THE COMPARATIVE METABOLISM OF SOME SUBSTITUTED

PHENYL-N-METHYLCARBAMATE INSECTICIDES

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Abstract

1. The metabolism of the N-methylcarbamates of 3-tertbutylphenol;
   3,5-ditertbutylphenol; and 2-isopropoxyphenol was investigated
   in insects and mammals.

2. The major degradative pathway in enzyme systems from insects and
   mice was oxidative. The major metabolites from tertbutyl
   substituted phenyl-N-methylcarbamates were N-hydroxymethyl
   derivatives and tertbutanol derivatives. Baygon yielded
   N-hydroxymethyl, ring hydroxyl and O-dealkyl derivatives as
   major metabolites.

3. The rates of oxidation of the three insecticides in each enzyme
   system were similar.

4. Oxidation was inhibited by piperonyl butoxide and Metopirone,
   apparent I_{50} for singly oxidised metabolites was 10^{-4}M, and for
   metabolites with two oxidations 10^{-5}M.

5. Enzymic hydrolysis of carbamate insecticides required reduced cofactor
   in insect and mouse systems. Mouse blood did not effect hydrolysis.

6. A wide variation of oxidising ability was found in live insects.
   Musca domestica was most active, Tenebrio molitor and Costelytra
   zealandica were least active.

7. Insecticide synergists reduced insects' ability to oxidise Baygon
   to acetone.

8. *Musca domestica* and *Lucilia sericata* larvae oxidised carbamate
    insecticides slower than the adult forms.

9. Mice excrete 3-tertbutylphenyl-N-methylcarbamate as phenolic metabolites,
    with only minor oxidative products.

10. Different rates of metabolism among insects could account for the
    selective toxicity of aryl-N-methylcarbamates.

Preface

The effects of various alkyl substituents on the insecticidal activity of substituted phenyl-N-methylcarbamates has been investigated in detail (Kolbezen et al., 1954). It has been suggested that the variation in the ease of metabolism of such alkyl substituents among insect species might confer selective action to the insecticide. Such differences in the metabolism of xenobiotics among animal species have been widely reported (Williams, 1959), and might be a major factor in the selectivity of carbamate insecticides.

A comparative study of the metabolism of tertbutyl and isopropoxyl substituted phenyl-N-methylcarbamates was undertaken in order to determine the influence of these groups on the overall metabolism of the insecticides and their rôle in conferring selective activity.

Acknowledgements

Chemical names of some carbamate insecticides, and insecticide synergists

Trade name	Chemical name
Banol	3,4-dimethyl-6-chlorophenyl-N-methylcarbamate
Baygon Propoxur Bayer 39,007	2-isopropoxyphenyl-N-methylcarbamate
Butacarb	3,5-ditertbutylphenyl-N-methylcarbamate
Carbaryl Sevin	1-naphthyl-N-methylcarbamate
Dimetilan	2-dimethylcarbamoyl-3-methyl-5-pyrazolyl dimethyl-carbamate
Furadan	2,3-dihydro-2,2-dimethyl-7-benzofuranyl-N-methyl-carbamate
H.R.S. 1422	3,5-diisopropylphenyl-N-methylcarbamate
Isolan	1-isopropyl-3-methyl-5-pyrazolyl dimethylcarbamate
Matacil	3-methyl-4-dimethylaminophenyl-N-methylcarbamate
Mesurol	3,5-dimethyl-4-methylthiophenyl-N-methylcarbamate
Temik	2-methyl-2-(methylthio)-proprionaldehyde-O-methyl-carbamoyloxime
UC.100854	3-isopropylphenyl-N-methylcarbamate
Zectran	3,5-dimethyl-4-dimethylaminophenyl-N-methylcarbamate
Metopirone, Metyrapone	3-methyl-1,2-bis(3-pyridyl)-1-propanone

Piperonyl butoxide   4-[2-(2-butoxyethoxy)ethoxy]methyl-5-propyl-1,2-
                              methylenedioxybenzene

S.K. & F.525-A.   2-diethylaminodiphenylpropylacetate

T.D.M.   Tetramethyldiaminodiphenylmethane

PART I

INTRODUCTION

History and development of the carbamate insecticides

The elucidation of the structure of physostigmine by Stedman in 1926 and the recognition of its potent inhibition of acetylcholinesterase (Engelhart and Loewi, 1930) has led to the development of the esters of carbamic acid into contact insecticides.
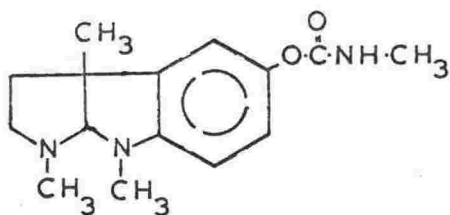
Physostigmine
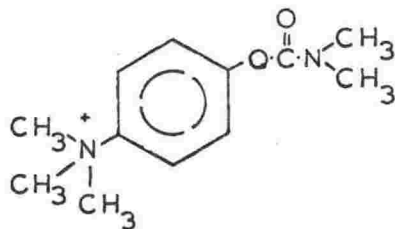
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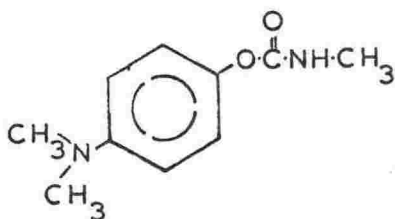
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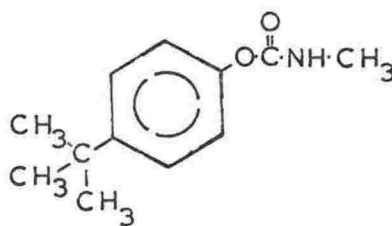
3-tertbutylphenyl-N-methylcarbamate

In 1954 it was shown that simple lipid soluble analogs of physostigmine such as 3-dimethylaminophenyl-N-methylcarbamate, and 3-tertbutyl phenyl-N-methylcarbamate were highly active insecticides and evidence was provided that the quaternary ammonium derivatives such as prostigmine were unable to penetrate the lipid sheaths surrounding insect nerves and were therefore inactive as insecticides (Kolbezen et al., 1954). About the same time Gysin (Gysin, 1954) described the strong insecticidal activity

of a group of N,N-dimethylcarbamates of a variety of heterocyclic enols
of which Isolan, Pyrolan, and Dimetilan have become commercial insecti-
cides.

The insecticidal action of phenyl-N,N-dimethylcarbamate (Meltzer,
1956) was noted, and both it and 1-naphthyl-N,N-dimethylcarbamate patented
(U.S. 2,854,374; 1958). Comparative studies of aryl carbamates have shown
that the N-methylcarbamates are generally more toxic but less stable to
hydrolysis than the N,N-dimethylcarbamates (Metcalf et al., 1962a, N-acyl-
N-methylcarbamates also show reduced insect toxicity (Hadaway and Barlow,
1966; Lewis, 1967).

The development of carbaryl (1-naphthyl-N-methylcarbamate) (Haynes
et al., 1957) showed that the carbamate insecticides can be stable, safe,
inexpensive, and have fairly broad spectrum effectiveness. Since carbaryl
was successfully introduced, carbamates have become a subject of growing
interest. As insecticides they represent alternatives to the chlorinated
hydrocarbons e.g. DDT, (1,1-bis(4-chlorophenyl)2,2,2-trichloroethane);
Dieldrin, (1,2,3,4,10,10-hexachloro-exo-6,7-epoxy-1,4,4a,5,6,7,8,8a-octa-
hydro-1,4-endo, exo-5,8-dimethanonaphthalene) and organophosphorus
compounds e.g. Parathion (O,O-diethyl-O-4-nitrophenylphosphorothioate);
Diazinon (O,O-diethyl-O-(2-isopropyl-4-methyl-6-pyrmidinyl)phosphorothioate
which may show high mammalian toxicity, or pose residue problems.

3.

Considerable impetus has been given to research and development of carbamate insecticides and a number have been synthesised and screened for insecticidal activity. Alkyl substituted phenyl-N-methylcarbamates have been exhaustively screened (Metcalf et al., 1962b; Metcalf et al., 1963; Fukuto et al., 1964; Kaeding et al., 1965; Lemin et al., 1965; Kohn et al., 1965; Metcalf and Fukuto, 1965; Fahmy et al., 1966; Metcalf and Fukuto, 1967; Knowles and Arthur, 1967). Alkoxyphenyl and alkylthiophenyl-N-methylcarbamates (Metcalf et al., 1960; Mahfouz et al., 1969); O-(methyl-carbamoyl)oximes (Bartley et al., 1966; Payne et al., 1966; Weiden et al., 1965); benzofuranyl-7-N-methylcarbamates (Bartley et al., 1966); benzo[b]thienylcarbamates (Kilsheimer et al., 1969); N-methyl and N,N-dimethyl-carbamates of 1,3-oxathiolan-2-yl; 1,3-dioxolan-2-yl; and 1,3-dithiolan-2-yl substituted phenols (Nikles, 1969; Durden and Weiden, 1969), have been prepared and a number show useful biological activity.

The increasing number of the types of carbamate insecticide has led to some correlation of chemical structure with insecticidal action within each group of compounds and some among the whole group of insecticides. Metabolic studies, however, have largely been confined to those compounds which possess high insecticidal action and are of commercial interest.

The mode of action of the carbamate insecticides

The carbamate insecticides exert their toxic action by inhibition of acetylcholinesterase (Metcalf and March, 1950). Inhibition can be

reversed by washing (Aldridge, 1953), dialysis (Kolbezen et al., 1954),
dilution or addition of substrate (Winteringham and Fowler, 1966). It
has been concluded that the primary mode of inhibition is competitive,
in view of the slow rates of carbamoylation when compared to the rapid
rate of complex formation (Metcalf and Fukuto, 1965). Inhibition of
cholinesterase by carbamates is thought to follow the reaction sequence:

$$EH + CX \xrightleftharpoons[]{K_a} EHCX \xrightarrow[k_2]{\quad HX + HOH \quad} EC \xrightarrow{k_3} EH + COH$$

$$k_i$$
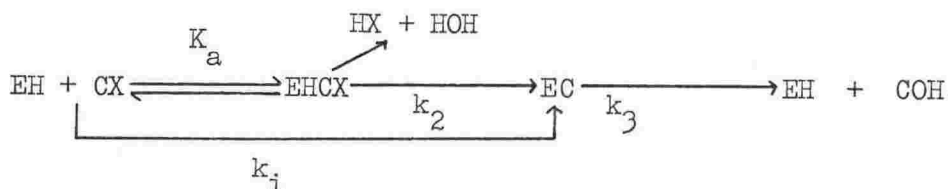
in which EH is the enzyme, CX is a carbamate, EHCX is a complex controlled
by an equilibrium affinity constant K_a, EC is the carbamoylated active
site, HX the leaving group, k_2 the carbamoylation rate constant and k_3
the regeneration constant (O'Brien et al., 1966; O'Brien, 1968). Overall
rates of inhibition are given by k_i ($k_i = {}^{k_2}/K_a$) (Main, 1964).

The carbamoylation (k_2) and binding (K_a) constants for a number of
N,N-dimethyl and N-methylcarbamates have been determined (Hastings et al.,
1970). For bovine erythrocyte cholinesterase the following data were
obtained.

TABLE I

Inhibition constants for some substituted carbamates.

N-methylcarbamate	K_a (mM)	k_2 (min^{-1})	k_i (M^{-1}min^{-1})	LD_{50} [1]
Phenyl	23.7 ± 2.0	6.8 ± 0.4	$2.9 \times 10^{+2}$	70 (a)
3-Isopropylphenyl	0.18 ± 0.06	80.7 ± 20.0	$4.6 \times 10^{+5}$	50 (a)
3-tertButylphenyl	0.07 ± 0.01	23.4 ± 2.0	$3.5 \times 10^{+5}$	50 (a)
1-Naphthyl	0.5	20	2.2×10^{4}	500 (a)
Eserine	0.003 ± 0.0003	10.8 ± 0.3	3.3×10^{6}	
Temik	10.3 ± 1.0	146 ± 10	1.6×10^{4}	5.5 (b)
Temik sulphone	17.0 ± 3.0	74 ± 8	4.4×10^{3}	20 (b)

(1) LD_{50} for Musca domestica in μg/g flies.

(a) Kolbezen et al., 1954.

(b) Metcalf et al., 1966.

It appears from these data that inhibitory activity is as much dependent on carbamoylation rates as on initial binding, but in general the smaller the K_a, or greater the 'fit' of the inhibitor molecule in the active site, the greater will be the overall inhibition. Exceptions are Temik and Temik sulphone, with low affinity for the enzyme and dependent on a rapid carbamoylation reaction for inhibition.

Studies on the inhibition and recovery of esterases from carbamate poisoning have been conducted with mammals (Baron et al., 1966) and houseflies (Mengle and Casida, 1958). In mice it was found that the toxic effects were primarily resultant from inhibition of brain cholinesterase enzymes, but liver aliesterases remained inhibited after signs of poisoning had disappeared. With houseflies, head cholinesterase was inhibited and near complete recovery of the enzymes, with non-lethal doses, was noted after 24 hours.

The anticholinesterase and psychopharmacological effects of 3-isopropylphenyl-N-methylcarbamate have been studied in rats. Male rats were three to four times more sensitive than females to the dose affecting avoidance behaviour. Atropine sulphate and methyl atropine blocked the effects on behaviour (Goldberg et al., 1963).

Although the action of the insecticidal carbamates is thought to be the same in all species tested, large variation in k_i and LD_{50} figures have been obtained, and a useful parameter of activity is I_{50}, the molar concentration of carbamate giving 50% inhibition of cholinesterase. But

since variation in LD_{50}'s between similar carbamates may be due largely to metabolic processes, a useful measure is the comparison of LD_{50}, and LD_{50} when applied with a synergist to halt oxidative metabolism. The resulting ratio, the synergistic ratio, gives an indication of the metabolism occurring. The relationship between this modified LD_{50} figure and I_{50} or k_i is more obvious.

Correlation between chemical structure and anticholinesterase activity

The conclusions of Metcalf and Fukuto (1965) were that 'close fit' of the carbamate to the enzyme is the major requirement for acetylcholinesterase inhibition, and that steric factors are most important. The optimal configuration seems to be close to that of acetylcholine, however the close analogs of acetylcholine are good anticholinesterases but poor insecticides, since they are polar in nature. Non-polar analogs may have insecticidal activity but show reduced anticholinesterase activity.
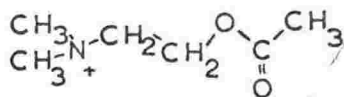
acetylcholine
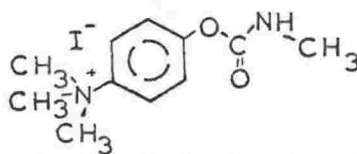
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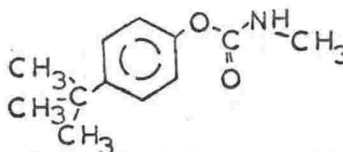
3-tertbutylphenyl-N-methylcarbamate

(a) The effect of aromatic alkyl substituents

The steric similarity of 3-tertbutylphenyl-N-methylcarbamate and acetylcholine leads to good anticholinesterase action, possibly because hydrophobic forces are involved in binding the aryl substituent to the anionic site. A decreasing order of activity, tertbutyl = isopropyl > ethyl > methyl > unsubstituted is found.

Where a single alkyl substitution is made the position is important. Substituents meta to the ester bond confer greater inhibitory activity than those ortho, while para substituted compounds are least inhibitory. In the case of alkoxy and alkylthio substituents the order becomes ortho > meta > para, since the alkyl part of the substituent is in a position sterically similar to a meta alkyl group.

Where more than one substituent is present, the position of the substitution markedly affects the anticholinesterase activity. The series of dimethylphenyl-N-methylcarbamates (Metcalf et al., 1963) tested showed 3,5-dimethyl substitution to lead to the greatest inhibitory effect. The same applies to dimethoxy, diisopropyl and ditertbutyl substituents. For trimethylphenyl-N-methylcarbamates, the 3,4,5-isomer is the most active.

With two different substituents, the 3,5- positions are best for alkyl groups whereas 2-alkoxy and 5-alkyl lead to maximum inhibitory activity.

Correlations on a similar basis have been made for other groups of carbamate insecticides (Nikles, 1969; Durden and Weiden, 1969; Kilsheimer et al., 1969).

(b) The effect of carbamate nitrogen substituents

The N-methylcarbamates are generally better inhibitors of acetyl-cholinesterase than the N,N-dimethylcarbamates (Metcalf et al., 1962a; Hastings et al., 1970), but are less stable to alkaline hydrolysis (Fukuto et al., 1967).

However, some compounds with a dimethyl substituted N, such as Isolan, where no N-methyl analogs have been made, show very high inhibitory and insecticidal activity.

Other N-alkyl substituents lead to less inhibitory carbamates than N-methyl substituents (Kolbezen et al., 1954).

Acylation of the N-methylcarbamate to N-acyl-N-methyl compounds yielded carbamates with reduced anticholinesterase activity (Lewis, 1967) and reduced toxicity towards mosquitoes (Hadaway and Barlow, 1966) and blowfly larvae (Fraser et al., 1967).

TABLE II

The effect of substitution position on anticholinesterase and insecticidal activity.

N-methylcarbamate of:		I_{50} M	Affinity	LD$_{50}$ Musca domestica	
				Unsynergised (μg/g)	P.B.O.[c] Synergised (μg/g)
2-tertbutylphenol	(a)	6.0×10^{-6}	30	75	–
3-tertbutylphenol	(a)	4.0×10^{-7}	500	50	8.0
4-tertbutylphenol	(a)	1.5×10^{-4}	0.8	500	–
2-isopropoxyphenol	(b)	6.9×10^{-7}	290	26	7
3-isopropoxyphenol	(b)	9.2×10^{-6}	22	180	19.5
4-isopropoxyphenol	(b)	8.8×10^{-6}	2.3	500	340
2-isopropylthio-phenol	(b)	1.4×10^{-7}	1420	23.0	–
3-isopropylthio-phenol	(b)	1.8×10^{-6}	110	46.5	–
4-isopropylthio-phenol	(b)	9.0×10^{-6}	22	700	–

I_{50}: molar concentration giving 50% inhibition of housefly head cholinesterase.

Affinity: ratio $\dfrac{I_{50} \text{ unsubstituted phenyl-N-methylcarbamate}}{I_{50} \text{ substituted phenyl-N-methylcarbamate}}$

effectively, the affinity is the reciprocal of the relative value of k_i, a primary variable in the enzyme inhibition.

(a) Data from Kolbezen et al., 1954; (b) Data from Metcalf & Fukuto, 1965; (c) Piperonyl butoxide

TABLE III

The effect of di- substitution on anticholinesterase and insecticidal activity.

N-methylcarbamate of:	I_{50} M	Affinity	LD_{50} Musca domestica Unsynergised (μg/g)	P.B.O. Synergised (μg/g)
2,4-ditertbutylphenol	1×10^{-3}	0.2	500	500
3,6-ditertbutylphenol	1×10^{-3}	0.2	500	500
3,5-ditertbutylphenol	7.8×10^{-8}	2551	39	6.0
3-tertbutyl-5-methylphenol	1.7×10^{-7}	1180	31	4.9
2-tertbutyl-5-methylphenol	8.0×10^{-4}	0.25	500	300
3-tertbutylphenol	4.0×10^{-7}	500	50	8.0
2-isopropoxyphenol	6.9×10^{-7}	290	25.5	7.0
2-isopropoxy-5-isopropylphenol	4.3×10^{-7}	466	48.5	9.5
2-isopropoxy-5-methylphenol	2.8×10^{-7}	715	40	6.0
2-isopropoxy-5-methoxyphenol	5.6×10^{-7}	358	6.5	3.0
3-isopropoxy-6-methoxyphenol	1.5×10^{-5}	13	150	10.0

Data from Kolbezen et al., 1954, and Metcalf and Fukuto, 1967.

Correlation between chemical structure and insecticidal activity

There appears to be a relationship between chemical structure and insecticidal activity for some insect species, but not for others. Kolbezen et al., (1954) showed that a relationship existed for thrips, but not for houseflies (Casida et al., 1960). However if the synergised toxicity for houseflies is compared, correlation is found (Metcalf and Fukuto, 1965).

The major factor involved in a good insecticide, aside from lipid solubility and acetylcholinesterase inhibition, is the ease with which the compound is metabolised. Thus insertion into the molecule of groups metabolised only slowly may enhance insecticidal activity (Chakraborty and Smith, 1967). Similarly, blocking positions in the aromatic nucleus which are normally hydroxylated during metabolism, such as the 5- position in Baygon (2-isopropoxyphenyl-N-methylcarbamate) (Shrivistava et al., 1969), may also increase insecticidal activity. The data given in Table III shows a marked difference in the LD_{50} values for Baygon and 5- substituted Baygon, although I_{50} values are similar.

Studies of the metabolism of carbamate insecticides may be of importance in determining why similar anticholinesterases are widely different in insecticidal action.

The metabolism of xenobiotics

During their lifetime both animals and plants come into contact with a wide variety of foreign compounds, compounds with varying degrees of toxicity towards the organism. These compounds, xenobiotics, are dealt with by one or several detoxication mechanisms, enzymic methods by which the organism can change the xenobiotic to a more readily excretable form and thus protect itself from the harmful effects of the compound. There are a number of such mechanisms available to the organism when confronted with such a xenobiotic, they include oxidation, reduction, hydrolysis, dehydrochlorination, and conjugation with a variety of materials.

Many of the detoxication reactions are carried out by vertebrate liver, and in insects by the fat body. Cellular fractionation of these tissues have led to the recognition of the involvement of the endoplasmic reticulum in these reactions. Preparations consisting of this organelle are known as microsomal preparations, and the reactions mediated by it as microsomal enzyme reactions.

The structure and nature of the components of the microsomal enzyme system have been studied in some detail, and a number of review articles have appeared (Kamin and Masters, 1968; Gram and Fouts, 1968; Remmer et al., 1968; Gillette et al., 1969).

The metabolism of foreign compounds have been the topic of several books (Kearney and Kaufman, 1969; O'Brien, 1967; Williams, 1959) and review articles (Gillette, 1963, 1965; Smith, 1963, 1968; Williams, 1964).

The metabolism of the carbamate insecticides has been studied in vertebrates, invertebrates and plants, and a number of reactions are involved; oxidation, hydrolysis and conjugation are the most important.

The in vitro metabolism of carbamate insecticides

The study of microsomal metabolism in liver preparations has been of considerable value in elucidating the pathways by which xenobiotic compounds may be eliminated.

Insect microsomal preparations have not been as widely used since reproducibility and enzyme stability have been difficult to obtain. This is caused in part by the release of endogenous inhibitors during homogenation of whole insects (Chakraborty et al., 1967; Krieger and Wilkinson, 1970). The use of microsomal preparations from housefly abdomens gave improved activities (Tsukamoto and Casida, 1967) and high activities and good reproducibility were obtained with homogenates of housefly and blowfly abdomens (Jordan and Smith, 1970). These latter systems have not yet been used for metabolic studies of carbamate insecticides.

Use of the microsomal preparation simplifies the metabolic study since conjugated metabolites can be avoided, and the rates of various reactions may be determined.

The carbamate insecticides have been shown to undergo a number of oxidative reactions and these appear to be more important than hydrolytic reactions in the in vitro systems. The metabolism of carbaryl has been investigated in detail, and the major metabolites from some other carbamate insecticides have been identified.

Epoxidation and hydroxylation of the aromatic nucleus

Initial studies with mammalian microsomal preparations indicated that the primary metabolic attack on carbaryl was oxidative rather than hydrolytic (Dorough et al., 1963). Oxidation was later shown to occur at the 4- and 5- positions to give 4-hydroxy carbaryl and 5-hydroxy carbaryl (Dorough and Casida, 1964). Further studies have shown that 5,6-dihydroxy-5,6-dihydro carbaryl was formed to an extent similar to 4-hydroxy carbaryl. This is apparently formed via an epoxidation on the 5- and 6- positions (Leeling and Casida, 1966), possibly similar to the epoxidation of aldrin to dielrin (Ray, 1967). A similar pattern of metabolites was obtained with fat body preparations from the blowfly larvae (Price and Kuhr, 1969).

Of a number of carbamates recently studied, only one was shown to undergo aromatic ring hydroxylation as a major step. Baygon (2-isopropoxy-phenyl-N-methylcarbamate) was hydroxylated at the 5- position in both rat liver microsomes (Oonithan and Casida, 1968) and housefly enzyme preparations (Shrivistava et al., 1969). The metabolism of carbamate insecticides to yield ring hydroxylated compounds as minor products has not been ruled out.

Oxidation of alkyl substituents on the aromatic nucleus

Oxidation of aromatic side chains takes place in many compounds and occurs widely among the carbamate insecticides.

(a) Methyl groups

Initial studies of Banol metabolism in both rat liver and cockroach (Blaberus giganteus) enzyme preparations showed that no oxidation of the aromatic methyl substituents occurred (Gemrich, 1967).

A number of methyl substituted carbamate insecticides studied by Oonithan and Casida (1968) in rat liver microsomes also showed no methyl group oxidation, these included Banol, Mesurol, Matacil, Zectran, Dimetilan and Isolan. In contrast, 3,4-dimethylphenyl-N-methylcarbamate was oxidised in rat liver microsomes to give a mixture of the 3- and 4-hydroxymethyl compounds (Miyamoto et al., 1969) which in the whole animal were oxidised further to the carboxylic acid. 3,4,5- and 2,3,5-trimethylphenyl-N-methyl-carbamates however were both oxidised in one of the methyl groups when incubated with rat or housefly abdomen enzymes. This reaction proceeded to both hydroxymethyl and carboxylic acid stage in vitro but only to the hydroxymethyl compound in live flies or rats (Slade et al., 1970).

(b) Ethyl groups

Ethyl substituted phenyl-N-methylcarbamates are poor insecticides (Kolbezen et al., 1954) and no metabolic studies have been reported. Ethyl substituents in compounds such as ethyl benzene are readily oxidised, mainly at the 2- carbon to yield methylphenylcarbinol (Smith et al., 1954a, b), and at the 1- carbon atom to yield phenylaceturic acid (El Masri and Smith, 1956).

(c) Isopropyl substituents

Isopropylbenzene is reported to be most easily oxidised at the 2-carbon atom (Chakraborty and Smith, 1967). The same is true of 3-isopropyl-phenyl-N-methylcarbamate (UC.10854) which in mammalian liver preparations is metabolised in major proportion to the carbinol 3-(2-hydroxy-2-methyl-ethyl)phenyl-N-methylcarbamate. A similar metabolite is reported to be formed from 3,5-diisopropylphenyl-N-methylcarbamate (HRS.1422), although there appears to be only minor oxidation of both isopropyl groups (Oonithan and Casida, 1968).

Oxidation of the isopropyl group at the 1- carbon atom has been reported with isopropylbenzene (Chakraborty and Smith, 1967), but has not been found with carbamate insecticides.

(d) tertiaryButyl substituents

Oxidation of tertbutyl substituted phenyl-N-methylcarbamates has not been reported, but Chakraborty and Smith (1967) showed that oxidation of tertbutylbenzene to yield 2,2-dimethylphenylethanol and 2,2-dimethylphenyl acetic acid occurs in rabbit enzyme preparations. The rate of these reactions was about three times slower than equivalent oxidations of iso-propylbenzene.

(e) Cyclic substituents

Furadan, (2,3-dihydro-2,2-dimethyl-7-benzofunanyl-N-methylcarbamate, Nia. 10242) is recorded as undergoing oxidation of the ring structure to give 3-hydroxy Furadan and 3-keto Furadan (Dorough, 1968). This reaction is apparently related to oxidation of the alkyl substituent of 3-isopropyl-phenyl-N-methylcarbamate.
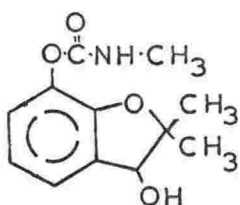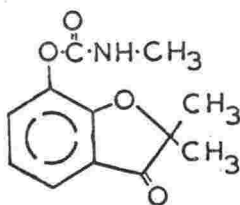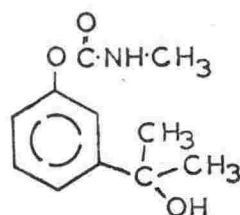
3-hydroxy Furadan   3-keto Furadan   3-(2-hydroxy-2-methylethyl)
                                                       phenyl-N-methylcarbamate

O-Dealkylation reactions

Ether cleavage is a major step in the metabolism of Baygon (2-iso-propoxyphenyl-N-methylcarbamate) in both mammalian (Oonithan and Casida, 1968; Dorough et al., 1963) and insect (Shrivistava et al., 1969) enzyme systems. The substituent is oxidised to yield acetone and 2-hydroxyphenyl-N-methylcarbamate. Oxidation is postulated to occur on the 2- carbon and lead to an unstable intermediate which breaks down to give acetone. No oxidation has been observed at the terminal methyl carbons (Casida et al., 1968).

Metabolism of nitrogen substituents

A variety of this type of reaction is encountered in the metabolism of carbamate insecticides.

(a) Oxidations of carbamoyl nitrogen substituents

Substituents on this nitrogen atom, methyl or dimethyl, are found to be oxidised. N,N-dimethylcarbamates are oxidatively demethylated to yield formaldehyde and N-methylcarbamate. This may then undergo further oxidation (Hodgson and Casida, 1961).

The oxidation of N-methylcarbamate groups could proceed by two routes:

$$R. \quad NH.CH_3 \longrightarrow R.NH.CH_2OH$$

$$R. \quad NH.CH_3 \longrightarrow R.NH_2 \quad + HCHO$$
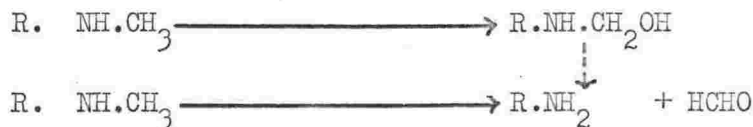
It is uncertain whether this oxidation is catalyzed by two separate enzyme systems or by one oxidation enzyme. The $NH.CH_2OH$, (N-hydroxymethyl) grouping is stable, but is cleaved under strong acid conditions (Balba et al., 1968). All of the carbamate insecticides studied produce the N-hydroxymethyl derivative as a major metabolite both in mammalian and insect enzyme systems.

The carbamate ($- OCONH_2$) is produced from Baygon, but not carbaryl, by rat liver enzyme (Oonithan and Casida, 1968).

Metabolic studies indicate that no N-oxide is formed from N-methylcarbamates (Oonithan and Casida, 1968) a reaction that occurs with phenacitin (4-phenetidine) and a number of other drugs (Uehleke, 1969).

(b) Oxidation of amino and methylamino aryl substituents

These groups are present in Matacil and Zectran, and are metabolised in a manner similar to the carbamoyl N-methyl substituent. Both compounds are metabolised to yield the 4-amino derivative, but the major metabolite in both cases from rat liver enzyme incubates is the 4-methylamino compound, with the concurrent formation of formaldehyde.

A metabolite of Zectran not found with Matacil is the 4-methyl-formamido compound, $(Ar-NH.CH_2OH)$, again similar to the oxidation of the N-methyl carbamate side chain (Oonithan and Casida, 1968).

(c) Metabolism of pyrazole nitrogen substituents

The pyrazole nitrogen in Isolan and Dimetilan appears to be stable to oxidation, alkyl substituted N atoms are also not oxidised (Oonithan and Casida, 1968).

Thioether oxidation

Mesurol is reported to be oxidised to both the sulphone and the
sulphoxide, however complete cleavage to the sulphate was not found
(Oonithan and Casida, 1968). Temik, 2-methyl-2-(methylthio)proprion-
aldehyde-O-(methylcarbamoyl)-oxime is also oxidised to sulphone and
sulphoxide (Metcalf et al., 1966a).

The hydrolysis of carbamate insecticides

Esterases promoting hydrolysis of carbaryl and Baygon have been obtained in the 25,000 g supernatant of mouse brain (Sakai and Matsumura, 1968), and homogenised cockroach tissue has been found to hydrolyse carbaryl (Matsumura and Sakai, 1968). In both of these studies a comparison with esterases hydrolysing simple esters and organophosphorus insecticides was made on agar gel electrophoresis.

Plasma from a number of mammalian species has been assayed for esterase activity towards carbaryl, mice plasma was found to be the least active, rabbit and swine plasma were the most active (Casida and Augustinsson, 1959).

Studies of the oxidative metabolism in mammals of carbaryl (Leeling and Casida, 1966) and several other carbamates (Oonithan and Casida, 1968) show hydrolysis by microsomal preparations in all cases to account for about ten percent of the total metabolism. However, using an incubation period of four hours, up to 80 percent of the total radioactivity was insoluble in ether, and probably represents conjugated metabolites which were not identified. Cofactor requirement experiments showed $NADPH_2$ to be required for oxidation, and resulted in an increase in the aqueous, unextractible metabolites, but on the basis of quantities of ether soluble metabolites it was concluded that $NADPH_2$ or $NADH_2$ were not required for hydrolysis.

Conjugation reactions

The conjugation of carbaryl metabolites with sulphate and glucuronide has been investigated *in vitro*, and glucuronidation found to take place primarily in the microsomal fraction of rat liver homogenates. Sulphate conjugation was shown to occur mainly with the 100,000 g supernatant fraction (Leeling and Casida, 1966), and glucuronide conjugation was found to be of greater significance in the system used. The results obtained from *in vivo* studies indicate the presence of at least 15 conjugate metabolites (Knaak *et al.*, 1965), but these results are not fully reflected by the *in vitro* experiments.

The in vivo metabolism of the carbamate insecticides

(1) Mammalian metabolism

Much of the mammalian metabolism data deals with the rate of excretion of the test compound. Urine, faeces and various tissue samples have been counted for radioactivity at intervals after administration of the dose.

Krishna and Casida (1966) studied the rate of ten radiolabelled carbamates in rats. All ten carbamates with the $[^{14}C]$-carbonyl label gave $^{14}CO_2$ which was almost wholly expired, but between two and ten percent remained in the body tissues for up to 48 hours after dosing. A greater proportion of $[^{14}C]$ label from N-methyl labelled carbamate was retained in the body. The results indicate that hydrolysis is the major degradative pathway; for N,N-dimethylcarbamates however, the reaction was slower than for N-methylcarbamates. Significant retention of radiocarbon in tissues has been ascribed to 'transcarbamoylation' of protein.

The metabolic fate of a few carbamate insecticides has been investigated, and is outlined below.

Carbaryl (1-naphthyl-N-methylcarbamate)

This is the most studied of the carbamates, and with goats, half of the administered dose was found in the urine and a trace in the milk

after 96 hours, (Dorough and Casida, 1964). Tentative identification of
the major urinary metabolites from rat and guinea pig showed the presence
of glucuronide and sulphate conjugates of 1-naphthol; 1,4-dihydroxy-
naphthalene; 1,5-dihydroxynaphthalene and 4-hydroxy carbaryl, and the
N-glucuronide of carbaryl (Knaak et al., 1965). The same study showed
47 to 57 percent to be excreted with an intact carbamate ester, and 39 to
44 percent to be excreted as 1-naphthol or its conjugates.

The N-methylcarbamate methyl group was found to be oxidised to
N-hydroxymethyl by rabbits, also found in the rabbit urine was 5,6-dihydro-
5,6-dihydroxynaphthol and 5,6-dihydro-5,6-dihydroxy carbaryl (Hassan et al.,
1966; Leeling and Casida, 1966). A similar pattern of metabolites was
found in bovine milk and urine (Dorough, 1967).

A comparative study with sheep, pig, monkey and man showed that
1-naphthylmethylimidocarbonate-O-glucuronide and 4-(methylcarbamoyloxy)-
1-naphthyl glucuronide were the major porcine urinary metabolites. The
sheep excreted in addition 4-(methylcarbamoyloxy)-1-naphthyl sulphate and
1-naphthyl glucuronide and sulphate. Monkey and man were shown to excrete
all of these metabolites (Knaak et al., 1968).

3,4-Dimethylphenyl-N-methylcarbamate

This compound was eliminated from rats almost entirely 48 hours
after oral dosing; 91.5 percent of the radiocarbon was found in urine and

5 percent in faeces (Miyamoto et al., 1969). With the $4-[^{14}C]-CH_3$ carbamate, 30 percent of the urine radiocarbon was 3-methyl-4-carboxy-phenyl-N-methylcarbamate, 10 percent was 2-methyl-4-hydroxybenzoic acid and 5 percent was 3-methyl-4-carboxyphenyl-N-hydroxymethylcarbamate.

The remaining urine radiolabel, about 40 percent of the administered dose, yielded four main groups of metabolites on DEAE cellulose chromato-graphy; one of these, a major metabolite was found to be the glucuronide of 4-methyl-3-hydroxymethylphenyl-N-methylcarbamate. Some N-hydroxymethyl compound was found as glucuronide, but no N- or O- glucuronide of the original carbamate was detected.

Banol (6-chloro-3,4-dimethylphenyl-N-methylcarbamate)

A dose of 5 mg/kg given orally to rats was eliminated completely by the kidneys within 48 hours. Banol (N-methyl-$[^{14}C]$) was excreted mainly as acidic compounds, about 35 percent as uronic acid conjugates. Some ten percent of the dose was excreted as oxidised metabolites, but hydrolysis of the carbamate ester was the major pathway of degradation (Baron and Doherty, 1967).

Zectran (4-dimethylamino-3,5-dimethylphenyl-N-methylcarbamate)

When administered orally to Beagle dogs, this carbamate was primarily eliminated in the urine (75%) and some radiolabel was obtained in the

faeces (25%). The urine contained 8.4 percent of ether extractible metabolites, two percent being Zectran phenol the remainder unidentified. The urine aqueous fraction 86 percent was identified as 4-dimethylamino-3,5-dimethylphenol and six percent as 2,6-dimethylhydroquinone (by exchange of the amino group for hydroxyl) conjugated as glucuronide or sulphate. Unchanged Zectran was not detected in the urine, and no ring hydroxylated or aryl-N-methyl oxidised metabolites were identified (Williams et al., 1964b).

Temik (2-methyl-2-(methylthio)-proprionaldehyde -O-(methylcarbamoyl)-oxime)

Following oral administration of Temik, rats excrete metabolites in urine (80%) and faeces (4%) within 24 hours. Trace amounts of unchanged carbamate were found in the urine, and 30 percent of the urinary metabolites were as the sulphoxide. Conjugates of Temik sulphoxide accounted for half of the aqueous soluble metabolites (Knaak and Sullivan, 1968).

Furadan (2,3-dihydro-2,2-dimethyl-7-benzofuranyl-N-methylcarbamate)

This insecticide has been studied in more detail than most others. Rats when orally dosed excreted 45 percent as hydrolysed compounds in 36 hours, 92 percent of ring-$[^{14}C]$ was found in the urine after this time, and four percent of the dose in the faeces.

Ten percent of the urine radioactivity was ether soluble, and found to be 3-hydroxy Furadan phenol and another minor unidentified metabolite. The aqueous fraction of the urine was found to contain conjugates of 3-keto Furadan phenol, (50%); Furadan phenol, (21%); 3-hydroxy Furadan, (15%); and 3-hydroxy-N-hydroxymethyl Furadan, (4%), (Dorough, 1968).

Mice were found to give similar metabolites, the major ones were 3-hydroxy Furadan phenol; 3-keto Furadan phenol and Furadan phenol (Metcalf et al., 1968).

The carbamate insecticides studied are metabolised in mammals primarily by hydrolytic reactions, irrespective of the route of administration. Furadan and carbaryl show appreciable oxidative metabolism, but the oxidation could take place after hydrolysis.

The carbamates are extensively metabolised to conjugated materials, β-glucuronides and sulphates of phenols are found in major proportion.

(2) Insect metabolism

Insects produce a number of oxidative metabolites from carbamate insecticides, and they are similar to the metabolites formed in vitro from mammal liver microsomes or insect microsomes. As a result of the introduction of hydroxyl groups, a variety of conjugated metabolites are also obtained. Conjugation in insects occurring to yield β-glucoside esters (Smith and Turbert, 1964); sulphate or phosphate esters (Binning et al., 1967) or glucoside-6-phosphate esters (Heenan and Smith, 1967).

The metabolism of carbamates has been studied in several insect species, and is similar to mammalian metabolism, although hydrolytic reactions are generally of less importance (Metcalf et al., 1967).

Some examples of carbamate insecticide metabolism by insects are given below.

Carbaryl (1-naphthyl-N-methylcarbamate)

Initial studies of carbaryl metabolism indicated that the critical detoxication step was hydrolysis to 1-naphthol, and when metabolism was inhibited by sesamex, the toxicity towards houseflies was increased (Eldefrawi and Hoskins, 1961).

A number of oxidative metabolites have been identified in insects and are similar to those from mammals (Camp and Arthur, 1967; Andrawes and Dorough, 1967; Ku and Bishop, 1967; Zayed et al., 1966). Similarly, a large proportion of the metabolites were found present as water soluble conjugated compounds.

Baygon (2-isopropoxyphenyl-N-methylcarbamate)

Oxidative processes are primarily responsible for Baygon metabolism in both resistant and susceptible houseflies. The percentage of the metabolites in the ether extract from topically dosed houseflies has been determined. The major metabolites have been identified as 5-hydroxy Baygon; N-hydroxymethyl Baygon and 2-hydroxyphenyl-N-methylcarbamate. The formation

of radioactive acetone from Baygon has been determined in eleven insect species, four hours and 20 hours after injection or topical dosing. The black blowfly (Phormia regina (Meigen)) was most active and the Naval orangeworm (Paramyelois transitella (Walker)) least active in ether cleavage (Shrivistava et al., 1969; Casida et al., 1968).

Genetic studies with houseflies indicate that resistance and high microsomal oxidative activity are conferred by the fifth and third chromosomes, and resistance but not enzyme activity by the second chromosome (Tsukamoto et al., 1968).

Furadan (2,3-dihydro-2,2-dimethyl-7-benzofuranyl-N-methylcarbamate)

Limited studies of Furadan metabolism in houseflies show the major metabolite to be 3-hydroxy Furadan; 3-keto Furadan and 3-hydroxy-N-hydroxy-methyl Furadan were present as minor metabolites.

Temik (2-methyl-2-(methylthio)proprionaldehyde-O-(methylcarbamoyl)-oxime)

In both boll weevils (Anthonomuo grandis (Boheman)) and tobacco budworms (Meliothis viriscens (F.)) oxidation of the sulphur atom was the predominant reaction, yielding primarily Temik sulphoxide and some sulphone. Hydrolytic processes accounted for less than ten percent of the total metabolism after 24 hours (Bull et al., 1967).

A similar pattern of metabolites was obtained from housefly metabolism experiments (Metcalf et al., 1966).

Insect and mammalian in vitro studies show remarkable similarity of metabolites formed from carbamate insecticides. This similarity is not seen in the in vivo situation, insects' major detoxication route appears to be oxidative with some hydrolysis. The oxidative metabolites may be excreted per se or be conjugated. Mammalian studies however, have indicated that oxidative processes are of minor significance and that hydrolysis and conjugation are the primary means of detoxication.

