METABOLISM OF C-10015, [2-(4,5-DIMETHYL-1,3-DIOXOLANE-2-YL)]-PHENYL METHYLCARBAMATE, BY RATS, HOUSE FLIES AND BEAN PLANTS

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
CHACHAWAL WONGPHYAT
1971

ABSTRACT

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Metabolism of C-10015 [2-(4,5-dimethyl-1,3-dioxoland-2-yl)]-phenyl methylcarbamate by rat liver microsomes fortified with reduced nicotinamide-adenine dinucleotide phosphate and by house flies and bean plants was examined. C-10015 was metabolized *in vitro* to yield at least three non-hydrolytic products. Two of these metabolites were tentatively proposed to be formed by enzymatic cleavage of the dioxolane ring to aldehyde and/or hydroxylation. It was apparent that decarbamylation was an important route for *in vivo* metabolism of C-10015 in house flies and bean plants.

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Entomology

1971

ACKNOWLEDGMENTS

I wish to express my since gratitude to Dr. Norman C. Leeling, my professor, for his tireless assistance rendered during this research.

I also wish to express my sincere thanks to Dr. Matthew Zabik for assisting me in determing the structures of metabolites from mass-spectrometry.

To Dr. Ronald E. Monroe goes my appreciation for making available to me the tissue combustion equipment used for part of my research.

Furthermore, I wish to convey my sincere thanks and appreciation to Dr. W. B. Drew, Dr. Jeffrey Granett, Mrs. Sandy Granett and Mrs. Karen Christlieb, who have in one way or another helped me and made my graduate study at Michigan State University a pleasant one.

I wish to acknowledge the partial financial support extended me through an assistantship from the Department of Entomology, Michigan State University.

My mother receives my deepest gratitude for her support and enthusiastic encouragement of my studies at Michigan State University.

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INTRODUCTION

I. Carbamate Insecticides And Their Mode Of Action

Carbamate insecticides have been widely used within the last decade, and have prompted numerous studies on their metabolic fate and their mode of action. Carbamate insecticides and many other organic insecticide chemicals are apolar and have adequate lipoid solubility to penetrate the lipoid nerve sheath and approach the site of action (Casida, 1969). Certain carbamates have a structural configuration resembling acetylcholine. Their inhibitory activity results from competition with acetylcholine for the active sites of the enzyme. The extent of inhibition is determined by the degree of stability of the carbamate insecticide to hydrolysis by the enzyme.

A. Carbamates As Insecticides

Development of carbamates as insecticides began with the work of H. Gysin of J. R. Geigy AG, of Switzerland, in 1947. Several well known, highly insecticidal N,N-dimethylcarbamates such as Isolan ® (1-isopropyl-3-methyl-5-pyrazolyl dimethylcarbamate), Pyrolan ® (1-phenyl-3-methyl-5-pyrazolyl

dimethylcarbamate), Dimetan ® (5,5-dimethyl-3-oxo-1-cyclohexane-1-yl dimethylcarbamate), Dimetilan ® [1-(dimethylcarbamoyl)-5-methyl-3-pyrazolyl dimethylcarbamate] and Pyromat ® (2-propyl-4-methyl-6-pyrimidyl dimethylcarbamate) were found. About 1950 work on N-methylcarbamates was initiated by Metcalf in California (Kolbezen et al., 1954). It is now known that the N-methyl compounds are generally more potent insecticides than the N,N-dimethyl analogues. The best known of the present N-methylcarbamate insecticides is carbaryl (1-naphthyl methylcarbamate) or Sevin ® insecticide (O'Brien, 1967). Most of the subsequent carbamates were aromatic and phenolic rather than naphthalic. In addition to carbaryl, some recent N-methylcarbamate insecticides are Zectran ® (4-dimethylamino-3,5-xylyl methylcarbamate), propoxur (o-isopropoxyphenyl methylcarbamate), Mesurol ® [4-(methylthio)-3,5-xylyl methylcarbamate] and Matacil ® (4-dimethylamino-m-tolyl methylcarbamate).

B. Correlation of Structure and Activity

The general formula of the current carbamate insecticides is $R_1R_2NC(O)OX$. For the present methylcarbamate insecticides, R_1 is methyl, R_2 is hydrogen and X is generally a substituted phenol. In present dimethylcarbamate insecticides X is a N-heterocyclic or hydroaromatic enol and R_1 and R_2 are both methyl groups. Other toxic compounds are known which contain thio- and thionocarbamate and which

have various aliphatic, alicyclic and aromatic substituents on the nitrogen or which have fluorine or various aliphatic alcohols or enols for X (Casida, 1963). Kolbezen et al. (1954) reported an investigation of 49 substituted phenyl N-methylcarbamates of which the o- and m-alkylated phenyl derivatives were found to be the most potent insecticides.

C. Mode of Action of Carbamate Insecticides

Most, if not all, carbamates are inhibitors of cholinesterases because of an ability to compete with acetylcholine for the active sites of cholinesterase. These compounds have generally been considered to be reversible competitive inhibitors (Myers, 1956; Wilson et al., 1960; Casida et al., 1960). It was first thought that the mechanism of inhibition of cholinesterase by carbamoyl fluoride was the formation of carbamoyl derivatives of cholinesterase at the esteratic site and that it was analogous to the phosphorylation reaction by dialkyl phosphate esters at the enzyme active centers (Myers, 1956; Myers and Kemp, 1954). From the available evidence it was shown, however, that the mechanism was competitive inhibition rather than carbamoylation (Myers et al., 1957). The inhibition is a time-dependent reaction as shown by the greater inhibition obtained when the inhibitor was added to the enzyme before the substrate than when the inhibitor and substrate were added at

the same time. In either case, as time progressed, an intermediate "equilibrium" value was approached (Augustinsson and Nachmansohn, 1949; Goldstein, 1951; Stedman and Stedman, 1931; Wilson et al., 1961; Wilson et al., 1960; Casida et al., 1960). It is now known that the mechanism of cholinesterase inhibition by carbamates is analogous to the reaction of organophosphates with acetylcholine (O'Brien, 1967). He described the mechanism of cholinesterase inhibition by using the following equation:

$$EOH + AX \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EOH{\cdot}AX \xrightarrow[X^- + H^+]{k_2} EOA \xrightarrow[H_2O]{k_3} EOH + A^+ + OH^-$$

Reversible complex

A is a symbol for either the acetyl group, the dialkyl phosphoryl group, or the methylcarbamyl group, EOH for cholinesterase and X for choline in acetylcholine, p-nitrophenol in paraoxon or 1-naphthol in carbaryl. The above reaction can be explained as follows: first, there is a complex formation between enzyme and substrate; second, acetylation, phosphorylation or carbamylation of the enzyme (at the serine hydroxyl site); third, hydrolysis, that is, deacetylation, dephosphorylation or decarbamylation. For carbamates, k_2 is very slow, k_3 is even slower, but k_a (k_{-1}/k_1) is exceptionally low. These values indicate that the EOH·AX complex is present at low levels and the carbamylated enzyme (EOA) is present at high levels. Carbamate

inhibition of cholinesterase thus can be explained as some of the inhibited enzyme being in the reversible form (EOH·AX) and some of it being in the carbamylated form (EOA).

II. Methylcarbamate Insecticide Metabolism

The metabolism of a compound is an important clue to understanding the toxicology and residue aspects of an insecticide chemical. Metabolism of methylcarbamate insecticides was carried out by a mechanism at a site other than initial hydrolysis at the carbamic ester site or toxophoric grouping (Dorough et al., 1963). It is known that microsomal mixed-function oxidases play an important role in insecticide metabolism. The microsomal enzymes attacked at least two sites of the methylcarbamate insecticides, the aryl ring and associated structures and the mono or dialkylated atoms (Terriere, 1968). There was also evidence of cleaving of the carbamate molecule at the ester and amide linkage (Plapp et al., 1964).

Casida and his colleagues have done extensive work on carbamate metabolism by mammals, insects and plants (Casida, 1963; Hodgson and Casida, 1960; 1961; Dorough and Casida, 1964; Krishna and Casida, 1966; Leeling and Casida, 1966; Oonnithan and Casida, 1968; Tsukamoto and Casida, 1967; Abdel-Wahab et al., 1966; Kuhr and Casida, 1967).

A. Metabolism In Mammals

Mammalian liver microsome enzymes metabolize methylcarbamate insecticides in many ways such as aromatic hydroxylation, aliphatic hydroxylation, N-dealkylation, O-dealkylation and sulfoxidation (Williams, 1959; Gillette, 1963). Study of the in vivo fate of the radiocarbon from ten variously labeled methyl- and dimethylcarbamate-^{14}C insecticides in rats showed that decarbamylation or hydrolytic removal of the $-OC(O)NHCH_3$ group was an important route of metabolism since much of the ^{14}C appeared as $^{14}CO_2$ (Krishna and Casida, 1966). There were five groupings of the carbamate insecticides known to be susceptible to oxidation or hydroxylation by rat liver microsomal enzymes: N-alkyl, N-formamide, O-alkyl, S-alkyl and the aromatic ring groups (Oonnithan and Casida, 1968).

