MFO activity differs qualitatively among these lineages (Georghiou 1972). Schonbrod et al. (1965) indicated that naphthalene oxidative enzymes were high in naphthalene resistant strain. Tsukamoto and Casida (1967a) indicated that methylcarbamate oxidation was much higher in resistance housefly strain than on susceptible ones.

Evolution of resistance to pesticides, resistance to different insecticides, cross-resistance, and causes of resistance have been
reviewed thoroughly by Folsom et al. (1970), Matthews and Casida (1970), Perry and Kintzias (1970), Philpot and Hodgson (1971), Oppenooorth and Welling (1976), Plapp et al. (1976) and Georghiou and Taylor (1977). They indicated that both oxidative and nonoxidative metabolic resistance genes are related to a variety of changes in total microsomal oxidase activity. These include increases in the quantity of cytochrome P-450, as well as several types of qualitative changes in cytochrome P-450.

High levels of MFOs characterized several other organophosphorous and carbamate resistant insects including Lygus hesperus (Leigh et al. 1977) and the tobacco budworm (Plapp 1973; Reed 1974). However, the same or similar resistance mechanisms probably occur widely among insect species.

Several studies have indicated that MFOs may be under hormonal control (Krieger and Wilkinson 1969; Benke and Wilkinson 1971; and Benke et al. 1972). It has been demonstrated that insecticide resistant strains of several species of insects may also exhibit varying degrees of cross-resistance to synthetic analogues of juvenile hormones (Plapp and Vinson 1973; Cerf and Georghiou 1974; and Plapp 1975). Georghiou et al. (1978) demonstrated that high levels of resistance to juvenoid methoprene have been induced in a dimethoate-resistant laboratory strains as well as in a field multiresistant strain of house-fly, Musca domestica, by exposure of mature larvae to methoprene treated pupation medium.

Resistance could be due to many other factors than MFOs such as alteration of acetylcholinesterase, low penetration, the metabolism of insecticides by esterases not associated with nervous system but which hydrolyze ester compounds in the presence of water and absence of
NADPH₂, selection of polyphenol oxidase which is usually associated with other insect tissues, and inert storage of insecticide or its metabolites in fatty tissues (Matsumura 1975).

Resistance due to acetylcholinesterase (ACHE) alteration was first reported in 1971 by Hama and Iwata in a population of the green rice leafhopper, *Nephotettix cincticeps*, resistant to carbamate insecticides. However, many studies suggest that high level of MFOs may result in resistant Hemipteran species. Leigh et al. (1977) indicated that *Lygus hesperus* insects have developed resistance to both organophosphorous and carbamate insecticides which is at least partially due to high MFO levels in that insect. On the other hand Brattsten and Metcalf (1970) demonstrated that the large milkweed bug, *Oncopeltus fasciatus*, have shown a moderate tolerance to carbaryl toxicity. Oppenoorth and Welling (1976) indicated that MFOs role in carbamate tolerance is much more important than any other cause of resistance.

**Factors Affecting MFO Activity**

**Physiological Factors**

It is well established that MFO activity shows considerable variations with respect to species and strain, sex, age, stage of development, and endogenous or exogenous factors (Wilkinson and Brattsten 1972; Wilkinson 1976; and Walker 1978).

Khan and Terriere (1968), Tsukamoto (1968), Casida (1969, 1970), Brattsten and Metcalf (1970), Gillet (1971) and Yang et al. (1971) indicated that there can be a good relationship between MFO activity and insecticide resistance which reflected that species or strains of
resistant insect had high in vitro levels of MFO activity. They also indicated that in most cases the high levels of MFO activity is always associated with increase in cytochrome P-450 levels. However, they found that houseflies selected for resistance with either a carbamate or DDT may simultaneously attain high epoxidation hydroxylation and O- and N-demethylation activities that in addition to metabolism enhancement of insecticide used, they also facilitate the metabolism of a large number of other unrelated insecticides by pathways involving microsomal oxidation. Call et al. (1977) demonstrated that significant differences have been found in enzyme activity levels between three species of corn rootworm.

Brattsten and Metcalf (1970) and Gillet (1971) pointed out that MFO activity in response to foreign compounds varies among insect species as well as among individuals of the same species and differences in MFO activity between resistant and susceptible species or strains of insects are quantitative rather than qualitative but it is quite possible to involve qualitative differences due to the change in the conformation of cytochrome P-450.

With respect to sex differences, it has become recently clear that in several insect species, MFO activity varies between sex and generally females have higher MFO activity.

El-Aziz et al. (1969) and Shrivastava et al. (1969) demonstrated that female houseflies have greater tolerance to many insecticides than the males and this tolerance is mainly caused by high MFO activity responsible for insecticide detoxication. Khan (1970) also indicated that microsomes from whole female houseflies have greater epoxidation
activity than those of males. In in vitro studies by Benke and Wilkinson (1971), epoxidation and hydroxylation activity have been found to be higher in microsomal preparations from cricket malpighian tubules of females than in males. El-Aziz et al. (1969) indicated that male of two housefly strains and German cockroaches, to some extent, were found to be more susceptible to carbamates than the females did. Lee and Brindley (1974) demonstrated that MFO activity in female alfalfa leaf-cutting bees was higher than in the males and males were much more susceptible to carbaryl insecticide than the females. Female western corn rootworms have been found to have much more MFO activity than the males (Call et al. 1977).

Differences observed between sexes are often small and may depend to some extent on the methods of preparation.

Insect age seems to play a critical role in the variations of MFO activity which usually correlates quite well with the level of cytochrome P-450. El-Aziz (1969) found that in some housefly strains, the MFO level was responsible for metabolizing carbaryl decreased as flies aged. Lee and Brindley (1974) found that MFO activity in alfalfa leafcutting bees decreased with the bees age. The level of MFO activity in the fat bodies of young American cockroach has been found to increase but decreased after 100 days of age (Turnquist and Brindley 1975). It has been demonstrated by Call et al. (1977) that MFO activity in the whole body homogenates of western and southern corn rootworm decreased as the insects aged. However, it is well known that dramatic changes in MFO activity occur during development and
metamorphosis of insect species (Benke and Wilkinson 1971; Benke et al. 1972; Yu and Terriere 1974; Terriere and Yu 1975). Recently, it has become clear that the nutritional status of the insect exhibits major variations in the level of MFO activity and the response of insects against insecticides. Way (1954), Bass and Cawson (1960) and Ahmad and Forgash (1978) indicated that feeding activity or food type can markedly influence the susceptibility of insect species to insecticides, and these differences in susceptibility may be the result of changes in the activity of detoxication enzymes brought about by the alterations in the insect's nutrition. Perry (1964) and Perry and Kintzias (1970) demonstrated that susceptibility of insects to insecticides is influenced by diet and that might be related to the changes in MFO activity and metabolism. Adult houseflies fed on sugar and water were found to be consistently more susceptible to m-isopropyl phenyl N-methyl carbamate than those fed on milk and that this may result from changes in MFO activity (El-Aziz et al. 1969). Ahmad and Forgash (1978) demonstrated that the level of MFO activity in the gut of gypsy moth, *Prothetria dispar*, increased markedly when they fed on wheat germ diet. More recently, Fuhremann and Lichenstein (1979) indicated that some components occurring naturally in food plants have the ability to alter the toxicity of insecticides as a result of their effects on MFO system.

**Environmental Factors**

The MFO system can be dramatically induced by sublethal levels of a wide variety of substances, including some of the insecticides
such as dieldrin, phosphoric acid esters, juvenile hormones and some secondary plant substances. However, the induction phenomenon is not genetically controlled and, in turn, is not inheritable. The increase in MFO activity has been found to be associated with increase of almost all MFO system components including cytochrome P-450. Induction by pesticides for different insect species has been pointed out by several investigators. Matthews and Casida (1970) indicated that dieldrin increased the cytochrome P-450 level in the Orlando-R strain. Yu and Terriere (1972) demonstrated that dieldrin increased the oxidase activity in Orlando R strain.

A decrease in parathion toxicity to the larval instars of wax moth *Galleria mellonella*, fed diet treated with chlorcyclizine, phenobarbital, aminopyrine has been reported by Ahmad and Brindley (1969). Turnquist and Brindley (1975) found that cytochrome P-450, p-nitroanisole, O-demethylation, EPN-detoxication, and NADPH-neotetrazolium reductase were induced in American cockroaches by chlorcyclizine. Aminopyrine decreased the carbaryl LD$_{50}$ against the male of alfalfa leafcutting bees while phenobarbital had no effect at all (Lee and Brindley 1974). Guirguis and Brindley (1975), indicated that amino pyrine made both male and female alfalfa leafcutting bees more susceptible to carbaryl.

Some secondary plant substances have been found to be good MFO inducers. Brattsten et al. (1977) demonstrated that secondary plant substances induced the MFO system in the larva of a polyphagous insect, the southern army worm, *Spodoptera eridania*, and thus enhanced the oxidative metabolism of natural toxicants.
The MFO system also could be thoroughly inhibited by a variety of inhibitors. The inhibition of MFO activity is a matter that has received more attention in insects than in mammals. This is derived from the fact that compounds that inhibit MFO activity have the potential to act as insecticide synergists \textit{in vivo} and thereby enhance the insecticide potency against insect pests. The best known of the potent synergists is piperonyl butoxide which will be discussed in the following section.

**Piperonyl Butoxide Synergism**

The toxicity of certain insecticides can be enormously increased by the addition of compounds which may not be insecticides at all. These compounds are called synergists. Synergistic compounds such as methylenedioxyphenyl derivatives have been used for many years as useful indicators of the detoxication mechanism by MFOs. It has been demonstrated by Wachs (1947) that methylene dioxyphenyl compounds (MDPs) effectively enhanced the toxicity of pyrethrum insecticides. Remarkable activating features of MDPs have been demonstrated by Moorefield (1958) which they used with carbaryl against \textit{Musca domestica} and \textit{Aphis fabae}.

Through several studies, it has become clear that resistant insect species are often associated with high level of MFOs which increase the detoxication potential of insects and resulted from selection of individuals containing adaptive genes. These MFOs could be inhibited by MDPs (Philleo et al. 1965; Metcalf et al. 1967). The inhibitory effects of the MDPs are to dramatically increase the toxicity of carbamate insecticides (Georghiou and Metcalf 1961; Casida 1963; Dorough et al.)