In neither the mammalian nor the insect dosing experiments has any complete identification of metabolites been undertaken, similarly a complete account of radioactive metabolites has not emerged for any carbamate. Studies on the rate of metabolism in the different species are also not reported.

(3) Plant metabolism

Carbamate insecticides are widely used on food crops, and consequently their metabolism in plants has been studied. Some insecticidal carbamates show marked phytotoxicity, and some such as 3-tertbutylphenyl-N-methyl-carbamate and 3-isopropylphenyl-N-methylcarbamate have quite effective systemic action (David et al., 1960).

Metabolism in the plant is by both hydrolytic and oxidative routes (Williams et al., 1964a; Coppedge et al., 1967). A number of carbamate insecticides have been studied in some detail (Kuhr and Casida, 1967) and the oxidised or hydrolysed carbamates have been found conjugated mainly as glycosides which persist in the plant tissues. The pattern of oxidative metabolism is similar to that found in both mammal and insect.

Inhibition of the metabolism of carbamate insecticides

In insects, carbamates are degraded by oxidative pathways. The inhibition of degradation causes an increase in toxicity of such insecticides and this can be accomplished by the simultaneous or prior addition of compounds such as sesoxane and piperonyl butoxide, the 'classic' pyrethin synergists. Moorefield (1958, 1960) first demonstrated that these synergists greatly increased the toxicity of several carbamates towards houseflies and German cockroach. Synergism, or the substantially more than additive toxic action of two substances used together, is of great use to the entomologist for the control of resistant insect strains or increasing the spectrum of activity of an insecticide, for example, the use of carbaryl plus piperonyl butoxide to control the body louse or houseflies.

A number of reports on the mode of action and structure-activity relationships of the carbamate insecticide synergists have been made (Weiden and Moorefield, 1965; Metcalf et al., 1966b; Wilkinson et al., 1966), and the subject recently reviewed by Metcalf (1967).

The primary mode of action of carbamate synergists is through the inhibition of oxidative degradation, but the effect of the inhibitor on each of the oxidation reactions has not been determined.

Selective insecticidal action

Several factors account for selectivity in its broadest sense, such as differences in exposure or behaviour of the fauna, and differences in physiological sensitivity after comparable contact between insecticide and organism. This latter has been defined by Albert (1965) as "the injury of one kind of living matter without harming some other kind with which the first is in intimate contact". The ideal insecticide would thus be highly toxic to the insect pest, but innocuous to other flora and fauna with which it came in contact. Although the 'ideal insecticide' doesn't exist, a number exhibit a high degree of interspecies selectivity, even at comparable tissue concentrations.

Differences in the biochemistry of insects and mammals suggest their possible exploitation in the development of selective insecticides (Winteringham, 1965).

Factors affecting selective toxicity of carbamate insecticides

As the carbamate insecticides are anticholinesterase agents, intoxication will be a function of the rate of arrival of the carbamate at the site of action. This rate will be the result of the competing processes of penetration, detoxication (and in the case of Temik, intoxication), inert storage and excretion.

LD_{50} figures for various insect species give an indication of selectivity, but provide no indication of the mechanisms involved in terms of the above factors.

(1) Penetration

Many carbamates are not selective at the cellular level, and if injected into mammals to achieve the same tissue concentration obtained in insects much 'selectivity' disappears.

In insects, selectivity depends in many cases on two factors, a high surface to volume ratio, and a high accessibility of its central nervous system to lipophilic contact insecticides. Lovell (1963) has shown that there is considerable variation in the injected-contact toxicity ratio from one insect species to another, and from one anticholinesterase to another, which suggests that the effective penetration barriers vary from one species to another and from one insecticide to another.

Winteringham (1965) suggests that only minor differences in cuticle permeability and activity of detoxication enzymes, are needed to account for marked selectivity.

Georgiou and Metcalf (1961) found that the ratio

$$\frac{LD_{50} \text{ resistant strain}}{LD_{50} \text{ susceptible strain}}$$

was greater than 50 for 3-isopropylphenyl-$\underline{N}$-methylcarbamate, but the corresponding ratio of unmetabolised carbamate after 18 hours was about three.

(2) Metabolism of insecticides

It has been demonstrated that the presence of synergists inhibits *in vivo* detoxication and also eliminates much of the great variation in toxicities of the carbamates. Thus, if differential toxicity between insect species and between insect and mammal is eliminated by a suitable synergist, such selectivity is likely to be caused by differences in oxidative metabolism.

Differences in the ease of oxidation of say alkyl substituents of substituted phenyl-$\underline{N}$-methylcarbamates may enhance their selective action (Chakraborty and Smith, 1967).

EXPERIMENTAL

PART II

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from commercial sources, as follows:

Aldrich Chemical Company Inc., U.S.A.

    2-tertbutylphenol (011361) N_D^{20} 1.5232

    3,5-ditertbutylcatechol (090491)   m.p. 95-96°

    3,5-ditertbutyl-4-hydroxybenzoic acid (101481) m.p. 207-209°

Ciba Laboratories Ltd., England

    Metopirone (Metyrapone) 3-methyl-1,2-bis(3-pyridyl)-1-propanone

Fluka A.G. Chemische Fabrik, Switzerland

    2-hydroxybenzylalcohol (54615)   m.p. 86°

    3-hydroxybenzylalcohol (54616)   m.p. 73°

    4-hydroxybenzylalcohol (337568/a)   m.p. 124-5°

K and K Laboratories Inc., U.S.A.

    2-sulphobenzoic anhydride (15486)

Koch-Light Laboratories Ltd., England

    3-tertbutylphenol (19312)

    4-N,N-dimethylaminocinnamaldehyde (25805)

Packard Instrument Co. Inc., U.S.A.

Hydroxide of Hyamine 10-X (1 molar solution in methanol)

P.P.O. (2,5-diphenyloxazole)

dimethyl P.O.P.O.P.(1,4-bis-2-(4-methyl-5-phenyloxazoyl)-benzene)

Rohm and Haas, U.S.A.

Triton X100

Sigma Chemical Co.

2,7-diaminofluorene (37B.2810)

β-NAD from yeast (17B.7750)

NADP from yeast (28B.7710)

β-NADH$_2$

NADPH$_2$ type 1

Glucose-6-phosphate (107B-5260)

Glucose-6-phosphate dehydrogenase

Smith, Kline and French Laboratories, U.S.A.

S.K. & F. 525-A

British Drug Houses Ltd.

This company supplied all other chemicals

Preparation and purification of compounds

(a)   tertButylphenyl-N-methylcarbamates

7.0 g (46.7 mM) of each of the three tertbutylphenol isomers was reacted with 2.85 g (50 mM) of methylisocyanate in sodium dried diethylether in the presence of a catalytic amount of triethylamine (0.05 g, 0.5 mM). The reaction mixtures were allowed to stand in the dark in stoppered vessels for 96 hours, (Kolbezen et al., 1954). The three N-methylcarbamates formed were recrystallised from ether at − 20° and then twice from dry benzene.

The following melting points were obtained −

2-tertbutylphenyl-N-methylcarbamate   95°-96°   lit: 95.5-96.5°

3-tertbutylphenyl-N-methylcarbamate   110°-111°   lit: 110-112°

4-tertbutylphenyl-N-methylcarbamate   143°-145°   lit: 144-145°

(b)   2-Hydroxyphenyl-N-methylcarbamate

1.5 g of catechol (13.6 mM) was reacted with 3.30 g (29.0 mM) of methylisocyanate in anhydrous ether with a catalytic amount (0.5 mM) of triethylamine, at room temperature for 72 hours.

The phenyl-1,2-bis(N-methylcarbamate) precipitated as rod-shaped crystals on standing and were recrystallised from cold ether: melting point 131°. Micro Kjeldahl analysis for nitrogen gave N = 12.2% (required 12.5%).

The carbamate ester was partially hydrolysed by adjusting the pH to 8.0 with sodium hydrogencarbonate solution (1.0 M), and shaking for 10 minutes. The solution was then acidified to pH 1.0 and extracted with ether. The extract was evaporated to dryness and the product recrystallised from benzene and ethylacetate until chromatographically pure: melting point 126°. Micro Kjeldahl analysis for nitrogen gave N, 8.5% (required 8.4%). Chromatography on silica gel G plates with ether:isooctane (7:3 ^{V}/v) as solvent gave R_f's –

2-isopropoxyphenyl-N-methylcarbamate	:	0.35
2-hydroxyphenyl-N-methylcarbamate	:	0.15
Phenyl-1,2-bis(N-methylcarbamate)	:	0.00

(c) 3-tertButylphenylcarbamate

1.54 g (10.3 mM) of 3-tertbutylphenol was dissolved in a toluene solution containing 1.0 g (10.5 mM) of phosgene. 1.3 g (10.3 mM) of N,N-dimethylaniline was added with stirring during 0.5 hours. Stirring was continued for 1.5 hours when the two layers were allowed to separate. The upper (toluene) layer was washed with dilute HCl then water to give a toluene solution of 3-tertbutylphenylchlorofomate (Raiford and Inman, 1934).

This solution was shaken with excess NH_4OH solution (sp.gr. 0.88) and a white crystalline material was precipitated. The precipitate was extracted into a toluene-ether solvent which was then evaporated to dryness

in vacuo. The residual crystals were then recrystallised from dry ether, melting point 167.5 - 168.5°.

Elemental analysis of a sample gave C, 68.22%; H, 7.89%; N, 7.17% (required C, 68.4%; H, 7.78%; N, 7.26%).

(d) 3,5-Ditertbutyl[^{3}H]phenyl-N-methylcarbamate

Tritiation was carried out by the method of Hilton and O'Brien (1964). 100 mCi of THO (100 mC/20 μl) and 180 mg P$_2$O$_5$ were stirred together in a 'snap cap' vial for 10 minutes. BF$_3$ was introduced through the vial cap for 30 minutes by means of a hypodermic needle. BF$_3$ was generated by heating BF$_3$NH$_4$ and 18M-H$_2$SO$_4$ in a flask placed in an oil bath. The BF$_3$ so produced was passed through a trap containing 18M-H$_2$SO$_4$ and thence to the tritiation vessel.

Stirring was continued for one hour, when 200 mg of Butacarb in 0.2 ml of cyclohexane was added through the previously placed hypodermic needle. The needle Luer joint was then sealed with a glass stopper and stirring continued for 36 hours.

The cyclohexane solution was extracted 5 times with small volumes of water and then dried over anhydrous Na$_2$SO$_4$, and evaporated to dryness in vacuo.

Radiochemical purity by dilution analysis was calculated to be 99.50%. Specific activity was 0.1335 mCi/mM.

Lability of tritium label. Treatment of tritium labelled compound with 2M-NaOH for six hours at 60°, followed by acidification, and extraction with ether gave 99.7% recovery of tritium. This indicates that the N-methyl-carbamate side chain was not labelled, and that any ring label was not exchanged in caustic soda.

(e)   3-tertButyl $[^3H]$ phenyl-N-methylcarbamate

This compound was a gift from Dr G. E. R. Hook and was tritiated by the method of Hilton and O'Brien. The sample had a specific activity of 0.89 mCi per mM. (4.3 μCi/mg).

(f)   2-Isoprop(1,3-$[^{14}C]$)oxyphenyl-N-methylcarbamate

$[^{14}C]$-labelled compound was obtained from "The Radiochemical Centre", Amersham, England with a specific activity of 10.2 mCi per mM. (48.8 μC/mg).

Dilution analysis gave the radiochemical purity in excess of 99%.

(g)   Acetone semicarbazone

0.01 moles (0.58 g) of acetone was reacted with 0.01 moles (1.12 g) of semicarbazide HCl in phosphate buffer at pH 7.0. The resultant white crystals were filtered at 0° and crystallised from ethanol three times. The melting point of the white needles of acetone semicarbazone was 190 - 190.5^O.

Animals

Mus musculus (mouse): 5 to 7 week old mice were used, and were obtained
from the National Health Institute, Wellington. They were fed on
'Diet 86', supplied by Westfield Freezing Works Ltd., Auckland.

Apis mellifera (honey bee, Italian strain): Kept in a hive, the worker
bees were netted prior to use.

Costelytra zealandica (grass grub): Larvae were collected in the field by
the Entomology Division, D.S.I.R., Nelson, and kept at 4° prior to
use.

Dermestes maculatus (hide beetle): Retained on a diet of fishmeal, bakers'
yeast, dog biscuits and water before use.

Epiphyas postvittana (light brown apple moth): Reared on a diet of sheep
nuts and lucerne meal.

Graphognathus leucoloma. Bok. (white fringed weevil): Larvae were kept
beneath damp turf, and allowed to feed on carrot.

Lucilia sericata (blowfly): Obtained originally from Wallaceville Animal
Research Centre, Upper Hutt, insects were reared on a diet containing
sucrose, milk powder, brewers' yeast, cholesterol and water. Larvae
were grown on a diet of milk powder, bakers' yeast, agar and water.

Musca domestica. L. (housefly): Strains V, A and Y were obtained originally

    from Dr D. Spiller, Plant Diseases Division, D.S.I.R., Auckland.

    Strain V, (SP2AB), a susceptible strain.

        V_C, V strain pressured with 3-tertbutylphenyl-N-

           methylcarbamate.

        V_D, V strain pressured with DDT.

        V_L, V strain pressured with Lindane (hexachloro-

           cyclohexane).

        A, (SPK.DDT.r) a DDT resistant strain.

        Y, a yellow-eyed mutant strain obtained initially

           from Canberra.

        M, a wild strain collected at Miramar, Wellington.

    Houseflies were reared on the same diet as blowflies.

Periplaneta americana (cockroach): Full grown nymphs were used in experiments,

    insects were reared on 'Tomoana' dog biscuits (supplied by Westfield

    Freezing Works, Auckland) and water.

Sitophilus granarius (grain weevil): Reared on wheat.

Sitophilus oryzae (rice weevil): Reared on wheat.

Tenebrio molitor (yellow mealworm): Reared on a diet of whole wheatmeal,

    wheatgerm and bakers' yeast.

Wiseana sp. (Porina): Larvae were collected in the field by the Entomology

    Division, D.S.I.R., Nelson, and kept at 4° before use.

Preparation of enzymes

1. Mouse liver enzyme

Six week old male mice were killed by cervical dislocation. Livers were removed and placed immediately into ice cold phosphate buffer solution at pH 7.4 (0.1 molar).

The liver tissue was rapidly blotted dry between filter paper discs, weighed and homogenised in a Potter-Elvehjem type homogeniser with a teflon pestle at 0° in nine volumes of phosphate buffer.

The homogenate was centrifuged at 12,000 g for 10 minutes at 0° to 2° to remove mitochondria and cell debris. The supernatant was made up to the required volume to give 1.0 g of wet tissue equivalent per 10 ml of enzyme solution.

2. Mouse liver microsomes

The 12,000 g supernatant from 1 g of mouse liver homogenate prepared as above was centrifuged at 100,000 g for one hour at $0 - 4^{\circ}$ in an 'M.S.E. High Speed 50' centrifuge. The 100,000 g sediment was resuspended in phosphate buffer (pH 7.4, 0.1 M) and used as the enzyme source.

3. Insect enzymes

Enzyme was prepared from housefly (<u>Musca</u> <u>domestica</u>) and blowfly (<u>Lucilia</u> <u>sericata</u>) abdomens.

After carbon dioxide anaesthesia abdomens were carefully removed from the insect and placed into ice cold phosphate buffer (pH 7.4, 0.1 M).

Homogenisation was conducted at 0° with a concentration of not more than three abdomens per ml, in a loose fitting Potter-Elvehjem homogeniser with a teflon pestle. Not more than four downward strokes of the pestle were used to give the homogenate.

The concentration of enzyme was two abdomens per ml of incubate.

<u>Incubation Procedures</u>

1. Mouse liver enzyme

Incubations were carried out in 50 ml Erlenmeyer flasks at pH 7.4 in a shaking water bath at 37°. To ensure adequate oxygenation, the depth of the incubate was kept less than 1 cm. The total volume of incubate was usually 10 ml, the enzyme solution was added to a flask containing cofactors to give the following final concentrations. Nicotinamide 10 mM; Mg^{2+}, 15 mM; NADP 0.1 mM; NAD 0.1 mM; glucose-6-phosphate, 4 mM.

Substrate $(1.0 \mu M/ml; 10^{-3}$ molar) was added in 0.05 ml of acetone to initiate the reaction.

Control reactions were similarly prepared, but substrate was added at the completion of the incubation period, or was incubated in buffer plus cofactors.

2. Mouse liver microsomes

Incubations were conducted as above, microsomes from 1 g wet weight of liver were used in each 10 ml incubation. Microsomes were incubated for 20 minutes at 37° with added cofactors to give final concentration NAD;

$NADH_2$; NADP; $NADPH_2$: 0.1 mM

glucose-6-phosphate : 4.0 mM

3. Insect enzymes

To initiate the reaction, enzyme was added to cofactor and substrate concentrations which were the same as those for mouse liver enzyme, except that nicotinamide and Mg^{++} were omitted from the reaction. Incubations were continued for 0.5 hours at $30-32^{o}$ and terminated by shaking with an equal volume of diethylether.

Extraction of metabolites from enzyme incubates

Incubates were extracted four times at pH 7.4 with equal volumes of ether, or in the case of radiotracer experiments, until no further radio-active material could be extracted. To facilitate extraction of carboxylic acid metabolites, the incubate was acidified (pH 2.0) and extracted a further three times with ether.

The ether layers from each extraction were bulked and reduced to a small volume in vacuo (20 mm Hg, 40°). The residues were then dried over anhydrous sodium sulphate, and the ether layer and washings reduced to dryness under vacuum. The residue was made up to a small known volume with ether, acetone or dioxane. This solution was then used for further investigations.

10-20 μl portions of these solutions were spotted onto silica gel loaded paper (Whatman, S.G. 81); thin layer plates on Whatman No.I on 3 MM paper for high voltage electrophoresis.

Topical dosing of insects

Insects were dosed topically on the dorsal thorax from a calibrated capillary tube with acetone solutions of radioactive carbamates.

3-tertButylphenyl-N-methylcarbamate

Insects received either 0.13 μl or 0.18 μl of a solution containing 6.95 μg per μl (68,000 $\pm$ 4,750 dpm per μl).

Butacarb

Adult houseflies and blowflies, and larval grass grubs received 1.0 μl of a solution containing 0.095 μg (1,050 $\pm$ 168 dpm). Larval houseflies and blowflies received one-tenth of the adult dose.

Extraction of metabolites

Dosed and control insects were killed after the appropriate time interval by carbon dioxide anaesthesia and the addition of ether.

The holding vessel was washed with ether, and the insects and ether rinsings homogenised with acid washed sand in a glass mortar and pestle. Ether rinsing and homogenisation were repeated four times. The ether layers were bulked and reduced to a small volume in vacuo (20 mm Hg, 40°). The residues were then dried over anhydrous sodium sulphate and the ether layer and washings reduced to dryness, again under vacuum. The residue was made up to a small known volume with ether or acetone.

The holding vessel was washed with an acetone-ethanol-water solution (1:1:2 $^v/v$) and this was added to the insect homogenate, thoroughly mixed, and transferred to 50 ml centrifuge tubes for separation (5000 g, 20 mins.). The supernatant was decanted, and the pellet rehomogenised in the above solvent and holding vessel rinsings before centrifugation. The procedure was repeated four times, and the aqueous extracts combined.

Portions of the ether and aqueous fractions, and the pellet from aqueous extraction were counted for radioactivity.

Treatment of aqueous extract from insects

The aqueous fraction was reduced in volume by evaporation _in vacuo_ at 40°, portions were then subject to hydrolysis.

(a) Enzyme hydrolysis

An active hydrolytic enzyme prepared from the viscera of Paua (_Haliotis australis_) as an acetone powder (a gift from Dr A. G. Clark) was used for enzymic hydrolysis of the aqueous extract. This enzyme was incubated at pH 6.0 and 37° for 12 hours with a portion of the aqueous metabolites.

After this time, the incubate was extracted with ether until no more radioactive compounds could be extracted from the aqueous phase. The bulked ether layers were reduced in volume, dried, and again evaporated to dryness. The residue was dissolved in a small volume of acetone.

(b) Acid hydrolysis

The aqueous layer remaining from (a) above, or a portion of the primary aqueous extract was refluxed with 5M-HCl for three hours. This was then extracted with ether until no further radioactive material could be removed.

Dosing of mice

Mice were dosed with 0.1 ml of an aqueous solution of carbamate insecticide in 1.0% (^{V}/v) Triton X-100 emulsifier (1 mg/ml). The dose was administered by stomach tube, or by intraperitoneal injection.

Extraction of metabolites

Urine was collected and monitored every 24 hours for radioactive compounds. The 96 hour bulked urine sample was exhaustively extracted with ether at pH 4.5 and the proportion of ether soluble metabolites determined. A sample of the ether extract chromatographed, and the quantity of metabolites measured by scintillation spectrometry.

The remaining urine sample was incubated at pH 6.0 with Paua viscera acetone powder for 36 hours at 37°. The ether extract of this incubate was monitored for tritium, chromatographed and the chromatograms counted for radioactive metabolites.

Microdiffusion techniques

O-Dealkylation of Baygon was measured by trapping in a suitable medium the radioactivity released from the isoprop$(1,3-[^{14}C])$oxy- side chain.

Enzymic procedures

1 ml of enzyme preparation, from either mouse liver or fly abdomen was added to the outer compartment of a Conway microdiffusion vessel, the substrate and cofactors were added to initiate the reaction. The centre well of the vessel contained the trapping agent. The incubation was conducted at room temperature in most experiments, while being swirled on a microdiffusion vessel shaking table.

At the completion of the required time, the reaction was terminated by the addition of 0.5 ml of 10% $(^{V}/v)$ perchloric acid. The aperture in the lid of the diffusion vessel was then closed, the vessels swirled until diffusion of volatile compounds was completed.

The trapping agent was then carefully removed from the centre well with a dropping pipette and placed into a scintillation counting vial. The centre well was washed four times with 0.5 ml portions of distilled water, the washings were transferred to the counting vial. The aqueous solution of trapping agent was counted for $[^{14}C]$ using the toluene-Triton X-100 scintillant solution.

In vivo procedures

Insects were dosed by injection into the abdomen or into the flight muscle with acetone solutions of $[^{14}C]$ labelled Baygon:

(1) Dosing procedure

(a) Injection

Baygon-$[^{14}C]$ was dissolved in a solution of acetone-phosphate buffer (20% ^{V}/v acetone, 80% ^{V}/v 0.1 M-phosphate buffer pH 7.4) to give a concentration of 0.1 μg per 1 μl (62,300 $\pm$ 790 dpm).

The insects were dosed with 0.1, 0.05 or 0.025 μg of insecticide by injection with a glass needle attached to an 'Agla' syringe held in a 'Burchard' microapplicator. The dose delivered was monitored by injection of insecticide onto filter paper which was then counted for radioactivity. Insects after dosing were transferred to the retaining vessel.

(b) Contact

When the insects to be dosed were very small, (about 2 mg mass) injection of small amounts (0.25 μl) of acetone solution of insecticide (0.025 μg) or acetone control was found to kill the insect. These insects were allowed to

walk on a film of insecticide spread on the surface of the
retaining vessel.   Ten insects were used for each experimental
point.

(2)   Diffusion vessels

The dosed insect was placed into a retaining vessel.

(a)   Conway microdiffusion vessels

The insect was placed into the outer compartment, the
inner chamber contained 0.5 or 1.0 ml of trapping agent.
The insect was prevented from reaching the centre well by
a cylinder of plastic gauze placed on the outer side of
the centre well.   The insect was killed at the termination
of the experiment by cooling the Conway unit for 30 minutes
at $- 10^{\circ}$, or by dropping 1 ml of 10% $(^{v}/v)$ perchloric acid
onto the insect.

The units were then swirled at room temperature or placed
in an incubator for diffusion to take place.   The trapping
agent was removed and counted as before.

(b) Scintillation vials

After dosing, the insect was placed into a 4 cu.cm.
vial inside a scintillation vial containing 1 ml of trapping
agent. A disc of plastic or stainless steel gauze was placed
on top of the vial to prevent the insect escaping. The
scintillation vial was closed and the insect retained for
the required time. The experiment was terminated by opening
the scintillation vial, dropping 0.1 ml of 10% $(^V/v)$ perchloric
acid onto the insect and closing the vial. The volatile
compounds from metabolism were allowed to diffuse into the
trapping agent at elevated temperature (40°).

The 4 cu.cm. vial was removed with forceps and the outside
washed with distilled water into the scintillation vial. The
vial was then filled with scintillant and counted for radio-
activity.

Trapping of volatile radioactive compounds

Various agents were used to trap volatile compounds produced from
Baygon metabolism, and the rates of diffusion from the insect or enzyme
incubate followed using a sample of radioactive acetone bisulphite prepared
from enzyme incubations.

The compounds used as trapping agents were:

(1) Sodium metabisulphite:- 0.15 molar solution in distilled water.

(2) Semicarbazide hydrochloride:- 20 millimolar solution in phosphate buffer pH 7.0.

(3) Sodium hydroxide:- 1.0 molar aqueous solution for $^{14}CO_2$ determination.

(4) Hydroxide of Hyamine 10-X:- 0.1 molar solution in aqueous methanol (0.1 ml of molar methanolic solution + 0.9 ml H_2O). This was used to trap any volatile organic material.

Determination of diffusion efficiency

The outer well of a Conway unit, or the 4 cu.cm. vial in a scintillation vial were charged with a solution of radioactive acetone-bisulphite addition compound. The centre well or scintillation vial contained 1.0 ml of the trapping agent under test. The radioactive solution was acidified with HCl and the vessel closed. The radioactivity in the trapping agent under test was measured at time intervals. The means from six determinations are plotted.

Bisulphite was found to be unsuitable for _in vivo_ experiments as the released SO_2 asphyxiated the insects and caused death within a short time. Semicarbazide HCl was the preferred trapping agent and was used for most purposes.

Efficiency of ^{14}C-acetone entrapment by semicarbazide-HCl solution and NaOH solution

1. Conway unit
2. Scintillation vial
3. Conway unit, NaOH

Time in hours

% ^{14}C-acetone transferred
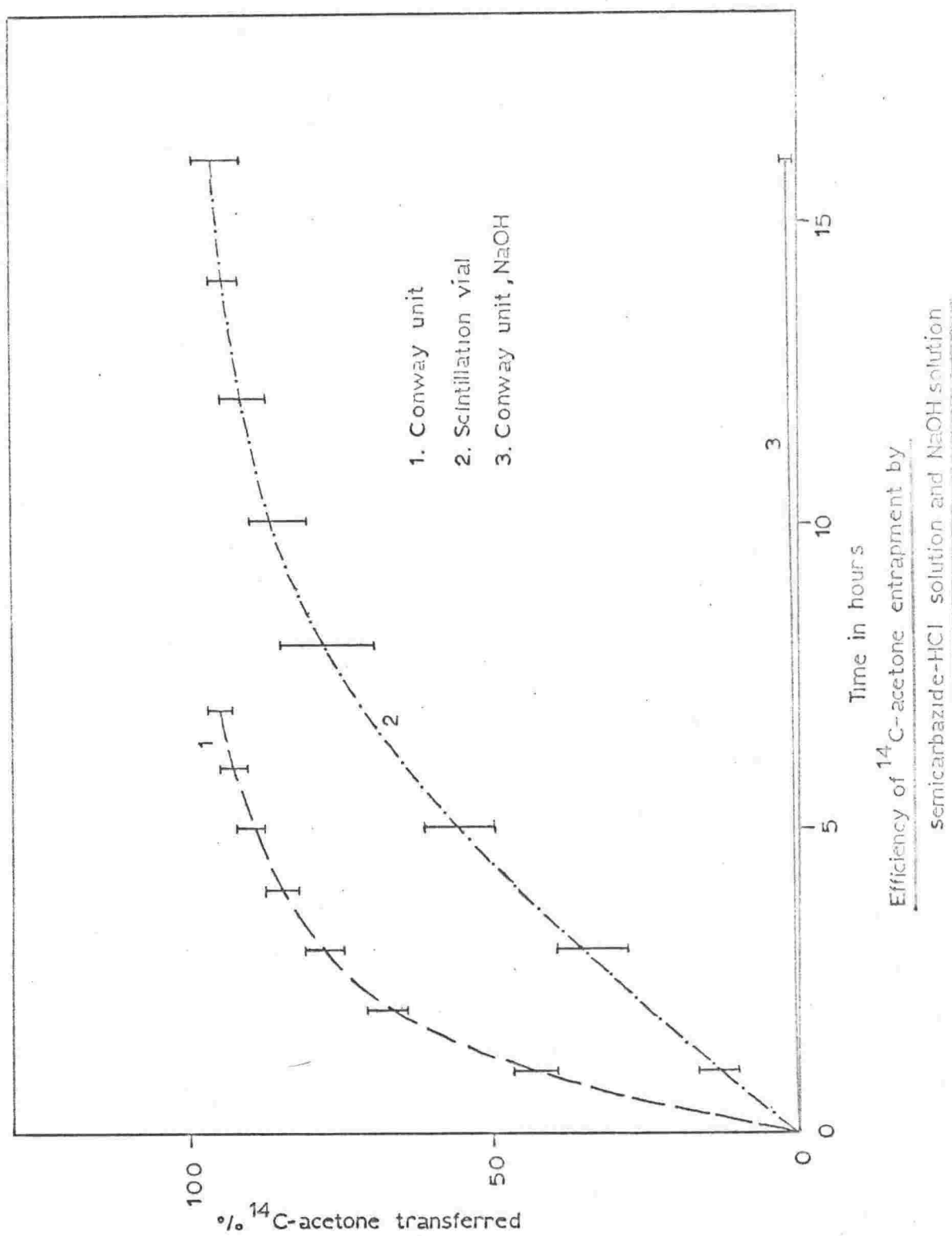
Techniques used in metabolite identification

Chromatography

(a) Thin layer chromatography

Chromatographic plates were prepared from Silica Gel G (Merck A.G., Darmstadt, Germany) with a thickness of wet slurry 0.25 mm. The plates were activated at 120° for 2 hours prior to use. Compounds and metabolite solutions were applied with 'Micro-cap' calibrated capilliaries.

Chromatograms were run for 15 cms in the solvent system.

Solvent systems used

I.	Diethyl ether	
II.	Ether : isooctane	4 : 1 by volume
III.	Ether : isooctane	5 : 1 by volume
IV.	Ether : isooctane	7 : 3 by volume
V.	Chloroform : acetonitrile	4 : 1 by volume
VI.	Benzene : dioxane : acetic acid	15 : 5 : 1 by volume
VII.	Toluene : ethyl fomate : formic acid	5 : 7 : 1 by volume

Two dimensional chromatography was used in the determination of the nature of the metabolites of the phenolic moiety of the various metabolites formed. The chromatogram was run in one direction then the solvent allowed to dry. The origin line was sometimes sprayed with reagent (D) and allowed

to stand for one hour prior to warming the plate at 100° for ten minutes. The plate on cooling was chromatographed in the second direction.

(b) Paper chromatography

(i) Water soluble compounds

Aqueous extracts from insects were chromatographed on Whatman No.4 or Whatman 3MM paper in the upper layer of butanol : acetic acid : water (4 : 1 : 5 ^{V}/v). These were allowed to run for 40 to 45 cms in a descending fashion.

(ii) Apolar compounds

Insufficient separation of metabolites was achieved on TLC for quantitative measurements to be made and an alternative procedure was used.

Metabolite solution was spotted onto a silica gel loaded paper (Whatman S.G. 81), without previous activation. The paper was allowed to develop in a descending manner for 45 cms in solvents I, II or III.

(c) Gas liquid chromatography

This was used to separate the various phenolic metabolites and the best separations were obtained with Carbowax 600 (5% ^{W}/w) columns. The columns were treated with 2.0% ^{V}/w phosphoric acid to help reduce tailing of the peaks (Kolloff et al., 1963).

The Perkin Elmer gas-liquid chromatograph (Model 811) with a flame
ionisation detector was used for the separation. 18 inch columns were
used and the flow rates and pressures were :- N_2, 40 lbs per ins^2.,
120 cu. cm/min through the column 24 cu. cm/min through the detector;
H_2, 15 lbs per ins^2.; Air, 35 lbs per ins^2.

Usually 1 μ l quantities of solvent were injected containing various
amounts of phenolic metabolites or standards.

Electrophoresis

High voltage paper electrophoresis

Whatman 3 MM or No.I paper was cut into strips 15 cm by
45 cms, compounds to be electrophoresed were spotted at the
mid point of the paper and allowed to dry. Buffer was run in
from each end of the paper and blotted dry before being
electrophoresed on the 'Shandon High Voltage Electrophoresis'
apparatus. Running times of up to 30 minutes were used and
at the completion of the run, papers were removed, dried while
in a horizontal plane and compounds detected with spray reagents,
or cut into strips and counted for radioactive compounds.

Electrophoresis at pH 12 to 14 was carried out with two
balancing strips each side of a 1 to 5 cm wide strip on which
compounds had been placed. This was necessary to obtain
sufficient voltage for adequate separation.

Spray reagents used in the detection of metabolites

The following reagents were used on thin layer and paper chromatograms, and electropherograms for visualisation of metabolites.

(A) N-hydroxymethyl compounds were detected by their decomposition to formaldehyde in concentrated H_2SO_4 (6M). Formaldehyde was coupled with chromotropic acid to yield red-purple coloured spots (Beroza, 1963).

0.2% ($^W/v$) Chromotropic acid in $6M-H_2SO_4$ was heated on the TLC plate at 100° to develop colours (chromotropic acid, 4,5-dihydroxy-2,7-naphthalene disulphonic acid).

(B) Phenolic metabolites were detected by the yellow or red colours produced when coupled with diazonium salts.

0.1% ($^W/v$) Aqueous 4-nitrobenzenediazonium fluoroborate (Brentamine fast red GG) couples both ortho and para to a free hydroxyl group, and more slowly to an esterified phenolic group.

(C) Phenolic metabolites were also detected by coupling with Gibb's reagent, 2,6-dichloro-p-benzoquinone-4-chloramine, para substituted compounds did not couple.

0.2% ($^W/v$) Ethanolic solution, oversprayed with 1.0M- sodium hydrogencarbonate gave blue spots on yellow background.

(D) Phenolic esters were hydrolysed on the chromatogram by spraying with ethanolic NaOH (50% ($^V/v$) ethanol; 50% ($^V/v$) 2M-NaOH) and warming at 50° for 15 to 30 minutes. The hydrolysed phenols were then detected by spraying with reagent (B) or (C).

Amino group detection

(E) Ninhydrin reacted with amines and substituted amines on heating to give red-purple spots (2% (^{W}/v) solution in 0.1% (^{V}/v) collidine) (Krishna et al., 1962).

(F) 4-N,N-dimethylaminobenzaldehyde

A saturated solution in glacial acetic acid reacted with primary amines in the cold and secondary amines on heating at 100° to give yellow coloured Schiff's bases (Feigl, 1956, p.300).

(G) 4-N,N-dimethylaminocinamaldehyde

Prepared as for (F) and having a similar reaction, but gave red spots with primary amines and yellow-orange with secondary amines.

Detection of dihydroxy compounds

(H) Phthalimide and H_2SO_4

A saturated solution of phthalimide in $18M-H_2SO_4$ was sprayed on to TLC plates and heated at 100° for 30 minutes. The plate was then made alkaline by overspraying with 10M-NaOH when 1,3-dihydroxy compounds were detected as fluoroscein derivatives (Chapman, 1922).

(I) Formaldehyde and NaOH

Condensation occurred to give coloured products when dihydroxy benzenes and formaldehyde were heated in alkaline conditions (Sen and Sakar, 1925).

40% (^{V}/v) Formaldehyde and 2M-NaOH were sprayed onto the plate and heated at 100° for 30 minutes.

(J) 2-Phthalaldehyde and H_2SO_4

A saturated solution of 2-phthalaldehyde dissolved in 18 M-H_2SO_4 and gave coloured compounds in the presence of dihydroxy phenols (Eegriwe, 1943).

(K) 2-Sulphobenzoic anhydride

2% (^{W}/v) solution in 18 M-H_2SO_4 was used as a spray reagent, the plate was warmed and then made alkaline by addition of 10M-NaOH. 1,3-dihydroxybenzenes yielded fluorescent compounds (Rosenthaler, 1939).

(L) Bials test for pentoses

Orcinol or resorcinol reacted with fructose in HCl and gave yellow-orange colours. Fructose and HCl when sprayed onto the plate gave coloured spots on gentle heating with 1,3-dihydroxy compounds.

(M) Catechol and alkali

Dilute aqueous solution of catechol was sprayed onto the plate followed by 0.5M-NaOH; dihydroxy compounds gave green-brown coloured complexes (Eegriwe, 1943).

(N) Sodium nitroprusside

Sodium nitroprusside in 5% (^{W}/v) aqueous solution was sprayed onto TLC plates, followed by 5M-NaOH. This was then oversprayed with glacial acetic acid to give coloured compounds with 1,3-dihydroxy benzenes and methylene ketones.

Detection of aldehydes (Wasicky and Frehden, 1927)

(O) 2-dianisidine

A saturated solution of 2-dianisidine in glacial acetic acid was sprayed onto the plate and when warmed gave yellow coloured complexes with aldehydes.

(P) 2,7-diaminofluorene

Prepared as above (O), but gave more intense red colours than 2-dianisidine.

Detection of alcoholic functions

(Q) Vanadium oxinate

Sprayed with benzene solution of vanadium oxinate, and warmed to about 70° for ten minutes. A red colour was produced by alcohols. No reaction was found with compounds containing phenol, carboxyl or basic nitrogen.

Vanadium oxinate:- solution of vanadium salt and 8-hydroxyquinoline in 6% ($^{V}/v$) acetic acid was shaken with benzene to give the oxinate solution in benzene (Buscarons et al., 1949).

Detection of carboxylic acids

(R) Rhodamine B and uranylacetate

Benzene solution of Rhodamine B shaken with 1% ($^{W}/v$) aqueous solution of uranyl acetate. When sprayed onto TLC plate and examined under ultra-

violet light, carboxylic acids caused increased fluorescence, whereas other compounds tended to quench (Fiegl, 1956, p.461).

Other tests used during identification

(S) Ammoniacal silver nitrate

Black spots were formed with reducing compounds (Tollens, 1881).

Oxidation of tertbutyl groups

Oxidation of alkyl side chains to the benzoic acid derivative can be brought about by potassium permanganate solutions (Fieser and Fieser, 1967).

This method was utilised in the identification of metabolites of 3-tertbutylphenyl-N-methylcarbamate, a ring hydroxylated metabolite would yield γ-resorcylic acid, a side chain oxidised metabolite, 3-hydroxybenzoic acid.

Oxidation of tertbutylphenols, and orcinol was accomplished by adding 1 ml of 5% ($^{v}/v$) phosphoric acid, 1 ml of 5% ($^{w}/v$) potassium permanganate to an acetone solution of 1 to 10 mg of compound to be oxidised. The reaction mixture was heated at 100° for 10 to 15 minutes. The solution was then decolourised by addition of solid sodium metabisulphite.

This solution was then extracted with ether. The ether solution was reduced in volume under vacuum, and portions applied to TLC plates. These were developed in solvents VI and VII and compared with standards, for R_f and colour reaction.

Mass spectral analysis

Sample preparation

Bulked ether extracted metabolites from a number of incubations using mouse liver enzyme were hydrolysed in 2M-NaOH. The hydrolysate was acidified, and phenolic compounds extracted into ether.

Purification of the phenolic extract was accomplished by preparative TLC, and the band of unknown material eluted. This was further purified by micro scale vacuum distillation and the distillate washed into a clean vial with redistilled ether.

Accurate mass measurements and spectra were run on an 'A.E.I. MS-902b' mass spectrometer by Professor R. Hodges.

Measurement of radioactivity

Radioactivity was determined by scintillation counting, using a 'Packard Tricarb series 4000 Liquid Scintillation Spectrometer' with the following channel settings:

Channel	Discriminator	Gain	Isotope
red	50-500	5%	^{14}C
green	50-1000	56%	^{3}H
blue	300- ∞	1%	A.E.S.

Scintillation fluids

For solid or non-polar compounds, a solution of PPO 5 g; dimethyl POPOP 0.3 g; dissolved in one litre of toluene was used. Aqueous counting was performed in a fluid containing 33% ($^V/v$) Triton X-100 and 66% ($^V/v$) toluene with the same PPO and dimethyl POPOP concentration (Turner, 1968).

Sample preparation

(1) Organic soluble fluids

These materials, usually metabolites dissolved in ether were added to a precounted vial containing 20 ml of scintillant fluid. A portion of the total material to be counted was added accurately by 'Microcap' to the vial.

(2) Aqueous materials

These were added to 19 ml of scintillant in a precounted vial.
Usually 1 ml of sample was added, and the vial shaken vigorously for 15 to
30 seconds to give an emulsion. Samples were shaken again when the tempera-
ture had been reduced to 4° prior to counting.

(3) Paper chromatograms

These were cut into strips 2 cm by 1 cm and placed in precounted
vials containing 2 ml of scintillation fluid.

(4) Thin layer chromatograms

Portions of thin layer of 1 cm^2 or less were scraped from the plate
and transferred to precounting vials containing 2 ml of scintillant fluid.

All counting was performed at less than 5°, the vials were held in
the dark for 3 hours prior to counting to allow phosphorescence to decay.

(5) Protein solutions

These were dissolved in either 98% formic acid, or 'Hydroxide of
Hyamine 10-X'.

(i) Formic acid

Aqueous solutions of protein (1 ml) were added to 1 ml of
98-100% formic acid and heated in the scintillation vial on a
hot plate till the solution became clear. These were then

allowed to cool and were neutralised by addition of NaOH. Scintillant was then added and the vial shaken.

(ii) Hydroxide of Hyamine 10-X

1 ml of aqueous material was dissolved with heating in 1 ml of 1M-methanolic Hydroxide of Hyamine 10-X. The solution on cooling was neutralised with HCl if necessary and scintillant added, the vial was shaken and counted.

Determination of counting efficiency

Quench curves of efficiency versus Automatic External Standardisation (AES) channel reading were constructed for $[^{14}C]$ and $[^{3}H]$. For consistent and accurate efficiency determinations a vial containing at least 15 ml of scintillation fluid was required.

Efficiencies for samples prepared as in (1), (2) and (5) above were determined in this manner.

Efficiency in chromatogram counting

Materials to be chromatographed were counted as above, (1), (2), an equal amount was spotted onto the chromatogram and this then developed.

The total counts on the chromatogram minus background was compared with the total counts obtained from the non-chromatographed sample, the counting efficiency of which was known. These results could then be compared to give overall efficiency of chromatogram counting.

The efficiency of paper chromatogram counting was also checked by elution of metabolites from the chromatogram with ether. These were counted as in (1) above, this when compared with figures obtained from paper chromatogram strips showed counting efficiency of each metabolite to be the same within two percent.