Rabbits treated with carbaryl excreted eleven metabolites in the urine and six of these were identified as 1-naphthyl N-hydroxymethylcarbamate; 4-hydroxy-1-naphthyl N-methylcarbamate; 5-hydroxy-1-naphthyl N-methylcarbamate; 5,6-dihydro-5,6-dihydroxy-1-naphthyl N-methylcarbamate; 1-hydroxy-5,6-dihydro-5,6-dihydroxynaphthalene; and 1-naphthol (Leeling and Casida, 1966). In the study of in vitro metabolism of carbaryl by rat liver microsomes with added $NADPH_2$, thin-layer chromatography showed seven metabolites. Four of these were identified as: 1-naphthyl-N-hydroxymethylcarbamate, 4-hydroxy-1-naphthyl

methylcarbamate, 5,6-dihydro-5,6-dihydroxy-1-naphthyl methylcarbamate and 1-naphthol (Leeling and Casida, 1966).

B. Metabolism In Plants

Enzymes in bean plants metabolized N-methylcarbamate insecticides into organosoluble products, water soluble products and insoluble residues via many reactions such as N-methyl hydroxylation, ring hydroxylation and thioether oxidation (Abdel-Wahab et al., 1966). The hydroxylation products or metabolites produced in bean plants from methylcarbamate insecticides were similar to those formed in the liver microsome-$NADPH_2$ systems, but in plants these hydroxylated carbamates were rapidly conjugated as glycosides. These glycosides were quite persistent and, in many cases, yielded anticholinesterase agents on hydrolysis by β-glucosidase (Kuhr and Casida, 1967). Carbonyl-^{14}C labeled aryl methylcarbamates such as Zectran® and Matacil® injected into the bean plants yielded several metabolites including the 4-methylamino, 4-amino, 4-methylformamido and 4-formamido analogs. Mesurol was oxidized in beans to the sulfoxide and sulfone analogs (Abdel-Wahab et al., 1966).

C. Metabolism In Insects

In vivo, insect microsomal enzymes metabolized carbamate insecticides and yielded products similar to those

obtained by *in vitro* incubation of these insecticides with the $NADPH_2$-dependent system of liver microsomes (Dorough and Casida, 1964). It has been shown that under *in vitro* conditions, insect enzyme preparations had lower activity than that of liver microsomes (Brodie and Maickel, 1962). Microsomes prepared from house fly abdomen homogenates were more active in metabolizing methylcarbamate insecticides in the presence of $NADPH_2$ than were homogenates of the head, thorax or whole house fly (Tsukamoto and Casida, 1967). It was also found that $NADPH_2$ was a more specific and effective cofactor for microsomal enzyme activity than $NADH_2$. Strong inhibitors appeared in the nuclei and debris fraction from head and thorax homogenates (Casida, 1969).

Insect microsomes metabolized methylcarbamate insecticides through a variety of chemical reactions such as aromatic hydroxylation, *O*-dealkylation, *N*-dealkylation and sulfoxidation (Tsukamoto and Casida, 1967).

III. Biological Oxidation As A Detoxification Mechanism

The chemical reactions involved in detoxification mechanisms could be classified mainly as syntheses, oxidations, reductions and hydrolyses (Williams, 1959). Most insecticide chemicals were susceptible to biological oxidation, which resulted in either activation (conversion to their derivatives of increased toxicity) or detoxification to form products of less toxicity (Casida, 1969). Insects

and mammals in many cases had the same detoxification mechanisms and most differences were quantitative rather than qualitative (Smith, 1962). Many different enzyme systems seemed to be involved in the detoxification of N-alkyl and N,N-dialkylcarbamates (Casida et al., 1960). Esterase enzymes such as cholinesterases were able to hydrolyze certain carbamates, but these enzymes could not be regarded as an important detoxification mechanism because the turnover number was very low (Goldstein and Hamlisch, 1952; Myers, 1956). Oxidation as a detoxification mechanism in mammals has been associated with enzyme systems in liver microsomes. This system required $NADPH_2$ and oxygen for its action and could be blocked by certain inhibitors (Dorough and Casida, 1964; Leeling and Casida, 1966; Oonnithan and Casida, 1968). Similar enzyme systems from homogenates of house fly abdomens also required $NADPH_2$ and oxygen for the oxidation of methylcarbamates (Tsukamoto and Casida, 1967; Casida, 1969; Dorough and Casida, 1964).

A. Oxidation And Hydroxylation Of Insecticides

Three majors classes of synthetic organic insecticides, the carbamates, the chlorinated hydrocarbons and the organophosphates undergo oxidation reactions. Among the commercial carbamate insecticides which undergo biological oxidation are carbaryl, Mesurol, Matacil and Zectran. The

oxidation of these compounds was detailed in the section on Metabolism of Carbamates.

Among the chlorinated hydrocarbon insecticides which undergo biological oxidation are DDT, 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane and several cyclodiene compounds including aldrin, 1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4-endo-exo-5,8-dimethanonaphthalene, isodrin, the 1,4-endo-endo form of aldrin, and heptachlor, 1,4,5,6,7,8,8-heptachloro-3a,4,7,7a-tetrahydro-4,7-methanoindene. Tsukamoto (1959) first showed that DDT was oxidized to dicofol, 4,4'-dichloro-a-(trichloromethyl) benzhydrol, in the domestic fruit fly Drosophila melanogaster. Gianotti et al. (1956) showed qualitatively that aldrin was converted to the 6,7-epoxy form, dieldrin, in the American cockroach. Isodrin was converted to the 6,7-epoxy form, endrin, in the house fly (Winteringham and Lewis, 1959) and heptachlor was converted to the 2,3-epoxy form, heptachlor epoxide, in the house fly (Perry et al., 1958).

Four types of organophosphates are oxidized in vitro: thionates, dialkylphosphoramidates, compounds with a thio- group in a side chain and a diethylaminoethyl phosphorothiolate (Heath, 1961). Phosphoramidates, $X_2P(O)N(CH_3)_2$, and phosphorothiolates, $(RO)_2P(S)OX$, were usually poor anticholinesterase agents in vitro (O'Brien, 1960). They are activated, that is converted into potent

anticholinesterases, *in vivo* in insects and mammals, *in vitro* by liver slices or several insect whole-tissue preparations and *in vitro* by specially fortified liver homogenates. The activity of liver is principally due to the microsomes, which also catalyze a great variety of other oxidation reactions. The product of phosphoramidate activation is the hydroxyalkyl derivative, $X_2P(O)N(CH_3)CH_2OH$, and the product of phosphorothionate oxidation is the phosphate, $(RO)_2P(O)OX$. The thionophosphate of demeton, mixture of *O*,*O*-diethyl *S*-(and *O*)-2-[(ethylthio)ethyl] phosphorothionate, can be oxidized to its sulfoxide and sulfone in several insect tissues and in mammalian liver (March *et al.*, 1955). Liver homogenates prepared from various mammalian species possessed an enzyme system capable of catalyzing the destruction of *O*,*O*-diethyl-*S*-2-diethylaminoethyl phosphorothiolate (DSDP) via the oxidative mechanism (Scaife and Campbell, 1959).

B. General Nomenclature And Distribution Of Oxygenases

The term "oxygenase," in a broad sense has been applied to any enzyme capable of catalyzing the activation of oxygen and the subsequent addition of either two atoms of oxygen to a substrate or one atom of oxygen while reducing the other to water. In a narrow sense, the term oxygenase was applied to those enzymes which catalyze the addition of two atoms of oxygen to a substrate and the term

hydorxylase to those enzymes which catalyzed the addition of one atom of oxygen to a substrate while the other atom is simultaneously reduced to water (Hayaishi, 1962).

Oxygenases were first described in mushrooms (Mason et al., 1955) and pseudomonads (Hayaishi, et al., 1955) and were found to be ubiquitously distributed in animals, plants and microorganisms. They are distributed in various cellular fractions such as microsomes, mitochondria and the soluble fraction of liver cells (Knox and Edwards, 1955; Mehler, 1956; Iaccarino et al., 1961; Saito et al., 1957; Halkerston, et al., 1961; Mitoma, et al., 1956).

C. Role Of Oxygenases In Biological Oxidation

Oxygenases play an important role in the metabolism of various aromatic and aliphatic compounds. The oxygenation reaction was primarily involved, in vivo, in detoxication of foreign compounds through the formation of various metabolites (Hayaishi, 1962). Oxygenases and hydroxylases also play a regulatory role in energy distribution in the cell by competing with the conventional electron transport system of cytochromes for reduced nicotinamide nucleotides and for oxygen. Oxygenase activity could be inhibited in the presence of the cytochrome system, because the conventional electron transport system via flavoprotein and

cytochromes was much more active than oxygenase under normal physiological conditions (Hayaishi, 1962).

D. Hydroxylation Reactions

1. Mechanism of single hydroxylation

In 1955, when the use of $^{18}O_2$ and $H_2{}^{18}O$ was made possible, it was discovered that the oxygen atom incorporated into 3,4-dimethylphenol yielding 4,5-dimethylcatechol was derived from an oxygen molecule rather than water (Mason, et al., 1955). The overall reaction was represented by the following equation:

$$S + \frac{1}{2} O_2 \longrightarrow SO$$

This type of reaction always needs an electron donor so that one atom of oxygen could be incorporated into the substrate molecule(s) while the other was reduced to H_2O (Hayaishi, 1962) as shown by the following equation:

$$S + O_2 + AH_2 \longrightarrow SO + H_2O + A$$

In most hydroxylation reactions, reduced nicotinamide nucleotides appear to be specific electron donors. The reactions discussed so far involve the fixation of osygen into a substrate molecule in the presence of an appropriate external electron donor. The substrate itself can also serve as an internal electron donor for certain reactions. Using $^{18}O_2$ and $H_2{}^{18}O$, the reaction catalyzed by a lactic oxidative decarboxylase which converted

l-lactate to acetate plus CO_2 could be shown by the following equation (Hayaishi and Sutton, 1957):

$$CH_3 \cdot CHOH \cdot COOH + {}^{18}O_2 \longrightarrow CH_3 \cdot CO^{18}OH + CO_2 + H_2{}^{18}O$$

l-Lactic acid accepted one atom of oxygen and simultaneously furnished the electrons to reduce another oxygen atom.

