THE OXIDATION OF SOME ORGANIC COMPOUNDS IN INSECTS AND MAMMALS

by

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ABSTRACT

The metabolism of p-nitrotoluene in both mammals and insects mainly involves oxidation of the methyl side chain to a carboxyl group. Among the various species of insects studied *in vivo*, the most rapid oxidation occurred in flies. In isolated locust organs, most oxidation took place in fat body and gut. Homogenates of insect tissues were assayed for enzymes capable of oxidizing p-nitrotoluene to p-nitrobenzoic acid. Only locust fat body and fly abdomen homogenate 10,000g. supernatant contained enzyme activity. Various properties of the locust fat body enzyme system were studied. The 90,000g. 'microsomal' sediment of this supernatant was inactive unless combined with 90,000g. supernatant and required no added cofactors. Homogenates of other insects or locust organs and 10,000g. sediment from locust fat body inhibited microsomal oxidations carried out by locust fat body or rabbit liver enzyme. Inhibitory power was high in homogenate of whole flies and it could not be reversed by the addition of excess amounts of $NADPH_2$. Inhibition appeared to involve both irreversible inactivation of the enzyme and the removal of essential cofactors.

Rates of oxidation of toluene, n- and iso-propylbenzene

and *n*- and *tert*-butylbenzene were measured in 10,000g. supernatants of homogenated locust fat body, fly abdomen and rabbit liver. Activity per g. of animal was of the same order in the different species. In the rabbit, the alkyl side chain hydroxylase was found to be located in the microsomal fraction of liver and required $NADPH_2$ for its activity. Methyl groups were oxidized more rapidly than the higher alkyl groups and in higher homologues, hydroxylation of the α-methylene group occured more readily than terminal or penultimate oxidation. Oxidations in vertebrates and insects were inhibited by piperonyl butoxide and similar insecticide synergists. The oxidation enzyme was stimulated by pretreatment with phenobarbitone or 3,4 benzpyrene in rats but not in insects.

PREFACE

This thesis describes work carried out in the Department of Biochemistry of St. Mary's Hospital Medical School between April 1962 and March 1965.

In the last decade, metabolic studies of foreign compounds in mammals have yielded a body of knowledge invaluable to the understanding of the relationship between the chemical structure and effectiveness of biologically active molecules. Moreover, these studies have turned up metabolic products which were found to be more potent than the parent compounds in clinical use. In recent years, attempts have been made to extend these investigations to the field of insecticides in order to find out compounds of predictable selectivity. Sometimes compounds with selectivity between different species of insects are desired to avoid the destruction of useful insects together with the pest's predators and parasites. The other type of selectivity concerns the most important requirement of an ideal insecticide- high insect and low mammalian toxicity. With the extensive use of insecticides, the effect of their presence in foodstuffs upon public health has become the most important of all considerations

in the design and use of such compounds. Our knowledge of selectivity is most advanced in its relation to metabolism. It is in the establishment and exploitation of metabolic differences that there is room for greatest ingenuity, since quite subtle modification of a molecule may profoundly alter its behaviour in biochemical systems and may lead to the development of new compounds with the desired properties.

I am most indebted to Prof. R. T. Williams for his interest and guidance throughout this work and for the excellent facilities made available to me in his department. I express sincere gratitude to Prof. J. N. Smith for invaluable advice, encouragement and patience in his supervision of this work and for his continued interest after leaving the department for Victoria University, Wellington, New Zealand. I wish to thank Dr. J. W. Bridges and Dr. D. V. Parke for their helpful suggestions at various stages of this work and during the preparation of the manuscript. My thanks are due to the members of the staff of the department with whom I have had many fruitful discussions. I would also like to thank Mr. Audas and his staff of technicians who have been always helpful and Miss H. B. Turbert for her assistance with the experiments on the stability of $NADPH_2$ in

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Finally, I wish to thank the Agricultural Research Council for the financial support and Anti-Locust Research Centre and Rothamstead Experimental Station for supplies of insects.

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CHAPTER I.

INTRODUCTION.

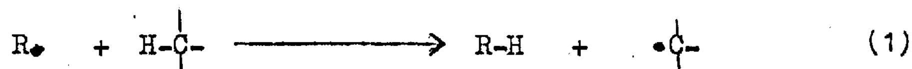
CHAPTER I. INTRODUCTION

Part I. Chemical oxidation of organic compounds.

The term 'oxidation' has been part of common chemical usage since the time of Lavoisier. During that period of close to two centuries, several definitions of the term have been offered. Transfer of oxygen, transfer of hydrogen or transfer of electrons have been the commonly used descriptions of oxidation and reduction processes, the last being the favoured definition at the present time. For practical purposes, organic chemists use the term oxidation to denote processes including oxygen addition or hydrogen abstraction, while inorganic chemists find it more convenient to picture oxidation as electron removal. The two descriptions are equivalent, in that in any oxidation process there is a net gain of electrons by the oxidizing agent, but since in practically all covalent compounds every atom is always surrounded by completed electron shells, it is often difficult to gauge the oxidation levels of organic compounds except by reference to their hydrogen or oxygen contents.

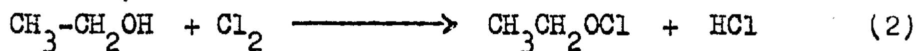
Classification of oxidation processes :- The study of oxidation reaction mechanisms is essentially an examination of ways by which electrons can be removed from organic compounds. Covalent bond fission is an essential feature of

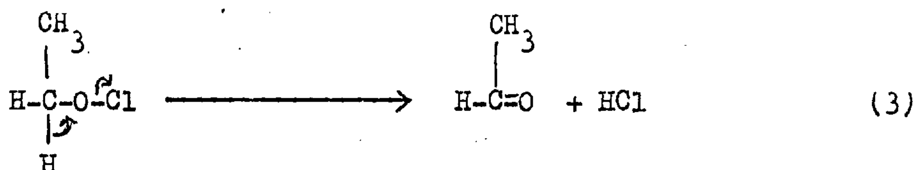
organic reactions and it can be effected by two different pathways, vis homolytic reactions in which electron pairs are symmetrically disrupted and heterolytic reactions in which electron pairs are transferred from one particle to another as an individual entity. These two pathways have distinguishable characteristics. In homolytic oxidations electrons are removed singly from organic molecules by active atoms or free radicals. It usually involves the removal from the molecule of one electron together with a hydrogen nucleus.



The initial product must undergo a reaction of similar type or combine with another free radical before stable entity results. Molecular oxygen forms peroxides by this reaction (see Eqs. 5 & 6). Homolytic electron-transfer reactions require considerably less activation energy than that needed for the direct breakage of covalent links, hence homolytic oxidations once started, proceed very rapidly indeed. The traces of free radicals required for the initiation of homolytic oxidation may be formed by the thermal dissociation of molecules which have weak covalent bonds by disruption of molecules by exposure to radiant energy, high energy particles or electrons or by single electron transfer from ions of

transition element having incomplete inner electron shells. Consequently, oxidations which are demonstrably prone to catalysis in these ways are homolytic in type. In heterolytic oxidations the carbon-hydrogen bond is cleaved by either hydride or proton abstraction. A heterolytic oxidant approaches an organic molecule at the point at which its valency electrons are most exposed. Further, heterolytic oxidations often involve sequences of reaction ending with the elimination from an organic complex of the oxidant together with its extra electrons as shown below :-


$$CH_3\text{-}CH_2OH + Cl_2 \longrightarrow CH_3CH_2OCl + HCl \qquad (2)$$

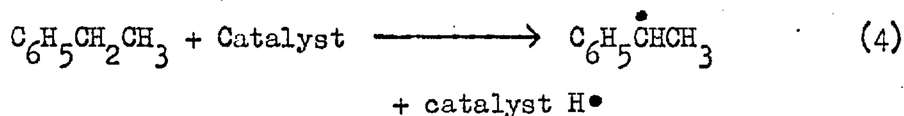


Heterolytic reactions yield stable molecules, or ionic products in one or at most two consecutive stages and very seldom lead to chain reactions. On the other hand, they usually require more activation energy than homolytic reactions and so tend to be slower processes.

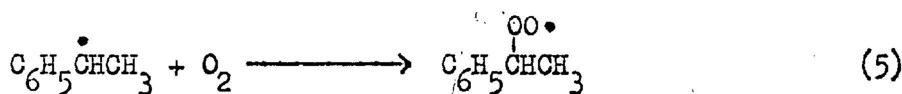
The number of oxidizing agents now known is so large that it is impossible to cover them in a short review. It is also difficult to classify them in terms of the mechanisms of their

action, as an oxidizing agent can react in more than one way under different conditions and with different compounds. The following survey will therefore be confined to some important oxidation reactions.

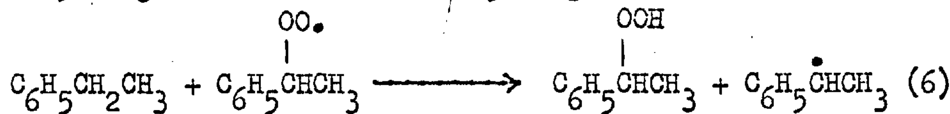
Autoxidations :- The term 'autoxidation' is applied generally to show oxidation which can be effected by free oxygen at moderate temperatures. It is promoted by light and small quantitites of many catalysts, notably the oxides and oil-soluble salts of heavy metals as well as by various peroxidic substances. In the initial stages, peroxides are characteristic products of almost all the autoxidations of organic compounds. As the oxidation proceeds these peroxides break down to complex mixtures of more stable products. It was suggested that the autoxidations are free radical processes. Thus the following mechanism has been proposed for the catalytic liquid phase oxidation of ethylbenzene by air, which gives acetophenone and methylphenylcarbinol (Emerson et al 1948).



$$C_6H_5CH_2CH_3 + \text{Catalyst} \longrightarrow C_6H_5\dot{C}HCH_3 + \text{catalyst H}\bullet \quad (4)$$

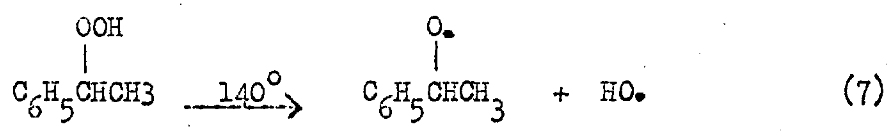


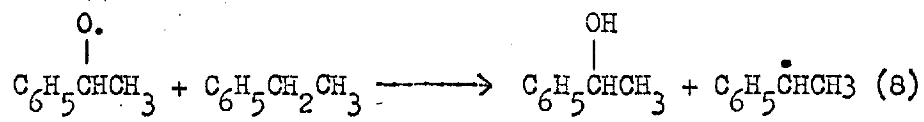
$$C_6H_5\dot{C}HCH_3 + O_2 \longrightarrow C_6H_5CH(OO\bullet)CH_3 \quad (5)$$

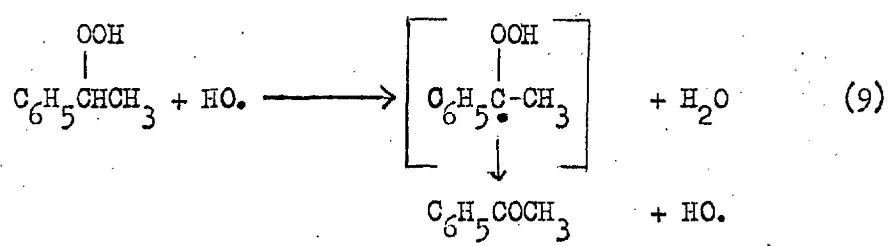


$$C_6H_5CH_2CH_3 + C_6H_5CH(OO\bullet)CH_3 \longrightarrow C_6H_5CH(OOH)CH_3 + C_6H_5\dot{C}HCH_3 \quad (6)$$

At 140° the α-phenylethyl hydroperoxide can decompose thermally into two free radicals.