Piperonyl butoxide is probably the most widely used and studied MDP. Sun and Johnson (1960) first demonstrated the importance of piperonyl butoxide as an inhibitor of the MFO enzymes in insects. This inhibition effect was confirmed by Casida et al. (1968). Metcalf et al. (1966) have pointed out the great effect of piperonyl butoxide in blocking the detoxication of carbamate-resistant houseflies. Casida (1970) and Wilkinson (1971) have reviewed the effects of synergists in inhibiting MFO enzymes and provided some structure activity relationships. Guirguis and Brindley (1975) reported that piperonyl butoxide has a dramatic effect on inhibition of the MFO system of alfalfa leafcutting bees and caused large enhancement of carbaryl toxicity.

Enzyme inhibition studies in Hemiptera have shown inconsistent trends. Gordon and Eldefrawi (1960) and Brattsten and Metcalf (1970, 1973a, 1973b) found that large milkweed bugs, Oncopeltus fasciatus, treated with piperonyl butoxide became more susceptible to carbaryl. On the other hand Gordon and Jao (1971) reported that piperonyl butoxide either had little effect or no effect at all on the toxicity of carbaryl to the large milkweed bug and Lygus hesperus. Among other Hemipteran species, piperonyl butoxide has a moderate effect on the toxicity of certain carbamate insecticides.

The inhibition of MFOs has been reported to be either competitive (Hansen and Hodgson 1971) in which the MDP compounds serve as alternative substrates for the MFO enzymes or non-competitive (Hennessy 1965; Hansch 1968; Ullrich and Schnable 1973) in which MDP compounds form carboxolium
ions, carbanions or free radicals which, in turn, interact with some MFO components to form a cytochrome P-450 complex. Hodgson and Tate (1976) suggested that the correlation between the formation of cytochrome P-450 complex and the inhibition of ethylmorphine N-demethylation is strongly supported classification of the inhibition as being non-competitive. However, Franklin (1972a) demonstrated that in in vivo MFO inhibition by MDPs was competitive and then changed to non-competitive after the formation of a cytochrome P-450 complex. Previous studies have indicated that MDP synergists undergo biotransformation by NADPH supplemented microsomal enzymes and the metabolite(s) formed bind to cytochrome P-450 (Philpot and Hodgson 1971). This complex blocks carbon monoxide binding to cytochrome P-450 and is believed to be responsible for synergist action (Hodgson and Philpot 1974). Kulkarni and Hodgson (1978) indicated that the mechanisms of synergistic action of MDPs appears to be related to the formation of a type III complex and consequent inhibition of co-binding to cytochrome P-450. They also demonstrated that cumene hydroperoxide mediated cytochrome P-450 oxidation and NADPH is not required in the presence of it to support the formation of type III spectra in vitro.

Piperonyl butoxide has been shown to have a great inhibitory effect in retarding the detoxication of insecticide-resistant insect species. Metcalf et al. (1966) indicated that piperonyl butoxide dramatically inhibited the detoxication of carbamate-resistant houseflies. The susceptible flies tended to accumulate greater amounts of intact carbaryl within their bodies than the resistant flies (Georghiou and
Metcalf 1961). The same relationship was demonstrated by Guirguis and Brindley (1975) in the case of alfalfa leafcutting bees.

The use of synergist may provide evidence that the resistance of insects to insecticides is due to the detoxication mechanism rather than insensitive target site. This suggestion has been confirmed by Brattsten and Metcalf (1970, 1973a, 1973b), and Guirguis and Brindley (1975). Using synergists to detect the changes in metabolism by resistant insects which effectively reduce the metabolic capacity, is another major reason for using synergists. However, Metcalf et al. (1960), Eldefrawi and Hoskin (1961), Georgiou and Metcalf (1961), El-Sebae et al. (1964) Fahmy and Gordon (1965), Metcalf and Fukuto (1965), Metcalf et al. (1967) El-Aziz et al. (1969), and Guirguis and Brindley (1975) have studied the effects of synergists on the metabolite rate of insecticides coming out with the fact that when the synergist is an effective MFO inhibitor, the metabolism rate is sharply slowed. Carbaryl and carbaryl with piperonyl butoxide toxicity were correlated well with microsomal epoxidase levels from adult house crickets, *Acheta domesticus* (L.), of different age and sex (Benke and Wilkinson 1971). Guirguis and Brindley (1975) indicated that MFO levels in the alfalfa leafcutting bees of different age and sex were correlated well with carbaryl and carbaryl plus piperonyl butoxide.

The so-called synergist ratio

\[
SR = \frac{LD_{50} \text{ of carbaryl}}{LD_{50} \text{ of carbaryl + synergist}}
\]

has been used by Brattsten and Metcalf (1970, 1973a, 1973b) to provide a rough estimation of MFO activity *in vivo*. They evaluated the *LD$_{50}$*
values of carbaryl with about 80 insect species. Wide ranges of variation in both carbaryl susceptibility and in the SR with piperonyl butoxide have been found. They attributed these differences to the levels of MFO activity among insects which is inhibited by piperonyl butoxide, so the higher the synergist ratio, the greater the MFO activity. Lee and Brindley (1974) indicated that the piperonyl butoxide - carbaryl synergist ratio for alfalfa leafcutting bees did not correlate well with decreasing microsomal detoxication in the male bees, nor with increasing tolerance to carbaryl of the females. Contradictions between SR values and the data obtained from honeybee, *Apis mellifera*, have been pointed out by Gilbert and Wilkinson (1974). Guirguis and Brindley (1975) reported that SR concept did not reflect obvious estimation of MFO activity in alfalfa leafcutting bees, *Mega-chile pacifica*. Brindley (1977) suggested that using synergist differences (LD$_{50}$ of carbaryl alone - synergized LD$_{50}$) rather than synergist ratios to estimate MFO activity in vivo, is more realistic and also the data could provide hypotheses that are clear, logical and useful in estimating of MFO levels. He plotted the SD values against LD$_{50}$ values for 70 insect species reviewed from Brattsten and Metcalf (1970, 1973a, 1973b), and data from his own laboratory for honeybees, and alfalfa leafcutting bees. A linear regression of Log LD$_{50}$ on Log SD had a perfect relationship for the leafcutting bees which carbaryl metabolism studies demonstrated to be insects that are dependent upon differing MFO levels for variations in carbaryl susceptibility.
Beside those factors discussed herein, there are some other factors such as temperature and pH, choice of tissue, and presence of inhibitors which are usually associated with microsomal preparations for in vivo studies. These factors have been thoroughly studied and are reported to affect MFO activity, although maximal activity may not reflect in vivo cellular conditions. Therefore, an in vivo approach to estimating MFO levels in insects may be very important.

**Evolution of the MFO System**

It is quite clear that the MFO system existed long before the advent of modern drugs and pesticides and the omnipresent occurrence of these enzymes throughout mammals and insects strongly suggests some common fundamental purposes for their existence. However, Brodie et al. (1958) and Brodie and Maickel (1962) first suggested that MFO systems have developed as a biochemical defense mechanism to protect organisms from the many naturally occurring lipophilic foreign compounds to which they are continually exposed. Freeland and Janzen (1974) have provided an excellent review of the role of the secondary plant substances and believed that MFO systems play a considerable role in feeding strategies of herbivorous animals. Secondary plant compounds are omnipresent in nature and their accumulation may serve as a defense mechanism against herbivores (Kingsbury 1964; Whittaker and Feeny 1971; Chapman 1974). The polyphagous insects should have evolutionarity adapted an effective MFO system to defend them against these toxic compounds that are frequently present in the diet. Krieger et al. (1971) indicated that the main
function of MFOs is the detoxication of secondary plant substances. Brattsten et al. (1977), demonstrated that MFO system in the larva of a polyphagous insect, the southern army worm, *Spodoptera eridania*, being effectively induced by secondary plant substances and thus enhanced the oxidative metabolism of natural compounds.

**Carbamate Insecticides**

Carbamate insecticides with the general structure of R-O-C-CH are derivatives of the naturally occurring alkaloid physostigime. Their effects as acetylcholinesterase inhibitors were well known long ago. (Matsumura 1975)

The reversible inhibitory effect of cholinesterase enzymes by carbamates could be generalized in two steps:

\[
K_d \quad K_2
E + CX \xrightleftharpoons{K_d} [E - CX] \rightarrow E - C + X
\]

The first step involving formation of the methylcarbamate-AChE complex (E-CX) is believed to be more important than the second step in fixing the inhibitory potency of methylcarbamates. (Wustner et al. 1978) In the equation (E - C) is the carbamylated enzyme, (X) is the leaving group, and \( K_2 \) is the carbamylation dissociation constant. The carbamylated enzyme rapidly splits by a hydrolytic process to regenerate the enzyme and is believed to be highly dependent on the N-alkyl substituents (O'Brien 1967, 1976).

The site of action of carbamates has been reviewed by O'Brien (1976) who demonstrated that the inhibition of AChE usually involves a reaction between the insecticide and the catalytic site (esteric site) of the
enzyme which is the hydroxyl group of an amino acid of the enzyme at which carbamylation of the inhibitory carbamate occurs.

Carbaryl Toxicity and Metabolism

Carbaryl (1-naphthyl N-methyl carbamate) is a carbamate insecticide, which has been widely used during the past 20 years. It has a lower mammalian toxicity than most organophosphorous insecticides and has relatively broad spectrum effectiveness (Crosby et al. 1965). It is highly specific among insects that are closely related phylogenetically but this specificity can always be minimized by the use of synergists (Fukuto et al. 1962; Georghiou and Metcalf 1962; Wilkinson et al. 1966). Carbaryl has been found to be effective against many insect species (Shaw 1959; Cowan et al. 1961; Morse 1961; Drummond 1962; Georghiou and Atkins 1964; Anderson and Atkins 1968; and Brattsten and Metcalf 1970, 1973a, 1973b). In contrast, it has not been effective against some insect species such as the housefly, Musca domestica (L.) (Eldefrawi and Hoskins 1961; and Georghiou et al. 1961) and the rice weevil, Sitophilus oryza (L.) (Strong and Sbur 1961).

Carbaryl has been quite effective against many hemipteran species. Gordon and Eldefrawi (1960), Jao and Gordon (1969), Brattsten and Metcalf (1970, 1973a, 1973b), and Gordon and Jao (1971) demonstrated that carbaryl showed a moderate toxicity to the large milkweed bug, Oncopeltus fasciatus. On the other hand, Leigh and Jackson (1968) and Leigh et al. (1977) indicated that carbaryl was effective against the bug Lygus hesperus. However, no carbaryl toxicity or metabolic data have been published about black grass bugs, the insects of concern in this study.
Carbaryl metabolism is a subject that attracts a great deal of attention due to its extensive use during the past 20 years. Carbaryl metabolism was first studied by Eldefrawi and Hoskins (1961) using the large milkweed bug, housefly, and German cockroach, Blattella germanica.