Metabolite extraction efficiency

The efficiency with which metabolites were extracted from _in vitro_ or _in vivo_ systems was determined by counting portions of aqueous and ether fractions in the above manner.

PART III

<u>EXPERIMENTAL</u>

THE METABOLISM OF 3-<u>tert</u>BUTYLPHENYL-<u>N</u>-METHYLCARBAMATE

CHAPTER I

IDENTIFICATION OF METABOLITES

Identification of the metabolites of 3-tertbutylphenyl-N-methylcarbamate

Initial identification

Mouse liver enzyme preparations were incubated with insecticide and cofactors. The ether extracted metabolites were chromatographed on thin layers for a distance of 15 cms.

Visualisation of phenolic compounds with spray reagents (D) and (B) showed that at least eight distinct spots were present.

Alkaline hydrolysis of the metabolites, and TLC of the extracted hydrolysate showed the presence of two phenolic compounds. The parent phenol, 3-tertbutylphenol, and one other.

These phenols and the unhydrolysed metabolites were subjected to a variety of tests in an effort to determine their structure.

To assist in identification, incubations were performed with 3-tertbutylphenylcarbamate and 3-tertbutylphenol as substrates, the metabolites from these compounds were compared with those from 3-tert-butylphenyl-N-methylcarbamate.

Substrate I;   3-tertbutylphenyl-N-methylcarbamate

Substrate II;   3-tertbutylphenylcarbamate

Substrate III;   3-tertbutylphenol

+ indicates a positive reaction.

- indicates no reaction, or a reaction not characteristic.

Chromogenic reagents gave the following results.

Colour reactions given by the metabolites of 3-tertbutylphenol derivatives

Metabolite	Solvent II Rf	Solvent II Hydrolysis Product Rf	Colour reactions with unhydrolysed metabolites												
			A	B	C	D	E	F	G	J	K	L	O	Q	R
Ia	0.06	0.47	+	-	-	+	+	-	-	+	+	+	-	-	-
Ib	0.13	0.46	-	-	-	+	+	+	+	-	-	-	-	-	-
Ic	0.20	0.47	-	-	-	+	+	-	-	-	-	-	-	-	-
Id	0.39	0.80	+	-	-	+	+	-	-	-	-	-	-	-	-
Ie	0.46	0.47	-	+	+	+	-	-	-	+	+	-	-	-	-
If	0.58	0.80	-	-	-	+	+	+	+	-	-	-	-	-	-
Ig	0.69	0.80	-	-	-	+	+	-	-	-	-	-	-	-	-
Ih	0.80	0.80	-	+	+	+	-	-	-	-	-	-	-	-	-
IIa	0.13	0.47	-	-	-	+	+	+	+	-	-	-	-	-	-
IIb	0.46	0.47	-	+	+	+	-	-	-	+	+	-	-	-	-
IIc	0.58	0.80	-	+	-	+	+	+	+	-	-	-	-	-	-
IId	0.80	0.80	-	+	+	+	-	-	-	-	-	-	-	-	-
IIIa	0.46	0.47	-	+	+	+	-	-	-	+	+	-	-	-	-
IIIb	0.80	0.81	-	+	+	+	-	-	-	-	-	-	-	-	-
I	0.68	0.81	-	-	-	+	+	-	-	-	-	-	-	-	-
II	0.57	0.80	-	-	-	+	+	+	+	-	-	-	-	-	-
III	0.80	0.80	-	+	+	+	-	-	-	-	-	-	-	-	-

Metabolites were hydrolysed to give a phenolic compound R_f. 0.47. This will be referred to as compound IV.

Interpretation of colour reactions

(1) Metabolites containing nitrogen

These were compounds which reacted with reagents (E), (F) and (G). Reagent (E) reacted with amines, (F) and (G) with primary amines. Thus metabolites Ie and Ih, IIb and IId, and IIIa and IIIb have no nitrogen in the side chain.

Metabolites Ib, If, IIa, IIc reacting with reagents (F) and (G) contain primary amine groups.

Metabolites Ia and Id, which react with reagent (A) contain an $\underline{N}$-CH$_2$OH group.

(2) The aromatic moiety

Metabolites that react with reagent (B) contain a free phenolic hydroxyl function with an unhindered ortho or para position. With reagent (C), a free para position is essential for coupling and colour development.

Thus, metabolites Ie, If, IIb, IId, IIIa, IIIb and compound III each have a free hydroxyl group, and contain no amino nitrogen.

A positive reaction obtained for metabolites Ia, Ie, IIb and IIIa with reagent (J) and (K) and in the case of Ia with reagent (L), indicates the presence of a 1,3-dihydroxy compound.

Negative reactions from reagents (O), (Q) and (R) indicated that aldehydic, alcoholic or carboxylic acid functions were absent. Reagent (Q) however was not particularly useful in this case as the presence of phenol, carboxyl or basic nitrogen causes non-reaction with alcohols; hydroxybenzyl alcohol isomers gave no reaction.

The nature of compound IV was investigated further as the above colour reactions gave doubtful identification.

Products of metabolite hydrolysis

Two phenolic compounds were detected, one with R_f. 0.80 in solvent II, and one with R_f. 0.47 in the same solvent.

The following metabolites yield identical phenols.

R_f. 0.80, 3-tertbutylphenol

Id, If, Ig, Ih, IIc, IId, IIIb, III.

R_f. 0.47, Compound IV

Ia, Ib, Ic, Ie, IIa, IIb, IIIa.

These two compounds were chromatographed and various spray reagents used in their characterisation.

TLC of 3t BPNMC metabolites
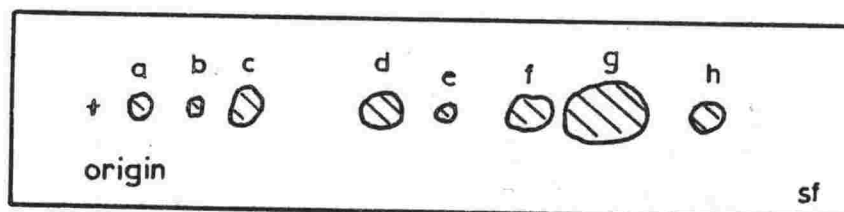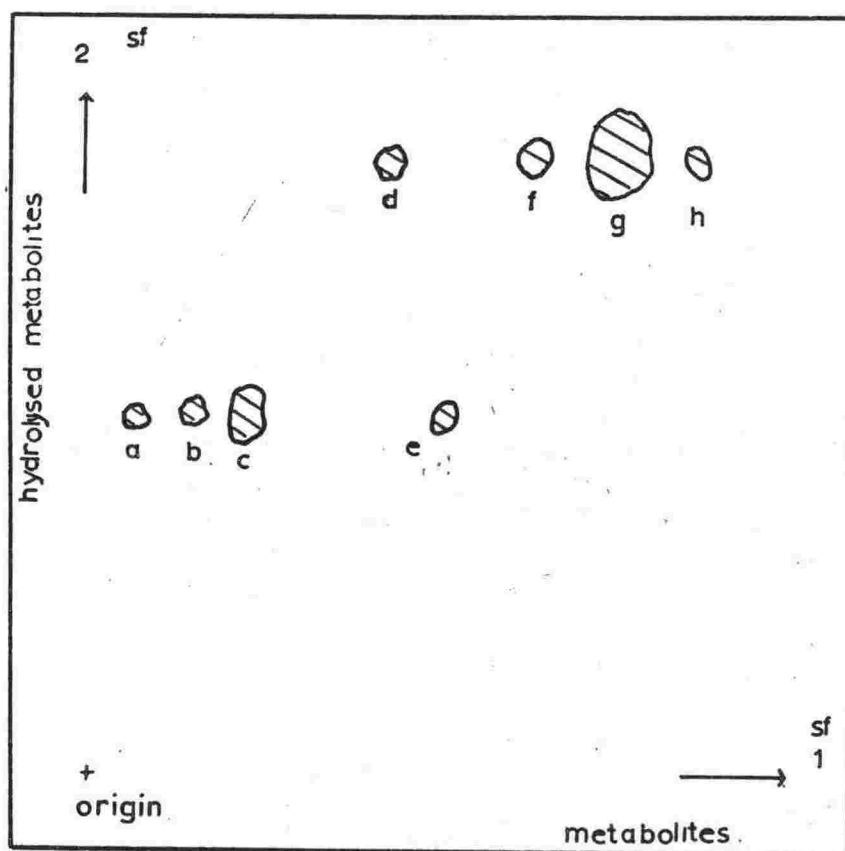
TLC in solvent II

Colour reactions given by hydrolysed metabolites

Compound[1]	R_f	Colour reagent												
		B	C	H	I	J	K	L	M	N	O	P	Q	R
III	0.80	+	+	-	-	-	-	-	-	-	-	-	-	-
IV	0.47	+	+	-	-	-	+	-	-	-	-	-	-	-
V	0.40	+	+	+	+	+	+	+	+	+	-	-	-	-
VI	0.40	+	+	+	+	+	+	+	+	+	-	-	-	-
VII	0.39	+	+	-	+	+	-	-	-	-	-	-	-	-
VIII	0.41	+	-	-	+	+	-	-	-	-	-	-	-	-
IX	0.41	+	+	-	-	-	-	-	-	-	-	-	-	-
X	0.30	+	+	-	-	-	-	-	-	-	-	-	-	-
XI	0.42	+	+	-	-	-	-	-	-	-	+	+	-	-

1.   V   3,5-dihydroxytoluene   (orcinol)

      VI   1,3-dihydroxybenzene   (resorcinol)

      VII   1,2-dihydroxybenzene   (catechol)

      VIII 1,4-dihydroxybenzene   (quinol)

      IX   3-hydroxybenzyl alcohol

      X   3-hydroxybenzoic acid

      XI   3-hydroxybenzaldehyde

      A positive test was obtained from compound IV with reagent (K) acting on 1,3-dihydroxybenzenes, indicating that this compound was possibly 1,3-dihydroxy-5-_tert_butylbenzene, other reagents for this group did not react.

Summary

Metabolite	Aromatic moiety	Carbamate side chain
Ia	Compound IV	$- NH.CH_2OH$
Ib	Compound IV	$- NH_2$
Ic	Compound IV	$- NH.CH_3$
Id	Compound III	$- NH.CH_2OH$
Ie	Compound IV	none
If	Compound III	$- NH_2$
Ig	Compound III	$- NH.CH_3$ (compound I)
Ih	Compound III	none
IIa	Compound IV	$- NH_2$
IIb	Compound IV	none
IIc	Compound III	$- NH_2$ (compound II)
IId	Compound III	none
IIIa	Compound IV	none
IIIb	Compound III	none

High voltage electrophoresis of metabolites

Compound	pH 2.0[a]	pH 7.4[b]	pH 10.0[c]	pH 12.0[d]	pH 13.0[e]	pH 14.0[f]
III	0.0 cm	0.0 cm	6.5 cm	6.6 cm	6.8 cm	6.9 cm
IV	0.0 cm	0.0 cm	6.5 cm	6.5 cm	6.9 cm	7.0 cm
V	0.0 cm	0.0 cm	6.4 cm	6.7 cm	10.8 cm	11.2 cm
VI	0.0 cm	0.0 cm	6.3 cm	6.8 cm	11.1 cm	11.1 cm
IX	0.0 cm	0.0 cm	6.6 cm	6.7 cm	7.1 cm	7.3 cm
X	0.4 cm	6.0 cm	10.6 cm	10.0 cm	10.2 cm	11.7 cm
XI	0.0 cm	0.0 cm	6.4 cm	6.6 cm	6.8 cm	7.0 cm

Compounds were electrophoresed for 15 to 20 minutes at 6000 v.

(a) 0.01M-HCl

(b) 0.1M mixed phosphate

(c) 0.05M-borate-NaOH

(d) 0.01M-NaOH

(e) 0.1M-NaOH

(f) 1.0M-NaOH

Electrophoresis results indicate a monohydroxy compound; the migration was similar to 3-tertbutylphenol, and quite unlike the dihydric compounds tested.

Ultraviolet spectra

Comparison of the unknown metabolite with known compounds was carried out in 0.1M-NaOH and 0.1M-HCl

Results obtained showed no marked differences between the B aromatic absorption band of the spectra of orcinol, 3-tertbutylphenol and 3-hydroxy-benzyl alcohol.

Oxidation of tertbutyl side chains

Results of the oxidation procedure indicated that compound IV was a monohydric phenol.

Oxidation of 3-tertbutylphenol yields 3-hydroxybenzoic acid.

Oxidation of orcinol yields γ-resorcylic acid (3,5-dihydroxybenzoic acid).

Compound[a]	Colour with reagent (B)	Solvent VI R_f	Solvent VII R_f
III	orange-red	0.62	0.71
V	orange	0.46	0.55
VI	red-brown	0.42	-
IX	orange	0.40	0.40
X	red	0.50	0.53
XI	orange	0.56	0.58
Oxidation products			
from III	red	0.48	0.52
from IV	red	0.48	0.53
from V	orange-yellow	0.30	0.33

(a) Designation of compounds, p.80.

Two spots were visible in the oxidation product chromatograms after spraying with Brentamine fast red GG (reagent (B)), one corresponded to unchanged substrate. No intermediate oxidation products were extracted.

From these results compound IV appears to be the side chain alcohol.

Gas liquid chromatography

The metabolites from mouse liver enzyme incubations were gas chromatographed along with standard compounds and gave the following results.

Retention time of various phenols (minutes)

Compound	Temperature		
	85°	125°	140°
tertButylbenzene	0.60	N.D.	N.D.
Benzyl alcohol	0.80	0.10	N.D.
3-Hydroxytoluene	0.90	0.20	N.D.
3-Hydroxybenzyl alcohol	N.D.	2.50	1.70
3-tertButylphenol	1.8	0.90	0.70
Metabolite IV	N.D.	2.70	1.90

Orcinol, resorcinol and catechol were not detected using the above column.

Mass spectral analysis

A sample of compound IV was prepared for analysis from the combined ether extractable metabolites of 50 mouse liver incubations. After hydrolysis and preparative TLC, the sample was distilled <u>in vacuo</u> to give a chromatographically pure product. Orcinol was treated in a similar manner and gave a white crystalline sublimate. The sample IV however yielded a clear, colourless oil, at 20 mm Hg pressure and 153°.

$^m/e$ Peaks were obtained at 166, 135, 107, 95, 91, 77 in major proportion. The reaction sequence suggested was
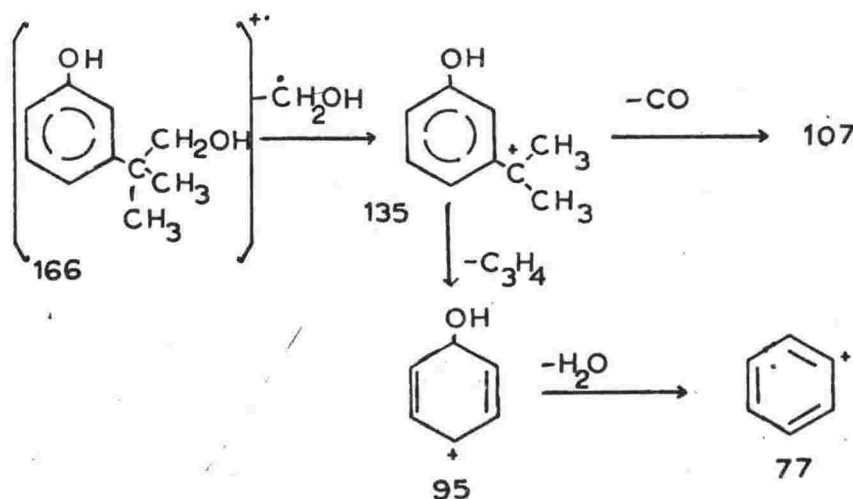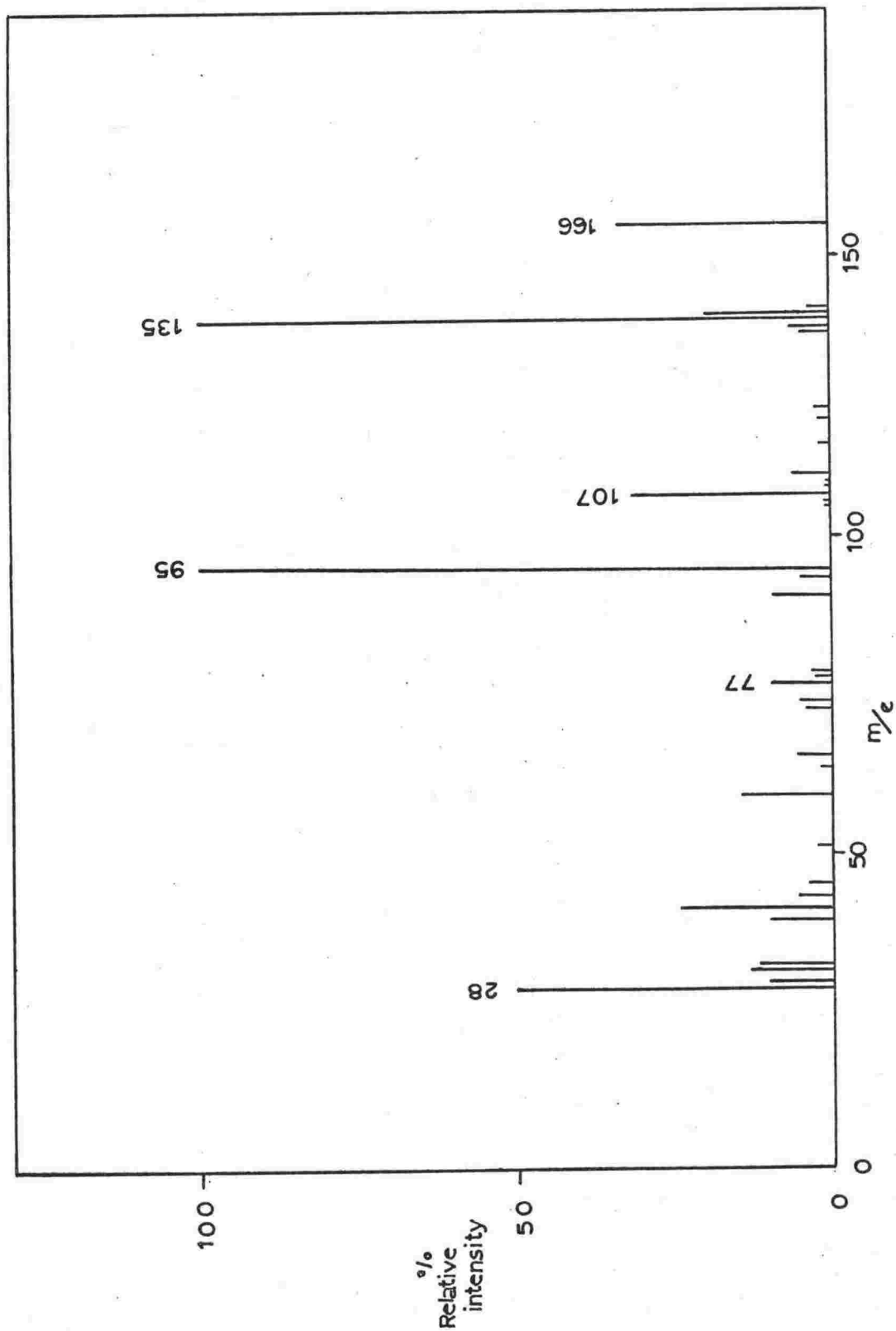
Mass spectrum of phenolic metabolite IV

The composition of the peaks was established by accurate mass measurements, and the pathways shown by metastable peaks.

The loss of $\cdot CH_2OH$ from $^m/e$ 166 would not be expected with 3,5-dihydroxy<u>tert</u>butylbenzene.

Thus compound IV appears to be 3-(1-hydroxy<u>tert</u>butyl)phenol.

Metabolite designation

The metabolites from mouse liver enzymes have been tentatively characterised as:

Ia :- 3-(1-hydroxy<u>tert</u>butyl)phenyl-<u>N</u>-hydroxymethylcarbamate

Ib :- 3-(1-hydroxy<u>tert</u>butyl)phenylcarbamate

Ic :- 3-(1-hydroxy<u>tert</u>butyl)phenyl-<u>N</u>-methylcarbamate

Id :- 3-<u>tert</u>butylphenyl-<u>N</u>-hydroxymethylcarbamate

Ie :- 3-(1-hydroxy<u>tert</u>butyl)phenol

If :- 3-<u>tert</u>butylphenylcarbamate

Ig :- 3-<u>tert</u>butylphenyl-<u>N</u>-methylcarbamate

Ih :- 3-<u>tert</u>butylphenol

IIa is the same compound as Ib.

IIb and IIIa are the same compounds as Ie and IV respectively.

Metabolites formed in other species

The species listed below gave the same metabolites as mouse liver enzyme preparations when incubated or dosed topically with 3-tertbutyl phenyl-N-methylcarbamate. Cochromatography, similarity of colour reactions and electrophoresis of hydrolysed metabolites were used to establish their identity.

The species used were:

Enzyme incubations -

Mus musculus, liver enzyme

Musca domestica, abdomen enzyme (seven strains)

Lucilia sericata, abdomen enzyme

Topical dosing -

Musca domestica (seven strains)

Lucilia sericata

Costelytra zealandica (larvae)(grass grub)

Periplaneta americana

Apis mellifera

Wiseana species (porina caterpillar)

Graphognathus leucoloma, Bok. (white fringed weevil)

Tenebrio molitor

The metabolism of 1-tertbutylphenyl-N-methylcarbamate and 2-tertbutylphenyl-N-methylcarbamate

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The metabolism of these two carbamates was studied in comparison with 3-tertbutylphenyl-N-methylcarbamate. Identification of metabolites was not attempted in detail.

The metabolites of the tertbutylphenyl-N-methylcarbamates were compared on TLC in solvent system II.

Metabolite	isomer		
	meta R_f	ortho R_f	para R_f
a	0.06	0.05	0.07
b	0.13	0.14	0.13
c	0.20	0.22	0.18
d	0.39	0.37	0.36
e	0.47	0.46	0.48
f	0.56	0.56	0.58
g	0.69	0.67	0.68
h	0.80	0.78	0.81

Metabolites were tentatively identified by means of spray reagents as used for the meta isomer, however in the case of the ortho isomer, metabolites a, b, c and e appear to be mixtures of at least two compounds. Hydrolysis and further TLC yield the parent phenol and a spot at R_f. 0.46 with a tailing streak. When treated with 2M-NaOH, this yielded a green-black colour, indicative of an ortho or para hydroxylation product.

Neither the meta or para isomers produced this metabolite on enzyme incubation and as the ortho isomer is less hindered in the 4, 5 and 6 positions, ortho or para hydroxylation is more likely to occur.

In other respects the metabolites of all three isomers are similar.

CHAPTER II

THE <u>IN VITRO</u> METABOLISM OF

3-<u>tert</u>BUTYLPHENYL-<u>N</u>-METHYLCARBAMATE

Rate of formation of metabolites of 3-tertbutylphenyl-
N-methylcarbamate

Mouse enzyme was incubated with substrate and cofactors for up to one hour. Incubates were removed at intervals, ether extracted, and radioactive compounds counted. The percentage of each metabolite on a chromatogram was calculated and expressed as μM formed per gram of mouse liver. The mean results of replicate experiments are given in the table below.

It can be seen that the reaction for all metabolites is linear for at least 30 minutes.

Linearity of the mouse enzyme reaction

Time minutes	0	10	20	30	45	60	Rate at 30 minutes μM/g/hr
Metabolite							
a	0.000	0.023	0.039	0.073	0.097	0.113	0.145
b	0.000	0.011	0.022	0.026	0.035	0.056	0.052
c	0.001	0.102	0.194	0.304	0.394	0.417	0.608
d	0.001	0.197	0.415	0.590	0.669	0.685	1.180
e	0.000	0.007	0.016	0.024	0.034	0.039	0.048
f	0.000	0.163	0.341	0.537	0.688	0.769	1.074
h	0.001	0.144	0.207	0.463	0.583	0.740	0.925
TOTAL	0.003	0.647	1.234	2.017	2.500	2.819	4.032

Paper chromatogram of 3tBPNMC metabolites from mouse enzyme and distribution of [³H]
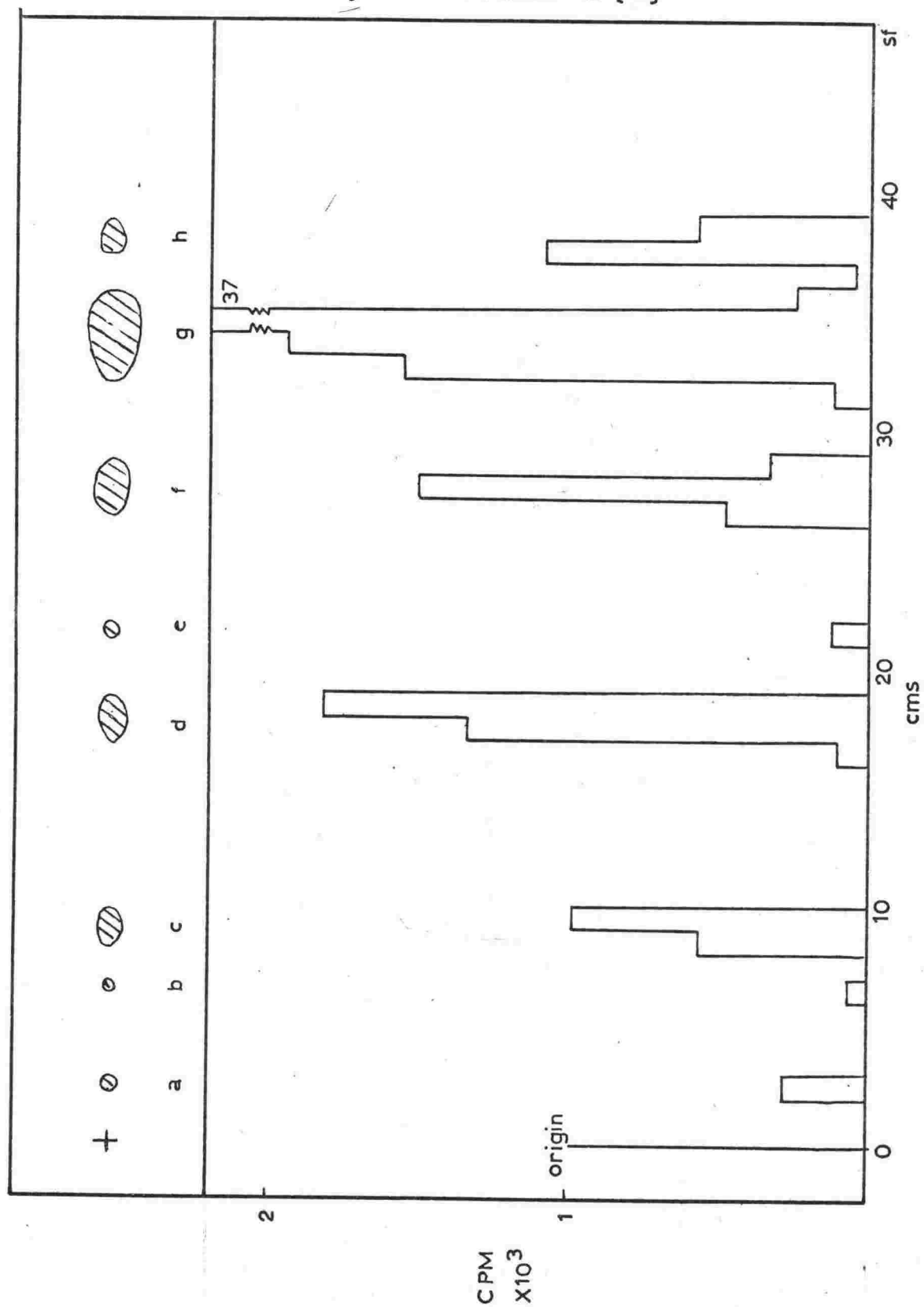
Mouse liver enzyme metabolism

The rates of formation of the various metabolites were determined in the mouse enzyme system with radioactive substrate. The mean results and range of three experiments are given.

Metabolite	Mean rate μM/g liver/hour	% radioactivity on chromatogram
a	0.105	0.54 ± 0.09
b	0.022	0.11 ± 0.06
c	0.616	3.08 ± 0.95
d	1.280	6.41 ± 0.25
e	0.046	0.23 ± 0.07
f	0.932	4.66 ± 1.42
h	0.674	3.37 ± 2.27
TOTAL	3.649	18.40 ± 0.65

Rate of metabolism of 3-tertbutylphenyl-N-methylcarbamate at various pH's
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Mouse liver enzyme was prepared and pH adjusted by addition of buffer. The pH was checked prior to and following incubation.

Phosphate buffer (0.1 M) was used for the experiment. A change in pH of up to 0.1 pH unit was noted during the course of the incubation. Mean results of replicate experiments are given.

The effect of pH on the metabolism of 3-tertbutylphenyl-N-methylcarbamate

pH

Metabolite	6.0		6.5		7.0		7.5		8.0		8.5	
	%[1]	rate[2]	%	rate	%	rate	%	rate	%	rate	%	rate
a	0.23	0.046	0.23	0.046	0.45	0.090	0.38	0.076	0.31	0.062	0.17	0.034
b	0.09	0.018	0.08	0.016	0.05	0.010	0.09	0.018	0.09	0.018	0.08	0.016
c	0.90	0.180	1.81	0.362	2.82	0.565	1.63	0.326	1.46	0.292	1.21	0.242
d	0.69	0.138	1.61	0.322	3.44	0.687	3.84	0.768	3.27	0.654	3.18	0.636
e	0.06	0.012	0.07	0.014	0.09	0.018	0.13	0.026	0.13	0.026	0.12	0.024
f	0.55	0.110	0.75	0.150	1.73	0.346	4.67	0.935	4.15	0.830	2.80	0.560
h	0.23	0.046	0.26	0.052	0.88	0.176	4.59	0.920	3.14	0.626	2.10	0.420
TOTALS	2.750	0.550	4.81	0.961	9.46	1.892	15.33	3.069	12.55	2.508	9.66	1.932

1. % of metabolite on chromatogram.

2. μM/g liver/hour.

Mouse enzyme metabolism of 3tBPNMC.
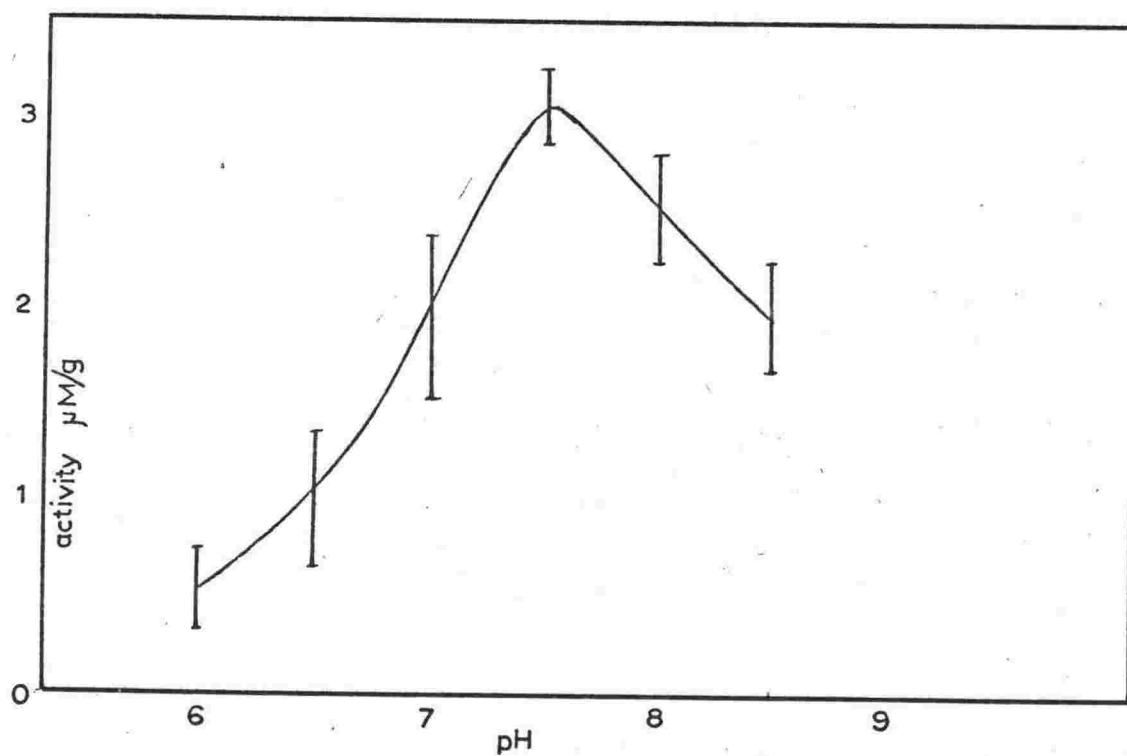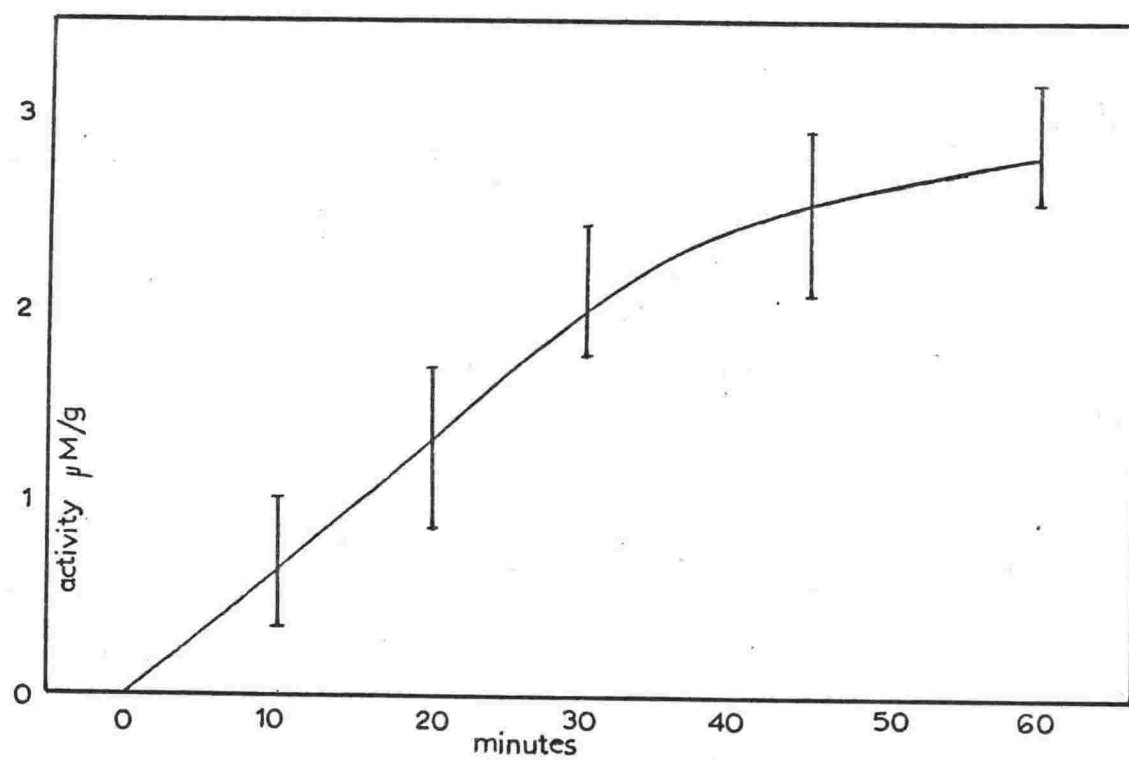
Inhibition of mouse liver enzyme metabolism

Mouse liver enzyme incubations were conducted for 30 minutes with the addition of the inhibitors piperonyl butoxide and Metopirone, in order to determine if the various oxidative pathways were inhibited to the same degree. The results were calculated as μ moles metabolite formed per gram of liver per hour and the percentage inhibition at each inhibitor concentration. The means of replicate experiments are given.

Metabolite	No Inhibitor Rate[1]	10^{-5} M-P.B.O. Rate	% I[2]	10^{-4} M-P.B.O. Rate	% I	10^{-3} M-P.B.O. Rate	% I
a	0.103	0.033	68.0	0.021	79.5	0.014	86.4
b	0.040	0.014	65.0	0.011	72.5	0.005	87.5
c	0.756	0.433	42.7	0.335	65.7	0.191	74.7
d	1.240	0.930	25.0	0.709	42.8	0.488	60.6
e	0.046	0.054	+17.4	0.061	+32.6	0.047	0.0
f	1.045	0.663	36.5	0.455	56.5	0.305	71.0
h	1.339	1.222	8.8	0.988	26.2	1.151	14.0
TOTALS	4.569	3.349	26.7	2.580	43.5	2.201	51.8
Non hydrolytic	3.184	2.073	34.9	1.531	51.9	1.003	68.5

1. μ M/g liver/hour.

2. % inhibition.

Inhibition of metabolism by Metopirone

| | No Inhibitor | Concentration of Metopirone | | | | | |
| | | 10^{-5} M | | 10^{-4}M | | 10^{-3}M | |
Metabolite	Rate[1]	Rate	% I[2]	Rate	% I	Rate	% I
a	0.140	0.067	52.1	0.067	52.1	0.062	55.7
b	0.056	0.038	32.1	0.021	62.5	0.014	75.0
c	0.584	0.234	60.0	0.206	64.7	0.086	85.3
d	1.094	0.852	22.1	0.669	38.8	0.449	59.0
e	0.024	0.022	8.4	0.033	+37.5	0.030	+25.0
f	0.773	0.394	49.0	0.304	60.7	0.214	72.4
h	0.553	0.213	60.2	0.383	31.0	0.140	74.7
TOTALS	3.224	1.820	43.5	1.683	47.8	0.995	69.1
Non hydro-lytic	2.647	1.585	40.2	1.267	52.1	0.825	68.8

1, 2. See previous footnotes.

The in vitro metabolism of 3-tertbutylphenyl-N-methylcarbamate
in Musca domestica strains and Lucilia sericata

Enzymes prepared from fly abdomens were used in these experiments. Substrate concentration was 1.0 millimolar.

Experiments were conducted to determine linearity of reaction with time, reaction rates in houseflies and blowflies, inhibition of metabolism by piperonyl butoxide and Metopirone, and reaction rates in various strains of Musca domestica.

Linearity of reaction

5 ml incubations were initiated by substrate addition, and terminated at the time intervals 0, 10, 20, 30, 45, 60 minutes. The metabolites were extracted, counted and chromatographed, the chromatograms were then also counted for radioactivity.

The following results were obtained from replicate experiments with Musca domestica (V) strain.

Time course of 3-tertbutylphenyl-N-methylcarbamate metabolism in housefly abdomen enzyme

Time (minutes)	0	10	20	30	45	60	Rate at 30 minutes μM/g/hr
Metabolite	I						
a	0.000	0.037	0.049	0.076	0.077	0.096	0.152
b	0.000	0.019	0.030	0.051	0.063	0.124	0.102
c	0.000	0.036	0.070	0.082	0.115	0.130	0.164
d	0.000	0.039	0.083	0.110	0.126	0.135	0.220
e	0.000	0.015	0.022	0.036	0.039	0.044	0.076
f	0.001	0.090	0.119	0.139	0.147	0.155	0.278
h	0.001	0.006	0.008	0.010	0.014	0.025	0.020
TOTALS	0.002	0.242	0.381	0.504	0.581	0.709	1.008

I, Figures are μM/g of flies (mean fly mass 11.5 mg).

Effect of pH on the metabolism of 3-tertbutylphenyl-N-methyl-
carbamate

Houseflies (Musca domestica (V)) were three days old when used
for preparation of abdomen enzyme. In these experiments pH was
adjusted from the 7.0 of homogenisation by addition of buffer. pH
was found to be stable during the course of incubation.

Effect of pH on the metabolism of 3-tertbutylphenyl-N-methylcarbamate in housefly abdomen preparations

pH

Metabolite	6.0		6.5		7.0		7.5		8.0		8.5	
	$\%^1$	rate^2	%	rate	%	rate	%	rate	%	rate	%	rate
a	0.071	0.052	0.076	0.056	0.114	0.034	0.095	0.070	0.057	0.042	0.054	0.040
b	0.021	0.016	0.031	0.023	0.071	0.053	0.058	0.043	0.045	0.033	0.045	0.033
c	0.084	0.062	0.089	0.066	0.145	0.107	0.125	0.093	0.121	0.087	0.101	0.075
d	0.068	0.050	0.103	0.076	0.128	0.095	0.182	0.135	0.134	0.099	0.103	0.076
e	0.004	0.003	0.025	0.019	0.037	0.027	0.027	0.020	0.024	0.018	0.012	0.009
f	0.173	0.128	0.209	0.155	0.344	0.254	0.269	0.199	0.208	0.154	0.163	0.121
h	0.128	0.095	0.237	0.175	0.323	0.239	0.304	0.225	0.157	0.116	0.154	0.114
TOTALS	0.549%	0.406	0.770%	0.570	1.162%	0.859	1.060%	0.785	0.746%	0.551	0.632%	0.468

1. % metabolite on chromatogram (mean of duplicate experiments).

2. μM/g flies/hour (mean mass fly = 13.5 mg).