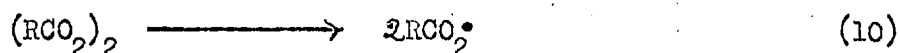


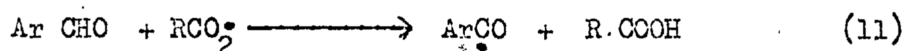


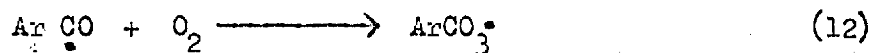
Paraffin hydrocarbons are much more resistant to auto-oxidation than are olefins; but the side chains of aromatic hydrocarbons are prone to attack in the α-position to give substituted benzyl radicals in which the odd electron can hybridize with the π electrons of the benzene ring.

<u>Peroxidation</u> :- The peroxy compounds of the general formula R-O-O-R are all valuable oxidizing agents. The reactions of these compounds depend to some extent on whether or not hydrogen is one of the attached groups. The hydro-peroxides, including hydrogen peroxide, have a wider range of reactions than do the ordinary peroxides. The central O-O bond of hydrogen peroxide and its analogues is a relatively

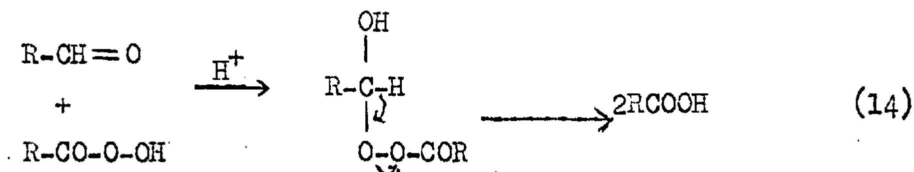
weak one (bond strength not more than 66K.cal) and consequently the compounds in this series can fairly easily split to give H-O. and R-O. radicals. Most oxidations in which the dialkyl, diaryl or mixed peroxides are involved are in fact autoxidations involving molecular oxygen, and the role of the peroxide is merely that of an initiator of the chain reaction.





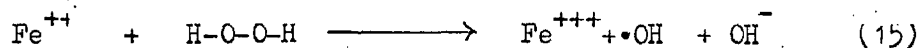


The per-acid can react with more aldehyde by an acid-catalysed heterolytic reaction.

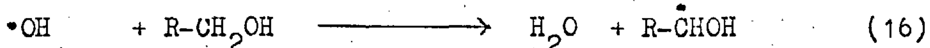


Alkyl and aryl peroxides can function as oxidants in their own right under certain circumstances (Stewart 1964). Dehydrogenation can be effected with radicals like phenyl, Ph., Methyl, Me., *tert*-butoxy, Me_3C-O., Benzoyloxy, PhCo.O., but with greatly differing degrees of reactivity, which decreases in the order given above. Fenton's reagent (1894) which is hydrogen peroxide containing a small quantity of a

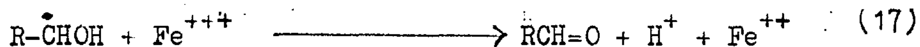
ferrous salt can oxidize a wide variety of organic compounds (Waters 1946a). This system has been extensively used for the studies on the mechanism of biochemical oxidations. It was found that in the aromatic hydroxylations and related oxidations by Fenton's reagent, the orientation of the entering hydroxyl group is in accord with a predominantly homolytic process involving free hydroxyl radical (Mason 1957). Primary and secondary alcohols, aldehydes and ethers oxidize by chain reaction which has now been proved to depend on the fact that these substances yield organic radicals that are capable of reducing ferric ions.



$$Fe^{++} + H\text{-}O\text{-}O\text{-}H \longrightarrow Fe^{+++} + \bullet OH + OH^{-} \qquad (15)$$



$$\bullet OH + R\text{-}CH_2OH \longrightarrow H_2O + R\text{-}\dot{C}HOH \qquad (16)$$



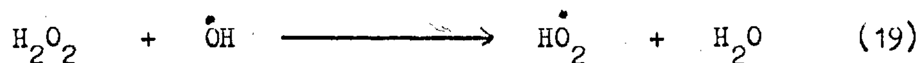
$$R\text{-}\dot{C}HOH + Fe^{+++} \longrightarrow RCH{=}O + H^{+} + Fe^{++} \qquad (17)$$

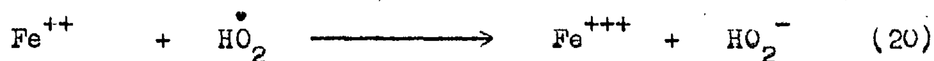
The hydroxylation of aromatic compounds depends upon the presence of a high concentration of ferric ions, for otherwise the oxidation of the primary adduct is much slower than dimerization (Waters 1964). It is generally accepted that in acid solution hydrogen peroxide is reduced to water in two one-electron steps. The overall reaction is as follows.

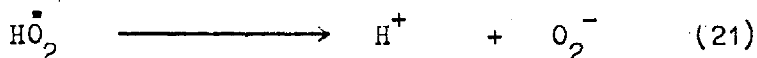
$$2Fe^{++} + H_2O_2 \rightleftharpoons 2Fe^{+++} + 2(OH)^{-} \qquad (18)$$

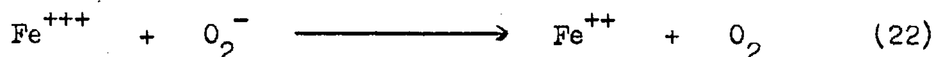
The hydroxyl radical set free in the first step of this reaction initiates its characteristic reactions. When the concentration of

hydrogen peroxide is large relative to that of ferrous ion initially present, and pH is higher, other reaction may occur including a chain which leads to evolution of oxygen(Baxendale1955).


$$H_2O_2 + \dot{O}H \longrightarrow H\dot{O}_2 + H_2O \quad (19)$$


$$Fe^{++} + H\dot{O}_2 \longrightarrow Fe^{+++} + HO_2^- \quad (20)$$


$$H\dot{O}_2 \longrightarrow H^+ + O_2^- \quad (21)$$


$$Fe^{+++} + O_2^- \longrightarrow Fe^{++} + O_2 \quad (22)$$

It was proposed that hydroperoxy radicals ($H\dot{O}_2$) are produced in the modified Fenton's reagent which is made of ascorbic acid acting in the presence of air, in a solution of ferrous ions and versene (EDTA) (Acheson & Hazelwood 1960; Norman & Radda 1962). This system will be discussed later (Part II, Sec. D). Which radical, $\dot{O}H$ or $H\dot{O}_2$ is predominantly concerned in several catalysed reactions that can be effected in nearly neutral solution of hydrogen peroxide is still uncertain.

Miscellaneous oxidations :- Chromic acid and potassium permanganate are two powerful and extensively used oxidizing agents in organic chemistry. The activity of both of them is a function of the acidity of the medium. Unlike permanganate the ability of chromic acid to oxidize organic compounds virtually diminishes in basic solution. MnO_2 is the usual product of reduction of permanganate in all but strongly basic solution; in the latter solution manganate fails to

disproportionate. The oxidation of substituted toluenes to the corresponding benzoic acid has been studied in acetic acid solution (Speroni and Barchielli 1941; Cullis and Ladbury 1955). Electron donating groups in the ring facilitate the oxidation rate but a Hammett plot for meta and para methyl, chloro, and nitro toluenes showed only a rough fit. It was suggested that both permanganate ion and manganese ions of lower valence were involved in these reactions. The most common lower oxidation state of chromium ion is Cr (III) and oxidations generally lead to this state. However, few if any reactions involve a three electron transfer in one step, and therefore, most reactions lead either to Cr (V) or Cr (IV) as an intermediate. These compounds may effect further oxidations and may lead to different products than those expected from Cr (VI) oxidations(Hampton *et al* 1956). Besides chromic acid the most commonly used Cr (VI) derivatives are chromyl chloride and chromyl acetate. The oxidation of aryl alkanes with chromic acid gives oxidation mostly at the carbon attached to the aromatic ring, whereas the oxidation using aq. Sodium dichromate gives oxidation principally at the end of the aliphatic chain rather than at the α-position (Wieberg 1965). The major products in both cases are acids. The oxidation of alkyl groups in substituted benzenes by chromyl chloride occurs preferentially at the carbon one-removed from the aromatic ring producing aldehydes or ketones (Wieberg *et al* 1962; Wieberg 1965). Oxidations of organic compounds by

by lead tetraacetate introduce hydroxyl group in the molecule in a protected form. With alkylbenzenes the reaction takes place on the α-carbon atom (Criegee 1965; Detilleax and Jadot 1955). Reactions of transition metal oxidants involve reactive cations which are stable in solutions of mineral acids, though unstable in neutral solution. The oxides of nitrogen, sulphur, and selenium are also frequently used for oxidizing organic compounds. There are many other oxidizing agents that are difficult to be classified either by structure, mechanism or function. They include carbonium ions, quinones, anodic oxidations and the halogens.

INTRODUCTION.

Part II. Enzymic Oxidations.

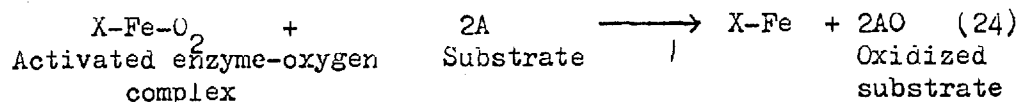
A wide variety of organic compounds, both exogenous and endogenous, are oxidized by living organisms. Before dealing with the oxidation reactions of compounds foreign to the body, a brief introduction is given in Section A to biological oxidation and classification of the enzymes involved in oxidation. The review is based on information obtained in mammals though a large number of the enzymes concerned have also been found in insects (Gilmour 1961). Section B covers in detail the biological oxidation of fatty acids. The chemical alterations alkyl side chains undergo, during the metabolism of fatty acids, are of special significance in relation to the metabolic fate of such groups in foreign substrates like alkylbenzenes. Here again, the information is confined to mammals, as in spite of the accumulation of analytical data on their fatty acid composition, very little is known of the metabolism of insect fats (Gilmour 1961). This absence of any information on the pathway of fatty acid oxidation represents a serious gap in our knowledge of insect biochemistry. In Section C, the known metabolic oxidations of foreign compounds in mammals and insects are reviewed, and Section D covers the microsomal enzyme system, responsible for the oxidative metabolism of foreign compounds. The various theories proposed on the mechanism of microsomal oxidations are

also included in the later section. In Section E the role of comparative metabolic studies in mammals and insects is discussed, specially with reference to the work presented in this thesis.

Section A. Biological oxidation and classification of the enzymes involved.