The vast majority of studies indicated that carbaryl may be metabolized via hydrolysis, oxidations, reductions, or conjugations (Kolbezen et al. 1954; Casida and Augustinsson 1959; Casida et al. 1960; Carpenter et al. 1961) demonstrated that hydrolysis rather than hydroxylation was the major metabolic route of carbaryl metabolism by MFO enzymes. For example, Casida (1963) reviewed data showing that hydrolysis of the ester linkage might be assumed to be the predominant metabolic pathway.

Hydroxylation rather than hydrolysis has been reported to be the major route of carbaryl metabolism (Dorough and Casida 1964; Zayed et al. 1966; Andrawes and Dorough 1967; Tsukamoto and Casida 1967a, 1967b; Kuhr 1970, 1971; Guirguis and Brindley 1975; Nakatsugawa and Morelli 1976).

A recent study by Hurst and Dorough (1978) indicated that hydrolysis is the major detoxication mechanism of carbamate insecticides by mammals.

Studies dealing with both mammals and insects reflected that the only major detoxication mechanism that is qualitatively different in insects and mammals is glucoside conjugation by insects (Smith 1962; Georghiou 1972; Devonshire 1973).
In all previous cases, MFO systems were the most important class of detoxication enzymes which showed a considerable variation in the rates of metabolism with respect to resistance, age, stage of development, sex, and species for carbamate insecticides in general, and carbaryl specifically.
MATERIALS AND METHODS

Chemicals

The 1-naphthyl-N-methylcarbamate (carbaryl) (Analytical grade, 99.9%) and small quantities of its derivatives, 4-hydroxy-1-naphthyl-N-methylcarbamate, 5-hydroxy-1-naphthyl-N-methylcarbamate, 1,4-naphthalenediol, 1,5-naphthalenediol, and 1-naphthol, were supplied by the Union Carbide Corporation, Olefins Division, New York. The synergist, piperonyl butoxide (a-[2-(2-butoxyethoxy)ethoxy]-4,5-methylenedioxy-2-propyltoluene), was purchased from K & K Laboratories, Incorporated, Plain View, New York. The 1-naphthyl-N-methyl[14C] carbaryl, with a specific activity of 21 mCi/mmol was purchased from California Bio-nuclear Corporation, San Fernando, California. Its radiochemical purity was 98%.

The scintillation mixtures (scintillation grade) used were: 2,5-diphenloxadazole (PPO), 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), and thixotropic gel powder (CAB-0-SIL). These were purchased from the Packard Instrument Company, Incorporated, Downers Grove, Illinois. Monoethanolamine, methylcellosolve, toluene, and phenethylamine were purchased from the J. T. Baker Chemical Company, Phillipsburg, New Jersey.

The iodine was obtained from Specialty Chemicals Division, New Jersey, while the silica gel G was purchased from E. Merck, Dramstadt, Germany.
Bioassay Procedures and Equipment

Chemicals and insects were introduced into the bioassay vials by the method described by Brindley (1975). The glass vials used were 5-dr capacity (27.25 x 55.00 mm) with plastic caps. A hole was drilled in the center of both the internal and external layer of each plastic vial cap and both holes were covered with soft screen. The bottom of each vial was labeled for individual recognition when arranged in holding boxes.

The 1-naphthyl-N-methyl-carbarylmate and piperonyl butoxide were dissolved in acetone and diluted to make several concentrations of carbaryl and 0.1 µg/0.5 ml of piperonyl butoxide. A volume of 0.5 ml of each concentration was transferred to each vial. The solutions containing the synergist, or the insecticide, were then allowed to evaporate by placing each vial sideways on a rack of two glass rods or on a breadboard using hands to shake the vials to allow the evaporation of the solution. The apparatus was placed in a fume hood and one of the glass rods was rotated with a rheostat-controlled drill, permitting the vials to rotate slowly. This action permitted the solution inside to evaporate and leave a residue on the inner walls of the vials. The vials were then capped and arranged in boxes separated by cardboard shelf packs so that each box contained 20 vials.

The insects used in this study came from the native populations of several range fields in the State of Utah. These insects were collected with nets during two consecutive years (1977 and 1978) and on two consecutive seasons each year (late spring and early summer). The bugs were collected only from grasses that were in good condition.
L. hesperius were collected from Monte Cristo, Salina and Cedar Breaks while both L. utahensis and L. hirtus were collected from Beaver Creek and Monte Cristo. Most collections were made between 1:00 and 4:00 p.m. The insects were divided into two groups. The first included adult males and females of the three species. These were pretreated with synergist by placing them in vials containing 0.1 µg residue of piperonyl butoxide. These insects were transferred after 1/2 hour (the time required for maximum MFO inhibition, Guirguis and Brindley 1975) into vials containing several concentrations of carbaryl alone. The second group of insects included all of the adult males and females of the three species. These were treated with carbaryl alone by placing them in vials that contained several concentrations of carbaryl only.

Five insects were used in each treatment vial, and each vial contained three grass leaves for food. After the vials were filled with grass and insects and recapped, each was placed in a slot of the 20-vial capacity boxes. Five vials of each box were used as controls. Eight of these boxes were placed in an incubator where the temperature was held at 20 ± 0.5°C. Several precautions were taken to insure that the insects were alive and healthy during each of the time-interval experiments.

The bioassay incubator is illustrated by Figure 2. This incubator consisted of a small plywood box within a larger styrofoam container (56 x 30 x 25 cm) covered with three lids of freezable liquid to maintain low temperature. The small interior box contained the temperature-regulating unit consisting of a small fan, thermostat, light bulb, and 6-volt rechargeable, motorcycle battery for power.
When the temperature exceeded 20°C, the fan became operable to cool the interior of the container. Temperatures below 20°C caused the thermostat to activate the light bulb for heat inside the incubator. The battery was usually replaced after 12 hours of use. This incubator provided excellent temperature control and never exceeded the range of 19.5 to 20.5°C. Caution was taken to protect the incubator from extreme heat such as direct sunlight. When field trips were extended overnight, extra laboratory materials and solutions were carried to prepare new bioassays the following day.

Data were recorded every 4, 8 and 12 hours on data sheets. Data on each sheet included the amount of insecticide residue per vial, the species of insect, time of vial preparation and application, location, grass type, and the observed mortality for each of the three time intervals. Death was considered as the total lack of insect movement.

The data collected from several separate experiments, and LC<sub>50</sub> values were calculated as micrograms of residual insecticide (with or without synergist) per vial. The results were plotted as percentage mortality relative to the logarithms of insecticide concentration. Synergist ratios and differences were also calculated and plotted against the LC<sub>50</sub>. Preliminary linear regressions were calculated to guide solution preparations. The final bioassay data were analyzed by regression analysis and slopes and correlation coefficients were calculated.

**Treatment of Insects with Labelled Carbaryl**

Only *L. hesperius* and *L. hirtus* were used in the metabolic study,
Fig. 2. Field incubator for conducting the bioassay experiments.
since L. utahensis was not available at the time of collection. Insects were collected from Beaver Creek and Monte Cristo during August 2 to August 9, 1978.

Prior to treatment with labelled carbaryl, several bioassays were conducted using high concentrations of unlabelled carbaryl to determine the maximum usable concentration without causing any mortality to individuals of the two species.

\[ N-\text{[^14}C]H_3\text{-carbaryl, with a specific activity of 21 mCi/mmol, was dissolved in acetone to yield concentrations of 10 \mu g/0.5 \text{ml, 20 \mu g/}\]
\[0.5 \text{ml, and 50 \mu g/0.5 ml in vials specified for the treatment of male L. hirtus, male L. hesperius, and both L. hirtus and L. hesperius respectively. The labelled carbaryl acetone solution was evaporated in the same manner as the bioassay vials, leaving the residue of the labelled carbaryl on the walls of each vial. Vials containing 0.1 \mu g/ vial piperonyl butoxide solution were also prepared.}\]

The insects were then divided into groups according to species and sex. Each group consisted of 50 adults. The groups were transferred into the vials designated for each species and sex for a period of 30 sec. One group was pretreated with piperonyl butoxide while another was left untreated to determine the amount of \[^{14}\text{C} \text{uptake by each group. After the 30-sec treatment, the groups were transferred into the metabolism chamber (175 ml flasks). Two grass leaves were added to each flask to provide food for the insects. The flasks were then attached to the volatile-metabolite collecting units as described by Casida et al. (1968), in order to trap CO}_2 \text{ and other volatile products. The temperature was controlled inside the metabolism.} \]
chamber by dipping the chamber in a water bath adjusted to 20°C (the temperature in the incubator where the bioassay vials were kept).

The insects were kept in the metabolism chamber for 2, 4, and 6 hours after treatment with the labelled carbaryl. The insects which were pretreated with piperonyl butoxide were kept in the metabolism chamber for only the 6 hour period. Two replicates of each treatment were used for each time interval.

The radioactivity of samples was determined with a Packard Tri-carb liquid scintillation spectrometer (model 527). The quench corrections were made by using external standard and channel ratio methods. The total percentage radiocarbon was calculated from the total disintegrate ions per minute (dPM) for the various fractions.

**Collecting CO$_2$ and Other Volatile Products**

The apparatus and the procedure used for collecting CO$_2$ and radioactive volatile organic compounds were those described by Casida et al. (1968). In operation, the impinger tube contained 0.5 ml of 2.5% (wt/vol) aqueous HCl (aqueous acid trap) placed directly in the tube. By using a motorized pump, appropriate air flow was drawn in sequence through the metabolism chamber, the impinger tube, and the scrubber column containing 6 ml of 2:1 methylcellosolve: monoethanolamine mixture (organic base trap) to trap $^{14}$CO$_2$. After each hour in the metabolism unit, fresh acid was introduced into the impinger tube. Immediately after each experimental period, the contents of the organic base trap and the aqueous acid trap were removed for direct measurement to scintillation vials containing 15 ml of scintillation mixture of 0.55% (wt/vol) of PPO in 2:1 toluene: methylcellosolve mixture (Jeffery and Alvarez 1961)
and 3 ml of 2:1 methylcellosolve: monoethanolamine mixture. Quench corrections were made by using the external standard.