Housefly enzme metabolism of 3tBPNMC.
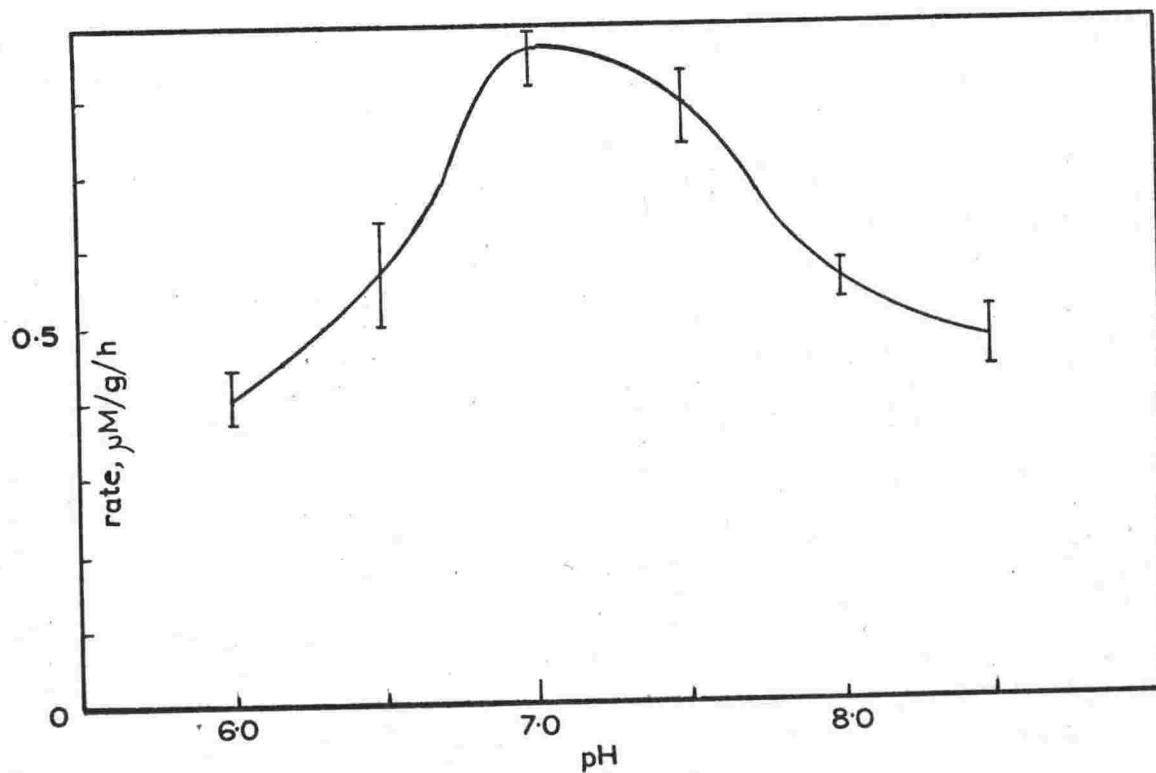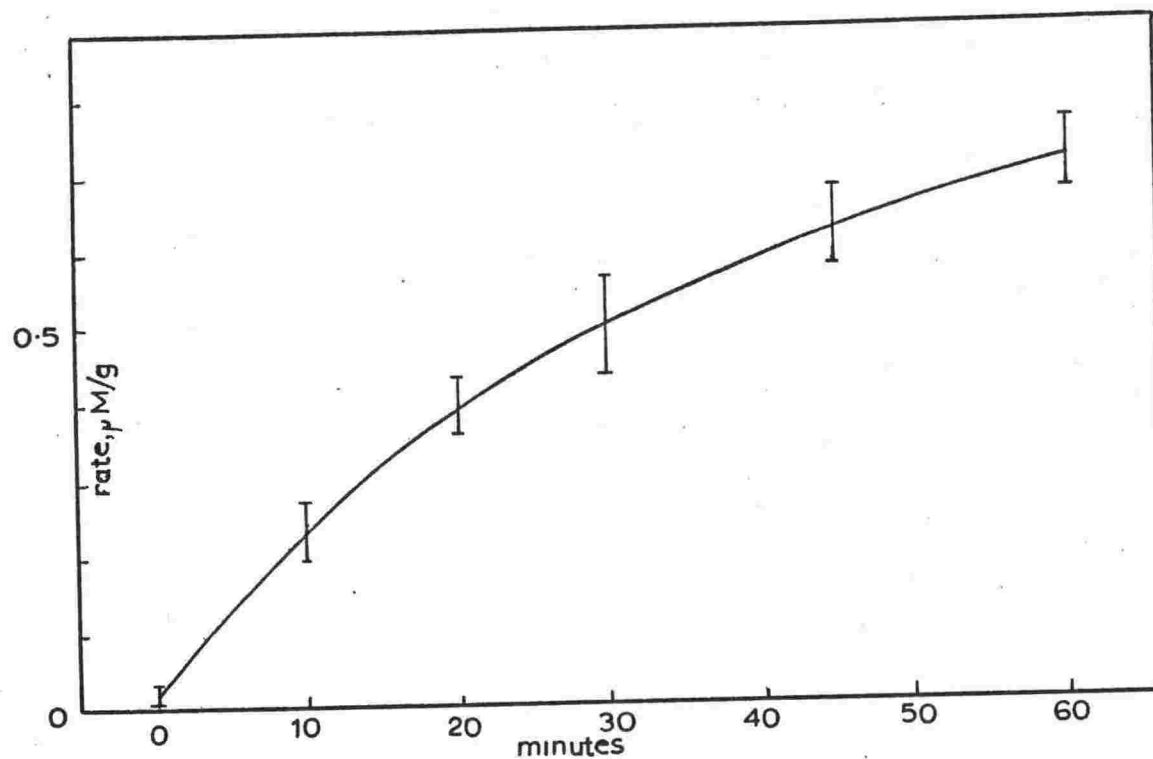
Inhibition experiments

Experiments were conducted to determine whether each oxidation reaction showed the same sensitivity to piperonyl butoxide and Metopirone, and the degree of inhibition if any, at various inhibitor concentrations.

For these experiments M. domestica (V) abdomen enzyme, and L. sericata abdomen enzyme were used.

Metabolites were extracted and radioactivity determined as before.

Equal numbers of male and female insects were used in the enzyme preparation to compensate for any sex differences in metabolism.

Inhibition by piperonyl butoxide of 3-tertbutylphenyl-N-methylcarbamate metabolism in Musca domestica enzyme.

Metabolite	Uninhibited Rate[1]	10^{-5}M-P.B.O.		10^{-4}M-P.B.O.		10^{-3}M-P.B.O.	
		rate	% inhibition[2]	rate	% inhibition	rate	% inhibition
a	0.112	0.016	86.7	0.008	92.8	0.001	99.1
b	0.082	0.024	66.8	0.007	91.5	0.002	97.6
c	0.428	0.338	21.0	0.212	50.5	0.119	72.2
d	0.439	0.376	14.5	0.245	44.2	0.136	69.0
e	0.019	0.017	10.5	0.012	36.8	0.008	57.9
f	0.646	0.519	19.7	0.359	44.5	0.132	79.7
h	0.077	0.092	+ 19.5	0.124	+ 61.0	0.150	+ 94.8
TOTALS	1.803	1.382	23.3	0.967	46.4	0.548	69.6

1. Mean rate of formation of metabolite in μM/g flies/hour (replicate experiments).
2. Mean percentage inhibition (in some hydrolytic reactions, an activation was noted.

Inhibition by Metopirone of 3-tertbutylphenyl-N-methylcarbamate metabolism in Musca domestica enzyme

Metabolite	Uninhibited rate[1]	10^{-5} M-Metopirone rate	% inhibition[2]	10^{-4} M-Metopirone rate	% inhibition	10^{-3} M-Metopirone rate	% inhibition
a	0.535	0.288	46.2	0.081	84.9	0.066	87.6
b	0.085	0.034	60.0	0.018	78.8	0.006	93.0
c	0.451	0.239	47.0	0.068	85.0	0.038	91.5
d	0.406	0.203	50.0	0.075	81.5	0.056	86.2
e	0.060	0.032	46.6	0.016	73.4	0.006	90.0
f	0.651	0.289	55.6	0.070	89.2	0.041	93.7
h	0.349	0.194	44.4	0.125	64.2	0.052	85.1
TOTALS	2.537	1.279	49.6	0.453	82.1	0.265	89.5

1.) See previous footnotes, p.104.
2.)

Inhibition of the metabolism of 3-tertbutylphenyl-N-methylcarbamate by piperonyl butoxide in *Lucilia sericata* enzyme.

Metabolite	Uninhibited rate[1]	10^{-5}M-P.B.O.		10^{-4}M-P.B.O.		10^{-3}M-P.B.O.	
		rate	[2]% inhibition	rate	% inhibition	rate	% inhibition
a	0.048	0.017	64.6	0.008	83.3	0.002	95.8
b	0.030	0.008	73.3	0.001	96.6	0.000	100.0
c	0.168	0.139	17.3	0.083	50.6	0.019	88.7
d	0.085	0.063	25.9	0.042	50.6	0.012	85.8
e	0.016	0.013	18.7	0.015	6.2	0.009	43.7
f	0.170	0.135	20.6	0.088	48.2	0.029	82.9
h	0.125	0.118	5.6	0.130	+ 4.0	0.164	+ 31.2
TOTALS	0.642	0.493	23.2	0.367	42.8	0.235	63.4

1.) See previous footnotes, p.104.
2.)

Inhibition of the metabolism of 3-tertbutylphenyl-N-methylcarbamate by Metopirone in $\underline{Lucilia}$ $\underline{sericata}$ enzyme.

Metabolite	Uninhibited rate[1]	10^{-5}M-Metopirone		10^{-4}M-Metopirone		10^{-3}M-Metopirone	
		rate	% inhibition[2]	rate	% inhibition	rate	% inhibition
a	0.119	0.051	57.1	0.016	86.6	0.006	95.0
b	0.089	0.037	58.4	0.009	89.9	0.001	98.8
c	0.396	0.219	44.7	0.094	76.3	0.051	87.1
d	0.441	0.237	46.2	0.106	75.9	0.027	93.9
e	0.033	0.019	42.4	0.004	87.9	0.004	87.9
f	0.594	0.318	46.5	0.112	81.1	0.042	92.9
h	0.110	0.089	19.1	0.077	30.0	0.017	84.5
TOTALS	1.782	0.970	45.6	0.418	76.5	0.148	91.7

1.) See previous footnotes, p.104.

2.)

[³H] Distribution on a chromatogram, of housefly enzyme 3tBPNMC metabolites.
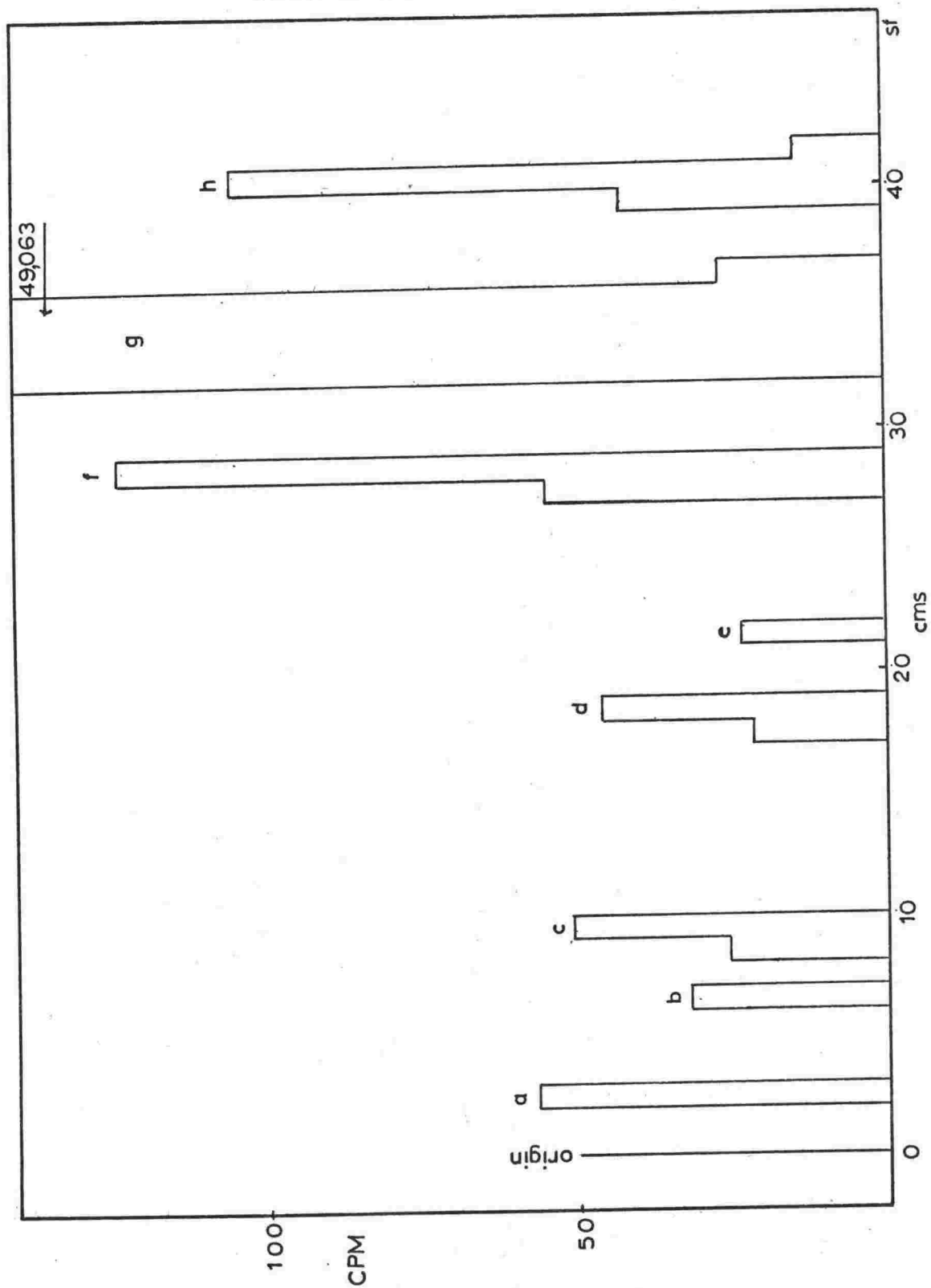
The <u>in vitro</u> rate of formation of metabolites in various
strains of <u>M</u>. <u>domestica</u>

Abdomen enzyme was prepared from six strains of housefly and
rates of enzymic oxidation were compared.

The mean total rates of metabolism were:

Strain	Mean mass of fly	μM/fly/hour	Total metabolism μM/g flies/hour	Non-hydrolytic metabolism μM/g/hour
V	13.5 mg	0.014	1.078	0.852
M	11.0 mg	0.029	2.649	1.819
Y	8.0 mg	0.021	2.666	2.269
V_C	9.0 mg	0.015	1.689	1.348
V_D	12.5 mg	0.011	0.873	0.745
V_L	15.0 mg	0.014	0.937	0.815

Musca domestica strains, in vitro rate of metabolism to each metabolite.

Strain	Metabolite							Total	Total butyl group oxidation	Total carbamate oxidation	% substrate metabolised
	a	b	c	d	e	f	h				
V	0.232	0.133	0.150	0.158	0.040	0.140	0.226	1.079	0.554	0.662	5.39
M	0.573	0.226	0.346	0.357	0.146	0.172	0.830	2.650	1.290	1.327	13.25
V_D	0.085	0.074	0.320	0.150	0.002	0.115	0.127	0.873	0.480	0.423	4.36
Y	0.340	0.123	0.664	0.555	0.074	0.577	0.399	2.732	1.201	1.594	13.40
V_C	0.172	0.112	0.618	0.175	0.059	0.214	0.341	1.691	0.959	0.671	8.50
V_L	0.050	0.057	0.499	0.140	0.025	0.045	0.121	0.937	0.631	0.292	4.69

Metabolites as previously designated. Mean rates of replicate experiments in μM/g flies/hour, after 30 minute incubation.

CHAPTER III

THE <u>IN VIVO</u> METABOLISM OF

3-<u>ter</u>tBUTYLPHENYL-<u>N</u>-METHYLCARBAMATE

The in vivo metabolism of 3-tertbutylphenyl-N-methylcarbamate

Insects were dosed topically and retained for up to 36 hours prior to extraction of metabolites.

Ether extractable metabolites were chromatographed on Whatman 'Chomedia' S.G. 81 paper, and aqueous fractions treated with hydrolytic enzymes and extracted with ether. Acid hydrolysis of the aqueous metabolites was avoided since chromatograms showed that this treatment caused some cleavage of the $N-CH_2OH$ group.

No attempt was made to identify the conjugated metabolites, instead, these metabolites were freed by hydrolysis and total conjugation of each metabolite measured.

In all cases extraction of radioactive material was in excess of 95% of the applied dose, indicating little binding of substrate or metabolites to protein.

The first group of experiments involved holding times of 30 to 36 hours in which time 50 to 98% of the substrate was metabolised, and calculated rates of metabolism are probably low. For the second set of experiments, the withholding time was 12 hours, and about 50% of the substrate remained unmetabolised with a resultant higher rate of metabolism on a per hour basis.

Insects dosed

Table	Species and Strain	Mean mass	Number dosed	Dose received	Withholding time	T°	$[^3H]$ recovery
I	C. zealandica	125 mg	25	1.25 μg	32 hrs	22°	98.8%
II	M. domestica (V) (3 day) adult	10 mg	50	0.90 μg	36 hrs	22°	98.0%
III	M. domestica (V) larvae	20 mg	50	0.90 μg	36 hrs	22°	97.2%
IV	L. sericata (3 day) adult	20 mg	40	0.90 μg	30 hrs	22°	98.6%
V	M. domestica (A) (3 day) adult	16 mg	50	0.90 μg	36 hrs	21°	97.4%
VI	A. mellifera (adult workers)	100 mg	25	0.90 μg	6 hrs (died)	21°	95.3%
VII	M. domestica (V) (3 day) adult	13.5 mg	50	0.90 μg	12 hrs	22°	102%
VIII	M. domestica (A) (3 day) adult	9.5 mg	50	0.90 μg	12 hrs	22°	97.4%
IX	M. domestica (M) (3 day) adult	11.0 mg	50	0.90 μg	12 hrs	22°	97.0%
X	M. domestica (Y) (3 day) adult	8.0 mg	50	0.90 μg	12 hrs	22°	96.8%
XI	M. domestica (V_D) (3 day) adult	12.5 mg	50	0.90 μg	12 hrs	22°	95.5%
XII	M. domestica (V_C) (3 day) adult	9.0 mg	50	0.90 μg	12 hrs	22°	97.7%
XIII	M. domestica (V_L) (3 day) adult	15.0 mg	50	0.90 μg	12 hrs	22°	96.5%

Insects dosed (continued)

Table	Species and Strain	Mean mass	Number dosed	Dose received	Withholding time	T°	$[^3H]$ recovery
XIV	T. molitor adult	70 mg	20	0.90 μg	12 hrs	22°	97.7%
XV	T. molitor pupae	120 mg	20	0.90 μg	12 hrs	22°	96.1%
XVI	T. molitor larvae	100 mg	20	0.90 μg	12 hrs	22°	98.4%

Explanation of Tables.

(i)   Metabolites: Refers to metabolite designation

                                       assigned previously.

(ii)   % R. Ether: The percentage of each metabolite on

                                       a chromatogram of the ether

                                       extractible metabolites.

(iii) % R. Aqueous: The percentage of each metabolite on

                                       a chromatogram of the ether

                                       extractible metabolites from enzyme

                                       treated aqueous soluble metabolite

                                       fraction.

(iv)   % T. Ether   Percentage of each metabolite in (ii)

                                       above corrected to percentage of

                                       total radioactivity.

(v)   % T. Aqueous: (iv) but for % R. Aqueous.

(vi)   Total: The sum of the corrected aqueous and

                                       ether totals, the total percentage

                                       metabolite formed and found in both

                                       fractions.

(vii) % conj.:   The percentage of the total of the

                                       aqueous metabolite conforms to the

                                       percentage of each metabolite that

                                       is in a conjugated state.

(viii) % metabolism: The percentage of each metabolite expressed as in terms of substrate used.

(ix)   Rate/insect: Rate of formation of each metabolite - expressed as nanomoles of substrate used per insect per hour.

(x)   Rate/gram: Rate as above but per gram of insects' tissue.

Histograms of housefly metabolites.

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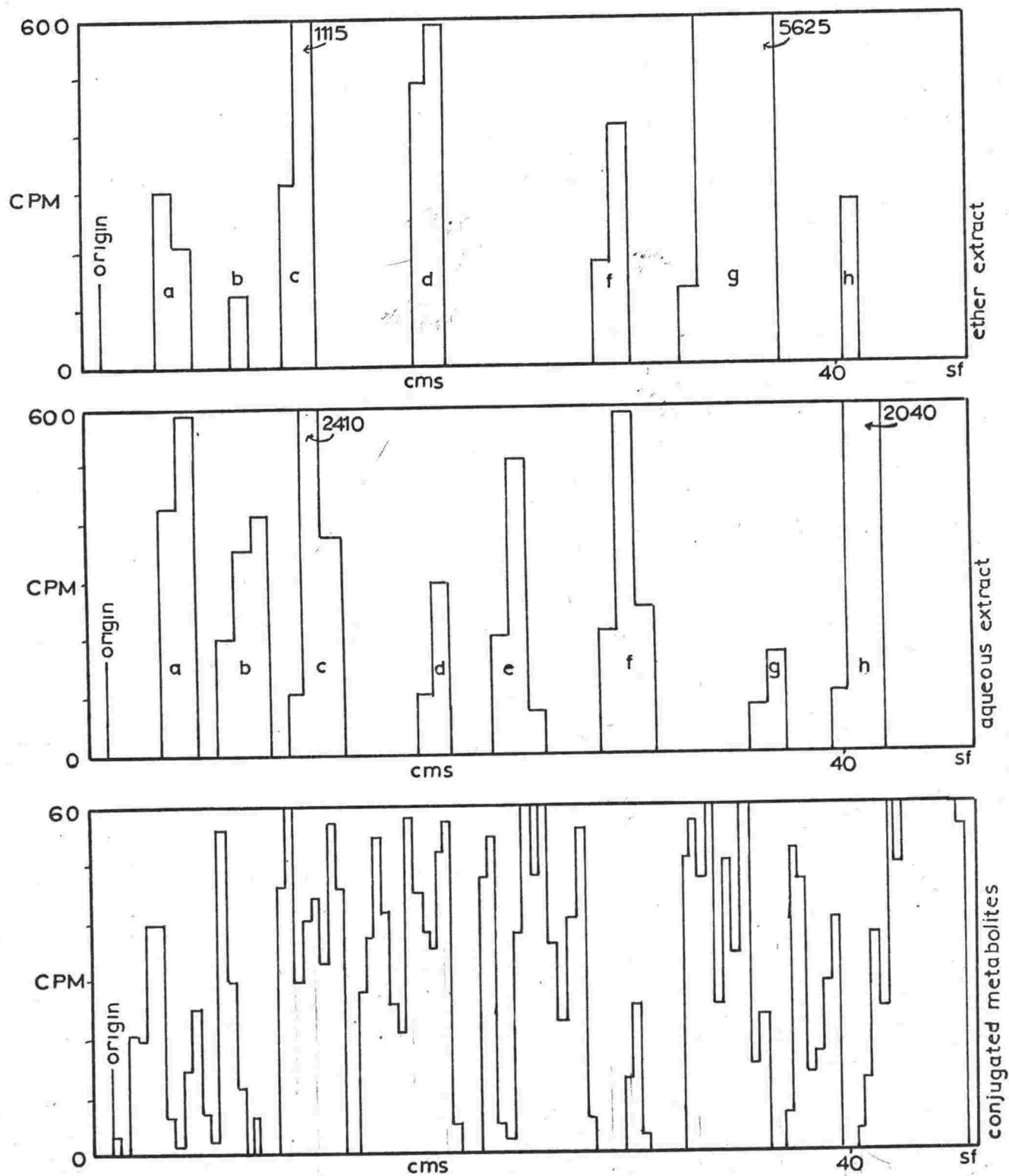
TABLE I

Metabolites	% R.Ether	% R.Aqueous	% T.Ether	% T.Aqueous	Total	% Conj.	% Metabolism	Rate/ Insect	Rate/g
a	15.70	3.71	10.05	1.16	11.21	10.3	21.20	0.0214	0.140
b	0.53	37.50	0.37	11.72	12.09	97.0	23.00	0.0230	0.151
c	0.50	8.00	0.32	2.50	2.82	88.8	5.38	0.0050	0.035
d	0.20	25.60	0.02	8.00	8.10	99.0	15.45	0.0155	0.115
e	0.01	2.46	0.07	0.77	0.85	90.6	1.62	0.0020	0.011
f	4.10	6.40	2.97	2.00	4.80	41.5	9.15	0.0090	0.060
g	63.80	7.70	45.20	2.40	47.58	–	–	–	–
h	15.15	8.80	9.70	2.75	12.45	22.1	23.80	0.0240	0.156
TOTALS	99.99	100.17	68.5	31.30	99.87	–	100.00	0.1000	0.668

TABLE II

Metabolites	% R.Ether	% R. Aqueous	% T.Ether	% T.Aqueous	Total	% Conj.	% Metabolism	Rate/ Insect	Rate/g
a	8.70	9.25	4.25	4.23	8.48	50.00	8.33	0.0099	0.99
b	0.83	1.96	0.83	0.92	1.75	51.60	1.72	0.0021	0.21
c	34.20	52.00	16.72	24.40	41.12	59.40	40.50	0.0483	4.83
d	16.90	8.96	7.83	4.20	12.03	34.90	12.01	0.0144	1.44
e	8.20	5.20	4.01	2.44	6.45	37.80	6.35	0.0075	0.75
f	29.20	19.90	14.30	9.38	23.68	39.60	23.30	0.0278	2.78
g	1.80	1.67	0.88	0.78	1.61	-	-	-	-
h	0.16	0.92	0.08	0.44	0.51	86.40	0.50	0.0006	0.06
TOTALS	99.99	99.86	48.90	46.97	95.70	-	100.00	0.119	11.06

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TABLE III

Metabolites	% R.Ether	% R.Aqueous	% T.Ether	% T.Aqueous	Total	% Conj.	% Metabolism	Rate/ Insect	Rate/g
a	9.96	1.42	9.06	0.13	9.19	14.2	9.64	0.0106	0.530
b	0.00	12.10	-	1.90	1.90	100.0	1.99	0.0020	0.100
c	59.60	28.60	54.30	2.58	56.88	4.5	58.60	0.0658	3.280
d	25.60	2.10	23.30	0.19	23.49	0.8	24.60	0.0276	1.345
e	-	0.45	-	0.04	0.04	100.0	0.04	0.0005	0.023
f	1.38	5.10	1.25	0.46	1.71	27.0	1.80	0.0020	0.099
g	2.26	28.50	2.06	2.56	4.62	-	-	-	-
h	1.15	21.60	1.05	1.95	3.00	65.0	3.15	0.0035	0.017
TOTALS	99.95	99.87	91.00	9.81	99.87	-	99.82	0.1114	5.394

TABLE IV

Metabolites	% R.Ether	% R.Aqueous	% T.Ether	% T.Aqueous	Total	% Conj.	% Metabolism	Rate/ Insect	Rate/g
a	2.75	1.97	1.73	0.78	2.51	31.1	2.57	0.0037	0.166
b	4.68	4.64	2.84	1.83	4.67	39.1	4.77	0.0069	0.347
c	69.00	78.00	41.80	30.80	72.60	42.4	74.50	0.1082	5.410
d	10.48	7.15	6.35	2.82	9.15	30.8	9.40	0.0137	0.685
e	0.99	0.90	0.61	0.36	0.96	37.5	0.98	0.0014	0.070
f	8.11	4.06	4.92	1.70	6.62	25.7	6.78	0.0099	0.463
g	2.42	1.51	1.97	0.51	2.48	-	-	-	-
h	0.50	0.94	0.31	0.37	0.68	54.4	0.70	0.0010	0.051
TOTALS	98.93	99.17	60.60	39.40	99.68	-	100.00	0.1449	7.193

TABLE V

Metabolites	% R.Ether	% R.Aqueous	% T.Ether	% T.Aqueous	Total	% Conj.	% Metabolism	Rate/ Insect	Rate/g
a	15.99	5.50	11.34	1.61	12.95	12.5	14.32	0.0132	0.823
b	0.00	13.20	39.20	3.82	3.82	100.0	4.24	0.0039	0.242
c	55.20	49.80	39.20	14.45	53.65	26.9	59.50	0.0545	3.410
d	9.55	2.58	6.78	0.75	7.53	10.1	8.34	0.0077	0.478
e	3.04	6.14	2.16	1.79	3.95	45.5	4.38	0.0040	0.250
f	2.44	0.00	1.73	–	1.73	0.0	1.91	0.0018	0.110
g	12.60	3.56	8.95	1.03	9.98	–	–	–	–
h	1.17	19.40	0.83	5.62	6.45	87.2	7.15	0.0066	0.410
TOTALS	99.99	100.18	70.99	29.07	100.06	–	99.84	0.0916	5.723

al	% Conj.	% Metabolism	Rate/ Insect	Rate/g
57	30.6	5.20	0.0125	0.125
22	36.8	4.04	0.0097	0.097
19	29.8	20.45	0.0492	0.492
45	16.1	24.60	0.0590	0.590
40	45.8	14.51	0.0348	0.348
47	42.2	11.47	0.0274	0.274
70	-	-	-	-
00	24.0	19.70	0.0472	0.472
99	-	100.00	0.2398	2.398

TABLE VII

Metabolites	% R.Ether	% R.Aqueous	% T.Ether	% T.Aqueous	Total	% Conj.	% Metabolism	Rate/ Insect	Rate/g
a	5.17	10.71	3.88	2.68	6.56	40.8	11.75	0.0239	1.760
b	1.31	9.15	0.98	2.29	3.27	70.0	5.85	0.0118	0.875
c	14.35	28.90	10.76	7.25	18.01	40.3	32.40	0.0656	4.860
d	11.95	4.15	8.97	1.13	10.10	11.2	18.10	0.0368	2.725
e	0.00	7.95	0.00	1.99	1.99	100.0	3.56	0.0073	0.541
f	6.77	10.44	5.08	2.51	7.59	33.1	13.58	0.0276	2.045
g	57.55	3.40	43.15	0.95	44.10	-	-	-	-
h	2.90	21.40	2.17	5.45	7.62	71.6	13.62	0.0277	2.050
TOTALS	100.00	100.06	74.99	24.15	99.24	-	98.86	0.2007	14.856

TABLE VIII

Metabolites	% R.Ether	% R.Aqueous	% T.Ether	% T.Aqueous	Total	% Conj.	% Metabolism	Rate/Insect	Rate/g
a	5.37	11.65	4.16	2.62	6.78	38.7	13.85	0.0247	2.600
b	1.04	6.15	0.85	1.38	2.23	62.0	4.57	0.0081	0.852
c	16.30	31.00	12.62	6.98	19.60	35.7	40.01	0.0714	7.510
d	6.20	4.19	4.80	0.95	5.75	16.5	11.78	0.0210	2.123
e	0.00	6.98	0.00	1.57	1.57	100.0	3.21	0.0057	0.599
f	3.74	11.80	2.90	2.68	5.58	47.9	11.45	0.0203	2.137
g	4.89	4.50	50.25	1.01	51.26	–	–	–	–
h	2.39	23.65	1.83	5.96	7.79	76.6	15.95	0.0284	2.700
TOTALS	99.93	99.92	77.41	22.15	100.56	–	100.82	0.1796	18.521

TABLE IX

Metabolites	% R.Ether	% R.Aqueous	% T.Ether	% T.Aqueous	Total	% Conj.	% Metabolism	Rate/ Insect	Rate/g
a	2.26	9.17	1.69	2.33	4.02	57.8	9.51	0.0146	1.327
b	1.01	6.56	0.75	1.67	2.42	69.0	5.74	0.0088	0.799
c	7.15	28.95	5.33	7.35	12.68	58.0	30.00	0.0462	4.199
d	8.00	3.47	5.96	0.88	6.84	12.0	16.20	0.0249	2.265
e	0.00	8.60	0.00	2.18	2.18	100.0	5.16	0.0079	0.717
f	4.59	7.24	3.42	1.84	5.26	35.0	12.45	0.0192	1.745
g	74.70	8.60	55.60	2.18	57.78	-	-	-	-
h	2.01	27.40	1.50	6.96	8.86	78.5	21.00	0.0322	2.922
TOTALS	99.72	99.99	74.25	25.39	100.04	-	100.06	0.1538	13.974

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TABLE X

Metabolites	% R.Ether	% R.Aqueous	% T.Ether	% T.Aqueous	Total	% Conj.	% Metabolism	Rate/ Insect	Rate/g
a	5.34	13.05	3.94	3.39	7.34	46.2	14.55	0.0267	3.335
b	1.07	6.00	0.79	1.56	2.35	66.5	4.66	0.0086	1.075
c	16.00	34.20	11.83	8.90	20.73	43.0	41.20	0.0755	9.440
d	7.22	2.23	5.34	0.58	5.92	9.7	11.72	0.0215	2.685
e	8.00	3.74	0.00	0.97	0.97	100.0	1.92	0.0035	0.437
f	3.72	13.35	2.75	3.47	6.22	55.8	12.34	0.0226	2.821
g	65.60	3.76	48.50	0.97	49.47	-	-	-	-
h	0.96	23.73	0.71	6.16	6.87	89.6	13.61	0.0250	3.121
TOTALS	99.91	100.06	73.87	26.00	99.87	-	100.00	0.1833	22.914

TABLE XI

Metabolites	% R.Ether	% R.Aqueous	% T.Ether	% T.Aqueous	Total	% Conj.	% Metabolism	Rate/ Insect	Rate/g
a	7.90	17.20	5.65	4.90	10.55	46.4	14.65	0.0382	3.051
b	2.19	2.52	1.57	0.72	2.29	31.4	3.18	0.0084	0.672
c	25.90	39.35	18.50	11.20	29.70	37.7	41.25	0.1083	8.660
d	15.85	2.64	11.32	0.75	12.07	6.2	16.75	0.0440	3.519
e	0.00	14.70	0.00	4.18	4.18	100.0	5.82	0.0152	1.215
f	6.15	3.84	4.40	1.09	5.49	19.9	7.63	0.0200	1.600
g	38.80	7.06	27.80	2.01	29.81	-	-	-	-
h	2.68	12.68	1.92	3.62	5.54	65.4	7.70	0.0202	1.615
TOTALS	99.47	99.99	71.16	28.47	99.63	-	96.98	0.2543	20.332

TABLE XII

Metabolites	% R.Ether	% R.Aqueous	% T.Ether	% T.Aqueous	Total	% Conj.	% Metabolism	Rate/ Insect	Rate/g
a	2.38	10.50	1.71	2.94	4.65	63.2	12.81	0.0169	1.878
b	0.56	4.82	0.43	1.34	1.77	75.8	4.64	0.0065	0.722
c	6.80	28.80	4.90	8.06	12.96	62.3	34.00	0.0472	5.240
d	3.93	9.70	2.83	2.72	5.55	48.9	14.51	0.0220	2.441
e	0.00	12.81	0.00	3.58	3.58	100.0	9.48	0.0130	1.445
f	2.88	8.93	2.07	2.50	4.57	54.6	11.98	0.0091	1.011
g	82.95	7.68	59.70	2.15	61.85	-	-	-	-
h	0.42	16.80	0.30	4.70	5.00	94.0	13.10	0.0182	2.201
TOTALS	99.92	100.04	71.94	27.99	99.93	-	99.89	0.1329	14.938

TABLE XIII

Metabolites	% R.Ether	% R.Aqueous	% T.Ether	% T.Aqueous	Total	% Conj.	% Metabolism	Rate/ Insect	Rate/g
a	8.90	11.05	6.61	2.83	9.44	30.0	17.85	0.0344	2.295
b	0.00	1.79	0.00	0.71	0.71	100.0	1.35	0.0026	0.175
c	18.35	32.40	13.64	8.30	21.94	37.8	41.50	0.0800	5.330
d	5.50	2.89	4.09	0.74	4.83	15.6	9.15	0.0176	1.172
e	0.93	9.00	0.69	2.30	2.99	77.0	5.66	0.0109	0.727
f	3.80	6.76	2.82	1.73	4.55	38.0	8.61	0.0166	1.108
g	60.30	5.02	44.90	1.28	46.18	–	–	–	–
h	2.20	30.40	1.63	7.78	9.41	82.6	17.80	0.0342	2.280
TOTALS	99.98	99.31	74.38	25.67	100.05	–	101.92	0.1963	13.087

TABLE XIV

Metabolites	% R.Ether	% R.Aqueous	% T.Ether	% T.Aqueous	Total	% Conj.	% Metabolism	Rate/ Insect	Rate/g
a	0.85	0.70	0.48	0.30	0.78	39.0	1.63	0.003	0.043
b	0.14	1.17	0.08	0.51	0.59	86.0	1.16	0.002	0.029
c	5.41	1.10	3.08	0.47	3.55	13.0	7.00	0.013	0.185
d	5.76	12.20	3.28	5.26	8.54	61.5	16.80	0.031	0.442
e	–	0.30	0.00	0.13	0.13	100.0	0.26	0.001	0.014
f	0.71	5.93	0.40	2.56	2.96	86.5	5.82	0.011	0.155
g	86.50	0.00	49.20	0.00	49.20	0.0	–	–	–
h	0.46	78.60	0.26	33.90	34.16	99.0	67.20	0.126	1.800
TOTALS	99.91	99.99	56.79	43.13	99.91	–	99.77	0.186	2.711

TABLE XVI

Metabolites	% R.Ether	% R.Aqueous	% T.Ether	% T.Aqueous	Total	% Conj.	% Metabolism	Rate/ Insect	Rate/g
a	1.15	0.58	0.76	0.19	0.95	20.0	1.95	0.004	0.040
b	0.11	0.02	0.07	0.01	0.08	12.5	0.16	0.001	0.010
c	9.50	0.51	6.22	0.17	6.39	2.5	13.10	0.024	0.240
d	10.15	13.10	6.88	4.38	11.26	39.0	23.10	0.042	0.442
e	0.00	0.07	0.00	0.02	0.02	100.0	0.04	0.001	0.010
f	3.36	3.76	2.20	1.26	3.46	36.5	7.10	0.012	0.120
g	73.70	0.00	51.50	0.00	51.50	–	–	–	–
h	2.04	82.00	1.34	27.40	28.74	96.0	59.00	0.105	1.050
TOTALS	100.01	100.00	68.96	33.43	102.40	–	104.40	0.189	1.890

133.

Summary of results

Table	Species	Total Metabolism nM/g/hr	nM/insect/hr	per gram rate of formation of: NH.CH$_2$OH	NH$_2$	tBuOH	N oxidation	Double oxidation	Hydrolysis
I	C.zealandica	0.67	0.10	0.26	0.21	0.34	0.47	0.29	0.17
II	M.domestica (V)	11.06	0.12	2.43	2.99	6.78	5.42	0.12	0.08
III	M.domestica (larvae)(V)	5.39	0.11	1.88	0.20	3.93	2.07	0.63	0.04
IV	L.sericata	7.19	0.15	0.85	0.80	5.99	1.66	0.52	0.12
V	M.domestica (A)	5.72	0.09	1.30	0.35	4.73	1.65	1.07	0.66
VI	A.mellifera	2.40	0.24	0.72	0.37	1.06	1.09	0.22	0.82
VII	M.domestica (V)	14.86	0.20	4.49	2.92	8.04	7.41	2.64	2.59
VIII	M.domestica (A)	18.52	0.18	4.72	2.99	11.56	7.71	3.45	3.30
IX	M.domestica (M)	13.97	0.15	3.95	2.54	7.04	6.14	2.13	3.64
X	M.domestica (Y)	22.91	0.18	6.02	3.90	14.29	9.92	4.41	3.56
XI	M.domestica (V$_D$)	20.33	0.25	6.57	2.27	13.60	8.84	3.72	2.83
XII	M.domestica (V$_C$)	14.94	0.13	4.32	1.73	9.29	6.05	2.60	3.65
XIII	M.domestica (V$_L$)	13.09	0.20	3.47	1.28	8.53	4.75	2.47	3.00
XIV	T.molitor(adult)	2.71	0.18	0.48	0.18	0.27	0.66	0.08	1.81
XV	T.molitor(pupa)	0.84	0.10	0.09	0.06	0.06	0.16	0.02	0.63
XVI	T.molitor(larvae)	1.89	0.19	0.48	0.13	0.30	0.61	0.06	1.06

The <u>in vivo</u> metabolism of 3-<u>tert</u>butylphenyl-<u>N</u>-methylcarbamate
in mouse

Three mice were dosed orally with 0.1 mg of radioactive carbamate, and three by intraperitoneal injection. Mice were retained in glass metabolism cages, and urine collected over a period of 96 hours.

Extraction of metabolites

Urine was taken at 24 hour intervals, and a portion monitored for radioactivity. The 96 hour bulked urine sample pH 4 to 5 was extracted with ether and the percentage of ether extractible metabolites determined.

A sample of this ether extract was run on Whatman S.G. 81 paper and radioactivity counted. Samples were also run on TLC, and by the use of various spray reagents, metabolites were shown to be similar to those obtained from <u>in vitro</u> studies.

The remaining urine sample was incubated at pH 6.0 with paua viscera acetone powder, for 36 hours at 37°. The ether extract of this incubate was monitored for $\left[^{3}H\right]$ and chromatographed both on Whatman S.G.81 paper and TLC.

Mean cumulative tritium excretion in urine as percentage
   of the applied dose

Time (hours)	oral dose	i.p. dose
24	75.3 ± 5.1	64.7 ± 7.2
48	83.2 ± 5.0	68.1 ± 9.0
72	84.9 ± 5.0	68.8 ± 9.0
96	85.2 ± 4.9	70.5 ± 8.7

Mouse	% dose in urine (96 hr)	% urine $[^3H]$ ether extractible	% urine $[^3H]$ released by enzyme	% urine $[^3H]$ not extracted	% dose not recovered
Mean: from orally dosed mice	85.2 ± 4.0	20.2 ± 2.5	70.4 ± 5.0	9.4 ± 8.0	14.8 ± 5.0
Mean: from i.p. dosed mice	70.5 ± 8.5	21.7 ± 4.0	70.5 ± 1.0	7.9 ± 4.5	29.5 ± 9.0

Ether extractible metabolites all corresponded to metabolites
previously obtained from in vitro metabolism in both TLC R_f. and colour
reactions. Faeces were not monitored, and probably contained the majority
of the unextracted radioactivity as conjugates excreted via the bile.

The non-extractible tritium in the urine may be conjugates of
carboxylic acid metabolites, which would not be hydrolysed by the enzyme
preparation used.

Alkaline hydrolysis of the ether extract, and electrophoresis and
TLC of the hydrolysed extract showed two major spots, corresponding to
metabolites e and h of the in vitro system.