2. Mechanism Of Double Hydroxylation

It has been shown that the oxidation of nicotinic acid to 6-hydroxynicotinic acid involves hydration of a double bond followed by dehydrogenation, the oxygen atom thus coming from water (Hughes, et al., 1960). It has been proposed that the nitrogen atom in the ring of purines, pteridines (Forrest, et al., 1956) and nicotine (Hochstein and Rittenberg, 1959) produced sufficient polarization, with the presence of a lone pair of electrons, that hydration of the double bond was facilitated in such molecules.

Another possible mechansim involved the enzymatic hydroxylation of kynurenic acid to 7,8-dihydroxykynurenic acid in the dehydration of dihydrodiol compounds (Kuno, et al., 1961). The available evidence showed that in the presence of $NADH_2$ or $NADPH_2$ and oxygen, kynurenic acid was converted enzymatically to the 7,8-dihydrodiol of kynurenic acid, which was then dehydrogenated to 7,8-dihydroxykynurenic acid by a NAD-linked dehydrogenase.

3. Ring Hydroxylation

One very common metabolic pathway in animals is the hydroxylation of the aromatic ring of compounds foreign to the body. An aromatic hydroxylating system has been found in liver microsomes which required $NADPH_2$ and oxygen for its activity as in the formation of 1-naphthol and 1,2-dihydro-1,2-dihydroxy naphthalene from naphthalene (Mitoma, et al., 1956). The mechanism of this system involved the formation of 1,2-dihydro-1,2-epoxy naphthalene as an intermediate which could then react either chemically or enzymatically with water yielding 1,2-dihydro-1,2-dihydroxy naphthalene or with reduced glutathion to give a mercapturic acid which was then excreted (Booth, et al., 1960). The available evidence suggested that with some cyclic compounds an epoxide may be an intermediate metabolite in ring hydroxylated (Boyland and Sims, 1960), but it has not yet been synthesized or isolated, so epoxide formation remained hypothetical.

A different hydroxylation mechanism is illustrated by the oxidation of nicotine, ℓ-1-methyl-2-(3-pyridyl)-pyrrolidine, in the presence of the liver microsome -$NADPH_2$- oxygen system. The first reaction is hydroxylation at the α-position of the pyrrolidine ring by which nicotine is converted to hydroxynicotine. This hydroxylation was similar to the conversion of quinoline to 2-hydroxyquinoline (Knox,

1946) and it has been proposed that a similar mechanism may be involved in the oxidation of purines by xanthine oxidase (Bergmann and Dikstein, 1956).

4. Oxidative Demethylation

Oxidation of the N,N-dialkylcarbamates by rat liver microsomes takes place at only one of the two alkyl groups, preferentially attacking the shorter of the two if different radicals are present. Methylcarbamates incubated with rat liver microsomes in the presence of $NADPH_2$ and oxygen give formaldehyde-yielding products (Hodgson and Casida, 1960; 1961). Thus, there is *in vitro* evidence for the first step in oxidative demethylation in which a methyl group is oxidized to a hydroxymethyl group. The *in vivo* N-demethylation of certain drugs such as N-methylbarbital and N-methylphenobarbital has also been illustrated in rat livers (Kuroiwa, 1963). Many other N-methylated drugs are known to be demethylated in the body. The two optical forms of N-methylglutathimide, N-methyl-α-ethyl-α-phenylglutarimide, for example, are oxidized at the N-methyl group to the corresponding N-hydroxymethyl form which was excreted as a glucuronide of a new type, that of a N-hydroxymethyl group (Williams and Parke, 1964). The formation of a N-hydroxymethyl glucuronide supports the view that N-demethylation proceeds by oxidation of the methyl group to hydroxymethyl, which can be removed as formaldehyde if not conjugated. Menzer and Casida (1965) reported the successive N-methyl

hydroxyiation and subsequent demethylation of the organophosphate insecticide Bidrin, ® 3-hydroxy-N,N-dimethyl-cis-crotonamide, dimethyl phosphate to 3-(dimethoxyphosphinyloxy)-N-methyl-N-hydroxymethyl-cis-crotonamide and 3-(dimethoxyphosphinyloxy)-N-methyl-cis-crotonamide, by house flies, bean plants and mammals. The same metabolites were formed in hen eggs with Bidrin injected into the yolk sac prior to incubation (Roger, et al., 1964).

5. N-hydroxylation

Another type of hydroxylation reaction has been demonstrated in vivo with the isolation of the aryl hydroxylamine, N-hydroxy-2-acetylaminofluorine, from the urine of rats fed 2-acetylaminofluorine. This metabolite appeared in the urine as a conjugate, probably an ether-type N-O glucuronide (N-O-C linkage) (Cramer, et al., 1960). Investigation of the metabolism of the hydroxylamine showed that this compound and the parent amine yielded the same urinary metabolites. The excretion of larger amounts of 1-hydroxy-2-acetylaminofluorene than 3-, 5-, and 7-hydroxy derivatives after injection of the hydroxylamine instead of acetylaminofluonine suggested that the hydroxylamine may be an intermediate in the formation of the ortho-hydroxylation product (Miller, et al., 1960). Administration of a mixture of 2-acetylaminofluorine-9-^{14}C and unlabeled hydroxylamine showed N-hydroxyacetylaminofluorine to be a direct precursor of the acetylaminophenol (Miller

and Miller, 1960). The *in vivo* conversion of an arylhdroxylamine to an aminophenol indicated that an enzyme system was present which could carry out this process. Although it has been suggested that *O*- and *P*- hydroxylations of aromatic amines are catalyzed by different enzymes, because of the different ratios which occurred in different species (Parke and Williams, 1956; Weisburger, *et al.*, 1957; Parke, 1960), it was probable that *ortho*-hydroxylation of aromatic amines occurred by rearrangement of *N*-hydroxy derivatives, probably through a quinolimide intermediate (Miller, *et al.*, 1960; Miller and Miller, 1960). That *N*-hydroxylation was a general reaction of aromatic amines (Miller, *et al.*, 1960) was supported by a further example of this type of reaction, the identification of *N*-hydroxy-4-acetylaminobiphenyl in the β-glucuronidase treated urine of rats treated with 4-acetylaminobiphenyl (Wyatt, *et al.*, 1961). In addition to these acetyl derivatives, *N*-hydroxylation has been demonstrated with primary amines such as 2-naphthylamine (Walpole and Williams, 1958; Boyland and Manson, 1962).

E. Major Factors Controlling Oxygenase And Oxidase Activities

One of the major factors controlling oxygenase and oxidase activities was their affinity for oxygen and reduced nicotinamide nucleotides (Hayaishi, 1962). It has been shown that cytochrome oxidase (electron carrier of the

conventional electron transport pathway) exhibited a 10-100 times higher affinity for oxygen than do the oxygenases, hydroxylases and other oxidases and that the affinity towards reduced nicotinamide nucleotides was of about the same order between the electron transport system and these enzymes (Hayaishi, 1962).

MATERIALS AND METHODS

Synthesis Of C-10015-Carbonyl-^{14}C, 2-(4,5-dimethyl-1,3-dioxolane-2-yl)-phenyl methylcarbamate-carbonyl-^{14}C

Authentic non-labeled C-10015 (analytical grade) was obtained from CIBA Agrochemical Corp., Vero Beach, Florida. C-10015-carbonyl-^{14}C was obtained by reacting acetyl-1-^{14}C chloride (New England Nuclear, Boston, Mass.) with sodium azide to yield methyl isocyanate-^{14}C which was then reacted with appropriate phenol (prepared by alkaline hydrolysis of C-10015). The reaction tube utilized consisted of two compartments separated by a break seal. This apparatus provided a separated chamber for preparation of methyl isocyanate which could be brought into contact with the phenol in the other chamber by rupture of the break-seal with the glass slug. The product consisted of two isomers and was purified by liquid chromatography on a Florisil® column (Krishna, et al., 1962). The purity was ascertained by thin-layer chromatography (TLC) on Silica Gel G (Brinkmann Instruments Inc., Westbury, New York) using 20 x 20 cm plates with a gel thickness of 0.25 mm. The plate was developed with chloroformacetonitrile (2:1). More than 99% of the radioactivity co-chromatographed with authentic non-labeled C-10015, using radioautography to detect the

carbon-14 material and a 10% sodium hydroxide spray followed by Gibb's (N,2,6-trichloro-p-benzoquinoneimine) reagent (Block, et al., 1958) for detection of the authentic non-labeled compound. The R_f value of the first isomer was 0.55 and that of the second was 0.45. It was later found that the isomer of lower R_f value was more stable than the higher. Therefore, only the isomer with the lower R_f value was used for the subsequent metabolism studies.