The insects of each group (including those of the untreated group) were rinsed three times with 5 ml portions of cold acetone. The washings were combined in a scintillation vial and evaporated to near dryness using a gentle stream of air. Then, 15 ml of a scintillation mixture consisting of 4 g PPO and 0.5 g POPOP in 1 liter toluene were added to each vial, and the radioactivity was measured directly.

**Homogenization**

After each specific period of treatment, the insects were transferred from the metabolism chamber and rinsed three times with 50-ml aliquots of cold acetone to remove the unabsorbed labelled carbaryl from the insect integument. All the washes were combined in a Erlenmeyer flask and evaporated to dryness at room temperature using a gentle stream of air. The radiocarbon content of each sample was determined from 15 ml of scintillation mixture containing 4 g PPO and 0.5 mg POPOP in 1 liter of toluene. Any radiocarbon in the insects' excreta was removed from the holding flasks by rinsing three times with 3-ml portions of diethyl ether. The surface-washed insects and the combined holding-flask rinses were placed in the homogenizing container and macerated for 2 min at room temperature with an Omni-Mixer (Ivan Sorval, Incorporated, Norwalk, Connecticut). The homogenates were transferred into 200 ml centrifuge tubes and centrifuged at suitable speed to separate the residue from the solvents and to facilitate filtration. The supernatant was then filtered through
coarse filter paper in a Buchner funnel and into 60 ml separatory funnels. The ether fraction (upper phase) was withdrawn with a pipet and placed in Erlenmeyer flasks. The residue left in the centrifuge tubes was again homogenized with 6 ml of diethyl ether for 2 min and then filtered. The ethers, which were filtered in the separatory funnels were withdrawn after thorough shaking with the water phase. Two portions of 5 ml of chloroform were used to rewash each of the water fractions and the insoluble unextractable residues. The combinations of the chloroform and ether extracts in the Erlenmeyer flasks represented the organosoluble extracts. The water was collected in other Erlenmeyer flasks to form the water-soluble extracts. By using a gentle stream of air, both the water- and organo-soluble fractions were evaporated to near dryness and their residues were dissolved in 5 ml of acetone. Aliquots of 100 µl of each of these dissolved residues were used to spot the thin-layer chromatography. The remaining portion was transferred to scintillation vials containing 15 ml of scintillation mixture consisting of 3.29 g PPO, 0.08 g POPOP, 40 g CAB-O-SIL in 1 liter of toluene. The radiocarbon contents of each organo- and water-soluble fraction were measured directly.

**Thin-Layer Chromatography**

Thin-layer chromatography was conducted in two dimensions on 20 x 20 cm plates with silica gel G (Brinkmann, Incorporated, Cantiague, New York). Each layer was prepared in the laboratory at 0.33 mm thickness. The plates were activated for 1 hr at 110°C and developed in the first direction with 4:1 (v/v) diethyl ether:n-hexane. Immediately after drying, the plates were developed in the second
direction to minimize decomposition (Dorough and Casida 1964) using a 4:1 mixture of methylene chloride:acetonitrile (r/r). The chromatograms were developed in chambers lined on the inside with filter paper.

Pure carbaryl, each of its authentic derivatives, and combinations of all of them were chromatographed with the same solvent system in order to determine their $R_f$ values (ratio of movement of solute to that of the solvent front). Iodine vapors were used as a detecting system for the metabolites on all thin-layer chromatography. The colored areas, original spot, and total gel were scraped from the plates and transferred into scintillation vials containing 15 ml of the same scintillation mixture used for the analysis of organo- and water-soluble fractions.

The radiocarbon content of the insoluble unextractable residue and filter paper determined by combustion did not make a significant difference in the total radioactivity recovered. (Guirguis and Brindley 1975). The combustion procedure was not used in this study.

**Identification of Carbaryl and Its Metabolites**

Carbaryl and its metabolites were identified by measuring the $R_f$ values of pure carbaryl and its authentic derivatives. This was accomplished by exposing the thin-layer chromatography plates to iodine vapors, comparing the values obtained with those from literature, and by identifying the characteristic color of each metabolite-iodine complex.

The method described by Dorough and Wiggins (1969) was used to free the aglycone portion of the conjugated metabolites. This procedure
involved acid hydrolysis brought about by adding 0.9 M HCl to the water-soluble fractions, then incubating them in a boiling-water bath for 1 hr to hydrolyze the conjugates. After the mixture cooled, the aglycones were extracted with dichloromethane. The extract was then analyzed by thin-layer chromatography. The methods used for separating and identifying the aglycones were the same as described for analysis of the organo-soluble fractions.
RESULTS AND DISCUSSION

Studies like the present research may be important for several reasons. Brattsten and Metcalf (1970, 1973a, 1973b) have suggested that the use of piperonyl butoxide which inhibits MFOs, could be used to alter the LD$_{50}$ of carbaryl, which is metabolized by MFOs to indicate MFO levels in insects in vivo. Brindley (1977) has proposed an alternative way of viewing the change in the LD$_{50}$. If his proposal is correct, synergist differences rather than synergist ratios may provide hypotheses that are clear and useful in estimating MFO levels in vivo. He plotted the synergist differences (LD$_{50}$ of carbaryl-LD$_{50}$ of carbaryl after piperonyl butoxide) against LD$_{50}$ for about 80 insect species. The points formed an orderly pattern (Figure 3. Brindley, 1977) relative to the equation:

(1) \( \log \text{LD}_{50} = 1.014 \log \text{SD} + 0.01 \)

The slope of this line is nearly 1 and when MFOs are fully important in detoxifying carbaryl and when piperonyl butoxide is effective in inhibiting them, the synergist difference should approach the LD$_{50}$ value. Brattsten and Metcalf also observed that if piperonyl butoxide is an effective synergist, the LD$_{50}$ values should be shifted to a small value in a relatively narrow range.

The so-called percent dependency which is the observed SDX100/ calculated SD could be used to estimate the role of MFO in establishing carbaryl tolerance. If the observed SD is equal to the expected, a reasonable hypothesis would be that the insect depends essentially on MFOs for its defense against carbaryl.
Synergist differences and LD$_{50}$ values for alfalfa leafcutting bees (●) with a regression line where Log LD$_{50}$ = 1.01 Log Synergist Difference - 0.01. Additional data (○) calculated from Brattsten and Metcalf for 70 additional species and for honey bees, Apis mellifera (△) have been added. (Brindley, 1977).
Since the development of resistance to insecticides appears to be often associated with a general increase in MFO activity (Folsom et al. 1970; Matthews and Casida 1970; Philpot and Hodgson 1971; Plapp 1973; Reed 1974; Plapp et al. 1976; Georghiou and Taylor 1977), the synergist difference approach may enable us to speculate whether insect populations in the field are going to be tolerant or susceptible to given insecticides detoxified by MFOs. This objective can probably not be achieved if only in vitro, laboratory oriented, techniques are available to characterize MFO levels. Also, by applying the SD approach, one can detect if oxidation by MFOs is the major mechanism for resistance and in turn refer to the most effective inhibitor for controlling the resistant insects. If the SD concept is correct, it will enable us to detect which detoxication is dominant and, therefore, which insecticide should be selected for pest control. It might also help us to choose a more suitable time for insecticide application if the MFO level is relatively minimal during one stage of development.

Moreover, the SD concept may help us to understand better how to protect beneficial insects and to conduct a successful biological control when it is used along with chemical control. If a beneficial insect such as a parasite has less dependency upon MFOs than the pest insect, switching to other insecticides which undergo different detoxifying mechanism by the beneficial insect, may be highly desirable.

Brindley (1975) indicated that insecticide bioassays can be conducted with great precision if the physiological and environmental factors are standardized. He also demonstrated that the bioassay
techniques could be useful if based on field population of agricultural insects with a simple test kit might indicate the early development of insect resistance to insecticides.

A combination of field bioassays and \textit{in vivo} metabolic studies would be of importance in confirming the hypotheses that bioassay data may be done with field populations of insects and if they also reflect the MFO levels in the insect.

This study was organized to test these objectives in grass bugs using the "percent dependency" as hypothetical approach to and interpretations of the data.

The \( \text{LC}_{50} \) data showed considerable variations in carbaryl lethality with respect to species, sex, age and treatment with piperonyl butoxide.

In terms of susceptibility, \textit{L. utahensis} was the most susceptible species while \textit{L. hirtus} showed a moderate susceptibility. \textit{L. hesperius} was the most tolerant among the three species (Table 1).

The males were more susceptible than females. \textit{L. utahensis} males were more susceptible than \textit{L. hirtus} and \textit{L. hesperius} males, respectively.

Based on data derived from experiments conducted in 1978, Figure 4 shows typical relationships between mortality and carbaryl toxicity to the three species.

As the populations aged, the grass bug species became more susceptible to carbaryl (Table 1). This was based on the fact that we were dealing with adults which may require only two weeks from the appearance of the first adults until the maximum adult populations are reached (Todd and Kamm 1974). So a logical conclusion would be that as the date of treatments become later, the insects present
Table 1a. Synergist differences, ratios, and percent dependencies as calculated from unsynergized and synergized LC$_{50}$'s of carbaryl to three species and sexes of *Labops* bugs on different locations and dates.