Histograms of mouse metabolites.
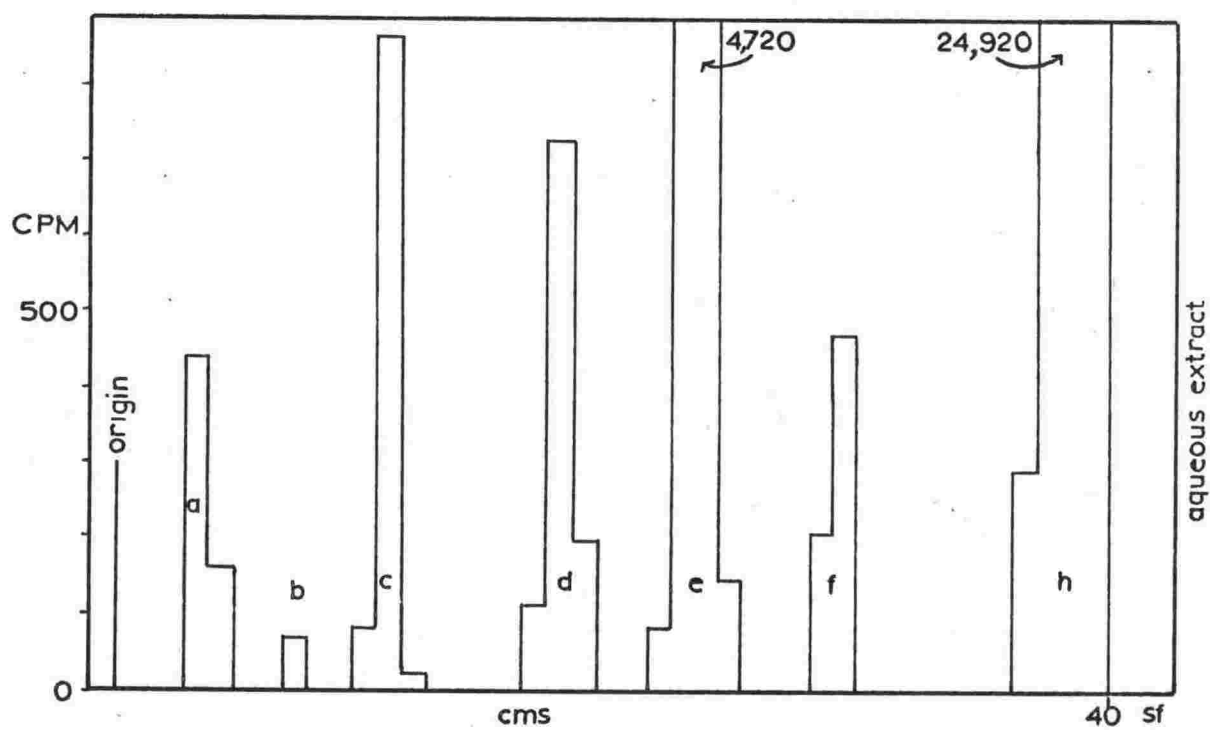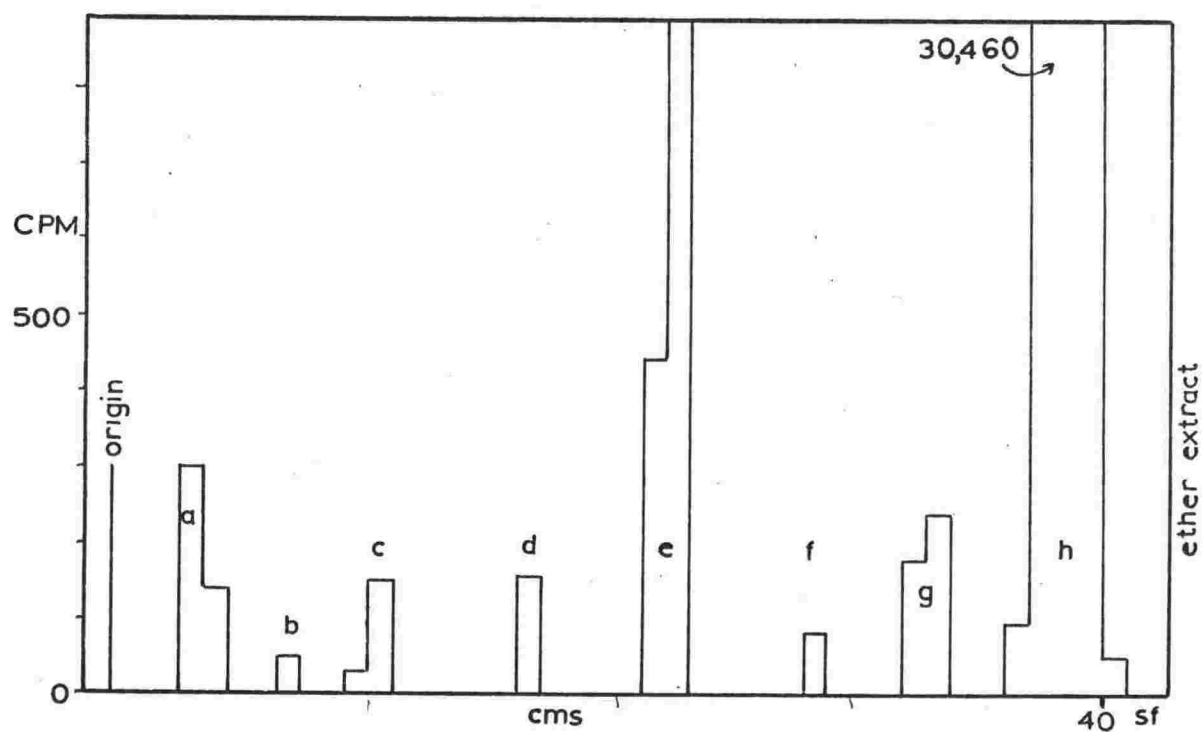
In vivo metabolism of 3-tertbutylphenyl-N-methylcarbamate in orally dosed mice

Metabolite	% R.Ether[1]	% T.Ether[2,4]	% R.Aqueous[1]	% T.Aqueous[2,5]	% Total	% Conjug.[3]
a	1.33	0.27	1.80	1.30	1.56	80.8
b	0.09	0.02	0.22	0.16	0.18	92.3
c	0.60	0.12	2.97	2.13	2.25	94.1
d	0.45	0.09	2.47	1.75	1.84	95.1
e	3.40	0.65	14.82	10.56	11.21	96.9
f	0.22	0.05	2.09	1.48	1.53	96.8
g	1.47	0.29	0.00	0.00	0.29	0.0
h	92.43	18.70	75.63	53.09	71.79	74.1
TOTAL	99.99	20.19	100.00	70.47	90.65	77.7

1. % of counts on chromatogram (mean from three experiments).

2. % of total in each fraction.

3. % of metabolite in conjugated form.

4. T.Ether: Ether extractible metabolite.

5. T.Aqueous: Ether extract of hydrolysed aqueous fraction.

In vivo metabolism of 3-tertbutylphenyl-N-methylcarbamate in intraperitoneally dosed mice

Mean results of three experiments are quoted.

Metabolite	% R.Ether [1]	% T.Ether	% R.Aqueous	% T.Aqueous	% Total	% Conjug.
a	2.34	0.53	4.20	2.96	3.49	84.9
b	0.61	0.09	0.24	0.17	0.26	71.2
c	1.70	0.39	2.19	1.55	1.94	80.1
d	1.71	0.38	2.86	2.00	2.38	80.8
e	1.09	0.25	14.00	9.87	10.12	97.6
f	1.02	0.23	1.75	1.23	1.46	85.0
g	0.07	0.01	0.00	0.00	0.01	0.0
h	91.45	19.80	74.74	52.56	72.36	72.8
TOTAL	99.99	21.68	99.98	70.34	92.02	76.6

1. See previous footnotes, p.137.

PART IV

THE METABOLISM OF BUTACARB

(3,5-di tert BUTYLPHENYL-N-METHYLCARBAMATE)

CHAPTER I

NATURE OF METABOLITES

Identification of the metabolites of 3,5-ditertbutylphenyl-N-
methylcarbamate

Initial identification

Metabolites from 50 mouse liver incubations were pooled for chromatographic and electrophoretic investigation.

TLC in solvent III and visualisation of the phenols with spray reagent (D) showed the presence of at least seven metabolites.

Alkaline hydrolysis of the metabolites, followed by extraction into ether, and chromatography in the same solvent indicated the presence of six phenolic compounds (metabolites I to VI) after visualisation with spray reagent (B).

The number of phenolic compounds indicated that the spots found in the initial TLC must in some cases be comprised of more than one compound.

Two dimensional TLC gave improved separation of metabolites, the first direction was developed in solvent III, then at right angles in solvent I. Eleven metabolite spots were seen after spraying with reagent (D).

(1) Characterisation of aromatic moiety

Hydrolysed metabolites were subjected to thin layer chromatography.

Colour reactions given by Butacarb metabolites

Known compound or metabolite	R_f solvent III	R_f solvent I	Spray reagent				
			B	O	P	R	S
I	0.06	0.30	+	−	−	+	−
II	0.13	0.43	+	−	−	+	−
III	0.18	0.61	+	−	−	−	−
IV	0.23	0.68	+	−	−	−	−
V	0.43	0.73	+	−	−	−	−
VI	0.59	0.80	+	−	−	−	−
VII	0.61	0.81	+	−	−	−	−
VIII	0.11	0.35	+	−	−	+	−
IX	0.80	0.87	+	−	−	−	+
X	0.31	0.76	+	+	+	−	+

VII   3,5-ditertbutylphenol

VIII   3,5-ditertbutyl-4-hydroxybenzoic acid

IX   3,5-ditertbutyl-1,2-dihydroxybenzene

X   3-hydroxybenzaldehyde

These results indicated the presence of two carboxylic acid metabolites, and the absence of aldehydes, or ring hydroxylated compounds. It was unlikely that ortho or para hydroxylation would occur since the bulky tertbutyl substituents would hinder the reaction, this was supported by the negative reaction of Gibbs' reagent, colour reagent (C).

TLC of Butacarb metabolites
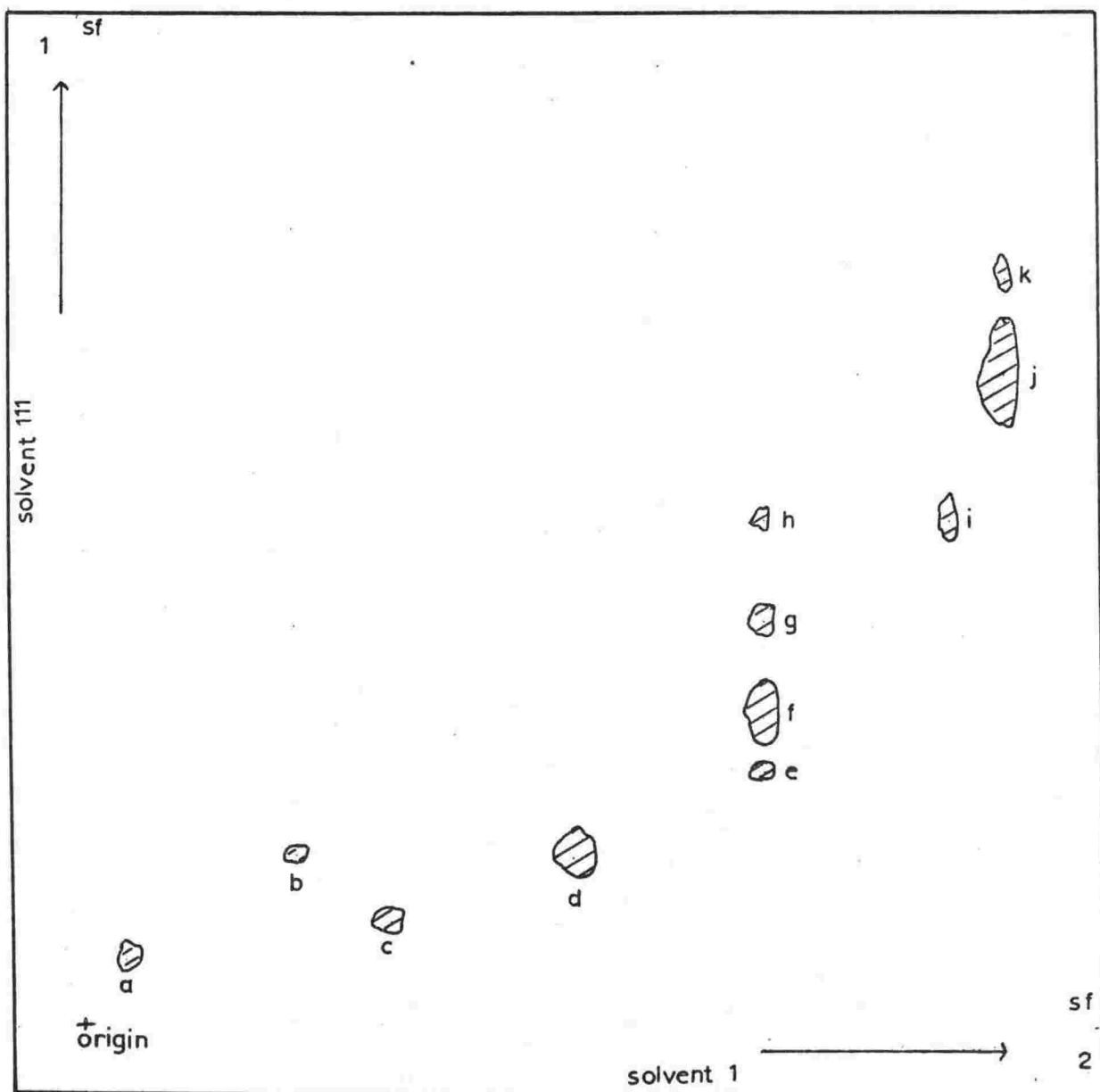
High voltage electrophoresis was conducted to determine the nature of the phenols from the hydrolysed metabolites.

| Metabolite | Migration at pH:(cms) towards anode | | | |
	$(a)_{2.0}$	$(b)_{7.0}$	$(c)_{12.0}$	$(d)_{9.0}$
I	0.0	6.1	9.7	7.3
II	0.0	6.6	10.9	7.7
III	0.0	0.0	6.3	6.2
IV	0.0	0.0	6.7	7.1
V	0.0	0.0	6.5	6.8
VI	0.0	0.0	6.6	7.3
VII	0.0	0.0	6.3	7.4
VIII	0.0	6.4	10.3	7.4
IX	0.0	0.0	5.8	6.2
X	0.0	0.0	6.1	7.1

4000 volts were applied for 20 minutes.

(a)   0.01M-HCl

(b)   0.1M mixed phosphate

(c)   0.01M-NaOH

(d)   0.1M-borax-boric acid

From the above data it would appear that hydrolysed metabolites I and II are carboxylic acids, whereas metabolites II and V and compound IX with reduced migration in borax, are possibly dihydroxy compounds. Metabolite VI was the parent 3,5-ditertbutylphenol.

Gas liquid chromatography

GLC of the hydrolysed metabolites on columns previously described showed 4 peaks at 160°.

Retention time (minutes) of the hydrolysed metabolites of Butacarb

Metabolite	Temperature		
	140°	150°	160°
I	N/D	N/D	N/D
II	N/D	N/D	N/D
III	N/D	N/D	4.25
IV	N/D	N/D	3.25
V	N/D	3.90	2.50
VI	1.50	1.10	0.75
VII	1.50	1.10	0.75
XI	1.40	1.30	1.20
XII	0.20	0.10	0.10

N/D   Not detected within 10 minutes.

VII   3,5-ditertbutylphenol.

XI   3-hydroxybenzylalcohol.

XII   3-hydroxytoluene (m-cresol).

Thus, it would appear that the metabolites III, IV and V are hydroxylated in the tertbutyl side chains. Metabolite V probably is the monohydric compound, metabolite IV the dihydric acid, III the trihydric compound. Orcinol and resorcinol were not detected on this column. Metabolites I and II showing acidic behaviour on electrophoresis were also not detected on this column.

Structure of the hydrolysed metabolites
_____

The aromatic moieties were tentatively assigned the following

structures.

I
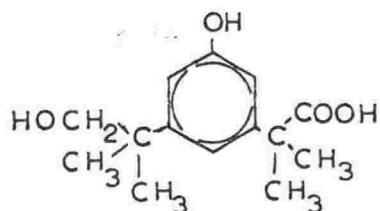
II

III
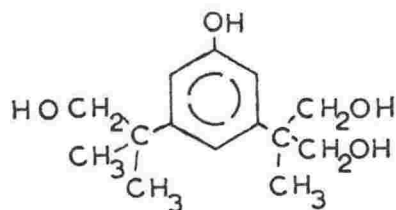
IV
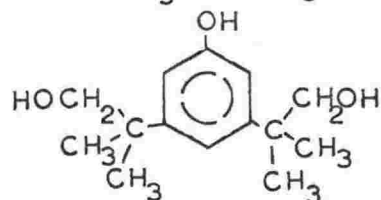
V
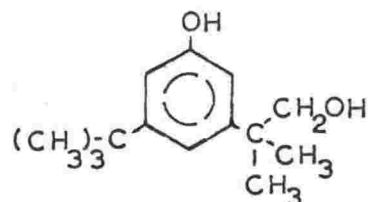
VI
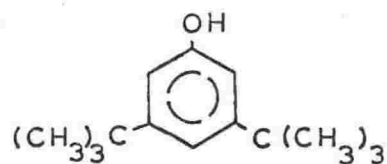
(2)   The carbamate ester side chain

Characterisation of this moiety was undertaken using colour reactions.

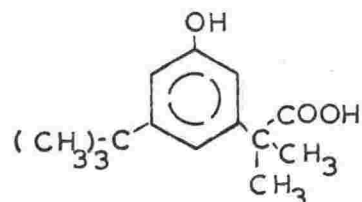| Metabolite	$R_f^{(1)}$	$R_f^{(2)}$	Phenol	$R_f^{(3)}$	Colour reaction								
					A	B	C	D	E	G	P	R	S
a	0.06	0.04	I	0.07	−	−	−	+	+	−	−	+	−
b	0.17	0.21	II	0.17	−	−	−	+	+	−	−	+	−
c	0.10	0.30	III	0.22	−	−	−	+	+	−	−	−	−
d	0.17	0.48	IV	0.29	−	−	−	+	+	−	−	−	−
e	0.25	0.67	IV	0.29	−	+	+	+	−	−	−	−	−
f	0.30	0.67	V	0.55	+	−	−	+	+	−	−	−	−
g	0.40	0.67	V	0.54	−	−	−	+	+	−	−	−	−
h	0.50	0.67	V	0.54	−	+	+	+	−	−	−	−	−
i	0.50	0.85	VI	0.75	+	−	−	+	+	−	−	−	−
j	0.64	0.90	VI	0.75	−	−	−	+	+	−	−	−	−
k	0.74	0.90	VI	0.75	−	+	+	+	−	−	−	−	−
Butacarb	0.64	0.90	VI	0.75	−	−	−	+	+	−	−	−	−
Butacarb phenol	0.74	0.90	VI	0.73	−	+	+	+	−	−	−	−	−

(1)   Ether:isooctane (5:1), solvent III

(2)   Ether, solvent I

(3) R_f. of phenolic part in solvent (1)

The colour reactions indicate the following structures.

Metabolite	Aromatic moiety	Carbamate side chain
a	I	$-OCONHCH_3$
b	II	$-OCONHCH_3$
c	III	$-OCONHCH_3$
d	IV	$-OCONHCH_3$
e	IV	None
f	V	$-OCONHCH_2OH$
g	V	$-OCONHCH_3$
h	V	None
i	VI	$-OCONHCH_2OH$
j	VI	$-OCONHCH_3$
k	VI	None

There appears to be no carbamate formed from Butacarb, and only minor amounts of hydrolytic products. Mono and dihydroxylated phenols were detected, but no free carboxylic acid was found in the ether extracts.

Metabolites from Butacarb formed in housefly and blowfly enzyme incubates

Metabolites obtained from 50 incubates from Musca domestica and Lucilia sericata enzyme were each pooled. They were chromatographed and electrophoresed in the same manner as the metabolites from mouse liver enzyme.

Qualitatively, the metabolites were found to be similar to those obtained from mice, with again almost no hydrolysis, and no detectable carbamate formation. The chromatographic and electrophoretic patterns were compared with mouse liver enzyme metabolites and found to match in all respects. A more rigid characterisation however was not undertaken.

Metabolites of Butacarb formed in grass grub

Grass grubs (20) were dosed topically with $1 \mu g$ of Butacarb in acetone ($1 \mu l$) and allowed to remain for 24 hours in a petri dish. They were then extracted as described previously. The ether layer from the enzyme hydrolysed portion was added to the initial ether extract.

Chromatography and electrophoresis showed a similar pattern to mouse and fly enzyme metabolites, but was further complicated by the presence of hydrolysis products. Two dimensional chromatograms showed that there were six hydrolytic products present in addition to the N-methylcarbamates and an additional metabolite at R_f. 0.08 giving a positive chromotropic acid test. When eluted from the chromatogram and hydrolysed, the hydrolysis product on further TLC had R_f. 0.23 (both R_f'.s in solvent III) indicating that it is the N-hydroxymethyl derivative of phenolic metabolite III.

CHAPTER II

IN VITRO METABOLISM OF BUTACARB

The metabolism of Butacarb by mouse liver enzyme

Mouse liver enzyme was incubated with radioactive Butacarb and the metabolites assayed by chromatogram and electropherogram counting.

Chromatograms of metabolites were developed in solvents I and III and solvent III was used for hydrolysed metabolites. Electropherograms were run at pH 7.0 and the carboxylic acid metabolites assayed.

The metabolism of Butacarb was compared with that of 3-tertbutyl-phenyl-N-methylcarbamate using the same enzyme preparations, and with the same cofactor and substrate concentrations.

Comparison of total metabolic rates

Percentage carbamate remaining after 30 minutes incubation

	3-tertButylphenyl-N-methylcarbamate	Butacarb
Mean % remaining	81.4 ± 2.2	76.9 ± 3.7
Mean rate	$3.91\ \mu M/g$ liver/hour	$4.61\ \mu M/g$ liver/hour

Extraction with ether was continued to completion, and recoveries of radioactive metabolites and substrate were determined for each experiment as being in excess of 99.9%.

Nine spots were evident when metabolites were chromatographed in solvent III, and seven spots in solvent I. Six phenolic spots were obtained on hydrolysis and two spots when the metabolites were electrophoresed.

TABLE I

The percentages of phenolic metabolites formed by mouse liver enzyme

Phenolic metabolite	Mean % chromatogram[1]	Mean rate μM/g liver/hour[2]
I	0.8	0.17
II	0.1	0.02
III	1.4	0.28
IV	6.2	1.30
V	12.1	2.42
VI	79.4	–
TOTALS	100.0%	4.19

1. % radioactivity on chromatogram mean from three experiments.

2. Rate production in μM/g liver/hour. Rate of formation of IV is excluded from the total since this is comprised mainly of unchanged substrate.

High voltage electrophoresis

High voltage electrophoresis gave the following data for carboxylic acids.

Mean % electropherogram   0.9

Mean rate μM/g liver/hour   0.19

    Migration of 8.6 cms was recorded as the mean migration at pH 7.0 and 4000 v.

TABLE II

Metabolites from mouse liver enzyme chromatographed in solvent III

Spot[1]	Metabolite[2]	Means: % Chromatogram[3]	Rate[4]
i	a	0.9	0.17
ii	b & d	5.5	1.09
iii	c	1.4	0.28
iv	e	0.6	0.11
v	f	9.3	1.85
vi	g	1.1	0.22
vii	h & i	2.7	0.54
viii	j	86.9	–
ix	k	1.7	0.33
Totals		100.0	4.61[5]

(1)   Spot designation on chromatogram.

(2)   Corresponding metabolites.

(3)   % of radioactivity on chromatogram.

(4)   Mean rate of formation μM/g liver/hour.

(5)   Total rate of formation (mean) excluding spot viii (Butacarb substrate).

TABLE III

Metabolites from mouse liver enzyme chromatographed in solvent I

Spot[1]	Metabolite[2]	% Chromatogram[3]	Means: Rate[4]
x	a	0.9	0.17
xi	b	0.1	0.02
xii	c	1.4	0.27
xiii	d	5.4	1.08
xiv	e,f,g,h	11.2	2.25
xv	i	2.4	0.48
xvi	j,k	78.6	-
Totals		100.0	4.28[5]

(1) See previous footnotes.

From Tables II and III the following data were obtained.

Metabolite	% (Mean)	Rate: μM/g liver/hour
a	0.9	0.17
b	0.1	0.02
c	1.4	0.27
d	5.4	1.08
e	0.6	0.11
f	9.3	1.86
g	1.1	0.22
h	0.3	0.06
i	2.4	0.48
j	76.9	-
k	1.7	0.33
Totals	100.0	4.61

From this table the percentage of the various phenols were obtained.

Phenolic metabolite	Metabolite	Percentage of total
I	a	0.9%
II	b	0.1%
III	c	1.4%
IV	d + e	5.9%
V	f + g + h	10.7%
VI	i + j + k	81.0%

The metabolism of Butacarb by <u>Musca domestica</u> enzyme

Housefly abdomen enzyme was prepared and used as previously described. Assay was conducted using the method as described for mouse liver enzyme. Mean recovery of radiolabel was determined as 99.97% of the added substrate.

Determination of hydrolysis products

TABLE I

Phenolic metabolites of Butacarb formed by housefly enzyme

Metabolite	% Chromatogram[1]	Mean: Rate[2]
I	0.13	0.10
II	0.01	0.07
III	0.16	0.12
IV	0.34	0.26
V	0.56	0.43
VI	98.80	-
Totals	100.00	0.97

(1) % radiolabel on chromatogram (mean of replicate experiments).

(2) Rate formation $\mu M/g$ fly/hour.

High voltage electrophoresis .

The acidic metabolites migrated a mean distance of 7.8 cms at pH 7.0 and 4000 v.

	% Chromatogram	Rate μM/g flies/hour
Mean of replicate experiments	0.14	0.10

Results obtained from chromatograms of metabolites

TABLE II

Metabolites of Butacarb formed by housefly enzyme, chromatographed in solvent III (ether:isooctane 5:1 ^{V}/v)

		Mean:	
Spot[1]	Metabolite[2]	% Chromatogram[3]	Rate[4]
i	a	0.13	0.10
ii	b & d	0.05	0.04
iii	c	0.17	0.13
iv	e	0.29	0.22
v	f	0.27	0.20
vi	g	0.17	0.13
vii	h & i	1.07	0.82
viii	j	97.57	-
ix	k	0.28	0.21
Totals		99.99	1.84[5]

See footnote on p.150.

155.

TABLE III

Butacarb metabolites formed by housefly enzyme, chromatography in
solvent I (diethyl:ether).

Spot[1]	Metabolite[2]	% Chromatogram[3]	Rate[4]
x	a	0.13	0.10
xi	b	0.01	0.01
xii	c	0.17	0.13
xiii	d	0.04	0.03
xiv	e,f,g,h	0.84	0.63
xv	i	0.96	0.73
xvi	j,k	97.86	-
Totals		100.00	1.62[5]

See previous footnotes.

Percentage of each metabolite was obtained from Tables II and III.

Metabolite	Mean %	Rate μM/g flies/hour
a	0.13	0.10
b	0.01	0.01
c	0.17	0.13
d	0.04	0.03
e	0.29	0.22
f	0.27	0.20
g	0.17	0.13
h	0.12	0.09
i	0.96	0.73
j	97.57	-
k	0.29	0.22
Totals	99.99	1.84

From this table, the percentages of the various phenolic metabolites were calculated.

Phenolic metabolite	Metabolites	%
I	a	0.13
II	b	0.01
III	c	0.17
IV	d & e	0.33
V	f & g & h	0.55
VI	i & j & k	98.82

The metabolism of Butacarb by <u>Lucilia sericata</u> enzyme

Blowfly abdomen enzyme was prepared and used as previously. Recovery from incubations of radioactive material:- 99.55% (mean of replicate experiments).

TABLE I

Determination of hydrolysis products

Metabolite	Mean: % Chromatogram[1]	Rate[2]
I	0.14	0.08
II	0.01	0.01
III	0.14	0.08
IV	0.23	0.13
V	0.45	0.26
VI	99.01	—
Totals	100.00	0.57

(1) % radiolabel on chromatogram.

(2) Rate of metabolite formation μM/g fly/hour.

High voltage electrophoresis

Acidic metabolites at pH 7.0 migrated 8.6 cms (4000 v, 20 min.).

Mean % chromatogram, 0.150

Mean rate, μM/g/hour, 0.087

TABLE II

Percentages of metabolites from blowfly enzyme on a chromatogram developed
in solvent III (ether:isooctane 5:1 ^{V}/v)

| Spot[1] | Metabolite[2] | Mean values | |
		% Chromatogram[3]	Rate[4]
i	a	0.14	0.08
ii	b & d	0.04	0.02
iii	c	0.14	0.08
iv	e	0.19	0.11
v	f	0.19	0.11
vi	g	0.16	0.09
vii	h & i	1.15	0.61
viii	j	97.77	-
ix	k	0.34	0.20
Totals		100.00	1.29[5]

(1) See previous footnotes, p.150.

TABLE III

Percentages of metabolites from blowfly enzyme on a chromatogram developed
in solvent I (diethyl:ether)

| Spot[1] | Metabolite[2] | Mean values | |
		% Chromatogram[3]	Rate[4]
x	a	0.15	0.08
xi	b	0.01	0.01
xii	c	0.14	0.08
xiii	d	0.03	0.02
xiv	e,f,g,h	0.64	0.37
xv	i	0.94	0.55
xvi	j & k	98.09	-
Totals		99.99	1.10

(1) See previous footnotes.

The percentage and rate of formation of each metabolite was calculated from the preceding tables.

Metabolite	Mean %	Rate μM/g flies/hour
a	0.14	0.08
b	0.01	0.01
c	0.14	0.08
d	0.03	0.02
e	0.19	0.11
f	0.19	0.11
g	0.16	0.09
h	0.10	0.06
i	0.94	0.55
j	97.77	-
k	0.34	0.20
Totals	100.00	1.29

The percentage of phenolic metabolites calculated from this table are:

Phenol	Metabolites	%
I	a	0.14
II	b	0.01
III	c	0.14
IV	d & e	0.22
V	f & g & h	0.45
VI	i & j & k	99.05

CHAPTER III

IN VIVO METABOLISM OF BUTACARB

The <u>in vivo</u> metabolism of $[^3\text{H}]$-Butacarb

The time course of Butacarb metabolism in flies

    Blowfly and housefly adults and larvae were topically dosed with 95 ng (362 pM) or 9.5 ng (36.2 pM) of $[^3\text{H}]$-Butacarb. Replicate batches of 50 insects were retained for up to 24 hours. The insects were extracted with ether as previously described and the extracts assayed for radio-activity. Chromatography on silica gel G thin layers and development in solvent system III separated the insecticide from metabolites, the percentage of $[^3\text{H}]$-Butacarb on the chromatogram was determined and expressed as a percentage of the applied dose.

The rate of disappearance of $[^3\text{H}]$-Butacarb from flies

Mean % $[^3\text{H}]$-Butacarb remaining

Species	Musca domestica			Lucilia sericata		
Stage and dose (time, hours)	adult (95ng)	adult (9.5ng)	larvae (9.5ng)	adult (95ng)	adult (9.5ng)	larvae (9.5ng)
0	97.4%	96.6%	97.3%	96.3%	97.9%	98.2%
4	79.2%	73.1%	90.0%	83.1%	74.8%	77.8%
8	-	51.9%	71.0%	58.4%	40.3%	64.2%
12	45.1%	26.0%	62.7%	46.2%	21.3%	48.3%
16	29.6%	11.1%	48.2%	30.1%	8.9%	36.3%
24	23.2%	4.3%	35.4%	9.8%	3.2%	21.7%

The results indicate that the adult insect is more capable of oxidation of Butacarb, the larvae dosed with 95 ng died within two hours of dosing. The first and second instar larvae were killed by the low dose of 9.5 ng per insect, and hence the data above was collected for prepupal larvae.

The metabolism of $[^3H]$ Butacarb in insects

Three day old <u>Musca domestica</u>, five day old <u>Lucilia sericata</u> and larval <u>Costelytra zealandica</u> were each dosed topically with $0.095\,\mu$g (0.362 nM) of $[^3H]$-Butacarb (0.1336 μCi/μM), in duplicate batches of 50 insects. Flies were retained for 12 hours at 30° in large test tubes, and grass grubs were held in petri dishes for 24 hours in the dark.

Larvae of <u>M</u>. <u>domestica</u> and <u>L</u>. <u>sericata</u> were dosed with 9.5 ng (36.2 pM) $[^3H]$-Butacarb. Duplicate batches of 50 were dosed and retained in small petri dishes in the dark for 12 hours.

Extraction and metabolite separation

The insects were extracted as previously described. Trial chromatograms indicated a large number of ether soluble metabolites from all species, and in view of the low specific activity of the Butacarb used, estimation of phenolic compounds only was attempted.

Both ether and aqueous fractions were counted for radioactivity and then subjected to hydrolysis, the residue was also counted.

The ether layer was hydrolysed in 2M-NaOH and re-extracted into ether, counted and applied to chromatograms, and electropherograms.

Extraction efficiencies

Species	% Ether (1)	% Aqueous (2)	% hydrolysis (3) residue	% tissue (4) residue	%(5) T
M.domestica adult	61.6	28.0	3.0	7.3	89.6
M.domestica larvae	41.5	52.5	1.3	4.7	94.0
L.sericata adult	62.5	29.9	3.0	4.5	92.4
L.sericata larvae	46.5	44.9	1.9	6.7	91.4
C.zealandica larvae	58.3	31.6	2.5	7.6	89.9

Results are means of replicate experiments.

(1) Percentage applied dose in ether extractible fraction.

(2) Percentage applied dose in aqueous fraction after hydrolysis.

(3) Percentage applied dose unextractible from hydrolysed aqueous fraction.

(4) Percentage applied dose unextractible from homogenised tissue.

(5) Percentage of applied dose that was recovered.

The metabolism of Butacarb in insects

| | M. domestica | | | | | | L. sericata | | | | | | C. zealandica | | |
| | adult | | | larvae | | | adult | | | larvae | | | larvae | | |
Metabolite	%E(1)	%A(2)	%T(3)	%E	%A	%T	%E	%A	%T	%E	%A	%T	%E	%A	%T
I	2.15	3.98	6.13	0.77	1.06	1.84	4.57	2.41	6.97	0.24	2.23	2.47	5.70	4.92	10.61
II	0.01	0.15	0.15	0.39	0.09	0.42	0.68	1.37	2.05	0.00	1.39	1.36	0.35	0.59	0.95
III	1.61	0.52	2.13	0.56	0.86	1.41	2.76	0.07	2.85	1.46	4.21	5.67	1.11	1.04	2.14
IV	1.54	1.62	3.17	3.66	2.44	6.09	3.49	3.74	7.23	1.52	3.70	5.22	1.61	1.66	3.27
V	10.88	10.50	21.39	7.36	13.32	20.68	11.19	5.79	17.03	9.08	11.40	20.57	4.89	7.71	12.56
VI	45.40	10.46	55.87	28.80	34.75	62.55	39.76	11.13	50.91	34.18	22.02	56.20	44.69	15.75	60.43
Total	61.59	27.23	88.84	41.54	52.52	93.99	62.45	24.51	87.02	46.48	44.95	91.39	58.35	31.67	89.95

(1) %E : Mean percentage of each metabolite in ether fraction as percentage of applied dose.

(2) %A : Mean percentage of each metabolite in aqueous fraction as percentage of applied dose.

(3) %T : Mean total percentage of each metabolite as percentage of applied dose.

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High voltage electrophoresis

At pH 8.0 and 4000 volts, acidic compounds migrated between 8.5 and 10.2 cms in 20 minutes.

Species	% acidic compounds on electropherogram[1]		
	Ether extract	Aqueous extract	Combined total
M. domestica adult	2.02	3.95	5.97
M. domestica larvae	0.85	0.98	1.83
L. sericata adult	5.23	3.72	8.95
L. sericata larvae	0.20	3.53	3.73
C. zealandica larvae	5.87	5.59	11.46

(1) Percentage on electropherogram is corrected to percentage

of total in each fraction.

Rates of oxidation

The approximate rate of oxidation for the various metabolites can be obtained since the previous experiments show that the reaction was linear during the withholding period.

The rate figures probably account for about half of the full rate as metabolites with an unchanged phenolic moiety are not included.

Rate of metabolism of Butacarb in some insects

Metabolite	M. domestica adult		M. domestica larvae		L. sericata adult		L. sericata larvae		C. zealandica larvae	
	%T(1)	Rate(2)	%T	Rate	%T	Rate	%T	Rate	%T	Rate
I	6.13	133.0	1.84	3.8	6.97	128.0	2.47	42.5	10.61	14.0
II	0.15	3.5	0.42	0.85	2.05	38.0	1.36	2.4	0.94	1.5
III	2.13	46.0	1.41	2.90	2.83	52.0	5.67	9.8	2.14	3.0
IV	3.17	69.0	6.09	12.6	7.23	133.0	5.22	9.1	3.27	4.5
V	21.39	465.0	20.68	42.7	17.03	313.0	20.47	35.5	12.56	17.0
VI	55.87	−	63.55	−	50.91	−	56.20	−	60.43	−
Totals	88.84	716.0	93.99	62.8	87.02	664.0	91.39	61.5	89.95	40.0

(1) Percentage of total applied dose.

(2) Rate of oxidation as pM/g insect/hour.

PART V

THE METABOLISM OF BAYGON

(2-ISOPROPOXYPHENYL-N-METHYLCARBAMATE)

CHAPTER I

NATURE OF METABOLITES

Identification of the metabolites of 2-isopropoxyphenyl-N-
                    methylcarbamate

Initial identification

Metabolites from mouse liver enzyme incubations were subjected to thin layer chromatography. The chromatograms were developed in solvent system VI (ether:isooctane 8:3 (^{v}/v)), and on treatment with spray reagent (D) ten spots appeared. These apparently consist of two phenolic compounds, one at R_f.0.8, which cochromatographs with 2-isopropoxyphenol, and the other at R_f.0.30 with similar properties to catechol.

Metabolites were removed from the plate, hydrolysed and re-chromatographed. Only two spots were visible, at R_f's 0.3 and 0.8.

The spot at R_f. 0.3 reacted with colour reagents as a 1,3-dihydroxy compound, that at R_f. 0.8 cochromatographed with 2-isopropoxyphenol and had similar colour reactions.

Poor separation of metabolites was obtained in other solvent systems tested.

Interpretation of colour reactions

(1) Metabolites containing nitrogen

Compounds reacting with reagents (E) and (G) contain carbamate-nitrogen and include all metabolite spots except If and Ij. Spots Ib, c and h reacting with ninhydrin, but not $\underline{N},\underline{N}$-dimethylaminocinnamaldehyde could be carbamates, whereas metabolites Ia and Ig, reacting with chromotropic acid have the $\underline{N}$-hydroxymethyl grouping. Metabolites If and Ij do not react with any of the reagents for nitrogen and are probably phenolic compounds.

Colour reactions given by Baygon metabolites

Metabolite	R_f (i)	R_f (ii)	(A)	(B)	(D)	(E)	(G)	(I)	(C)	(L)	(N)	(NaOH)	^{14}C *
Ia	0.05	0.31	+	+	+	+	−	+	+	+	+	+	+
Ib	0.08	0.31	−	+	+	+	+	+	+	−	−	+	−
Ic	0.11	0.29	−	+	+	+	+	+	−	−	+	−	+
Id	0.16	0.30	−	+	+	+	−	+	−	+	+	−	+
Ie	0.26	0.31	−	+	+	+	−	+	+	−	−	+	−
If	0.31	0.30	−	+	+	−	−	+	+	+	+	+	+
Ig	0.41	0.79	+	−	+	+	−	−	−	−	−	−	+
Ih	0.61	0.81	−	−	+	+	+	−	−	−	−	−	+
Ii	0.65	0.80	−	−	+	+	−	−	−	−	−	−	+
Ij	0.80	0.80	−	+	+	−	−	−	+	−	−	−	+
catechol	0.31	−	−	+	+	−	−	+	+	+	−	+	−
Baygon (I)	0.65	0.80	−	−	+	+	−	−	−	−	−	−	−
II	0.26	0.30	−	+	+	+	−	+	+	−	−	+	−
III	0.80	−	−	+	+	−	−	−	+	−	−	−	−
orcinol	0.33	−	−	+	+	−	−	+	+	+	+	−	−

*   Metabolites with $\left[^{14}C\right]$-label attached.

(i) R_f. of metabolite.

(ii) R_f. of hydrolysed metabolite.

II   2-hydroxyphenyl-N-methylcarbamate.

III   2-isopropoxyphenol.

(2)   The aromatic moiety

Colour reactions indicated that all metabolites except Ig, h and i possess a free ring hydroxyl function, and that metabolites a, b, e, f, and j also have free para positions.   Hence metabolites c and d contain a free ring hydroxyl group, but no position para to it for coupling with reagent (C).   This would indicate a hydroxyl group para to the isopropoxyl group, that is, a 5-hydroxylated compound.

Metabolites a, c, d and f and orcinol reacted with reagent (N) to give similar coloured compounds.   This indicates that they are 1,3-dihydroxy compounds.

Catechol when sprayed with 2M-NaOH gave a greenish brown colour, which was also observed with metabolites Ia, b, e and f.   This conflicts with the previous test, and indicates that metabolites Ia and If may be mixtures.

O-Dealkylation of $\left[^{14}C\right]$-Baygon causes a loss of label.   The metabolites Ib and Id have no associated radioactivity and can be assumed to have a free 2-hydroxyl group, while other metabolites retain an intact ether group indicating that metabolites a, e, d, f are hydroxylated elsewhere.

Three aromatic groups are involved:

II   2-isopropoxyphenol

III   1,2-dihydroxybenzene (catechol)

IV   5-hydroxy-2-isopropoxyphenol

High voltage electrophoresis

Metabolites were hydrolysed in 2.0M-NaOH prior to electrophoresis at 6000 v for 15 to 20 minutes.

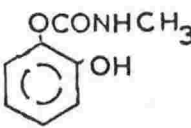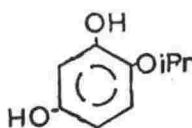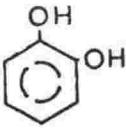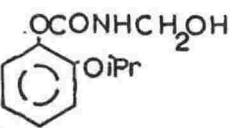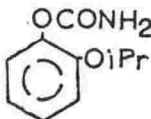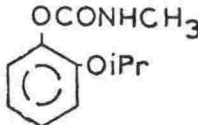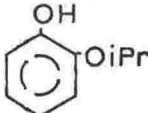| Hydrolysed metabolite	Distance travelled at pH (in cm)[1]					
	2.0	7.4	10.0	12.0	13.0	14.0
a	0.0	0.0	6.2	6.3	10.6	10.7
b	0.0	0.0	5.9	6.2	10.4	10.7
c	0.0	0.0	6.0	6.3	10.9	11.1
d	0.0	0.0	5.8	5.8	11.1	10.9
e	0.0	0.0	7.1	6.1	10.6	11.1
f	0.0	0.0	6.8	7.0	11.2	10.9
g	0.0	0.0	6.4	6.6	6.4	6.5
h	0.0	0.0	6.3	6.4	6.6	6.4
i	0.0	0.0	5.9	6.2	6.8	6.7
j	0.0	0.0	6.1	6.6	6.7	6.6
catechol	0.0	0.0	6.3	6.4	10.7	10.9
orcinol	0.0	0.0	6.2	6.3	10.1	10.3
2-isopropoxyphenol	0.0	0.0	5.8	5.9	6.8	6.8
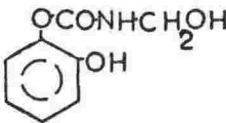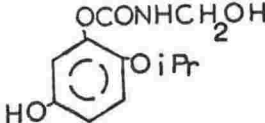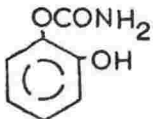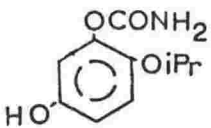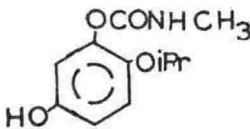
(1) Buffers used are described on p.82

From these results it can be concluded that metabolites a, b, c, d, e and f contain a doubly ionisable function.