Preparation of Liver Fraction And Enzyme Incubation of C-10015-Carbonyl-^{14}C

Methods used for liver microsome preparation and enzyme incubation studies were previously described by Leeling and Casida (1966) and Oonnithan and Casida (1968). Male albino rats (Wistar strain) were killed by cerebral concussion and the livers removed as quickly as possible and immersed in 0.5 M phosphate (Na_2HPO_4-KH_2PO_4) buffer (pH 7.4) at 0° C. After removing any adhering tissues, each liver was rinsed one more time with chilled 0.5 M phosphate buffer, cut into pieces and homogenized in 0.5 M phosphate buffer for 1 min at 0° C with a glass-Teflon® homogenizer (Potter-Elvehjem type) to yield a 20% (w/v) homogenate, on a fresh liver basis. The homogenate was centrifuged at 0° for 25 minutes at 10,000g resulting in sedimentation of the cell debris (undisrupted cells, nuclei, mitochondria, erythrocytes, etc.) The supernatant, or microsome plus soluble fraction, was further centrifuged

at 105,536g for 60 minutes at 0° C to sediment the microsome fraction from which the 'soluble fraction' was decanted. The microsomal pellet was washed twice by resuspension in 0.5 M phosphate buffer and recentrifugation.

An aliquot containing 100,000 cpm of ^{14}C-carbonyl-C-10015 (about 16 ug) was added to the bottom of a 25 ml Erlenmeyer flask in 10 ul of ether and the solvent was allowed to evaporate at room temperature. To the flask was then added 0.5 ml containing each of the following components: liver microsomes (equivalent to 200 mg of liver); 2 umoles of reduced pyridine nucleotide cofactor ($NADPH_2$); 16 umoles of nicotinamide; 2 umoles of barium chloride in 0.5 M phosphate buffer, thus each flask contained a total volume of 2 ml. In each experiment, fresh enzyme preparations and fresh cofactor solutions were used. The flasks were shaken aerobically at 37° C for three hrs. on a Dubnoff metabolic shaking incubator. After incubation the incubation mixtures were immediately either extracted or frozen. Frozen samples were stored at -15° C for not more than 24 hrs. before extraction.

Analysis Of Metabolites

The incubation mixtures were extracted with three 5 ml portions of anhydrous ethyl ether. The combined ether extract was dried over anhydrous sodium sulfate and evaporated under reduced pressure in a rotary evaporator to

approximately 0.5 ml, quantitatively transferred to a grad uated centrifuge tube and reduced to 0.2 ml with a fine stream of air. One hundred ul of the ether extract was added to 15 ml of liquid scintillation fluor for counting. The fluor consisted of toluene-methylcellosolve (2:1), 5.5 g PPO/ℓ and 0.1 g POPOP/ℓ. The remainder was spotted on a Silica Gel G chromatoplate, of 0.25 mm thick, for resolution and quantitation of radioactive components. The TLC plates were developed with chloroform:acetonitrile (2:1). Detection of resolved radioactive compounds on the plates was accomplished by radioautography using no-screen medical X-ray film (Eastman Kodak Company, Rochester, N.Y.) The radioactive regions on the plate corresponding to darkened areas on the film were scraped into scintillation vials and counted. This procedure for radioassay of resolved metabolites by counting the scraped regions gave an average ^{14}C-recovery of 92% of that originally spotted on the plates. The counts for each metabolite component were related, on a percentage basis, to the total radiocarbon recovered from scraping TLC plates.

Characterization of C-10015 Metabolites From The Rat Liver Microsome System

Larger quantities of C-10015 metabolites needed for characterization studies were obtained with slight changes of the procedures described above. Two-dimensional

TLC was used and the plates were developed first with the ether:hexane:ethanol (77:20:3) mixture and then with chloroform:acetonitrile (2:1). Each metabolite scraped from TLC plates was extracted with 7-8 ml of acetone and centrifuged in a graduated centrifuge tube. The acetone was transferred by Pasteur pipet to another graduated centrifuge tube and reduced to approximately 100 ul with a fine stream of air. The concentrated acetone extract was transferred to a melting point tube using a 50 ul syringe. The extract was evaporated to dryness in a lyophilizer for 1-2 hours. The metabolite in each melting point tube was analyzed by direct probe mass spectrometry (Du Pont mass spectrometer 21-490). All mass spectra were obtained at an ionizing voltage of 70 eV.

Metabolism of C-10015-Carbonyl-^{14}C in Bean Plants

The methods and materials used for the study of metabolism of C-10015 -carbonyl-^{14}C by bean plants (Phaseolus nulgaris, var. Bountiful) were the same as the applicable ones described by Abdel-Wahab, et al. (1966), with certain exceptions and changes as noted in the following procedures.

About 100,000 cpm (about 16 ug) of C-10015 -carbonyl-^{14}C were individually injected into the stems of 14 day-old, growing bean plants (Abdel-Wahab, et al., 1966). After injection, the growing plants were held in

the greenhouse for 0, 1/3, 1, 2, 3, 4 and 6 days, respectively. Each replicate of three identically treated plants was cut, stored at -15° C until analyzed 1-4 weeks later and extracted with acetone once and chloroform twice (each time with 30 ml) using the described procedure. This divided the components into three fractions: the acetone-chloroform or organic phase, the acetone-water or aqueous phase and the insoluble residue. The total radioactivity of the organic and aqueous fractions was determined using previously published procedures (Abdel-Wahab, et al., 1966). The total radioactivity of the insoluble residue fraction was analyzed by placing 100 mg samples into bags prepared from 2·54 cm dialysis casing and combusting them to $^{14}CO_2$ in a combustion flask according to the technique previously reported by Hopkins and Lofgren (1968). The $^{14}CO_2$ was trapped in 10 ml monoethanolamine:ethylene glycol monomethylether (1:2 v/v) and a 3.0 ml portion of the liquid was transferred to a scintillation vial for radioassay.

House Fly In Vivo And In Vitro Metabolism Of C-10015-carbonyl-^{14}C

Methods used for the study of house fly metabolism of methylcarbamate insecticides in vivo and in vitro were previously described by Tsukamoto and Casida (1967) and Shrivastava, et al. (1969).

Procedures For *In Vivo* House Fly Metabolism Studies

To a group of 30-40 female flies (*Musca domestica* L.), 3-5 days old, 1 ul of acetone containing 0.2 - 0.3 ug ^{14}C-carbonyl labeled C-10015 was applied topically on the ventral side of each abdomen. Groups of treated flies were placed in the metabolism chamber which was connected to a series of collecting traps. The collecting traps consisted of a chamber that was kept as acetone-dry ice temperature for trapping volatile products and two $^{14}CO_2$ trapping tubes. The $^{14}CO_2$ respired was bubbled through 5 ml of monoethanolamine:ethylene glycol monomethylether (1:2 v/v) and a 1.0 ml portion of the liquid was transferred to a scintillation vial for radioactive assay. Any $^{14}CO_2$ that escaped from this tube was trapped in the scrubber tube consisting of a scintered glass tube bubbling in 30 ml of monoethanolamine: ethylene glycol monomethylether (1:2 v/v). Air was pulled through the respirometer with a vaccuum pump. The $^{14}CO_2$ and the volatile products were collected at appropriate time intervals and the trapping solutions were replaced with fresh solutions. At the end of the experimental periods the flies in the chamber were either immediately analyzed or frozen at -15° C for not longer than 24 hrs. before analysis.

For analysis, the flies were transferred to another tube and surface-washed over the body with 10 ml of cold

acetone. The resulting acetone solution was used to wash the metabolism chamber and to dissolve any excreta in it. This procedure of washing the metabolism chamber was repeated 3 more times, using 10 ml portions of acetone, and the four wash solutions were combined to form the acetone-soluble fraction.

Acetone extracts of each group of 30-40 flies were prepared by homogenizing the flies in 10 ml of acetone at 5° C, using a Pyrex® tissue grinder in a 50 ml tube. The homogenizing pestle was rinsed with 2-3 ml of acetone, the homogenate centrifuged in a graduated centrifuge tube and the supernatant removed with a Pasteur pipet. The sediment was transferred back to the homogenizing tube and the acetone extraction was repeated in an identical manner two additional times, so that the final acetone extract (36-40 ml) represented the combination of three acetone extractions. The extract was evaporated under reduced pressure in a rotary evaporator to approximately 0.3 ml and quantitatively transferred to a graduated centrifuge tube (final volume 0.5 ml). Fifty ul of the concentrated extract was added to 15 ml of liquid scintillation fluor for counting. The combined excrement extract was concentrated and counted in an identical manner.

The dried sediment samples were analyzed by combusting them to $^{14}CO_2$ in a combustion flask as previously described (Hopkins and Lofgren, 1968).

Procedure For *In Vitro* House Fly Metabolism Studies

Enzyme preparation consisted of homogenization of fly abdomens in 0.25 M sucrose and 0.15 M phosphate buffer (pH 7.4) in an ice bath using a glass-Teflon ® homogenizer. In each experiment, bovine serum albumin (BSA) was used at 1.5% (w/v) in both the homogenization and incubation mixtures.

Fractions of homogenates of house fly abdomens were obtained by differential centrifugation, as follows: nuclei, cell debris and mitochondria at 10,000g for 25 min. and microsomes at 105,536g for 60 minutes. All differential sedimentation was at 0° C.