<table>
<thead>
<tr>
<th>Date</th>
<th>Species and Location</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Unsynergized</td>
<td>Synergized</td>
</tr>
<tr>
<td>July 16, 1977</td>
<td>L. hesperius (1)</td>
<td>0.51</td>
<td>0.24</td>
</tr>
<tr>
<td>July 18, 1977</td>
<td>L. hesperius (1)</td>
<td>0.49</td>
<td>0.26</td>
</tr>
<tr>
<td>August 5, 1977</td>
<td>L. hesperius (2)</td>
<td>0.31</td>
<td>0.17</td>
</tr>
<tr>
<td>August 6, 1977</td>
<td>L. hesperius (3)</td>
<td>0.625</td>
<td>0.36</td>
</tr>
<tr>
<td>June 23, 1978</td>
<td>L. hesperius (3)</td>
<td>0.65</td>
<td>0.26</td>
</tr>
<tr>
<td>June 24, 1978</td>
<td>L. hesperius (3)</td>
<td>0.62</td>
<td>0.29</td>
</tr>
<tr>
<td>August 2, 1978*</td>
<td>L. hesperius (1)</td>
<td>0.31</td>
<td>0.17</td>
</tr>
<tr>
<td>July 26, 1977</td>
<td>L. hirtus (4)</td>
<td>0.29</td>
<td>0.16</td>
</tr>
<tr>
<td>July 29, 1977</td>
<td>L. hirtus (4)</td>
<td>0.26</td>
<td>0.16</td>
</tr>
<tr>
<td>July 30, 1977</td>
<td>L. hirtus (4)</td>
<td>0.21</td>
<td>0.13</td>
</tr>
<tr>
<td>August 2, 1977</td>
<td>L. hirtus (4)</td>
<td>0.16</td>
<td>0.10</td>
</tr>
<tr>
<td>August 3, 1978</td>
<td>L. hirtus (4)</td>
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<td>0.05</td>
</tr>
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<td>July 16, 1977</td>
<td>L. utahensis (1)</td>
<td>0.14</td>
<td>0.09</td>
</tr>
<tr>
<td>July 18, 1977</td>
<td>L. utahensis (1)</td>
<td>0.10</td>
<td>0.06</td>
</tr>
<tr>
<td>July 29, 1977</td>
<td>L. utahensis (1)</td>
<td>0.07</td>
<td>0.05</td>
</tr>
<tr>
<td>August 2, 1978</td>
<td>L. utahensis (1)</td>
<td>0.03</td>
<td>0.02</td>
</tr>
</tbody>
</table>

(1) = Monte Cristo  
(2) = Cedar Breaks  
(3) = Salina  
(4) = Beaver Creek  
*Metabolic study has been done on it
Table 1b. Correlation coefficient (r), slopes (m), and intercepts (b) as calculated from the bioassay data where % mortality = m log µ/vial (5 insects) (contrations used) + b.

<table>
<thead>
<tr>
<th>Species</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>hesperius</td>
<td>100.15</td>
<td>79.16</td>
<td>61.88</td>
<td>64.82</td>
<td>78.94</td>
<td>95.77</td>
<td>87.99</td>
<td>104.53</td>
<td>0.88</td>
<td>0.85</td>
<td>0.89</td>
<td>0.96</td>
</tr>
<tr>
<td>hesperius</td>
<td>108.42</td>
<td>73.90</td>
<td>72.76</td>
<td>66.95</td>
<td>83.20</td>
<td>99.64</td>
<td>92.58</td>
<td>111.41</td>
<td>0.92</td>
<td>0.88</td>
<td>0.81</td>
<td>0.91</td>
</tr>
<tr>
<td>hesperius</td>
<td>74.04</td>
<td>77.71</td>
<td>68.76</td>
<td>59.70</td>
<td>88.17</td>
<td>108.53</td>
<td>103.07</td>
<td>108.33</td>
<td>0.89</td>
<td>0.92</td>
<td>0.93</td>
<td>0.87</td>
</tr>
<tr>
<td>hesperius</td>
<td>88.96</td>
<td>59.66</td>
<td>81.78</td>
<td>58.15</td>
<td>67.09</td>
<td>77.30</td>
<td>86.14</td>
<td>88.96</td>
<td>0.84</td>
<td>0.91</td>
<td>0.72</td>
<td>0.93</td>
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<tr>
<td>hesperius</td>
<td>71.35</td>
<td>75.18</td>
<td>75.91</td>
<td>61.88</td>
<td>63.40</td>
<td>72.62</td>
<td>93.84</td>
<td>87.99</td>
<td>0.84</td>
<td>0.13</td>
<td>0.88</td>
<td>0.89</td>
</tr>
<tr>
<td>hesperius</td>
<td>88.83</td>
<td>86.06</td>
<td>60.41</td>
<td>66.92</td>
<td>68.64</td>
<td>80.0</td>
<td>82.42</td>
<td>88.60</td>
<td>0.92</td>
<td>0.91</td>
<td>0.80</td>
<td>0.93</td>
</tr>
<tr>
<td>hesperius</td>
<td>67.38</td>
<td>83.52</td>
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<td>67.80</td>
<td>84.75</td>
<td>113.56</td>
<td>96.85</td>
<td>116.28</td>
<td>0.89</td>
<td>0.94</td>
<td>0.76</td>
<td>0.94</td>
</tr>
<tr>
<td>hirtus</td>
<td>64.34</td>
<td>64.33</td>
<td>65.07</td>
<td>69.55</td>
<td>84.03</td>
<td>104.03</td>
<td>101.99</td>
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<td>0.89</td>
<td>0.92</td>
<td>0.90</td>
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<tr>
<td>hirtus</td>
<td>70.92</td>
<td>63.60</td>
<td>65.07</td>
<td>63.21</td>
<td>91.24</td>
<td>107.73</td>
<td>101.99</td>
<td>118.99</td>
<td>0.90</td>
<td>0.92</td>
<td>0.92</td>
<td>0.90</td>
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<tr>
<td>hirtus</td>
<td>64.01</td>
<td>64.34</td>
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<td>93.92</td>
<td>108.41</td>
<td>99.95</td>
<td>115.58</td>
<td>0.94</td>
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<td>0.89</td>
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<tr>
<td>hirtus</td>
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<td>39.46</td>
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<td>109.74</td>
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<td>0.80</td>
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<td>hirtus</td>
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<td>36.80</td>
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<td>109.79</td>
<td>120.55</td>
<td>112.48</td>
<td>0.92</td>
<td>0.80</td>
<td>0.82</td>
<td>0.77</td>
</tr>
<tr>
<td>utahensis</td>
<td>75.98</td>
<td>77.51</td>
<td>69.67</td>
<td>70.68</td>
<td>115.90</td>
<td>129.51</td>
<td>122.98</td>
<td>131.95</td>
<td>0.93</td>
<td>0.94</td>
<td>0.96</td>
<td>0.94</td>
</tr>
<tr>
<td>utahensis</td>
<td>75.98</td>
<td>76.32</td>
<td>92.56</td>
<td>83.04</td>
<td>125.90</td>
<td>130.94</td>
<td>160.04</td>
<td>148.04</td>
<td>0.97</td>
<td>0.94</td>
<td>0.90</td>
<td>0.94</td>
</tr>
<tr>
<td>utahensis</td>
<td>52.09</td>
<td>61.07</td>
<td>65.07</td>
<td>53.36</td>
<td>111.50</td>
<td>137.38</td>
<td>137.10</td>
<td>137.28</td>
<td>0.83</td>
<td>0.83</td>
<td>0.92</td>
<td>0.96</td>
</tr>
<tr>
<td>utahensis</td>
<td>45.65</td>
<td>45.25</td>
<td>49.60</td>
<td>46.70</td>
<td>117.53</td>
<td>129.90</td>
<td>131.89</td>
<td>137.41</td>
<td>0.90</td>
<td>0.74</td>
<td>0.80</td>
<td>0.88</td>
</tr>
</tbody>
</table>

1 = of female, unsynergized LC\(_{50}\)
2 = male, unsynergized LC\(_{50}\)
3 = female, synergized LC\(_{50}\)
4 = male, synergized LC\(_{50}\)
Fig. 4. The relationship between mortality and carbaryl toxicity in three species of Labops with and without piperonyl butoxide based on 1978 data.
were generally older. Table 1 shows the changes in carbaryl toxicity with date of insecticide bioassay.

It should also be understood that the terms "resistance" or "tolerance" or "susceptibility" used herein are attributed to the toxicity of carbaryl against the grass bugs only. In comparing toxicity against grass bugs with the many species of insects studied by Brattsten and Metcalf (1970, 1973a, 1973b), the grass bugs tend to be among the most susceptible insects to carbaryl poisoning. For example, adult honeybees and stable fly, *Stomoxys calcitrans*, are very susceptible to carbaryl (LD$_{50}$ 2.3 mg/gm, 0.66 mg/gm) (Brattsten and Metcalf 1970) whereas *L. hesperius* adult female LC$_{50}$ ranged from 0.31 to 0.65 µg carbaryl/25 gm (0.012-0.026 µg/gm).

Brindley (1975) used a biological assay approach similar to this one which, indeed, inspired the methods of this study. Alfalfa weevil adults and larvae survived more than 1280 µg carbaryl/vial while LC$_{50}$ values for a feca beetle and a wasp (*Monodontomterus*) were 10.1 and 0.82 µg carbaryl/vial, respectively.

Treatment with piperonyl butoxide enhanced the toxicity of carbaryl against the three species of grass bug. However, piperonyl butoxide had the great effect on enhancement of carbaryl toxicity against *L. hesperius* while it showed a moderate effect on *L. hirtus*. Treatment of female *L. hesperius* with piperonyl butoxide exhibited the best synergistic effect whereas synergism was less pronounced in the case of *L. utahensis* (Table 1).

If MFOs are dominant in controlling carbaryl toxicity against *Labops* bugs, the carbaryl-piperonyl butoxide synergist difference
should approach the LD$_{50}$ of carbaryl alone (Brindley 1977). *L. hesperius* apparently depends most on MFO enzymes in detoxifying carbaryl (about 60% dependency) and has the highest synergist difference (0.38) relative to carbaryl LC$_{50}$ (0.648 µg carbaryl/vial) while *L. utahensis* males which do not show high dependency on MFOs (13%) has a very low synergist difference (0.004) while its LC$_{50}$ is 0.013 µg carbaryl/vial.

Plots of LC$_{50}$ values of carbaryl to the three species against the synergist difference values are shown in Figure 5. On the other hand, plot log LC$_{50}$ values against the synergist ratio values show that there is no highly correlated simple relationship between LC$_{50}$ values and synergist ratio values (Figure 6).

The decrease in the susceptibility of the grass bug species to carbaryl was associated with a general increase in the percent dependency of insects upon MFOs for carbaryl detoxication. For the most part, *L. hesperius* required higher dose (0.65 µg carbaryl/vial) to provide 50% mortality are found to be highly dependent on MFO system (about 60% dependency) while *L. utahensis* with low LC$_{50}$ (0.33 µg carbaryl/vial has low percent dependency (13%). So it can be concluded that the percent dependency of the insects upon MFOs plays a major role in determining the tolerance or susceptibility of these insects to carbaryl. The tolerance of *L. hesperius* in comparison with the other two species could be attributed to some extent to its higher percent dependency upon MFOs for carbaryl detoxication and the increase in synergistic differences with piperonyl butoxide-carbaryl combination.
Fig. 5. Relationship between LC$_{50}$ and synergist difference (SD) in 3 species of Labops with a regression line where Log LC$_{50} = 1.82 \log SD + 0.05.$
Fig. 6. Relationship between LC$_{50}$ and synergist ratio (SR) in three species of Labops where

$$SR = \frac{LC_{50} \text{ of carbaryl alone}}{\text{synergized } LC_{50}}$$

$$LC_{50} = 0.24 \text{ SR }+ 0.15$$

$$r^2 = 0.37$$
Based on synergist differences and percent dependency, Figure 7 shows the ranking of the three species according to carbaryl LC$_{50}$ and estimated percent dependency upon MFOs.