Summary

Metabolite	Carbamate side chain ester	aromatic moiety (see p.173)
Ia	$- NH.CH_2OH$	IV & III
Ib	$- NH_2$	III
Ic	$- NH_2$	IV
Id	$- NHCH_3$	IV
Ie	$- NHCH_3$	III
If	$-$ none	IV & III
Ig	$- NH.CH_2OH$	II
Ih	$- NH_2$	II
Ii	$- NHCH_3$	II
Ij	$-$ none	II

The structures below have been tentatively assigned to the
metabolites of Baygon

Metabolite	Solvent I R_f	Structure
a	0.05	
b	0.08	
c	0.11	
d	0.16	
e	0.26	
f	0.31	
g	0.40	

Metabolite	Solvent I R_f	Structure
h	0.61	
i	0.65 (Baygon)	
j	0.80 (2-isopropoxyphenol)	

Identification of the volatile radiolabelled metabolite(s)

Baygon, labelled with $\left[^{14}C\right]$ in the 1 and 3 positions of the isopropoxyl side chain loses radioactivity in the form of either $^{14}CO_2$ or acetone.

The volatile radioactive metabolites were readily trapped in aqueous sodium bisulphite, and in buffered semicarbazide hydrochloride. No appreciable radioactivity was collected in 2M sodium hydroxide.

Trapped volatile radioactive material from mouse liver enzyme incubations was bulked, cooled to 0° and the resultant crystalline precipitate filtered. This was recrystallised twice from cold ethanol.

Mixed melting points of the trapped material with acetone semi-carbazone gave no depression from the acetone semicarbazone melting point of 190°.

The volatile metabolites from ten, one ml mouse liver enzyme incubations were trapped in semicarbazide hydrochloride, the samples were bulked and a portion counted (total dpm = 65,136). The precipitate at 0° was filtered, and crystallised twice from cold ethanol. A sample of this was counted for radioactivity (total dpm in precipitate = 63,294). This precipitate was then diluted with unlabelled acetone semicarbazone, recrystallised four times and the specific activity of the mixture determined.

Specific activity of labelled compound = 2.38×10^{-4} mCi/mM

Calculated specific activity of mixture = 1.635×10^{-5} mCi/mM

Actual specific activity of recrystallised mixture = 1.645×10^{-5} mCi/mM

Radioactive purity = 100.6%

The isopropoxyl side chain of Baygon in mice appears to be released as $\left[{}^{14}C\right]$-acetone, indicating attack by oxidation on the 2 carbon atom rather than the terminal methyl groups.

CHAPTER II

IN VITRO METABOLISM OF BAYGON

The O-dealkylation of $[^{14}C]$-Baygon *in vitro*

Incubations using mouse liver enzyme, M. *domestica* and L. *sericata* abdomen enzyme preparations were conducted to determine the optimal conditions for Baygon O-dealkylation.

(a) The stability of $[^{14}C]$-Baygon in buffer solutions

$[^{14}C]$-Baygon was incubated at 21° in Conway microdiffusion vessels, any $[^{14}C]$-acetone produced was trapped as the semicarbazone. Buffers used were 0.1M-tris-HCl buffer and 0.1M-phosphate buffer both at pH 7.4.

At a substrate concentration of 1.0 millimolar no $[^{14}C]$-acetone was formed in up to 36 hours of incubation in either buffer.

(b) Cofactor requirements for Baygon metabolism

Enzyme preparations were incubated for 30 minutes with the cofactor additions given in the following table.

Phosphate buffer allowed higher rates of O-dealkylation than did tris-HCl buffer for the housefly enzyme, it was not tested in the blowfly. There appears to be no difference between the two buffers with mouse liver enzyme.

Nicotinamide gave slight inhibition in the fly incubations and was not generally included in enzyme fortification. The carbon monoxide inhibition and cofactor requirements indicated that the system was micro-somal in nature.

Cofactors added	Mouse		Housefly		Blowfly
	Phosphate Rate	Tris-HCl Rate	Phosphate Rate	Tris-HCl Rate	Phosphate Rate
None	0.01	0.01	0.0	0.0	0.0
NAD.G6P.	0.21	0.20	0.05	0.02	0.11
NADP. G6P.	0.43	0.45	0.22	0.19	0.18
NAD.NADP. G6P.	-	-	0.30	0.22	0.20
NAD.NADP. G6P. Mg^{++}.	0.42	0.41	0.31	0.22	0.21
NAD.NADP. G6P. Nicotinamide.	0.44	0.46	0.25	0.21	0.19
NAD.NADP.G6P. KCN.	-	-	0.31	0.20	0.22
NAD.NADP. G6P. KCN. BSA.	-	-	0.31	0.23	0.23
NAD.NADP. G6P. BSA.	-	-	0.30	0.21	0.21
NAD.NADP. G6P. CO.	-	-	0.05	0.04	0.06

KCN. 10^{-4} molar potassium cyanide.

BSA. 1% ^{w}/v bovine serum albumin.

CO. Carbon monoxide was bubbled into the buffer solution prior

to incubation, the Conway unit was incubated in the dark.

Cofactor concentrations were as described on p.48

Rate. Rate of O-dealkylation at 30 minutes, M/g liver or flies/hour.

(c) Effect of substrate concentration on Baygon metabolism

Enzymes were prepared and fortified with cofactor as described previously. Various amounts of substrate were added to initiate the reaction. O-Dealkylation was measured by removing $\left[^{14}C\right]$-acetone semi-carbazone after a suitable diffusion time and counting for radiocarbon in an aqueous-holding scintillant fluid. Incubations were continued for 30 minutes at a temperature of 22°, incubate volumes were 1.0 ml.

Substrate added μg	Molarity of substrate	Mean rate of O-dealkylation $\mu M/g$ flies or liver/hour		
		Musca domestica	Lucilia sericata	Mus musculus
50	0.25×10^{-3}	0.08	–	–
100	0.5×10^{-3}	0.16	0.08	0.31
150	0.75×10^{-3}	0.23	–	–
200	1.0×10^{-3}	0.29	0.20	0.65
250	1.25×10^{-3}	0.32	–	–
500	2.5×10^{-3}	0.32	0.20	0.69
750	3.75×10^{-3}	0.33	0.17	0.71
1000	5.0×10^{-3}	0.32	0.17	0.71

(d) The effect of enzyme concentration on Baygon metabolism

Enzyme concentration required for optimal O-dealkylation was investigated. One ml incubations were used, with constant substrate concentration of 1.0 millimolar. The incubation time was 0.5 hours and temperature 22°.

Mean rate of O-dealkylation nM/hour

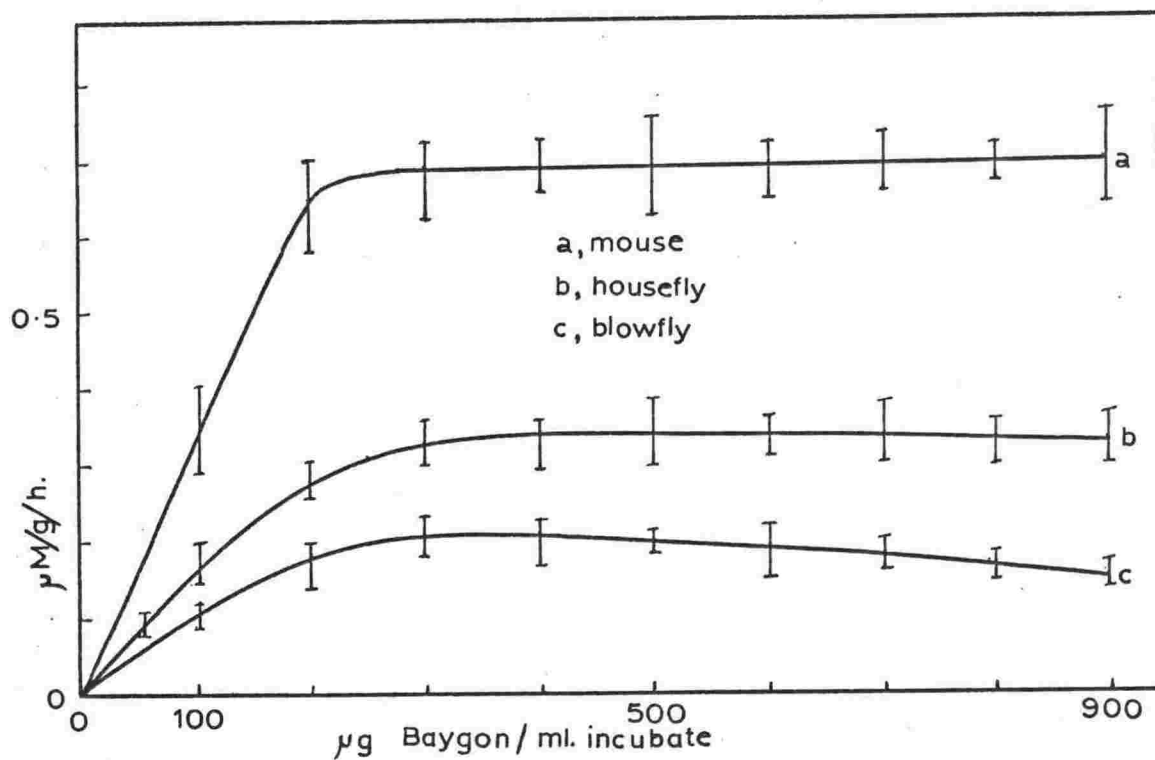| Abdomens	M. domestica	L. sericata	g liver[1]	M. musculus
0.5	1.0	-	0.005	4.2
1.0	2.1	3.0	0.010	9.3
2.0	2.9	4.2	0.025	22.1
3.0	2.6	3.8	0.050	42.0
4.0	2.0	3.5	0.100	70.1
5.0	1.9	3.2	0.500	84.0
6.0	1.6	-	1.000	88.3
7.0	1.5	-		

(1) 10,000 g supernatant from quoted amount of liver.
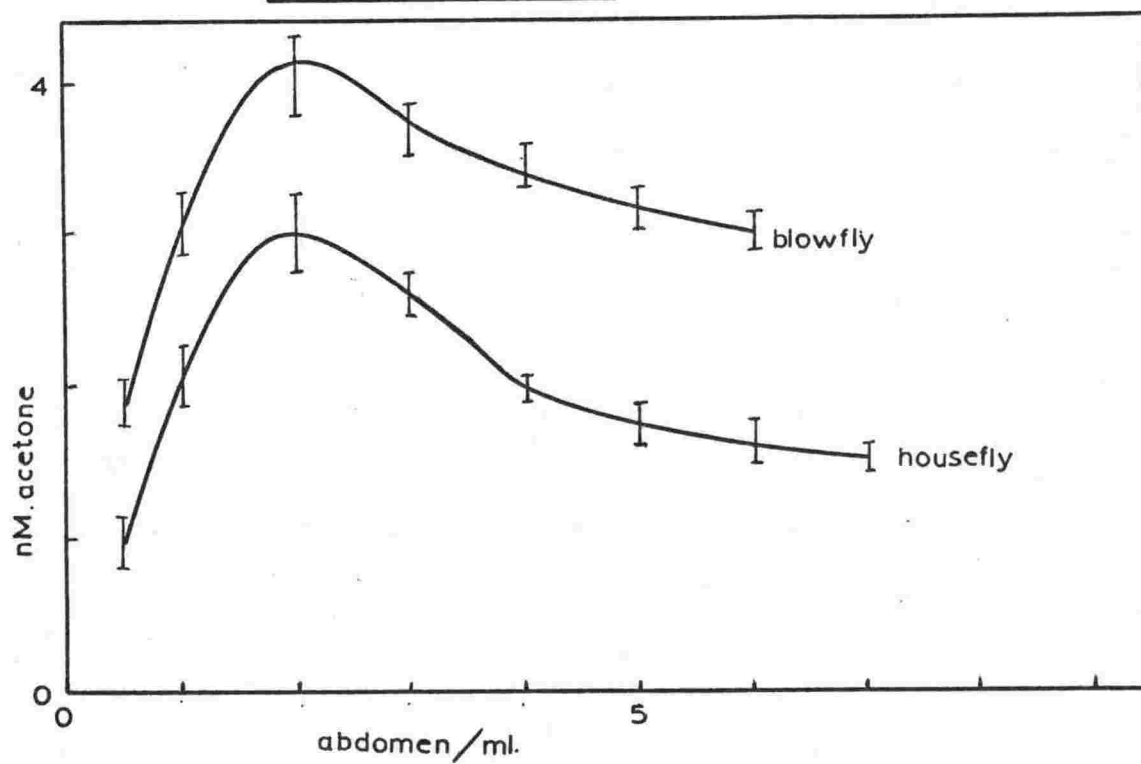
(e) O-Dealkylation of $[^{14}C]$-Baygon with time

The time course of the O-dealkylation was followed to ensure that
incubations were stopped during a linear part of the reaction. The
incubations of 1.0 ml were continued for up to 1 hour at 22° with substrate
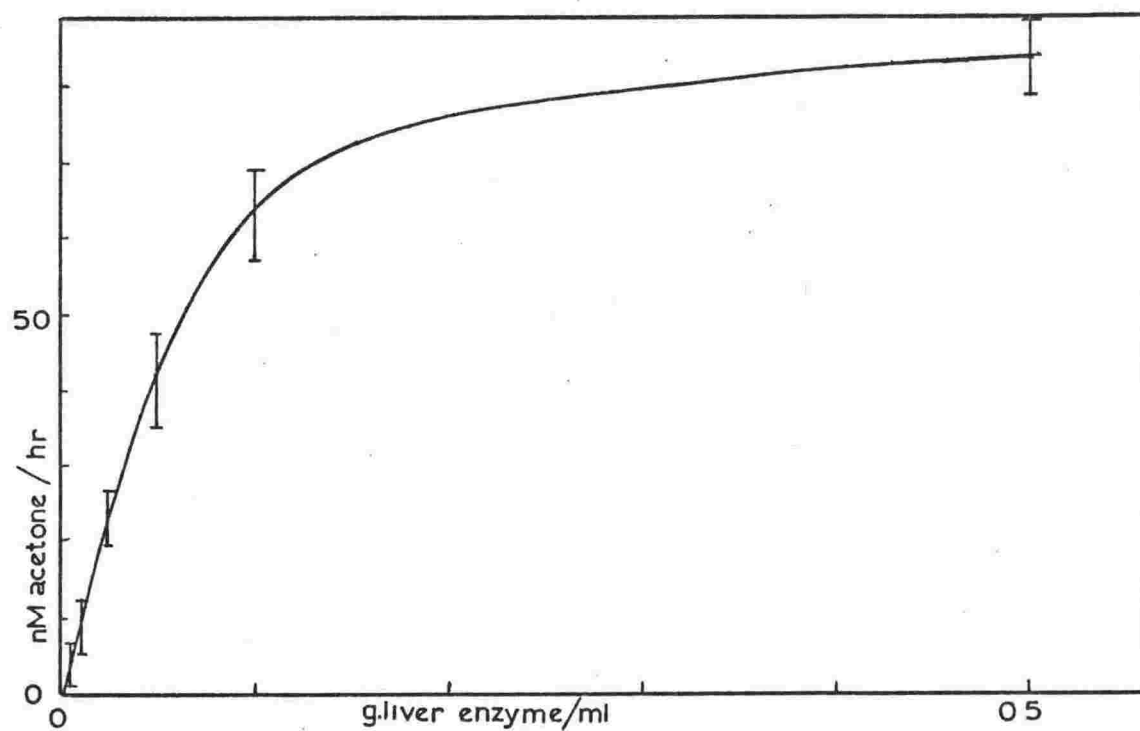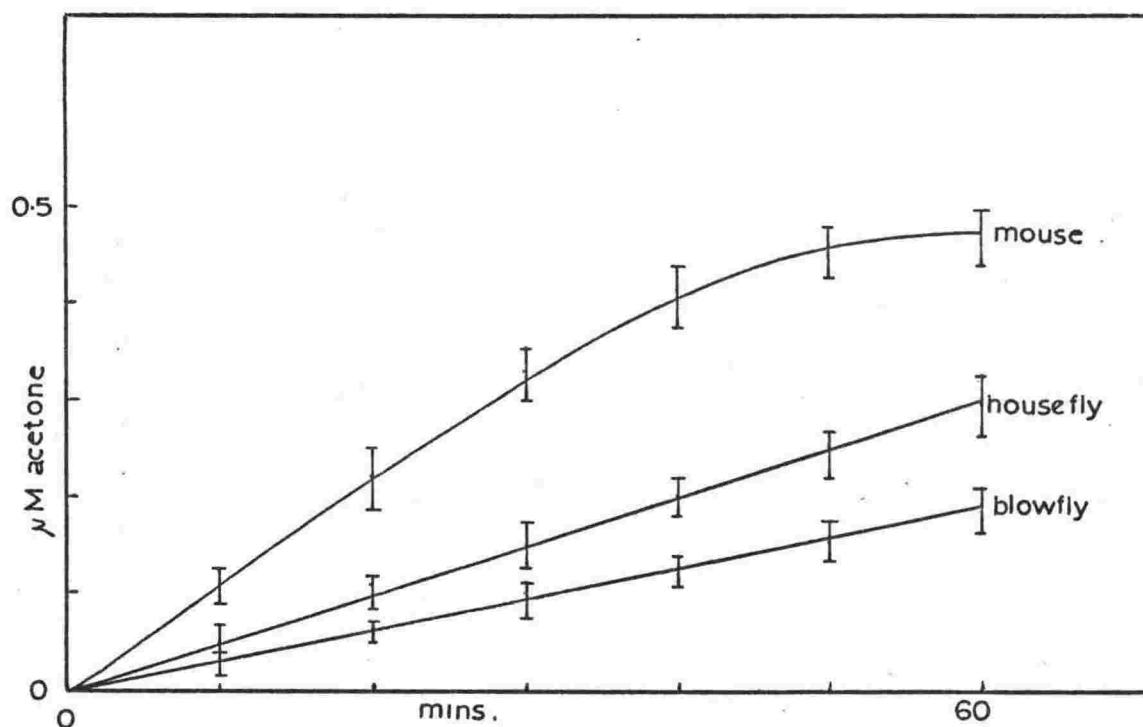concentration 1.0 millimolar.

Time minutes	Amount of $[^{14}C]$-acetone produced per liver or whole insect					
Species:	10	20	30	40	50	60
M. domestica	0.051	0.105	0.163	0.203	0.250	0.285
μM/g/hour	0.306	0.315	0.326	0.304	0.300	0.285
L. sericata	0.033	0.067	0.098	0.122	0.155	0.178
μM/g/hour	0.198	0.201	0.196	0.183	0.186	0.178
M. musculus	0.120	0.218	0.330	0.412	0.468	0.472
μM/g/hour	0.720	0.654	0.660	0.620	0.562	0.472

[S] vs O-dealkylation

a, mouse
b, housefly
c, blowfly

μM/g/h.

μg Baygon/ml. incubate

[E] vs O-dealkylation

nM. acetone

blowfly

housefly

abdomen/ml.

Mouse [E] vs O-de-alkylation

Time course of O-dealkylation, (enzymic).
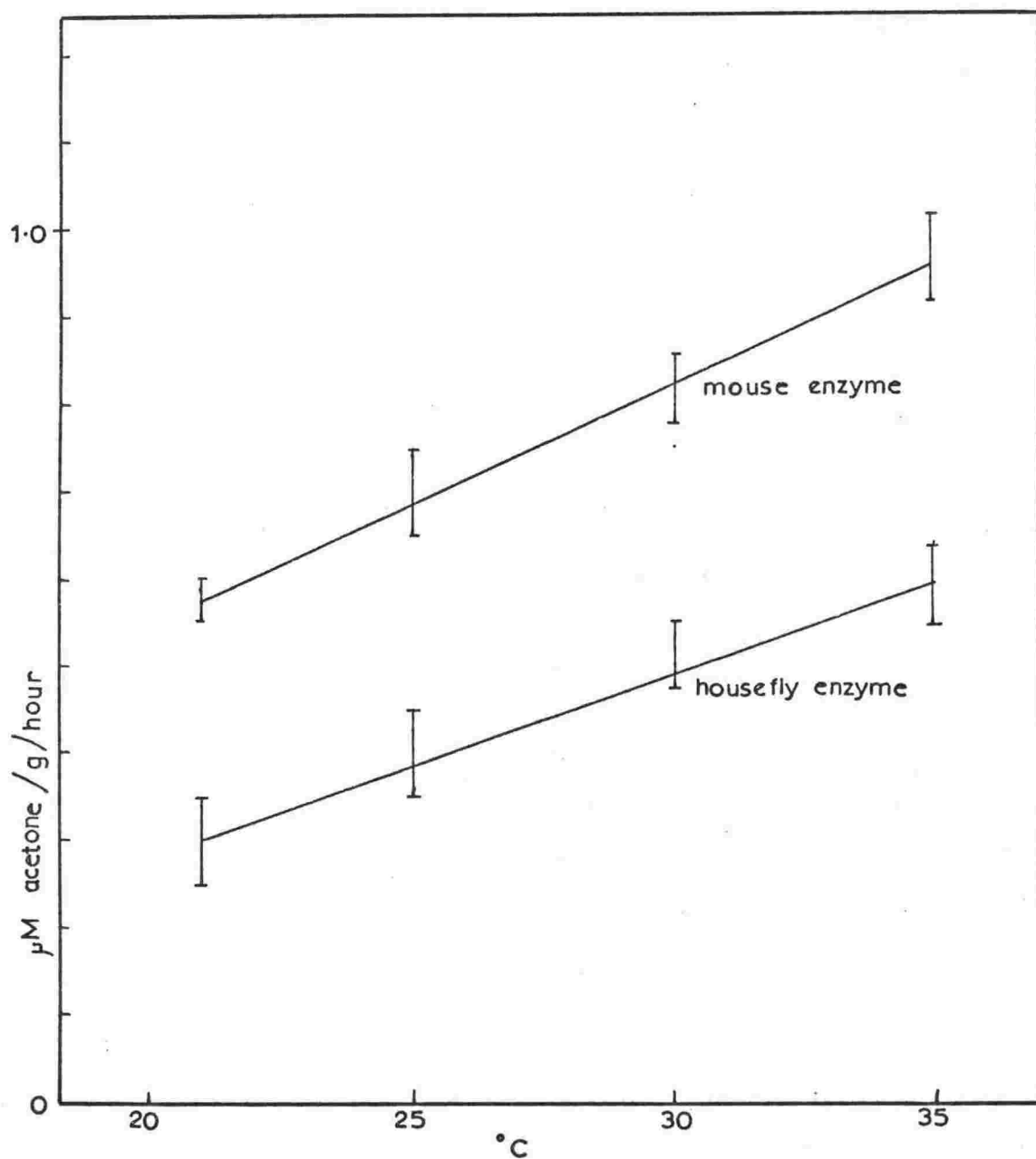
pH   vs   O-dealkylation
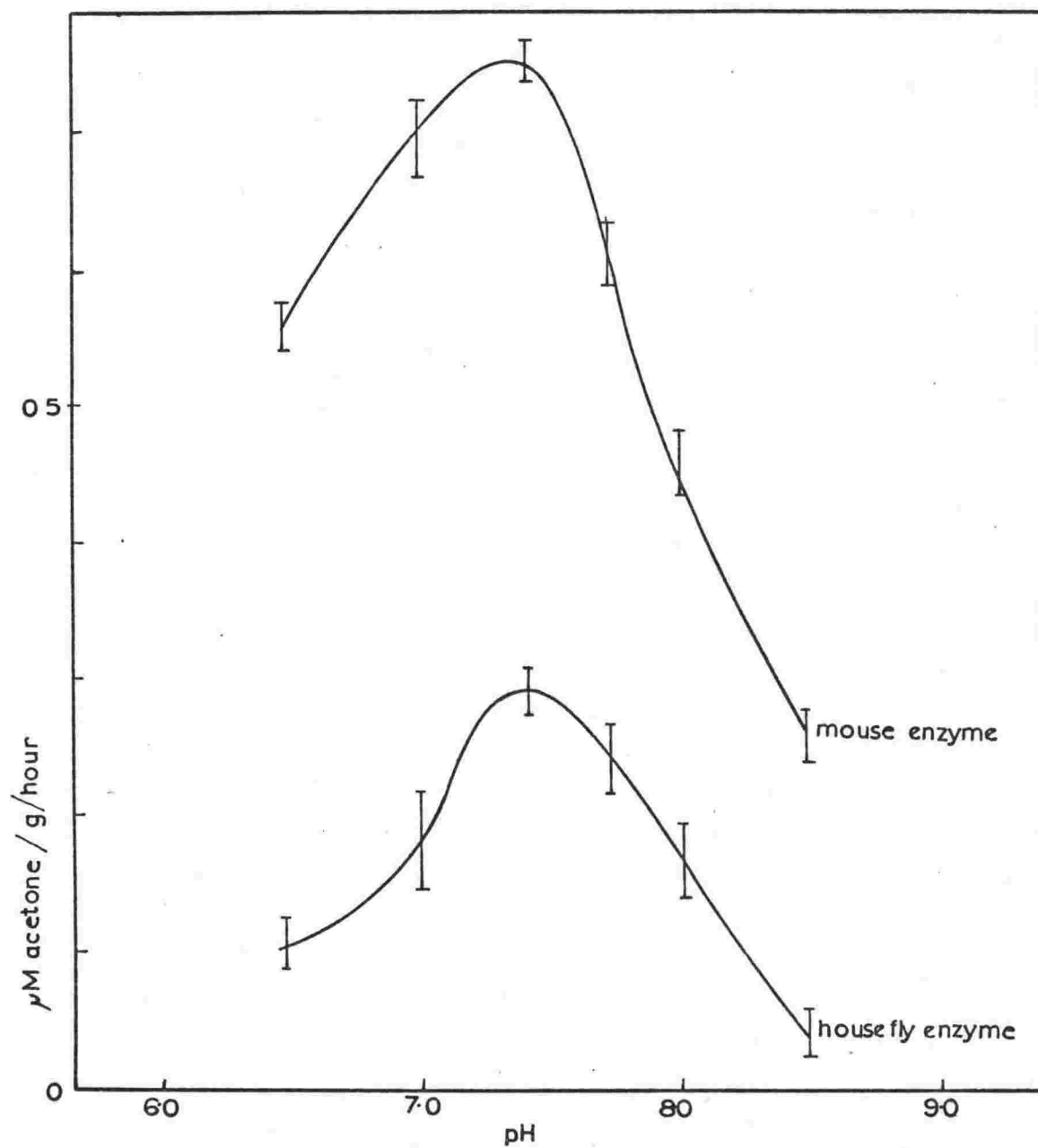
(f) The effect of pH on the O-dealkylation of Baygon

Optimal pH for microsomal reactions is reported in the literature to range from pH 7.0 to pH 8.5. The optimal pH for O-dealkylation was measured for mouse and *Musca domestica* enzymes. pH was measured both before and after the 30 minute incubation period.

(g) O-Dealkylation of Baygon at various temperatures

Since O-dealkylation rates were mainly conducted at ambient temperature, 20 to 22° on a microdiffusion vessel shaking table, the need to compare oxidation rates with those of other carbamate insecticides at different temperatures necessitated incubations at a range of temperatures. These experiments were carried out at ambient temperature, and above, in incubators with occasional swirling by hand, other conditions were held constant. Mouse liver enzyme and fly abdomen enzyme were used, the same enzyme preparation was used for all temperatures. The results are plotted on the following graph.

Temperature vs O-dealkylation

Inhibition of Baygon O-dealkylation

Experiments were conducted to determine the degree of inhibition of the enzymic O-dealkylation of Baygon in mouse liver enzyme, housefly, and blowfly abdomen enzymes.

One ml incubations were carried out for 30 minutes at 21° in Conway microdiffusion dishes, $[^{14}C]$-acetone was trapped as the semi-carbazone and counted after a six hour diffusion time.

Inhibition experiments

Fly abdomen enzyme		Fly abdomen enzyme			
		Musca domestica[1]		Lucilia sericata[2]	
Inhibitor	Concentration	Rate[3]	% inhibition	Rate	% inhibition
None	-	0.270	-	0.202	-
Piperonyl-butoxide	10^{-3}M	0.051	81.3	0.041	79.7
	10^{-4}M	0.097	64.2	0.083	59.0
	10^{-5}M	0.156	43.3	0.106	47.4
TMD	10^{-3}M	0.033	88.0	0.038	81.3
	10^{-4}M	0.092	66.0	0.081	60.0
	10^{-5}M	0.107	60.3	0.103	49.0
SKF-525-A	10^{-3}M	0.067	75.2	0.056	71.5
	10^{-4}M	0.114	57.9	0.096	52.0
	10^{-5}M	0.180	33.3	0.139	31.2
Sesoxane	10^{-3}M	0.054	80.0	0.038	81.7
	10^{-4}M	0.089	63.2	0.078	61.2
	10^{-5}M	0.157	42.0	0.105	48.1

(1) Mean of three experiments.

(2) Mean of replicate experiments.

(3) Rate as μM/g insect/hour.

Mouse liver enzyme

Inhibitor	Concentration	0.1 g liver equivalent per ml		0.005 g liver equivalent per ml	
		Rate[1]	% inhibition[2]	Rate	% inhibition
None	-	0.676	-	0.817	-
P.B.O.	10^{-3}M	0.125	81.4	0.172	79.0
	10^{-4}M	0.356	47.3	0.427	47.2
	10^{-5}M	0.498	26.1	0.621	23.8
T.M.D.	10^{-3}M	0.134	80.2	0.181	77.8
	10^{-4}M	0.416	37.7	0.529	35.1
	10^{-5}M	0.529	23.0	0.687	15.8

(1), (2), see footnotes on previous page.

No marked differences were observed between the degree of inhibition in incubates containing high and low liver enzyme concentrations.

Metabolism of Baygon to 5-hydroxy-2-isopropoxyphenyl-N-methylcarbamate

Enzymes prepared from houseflies, blowflies and mouse liver were incubated with $\begin{bmatrix} 14 \\ C \end{bmatrix}$-Baygon for 30 minutes at 21°. Incubations (1 ml) were carried out in 10 ml conical flasks.

The incubates were extracted with ether, which was taken down to dryness, unlabelled acetone added and evaporated under vacuum twice to remove $\begin{bmatrix} 14 \\ C \end{bmatrix}$-acetone produced during metabolism.

A measure of acetone-$\begin{bmatrix} 14 \\ C \end{bmatrix}$ production was obtained by using the same enzyme preparations and incubation conditions in Conway micro-diffusion vessels.

The ether extracts from the enzyme incubations were subjected to alkaline hydrolysis for 3 hours at 60°. These were then acidified, extracted with ether which was evaporated to dryness, and made up to a small volume, part of which was counted for radioactivity, and part of which was chromatographed on thin layers and then counted for radio-activity.

During all the operations, losses of radioactivity were minimised by multiple extractions and rinsings with ether.

Rate of Baygon ring-hydroxylation _in vitro_

	Mus musculus	Musca domestica	Lucilia sericata
[14C]-Baygon added to incubation (dpm)	178,150	178,150	178,150
[14C]-Acetone produced (dpm)	6,245 (3.50%)	539 (0.30%)	272 (0.15%)
[14C]-Label recovered in ether extract of incubate (dpm)	171,875	177,450	177,875
Recovery of [14C]-label (dpm)	178,120	177,989	178,147
Recovery of [14C]-label (%)	99.98%	99.91%	100.00%
Ring hydroxy Baygon; [14C]			
as percentage of [14C] on a chromatogram	3.97	0.32	0.18
as percentage of total added [14C]	3.82	0.31	0.18
Rates of oxidation of Baygon, (μM/g liver or flies/hour)			
to [14C]-acetone	0.07	0.30	0.19
to ring hydroxy Baygon	0.77	0.31	0.23

The above results are the mean of four experiments. Mean masses of animals used, _M. domestica_, 20 mg, _L. sericata_, 25 mg, _M. musculus_, 26 g.

Substrate (1 μmole, 208 μg) was delivered in 0.1 ml of acetone solution giving 178,150 $\pm$ 50 dpm with quench correction.

Inhibition of the Baygon 5-hydroxylation reaction

Piperonyl butoxide and sesoxane were used to inhibit the hydroxylation of Baygon in housefly and blowfly enzymes. No account was taken of the volatile $[^{14}C]$ labelled metabolites.

Inhibition of the Baygon ring hydroxylation reaction

Inhibitor	Concentration	Musca domestica		Lucilia sericata	
		Rate[1]	% I	Rate[1]	%I
None	–	0.288	–	0.212	–
P.B.O.	10^{-3}M	0.056	80.5	0.051	75.7
	10^{-4}M	0.102	64.3	0.090	56.3
	10^{-5}M	0.190	34.3	0.150	28.5
Sesoxane	10^{-3}M	0.050	82.5	0.053	75.9
	10^{-4}M	0.087	70.0	0.101	52.0
	10^{-5}M	0.171	40.7	0.151	28.7

(1) Rate as μM/g flies/hour. Mean values from replicate experiments calculated from % radioactivity on chromatogram corresponding to 5-hydroxy Baygon, and this expressed as % of total substrate added.

The metabolism of 2-isoprop(1,3-$[^{14}C]$)oxyphenol

Incubations were conducted using mouse liver enzyme, housefly enzyme and blowfly enzyme with 2-isopropoxyphenol as substrate. Ether extraction of the incubates followed by chromatography of the extracted material showed no apparent metabolism of this compound.

The above enzymes were incubated with radioactive 2-isopropoxyphenol as substrate. No transfer of volatile radiocarbon into Hydroxide of Hyamine 10-X trapping agent could be detected after incubation in Conway microdiffusion vessels.

CHAPTER III

IN VIVO METABOLISM OF BAYGON

Metabolism of $[^{14}C]$-Baygon in vivo

Insects were dosed topically with $[^{14}C]$-Baygon in acetone solution and retained in Conway diffusion vessels. The rate of O-dealkylation was measured as evolution of $[^{14}C]$-acetone.

Initial plots of time versus acetone released from houseflies indicated a very low rate of metabolism, up to five percent of the applied dose was excreted as $[^{14}C]$-acetone after six hours.

Rate of absorption of $[^{14}C]$-Baygon

Immediately after dosing, the insect was placed into a screw cap scintillation vial and retained for the required time. The vial was then opened, and 18 ml of scintillant solution run in. The insect was washed with 1 ml portions of toluene after extraction from the vial, the washings were added to the vial, which was then counted for radioactivity.

Radiocarbon remaining outside the insect was not checked for metabolites, but penetration rates were calculated from the amount of $[^{14}C]$ on the vial and exterior of the insect. The following graphs indicate these rates.

Penetration of $[^{14}C]$-Baygon.

1. grass grub
2. ♂ ⎤ housefly (V)
3. ♀ ⎦

4. ♂ ⎤ housefly (Vc)
5. ♀ ⎦

% absorption (of 0·1 μg dose)

Time (hours)
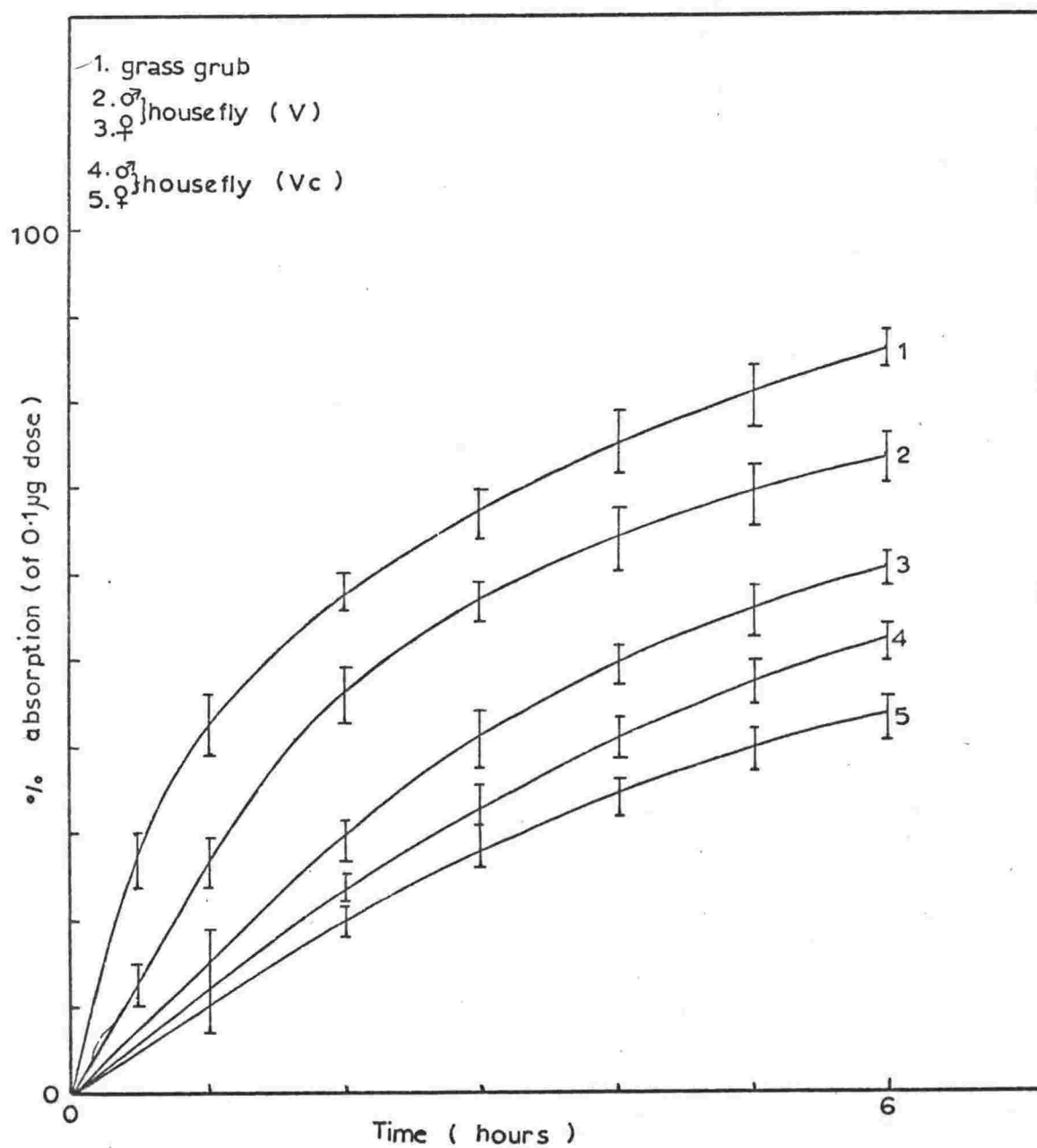
To avoid variation in penetration rates $[^{14}C]$-Baygon was injected into the flight muscle of adult, or abdominal cavity of larval insects.

In vivo metabolism

A number of insect species were injected with $[^{14}C]$-Baygon, and the rates of production of $[^{14}C]$-acetone measured. The insects were held individually in the diffusion chambers.

Musca domestica metabolism

(a) Time course of the O-dealkylation of Baygon

Three day old adult flies were injected with $[^{14}C]$-Baygon and retained as individuals for up to 24 hours. Five flies were dosed at each time point. The insects were not sexed before dosing.

The mean results of three experiments are presented.

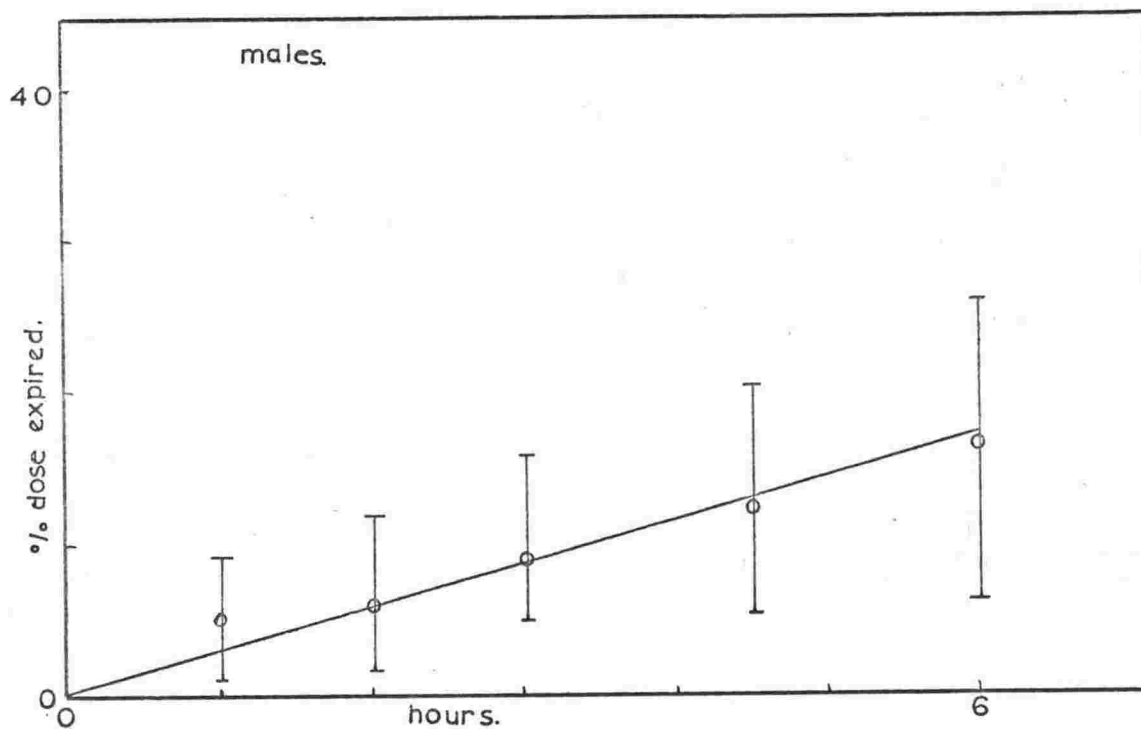| Time hours	Mean % dose metabolised	Rate/insect (nM/insect/hour)	nM/g insect/hour
0.0	0.8	–	–
1.0	4.7	0.023	1.97
2.0	8.4	0.020	1.76
3.0	12.0	0.019	1.67
4.0	14.7	0.018	1.52
5.0	22.0	0.021	1.83
6.0	22.3	0.018	1.56

Replicate experiments where flies were retained for up to 24 hours were conducted to determine the maximum amount of O-dealkylation.

Time hours	Mean % dose metabolised	Rate/insect (nM/insect/hour)	nM/g insect/hour
0.0	0.7	–	–
4,0	15.3	0.018	1.66
8.0	23.0	0.014	1.26
12.0	24.9	0.010	0.94
16.0	23.6	0.007	0.65
20.0	23.3	0.006	0.53
24.0	24.6	0.005	0.45

In these experiments, the range of dealkylation was very large, flies were therefore sexed and each point obtained from the acetone expired by each of five flies.