A typical incubation mixture, each in a 25 ml Erlenmeyer flask, consisted of 100,000 cpm (about 16 ug) of C-10015-carbonyl-^{14}C; 5 umoles of $NADPH_2$ and an amount of enzyme preparation equivalent to 15 fly abdomens in a 2 ml final incubation volume. The substrate-enzyme system was shaken aerobically for two hrs. at 30° C on a Dubnoff metabolic shaking incubator. After incubation, the mixtures were either held at -15° C for subsequent extraction or were immediately successively extracted with three 5 ml portions of ether. The combined ether extracts were evaporated under reduced pressure in a rotary evaporator and spotted on a TLC plate. Two dimensional TLC analyses were performed, the solvent being ether:hexane:ethanol (77:20:3) and chloroform:acetonitrile (2:1).

Quantitation studies of metabolites were accomplished by radioautography and scraping the TLC plates as previously described.

RESULTS AND DISCUSSION

Metabolism of C-10015 by Rat Liver Microsomes

Metabolism of C-10015 by rat liver microsomes produces at least three non-hydrolytic products. These three metabolites were designated as A, B and D based on autoradiograms of the TLC resolution of labeled components in incubation mixtures as indicated in Figure 3. Unchanged C-10015 appeared as spot C. The R_f values for these compounds (A to D) in ether:hexane:ethanol (77:20:3) were 0.15, 0.21,0.30 and 0.42, respectively. In chloroform: acetonitrile (2:1), the R_f values were 0.22, 0.33, 0.45 and 0.53, respectively. Reactions involved in this metabolism include hydroxylation (metabolites A and B) and enzyme cleavage of the dioxolane ring to the aldehyde (metabolite A) as will be discussed later.

The pH optimum for non-hydrolytic metabolism was 7.4 for rat liver microsomes when fortified with $NADPH_2$ (Table 1-A).

The optimum incubation time for liver microsome metabolism of C-10015 was three hrs. as shown in Table 1-B.

The effect of selected divalent cations at 2 mmoles per flask on metabolism was ascertained (Table 1-C). The

Table 1. Metabolism Of C-10015-Carbonyl-^{14}C by Rat Liver Microsomes

Experiment and Variables[a)]	Replicates	Relative % of Each ^{14}C-Component in Ether Extract A	B	C	D
A. Microsomes + $NADPH_2$ + Ba^{2+} with pH varied[b)]					
6.8	2	6.4	18.9	72.4	2.3
7.0	2	8.5	26.5	62.2	2.8
7.2	2	9.7	30.3	57.7	2.3
7.4	2	10.8	37.7	47.9	3.5
7.6	2	8.2	36.4	52.8	2.6
7.8	2	9.1	33.1	55.6	2.1
B. Microsomes + $NADPH_2$ + Ba^{2+} at pH 7.4 with incubation time varied (hrs.)					
0.5	2	7.0	21.6	71.2	0
1	2	7.9	23.9	67.8	0
2	2	8.4	25.8	64.6	1.2
3	2	9.4	32.8	56.2	1.6
4	2	6.6	27.0	65.1	1.3

Table 1. Continued

Experiment and Variables	Replicates	Relative % of Each ^{14}C-Component in Ether Extract A	B	C	D
C. Effect of divalent cations at 2mmoles and nicotinamide at 16 umoles per flask					
No divalent cation	2	4.9	12.1	82.0	1.0
Ba^{2+} (no nicotinamide)	2	9.8	28.6	57.8	3.7
Ba^{2+} + nicotinamide	2	10.6	29.4	57.2	2.8
Ca^{2+} + nicotinamide	2	3.2	8.4	87.3	1.1
Mg^{2+} + nicotinamide	2	9.9	23.2	64.9	1.8
Mn^{2+} + nicotinamide	2	6.2	13.9	78.4	1.5
Pb^{2+} + nicotinamide	2	5.6	15.6	77.2	1.6
Sn^{2+} + nicotinamide	2	2.3	6.1	90.5	1.1

a) Except where noted otherwise, each reaction mixture contained the following: 16 ug C-10015-carbonyl-^{14}C, 0.5 M pH 7.4 Na_2HPO_4-KH_2PO_4 buffer, 2.0 umoles $NADPH_2$, 16 umoles nicotinamide, 2.0 mmoles barium chloride, rat liver microsomes in 0.5 ml of 0.5 M pH 7.4 phosphate buffer (equivalent to 200 mg liver). All reaction mixtures were incubated at 37° C for 3 hrs.

b) The pH was varied for the phosphate buffer system. For this experiment rat liver microsomes were prepared in 0.25 M sucrose.

Figure 1. Autoradiogram of one-dimensional TLC of C-10015-carbonyl-^{14}C metabolism by rat-liver microsomes. Development of TLC was with chloroform:acetonitrile, 2:1.

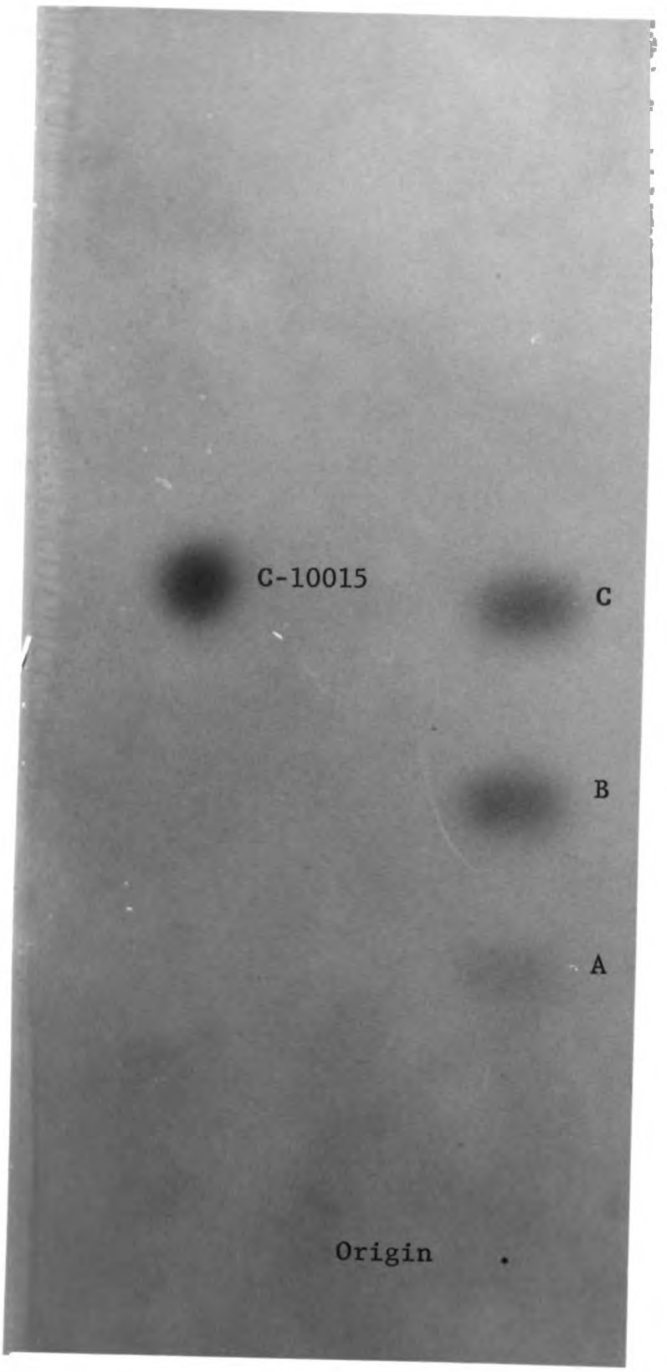


Figure 1.

Figure 2. Autoradiogram of two-dimensional TLC of C-10015-carbonyl-^{14}C metabolism by house fly microsomes. Development of TLC was with ether:hexane:ethanol, 77:20:3 and then chloroform:actonitrile, 2:1.

Figure 3. Autoradiogram of two-dimensional TLC of C-10015-carbonyl-^{14}C metabolism by rat-liver microsomes. Development of TLC was with ether:hexane:ethanol, 77:20:3 and then chloroform:acetonitrile, 2:1.

Figure 2.