As a rule of thumb, the term high or low MFO levels is relative and subject to different factors (see factors affecting MFO levels). So it can be concluded that the actual percentage of MFOs in insect that contributed in the tolerance of insect to insecticides will give better understanding in comparative studies than the level of MFOs. There are examples in which insects have very low MFO enzyme levels (where LD$_{50}$ values were low) but the synergist differences showed that the insects have high percent dependency upon the MFO enzymes in detoxication mechanism.

The following examples are based on calculations of other person's data (Table 2) using the synergist differences and percent dependency concepts.

Metcalf et al. (1966), and Brattsten and Metcalf (1970) demonstrated that honeybees are very susceptible to carbaryl insecticide. This might indicate that honeybees have very low MFO levels but the calculated percent dependency ranged from 65 to 86% which means that honeybees depend to some extent on MFO enzymes in detoxifying carbaryl but with low LD$_{50}$ values, must have very little MFOs. Considering carbaryl toxicity against the black grass bug, which reflects that the black grass bug is one of the most susceptible insects to carbaryl, one could understand that black grass bugs have very low MFO levels. The percent dependency values ranged from 13-60% which indicates that some species (L. hesperius) depend to some extent on MFOs in carbaryl detoxication.
Fig. 7. Ranking the three species of Labops according to carbaryl toxicity and estimated percent dependency upon MFOs.
Table 2. Data and calculations for effect of Piperonyl butoxide synergist on carbaryl toxicity in insects to show synergist differences and estimated percent dependencies upon MFOs for detoxication.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sex</th>
<th>Age</th>
<th>Insecticide</th>
<th>Synergist</th>
<th>LD$_{50}$ mg/g</th>
<th>SD</th>
<th>% Dep.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Honeybee</td>
<td>Female</td>
<td>Adults</td>
<td>Carbaryl</td>
<td>Piperonyl-butoxide</td>
<td>2.3</td>
<td>1.5</td>
<td>65</td>
<td>Brattsten &amp; Metcalf 1970</td>
</tr>
<tr>
<td>Honeybee</td>
<td>Female</td>
<td>4 days</td>
<td>Carbaryl</td>
<td>Piperonyl-butoxide</td>
<td>0.113</td>
<td>0.102</td>
<td>86</td>
<td>Guirguis &amp; Brindley unpublished data</td>
</tr>
<tr>
<td>German Cockroach</td>
<td>Female</td>
<td>Adults</td>
<td>Carbaryl</td>
<td>Piperonyl-butoxide</td>
<td>22</td>
<td>16.7</td>
<td>77</td>
<td>Brattsten &amp; Metcalf 1970</td>
</tr>
<tr>
<td>Sacophaga bullata</td>
<td>Female</td>
<td>Adults</td>
<td>Carbaryl</td>
<td>Piperonyl-butoxide</td>
<td>4000</td>
<td>3990</td>
<td>105</td>
<td>Brattsten &amp; Metcalf 1970</td>
</tr>
<tr>
<td>Phormia regina</td>
<td>Female</td>
<td>Adults</td>
<td>Carbaryl</td>
<td>Piperonyl-butoxide</td>
<td>29</td>
<td>24.7</td>
<td>78</td>
<td>Brattsten &amp; Metcalf 1970</td>
</tr>
<tr>
<td>Musca domestica</td>
<td>Male</td>
<td>Adults</td>
<td>Carbaryl</td>
<td>Piperonyl-butoxide</td>
<td>900</td>
<td>887.5</td>
<td>103</td>
<td>Brattsten &amp; Metcalf 1970</td>
</tr>
<tr>
<td>Drosophila melanogaster</td>
<td>Male</td>
<td>Adults</td>
<td>Carbaryl</td>
<td>Piperonyl-butoxide</td>
<td>2.0</td>
<td>1.7</td>
<td>85</td>
<td>Brattsten &amp; Metcalf 1970</td>
</tr>
<tr>
<td>American cockroach</td>
<td>Female</td>
<td>Adults</td>
<td>Carbaryl</td>
<td>Piperonyl-butoxide</td>
<td>190</td>
<td>197.5</td>
<td>98</td>
<td>Brattsten &amp; Metcalf 1970</td>
</tr>
<tr>
<td>Murgantia histronica</td>
<td>Female</td>
<td>Adults</td>
<td>Carbaryl</td>
<td>Piperonyl-butoxide</td>
<td>120</td>
<td>118.35</td>
<td>99</td>
<td>Brattsten &amp; Metcalf 1970</td>
</tr>
<tr>
<td>Oncopeltus fasciatus</td>
<td>Female</td>
<td>Adults</td>
<td>Carbaryl</td>
<td>Piperonyl-butoxide</td>
<td>32.5</td>
<td>32.06</td>
<td>97</td>
<td>Brattsten &amp; Metcalf 1970</td>
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<tr>
<td>Allocaris pullicaria</td>
<td>Female</td>
<td>Adults</td>
<td>Carbaryl</td>
<td>Piperonyl-butoxide</td>
<td>30</td>
<td>29.59</td>
<td>71</td>
<td>Brattsten &amp; Metcalf 1970</td>
</tr>
</tbody>
</table>
German cockroaches, *Blattella germanica*, which are considered to be susceptible to carbaryl \( \text{LD}_{50} = 22 \text{ mg/gm} \) (Brattsten and Metcalf 1970) showed quite high percent dependency (77%) in carbaryl detoxication.

Some Dipteran species are resistant to carbaryl toxicity while others are susceptible. (Brattsten and Megcalf 1973a) The calculated percent dependency values were 105 and 103% for the resistant species, *Sargophaga bullata*, \( \text{LD}_{50} = 4000 \text{ mg/g} \) and *Musca domestica* \( \text{LD}_{50} = 900 \text{ mg/g} \) and 78 and 85% for the susceptible species, *Phormia regina* \( \text{LD}_{50} = 29 \text{ mg/g} \) and *Drosophila melanogaster* \( \text{LD}_{50} = 2 \text{ mg/g} \) respectively.

American cockroaches are very tolerant to carbaryl insecticide \( \text{LD}_{50} = 190 \text{ mg/g} \) (Brattsten and Metcalf 1970) which correlated with the fact that they have high MFO levels. (Turnquist and Brindley 1975). The calculated percent dependency on MFOs for carbaryl detoxication is 98%.

Among the Hemipteran species, *Murgantia histrionica*, is quite tolerant to carbaryl \( \text{LD}_{50} = 120 \text{ mg/g} \) while *Oncopeltus fasciatus*, and *Allocoris pullicaria* are considered to be susceptible \( \text{LD}_{50} = 32.5 \text{ and } 30 \text{ mg/g, respectively} \). (Brattsten and Metcalf 1970). The percent dependency values are 99, 97 and 71%, respectively.

Table 2 shows those calculated percent dependency values along with \( \text{LD}_{50} \)'s and references.

Insects with high MFO levels may not depend largely on MFOs while others with low MFO levels could depend largely on MFOs for insecticide detoxication.
L. hesperius tolerance, could be due to MFO dependency in conjunction with some other factors.

It is well known that tolerance of insects to insecticides is influenced by diet (Way 1954; Bass and Cawson 1960; Perry 1964; Perry and Kintzins 1970; and Ahmad and Forash 1970). Smolenski et al. (1974) indicated that Agropyron intermedium, the grass on which L. hesperius feeds contains a moderate level of alkaloids. However, they did not specify what kind of alkaloids the grasses have. Brattsten et al. (1977), have emphasized the importance of dietary components of secondary plant chemicals in possible induction of insect MFO levels. It has not been demonstrated that induction of MFOs can occur in grass bugs.

The main function of the MFO system is the detoxication of secondary plant substances in food plants (Krieger et al. 1971). Due to the fact that secondary substances are omnipresent in nature, the phylophagous insects are equipped with very effective oxidative enzymes to handle a wide range of structures and concentrations of natural toxicants (Freeland and Janzen 1974).

Another possible explanation of L. hesperius tolerance might be that L. hesperius as any other Hemipteran insect has the ability to secrete polyphenol oxidase which could be expected to aid the oxidative process by MFO system (Miles 1974). However, it should not be understood that those are the only possible explanations for development of tolerance (See Review of Literature). On the other hand, low LC$_{50}$ values of carbaryl to L. utahensis may indicate that carbaryl proceeds directly to the site of action. The low percent dependency could also enable us to conclude that there might be
other detoxication mechanisms or barriers than the MFO system (Winteringham 1969) but which are apparently, ineffective in raising carbaryl tolerance very high in the grass bugs.

Because the short season of *L. utahensis* we could not do any metabolic study on it to confirm the possibility that might be other detoxication mechanisms than MFO enzymes involved.

The design of the metabolic study was derived from the fact that carbaryl, which has penetrated into the insect's body, is often first metabolized due to oxidation or hydrolysis into more polar products which are still soluble in organic solvents and so may be recovered as organo-soluble extracts. These products are more readily eliminated when they are conjugated to hydrophilic products that could be extracted as water-soluble extracts.

If the insects under study have high and active MFO systems in carbaryl detoxication, several metabolites should be identified and measured.

Thin layer chromatography, iodine vapors, and $R_f$ values were used to detect and assure the presence of such metabolites.

Data from the present metabolic study seemed to confirm the biological assay data. Even though we could not study the differences in the metabolic rate between the most susceptible (*L. utahensis*) and the most tolerant (*L. hesperius*), the metabolic studies on *L. hesperius* and *L. hirtus* reflected the relative differences between the two species. Male *L. hesperius* were the most susceptible insects among the tested insects for the metabolic studies while female *L. hirtus* were the most tolerant ones.
The percent of organo- and water-soluble fractions collected showed considerable variations with respect to insects susceptibility and time after treatment. Radioactivity in the organo-soluble fractions decreased with time in the case of females *L. hesperius* and *L. hirtus* whereas the activity increased in the water-soluble fractions. Male *L. hirtus* showed high increase in the organo-soluble fractions and less in the water-soluble fractions (Table 3).