Time course of Baygon O-dealkylation (houseflies, dose 0·1µg)
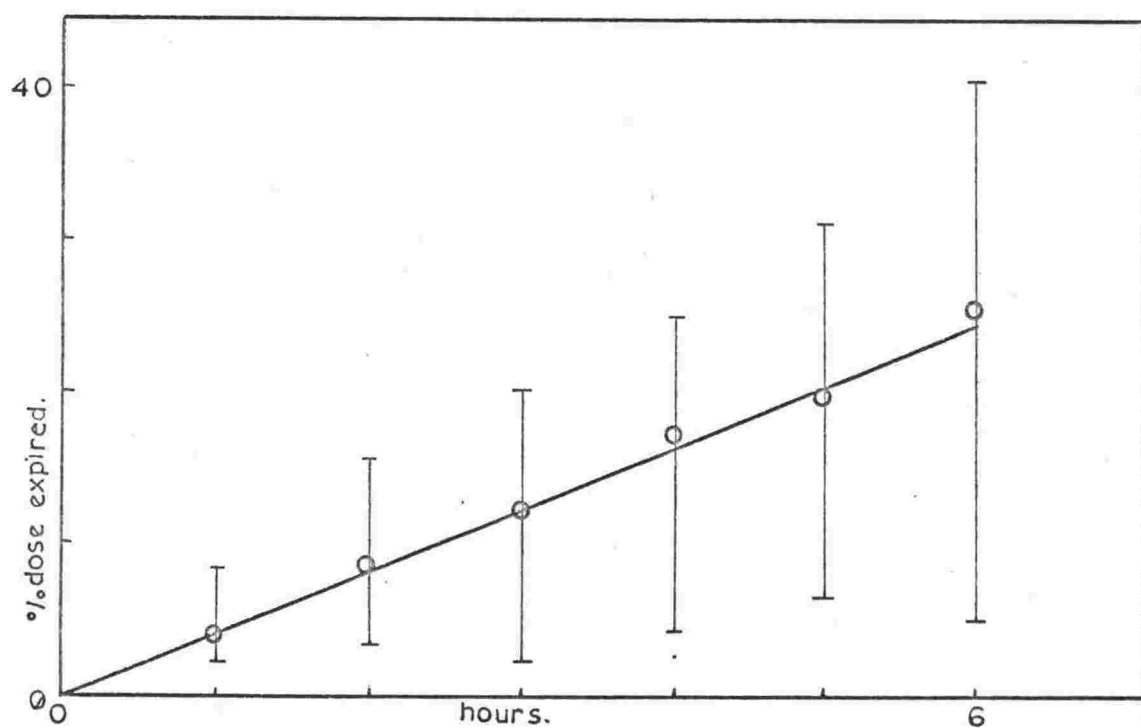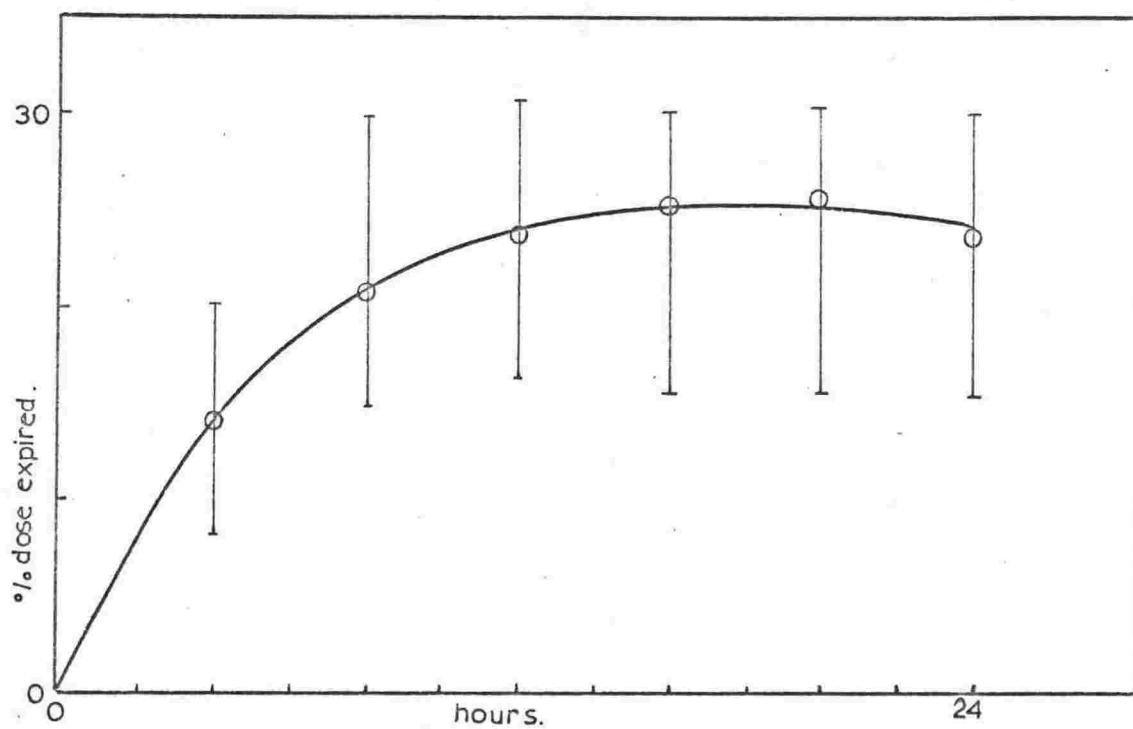
Time course of Baygon O-dealkylation (houseflies dose, 0.1 μg)
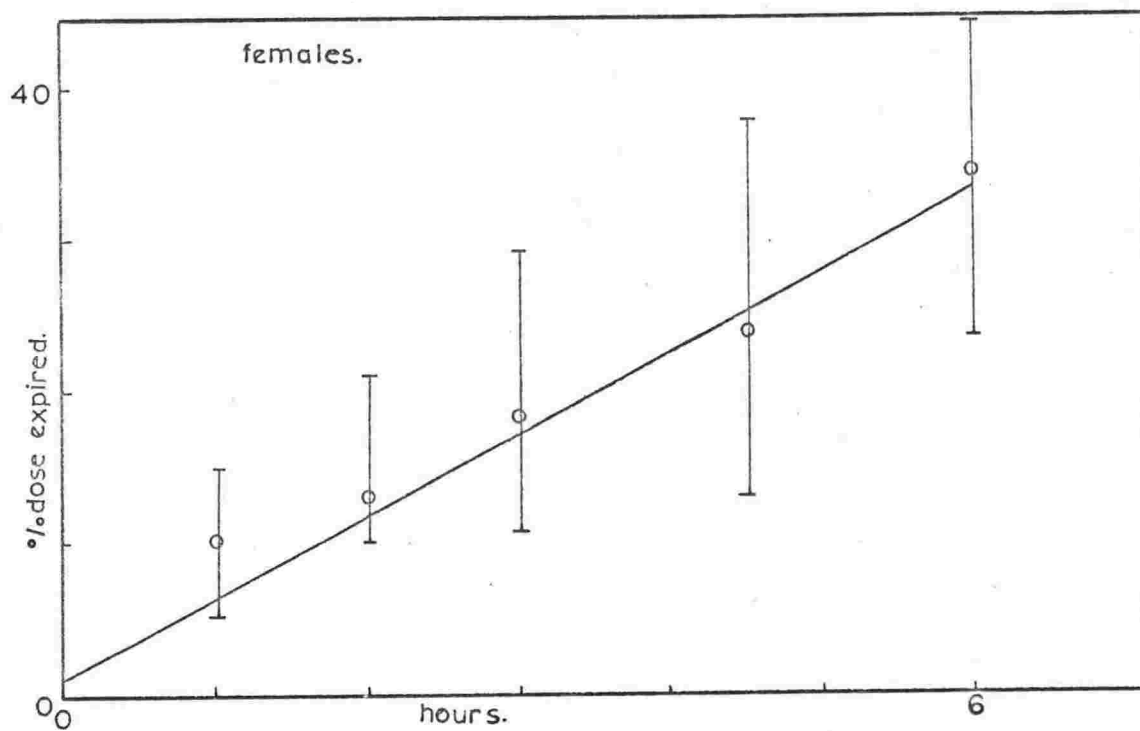
Male houseflies, mean $[^{14}C]$-acetone formation

| Time hours	Mean % dose metabolised	Mean rate/insect (nM/insect/hour)	(nM/g insect/hour)
0	0.5	–	–
1.0	4.0	0.019	2.10
3.0	7.2	0.012	1.40
4.5	10.6	0.012	1.40
6.0	14.3	0.012	1.40

Female houseflies, mean $[^{14}C]$-acetone formation

Time hours	Mean % dose metabolised	Mean rate/insect (nM/insect/hour)	(nM/g insect/hour)
0	0.7	–	–
1.0	9.2	0.044	3.80
3.0	18.4	0.031	2.50
4.5	27.5	0.029	2.20
6.0	35.1	0.028	2.15

(b)   The effect of housefly age on O-dealkylation of Baygon

Insects were dosed with 0.1 μg (0.475 nmoles) of $[^{14}C]$-Baygon. Ten individuals were dosed for each age group in each experiment by injection with an acetone-phosphate buffer solution.

Insects were dosed at various times after hatching, day one was the day of emergence of the adult. The insects were held for six hours.

Maximal rates of metabolism were found in the early adult stages.

Housefly dose vs activity.
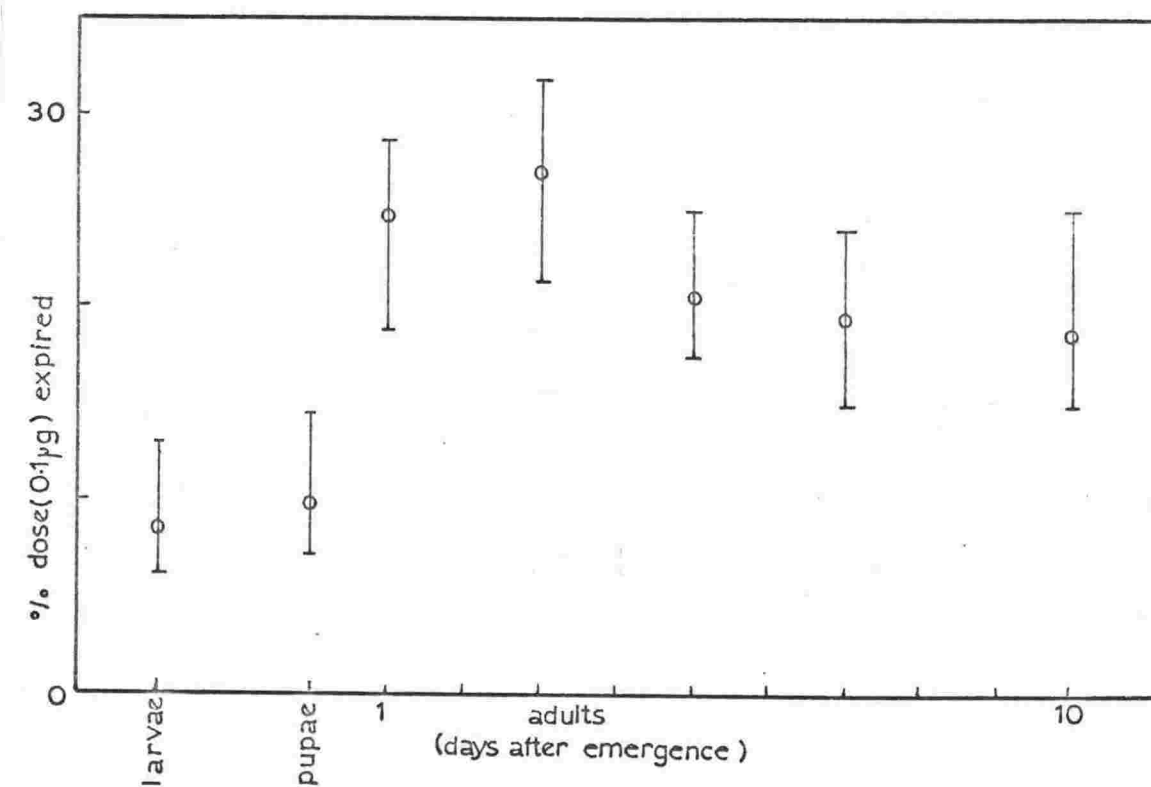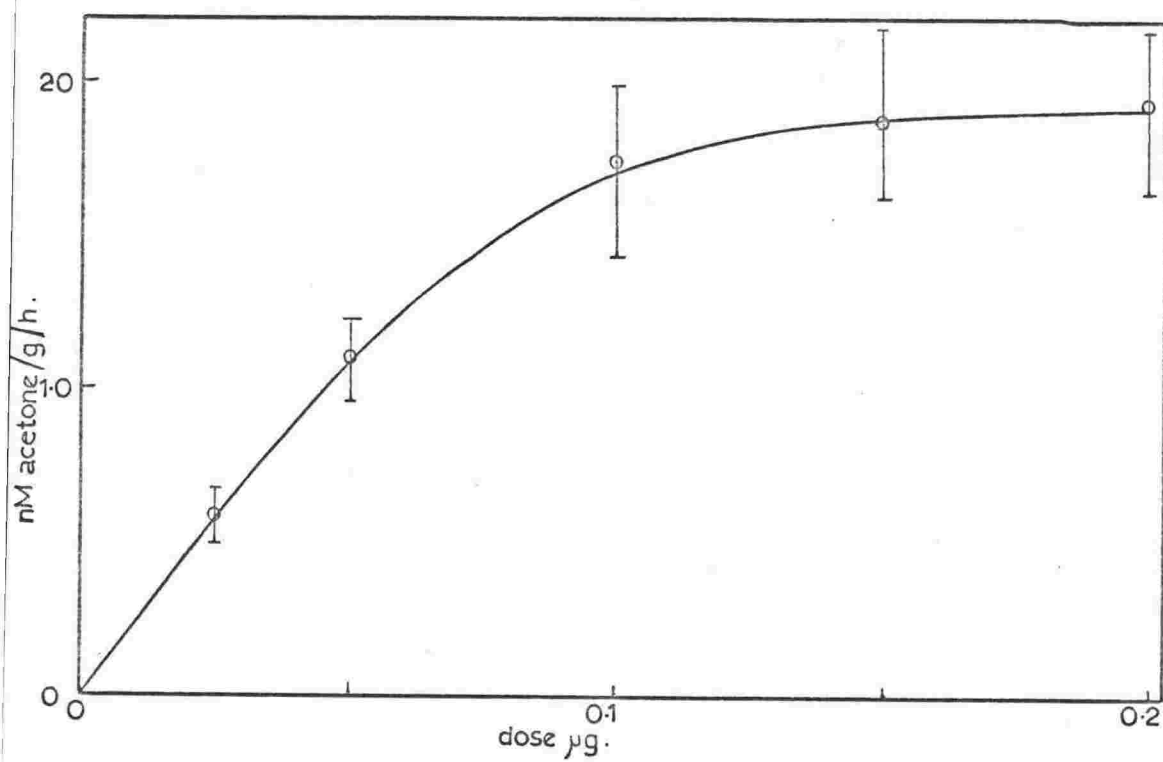
Metabolism vs housefly age

	Age	Mass mg [1]	% dose [2]	Rate/insect [3]	Rate/g [4]
Larvae	- 5	16.5	10.3	8.2	0.48
Pupae	- 1	17.0	11.1	9.3	0.45
Male	+ 1	12.0	29.6	23.5	1.95
	3	11.5	30.3	24.0	2.08
	5	11.5	29.3	23.3	2.02
	7	11.5	24.9	19.7	1.71
	10	11.0	24.2	19.1	1.74
Female	+ 1	9.0	19.5	15.4	1.71
	3	8.0	21.7	17.2	2.15
	5	9.5	19.0	15.0	1.57
	7	9.0	16.6	13.1	1.45
	10	8.5	15.5	12.2	1.43

(1) Mean mass of flies used

(2) Mean % dose recovered as $[^{14}C]$-acetone

(3) Rate/insect expressed as pmoles per insect per hour

(4) Rate/g as nmoles/gram insect/hour

The effect of the applied dose level on O-dealkylation of Baygon

Houseflies were dosed by injection with $[^{14}C]$-Baygon and retained for six hours. The rate of O-dealkylation at each dose level was graphed.

Dose (µg)	Mean % metabolism	Rate of [1] metabolism/fly	Rate of [2] metabolism
0.025	33.5	6.80	0.65
0.050	28.7	11.60	1.11
0.100	22.5	18.00	1.72
0.150	16.2	19.50	1.86
0.200	11.6	18.60	1.77

(1) pmoles/insect/hour

(2) nmoles/gram insect/hour (mass insects 10.5 mgs)

With high dose levels, in excess of the topical LD_{50} values, flies were knocked down and did not recover, the percentage dose expired as $\begin{bmatrix}^{14}C\end{bmatrix}$-acetone was found to be very low after a six hour retention period.

Dose (µg)	Mean % metabolism
0.250	1.60 (0.2)
0.500	0.4 (0.2)
1.000	0.2 (0.1)
2.000	0.1 (0.1)

The figures obtained for doses greater than 0.5 µg per fly are not sufficiently different from control values (in parenthesis) to be meaningful. As repeated experiments gave similar results, the flies were assumed to be rapidly killed by the applied doses.

The effect of inhibitors on Baygon O-dealkylation

Three day old unsexed houseflies were dosed topically with inhibitor three hours before injection of insecticide. The insects were given $0.05\,\mu g$ Baygon and $0.05\,\mu g$ or $0.025\,\mu g$ of inhibitor.

Following injection the insects were placed in diffusion units and $\left[^{14}C\right]$-acetone production measured at various times.

Inhibition of Baygon <u>O</u>-dealkylation in live houseflies

Time hours	Uninhibited % dose metabolised to [^{14}C]-acetone	Piperonyl butoxide Mean % dose metabolised[1]		Mean %[2] inhibition		T.D.M. Mean % dose metabolised		Mean % inhibition		SKF.525-A Mean % dose metabolised		Mean % inhibition	
		1:5	1:1	1:5	1:1	1:5	1:1	1:5	1:1	1:5	1:1	1:5	1:1
1.0	7.9	0.8	2.5	90%	68%	0.6	2.6	93%	67%	0.8	2.9	90%	63%
3.0	11.1	2.4	5.5	78%	50%	1.7	4.7	85%	58%	2.6	3.7	77%	67%
4.5	13.6	3.0	8.8	78%	36%	2.8	6.2	80%	54%	3.7	7.6	73%	45%
6.0	22.8	4.0	9.8	83%	57%	3.4	7.9	85%	65%	4.4	10.2	81%	55%

(1) Ratio of insecticide to inhibitor

(2) As % of uninhibited acetone production

P.B.O.   Piperonyl butoxide

T.D.M.   tetramethyldiaminodiphenylmethane

SKF.525-A   2-diethylaminodiphenylpropyl acetate

Mean % inhibition of Baygon $\underline{O}$-dealkylation in houseflies

Dose	P.B.O.	T.D.M.	SKF.525-A
1:1	53%	61%	58%
1:5	82%	86%	80%

Metabolism of Baygon by grass grub (Costelytra zealandica)

The effect of dose levels on grass grub O-dealkylation of Baygon

Grass grubs were dosed with $[^{14}C]$-Baygon by injection and retained for six hours (five insects were used for each experimental point).

Dose μg	Mean % metabolism	Rate metabolism/ grub (1)	Rate metabolism/ gram (2)
0.025	20.7	4.2	0.042
0.05	19.9	7.9	0.079
0.100	18.4	14.7	0.147
0.200	7.7	12.3	0.123
0.500	2.8	11.3	0.113
1.000	1.5	12.0	0.120

(1) pM/insect/hour

(2) nM/gram/hour

As in the previous experiment with houseflies, the rate of metabolism increases linearly up to $0.1 \mu g$ dose and then levels off. With the $0.1 \mu g$ dose, however, the grubs appeared to be dead, but the rate of metabolism found suggests 'knock down' with metabolism occurring at maximal rate.

When compared with houseflies on a dose/body weight basis the following results were obtained.

	Musca domestica		Costelytra zealandica	
Dose μg	Dose $\mu g/g$[1]	Rate/g[2]	Dose $\mu g/g$[1]	Rate/g[2]
0.025	2.5	0.65	0.25	0.042
0.050	5.0	1.11	0.5	0.079
0.100	10.0	1.72	1.0	0.147
0.150	15.0	1.86	1.5	
0.200	20.0	1.77	2.0	0.123
0.500	50.0	–	5.0	0.113
1.000	100.0	–	10.0	0.120

(1) μg Baygon per gram insects

(2) nmoles/gram/hour

At each dose level there is a ten fold difference in oxidation rates between housefly and grass grubs.

Grass grubs were injected with 0.1 μg Baygon three hours after topically applying 0.1 μg or 0.5 μg of inhibitor. The larvae were placed in the diffusion chambers and retained for the appropriate time.
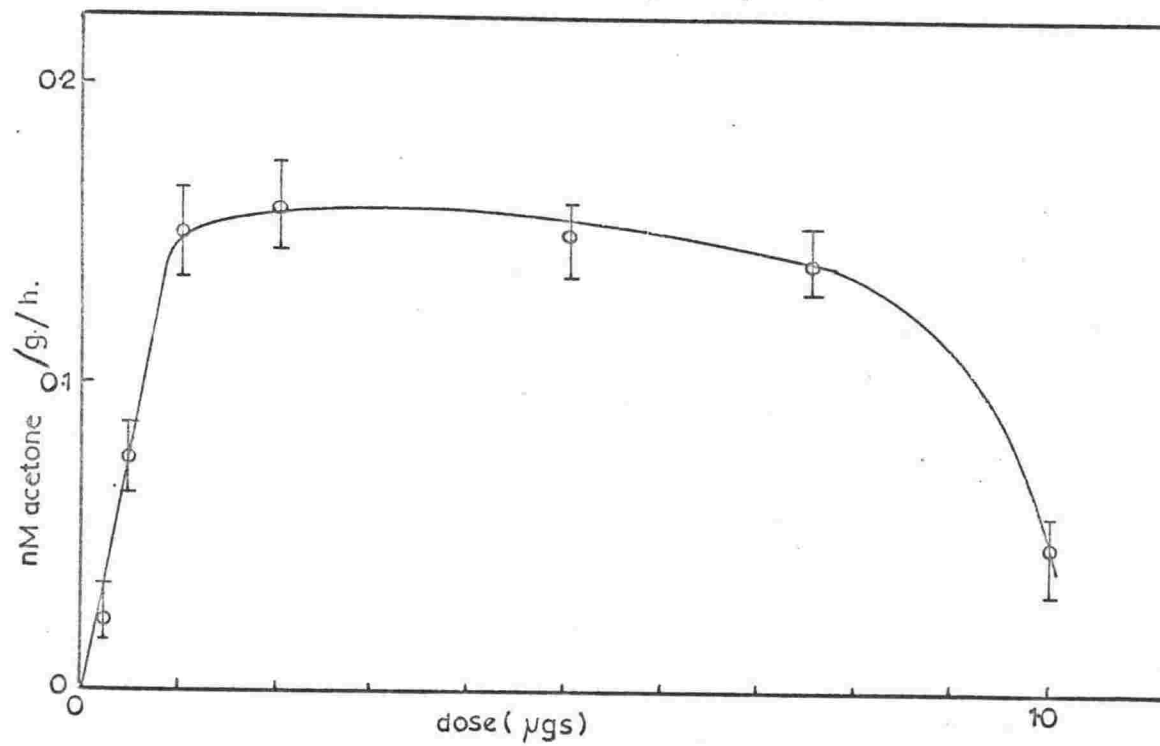
Uninhibited metabolism

Time hours	Mean % dose metabolised	Rate/grub/hour[1]	Rate/gram/hour[2]
1.0	4.8	23.1	0.231
2.0	5.2	12.5	0.125
3.0	7.5	12.0	0.120
4.0	11.9	14.3	0.143
5.0	13.8	13.3	0.133
6.0	18.4	13.1	0.131

(mean data from four experiments). (1) pM/grub/hour (2) nM/g/hour

Time course of grass grub metabolism.
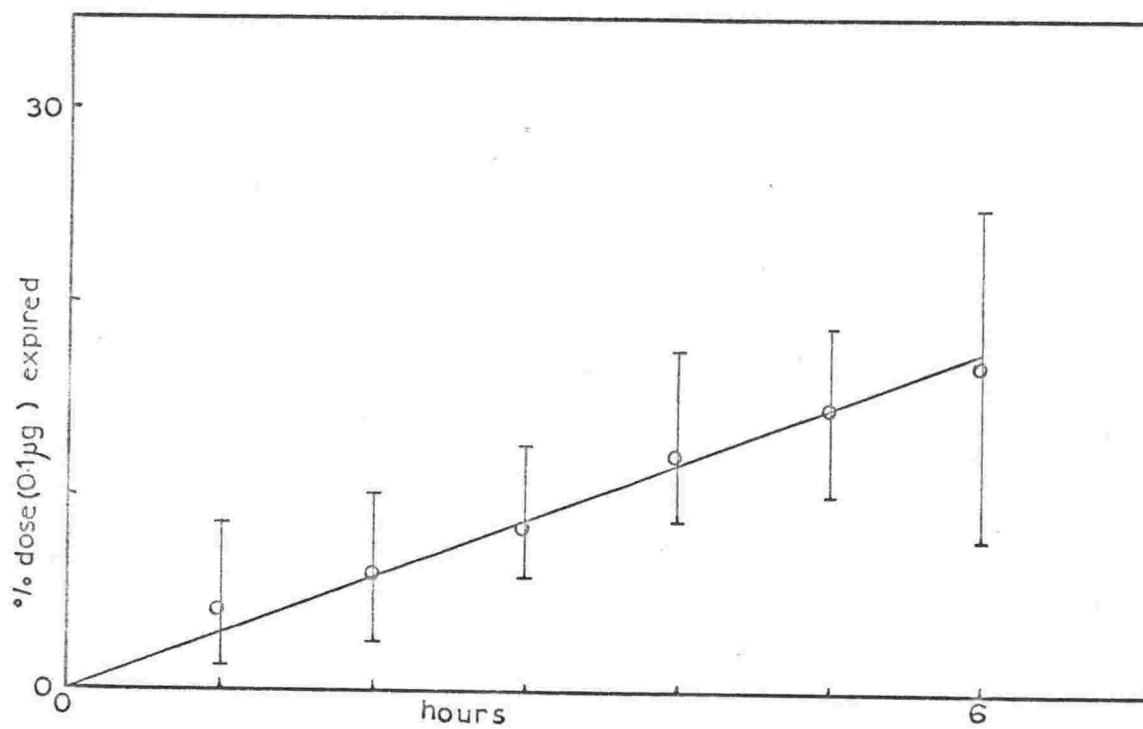
Dose response curve   (grass grub)

Inhibition of Baygon O-dealkylation in grass grubs

Time hours	Uninhibited % dose metabolised	Piperonyl butoxide		T.D.M.		SKF.525-A		Sesoxane	
		Mean % dose metabolised	Mean % inhibition	Mean % dose metabolised	Mean % inhibition	Mean % dose metabolised	Mean % inhibition	Mean % dose metabolised	Mean % inhibition
(1) 1.0	4.8	1.9	60.5	1.7	64.5	2.1	56.4	1.8	62.5
3.0	7.5	3.1	58.8	2.7	64.1	3.3	56.1	2.9	61.4
4.5	13.3	4.0	70.0	3.3	75.2	4.1	69.1	3.5	72.7
6.0	18.4	5.3	71.2	4.8	73.8	6.3	65.8	5.0	72.8
(2) 1.0	4.8	0.4	91.6	0.4	91.6	0.5	89.5	0.5	89.5
3.0	7.5	1.0	86.5	0.8	89.3	1.5	80.0	0.9	88.0
4.5	13.3	1.5	87.3	1.1	90.4	1.9	35.7	1.4	88.1
6.0	18.4	2.3	87.5	2.0	89.1	2.4	86.0	2.1	88.6

(1) Ratio of inhibitor to insecticide 1:1

(2) Ratio of inhibitor to insecticide 5:1

Mean % inhibition of Baygon O-dealkylation in grass grubs

Dose	P.B.O.	T.D.M.	SKF.525-A	Sesoxane
1:1	65.1	69.4	61.8	67.4
1:5	88.2	90.1	82.8	88.3

The O-dealkylation of Baygon by blowflies (Lucilia sericata)

Insects were dosed with 0.1 µg or 0.2 µg of Baygon, and retained for up to six hours. Insects were also dosed topically with P.B.O. three hours prior to injection of insecticide.

For these experiments the dose of P.B.O. was 0.1 µg and 0.5 µg and insecticide 0.1 µg. With 0.2 µg of insecticide, the insects were rapidly killed with no measurable production of $[^{14}C]$-acetone.

Mean % inhibition by P.B.O. of Baygon O-dealkylation in blowflies

Insecticide to P.B.O. ratio	1:1	1:5
Males	44%	78.9%
Females	46.0%	88.9%

Inhibition of the O-dealkylation of Baygon in _Lucilia sericata_

Dose sex	Time hours	Mean % metabolism	Mean rate per insect (1)	Mean rate per gram (2)	Piperonyl butoxide inhibition			
					P.B.O./Baygon, 1/1		P.B.O./Baygon, 1/5	
					% metabolism	% inhibition	% metabolism	% inhibition
0.1 μg female	0	0.4	-	-	0.3	-	0.1	-
	2	13.1	31.2	1.25	6.8	48.0	1.5	88.7
	4	26.6	32.0	1.28	14.9	44.0	3.1	88.3
	6	40.9	32.6	1.31	22.5	45.0	4.2	89.8
0.1 μg male	0	0.4	-	-	0.2	-	0.4	-
	2	5.9	14.2	0.85	3.0	49.1	2.3	61.2
	4	13.6	16.4	0.98	7.4	45.6	1.7	87.6
	6	27.5	22.2	1.34	17.3	37.1	3.3	88.1
0.2 μg female	0	0.5	-	-				
	2	7.9	38.0	1.52				
	4	12.2	29.4	1.18				
	6	22.2	35.8	1.43				
0.2 μg male	0	0.5	-	-				
	2	4.6	22.2	1.34				
	4	6.8	16.4	0.98				
	6	12.0	19.2	1.15				
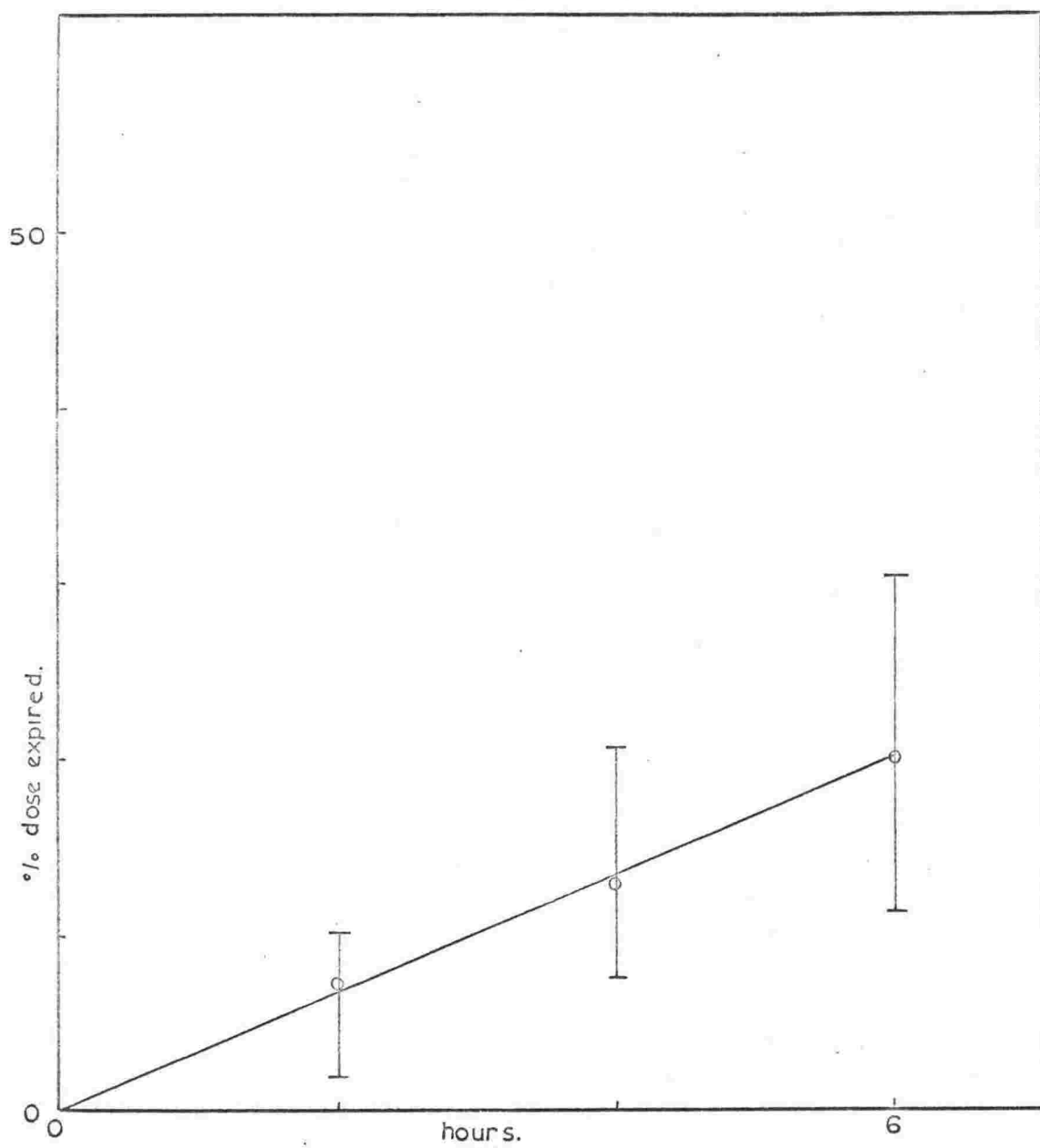
(1) pmoles/insect/hour

(2) nmoles/g insect/hour
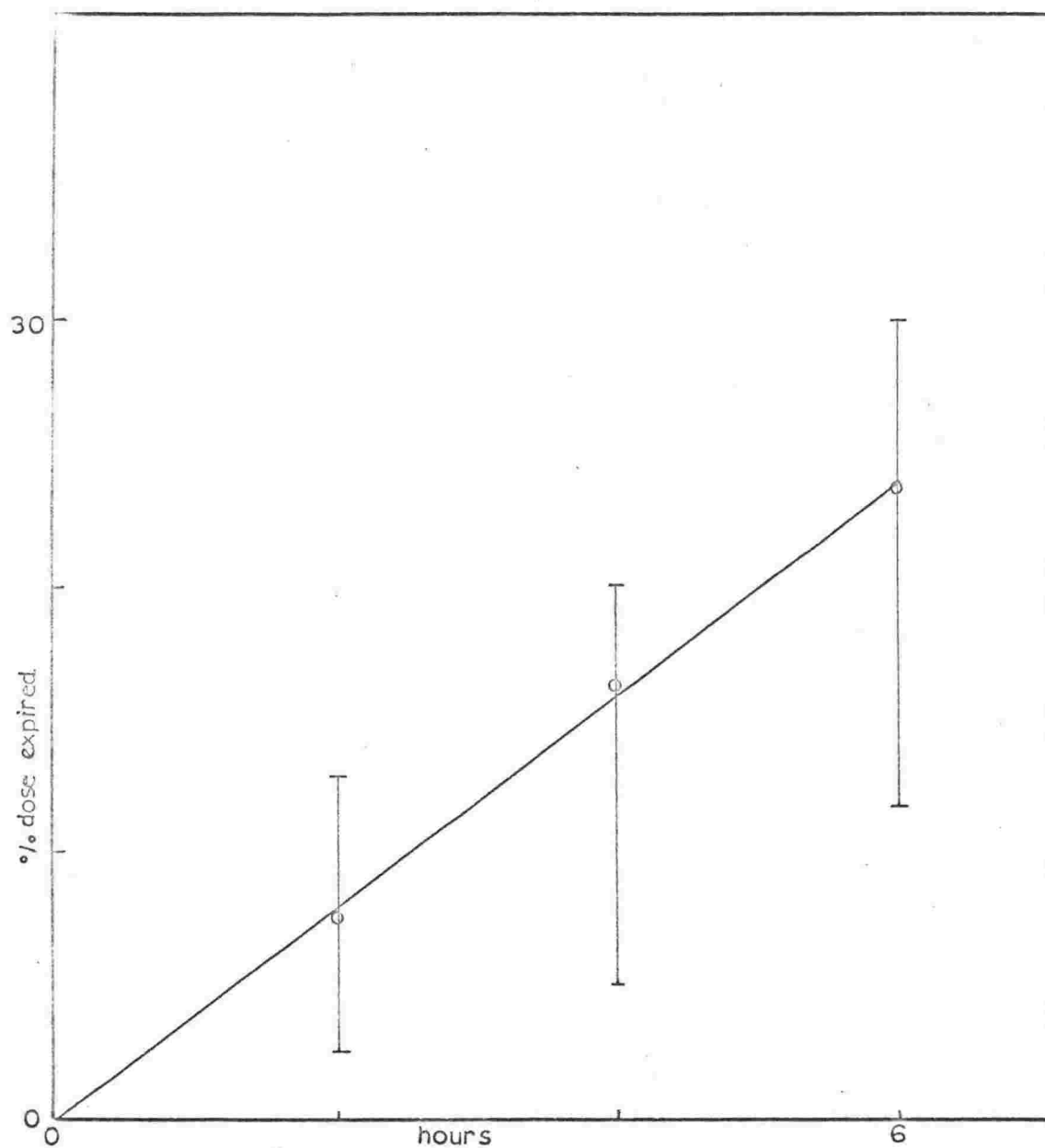
O-Dealkylation of Baygon (0·1µg) by male blowflies.

O-Dealkylation of Baygon (0·1 µg) by female blowflies.

O-Dealkylation of Baygon (0.2µg) by male blowflies.

O-Dealkylation of Baygon (0·2µg) by female blowflies.

O-Dealkylation of $\left[^{14}C\right]$-Baygon in various species

Individual insects were injected with $\left[^{14}C\right]$-Baygon in acetone-phosphate buffer solution and allowed to metabolise the insecticide for up to six hours. Results from five insects were used for each time point. The mean figures for replicate experiments are presented.

Species & dose	Time hours	Mean % metabolism	Rate/insect[1]	Rate/g[2]
Apis mellifera 70 mg mass 0.5 μg (0.72 μg/g)	2	7.1	8.5	0.059
	4	16.2	9.9	0.069
	6	23.9	9.3	0.065
0.10 μg (1.43 μg/g)	2	3.7	8.9	0.062
	4	8.9	10.7	0.075
	6	16.4	13.2	0.093
0.20 μg (2.86 μg/g)	2	1.7	8.2	0.093
	4	3.2	7.7	0.054
	6	5.6	8.9	0.063
Graphognathus leucoloma 105 mg/larvae 0.1 μg (0.95 μg/g)	2	10.8	26.0	0.248
	4	22.9	27.5	0.262
	6	32.3	26.0	0.248
0.1 μg (1.9 μg/g)	2	4.9	23.5	0.224
	4	10.5	25.2	0.240
	6	17.3	27.6	0.263

(1) pM/insect/hour

(2) nM/g insect/hour

Species & dose	Time hours	Mean % metabolism	Rate/insect[1]	Rate/g[2]
Periplaneta americana 60 mg mean mass 0.1 µg (1.67 µg/g)	2	16.0	38.5	0.230
	4	27.6	33.2	0.200
	6	64.6	50.2	0.300
0.2 µg (3.34 µg/g)	2	11.3	54.0	0.330
	4	17.4	41.9	0.250
	6	31.3	50.2	0.30
Wiseana sp. (porina) 163 mg per larva 0.1 µg (0.62 µg/g)	2	10.5	25.7	0.157
	4	21.5	25.7	0.157
	6	29.8	24.0	0.146
0.2 µg (1.24 µg/g)	2	3.9	18.7	0.115
	4	9.4	22.6	0.138
	6	14.1	22.6	0.139

(1) pM/insect/hour

(2) nM/g insect/hour

Other species were dosed by injection or allowed to come into contact with Baygon, and retained for 6 hours before measurement of $[^{14}C]$-acetone production.

Species & dose	Application of dose	Mean % metabolism	Rate/ insect [1]	Rate /g [2]
Dermestes maculatus Dose, 0.1 μg				
Adult 26 mg	Injection	18.7	14.90	0.576
Larvae 34 mg	Injection	22.1	17.80	0.521
Epiphyas postvittana Dose, 0.1 μg				
Adult 26.5 mg	Injection	14.1	11.20	0.425
Sitophilus granarius Dose, 0.1 μg				
Adult (1.87 mg)	Contact	1.61	1.29	0.692
Sitophilus oryzae Dose, 0.1 μg				
Adult (1.21 mg)	Contact	1.10	0.86	0.714
Tenebrio molitor Dose, 0.1 μg				
Adult 70 mg	Injection	14.41	11.50	0.081
Pupae 120 mg	Injection	13.21	10.57	0.088
Larvae 100 mg	Injection	5.95	4.77	0.050

(1) pM/hr/insect

(2) nM/g insect/hour

Metabolism of $[^{14}C]$-Baygon to $^{14}CO_2$

Insects were dosed with $0.1\,\mu g$ $[^{14}C]$-Baygon by injection, and retained in diffusion chambers for six hours. The trapping agents used were sodium hydroxide (2M); Hydroxide of Hyamine 10-X, and semicarbazide hydrochloride.

Species	NaOH	I HH10X[1]	II Semicarbazide	I - II
C. zealandica, larvae	0.3	17.7	17.5	0.2
M. domestica, male	2.1	17.1	16.2	0.9
M. domestica, female	2.3	35.3	34.9	0.4
L. sericata, male	2.6	23.1	22.2	0.9
L. sericata, female	2.0	39.0	38.4	0.6

Mean % applied dose recovered in trapping agent

(1) Hydroxide of Hyamine 10-X

From these results it appears that between zero and three percent of the injected dose is metabolised to $^{14}CO_2$. However some acetone will be trapped in NaOH, up to 2% of the total present, the difference between I and II will probably give a more accurate figure for $^{14}CO_2$ production. In each of the species tested this would account for less than 1% of the total dose.

The metabolism of 2-isoprop(1,3-$[^{14}C]$)oxyphenol

Radiolabelled phenol, 1.0 μg in 0.5 μl of acetone, was injected into housefly flight muscle and insects were retained in the metabolism units until death (about 36 hours). No radiocarbon was trapped in semicarbazide hydrochloride or in Hydroxide of Hyamine 10-X.

The conclusion drawn is that no metabolism occurs with 2-isopropoxyphenol, possibly because of its polar nature it is unable to reach the oxidation enzyme system.

PART VI

HYDROLYSIS OF N-METHYLCARBAMATE INSECTICIDES AND

THEIR MICROSOMAL SOLUBILITY

The hydrolysis of substituted phenyl-N-methylcarbamates

Experiments were conducted to determine the nature of, and cofactor requirements of, the enzyme systems hydrolysing Baygon, Butacarb, and 3-tertbutylphenyl-N-methylcarbamate.

The enzyme systems used in this study were prepared as previously described, radioactive substrate concentrations of 1.0 mM were used. Each carbamate was tested with the same enzyme system, but different enzymes were used for the different carbamates.