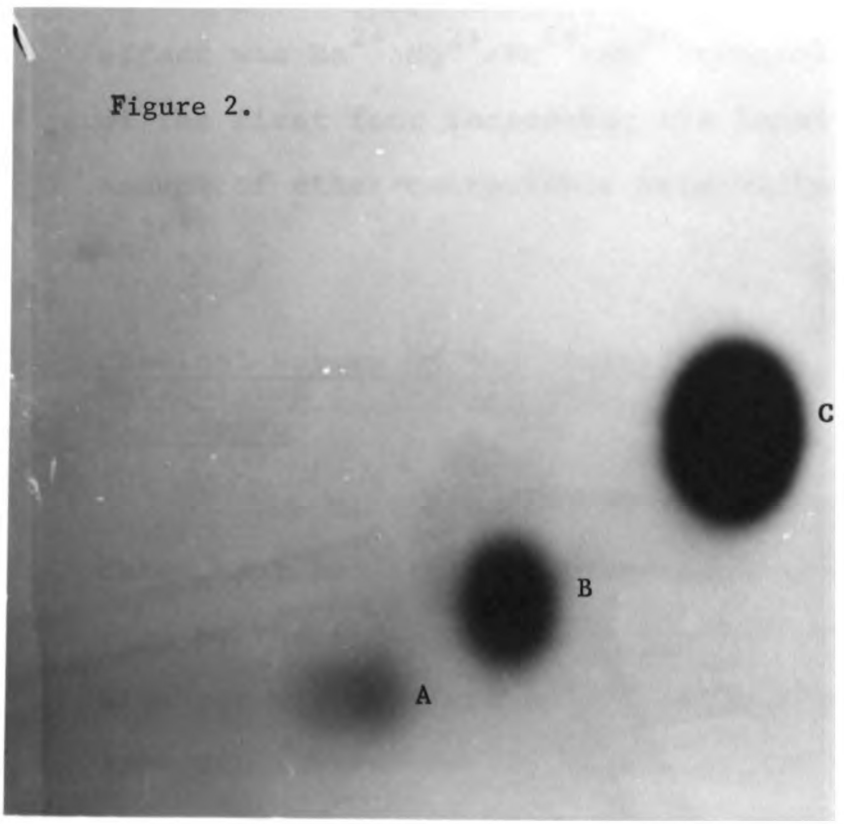
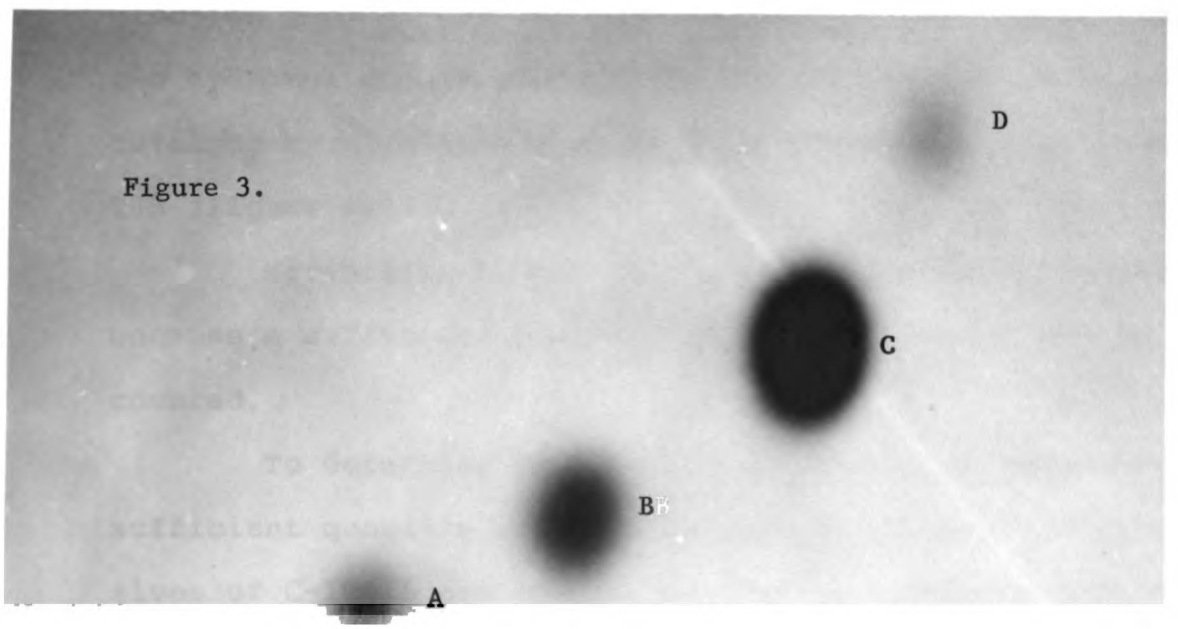


Figure 3.



. Origin

effect was $Ba^{2+}>Mg^{2+}>Pb^{2+}>Mn^{2+}>$control$>Ca^{2+}>Sn^{2+}$, with each of the first four increasing the total metabolism. The amount of ether-extractable metabolites was greatest with Ba^{2+}.

Chemical Nature Of The Carbamate Metabolites of C-10015 From Liver Microsomes

The mass spectrum of metabolite A (Figure 4) indicated that the starting material (C-10015) had been modified by the addition of one hydroxyl group and that an aldehyde was obtained by enzymatic cleavage of the dioxolane ring, based on the molecular ion peak at m/e 195.

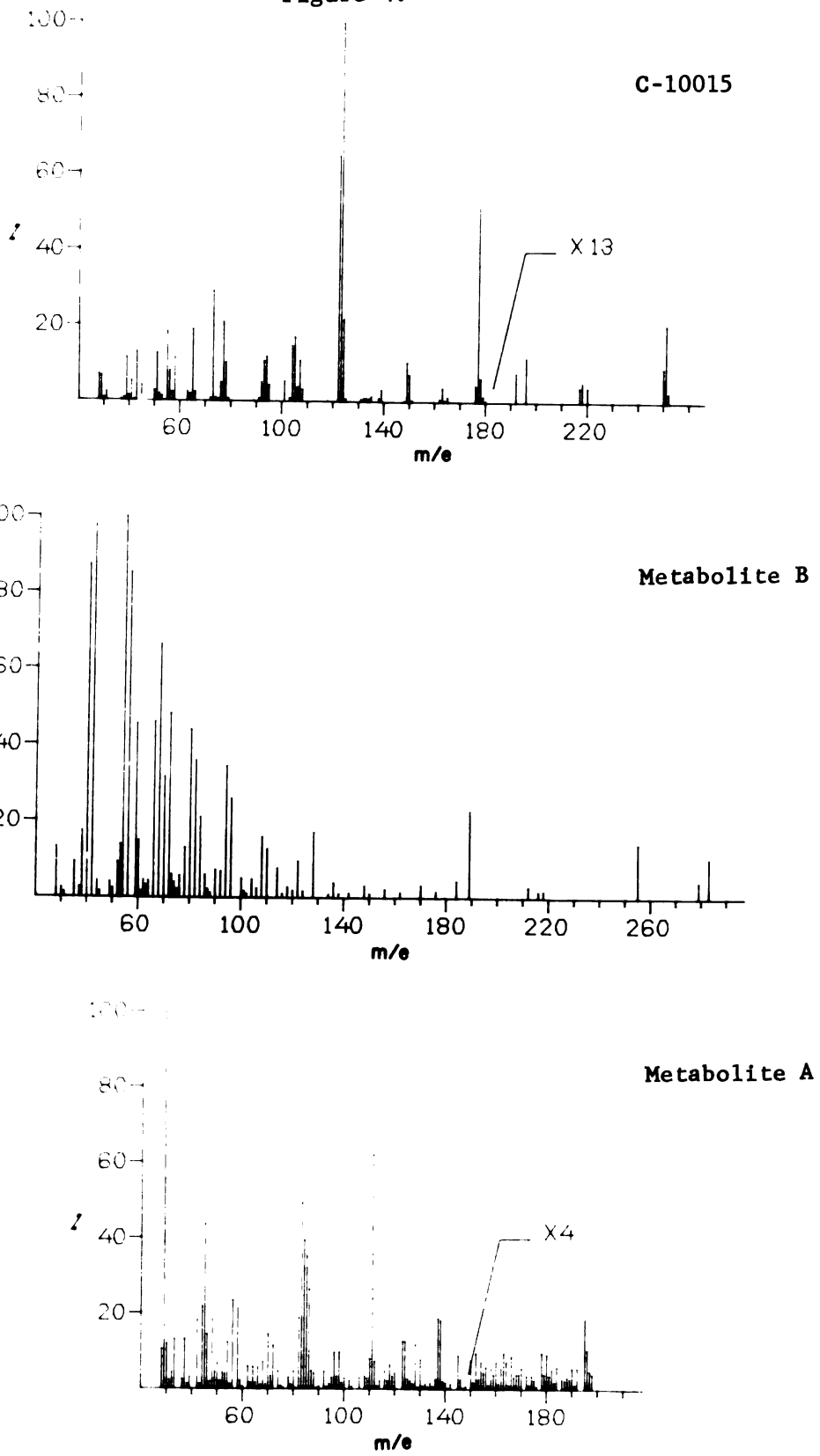
Mass spectrometry of metabolite B showed a molecular ion peak at m/e 283 indicating that the C-10015 had two hydroxyl groups added. The ion at m/e 255 could be obtained by elimination of CO from the parent peak at m/e 283 (Figure 4).

Metabolite D was not analyzed by mass spectrometry because a sufficient quantity of material could not be recovered.

To determine the precise structure of metabolites, sufficient quantity of metabolites and authentic derivatives of C-10015 are needed for analysis by mass spectrometry, infrared spectrometry and nuclear magnetic resonance spectrometry.

Figure 4. The mass spectra of C-10015 and Metabolites A and B.

Figure 4.


C-10015
X 13
100
80
60
I
40
20
60
100
140
180
220
m/e
Metabolite B
100
80
60
I
40
20
60
100
140
180
220
260
m/e
Metabolite A
80
60
I
40
X4
20
60
100
140
180
m/e

Metabolism of C-10015-carbonyl-^{14}C in Bean Plants

Table 2 lists the recovery results obtained when ^{14}C-carbonyl labeled C-10015 was injected into the stem of 14-day-old growing bean plants. The percentage of injected radioactivity recovered in each fraction was calculated and that not accounted for was tabulated as lost. Six days after injection into the stem, loss of radioactivity was 100%. It is apparent that C-10015 disappeared rapidly from the leaf; volatilization of the chemical or loss as $^{14}CO_2$ is probably involved. The rapid disappearance of C-10015 from bean plants is comparable to that of Mesurol, of which 64% was lost by six days after injection into the stem of bean plants (Abdel-Wahab, et al., 1966). Table 2 also shows that C-10015 was metabolized into organosoluble products, water-soluble products and insoluble residues following injection into the stem of growing bean plants.