These results, apparently, indicated that the ability of female *L. hesperius* and *L. hirtus* to degrade carbaryl and the transfer of the metabolites achieved into the water-soluble fraction could partially account for their carbaryl tolerance. On the other hand, the less tolerant species (male *L. hirtus*) tended to accumulate carbaryl and its metabolites in the organo-soluble fraction and very slowly transfer them into water-soluble fractions by conjugation processes.

It was possible to detect some metabolites in the organo-soluble fractions beside the unmetabolized carbaryl which was dominant in each insect species and sex. Those metabolites were 4-hydroxycarbaryl, 5-hydroxycarbaryl, and two unidentified metabolites. These should not be understood as the only metabolites associated with carbaryl degradation by *Labops*. Scraping all remaining silica gel and the original spots from the plates after the identifications were removed did not show any radioactivity. This led us to believe that those collected metabolites could be the only ones resulting from the hydroxylation of carbaryl molecule by insects (Table 4). The major compound was the unmetabolized carbaryl which decreases with time in all insects. The decrease in carbaryl concentration was much greater.
Table 3. Percentage distribution of $^{14}$C in fractions of two species of adult *Labops* at varying intervals (hr) after contact treatments with 10 µg/vial, and 50 for male and female *L. hirtus* and 20, and 50 µg/vial for male and female *L. hesperius*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sex</th>
<th>Date of treatment</th>
<th>hr.</th>
<th>External rinse</th>
<th>CO$_2$ trap</th>
<th>Volatile trap</th>
<th>Organic solvent fraction</th>
<th>Aqueous fraction</th>
<th>% total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. Hesperius</em></td>
<td>Male</td>
<td>August 2, and 2</td>
<td>2</td>
<td>69</td>
<td>0.05</td>
<td>0.03</td>
<td>10</td>
<td>4.8</td>
<td>83.88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>August 3, 1978</td>
<td>4</td>
<td>67</td>
<td>0.07</td>
<td>0.05</td>
<td>12</td>
<td>4.9</td>
<td>84.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td>65</td>
<td>0.08</td>
<td>0.05</td>
<td>13</td>
<td>5</td>
<td>83.13</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>August 4, and 2</td>
<td>2</td>
<td>74</td>
<td>0.1</td>
<td>0.06</td>
<td>5</td>
<td>4.5</td>
<td>83.66</td>
</tr>
<tr>
<td></td>
<td></td>
<td>August 5, 1978</td>
<td>4</td>
<td>70</td>
<td>0.3</td>
<td>0.07</td>
<td>4.5</td>
<td>7</td>
<td>81.87</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td>65</td>
<td>0.5</td>
<td>0.08</td>
<td>3</td>
<td>14</td>
<td>82.58</td>
</tr>
<tr>
<td><em>L. hirtus</em></td>
<td>Male</td>
<td>August 6, and 2</td>
<td>2</td>
<td>69</td>
<td>0.04</td>
<td>0.03</td>
<td>12</td>
<td>5</td>
<td>86.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>August 7, 1978</td>
<td>4</td>
<td>66</td>
<td>0.06</td>
<td>0.04</td>
<td>14</td>
<td>5.5</td>
<td>85.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td>62</td>
<td>0.07</td>
<td>0.05</td>
<td>15</td>
<td>6</td>
<td>83.12</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>August 8, and 2</td>
<td>2</td>
<td>72</td>
<td>0.1</td>
<td>0.005</td>
<td>8</td>
<td>4.2</td>
<td>84.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>August 9, 1978</td>
<td>4</td>
<td>69</td>
<td>0.2</td>
<td>0.07</td>
<td>6</td>
<td>6</td>
<td>81.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td>64</td>
<td>0.4</td>
<td>0.08</td>
<td>5</td>
<td>12</td>
<td>81.48</td>
</tr>
</tbody>
</table>
in the case of the female *L. hesperius* (tolerant) than in the case of *L. hirtus* (susceptible). Six hours after treatment, the unmetabolized carbaryl quantity was 36.7% of the total metabolites in female *L. hesperius* while it was 71.43% in the case of male *L. hirtus* (Table 4). Figures 8-10 show the relationship among organo, water-soluble fractions and unmetabolized carbaryl.

Three unidentified metabolites were found in the water-soluble fractions. They did not have the characteristics of the metabolites found in the organo-soluble fractions. Perhaps the methods for hydrolyzing the water-soluble fraction might not have been completed. The metabolites increased with time in all insects and the higher concentration was in the female *L. hesperius* > female *L. hirtus* > male *L. hesperius* > male *L. hirtus* and the most dominant metabolites was metabolite number 1.

These results apparently reflected a good correlation between the unmetabolized amount and the toxicity of carbaryl to Labops bugs. The susceptibility of the insects was associated with the increase in unchanged carbaryl in the insect's body. The data also suggested that carbaryl persistence controls the response of Labops to carbaryl.

Guirguis and Brindley (1975) found that the amount of unchanged carbaryl was much more in the susceptible alfalfa leafcutting bees (4-day-old males) which were less capable of degrading carbaryl, than in the more tolerant bees (4-day-old females and 1-day-old males).

The rest of the metabolites were found in small quantities and decreased with time in the case of female *L. hesperius* and *L. hirtus* while they increased in the case of male *L. hirtus* and *L. hesperius*,
Table 4. The metabolic products of radio labelled carbaryl collected in the organo-soluble fraction of L. hesperus on percentage basis of a total $^{14}$C applied in 0 time treatments and B total metabolites collected in organo-soluble fractions.

<table>
<thead>
<tr>
<th>Products</th>
<th>% collected of</th>
<th>L. hesperus-Male</th>
<th>L. hesperus-Female</th>
<th>L. hirtus-Male</th>
<th>L. hirtus-Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 hr</td>
<td>4 hr</td>
<td>6 hr</td>
<td>2 hr</td>
</tr>
<tr>
<td>4-hydroxy-carbaryl</td>
<td>a</td>
<td>0.15</td>
<td>0.21</td>
<td>0.18</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>0.54</td>
<td>0.66</td>
<td>0.70</td>
<td>0.24</td>
</tr>
<tr>
<td>Carbaryl</td>
<td>a</td>
<td>1.08</td>
<td>0.58</td>
<td>0.41</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>3.80</td>
<td>2.00</td>
<td>1.70</td>
<td>2.00</td>
</tr>
<tr>
<td>5-hydroxy-carbaryl</td>
<td>a</td>
<td></td>
<td></td>
<td></td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td></td>
<td></td>
<td></td>
<td>0.42</td>
</tr>
<tr>
<td>Unknown (1)</td>
<td>a</td>
<td>0.17</td>
<td>0.19</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>0.68</td>
<td>0.75</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>Unknown (2)</td>
<td>a</td>
<td></td>
<td></td>
<td></td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td></td>
<td></td>
<td></td>
<td>0.52</td>
</tr>
<tr>
<td>Total organo-soluble</td>
<td>a</td>
<td>1.40</td>
<td>0.98</td>
<td>0.81</td>
<td>0.62</td>
</tr>
<tr>
<td>fraction</td>
<td>b</td>
<td>5.02</td>
<td>3.41</td>
<td>3.24</td>
<td>3.18</td>
</tr>
</tbody>
</table>
Fig. 8. Relationship between the percent of organo soluble fraction and the time after treatment in 2 species of Labops.
Fig. 9. Relationship between the percent of water soluble fraction and the time after treatment in 2 species of <i>Labops</i>. 
Fig. 10. Relationship between the percent of unmetabolized carbaryl and the time after treatment in 2 species of Labops.
suggesting the accumulation of the metabolites in the susceptible insects, due probably to a decrease in the effectiveness of the metabolite conjugation.

Guirguis and Brindley (1975) found that carbaryl metabolites were accumulated with time in less tolerant alfalfa leafcutting bees (4-day-old males) and decreased with time in the more tolerant 4-day-old females and 1-day-old males. Eldefrawi and Hoskins (1961) and Metcalf et al. (1966, 1967), indicated that resistant houseflies excreted the metabolites of carbaryl faster than susceptible flies and they related the increase in the toxicity of carbaryl to the accumulation of the metabolites with time in the susceptible houseflies.

There were some variations in the kind of metabolites detected in respect to insect species and sex. 5-hydroxycarbaryl was found only in the organo-soluble fractions of female *L. hesperius* while 4-hydroxycarbaryl was found in the organo-soluble extracts of both *L. hesperius* and *L. hirtus*. On the other hand, metabolites 1 and 2 from the water-soluble extracts were found in both females and males *L. hesperius* and *L. hirtus* while metabolite 3 was found only in case of female *L. hesperius* supporting the observation that female *L. hesperius* have more ability to degrade carbaryl and transfers the metabolites to the water-soluble fraction very rapidly.

The data showed that there were quantitative and qualitative differences in the metabolic products by both species and sex but since the methods of detection on the plates were not very sensitive, this should be investigated further. However, Eldefrawi and Hoskins (1961), El-Aziz et al. (1969), Kuhr (1970), Fukuto (1972), Kuhr and
Davis (1975), Fukuto (1976) and Hollingworth (1976), indicated that different metabolic products may be found in the various insect species. On the other hand, differences in metabolism of the same compound in different insects can be appreciated from the following examples. Carbaryl is metabolized to at least six products in German cockroach (Kuhr, 1970) and in alfalfa leafcutting bees (Guirguis and Brindley (1975), three in housefly (Eldefrawi and Hoskins (1961) and European corn borer (Kuhr and Davis, 1975) and only one polar product in the milkweed bug (Eldefrawi and Hoskins 1961). Hollingworth (1971, 1976), Gillet (1971) and Gilbert and Wilkinson (1974) found a wide species variation in aldrin epoxidase activity in tissues from numerous insect species and related that to their different oxidative capacity and metabolic pathways.

Studies of the metabolic rate of carbamate insecticides including carbaryl in mammals have also been determined showing considerable variations in the metabolic capacity and products among different species (Dorough and Wiggins 1969; Dorough 1970; Fukuto 1972, 1976; Hollingworth 1976; Yang 1976; Baron 1978).

Since we have been able to detect 4- and 5-hydroxylcarbaryl only, it might be suggested that oxidation mechanisms specially aromatic ring hydroxylation in Labops bugs is dominant, but this should not be understood as the only metabolites produced by these insects.