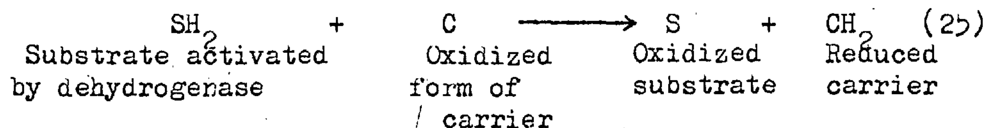
The investigation of biological oxidation was begun by Lavoisier about two hundred years ago. Since then, oxidation of organic substances by living organisms has remained one of the most important topics in biological science. Lavoisier and his contemporaries defined the term "oxidation" as the addition of oxygen atoms to a substrate, but now it is employed to signify removal of electrons, removal of hydrogen atoms or incorporation of oxygen. It soon became apparent that living organisms contain a number of enzymes which catalyse the oxidation of various biological substances; these were designated "oxydases" (Bertrand 1896). The early worker generally presumed that by some means the oxygen molecules were activated, and then combined with substrates. Otto Warburg (1949) suggested that the essential process in cell respiration was the activation of oxygen and that this activation was catalysed by iron-containing compounds which he referred to as the "atmungsferment" meaning respiratory enzyme. According to him, the iron complex, X-Fe, functions in oxidations as follows :-

$$X\text{-}Fe + O_2 \longrightarrow X\text{-}Fe\text{-}O_2 \qquad (23)$$



$$\underset{\text{Activated enzyme-oxygen complex}}{X\text{-}Fe\text{-}O_2} + \underset{\text{Substrate}}{2A} \longrightarrow X\text{-}Fe + \underset{\text{Oxidized substrate}}{2AO} \qquad (24)$$

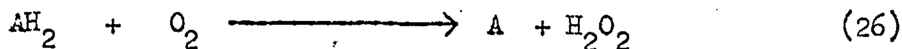
This role of oxygen molecules *per se* in biological oxidation processes was questioned upon the discovery by Schardinger of an enzyme in milk which oxidizes aldehydes to acids in the presence of methylene blue, which itself is reduced in the process, under anaerobic conditions. This finding prompted Wieland to put forward his theory of 'hydrogen activation' according to which certain hydrogen atoms of the substrate are activated by an enzyme (dehydrogenase) so that they may be removed by a 'hydrogen acceptor' (carrier) which can be molecular oxygen or other oxidizing agent. A carrier may be defined as a reversible oxidation-reduction system, the oxidized form of which can accept hydrogen or electrons and be reduced, and the reduced form can be oxidized by giving hydrogen or electrons to another carrier or to oxygen. The function of a simple carrier may be represented as follows :-



$$\underset{\text{Substrate activated by dehydrogenase}}{SH_2} + \underset{\text{Oxidized form of carrier}}{C} \longrightarrow \underset{\text{Oxidized substrate}}{S} + \underset{\text{Reduced carrier}}{CH_2} \qquad (25)$$

This 'dehydrogenation' theory gained experimental support through ingenious experiments by Thunberg, and numerous

dehydrogenases have been isolated from mammal, insect and plant sources as well as from micro-organisms. Pyridine nucleotides, flavin nucleotides and cytochromes have been found to act as carriers for various dehydrogenases. In cases when the oxygen molecules serve as the immediate electron acceptor, without involving the electron transport chains the enzymes have been called oxidases. Classical oxidases can be divided into two categories. In the first category, the enzyme catalyses the transfer of two electrons to one molecule of oxygen forming hydrogen peroxide (Eq. 26). Some flavin containing enzymes, such as D-amino acid oxidase, glucose oxidase, xanthine oxidase belong to this group of enzymes. In the second type, two electrons are transferred to an atom of oxygen to produce water (Eq. 27). Cytochrome oxidase and ascorbic acid oxidase are examples of this class of oxidase. Mason (1957) called these two types of enzymes electron transfer oxidases.



$$AH_2 + O_2 \longrightarrow A + H_2O_2 \qquad (26)$$

$$AH_2 + \tfrac{1}{2}O_2 \longrightarrow A + H_2O \qquad (27)$$

where AH_2 is the substrate and A is its oxidized form.

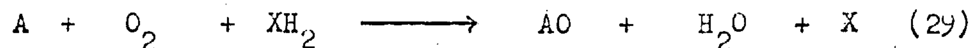
The other two groups of oxidases according to Mason's classification (1957) are oxygen transferases and mixed function oxidases. Oxygen transferases catalyse the consumption of one molecule of oxygen per molecule of substrate

(Eq. 28) and both atoms of oxygen appear in the product.

$$A + O_2 \longrightarrow AO_2 \qquad (28)$$

where A is the substrate.

Well known examples are pyrocatechase, homogentisate oxidase, tryptophan oxidase, indole oxidase, lipoxidase and dihydroxyfumaric oxidase. Mixed function oxidase catalyses the consumption of one molecule of oxygen per molecule of substrate. One atom of this oxygen appears in the product and the other is reduced to H_2O in the presence of an appropriate electron donor (Eq. 29).



$$A + O_2 + XH_2 \longrightarrow AO + H_2O + X \qquad (29)$$

where A is the substrate and XH_2 is the electron donor.

These two types of reaction (Eqs. 28 &29) both involve oxygen fixation into a substrate molecule, and therefore they are different from the oxidase reactions represented in Eqs. 26 & 27. They are similar to the oxygenation reactions known to occur by chemical and photochemical processes, and Hayaishi _et al_ (1956) proposed the term "oxygenase" to designate the enzymes responsible for these reactions.

Mixed function oxygenases :- These oxygenases have two interdependant activities, reduction of one atom of O_2 coupled to specific oxygenation or hydroxylation of the substrate. As shown in Eq.29, this type of enzyme is characterised by apparently paradoxical requirements :

(i) O_2 is necessary, as a specific oxidizing agent.

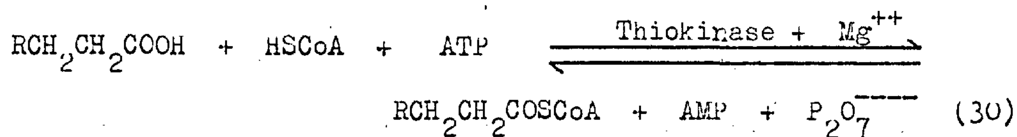
(ii) a source of electrons is needed to reduce one atom of O_2 to O^{--}. In most hydroxylation reactions $NADPH_2$ appears to be a specific electron donor, but in certain instances compounds including $NADH_2$, ascorbic acid, o-diphenols and dihydroxyfumaric acid also act as sources of electrons (Hayaishi 1962). It is now known that the liver enzymes systems which are responsible for the hydroxylation of foreign compounds in mammals are of the mixed function oxygenase type. The proposed mechanisms of action of these enzymes will be dealt with in Section D. Other examples of mixed function oxygenases are the phenolase complex, imidazole-acetic acid oxidase, phenylalanine hydroxylase, p-hydroxyphenylpyruvate oxidase, steroid hydroxylase and the peroxidase hydroxylating system.

Section B. Biological oxidation of fatty acids.

The fatty acids in the body are broken down into two carbon units as acetyl CoA, CH_3-CO-S-CoA, by an overall process known as "β-oxidation". Knoop (1905) tagged the hydrocarbon ends of even and odd fatty acids with the phenyl group, fed these phenyl-substituted fatty acids to dogs and examined the urine for end products of oxidation. He found that phenyl-substituted even carbon acids were oxidised to phenylacetic acid in the body and odd carbon acids were converted to benzoic acid. He explained this by proposing that oxidation of the fatty acids was taking place at the

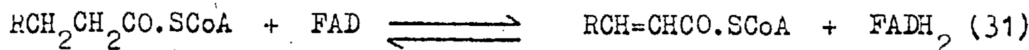
carbon atom in the β-position to the carboxyl group. Dakin (1921) extended and confirmed Knoop's observations and concluded that fatty acids in general are oxidised at the β-carbon atom with the splitting off of the two terminal carbon atoms, leaving a fatty acid chain shorter by two carbons than the original acid. As a result of many investigations over a long period of time, the detailed mechanisms of the β-oxidation of fatty acids have been established. The reactions involved are outlined below :-

I. Activation of fatty acids, formation of acyl coenzyme A derivatives.



$$RCH_2CH_2COOH + HSCoA + ATP \xrightleftharpoons{\text{Thiokinase} + Mg^{++}} RCH_2CH_2COSCoA + AMP + P_2O_7^{----} \quad (30)$$

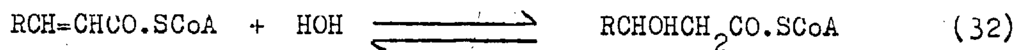
II. Formation of α,β-unsaturated fatty acyl CoA derivatives by acyl dehydrogenase.



$$RCH_2CH_2CO.SCoA + FAD \rightleftharpoons RCH{=}CHCO.SCoA + FADH_2 \quad (31)$$

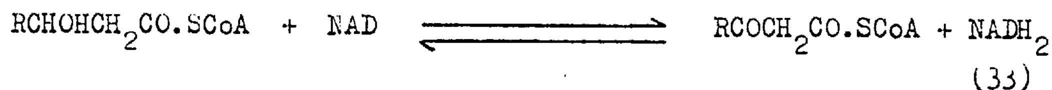
FAD and $FADH_2$ represent the oxidised and reduced forms of the flavoprotein acyl dehydrogenase enzyme respectively.

III. Hydration of α,β-unsaturated acyl CoA derivatives to form β-hydroxyl acyl CoA derivatives by enoyl hydrase or crotonase.



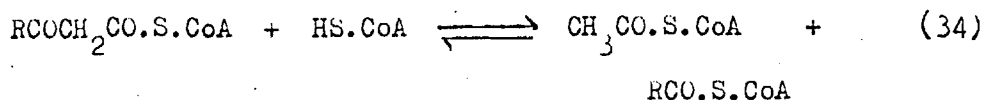
$$RCH{=}CHCO.SCoA + HOH \rightleftharpoons RCHOHCH_2CO.SCoA \quad (32)$$

IV. Oxidation of β-hydroxy acyl CoA derivatives to β-ketoacyl CoA derivatives by β-hydroxy acyl dehydrogenase.



$$RCHOHCH_2CO.SCoA + NAD \rightleftharpoons RCOCH_2CO.SCoA + NADH_2 \quad (33)$$

V. Thiolytic cleavage of β-ketoacyl CoA derivatives by thiolases.



$$RCOCH_2CO.S.CoA + HS.CoA \rightleftharpoons CH_3CO.S.CoA + RCO.S.CoA \qquad (34)$$

All these processes of fatty acid oxidation take place in cell mitochondria of tissues in general (Green 1954). The β-oxidation scheme discussed above accounts for the major portion of oxidative reactions of fatty acids. In addition to β-oxidation, α-oxidation and ω-oxidation yield products of some metabolic importance (Mahler & Cordes 1966a).

Section C. Oxidative metabolism of foreign compounds in mammals and insects.