The water soluble products of C-10015 presented in Table 2 are rather small when compared with the water soluble products of other N-methylcarbamate insecticides such as carbaryl, propoxur, UC 10854 and Banol® (Abdel-Wahab, et al., 1966). Percent recovery of water-soluble products of ^{14}C-carbonyllabeled carbaryl, propoxur, UC 10854 and Banol® were 35.2, 59.0, 64.4 and 45.8, respectively, at the third day after injection into the stem of growing bean plants (Abdel-Wahab, et al., 1966). The recovery of the

water-soluble products of C-10015 carbonyl-^{14}C was 8% at the third day after injection into the stem of the bean plants. Differences in the chemical nature of the substituted groups of each phenyl methylcarbamate are probably involved.

Metabolism of C-10015 by the $NADPH_2$-dependent Enzyme System from the House Fly

TLC autoradiograms (Figure 2) of organosoluble metabolites of C-10015-carbonyl-^{14}C plus house fly microsomes, $NADPH_2$ and BSA showed that at least three metabolites have the same two dimensional TLC R_f's as organosoluble C-10015 metabolites produced by rat liver microsomes. The metabolites were designated as A, B and D, respectively, (Figure 2) as previously described for the metabolism by rat liver microsomes.

The quantity of metabolites produced by house fly microsome-$NADPH_2$ systems was as follow (from A to D): 1.8%, 7.6% and 1.5%, respectively. The original compound, C-10015-carbonyl-^{14}C (spot C), constituted 89.1% of the radioactivity. Therefore, under in vitro conditions, the house fly preparation showed very little metabolism in comparison to that of liver microsomes. Brodie and Maickel (1962) also reported that insect enzyme preparations had low activity in comparison to that of liver microsomes.

Table 2. Fractions Recovered From Growing Bean Plants Injected With C-10015-carbonyl-^{14}C at Intervals After Treatment

Fractions	Replicates	Percent of injected radioactivity found for indicated time, in days.[a]						
		0	1/3	1	2	3	4	6
Organic phase	2	67.5	52.6	14.3	8.5	7.0	-	-
Aqueous phase	2	0.2	1.7	4.0	4.5	8.0	-	-
Insoluble residues	2	3.7	6.0	6.6	10.2	3.5	2.4	-
Lost	2	28.6	39.7	75.1	76.8	81.5	97.6	100

a) Total dpm injected into each bean plant was 125,000 dpm.

Identification of Organosoluble Metabolites Produced by the $NADPH_2$-dependent Enzyme System from House Flies

An insufficient quantity of these metabolites was obtained for analysis by mass spectrometry, however, all the spots obtained from TLC have the same two dimensional TLC R_f's as organosoluble metabolites produced by rat-liver microsomes. Therefore, they may have molecular structures identical to the metabolites produced by liver microsomes.

In vivo Metabolism of C-10015 by House Flies

Preliminary studies involving topical application of carbonyl-^{14}C labeled C-10015 to the ventral surface of the abdomen and subsequent metabolism were made. Radiocarbon recoveries in each fraction are presented in Table 3.

Table 3. Fractions Recovered From House Flies After Topical Application Of 0.2 - 0.3 ug of ^{14}C-carbonyl labeled C-10015

Recovered compound or fraction containing radiocarbon	Percent of total radiocarbon recovered in each fraction at varying intervals (hours) after treatment.[a]					
	1	4	8	16	24	Summation
$^{14}CO_2$	4.2	10.6	7.0	5.0	1.9	28.7
Other volatile products	-	0.18	0.08	0.07	0.06	0.39
Insoluble residue[b]	-	-	-	-	8.4	8.4
Excreted components[c]	-	-	-	-	10.5	10.5
Components in flies[d]	-	-	-	-	43.4	43.4
$^{14}CO_2$ trapped in the scrubber tube[e]	-	-	-	-	1.4	1.4
Total % radiocarbon recovered						92.79
Loss (% radiocarbon)						7.14

a) Total dpm applied topically onto the flies was 77033 dpm which was used for the 100% radiocarbon value.

b),c),d), and e) Analyzed at the end of the experiment (24 hrs. after treatment) only.

SUMMARY

The investigation of C-10015-carbonyl-^{14}C metabolism by rat-liver microsomes, house flies and bean plants has been conducted. Therefore, in the qualitative and quantitative analytical measurements of radioactivity, only products containing this carbon atom in their structure were found. Thus, certain hydrolysis products, such as the phenol and its derivatives were not detected.

Evidence from in vitro experiments suggested that hydroxylation and enzymatic cleavage of the dioxolane ring to an aldehyde were the major detoxication mechanisms for C-10015.

The rat-liver mecrosome fraction was selected for a detailed study of in vitro metabolism. The amount of metabolism was related to the presence of certain divalent cations, the optimum pH was 7.4 and the optimum incubation time was three hrs.

In vitro experiments suggested that house flies produced the same metabolic products of C-10015 as did the liver microsomes.

When C-10015 was applied topically onto the fly abdomens, about 30% of the C-10015 was degraded and expired as $^{14}CO_2$ within 24 hrs.

Six days after injection of C-10015 into growing bean plants, all of the C-10015 was lost from the plants. Rapid degradation of C-10015 with expiration of CO_2 was probably involved.

LIST OF REFERENCES

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Abdel-Wahab, A. M.; Kuhr, R. J.; and Casida, J. E. "Fate of ^{14}C-carbonyl-labeled aryl methylcarbamate insecticide chemicals in and on bean plants." J. Agr. Food Chem. 14 (1966) 290-298.

Augustinsson, K. B., and Nachmansohn, D. "Studies on cholinesterase. VI. Kinetics of the inhibition of acetylcholinesterase." J. Biol. Chem. 179 (1949) 543-549.

Bergmann, F., and S. Dikstein. "Studies on uric acid and related compounds. III. Observations on the specificity of mammalian xanthine oxidases." J. Biol. Chem. 223 (1956) 765-780.

Block, R. J.; Durrum, E. L.; and Zweig, G. A manual of paper chromatography and paper electrophoresis. Academic Press, New York, (1958) p. 305.

Booth, J.; Boyland, E.; and Sims, P. "Metabolism of polycyclic compounds. 15. The conversion of naphthalene into a derivative of glutathione by rat-liver slices." Biochem J. 74 (1960) 117-122.

Boyland, E., and Sims, P. "Metabolism of polycycyclic compounds. 16. The metabolism of 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene." Biochem. J. 77 (1960) 175-181.

Boyland, E., and Manson D. "The reaction of phenylhydroxylamine and 2-naphthylhydroxylamine with thiols." J. Chem. Soc. Unpublished work, cited in Boyland, E.; Manson, D.; and Nery, R. 1962.

Brodie, B. B., and Maickel, R. P. Comparative biochemistry of drug metabolism. Proc. Intern. Pharmacol. Meeting, 1st. 6 (1962) 299-329.

Casida, J. E. Mode of action of carbamates. Ann. Rev. Entomol. 8 (1963) 39-58.

Casida, J. E. Insect microsomes and insecticide chemical oxidations, in Microsomes and Drug Oxidations. Edited by J. R. Gillette, et al. Academic Press, New York, 1969.

Casida, J. E.; Augustinsson, K. B.; and Jonsson, G. "Stability, toxicity, and reaction mechanism with esterases of certain carbamate insecticides." J. Econ. Entomol. 53 (1960) 205-212.

Cramer, J. W.; Miller, J. A.; and Miller, E. C. "N-hydroxylation: a new metabolic reaction observed in the rat with the carcinogen 2-acetylaminofluorene." J. Biol. Chem. 235 (1960) 885-888.

Dorough, H. W.; Leeling, N. C.; and Casida, J. E. "Non-hydrolytic pathway in metabolism of N-methylcarbamate insecticides." Science, 140 (1963) 170-171.

Forrest, H. S.; Glassman, E.; and Mitchell, H. K. "Conversion of 2-amino-4-hydroxypteridine to isoxanthopterin in D. melanogaster." Science, 124 (1956) 725-726.

Giannotti, O.; Metcalf, R. L,; and March, R. B. The mode of action of aldrin and dieldrin in Periplaneta americana (L). Ann. Entomol. Soc. Am. 49 (1956) 588-592.

Gillette, J. R. Metabolism of drugs and other foreign compounds by enzymatic mechanisms. Prog. in Drug Res. 6 (1963) 11-73.

Goldstein, A. Properties and behavior of purified human plasma cholinesterase. III. Competitive inhibition by physostigmine and other alkaloids with special reference to differences in kinetic behavior. Arch. Biochem. Biopays. 34 (1951).

Goldstein, A. and Hamlisch, R. E. Properties and behavior of purified human plasma cholinesterase. IV. Enzymatic destruction of the inhibitors prostigmine and physostigmine. Arch. Biochem. Biophys. 35 (1952) 12-22.

Halkerston, I. D. K.; Eichhorn, J.; and Hechter, O. "A requirement for reduced triphosphopyridine nucleotide for cholesterol side-chain cleavage by mitochondrial fractions of bovine adrenal cortex." J. Biol. Chem. 236 (1961) 374-380.

Hayaishi, O. Biological oxidation. Ann. Rev. Biochem. 31 (1962) 25-46.

Hayaishi, O.; Katagari, M.; and Rothberg, S. "Mechanism of pyrocatechase reaction." J. Am. Chem. Soc. 77 (1955) 5450-5451.

Hayaishi, O. and Sutton, W. B. "Enzymatic oxygen fixation into acetate concomitant with the enzymatic decarboxylation of L-lactate." J. Am. Chem. Soc. 79 (1957) 4809-4810.