5-hydroxylcarbaryl has been reported as more toxic to some animals than carbaryl itself (Oonnithan and Casida 1968; Dorough 1970) but it seems not to have any noticeable correlation with Labops lethality after carbaryl exposure. In contrast, it has been found
in *L. hesperius*, which is considered to be more tolerant in comparison with the other species, and appears to be less accumulated in the insect's body.

The same result is echoed by Guirguis and Brindley (1975) with carbaryl synergized by piperonyl butoxide against alfalfa leafcutting bees.

Figures 11-12 show the chromatographic diagrams of the presence and positions of carbaryl metabolites in organo- and water-soluble fractions in *Labops* as detected by this study.

Treatment with piperonyl butoxide effectively increased the organo-soluble fraction activity and decreased it in the water-soluble fractions for the tolerant insects (females of *L. hesperius*). In males of *L. hesperius* and *L. hirtus*, piperonyl butoxide showed a little decrease in both organo- and water-soluble fractions (Table 5).

Piperonyl butoxide blocked the MFO systems of all insect sexes and species as showed by the disappearance of all organo- and water-soluble metabolites. In the same way, piperonyl butoxide, raised the unmetabolized carbaryl by 12.3 and 2.5 fold after six hours from treatment for female *L. hesperius* and male *L. hirtus*, respectively (Table 6). Apparently piperonyl butoxide effectively inhibited MFO enzymes and greatly decreased the metabolic rates of carbaryl to its hydroxylated metabolites. It exhibited the best synergist effect with insects highly dependent on MFOs as indicated by the persistence of carbaryl on the most tolerant insects and disappearance of its metabolites.

The evolution of $^{14}$CO$_2$ after treatment with N-$^{14}$CH$_3$ carbaryl was slightly higher from the more tolerant bugs than the less tolerant
Fig. 11. Thin-layer chromatographic pattern of metabolites in organo soluble fractions from carbaryl-treated Labops.
Fig. 12. Thin-layer chromatographic pattern of metabolites in water soluble fractions from carbaryl-treated Labops.  

<table>
<thead>
<tr>
<th>Unknown metabolites</th>
<th>1st Rf values</th>
<th>2nd Rf values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.62</td>
<td>0.86</td>
</tr>
<tr>
<td>2</td>
<td>0.49</td>
<td>0.59</td>
</tr>
<tr>
<td>3</td>
<td>0.54</td>
<td>0.65</td>
</tr>
</tbody>
</table>
Table 5. Percentage distribution of $^{14}\text{C}$ in fractions of two species of adult *Labops* synergist with piperonyl butoxide after 6 hr from treatments with labelled carbaryl.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sex</th>
<th>Treatment with carbaryl</th>
<th>External rinse</th>
<th>CO$_2$ trap</th>
<th>Volatile trap</th>
<th>organic solvent fraction</th>
<th>Aqueous fraction</th>
<th>% total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. hesperius</em></td>
<td>Male</td>
<td>control</td>
<td>65</td>
<td>0.08</td>
<td>0.05</td>
<td>13</td>
<td>5</td>
<td>83.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+piperonyl butoxide</td>
<td>58</td>
<td>0.08</td>
<td>0.03</td>
<td>12.5</td>
<td>5</td>
<td>75.61</td>
</tr>
<tr>
<td><em>L. hesperius</em></td>
<td>Female</td>
<td>control</td>
<td>65</td>
<td>0.50</td>
<td>0.08</td>
<td>3</td>
<td>14</td>
<td>82.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+piperonyl butoxide</td>
<td>71</td>
<td>0.40</td>
<td>0.07</td>
<td>10</td>
<td>8</td>
<td>89.47</td>
</tr>
<tr>
<td><em>L. hirtus</em></td>
<td>Male</td>
<td>control</td>
<td>62</td>
<td>0.07</td>
<td>0.05</td>
<td>15</td>
<td>6</td>
<td>83.12</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>+piperonyl butoxide</td>
<td>58</td>
<td>0.06</td>
<td>0.03</td>
<td>14.50</td>
<td>5</td>
<td>77.59</td>
</tr>
<tr>
<td><em>L. hirtus</em></td>
<td>Female</td>
<td>control</td>
<td>64</td>
<td>0.40</td>
<td>0.08</td>
<td>5</td>
<td>12</td>
<td>81.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+piperonyl butoxide</td>
<td>69.5</td>
<td>0.30</td>
<td>0.05</td>
<td>12</td>
<td>7</td>
<td>88.85</td>
</tr>
</tbody>
</table>
Table 6. The metabolic products of radio labelled carbaryl collected in the organo-soluble fraction of *Labops* synergist with piperonyl butoxide after 6 hr from treatments with labelled carbaryl on percentage basis of a total $^{14}$C applied in 0 time and b total metabolites.

<table>
<thead>
<tr>
<th>Products</th>
<th>Percentage collected of</th>
<th><em>L. hesperius</em>-male</th>
<th><em>L. hesperius</em>-female</th>
<th><em>L. hirtus</em>-male</th>
<th><em>L. hirtus</em>-female</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-hydroxycarbaryl</td>
<td>a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Carbaryl</td>
<td>a</td>
<td>1.15</td>
<td>1.28</td>
<td>1.25</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>4.60</td>
<td>4.90</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>5-hydroxycarbaryl</td>
<td>a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Unknown (1)</td>
<td>a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Unknown (2)</td>
<td>a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total organosoluble fraction</td>
<td>a</td>
<td>1.15</td>
<td>1.28</td>
<td>1.25</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>4.60</td>
<td>4.90</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>
bugs, range from 0.07-0.5% within the first 6 hours (Table 3) and appeared to be more related to time after application. The most tolerant insects (female *L. hesperius*) evolved more $^{14}\text{CO}_2$ than the least tolerant (male *L. hirtus*) suggesting a possibly higher metabolic rate associated with tolerance. Entrapment of other volatiles was similar and seemed to be not related to either time or tolerance. Piperonyl butoxide treatment had no significant effect on $^{14}\text{CO}_2$ evolution (Table 5).

Generally, the *in vivo* importance of MFO enzymes in carbaryl detoxication is indicated by the fact that these patterns of enzyme activity correlate well with observed changes in the toxicity of insecticide and its synergism by piperonyl butoxide to *Labops* bugs of different species and sex and thus, synergist differences are found to be useful in establishing some important conclusions regarding the MFO state and their effects in various insect species and sex.

The metabolic studies not only showed a good correlation between the amount of unmetabolized carbaryl in the organo-soluble fractions and carbaryl toxicity and susceptibility of insects but also correlated well with the bioassay data including the calculated synergist difference and percent dependency recording the effectiveness of the insecticide bioassays when used with standardized physiological and environmental conditions to reflect a quite precise and fast estimate of the major mechanism associated with field populations. The study also reflected the ability of those techniques to provide early cautions regarding the development of insect resistance to
insecticides, the same time promoting use of the simple bioassay methods and kit in further field studies along with other insecticides to control the pest insects.
SUMMARY

This study was designed to determine the toxicity, metabolism and distribution of carbaryl in adult Labops grass bugs with respect to their species, sex, and piperonyl butoxide treatment. These data would permit calculations of percent dependencies upon MFOs for detoxication of carbaryl in these insects.

A combination of bioassays and metabolic studies were conducted to show and confirm the ability of simple bioassays to predict MFO levels in vivo.

The insects used in this study came from the native populations of several range fields in the state of Utah during two consecutive years (1977 and 1978) and on two consecutive seasons each year (late spring and early summer).

The bioassay experiments were conducted in the field using a field incubator with a precise temperature control (20°C ± 0.5).

The LC\textsubscript{50} data showed considerable variations with respect to species, sex and treatment with piperonyl butoxide.

\textit{L. utahensis} was the most susceptible species (LC\textsubscript{50} 0.02 - 0.14 µg/vial) while \textit{L. hirtus} showed a moderate susceptibility (LC\textsubscript{50} 0.03-0.3 µg/vial). The most tolerant species tested was \textit{hesperius} (LC\textsubscript{50} 0.2-0.65 µg/vial).

In comparing the carbaryl toxicity to grass bugs with other insect species, grassbugs are one of the most susceptible insects to carbaryl poisoning.
In all three species, the males were more susceptible to carbaryl than the females. *L. utahensis* males were more susceptible than *L. hirtus* and *L. hesperius*.

Treatments with piperonyl butoxide enhanced carbaryl toxicity against the three species. It had the greatest effect on enhancement of carbaryl toxicity against *L. hesperius* (synergized LC$_{50}$ 0.1-0.26 µg/vial) while it showed a moderate effect on *L. hirtus* (synergized LC$_{50}$ 0.02-0.16 µg/vial). Its effect was less pronounced in *L. utahensis* (synergized LC$_{50}$ 0.013-0.9 µg/vial).

A linear relationship supposedly representing a correlation between carbaryl LD$_{50}$ and carbaryl-piperonyl butoxide synergist differences in leafcutting bees (Brindley 1977) was used to calculate what synergist differences should have been in the bugs if they were totally dependent upon MFOs for their tolerance.

The calculated percent dependency values were 38-59%, 25-46%, and 13-33% for *L. hesperius*, *L. hirtus*, and *L. utahensis*, respectively.

Males of *L. utahensis* had low percent dependency (13%) indicating that carbaryl may interact with other potential defense mechanisms such as penetration, storage in inert tissues, detoxication by enzyme systems not inhibited by piperonyl butoxide, or may be due to the insensitivity of the active site of intoxication. Unfortunately, we were not able to conduct a metabolic study on it since its seasonal life cycle was so short.

The metabolic study reflected a good correlation between the amount of unmetabolized carbaryl in the insect's bodies and the carbaryl toxicity and susceptibility of insects. Males of *L. hirtus* were less
capable in degrading carbaryl and tended to accumulate unmetabolized carbaryl in larger quantities than in females of *L. hesperius* and *L. hirtus*.

The mechanism of detoxication was apparently much the same for both species and sexes. 4- and 5-hydroxycarbaryl were the only metabolites found. Since the methods of detection were not very sensitive, this should be investigated further. Pretreatment with piperonyl butoxide prevented the appearance of all carbaryl metabolites in the organo soluble fraction and resulted in the accumulation of unmetabolized carbaryl. $^{14}CO_2$ evolution from the methyl-labelled carbaryl was much higher in the female bugs and was relatively unaffected by piperonyl butoxide.

In general, this study showed a good correlation between the bioassays and metabolic studies and reflecting the effectiveness of the bioassay with synergist difference and percent dependency concepts in establishing some important conclusions regarding the MFO state and their effects in both species and sexes of *Labops*. 
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