The incubates were extracted after 30 minutes with ether until no further radioactivity could be removed and the extract treated as before. The extract was run on a chromatogram (Whatman S.G. 81) to separate the phenol from the substrate and other metabolites.

The effect of added cofactor on in vitro hydrolysis of some carbamate insecticides

Addition[1]	M. musculus			M. domestica			L. sericata		
	3tBPNMC[2]	Baygon	Butacarb	3tBPNMC	Baygon	Butacarb	3tBPNMC	Baygon	Butacarb
None [3]	0.02%	0.03%	0.01%	0.01%	0.02%	0.00%	0.00%	0.02%	0.00%
NAD. G6P	0.43%	—	—	0.11%	—	—	0.16%	—	—
NADP. G6P	1.13%	—	—	0.28%	—	—	0.22%	—	—
NAD.NADP.G6P	1.41%	2.11%	1.61%	0.34%	0.43%	0.27%	0.31%	0.37%	0.23%
NAD.NADP.G6P PBO	0.51%	1.43%	0.73%	0.31%	0.49%	0.29%	0.39%	—	0.24%
NAD.NADP.G6P Met.	0.36%	1.01%	0.53%	0.08%	0.20%	0.11%	0.10%	—	0.07%

(1) NAD,NADP,   0.1 millimolar
     G6P; Glucose-6-phosphate, 4.0 millimolar
     PBO; Piperonyl butoxide, millimolar
     Met.; Metopirone, millimolar
     3tBPNMC; 3-tertbutylphenyl-N-methylcarbamate

(2) Percentage of phenol on a chromatogram

(3) No hydrolysis products could be detected after 30 minutes incubation in 0.1M phosphate buffer at pH 7.4

Mouse liver supernatant showed a requirement for NADP and NAD for hydrolysis to occur. A microsomal preparation using all three carbamates gave similar results.

Microsomes were prepared from 10,000 g supernatant from mouse liver homogenate. The microsomes from 1 g wet liver were used in each 10 ml incubation.

Addition[1]	3tBPNMC[2][3]	Butacarb	Baygon
None	0.00	0.00	0.00
NAD.NADP.G6P	0.01%	0.02%	0.01%
NAD.NADP.G6P-D'ase	1.75%	2.14%	2.26%
NADPH	1.68%	1.92%	2.14%
NADH	0.38%	0.47%	0.30%
NADPH. NADH	2.16%	2.33%	1.95%
NADPH + P.B.O.	0.47%	0.41%	0.53%
NADPH + Met.	0.36%	0.30%	0.37%

(1) NAD. NADP. NADPH. NADH   0.1 millimolar final concentration

  G6P; Glucose-6-phosphate   4.0 millimolar final concentration

  G6P-D'ase; Glucose-6-phosphate
              drhydrogenase   2 units

  P.B.O.; Piperonyl butoxide   1.0 millimolar

  Met.; Metopirone   1.0 millimolar

(2) 3tBPNMC; 3-tertbutylphenyl-N-methylcarbamate

(3) Percentage phenol on chromatogram

These data indicate a requirement for reduced cofactor, with NADH providing some 15% of the oxidation capacity.

Hydrolysis of carbamates by mouse blood

0.5 ml of mouse blood was diluted to 5 ml with phosphate buffer (0.1M, pH 7.4) and incubated with radioactive substrate (1 millimolar) for 30 minutes at 37°.

Extraction of incubate with ether, and chromatography of the extract showed that no hydrolysis occurred.

Distribution of radiolabelled carbamates

The distribution of carbamates between 10^5 g supernatant and 10^5 g pellet, and between 10^5 g pellet and phosphate buffer was determined to evaluate the effect of lipid solubility on metabolism.

Experiments were performed with conditions the same as for incubation experiments. Protein was determined by the biuret method (Lowry et al., 1951). Mean results of duplicate experiments are given.

Distribution of radioactive carbamates in mouse enzyme systems

Microsomes	Butacarb		3tBPMMC		Baygon	
	% in fraction	mg carbamate	% in fraction	mg carbamate	% in fraction	mg carbamate
Supernatant	71.25	1.425	34.4	0.687	91.0	1.82
Pellet	28.76	0.575	65.7	1.313	8.9	0.18
Total	100.01	2.00	100.10	2.00	99.90	2.00
Resuspended microsomes						
Supernatant	51.4	1.030	32.0	0.640	88.6	1.77
Pellet	48.6	0.970	67.9	1.360	11.4	0.23
Total	100.00	2.00	99.90	2.00	100.00	2.00

Mean mass of protein in each fraction

Wet weight of mouse liver: 1.000 grams
10,000 g supernatant: 0.150 grams
100,000 g supernatant: 0.088 grams
100,000 g pellet: 0.062 grams
100,000 g supernatant from resuspended microsomal pellet: 0.007 grams
100,000 g pellet from resuspended microsomal pellet: 0.050 grams

PART VII

DISCUSSION

CHAPTER I

THE NATURE OF THE METABOLITES OF SUBSTITUTED

PHENYL-N-METHYLCARBAMATE INSECTICIDES

The nature of the metabolites of aryl-N-methylcarbamates

The carbamate insecticide studied were quite resistant to non-enzymic degradation, as portrayed by their stability in buffer and buffer plus cofactor solutions at pH 7.4. The stability of carbamate insecticides has been reported to be pH dependent, they show increased rates of hydrolysis at high pH (Fukuto et al., 1967).

Extraction of metabolites from enzyme incubations and whole animals

Recoveries in excess of 98 percent of the added radiolabel were achieved from enzyme incubations of the three carbamates studied. Oonithan and Casida (1968) however reported radiocarbon losses of up to 30 percent after incubation for four hours with rat liver microsomes. This was caused in part by the formation of ether insoluble conjugate metabolites.

With live animals the recovery of radiolabel was lower than with enzyme incubations. For 3-tertbutylphenyl-N-methylcarbamate the tritium recovered from insects ranged from 95.3 to 102.0 percent, for Butacarb, 89.5 to 94.1 percent. For insects dosed with $[^{14}C]$ Baygon, it was assumed that all volatile radioactive metabolites would be expired. This was not checked by determination of the elimination of applied radioactive acetone but other workers (Casida et al., 1968) have found that up to 75 percent of the injected radiolabelled acetone was expired from living houseflies. Thus quantitative results obtained for this reaction were probably low.

Recovery of tritium label from mice dosed with 3-tertbutylphenyl-N-methylcarbamate was considerably lower than the recovery from insects. The urine was found to contain 70 percent of the radiolabel from i.p. dosed mice whereas for orally dosed animals urine contained 85 percent of the dosed radioactivity. Since the mouse faeces were not examined, any metabolites excreted in the bile were not monitored.

Separation and identification of the metabolites

The metabolites of 3-tertbutylphenyl-N-methylcarbamate that were ether extractible could be separated on thin layer chromatograms. The ether soluble metabolites of Butacarb and Baygon were not resolved by TLC into one component spots. Quantitative data for O-dealkylation, ring hydroxylation and hydrolysis were obtained for Baygon metabolism. Data were obtained for Butacarb by means of composite chromatograms run in different solvent systems. Few solvent systems gave adequate separation of the metabolites of any insecticide, and the in vivo ether soluble metabolites of Butacarb were too numerous to allow more than the assay of phenolic compounds formed after chemical hydrolysis.

The water soluble metabolites formed in vivo were unable to be separated in the systems used. The number of conjugate metabolites formed precluded both identification and quantization.

Identification of metabolites involved mainly the use of specific colour reactions, but for some metabolites ambiguous results have ensued. In the absence of authentic synthetic metabolites, any identifications made can only be tentative but when compared with previous workers' results, the present metabolite identifications appear to be consistent.

Metabolic alterations of the N-methylcarbamoyl ester group

N-methyl group oxidation and cleavage

3-tertButylphenyl-N-methylcarbamate, Butacarb and Baygon all yield at least one metabolite both in vivo and in vitro that reacts with chromotropic acid in sulphuric acid to give a red-purple coloration. This is indicative of the formaldehyde that is a breakdown product of the N-hydroxymethylcarbamates. Other workers (Oonithan and Casida, 1968; Dorough, 1970; Kuhr, 1970) have reported that aryl-N-hydroxymethyl-carbamates were readily formed from the N-methylcarbamates. These compounds are known to be markedly less toxic to mice than the original insecticides (Balba et al., 1968).

No evidence for the formation of N-hydroxy-N-methylcarbamates was found in a study by Oonithan and Casida (1968), but such metabolites might be indistinguishable from N-hydroxymethylcarbamates on the basis of colour reactions. Although no apparent N-hydroxylase activity is

shown towards carbamate insecticides a number of other compounds do act as substrates, and include p-phenetidine (Uehleke, 1969) and N-methylanilines (Das and Ziegler, 1970). The enzyme would thus appear to exhibit some substrate specificity.

N-Demethylation of 3-tertbutylphenyl-N-methylcarbamate and Baygon was found to take place both in vitro and in vivo, 2-isopropoxyphenylcarbamate was present as a minor metabolite, whereas 3-tertbutylphenylcarbamate was a major metabolite.

N-Demethylation of the carbamoyl grouping is not common among the N-methylcarbamate insecticides, but occurs widely among the N,N-dimethylcarbamate group of insecticides (Hodgson and Casida, 1961).

N-Demethylation of both the carbamoyl group and aryl amino groups appears to take place via the N-hydroxymethyl derivative (Oonithan and Casida, 1968), but the nature of the enzyme involvement is not known. In a number of compounds the N-methyl substituent is oxidatively cleaved, with the resultant formation of formaldehyde. This could take place through the breakdown of an unstable N-hydroxymethyl derivative, or be mediated entirely by an enzyme system. Aryl-N-hydroxymethylcarbamates are unusual in that they are quite stable, but they may slowly decompose to yield the carbamate derivative, although this might not account for the quantity of carbamate formed from 3-tertbutylphenyl-N-methylcarbamate. A rapid breakdown of this nature may however account for demethylation of compounds such as N-methyl aniline (Das and Ziegler, 1970), ethylmorphine

(Sladek and Mannering, 1969), and aminopyrine (Pederson and Aust, 1970).

Carbamate ester hydrolysis

Hydrolysis of the carbamate ester to yield the parent phenol took place with the aryl-N-methylcarbamates in enzyme incubations and whole animals. Some hydrolysis of oxidative metabolites may have occurred since phenols with altered tertbutyl, isopropoxyl, or aromatic groups were formed, but these may also have been produced by the oxidation of the parent phenols, since incubation of the phenols with enzyme preparations gave a small amount of metabolites. Low enzyme activity may be caused through slow penetration to the enzyme oxidation site, as the phenols are more polar than the carbamate insecticides.

Hydrolytic pathways appear to play a more important rôle in carbamate insecticide detoxication in live insects than in enzyme systems. In mice also, hydrolysis is the major route of detoxication of 3-tert-butylphenyl-N-methylcarbamate.

Metabolic alterations of the aromatic nucleus and its substituents

tertButyl group oxidation

The tertiary butyl groups of both Butacarb and 3-tertbutylphenyl-N-methylcarbamate were oxidised by insects and mice.

The tertbutyl substituent of 3-tertbutylphenyl-N-methylcarbamate was oxidised at one terminal methyl group to give the alcohol both in vitro and in vivo. In vivo it might also be oxidised to the carboxylic acid derivative to give a metabolite that was not recovered from mouse urine. No indication of a carboxylic acid metabolite was found with live insect experiments.

Butacarb was metabolised in vivo and in vitro to give metabolites which, when hydrolysed in sodium hydroxide, released six phenolic compounds. Identification based on electrophoretic migration, thin layer chromatography, and GLC retention times indicated the presence of mono, di, and tri alcoholic groups, carboxylic acid plus alcoholic group, and a carboxylic acid metabolite, 3,5-ditertbutylphenol was also present. Aldehydic functions could not be detected among the metabolites in this study.

These metabolites compare with those obtained by Chakraborty and Smith (1967) for tertbutylbenzene, where alcoholic and carboxylic metabolites were detected. Dacre (1961) found that 3,5-ditertbutyl-4-hydroxytoluene was oxidised at one butyl group to give an alcoholic function but this was not found in a later study (Daniel et al., 1968) in which it was reported that one tertbutyl group was oxidised to carboxylic acid, and another to an aldehydic function. The formation of an aldehydic grouping from 3,5-ditertbutyl-4-hydroxytoluene has been disputed by other workers (Holder et al., 1970).

Isopropoxyl substituent oxidation

Baygon was metabolised both in vivo and in vitro in insects, and in vitro in mice to acetone and 2-hydroxyphenyl-N-methylcarbamate. Oxidation appears to occur at the secondary carbon atom of the isopropoxyl group to give the unstable 2-(2-hydroxyisopropoxy)phenyl-N-methylcarbamate. This reaction is analagous to the oxidation of the isopropyl substituent of 3-isopropylphenyl-N-methylcarbamate to give the stable carbinol derivative, and to the oxidation of Furadan to yield 3-hydroxy and 3-keto Furadan.

Initial studies by Metcalf et al. (1967) indicated that Baygon was deisopropoxylated to give $^{14}CO_2$. Later work however (Casida et al., 1968) showed that the substituent was removed as acetone from live insects. The same study reported that 1-hydroxyacetone was not volatilised from live flies after injection, and could not be demonstrated to be formed from Baygon.

O-Dealkylation of drugs such as codeine to yield morphine and formaldehyde, and p-ethoxyacetanilide to p-hydroxyacetanilide and acetaldehyde (Gillette, 1963) may be mediated by the same enzyme system that dealkylates Baygon. The dealkylation of compounds such as Sumithion to yield desmethylSumithion however appears to require glutathione as a cofactor (Fukami and Shishido, 1966) and are not mediated by microsomal enzymes.

Aromatic ring hydroxylation

Metabolites of Baygon were found to contain ring hydroxyl substituents. 2-Hydroxyphenyl-N-methylcarbamate was formed through O-dealkylation, 2-isopropoxyphenol was formed through hydrolytic action and a combination of these two modes of attack led to the formation of catechol in small amounts. Other metabolites appear to be the result of insertion of hydroxyl groups. The substitution position was not definitely established, but is likely to be at the 5- position to give 5-hydroxy-2-isopropoxyphenyl-N-methylcarbamate and derivatives of this. Such a metabolite found in major proportion has been reported to be formed from Baygon in live insects and microsomal enzyme preparations (Shrivistava et al., 1969). A number of similar compounds were synthesised by the same research group, and their biological activity was found to be less than that of Baygon (Balba and Casida, 1968).

No ring hydroxylated metabolites were obtained from either Butacarb or 3-tertbutylphenyl-N-methylcarbamate. A metabolite from 2-tertbutyl-phenyl-N-methylcarbamate which was apparently hydroxylated in the 6-position of the aromatic ring was formed however.

In the case of Butacarb, steric hindrance of the 2-, 4-, and 6-positions by the ring substituents would reduce the likelihood of ring hydroxylation.

3-tertButylphenyl-N-methylcarbamate, which has a similar steric
configuration to Baygon might be expected to be hydroxylated in the
aromatic nucleus. Such an oxidation might result in the release of
tritium from the ring positions oxidised as has been reported for many
compounds, or a migration of the label, the so called "NIH" shift (Daly
et al., 1968). No release of label was found to occur, and provided
evidence that no ring hydroxylation occurred.

The major difference between the two insecticides is the presence
of the ether oxygen atom, and this may exert a strong orienting influence
on the ester group reducing any steric hindrance of the ring 5- position
of Baygon. Electronically, the two insecticides are similar and such
factors are not likely to influence ring hydroxylation reactions.

Multiple oxidation reactions

Butacarb, Baygon, and 3-tertbutylphenyl-N-methylcarbamate all gave
metabolites that were oxidised in more than one substituent group. These
include metabolites oxidised in the aromatic moiety alone, and in both
aromatic and ester moieties. Similar multiply altered metabolites are
reported to be formed from Furadan (Dorough, 1968).

The mechanism of formation of such compounds has not yet been
elucidated but such oxidations could be either sequential or concurrent.

Time course experiments have shown no time lag in the formation of multiply oxidised metabolites in vitro, and this implies that metabolites with a single oxidised function are not substrates for the enzyme system. The indication is that the molecule is oxidised in both substituents simultaneously, or that a second or third oxidation takes place without release of the molecule from the enzyme.

Conjugation reactions

Although a number of conjugated metabolites were formed in in vivo experiments, no identification of them was undertaken. The conjugates were hydrolysed by incubation with the paua viscera enzyme preparation and the released carbamate derivatives assayed. These released metabolites were similar to those formed in vitro, or to those that were ether soluble from in vivo experiments. In the case of mice dosed with 3-tertbutyl-phenyl-N-methylcarbamate and insects dosed with Butacarb, recoveries of radiolabel were low and might indicate the presence of carboxylic acid metabolites conjugated with amino acids. Such conjugates would not be hydrolysed by the hydrolytic enzymes used.

CHAPTER II

THE <u>IN VITRO</u> METABOLISM OF SOME SUBSTITUTED

PHENYL-<u>N</u>-METHYLCARBAMATES

The <u>in vitro</u> metabolism of some substituted phenyl-<u>N</u>-
    methylcarbamates

The conditions for optimal enzyme activity towards the insecticide
substrate were investigated for mammalian and insect enzyme systems,
and the results discussed below.

Effect of cofactor

Microsomal enzyme preparations frequently show activity towards
foreign compounds only when reduced cofactor $(NADPH_2)$ is present in
the system (Gillette <u>et al</u>., 1969). In the present study, the effect
of presence or absence of cofactor was determined, the concentrations
of cofactors were similar to those employed by other workers
(Chakraborty and Smith, 1967; Jordan and Smith, 1970; Leeling and
Casida, 1966).

Many studies have been conducted using 100,000 <u>g</u> microsomal
preparations, but a similar enzymic activity is obtained with 10,000 <u>g</u>
supernatant enzyme from mammalian liver. The latter preparation has
the advantages of ease of preparation, and possession of an enzyme
system that generates $NADPH_2$ from NADP and glucose-6-phosphate.

For oxidative metabolism mouse liver enzyme showed a requirement
for NADP, addition of NAD to this system increased activity by some
fifteen percent.

Insect abdomen enzyme systems also showed a requirement for NADP, NAD and glucose-6-phosphate. Nicotinamide was not added as this compound produced a slight inhibition. Addition of potassium cyanide (Jordan and Smith, 1970), or albumin (Tsukamoto and Casida, 1967), reported to improve the rates of oxidation of various substrates by insect enzymes, gave no marked benefit in the present study.

Oxidation by both mouse liver and insect abdomen enzymes was inhibited by the addition of carbon monoxide and microsomal oxidation inhibitors such as piperonyl butoxide.

Experiments using microsomal preparations from mouse liver, and enzymes from fly abdomens and mouse liver indicated that hydrolysis of carbamate insecticides requires reduced cofactors, the reaction was also inhibited by piperonyl butoxide and Metopirone.

Leeling and Casida (1966) reported that for carbaryl, hydrolysis by rat liver microsomes was independent of cofactor. However, only the portion of the metabolites that was ether soluble was assayed, the metabolites not extracted into ether, up to 85 percent of the added radiolabel, were not assayed.

Recently oxidative hydrolytic systems have been described that are involved in the rat microsomal metabolism of parathion (Nakatsugawa et al., 1968, 1969a) and diazinon (Nakatsugawa et al., 1969b). A system has been described for housefly metabolism (Nakatsugawa et al., 1969c) that is also cofactor dependent and inhibited by sesamex.

This pathway of oxidative hydrolysis appears to act on the carbamate insecticides, since with no added cofactors only minimal hydrolysis took place. The possibility must also be entertained that hydrolase activity is greater towards the oxidative metabolites of carbamates than towards the carbamate insecticide. If this were the case, a delay in the formation of phenols would be expected, but no such delay was found to occur.

Effects of buffer and pH

Housefly abdomen enzyme prepared in phosphate buffer at pH 7.4 allowed slightly higher rates of Baygon O-dealkylation than did the corresponding enzyme prepared in tris-HCl buffer at the same pH. This agrees with results published by Schonbrod and Terriere (1966). No difference in oxidation rates was found between phosphate and tris-HCl buffers for mouse liver enzyme.

pH Optima between 7.0 and 7.5 were obtained for the metabolism of 3-tertbutylphenyl-N-methylcarbamate and Baygon O-dealkylation. No correlation between the oxidation type and pH optimum could be found.

Effects of substrate concentration and enzyme concentration

The Baygon O-dealkylation reaction was used as a model system for the determination of optimal concentration of enzyme and substrate. The rate of oxidation of Baygon increases up to a maximum at about one

millimolar substrate concentration in both standard mammalian and insect enzyme systems. At this substrate concentration, a preparation of insect enzyme containing two abdomens per millilitre gives maximum oxidation rate. This is in agreement with the maximal housefly hydroxylation of biphenyl obtained by Jordan and Smith (1970). Mouse liver enzyme concentrations up to the 10,000 g supernatant from 0.1 grams of liver per millilitre gave maximum oxidation rates.

The distribution of radiolabelled insecticide between the 100,000 g supernatant and resuspended microsomes showed that the microsomes were saturated with substrate. The distribution between 100,000 g supernatant and microsomes in comparison to the above gave a measure of the binding of substrate to the supernatant protein. Butacarb alone appears to bind to any extent to supernatant protein or lipid; 71 percent of the radioactivity is associated with the 100,000 g supernatant from 10,000 g enzyme whereas 51 percent of radioactivity is in the supernatant of resuspended microsomes. The apparent order of microsomal solubility of the insecticides was 3-tertbutylphenyl-N-methylcarbamate $>$ Butacarb $>$ Baygon, but in each case, the system was saturated with insecticide. The solubility in mouse liver microsomes is inversely proportional to the housefly LD_{50} for the carbamates, and might have some influence on metabolism rates.

Effects of incubation time on metabolism

Reactions taking place in vitro with both mouse liver enzyme and fly abdomen enzymes were linear for at least 30 minutes. The reactions were halted after 30 minutes to enable comparisons of rates of metabolism to be made among the three carbamates studied. The mouse liver microsomal preparations gave linear hydrolysis reactions for less than 30 minutes and were terminated after 20 minutes. Other workers (Oonithan and Casida, 1968) appear not to have considered the linearity of metabolite formation, and compared the percentages of various metabolites present in rat liver microsome incubations after four hours.

The rate of metabolism of carbamate insecticides in vitro

The rate of metabolism of 3-tertbutylphenyl-N-methylcarbamate and Butacarb were found to be similar in each enzyme system tested. Butacarb, however, was more extensively oxidised than 3-tertbutylphenyl-N-methyl-carbamate yielding multiply oxidised metabolites that utilise a greater oxidative capacity.

Enzyme	Rate[1]	Butacarb	3-tertbutylphenyl-N-methylcarbamate
Mouse	μM/g/hour	4.10	2.96
Housefly	μM/g/hour	1.33	0.81
Blowfly	μM/g/hour	1.06	0.94
Mouse	x.μM/g/hour	7.94	3.09
Housefly	x.μM/g/hour	2.02	1.17
Blowfly	x.μM/g/hour	1.41	1.20

(1) x is the number of substituent oxidations made.

With mouse liver enzyme the expenditure of oxidative capacity was about twice as great for Butacarb as it was for 3-tertbutylphenyl-N-methylcarbamate, similarly, with fly enzyme the expenditure of oxidation capacity is about one and a half times greater.

The results summarised in the following table show that the major metabolites of 3-tertbutylphenyl-N-methylcarbamate and Butacarb are the N-hydroxymethyl and N-demethyl derivatives. Oxidation in the tertbutyl substituents also yield major metabolites. The major metabolites formed by mouse enzyme from Butacarb is the doubly oxidised derivative 3-tert-butyl-5-(1-hydroxytertbutyl)phenyl-N-hydroxymethylcarbamate but with insect enzyme, the 3,5-ditertbutylphenyl-N-hydroxymethylcarbamate was the most abundant.

Summary of reaction rates obtained for various carbamate insecticide metabolites

Reaction[1]	Carbamate	Enzyme		
		Mouse	Housefly	Blowfly
tBuOH	3tBPNMC[2]	0.62[3]	0.15	0.28
tBuOH	Butacarb	0.22	0.13	0.09
O-dealkylation	Baygon	0.70	0.30	0.19
ring hydroxy-lation	Baygon	0.77	0.31	0.23
tBuCOOH	Butacarb	0.02	0.01	0.01
$NH.CH_2OH$	Butacarb	0.48	0.73	0.55
$NH.CH_2OH$	3tBPNMC	1.28	0.16	0.26
NH_2	3tBPNMC	0.93	0.14	0.38
multiple oxidations				
tBuOH,tBuCOOH	Butacarb	0.17	0.10	0.08
tBuOH,tBuOH	Butacarb	1.08	0.03	0.02
tBuOH,tBuOH, tBuOH	Butacarb	0.27	0.13	0.08
$tBuOH,NH.CH_2OH$	Butacarb	1.86	0.20	0.11
$tBuOH,NH.CH_2OH$	3tBPNMC	0.11	0.23	0.08
$tBuOH,NH_2$	3tBPNMC	0.02	0.13	0.06
Hydrolysis				
-	3tBPNMC	0.67	0.23	0.12
-	Butacarb	0.33	0.21	0.20
-	Baygon	0.42	0.26	0.19

(1) tBuOH - oxidation to the side chain alcohol.
    tBUCOOH - oxidation to a carboxylic acid.
    $NH.CH_2OH$ - oxidation to N-hydroxymethyl derivative.
    NH_2 $\stackrel{\sim}{=}$ N-demethylation to the carbamate.

(2) 3tBPNMC - 3-tertbutylphenyl-N-methylcarbamate.

(3) Mean rates from at least two experiments $\mu M/g$ liver or flies/hour.

The ratio of <u>tert</u>butyl oxidation to <u>N</u>-methyl oxidation can be determined.

Substrate	Ratio	mouse enzyme	housefly enzyme	blowfly enzyme
Butacarb	tBuOH/NH.CH$_2$OH	1.54	0.63	0.58
3tBPNMC	tBuOH/NH.CH$_2$OH	0.54	1.31	1.11
3tBPNMC	tBuOH/NH.CH$_2$OH + NH$_2$	0.53	0.98	1.05

Inclusion of the rate of formation of 3-<u>tert</u>butylphenylcarbamate into the ratio makes the ratio for fly enzyme closer to unity, but has little effect on the mouse enzyme result.

The relative importance of each oxidation type is reversed between mouse and insect enzymes. The reversal in the ratio between the two insecticides might also be an indication of enzyme substrate specificity reflecting the bulky nature of the <u>tert</u>butyl substituents of Butacarb.

The rate of formation of the doubly oxidised derivatives of Butacarb and 3-<u>tert</u>butylphenyl-<u>N</u>-methylcarbamate, those with both an altered <u>tert</u>-butyl group and an <u>N</u>-hydroxymethyl structure, are remarkably similar in insects, but widely different in the mouse enzyme system. This latter oxidation appears to proceed about 15 times faster in mouse enzyme with Butacarb than with 3-<u>tert</u>butylphenyl-<u>N</u>-methylcarbamate.

Metabolism of the tertbutyl substituents to carboxylic acid is most evident for Butacarb, from the rate figures it might appear that the carboxylic acid is formed in any quantity only as a second oxidation reaction, possibly as a result of a second oxidation on one tertbutyl methyl group, whereas a temporary release from the enzyme's oxidative site might lead to the oxidation of a second methyl group to give the di or tri hydric alcohol derivative.

The rates of oxidation of 3-tertbutylphenyl-N-methylcarbamate to the side chain alcoholic derivative in the enzyme systems used were similar to the rates of formation of 2-hydroxyphenyl-N-methylcarbamate and 5-hydroxy-2-isopropoxyphenyl-N-methylcarbamate from Baygon. This however is some five times slower than the total rate of oxidation of Butacarb to the alcoholic derivative. The rate of oxidation of Baygon in fly enzymes corresponds to the rate of formation of the N-hydroxymethyl derivative of 3-tertbutylphenyl-N-methylcarbamate, but not to that of Butacarb. With no data about the rate of oxidation of Baygon to N-hydroxymethyl derivatives comparisons of this nature are difficult. Hook and Smith (1967) studied the rates of N-hydroxymethyl carbamate formation from a number of N-methylcarbamates, but the method used measured the total of N-demethylation and N-hydroxymethylcarbamate metabolites. With a mouse liver enzyme preparation a rate of 1.44 μM/g/ hour was obtained for Baygon metabolism to the N-hydroxymethyl derivative.

A rate of 0.36 μM/g/hour obtained for 3-isopropylphenyl-N-methylcarbamate N-hydroxymethyl formation was lower than the rate obtained in the present study for 3-tertbutylphenyl-N-methylcarbamate metabolism (2.34 μM/g/hour). If these data are strictly comparable, it suggests that the aromatic alkyl substituents confer considerable variation on the overall metabolism of such compounds.

A similar comparison may be made with the rates of oxidation of the alkyl substituents of the aromatic ring. Rates of metabolism for various aromatic alkyl substituents have been determined (Hook and Smith, 1967; Chakraborty and Smith, 1967; Chakraborty and Smith, 1964), and with mouse liver enzyme a wide variation in the rate of oxidation of methyl groups in compounds such as 4-nitrotoluene (1.73 μM/g/hour) and 4-toluidine (0.12 μM/g/hour) was found. Ring hydroxylation of biphenyl to 4-hydroxybiphenyl was determined by Jordan and Smith (1970) and a rate of reaction for enzyme preparations from houseflies and blowflies of 0.18 to 0.30 μM/g/flies/hour obtained. These data correlate closely to the rates of Baygon ring hydroxylation and O-dealkylation obtained from the present study.

It has been suggested (Chakraborty and Smith, 1967) that the insertion into a carbamate insecticide of an alkyl substituent that was only slowly metabolised as an alkyl benzene might confer on the molecule greater activity and a reduced rate of detoxication. The rate of

metabolism of the suggested alkyl group as in tertbutylbenzene was found to be 0.006 μM/g mouse liver/hour, this compares with rates 100 to 500 times higher when incorporated into the N-methylcarbamate.

Thus, until more information is obtained about the oxidation enzymes involved, the effects of the introduction of groups such as tertbutyl into a molecule will be difficult to predict with accuracy.

Inhibition of the in vitro metabolism of carbamate insecticides

The metabolism of 3-tertbutylphenyl-N-methylcarbamate and Baygon were inhibited by insecticide synergists such as piperonyl butoxide and sesoxane. Inhibition was also found with inhibitors of microsomal oxidations such as Metopirone and SKF.525-A.

With both mouse liver and fly abdomen enzymes an inhibitor concentration of 10^{-4} molar gave about 50 percent inhibition of metabolism for singly oxidised metabolites. The doubly oxidised metabolites of 3-tertbutylphenyl-N-methylcarbamate however showed 50 percent inhibition of formation rates at an inhibitor concentration of 10^{-5} molar.

Inhibitor molecules such as piperonyl butoxide are known to bind to the microsomal oxidising entity and are metabolised only slowly (Matthews et al., 1970) and hence act as alternative substrates.

If an array of 'oxidising centres' is postulated for the microsomal oxidation enzymes, and a number of such centres were involved in multiple oxidation reactions then fewer inhibitor molecules would be required to give the same inhibition of multiple oxidation as single oxidations.

CHAPTER III

THE IN VIVO METABOLISM OF SOME SUBSTITUTED

PHENYL-N-METHYLCARBAMATES

The in vivo metabolism of 3-tertbutylphenyl-N-methylcarbamate
in mice

The metabolites formed from 3-tertbutylphenyl-N-methylcarbamate by both orally and intraperitoneally dosed mice showed a similar quantitative distribution. The major metabolites were from hydrolytic reactions, these accounted for about 75 percent of the recovered radio-activity from mice dosed by either route. The similarity of the amount of hydrolysis of the insecticide by both administration routes indicates that the intestinal esterases do not hydrolyse 3-tertbutylphenyl-N-methylcarbamate.

Oxidative metabolites accounted for about 25 percent of the recovered dose, of which the major portion, about ten percent of the recovered dose, was 3-(1-hydroxytertbutyl)phenol.

Recovery of radioactive material in the urine was about 80 percent from the orally dosed mice and slightly less from the i.p. dosed mice after four days. A lower recovery may indicate more protein binding or lipid absorption of the insecticides for the i.p. dosed mice, than for the orally dosed mice. A greater fraction of the dose might also be excreted via the bile.

The in vivo metabolism of carbamate insecticides in insects

Housefly and blowfly experiments with Butacarb show that the metabolism of topically applied insecticide continues linearly for about fifteen hours. A retaining period of twelve hours was used for most experiments in which insecticide was topically applied to the insect. The rates of metabolism of carbamate insecticide in various species were obtained, but they may not be strictly comparable as the flies were the only species tested for linearity of reaction.

The metabolism of 3-tertbutylphenyl-N-methylcarbamate in insects

The metabolism of this carbamate in the insect species tested more closely reflects the in vitro metabolism than does the mouse in vivo metabolism. Unlike mice, insects are generally less able to metabolise by hydrolytic pathways, oxidative metabolites being of major importance. Grass grubs and yellow mealworms exhibit most hydrolytic activity, whereas flies metabolise 3-tertbutylphenyl-N-methylcarbamate extensively by oxidative attack.

Houseflies completely metabolise a topical dose of one microgram of insecticide within 36 hours, and about half of the dose in twelve hours. There was essentially no difference between adult and larval housefly metabolism of 3-tertbutylphenyl-N-methylcarbamate, but the

larval forms were more susceptible to a topical applied dose. This susceptibility might be caused through differences in penetration rates or target organs in adult and larval insects, since no major differences in metabolism were found.

Results obtained from seven strains of housefly suggest that the resistance shown by some strains was due mainly to factors other than metabolism. Tsukamoto and his colleagues (1968) found that for houseflies, resistance was in part conferred by factors on the second chromosome. This resistance was not caused by increased metabolic activity, and possibly resulted from factors such as thickened cuticle or reduced membrane permeability, and resistance in the houseflies used in this study might be conferred by this chromosome.

The rates of metabolism by houseflies of 3-tertbutylphenyl-N-methylcarbamate to the N-hydroxymethyl and N-demethyl derivatives are slower than the rate of tertbutyl group oxidation in the ratio of one to 0.56 to 0.92. The rate of tertbutyl group oxidation was up to four times faster than the rate of hydrolysis, which corresponded to about 20 percent of the metabolism.

Blowflies show a similar pattern of metabolism to that found for houseflies, but the rate of metabolism was lower.

Honey bees were retained for six hours only, since they were particularly susceptible to the insecticide. In six hours the insects metabolised 20% of the applied dose, 60 percent of the metabolites were water soluble, and about 30 percent of the total metabolites were formed through hydrolysis. The ratio of oxidation of tertbutyl to N-methyl groups was similar to that obtained for houseflies.

Grass grubs and all stages of yellow mealworms metabolise 3-tert-butylphenyl-N-methylcarbamate primarily by esterase action. Grass grubs produce up to 25 percent of their metabolites by hydrolytic action, whereas for yellow mealworms the figure is 75 percent. In these two species the relative importance of tertbutyl group oxidation and N-methyl oxidation is the reverse of that found for flies. The tertbutyl group is oxidised at about half the rate of the N-methyl group. These two species also formed less ether extractible metabolites than other insects, indicative of a more efficient conjugating system.

The rates of metabolism of 3-tertbutylphenyl-N-methylcarbamate in all insects tested were within the range 0.09 to 0.25 nM/insect/hour, but on a weight of insect basis ranged from 0.67 nM/g/hour for grass grubs to 22.9 nM/g/hour for Y strain houseflies. This wide variation might account for some of the selectivity of carbamate insecticides.

The metabolism of Butacarb in insects

Butacarb has been proposed as an insecticide to control the sheep blowfly (Lucilia sericata), the insecticide was found to be quite toxic

to first and second instar blowfly larvae $(1.5 \mu g/g$ insect) but less

toxic to the adult housefly $(39 \mu g/g$ insect) (Fraser et al., 1967).

These data are comparable on an individual insect basis since the first

and second instar larvae weigh between one and two milligrams, and the

adult up to 30 milligrams.

In the present study, the first and second instar larvae of both

housefly and blowfly were found to be susceptible to ten nanograms of

Butacarb per larva $(5 \text{ to } 10 \mu g/g)$. Smaller doses were not administered

because the specific activity of Butacarb was too low to give adequate

counting statistics. Prepupal larvae withstood 10 ng/insect $(0.5 \mu g/g$

insects), and the course of metabolism indicated that they were less able

than the adult to eliminate the insecticide. Although the masses of larvae

and adult were similar, the adult insect eliminated the insecticide up to

ten times faster than the larval stage. These results differ from those

obtained for 3-tertbutylphenyl-N-methylcarbamate where the metabolism in

larvae and adult flies was similar. This effect on Butacarb metabolism

might be caused by its lower lipid solubility, or by the number of

electrons that can be expended in oxidising the tertbutyl groups.

Phenols only were assayed in the metabolism of Butacarb; hydrolysed

metabolites yielded six phenols each with sufficient radioactivity to

be measured.

In the case of flies and grass grubs, about 30 percent of the dose

applied was recovered as altered phenolic compounds, this would indicate

that only a small amount of oxidation to N-hydroxymethyl derivatives took place since after the twelve hour retention time only 50 percent of the applied dose would be metabolised.

Adult flies produced up to three times the amount of carboxylic acid metabolites that were formed by larvae. In grass grubs, eleven percent of the applied dose (about 20 percent of the metabolites) was eliminated as carboxylic acids.

No measure of the alterations to the carbamoyl moiety were obtained, and hence the importance of such reactions was not ascertained.

The metabolism of Baygon in insects

The O-dealkylation of Baygon was used as an indication of the oxidising ability of live insects.

Topical application of the insecticide gave a low metabolic rate in houseflies, about five percent was eliminated as $[^{14}C]$-acetone after six hours. The low rate of metabolism was caused in part by the relatively slow penetration of insecticide, about 50 percent of the applied radioactivity remaining outside the insect after four hours. When the insecticide was administered by injection, a marked increase in the rate of metabolism was noted.

Houseflies, blowflies and grass grubs all gave wide individual variation in the percentage $[^{14}C]$-acetone excreted. Female insects gave higher percentages than did male insects, and dosing of separate sexes reduced the variation of metabolism. When the rates of metabolism were

calculated on a mass of insect basis, the difference between the rates of oxidation between male and female flies was reduced, since male flies were always of less mass than the female flies of the same age.

Dose response curves with houseflies, blowflies and grass grubs showed that the rate of metabolism was similar at each dose level employed. The percentage of the dose eliminated as $[^{14}C]$-acetone decreased with higher doses, indicating that the metabolic systems involved were all operating at maximum efficiency.

The metabolism of Baygon to acetone was found to be greatest with houseflies over two days old. Three day old houseflies were two to three times more efficient at oxidising Baygon than were the larvae or pupae. These results support those of Perry and Buckner (1970) who found that cytochrome P 450 levels increase with housefly age.

Application of insecticide synergist, or microsomal oxidation inhibitor to the insect prior to injection of insecticide caused a reduction in the rate of O-dealkylation of Baygon. A ratio of one part synergist to one part of insecticide caused about 50 percent inhibition of metabolism whereas a five to one ratio of synergist to insecticide gave about 80 to 85 percent inhibition of oxidation.

Shrivistava et al., (1969) found that application of piperonyl butoxide prior to the insecticide to houseflies caused no reduction in Baygon O-dealkylation.

The rates of oxidation of Baygon were determined for a number of species of insects. Houseflies, grain weevil and rice weevil gave the greatest rates of oxidation on a weight basis, whereas the lowest rates were found with honey bees, yellow mealworms and grass grubs. For Baygon as with 3-tertbutylphenyl-N-methylcarbamate the wide variations in metabolic rates among the various insects will ensure some selective insecticidal action.

CHAPTER IV

FACTORS AFFECTING THE SELECTIVITY OF SOME SUBSTITUTED

PHENYL-N-METHYLCARBAMATES

Factors affecting selective insecticidal action

The structure of the carbamate insecticides greatly influences the compound's effectiveness as a selective poison. Factors that are structure dependent include penetration of internal and external barriers in the animal, the interaction with the acetylcholinesterase target enzyme, and the ease of detoxication of the carbamate by the particular animal species.

Interaction of acetylcholinesterase with the inhibitor is thought to be similar for all species for a particular carbamate, but a large variation is found between different carbamate insecticides in both I_{50} and enzyme affinity (see tables pp. 11,12) but these data do not correlate to the observed LD_{50} values in the case of houseflies (Kolbezen et al., 1954). These factors are unlikely therefore to greatly influence the selective action of carbamate insecticides.

The polarity of the carbamate must be such that it is able to penetrate the cuticle and internal barriers of the animal. To be an effective insecticide, the compound should be highly lipid soluble. The order of mouse microsomal solubility for the carbamates studied was 3-tertbutylphenyl-N-methylcarbamate > Butacarb > Baygon which surprisingly is the reverse of their housefly LD_{50} values. The requirement of lipid solubility for cuticular penetration would also mean that the insecticide would arrive rapidly at the site of detoxication. Differences in lipid

solubility would allow the carbamate insecticides to be metabolised at different rates, and differences in the external and internal barriers among insect strains and species would allow a variation in the penetration rate of a particular poison and might lead to some selectivity.

It has been suggested that the effectiveness and rate of detoxication of insecticides might be governed by the ease of oxidation of the alkyl substituents on the aromatic nucleus, and that these alkyl groups might act as "selectophores" (Chakraborty and Smith, 1967; Hook and Smith, 1967).

The metabolism of three insecticidal carbamates was studied in a number of species, and in all species tested the metabolites formed from each carbamate were similar. The rates of metabolism were widely different however, with some species, mice and yellow mealworms, hydrolytic metabolism was the major pathway, whereas with other insects, oxidative metabolites were preponderant. Although a wide variation in rates of metabolism was found no oxidation reaction was truly species specific.

Inhibition of metabolism was brought about by application of a synergist prior to application of insecticide, or by addition of synergist or microsomal inhibitor to the in vitro system. In vitro all single oxidations were inhibited to the same extent with a particular inhibitor concentration, similar inhibition resulted from all the inhibitors used. Thus it would appear that the mechanism of inhibition of the various single oxidation reactions was similar, and that this was different from the

inhibition of the reactions giving multiply oxidised metabolites. In vivo a marked reduction in Baygon O-dealkylation was caused by the synergists, the reduction in metabolism corresponding to reduction in LD_{50} values.

The ease of oxidation of a substituent alkyl group in carbamate insecticides appears to affect the selectivity of the compound. Oxidation of such substituents is influenced by the presence of other groups in the molecule, 3-tertbutylphenyl-N-methylcarbamate and Butacarb are both oxidised in the tertbutyl substituents, but the second tertbutyl substituent in Butacarb allows an increased rate of oxidation.

An understanding of the structural factors influencing metabolism of carbamate insecticides is necessary before a compound's activity may be predicted. Studies of the metabolism of carbamate insecticides would enable compounds of this class to be designed for selective activity, but could not be applied to the design of other classes of drugs since the factors affecting metabolism would not be the same.

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