Heath, D. F. Organophosphorus Poisons. Pergamon Press, New York, 1961, 403 pp.

Hochstein, L. I., and Rittenberg, S. C. "The bacterial oxidation of nicotine. I. Nicotine oxidation by cell-free preparations." J. Biol. Chem. 234 (1959) 151-155.

Hodgson, E., and Casida, J. E. Biological oxidation of N:N-dialkyl carbamates. Biochem. Biophys. Acta 42 : (1960) 184-186.

Hodgson, E., and Casida, J. E. "Metabolism of N:N-dialkyl-carbamates and related compounds by rat-liver." Biochem. Pharmacol. 8 (1961) 179-191.

Hopkins, T. L. and Lofgren, P. A. "Adenine metabolism and urate storage in the cockroach, Leucophaea maderae." J. Insect Physiol. 14 (1968) 1803-1814.

Hughes, D. E.; Hunt, A. L.; Rodgers, A.; and Lowenstein, J. M. The hydroxylation of nicotinic acid by a cell-free system from Pseudomonas fluorescens. Proc. Intern. Congr. Biochem., 4th Congr. Vienna, 1958, Colloquia 13 (1960) 189-193.

Iaccarino, M.; Boeri, E.; and Scardi, V. "Preparation of purified 3-hydroxyanthranilic acid oxidase from rat and ox liver." Biochem. J. 78 (1961) 65-69.

Knox, W. E. "The quinine-oxidizing enzyme and liver aldehyde oxidase." J. Biol. Chem. 163 (1946) 699-711.

Knox, W. E., and Edwards, S. W. "Homogentisate oxidase of liver." J. Biol. Chem. 216 (1955) 479-487.

Kolbezen, M. J.; Metcalf, R. L.; and Fukuto, T. R. "Insecticidal activity of carbamate cholinesterase inhibitors." J. Agr. Food Chem. 2 (1954) 864-970.

Krishna, J. G., and Casida, J. E. "Fate in rats of the radiocarbon from ten variously labeled methyl-and dimethylcarbamate-C^{14} insecticide chemicals and their hydrolysis products." J. Agr. Food Chem. 14 (1966) 98-105.

Krishna, J. G.; Dorough, H. W.; and Casida, J. E. "Synthesis of N-methylcarbamates via methyl isocyanate-C^{14} and chromatographic purification." J. Agr. Food Chem. 10 (1962) 462-466.

Kuhr, R. J., and Casida, J. E. "Persistent glycosides of metabolites of methylcarbamate insecticide chemicals formed by hydorxylation in bean plants." J. Agr. Food Chem. 15 (1967) 814-824.

Kuno, S.; Tashiro, M.; Taniuchi, H.; Horibata, K.; Hayaishi, O.; Seno, S.; Touyama, T.; and Sakan, T. Enzymatic degradation of kynurenic acid. Abstract in Fed. Proc. 20 (1961) 3.

Kuroiwa, Y. "Studies on the metabolic N-demethylation. I. The metabolic fate of hexobarbital by rabbit liver slices." Chem. Pharm. Bull. 11 (1963) 160-163.

Leeling, N. C., and Casida, J. E. "Metabolites of carbaryl (1-naphthyl methylcarbamate) in mammals and enzymatic systems for their formation." J. Agr. Food Chem. 14 (1966) 281-290.

March, R. B.; Metcalf, R. L,; Fukuto, T. R.; and Maxon, M. G. "Metabolism of systox in the white mouse and american cockroach." J. Econ. Entomol. 48 (1955) 355-363.

Mason, H. S.; Fowlks, W. L.; and Peterson, E. W. "Oxygen transfer and electron transport by the phenolase comples." J. Am. Chem. Soc. 77 (1955) 2914-2915.

Mehler, A. H. "Formation of picolinic and quinolinic acids following enzymatic oxidation of 3-hydroxyanthranilic acid." J. Biol. Chem. 218 (1956) 241-254.

Menzer, R. E., and Casida, J. E. "Nature of toxic metabolites formed in mammals, insects and plants from e-(dimethoxyphosphinyloxy) N,N-dimethyl cis-crotonamide and its N-methyl analog." J. Agr. Food Chem. 13 (1965) 102-112.

Miller, J. A.; Cramer, J. W.; and Miller, E. C. The N- and ring-hydroxylation of 2-acetylaminofluorene during carcinogenesis in the rat. Cancer Research. 20 (1960) 950-962.

Miller, E. C., and Miller, J. A. "A mechanism of ortho1-hydroxylation of aromatic amimes in vivo." Biochem. Biophys. Acta 40 (1960) 380-382.

Mitoma, D.; Posner, H. S.; Reitz, H. C.; and Udenfriend, S. "Enzymatic hydroxylation of aromatic compounds." Arch. Biochem. Biophys. 61 (1956) 431-441.

Myers, D. K. "Studies on cholinesterase. 10. Return of cholinesterase activity in the rat after inhibition by carbamoyl fluorides." Biochem. J. 62 (1956) 556-563.

Myers, D. K., and Kemp, A. Jr. "Inhibition of esterases by the fluorides of organic acids." Nature 173 (1954) 33-34.

Myers, D. K.; Kemp, A. Jr.; Tol, J. W.; and de Jonge, M. H. T. "Studies on aliesterases. 6. Selective inhibitors of the esterases of brain and Saprophytic mycobacteria. Biochem. J. 65 (1957) 232-241.

O'Brien, R. D. Toxic Phosphorus Esters. Academic Press, New York, New York, 1960, 434 pp.

O'Brien, R. D. Insecticide-Action and Metabolism. Academic Press, New York, New York, 1967, 332 pp.

Oonnithan, E. S., and Casida, J. E. "Oxidation of methyl- and dimethylcarbamate insecticide chemicals by microsomal enzymes and anticholinesterase activity of the metabolites." J. Agr. Food Chem. 16 (1968) 28-44.

Parke, D. V. "Studies in detoxication. 85. The metabolism of [^{14}C] aniline in the rabbit and other animals." Biochem. J. 77 (1960) 493-503.

Parke, D. V., and Williams, R. T. "Species differences in the o- and p-hydroxylation of aniline." Biochem. J. 63 (1956) 12p.

Perry, A. S.; Mattson, A. M.; and Buckner, A. J. "The metabolism of heptachlor by resistant and susceptible house flies." J. Econ. Entomol. 51 (1958) 346-351.

Plapp, F. W.; Chapman, G. A.; and Bigley, W. S. "A mechanism of resistance to Isolan in the House fly." J. Econ. Entol. 57 (1964) 692-698.

Roger, J. C.; Chambers, H.; and Casida, J. E. "Nicotinic acid analogs: Effects on response of chick embryos and hens to organophospahte toxicants." Science 144 (1964) 539-540.

Saito, Y.; Hayaishi, O.' and Rothberg, S. "Studies on oxygenases: Enzymatic formation of 3-hydroxy-l-kynuronine from l-kinuronine." J. Biol. Chem. 229 (1957) 921-934.

Scaife, J. F., and Campbell, D. H. "The destruction of o,o-diethyl-s-2-diethylaminoethyl phosphorothiolate by liver microsomes." Canad. J. Biochem. Physiol. 37 (1959) 297-305.

Shrivastava, S. P.; Tsukamoto, M.; and Casida, J. E. "Oxidative metabolism of C^{14}-labeled Baygon by living house flies and by house fly enzyme preparations." J. Econ. Entomol. 62 (1969) 483-498.

Smith, J. N. "Detoxication mechansims." Ann. Rev. Entomol. 7 (1962) 465-480.

Stedman, E., and Stedman, E. "Studies on the relationship between chemical constitution and physiological action. III. The inhibitory action of certain synthetic urethanes on the activity of liver esterase." Biochem. J. 25 (1931) 1147-1167.

Terriere, L. C. "Insecticide-cytoplasmic interactions in insects and vertebrates." Ann. Rev. Entomol. 13 (1968) 75-98.

Tsukamoto, M. Metabolic fate of DDT in Drosophila melanogaster. I. Identification of a non-DDE metabolite. Botyu-Kagaku. 24 (1959) 141-151.

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

Walpole, A. L., and Williams, M. H. C. "Aromatic amines as carcinogens in industry." British Med. Bull. 14 (1958) 141-145.

Weisburger, J. H.; Weisburger, E. K.; and Morris, H. P. "Orientation of biochemical hydroxylation in aromatic compounds." Science 125 (1957) 503.

Williams, R. T. Detoxication Mechanisms. 2nd Edition. John Wiley and Sons, Inc., New York, 1959, 796 p.

Williams, R. T., and Parke, D. V. "The metabolic fate of drugs." Ann. Rev. Pharmacol. 4 (1964) 83-114.

Wilson, I. B.; Harrison, M. A.; and Ginsburg, S. "Carbamyl derivatives of acetylcholinesterases." J. Biol. Chem. 236 (1961) 1498-1500.

Wilson, I. B.; Hatch, M. A.; and Ginsburg, S. "Carbamylation of acetylcholinesterase." J. Biol. Chem. 235 (1960) 2312-2315.

Winteringham, F. P. W., and Lewis, S. E. "On the mode of action of insecticides." Ann. Rev. Entomol. 4 (1959) 303-318.

Wyatt, C. S.; Miller, J. A.; and Miller, E. C. "The N-hydroxylation of 4-acetylaminobiphenyl in the rat. Proc. Am. Assoc. Cancer Research 3, 1961, 279.