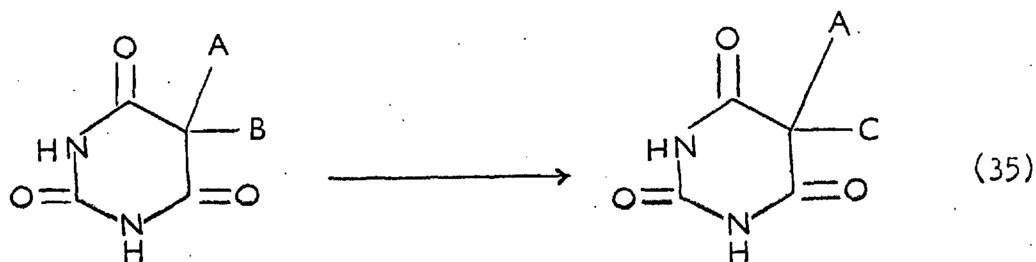
One of the important factors governing biological activity of a compound foreign to the body is metabolism. The majority of compounds undergo transformation in the body, which on the whole helps the body to get rid of the compound. In most cases oxidation leads to the formation of less active or biologically inert products, however, in some instances, more active products are produced and these can cause a toxic effect. Therefore, the duration of action and toxicity of a compound are often related to the rate and route of its metabolism in the body. In the last decade, development of new techniques, gas-liquid chromatography, spectrofluorometry for example, has made enormous contribution to metabolic studies. Metabolic pathways of a wide variety of compounds including drugs, pesticides,

food additives, cosmetics and detergents are now known and many more are being investigated. All information on this subject, until 1958 has been covered by R.T. Williams in his book 'Detoxication Mechanisms' (Williams 1959). The work done in the last few years had been reviewed in a number of articles (Meynert 1961; Williams 1962, 1964; Boyland & Booth 1962; Gillette 1963; Shideman & Mannering 1963; Shuster 1964; Williams & Parke 1964; Ariens & Simonnis 1964; Remmer 1965). Numerous studies have shown that metabolism of a large number of chemical substances follow a very few chemical pathways. The chemical changes involved in the metabolism of foreign compounds in mammals can be divided into oxidations, reductions, hydrolyses, and syntheses which are mainly conjugation reactions with carbohydrates and amino acids (Williams 1959). A compound may undergo any of these reactions or any combination of them, consecutively. Many of the oxidations, reductions or hydrolyses are followed by synthetic reaction. Until a few years ago, most of the metabolic work was mainly done in mammals. During the past few years there has been increasing interest in similar studies in insects, though the compounds selected for study were largely confined to insecticides. The different types of metabolic processes found to occur in insects have been reviewed by various authors (Smith 1955, 1962, 1964; Casida 1959; Perry 1960). It appears that the nature of the metabolic reactions in insects and mammals are very similar. Most differences are quantitative rather than qualitative. General

comparative aspects of these mechanisms have also been reviewed recently (Smith 1964$_b$). Among the known metabolic pathways, oxidation is one of the major changes foreign compounds undergo both in insects and mammals. The various types of oxidation reactions are carried out by enzyme systems, which in mammals are located in the microsomes of liver cells. Similar particulate systems from insect fat body and whole homogenate preparations have been reported (Fenwick 1958; Agosin et al 1961; Terrier et al 1962, 1965). The enzyme systems require reduced NADP and oxygen for their activity. The oxidative metabolic reactions which occur in mammals and insects can be classified into the following types. Each type of reaction is illustrated with examples.

(i) Alkyl side-chain oxidation :- A number of important drugs, barbiturates for example, are metabolized by alkyl side chain oxidation. Oxidation at both terminal (ω) and penultimate (ω-1) carbon atoms has been demonstrated with the barbiturates in dog, rat, rabbit , and man (Williams 1959). In a typical barbiturate structure there are usually two substituents in position 5, but only one undergoes biochemical change. Any extensive change which the molecule undergoes *in vivo* has been found to be located in this substituent. The metabolites are the alcoholic and ketonic products of alkyl side chain oxidation. Recently, Maynert (1965) has shown that though the ω and (ω-1) carbon atoms of alkyl substituents in barbiturates bear the brunt of oxidative attack, the reaction is not restricted to these two atoms in the chain.

Metabolism of barbiturates in mammals.



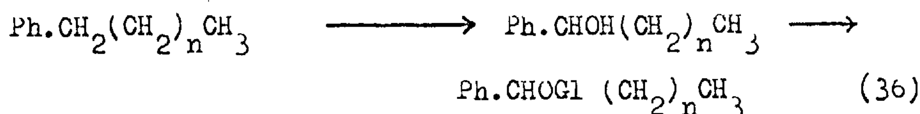
(where A and B are the substituents and C is the metabolized form of B).

The enzymic aspects of these reactions have not yet been studied in detail. Cooper and Brodie (1957) showed that pentobarbital and thiopental are oxidized by the action of enzymes in microsomes that have the requirement for both $NADPH_2$ and oxygen. Pentobarbital forms about equal amounts of an alcohol (penultimate oxidation) and an acid (terminal oxidation). Thiopental yields mainly a compound with a carboxylic group on the terminal carbon together with a small amount of an alcohol (penultimate oxidation).

Gillette (1958) demonstrated that rabbit liver microsomes oxidize thiopental at the terminal carbon atom to yield a primary alcohol, which is further oxidized to the corresponding acid by enzymes in the soluble part of the

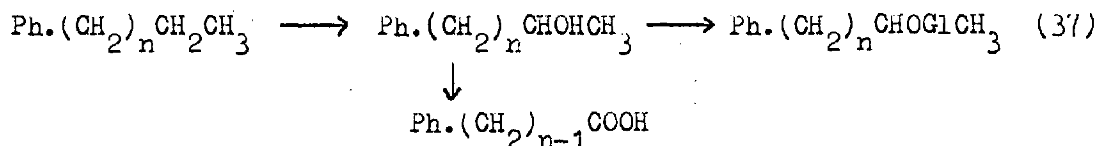
cell. Oxidation of a methyl group to a carboxyl group is a common reaction of methylbenzenes in mammals. The metabolic changes of higher alkylbenzenes in mammals *in vivo*, so far reported are limited to the following reactions (Williams 1959).

(1) Hydroxylation of the activated methylene group next to the benzene ring to form an alkyl phenyl carbinol which is normally excreted as glucuronide.



$$Ph.CH_2(CH_2)_nCH_3 \longrightarrow Ph.CHOH(CH_2)_nCH_3 \longrightarrow Ph.CHOGl\ (CH_2)_nCH_3 \qquad (36)$$

(2) Hydroxylation of the penultimate carbon atom to form a methylcarbinol which may be conjugated or further oxidized to an acid with two carbon atoms less than the original molecule.



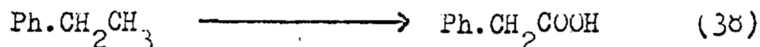
$$Ph.(CH_2)_nCH_2CH_3 \longrightarrow Ph.(CH_2)_nCHOHCH_3 \longrightarrow Ph.(CH_2)_nCHOGlCH_3 \qquad (37)$$

$$Ph.(CH_2)_nCHOHCH_3 \downarrow Ph.(CH_2)_{n-1}COOH$$

The phenyl fatty acid produced could then be oxidized in the β-position to yield benzoic acid or phenylacetic acid according to the number of carbon atoms in the alkyl chain.

(3) The third reaction is ω-oxidation leading to the formation of phenyl fatty acids which are then converted to benzoic or phenylacetic acid according to the number of carbon atoms in the

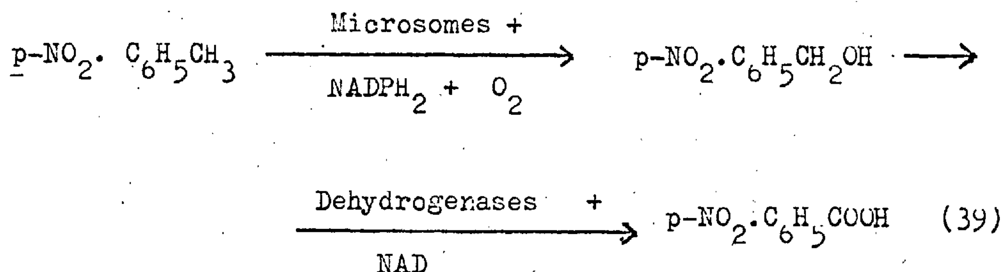
side chain.



$$Ph.CH_2CH_3 \longrightarrow Ph.CH_2COOH \quad (38)$$

The available information on the metabolism of the alkylbenzenes is presented in detail in Chapter III.

The mammalian enzyme system responsible for the oxidation of the alkyl side chain of p-nitrotoluene has been studied by Gillette (1959). He showed that a rabbit liver microsomal enzyme system converted p-nitrotoluene to p-nitrobenzyl alcohol in presence of $NADPH_2$ and oxygen. The alcohol is then oxidized to p-nitrobenzoic acid by the alcohol and aldehyde dehydrogenases in the soluble fraction.



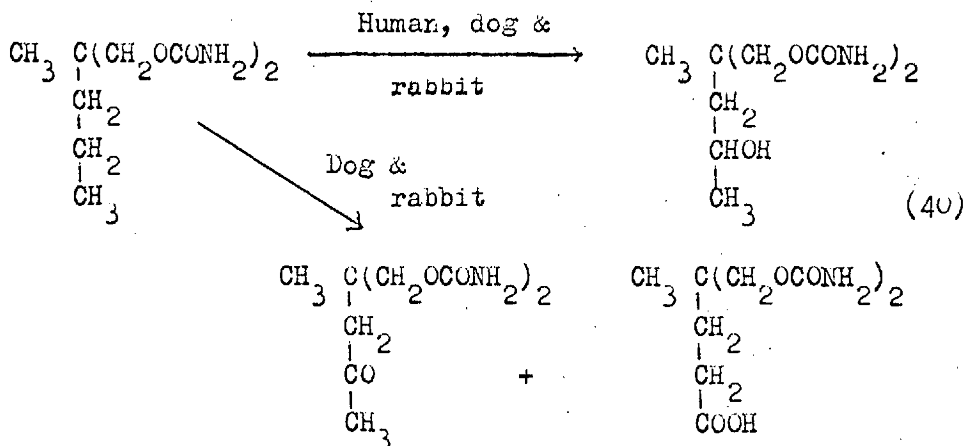
$$p\text{-}NO_2.\,C_6H_5CH_3 \xrightarrow[NADPH_2\ +\ O_2]{\text{Microsomes}\ +} p\text{-}NO_2.C_6H_5CH_2OH \longrightarrow$$

$$\xrightarrow[NAD]{\text{Dehydrogenases}\ +} p\text{-}NO_2.C_6H_5COOH \quad (39)$$

Meprobamate and the related carisoprodol and mebutamate are also metabolized in mammals by oxidation, the site of oxidation being the longer substituent attached to C_2 of the propan-1,3-diol portion of the molecule (Walkenstein et al 1958; Berger 1954). In fact, all the metabolites of meprobamate except the N-glucuronide

are those which could be expected as a result of ω and ($\omega-1$) oxidation of the n-propyl chain of the drug (Ludwig et al 1961; Yamamoto et al 1962).

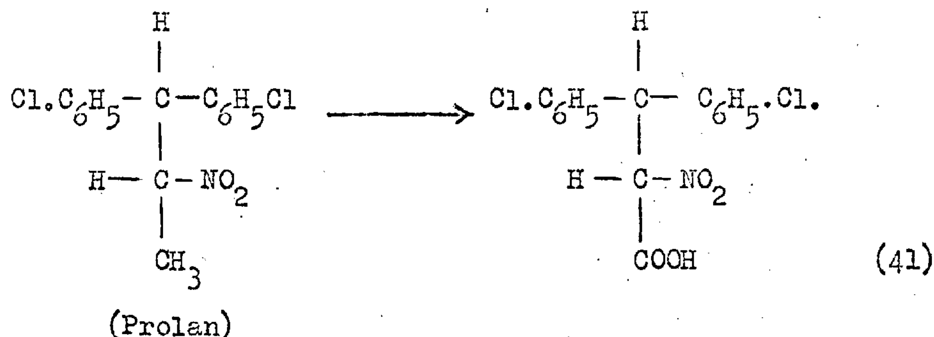
Metabolism of Meprobamate in mammals.



The alkyl side chains of sulphonylureas are also the sites of oxidative metabolic attack in man, rat, rabbit and guinea pig. These species oxidize the p-methyl group of tolbutamide to the carboxylic acid which is excreted. In some cases, small amounts of alcohol are also found (Scholz & Haussler 1964).

Metabolism of a number of compounds in insects also involves alkyl side chain oxidation. Terminal oxidation of an aliphatic chain probably occurs in locust's cuticle. After hydrolysis of the cuticle which had been tanned with 3,4-dihydroxyphenylpropionic acid, some 4-methylcatechol was found, as well as the 4-ethylcatechol. This suggests that the following sequence $RCH_2CH_2COOH \longrightarrow RCH_2CH_3 \longrightarrow RCH_2COOH \longrightarrow RCH_3$ occured (Malek 1960). Another example of

terminal oxidation of a methyl group is found in the metabolism of prolan [1,1-bis (p-chlorophenyl)-2-nitropropane] in house-flies (Perry & Buckner 1959). It has been suggested that the reaction goes as follows :-



(41)

Oxidation of an aliphatic position adjacent to a benzene ring (α-methylenic oxidation), has been observed in the metabolism of DDT in various insect species (Smith 1962). It has been reported (Agosin et al 1961)that a cockroach microsomal preparation oxidizes DDT [1, 1-(p-chlorophenyl) 2, 2, 2-trichloroethane] to kelthane [1, 1-bis-(p-chlorophenyl) 2, 2, 2-trichloroethnol] in the presence of $NADPH_2$ and oxygen. Another similar compound DMC [bis-(p-chlorophenyl)-methyl-carbinol] , an insecticide synergist is metabolized by resistant house-flies and excreted as a product tentatively identified as bis-(p-chlorophenyl)-acetic acid (Perry et al 1953). Recently, it has been suggested that alkyl chain oxidation play an important role in the metabolism of pyrethroids in house-flies (Fine 1963).

(ii) Aromatic ring hydroxylation :- The introduction of a hydroxyl group into an aromatic ring is a common reaction in mammals (Williams 1959). In vivo this is usually followed by conjugation producing glucuronide (mammals), glucoside (insects) or sulphate (insects and mammals). This reaction has been extensively studied in vitro with liver microsomal preparations and the results show that the enzyme system requires $NADPH_2$ and oxygen for its activity. The aromatic compounds which are hydroxylated by liver microsomes include acetanilide, aniline, quinoline, naphthalene, benzene, 3,4-benzpyrene, salicylic acid, 2-aminofluorene, 2-naphthylamine, diphenyl and coumarin (Brodie et al 1958 ; Creaven et al 1965 a, b). Aromatic rings can be hydroxylated in more than one position (Mitoma 1956 ; Boyland & Booth 1957 ; Williams 1959 ; Creaven et al 1965 b). It has been suggested that hydroxylation at different positions may involve different enzymes (Parke & Williams 1956 ; Creaven et al 1965 b). The following table shows how aromatic hydroxylation can vary from species to species and from compound to compound in the same species.

Table 1. Aromatic hydroxylation *in vivo*. (Williams 1964)

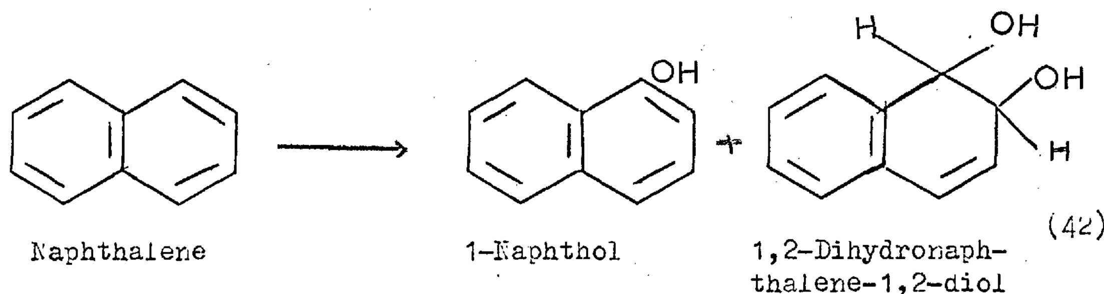
Compound	Extent of hydroxylation in					
	Man	Dog	Rabbit	Rat	Mouse	G.Pig
Amphetamine[1]	L	M	L	M	–	L
Ephedrine[2]	–	L	L	M	–	L
Imipramine[2]	M	–	H	L	–	–
Diethyl trytamine[2]	M	–	–	H	H	L
LSD[2]	–	–	–	M	–	L
Aniline[3]	–	H	H	H	H	H

H, 25-50% or more ; M, 5-20% ; L, less than 5% .

Alternative metabolic reaction : 1, deamination; 2, dealkylation; 3, none.

Hydroxylation of aromatic rings also occur in insects. Microsomal preparations have been made from insect tissues which have similar properties to the liver microsomal enzymes of mammals (Terriere & Arias 1962 ; Terriere *et al* 1965). *In vivo* naphthalene is converted to a number of ring hydroxylation products and their conjugates in g. pigs, rats, rabbits and houseflies (Williams 1959 ; Terriere 1961). However, only metabolites found *in vitro* using microsomal preparations of rat and housefly were

1-naphthol and 1,2-dihydronaphthalene-1, 2-diol (Boyland and Booth 1958; Terriere & Arias 1962).

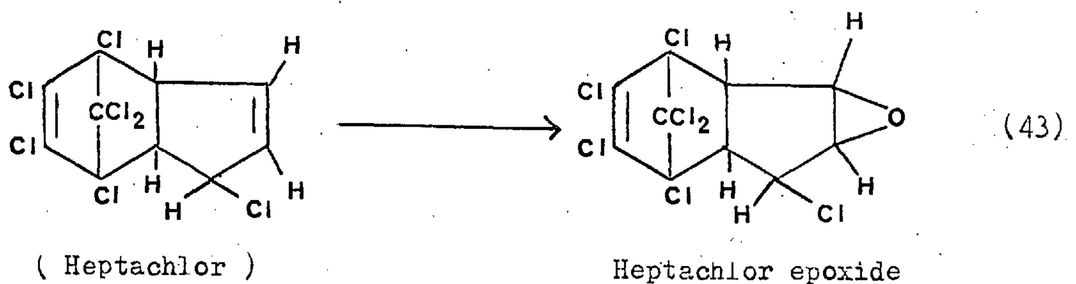


Naphthalene — 1-Naphthol — 1,2-Dihydronaphthalene-1,2-diol

Recently it has been reported that two of the metabolites of Sevin (1-naphthyl N-methyl carbamate) in rats, cockroaches and house-flies are 4-hydroxy-1-naphthyl N-methyl carbamate and 5-hydroxy-1-naphthyl N-methyl carbamate (Dorough and Casida 1964). Locusts excrete chlorobenzene as conjugates of o- , m- , and p-chlorophenol and 4-chlorocatechol (Smith and Gessner 1960). Spiders can also hydroxylate the aromatic ring, since umbelliferone is excreted by them as the ethereal sulphate of its hydroxylation product, esculetin (Smith 1962).

(iii) Epoxidation :- Epoxidation has been suggested as an intermediate step in the aromatic ring hydroxylation. According to Boyland and Booth (1962) the liver microsome

-NADPH$_2$- O$_2$ system converts naphthalene to 1,2-dihydro-1,2-epoxynaphthalene which then rearranges and reacts with water forming 1-naphthol and 1,2-dihydro-1,2-dihydroxynaphthalene respectively. Epoxidation has been mainly encountered in the metabolism of cyclodiene insecticides. For example, Heptachlor is converted to its epoxide in the dog, rat and cow (Davidow *et al* 1953$_{a,b}$; Ely *et al* 1955).

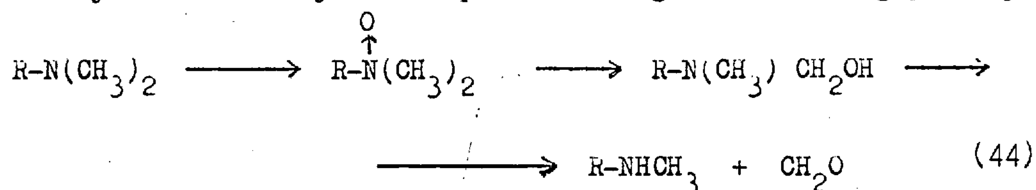


(43)

The metabolism of cyclodiene insecticides follow the same route in insects. A number of such compounds have been found to be metabolised to their epoxides in houseflies and American cockroaches (Metcalf *et al* 1956 ; Perry *et al* 1958; Brooks 1960 ; Perry 1960 ; Cohen and Smith 1961; Smith 1962).

(iv) N-dealkylation:- The oxidative removal of *N*-alkyl groups from foreign compounds is a general reaction in mammals

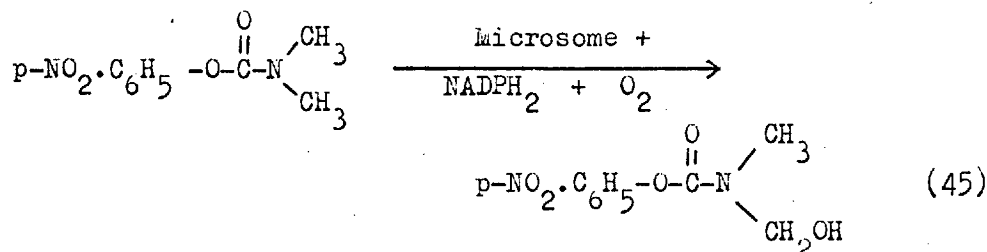
(Brodie *et al* 1958). In recent years considerable advances have been made in outlining the enzyme system involved in these reactions. Liver microsomal preparations have been shown to dealkylate a variety of *N*-alkylamines including aminopyrine, methylaniline, dimethylaminoazobenzene, methylamphetamine, methadone, diacetylmorphine, and codeine (Mueller & Miller 1953 ; La Du *et al* 1955 ; Axelrod 1956b; Gaudette and Brodie 1959). Demethylation in the body of *N*-methyl hydantoins and barbiturates has been established as a general step in their metabolism (Fishman 1956). Uehleke (1961, 1963) suggested that *N*-dealkylation proceed via *N*-hydroxylation followed by rearrangement. Such a scheme suggests a close relationship between *N*-dealkylation , *N*-hydroxylation and the formation of *N*-oxides, such as those obtained from trimethylamine, chloropromazine , imipramine, and nicotinamide (Baker & Chaykin 1962 ; Fishman *et al* 1962a, b; Kirchner *et al* 1963). According to Fish *et al* (1955, 1956) dealkylation of alkylamines proceed along the following pathway:-



$$R\text{-}N(CH_3)_2 \longrightarrow R\text{-}\overset{O}{\overset{\uparrow}{N}}(CH_3)_2 \longrightarrow R\text{-}N(CH_3)\ CH_2OH \longrightarrow$$

$$\longrightarrow R\text{-}NHCH_3 + CH_2O \qquad (44)$$

Schradan (Octamethyl pyrophosphoramide), an organophosphorous insecticide is converted to a potent cholinesterase inhibitor by microsomal enzyme from mammalian liver and locust fat body. The product is now believed to be hydroxymethyl Schradan (Heath *et al* 1955; Fenwick 1958; O'Brien 1960). It is now known that *N*-alkyl oxidation also plays a significant role in the metabolism of carbamate insecticides (Casida 1963). The rat liver microsome-$NADPH_2$-oxygen system hydroxylates *p*-nitrophenyl *N*,*N*-dimethyl carbamate, probably yielding *p*-nitrophenyl-*N*-methylol carbamate (Hodgson & Casida 1960, 1961). It has been suggested that analogues of this carbamate with different alkyl groups follow similar metabolic pathway.



(45)

Flies and cockroaches form an unstable intermediate from *p*-nitrophenyl *N*,*N*-dimethyl carbamate which is probably the *N*-methylol derivative (Hodgson & Casida 1960, 1961). Recently metabolic studies on the carbamate insecticide Sevin (1-naphthyl *N*-methyl carbamate)in rat liver,flies and cockroaches

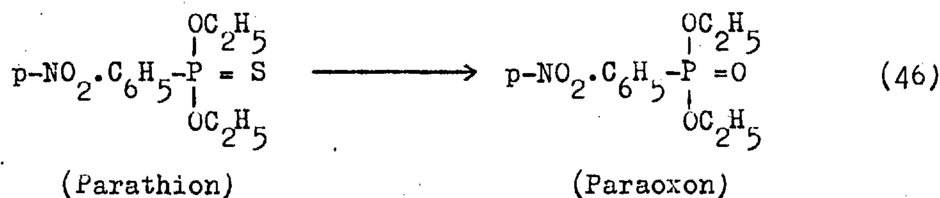
have shown that one of the metabolites is the N-hydroxymethyl derivative (Dorough and Casida 1964).

(v) O-dealkylation :- Aromatic ethers undergo O-dealkylation forming phenols in mammals and insects (Williams 1959; Brodie et al 1958; Smith 1964). Bray (1955) demonstrated that the demethylation of substituted anisoles occur in rabbit liver slices and that the methyl group is oxidatively split to form aldehyde. Axelrod (1956_a) showed that the ether cleavage enzyme system is localized in microsomes and require $NADPH_2$ and oxygen. He also suggested that more than one ether cleavage enzyme is present in liver microsomes.

(vi) N-hydroxylation :- A new type of hydroxylation reaction in vivo has been demonstrated by the isolation of the arylhydroxylamine, N-hydroxy-2-acetylaminofluorene from the urine of rats which had been fed 2-acetylaminofluorene (Boyland and Booth 1962). It has also been shown that the hydroxylamine is a precursor of the ortho-hydroxylation product, 1-hydroxy-2-acetylaminofluorene (Miller et al $1960_{a,b}$). Further work published in recent years suggest that orthohydroxylation of aromatic amines take place by N-hydroxylation, followed by rearrangement of N-hydroxy derivatives, probably via the corresponding quinonimide

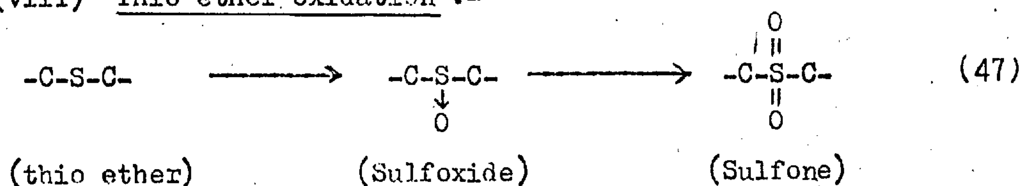
(Miller *et al* 1960 a,b; Boyland & Booth 1962). This reaction has not been reported in insects.

(vii) Phosphorothionate oxidation :- Phosphorothionate insecticides are metabolized by mammals to potent cholinesterase inhibitors by oxidation of the phosphorothionyl groups to phosphates (O'Brien 1960; Gillette 1963). *In vitro* studies have shown that the microsomal fraction of liver carries out this activation in the presence of cofactors (Davison 1955; Murphy & DuBois 1957; O'Brien 1959).



This reaction was first shown to occur in insect tissues by Metcalf & March (1953). They showed that cockroach gut oxidized a number of organophosphorous compounds including Parathion, methyl Parathion and Acethion Amide. It was reported that in the cockroach the fat body was the most effective of all tissues in oxidizing Parathion and Malathion (Kok & Walsop 1954; O'Brien 1957). As with liver preparations, homogenization of insect tissues eliminates oxidizing capacity. Addition of NAD, Magnesium and nicotinamide to a homogenate of cockroach gut preparation was found to restore a little of the Malathion oxidizing enzyme activity (O'Brien 1957).

(viii) Thio ether oxidation :-



Thio ethers such as Chloropromazine and 4,4-diaminodiphenyl-sulfide are oxidised by mammals to the corresponding sulfoxide derivatives (Gilette 1963). Thio ether oxidation is also involved in the metabolism of some organophosphorous insecticides of the Systox type (March et al 1955; Benjamini et al 1959). Thiono-Systox, for example, is converted by mouse liver and American cockroach tissues to its sulfone derivative.

Section D. Microsomal oxidising enzyme system.

Studies with tissue preparations have revealed that the enzymes responsible for metabolic oxidations in mammals are present mainly in the liver; in fact most are found only in this organ. These enzyme systems are localized in the microsomes of liver cells and require $NADPH_2$ and oxygen for their activity(Gillette 1963). Although the examples are very few, enzymes with similar properties have also been prepared from locust fat body, houseflies and cockroaches (Fenwick 1958, Agosin et al 1961, Terrier 1962). Attempts to define

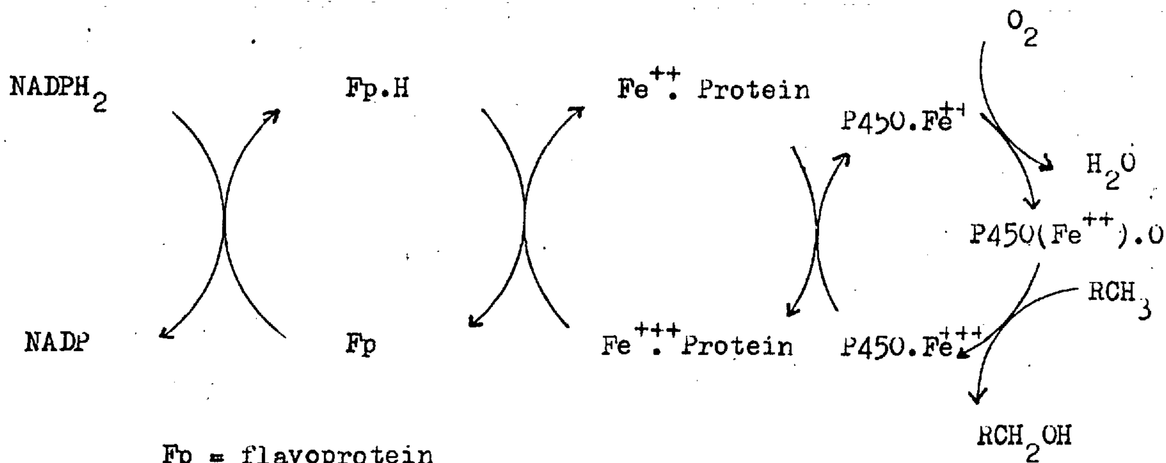
microsomal enzyme systems have been beset by numerous difficulties. Very few of these enzymes have been isolated in a form pure enough to allow proper characterization. Liver microsomes, as isolated by differential centrifugation, appear to consist of fragments arising from the endoplasmic reticulum and other membranes during cell rupture. The reticulum comprises two major components: a rough-surfaced form consisting of small, dense particles called ribosomes and a smooth-surfaced form devoid of ribosomes. On homogenization, the network of tubules is broken and forms small vesicles which can be isolated as "rough" and "smooth" microsomes.

Although the ribosomes play an essential role in protein synthesis, it was found that these particles are not important in metabolic oxidations. Fouts (1961) separated the "smooth" and "rough" microsomes and showed that the enzymes dealing with foreign compounds are concentrated mainly in "smooth" microsomes. Since the smooth endoplasmic reticulum of liver cells is associated with glycogen, attempts were made to correlate the liver glycogen content and the enzyme activity of the liver microsomes (Fouts 1962). However, such correlations are quite poor, especially since it is very difficult to alter glycogen content without producing other changes as well. It is not clear

whether the oxidative microsomal enzymes are localised in the lumen of the endoplasmic reticulum or in the lipoid membrane. The fact that treatment of microsomes with sonic osillations or hypotonic solutions fail to solubilize the enzyme system even though these treatments rupture the microsomal vesicles, indicates that these enzymes are firmly attached to the membranes. There has been no lack of effort to bring microsomal enzymes in solution. Conventional methods of solubilization were found to destroy the activity of many of the microsomal enzymes.

In recent years it has been suggested that one or more enzymes from the lipid-containing microsomal membranes can be separated by means of a phospholipase from snake venom. The enzyme preparation was reported to be capable of oxidizing aniline to p-aminophenol and its activity was not reduced (Imai & Sato 1960). Krisch (1962) used pancreatic lipase for solubilizing acetanilide hydroxylase of pig liver. Other microsomal enzymes which are reported to have been solubilized include glucoronyl tranferase, esterase, N-demethylase, nitroreductase and azoreductase (Schuster 1964). The common requirements of $NADPH_2$ and oxygen for various oxidation reactions in microsomes suggest that the enzyme systems are

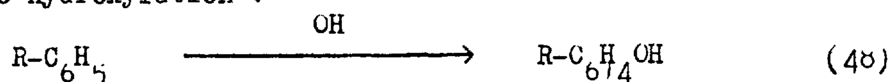
closely related. Other enzyme systems requiring $NADPH_2$ and oxygen include those which catalyse the oxidation of phenylaniline to tyrosine and the hydroxylation of steroids to steroid hormones (Mason 1957; Kaufman 1957). It has been shown that like these enzymes the liver microsomal enzyme system also incorporate atmospheric oxygen into the substrate (Mason 1957 ; Posner <u>et</u> <u>al</u> 1961 ; Baker & Chaykin 1962). How $NADPH_2$ is involved in these reactions is not clearly known. It has been suggested that P450, a cytochrome of unusual structure , found in the microsomal fractions from a large number of tissues may constitute the terminal oxidase, responsible for interaction with oxygen in the microsomal oxidizing enzyme system (Mahler & Cordes 1966 b). The sequence postulated is shown below.



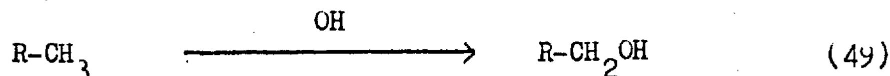
Fp = flavoprotein

All oxidation reactions in microsomes may be written as hydroxylations and the enzyme systems considered as hydroxylases (Gillette 1963).

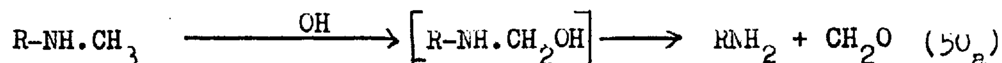
Aromatic hydroxylation :



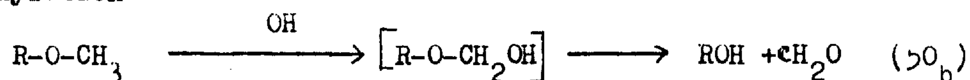
Alkyl chain oxidation :



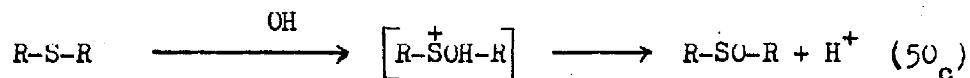
N-dealkylation :



O-dealkylation :



Sulfoxidation :



N-Oxidation :

Gillette *et al* (1957) found that liver microsomes contain an enzyme system that oxidizes $NADPH_2$ even in the absence of any foreign substrate, generating hydrogen peroxide. This finding and further evidence produced by other workers (Baker & Chaykin 1962 ; Gillette 1963) indicate that a $NADPH_2$ oxidase is an integral part of the microsomal oxidation enzyme system. The fact that peroxide is formed by the oxidation of $NADPH_2$ suggests that hydrogen peroxide may be utilized by non-specific enzymes in microsomes to catalyse the various metabolic reactions. An objection to this concept is that cyanide, which inhibits most peroxidases, does not effect the oxidation of hexabarbital

(Cooper & Brodie 1955), monomethyl-4-amino antipyrene (Gillette *et al* 1957) or the sulfoxidation of chloropromazine (Gillette & Kamm 1960). Moreover, a hydrogen peroxide generating system cannot replace the requirement for $NADPH_2$ in the various metabolic oxidations (Gillette *et al* 1957). It was suggested that the "active oxygen" formed in microsomes is probably not hydrogen peroxide but an intermediate leading to the formation of this peroxide (Gillette 1963). According to Mason's classification of oxidases (Mason 1957), the liver hydroxylating systems are considered as mixed function oxidases as they require molecular oxygen and a specific electron donor. Since "active oxygen" is derived from molecular oxygen, it must possess 4, 3, 2 or 1 oxidizing equivalents. Using this reasoning the following mechanisms were proposed for the formation of hydroxylating intermediates.

1. Four equivalents, O_2

a) Quaternary complex (Type I)

$$O_2\text{-}E\text{-}AH\text{-}NADPH_2 \longrightarrow AOH + NADP + E + H_2O \quad (51)$$

b) Active O_2 (Type II)

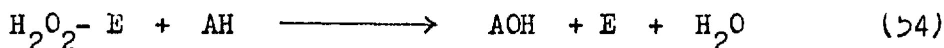
$$O_2\text{-}E + AH \longrightarrow AOH + EO \quad (52)$$

2. Three equivalents, HO_2 free radical (Type V)

$$HO_2\text{-}E + AH \longrightarrow AOH + EOH \quad (53)$$

3. Two equivalents

a) Peroxide (Type IV)



$$H_2O_2 - E + AH \longrightarrow AOH + E + H_2O \qquad (54)$$

b) Atomic oxygen (Type III)

$$O - E + AH \longrightarrow AOH + E \qquad (55)$$

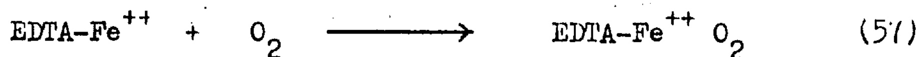
4. One equivalent, OH free radical

$$HO - E + AH \longrightarrow AOH + H^+ + e^+ + E \qquad (56)$$

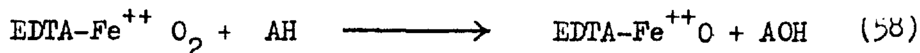
where E represents the enzyme and AH is the substrate oxidized. It was observed that non-specific non-enzymic hydroxylation occurs in the presence of O_2, ascorbic acid, ferrous ions and EDTA. The products obtained from a number of aromatic compounds are similar to those produced biologically (Udenfriend et al 1954; Brodie et al 1955; Mead et al 1958). Studies with this model hydroxylating systme were undertaken to aid in the elucidation of microsomal enzyme systems. A hydroxyl free radical mechanism for this system was advanced by Kreuger (1956), but such mechanism could not explain the orientation of the entering hydroxyl group. Aniline and p-cresol are oxidized to condensed products by free hydroxy radical generating system whereas the model system forms only simple hydroxylated derivatives (Brodie et al 1955; Mason 1957). Udenfriend et al (1954) postulated that the hydroxylating intermediate is the cation OH^+. The OH^+ theory explains orientation of the entering

hydroxyl group in the model reaction. Doubts have been raised concerning the existence of OH^{+} in aqueous solution (Burton *et al* 1956; Coulson 1956). Udenfriend *et al* proposed an alternative theory that the hydroxylation reagent is a product of hydrogen peroxide and ascorbic acid (Udenfriend *et al* 1954). The behaviour of the hydroxylating intermediate of the model system as a cation was explained by Mason(1957) in terms of type II and type III mechanisms put forward for mixed function oxidases.

Type II mechanism



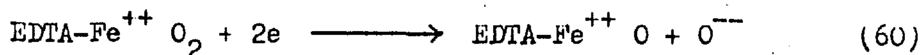
$$\text{EDTA-Fe}^{++} + O_2 \longrightarrow \text{EDTA-Fe}^{++}\,O_2 \qquad (57)$$



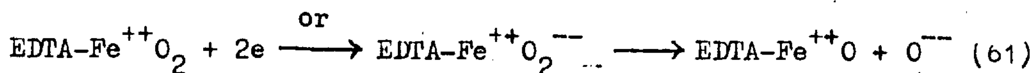
$$\text{EDTA-Fe}^{++}\,O_2 + AH \longrightarrow \text{EDTA-Fe}^{++}O + AOH \qquad (58)$$

$$\text{EDTA-Fe}^{++}O + 2e \longrightarrow \text{EDTA-Fe}^{++} + O^{--} \qquad (59)$$

Type III mechanism



$$\text{EDTA-Fe}^{++}\,O_2 + 2e \longrightarrow \text{EDTA-Fe}^{++}\,O + O^{--} \qquad (60)$$



or

$$\text{EDTA-Fe}^{++}O_2 + 2e \longrightarrow \text{EDTA-Fe}^{++}O_2^{--} \longrightarrow \text{EDTA-Fe}^{++}O + O^{--} \qquad (61)$$

$$\text{EDTA-Fe}^{++}\,O + AH \longrightarrow \text{EDTA-Fe}^{++} + AOH \qquad (62)$$

where AH is the substrate.

According to Braslow and Lukens (1960) there is not enough evidence to support the view that an electrophilic hydroxylating species is formed in the model system. They

observed that the nature of hydroxylation by this system is very similar to the pattern found with Fenton's reagent, and does not show any special preference for attack on electron-rich positions. They postulated a free radical mechanism similar to the reaction of Fenton's reacent as shown below.

$$Fe^{++} + H_2O_2 \longrightarrow FeOH^{++} + OH \qquad (63)$$

Mason et al (1957, 1958) studied the hydroxylation of some aromatic compounds in a system consisting of horseradish peroxidase, dihydroxyfumarate and oxygen. Since this system hydroxylates in electropositive in addition to electronegative positions, it is probable that the reaction occurs through a free radical mechanism (Buhler & Mason 1961). Moreover, the finding that hydrogen peroxide could not replace oxygen in the system suggests that the attacking species is a perhydroxy radical ($H\dot{O}_2$) and not a free hydroxyl radical. Participation of the perhydroxy radical in the model system was also suggested by other workers (Acheson & Hazelwood 1960 ; Norman & Radda 1962). All these reports indicate that the microsomal oxidative enzyme systems may act through the formation of free radicals. Indeed, many reactions carried out by microsomal enzymes for example the oxidation of p-nitrotoluene (Gillette 1959) are difficult to explain by ionic mechanism.

The pattern of hydroxylations of monosubstituted benzenes in the animal body suggests that these reactions are carried out by an enzyme-generated free hydroxyl radicals (Smith 1950 ; Parke & Williams 1958). More recently Diner (1964) suggested that activated molecular oxygen is involved in these reactions rather than free radicals of the hydroxyl or perhydroxyl type. He considered some electronic indexes of the perturbed molecules and proposed that the mechanism for metabolic ring hydroxylation was through the epoxidation of a double bond of the substrate molecule in an excited state followed by a rearrangement leading to a phenol. The opening of an epoxide ring occurs according either to the relative affinities of carbon atoms for oxygen atom, or to the relative stability of the end products, depending on the conjugation strength between the OH group and the aromatic ring.

Section E . Comparative metabolic studies in mammals and insects

It is now a commonly used practice to look into the metabolism of any new compound in a number of animal species before it is considered for human use. As a result, metabolic fate in mammals of a wide variety of foreign organic compounds are known and this knowledge has been profitably used in the design of newer drugs.

In contrast, inadequate studies on the metabolic processes of foreign compounds in insects have been the main limiting factor in the rational development of insecticides. Until a few years ago, the tedious wasteful methods of large-scale semi-random synthesis and screening were the usual features of research for producing effective insecticides. With the use of these compounds of various kind on an ever increasing scale, their effect upon human health, animal and plant life has become a matter of major concern. The history of a number of well known insecticides is marked by an early and enthusiastic acclaim for their potency, often followed by condemnation due to their devastating action on plant and animal life. Although a number of human diseases including allergy, Sinusitis, Alkalosis, Gastro-intestinal upset, Pneumonitis and insanity have been attributed to the intoxicating action of insecticides (Wayland 1960) without sufficient evidence, these reports led the Federal Authorities in the United States and increasingly in other countries to demand extensive data upon the metabolism of new insecticides before permitting them to be introduced in the market. In recent years, efforts have been made to understand the factors which contribute to the selectivity in the toxicity of a compound. It appears that the present day knowledge

of selectivity is most advance in its relation to metabolism. It is in the establishment and exploitation of metabolic differences that there is room for ingenuity, since quite subtle modification of a molecule may profoundly alter its behaviour in biochemical systems and may lead to the development of compounds of high insect and low mammalian toxicity. Results from metabolic studies in mammals and insects have been used with some success in the designing of some phosphorous insecticides. The best example of this is found among those organophosphates which contain carboxyester amide groups (Kreuger and O'Brien 1959 ; Dauterman & O'Brien 1964 ; Smith 1964_a). The low mammalian toxicity of these compounds results from the more extensive hydrolysis of ester or amide links in these species. The term 'selectophore' has been applied to those organic functional groups which offer a point of selective attack for a detoxication mechanism. It should be possible, if an extensive enough search is made, to find out other selectophoric groups which will act through metabolic processes other than hydrolysis. It is obvious that such studies will be more fruitful if simple compounds are used in the beginning. Once a difference is found, it will be necessary to determine the specificity of the enzyme(s) responsible for the differences. If it is not highly specific, it may form

the basis for a selectophore to be built into a known toxophoric nucleus. Another important requirement for a selectophore is that it should not increase the polarity of the molecule.

From these considerations it appears that alkyl groups may function as ideal selectophores. Introduction of such groups is a common feature in the constitution of a large number of insecticides. One of the reasons for the incorporation of an alkyl group in an insecticide is to increase its lipid solubility which permits it to pass through the insect cuticle and nerve sheath. This largely influences the biological activity of a molecule : the greater the lipid solubility, the higher the efficiency. Some vertebrates can oxidize the alkyl chain in alkylbenzenes with great ease (Williams 1959), and since little is known of their fate in insects, we have studied the metabolism of a series of alkylbenzenes in insects *in vivo* and *in vitro* and compared these results with those from similar experiments using vertebrate material.

CHAPTER II.

METABOLISM OF p-NITROTOLUENE AND p-NITROETHYLBENZENE IN MAMMALS AND INSECTS.

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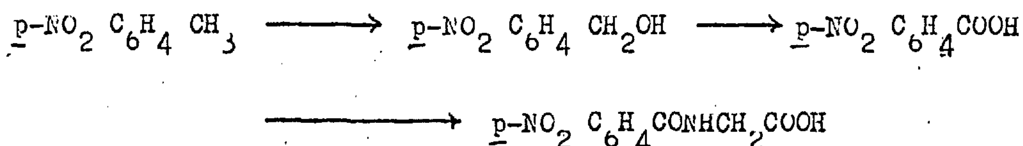
Section A. Chemical oxidation and metabolism of p-nitrotoluene and p-nitroethylbenzene.

p-Nitrobenzoic acid is the common product of the oxidation of p-nitrotoluene by a number of oxidizing agents, including nitric acid, chromic acid and permanganate. 82-94% of m- and p-nitrotoluene is oxidized to the corresponding benzoic acid by aqueous chromic acid at 250° . o-Nitrotoluene is degraded under the same conditions (Wiberg 1965). Oxidation with chromium trioxide in acetic anhydride in the presence of a strong acid gives the corresponding benzal diacetates. Chromyl chloride converts nitrotoluene to nitrobenzaldehyde. The effiency of the reaction has the order p-> m-> o- . Lead tetraacetate oxidation of p-nitrotoluene produces p-nitrobenzyl acetate (Cavill and Solomon 1954). Electrolytic oxidation of p-nitrotoluene using platimum electrodes yields p-nitrobenzyl alcohol, p-nitrocresol , whereas with PbO_2 electrodes it yields p-nitrobenzoic acid. p-Nitroacetophenone is the common product of various oxidation reactions of p-nitroethylbenzene. Oxidation of p-nitroethylbenzene carried out at molecular ratio with $KMnO_4$ at 65-75°

yields 60-62% *p*-nitroacetophenone . Liquid phase oxidation with oxygen at atm. pressure at approximately 135° also gives the same product.

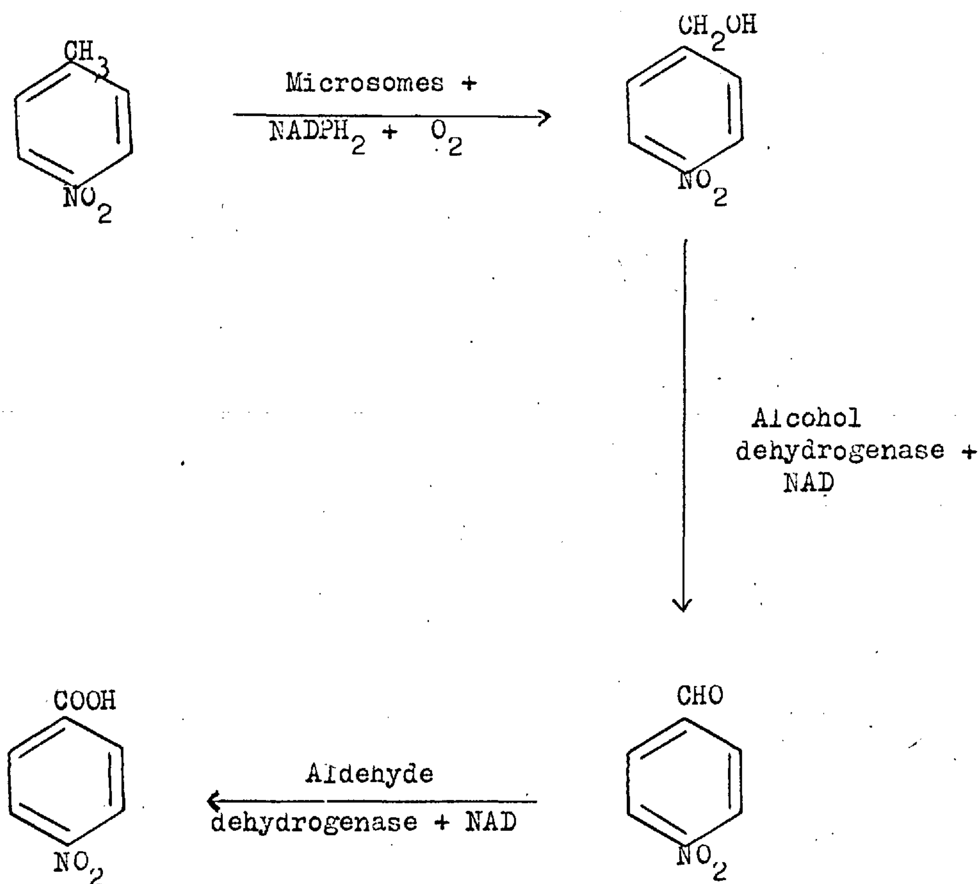
The metabolic fate of *o*- and *p*-nitrotoluene was studied at an early date by Jaffe (1874; 1878-9) who found that in dogs *o*-nitrotoluene was oxidized at the methyl group to yield both *o*-nitrobenzyl alcohol and *o*-nitrobenzoic acid. At least 25% of the dose fed was excreted in urine as a glucuronide of the alcohol, while *o*-nitrobenzoic acid formed amounted to about 10%. The end products of *p*-nitrotoluene metabolism were identified as *p*-nitrobenzoic acid and *p*-nitrohippuric acid.



In rabbits nitrobenzoic acids (*o*-, *m*-, *p*-) are largely excreted unchanged (Bray *et al* 1949). Gillette (1959) showed that *p*-nitrotoluene is oxidized to the corresponding alcohol by a $NADPH_2$ dependant enzyme system in rabbit liver microsomes. The *p*-nitrobenzyl alcohol is then

further oxidized to *p*-nitrobenzoic acid by NAD dependant enzyme systems localised in the soluble fraction of the liver (Fig. 1). No information is available on the metabolism of *p*-nitroethylbenzene in mammals or insects. The metabolism of ethylbenzene have been studied by Thierfelder & Daiber (1923), Smith *et al*(1954 $_{a,b}$) and El Masri *et al* (1956). In the rabbit *in vivo*, ethylbenzene is mainly metabolised to methylphenylcarbinyl glucuronide and hippuric acid. These compounds are formed in roughly equal amounts and account for about 60-70% of the dose. In addition to these, two other metabolites found are phenaceturic acid (15-25%) and mandelic acid(1-2%). No oxidation of the aromatic ring has been reported.

Fig. 1 . Metabolism of p-nitrotoluene in the rabbit *in vitro* (Gillette 1959).



CHAPTER II

Section B. Materials and Methods .

Compounds :- p-Nitrotoluene was crytallised from ethanol-water mixture (8:2), m.p. 53-54°. p-Aminobenzoic acid, m.p. 187°, p-nitrophenylacetic acid, m.p. 155°, p-aminohippuric acid, m.p. 199° and p-nitroethylbenzene, b.p. 245°/ 760 mm., n_D^{20} 1.54 were all commercial samples, which on paper chromatographic examination were found to be free of impurities. p-Nitrohippuric acid, m.p. 135° was prepared by the Schotten-Bauman reaction using glycine and p-nitrobenzoyl chloride. 1-(p-Nitrophenyl)ethanol b.p. 162° /16 mm. and 2-(p-nitrophenyl)-ethanol m.p. 56° were prepared by sodium borohydride reduction of p-nitroacetophenone and methyl p-nitrophenylacetate respectively.

Animals and dosing :- Fifth instar locusts (*Schistocerca gregaria*) were obtained from the Anti-locust Research Centre, London, and were kept as described by Myers and Smith (1953). The insects were fed on fresh grass and water *ad lib*. Normal strain of houseflies was obtained from the Rothamstead Experimental Station as pupae and was used within 3 days of emergence. Other insects were obtained from the same source and used immediately. The resistant strains of flies were obtained from the Department of Entomology , London School of Hygiene and Tropical Medicine. Compounds were administered at 200µg./g. in

acetone (0.01-02 ml.) by injection with Agla Micrometer syringe (Burroughs Wellcome Ltd.). Flies and mustard beetles (Phaedon) were dosed topically at 100 µg./g. and 200 µg./g. respectively.

Female New Zealand white rabbits maintained on a diet of rabbit cubes (S.G.1, J.Bark Ltd.) and water, were used. The compound was administered by stomach tube as suspension in 10 ml. of water. Female albino rats maintained on a diet of rat cubes were used. p-Nitrotoluene in arachis oil (4 ml.) was given to rats orally using a syringe with a long blunt-ended needle.

Tissue preparations :-

(i) Intact tissues :- The tissues were removed from the insect and placed in an ice-cooled beaker. The contents of the gut were removed before use.

(ii) Homogenate and sub-cellular fractions :- The tissues (rabbit, rat or insect) or in some cases, the whole insects were homogenized in 0.25M sucrose for 1-2 mins. at 0-3°. A Potter-Elvehjem homogenizer with a loose-fitting teflon pestle driven by a Towers motor was used. The homogenate was centrifuged in a M.S.E. 'High Speed 17' refrigerated centrifuge at 3° at 10,000g. for 10 mins. . The supernatant (10,000g. sup.) was used for routine assays. For the preparation of microsomes, the 10,000g.

supernatant was centrifuged in a M.S.E. 'Superspeed 40' centrifuge at 90,000g. for 1 hr. at 0-5° . The soluble fraction was poured off and the microsomal pellet was resuspended in the required volume of 0.25M sucrose by gentle homogenization. All homogenates and sub-cellular fractions were prepared to give a set weight of 250 mg.tissue/ml. or 1.5-2 locusts' fat body/ml. . In some preparations, especially those from beetles, flies and caterpillars, higher tissue concentration was used. During the later part of the work only abdomens were used for fly enzyme preparation.

Qualitative examination of the metabolites :-

Dosed insects were ground in a homogenizer with 5 ml. of acetone/water (80% V/V) and the mixture was centrifuged. The supernatant was concentrated in vacuo at 45° and examined by paper chromatography. Urine (5 ml.) from dosed rats and rabbits was hydrolysed by 1 ml. β-glucuronidase (Ketodase, W.R. Warner & Co. Ltd. , 5,000 units/ml.) using 0.2M acetate buffer, pH 4.6 (5 ml.). The incubations were carried out at 38° for 16 hrs. . Some urine samples had to be concentrated in vacuo at 45° before paper chromatographic analysis. In descending solvent systems unknown were run against reference compounds on Whatman No. 1, 4 or 3MM paper strips. R_f values of the compounds used are

quoted in Table 2.

Detection methods :- The acids were conveniently located on the papers by illumination with U-V light of 254 mμ . from a Hanovia Chromatolite lamp. In this light p-aminobenzoic acid and p-aminohippuric acids are feebly purple, while p-nitrobenzoic acid and p-nitrohippuric acid strongly quench the background fluorescence of the paper and appear as dark spots. These compounds were also located as pink spots, when the paper was sprayed with Titanous chloride solution in HCl (0.15% W/V) and then oversprayed with p-dimethylaminocinnamaldehyde (0.5% solution in a 1:1 mixture of ethanol and glacial acetic acid). Yellow spots were obtained by spraying with p-dimethylaminobenzaldehyde under the same conditions.

Quantitative examination of the metabolites :-

Estimation of p-nitrobenzoic acid in various preparations was done by the following method. Single large insects or 1g. batches of insects were homogenized in 5 ml. water containing 0.5 ml. of 0.5N NaOH. Protein was removed by the addition of 0.5 ml. of 10% (W/V) $ZnSO_4$ solution and 10 ml. of carbon tetrachloride was added to extract unchanged p-nitrotoluene. After shaking (3 mins.) and centrifugation, 3 ml. of the supernatant was acidified with 0.1 ml. of 2N HCl and shaken for

half minute with 1 ml. of 1% (W/W) Zinc amalgam. The p-amino-benzoic acid formed was then determined according to Bratton and Marshall's method (1939). The method consisted of diazotising p-aminobenzoic acid , destroying the excess of sodium nitrite by using ammonium sulphamate and coupling the diazonium salt with N-(1-naphthyl) ethylenediamine. The colour was measured at 555 mμ (Fig. 4). In some insects, particularly flies, large blank values were obtained from untreated insect extracts. This was minimised by extracting the dye in 5 ml. of amyl alcohol in which the interfering material was not soluble. In experiments where p-nitrobenzyl alcohol was the substrate, ether was used instead of carbon tetrachloride to extract excess substrate and the diazo colours were measured within 10 mins. of coupling. p-Nitrobenzyl alcohol gave the same intensity of diazo colour in the procedure for assaying p-nitrobenzoic acid but the speed of azo-coupling was considerably slower (Fig. 2). Recoveries of p-nitrobenzoic acid added to 1g. of locust homogenate were 83.2 S.E.M.± 1.9 (10). In the case of rats and rabbits, urine samples (0.2-0.4 ml.) were banded across 8 inches wide Whatman 3MM paper. The chromatograms were developed in butan-1-ol/NH_3 (sp.gr. 0.88)/ water (4:1:5 V/V) for 5 hrs. . The appropriate strips of the paper (their position determined with reference spots of the known compounds) were cut out and eluted with dil. ammonia solution .

p-Nitrobenzoic acid and p-nitrohippuric acids in the eluates were then determined by the method described earlier. Hereafter, this p-nitrobenzoic acid estimation procedure will be referred to as the standard method.

Estimation of 1-(p-nitrophenyl)ethanol :- The reaction mixture (5 ml.) was acidified with 3 ml. of 10N HCl and extracted with 3 x 5 ml. of ether. The ether layer was evaporated to small bulk and transferred to a paper chromatogram and run in solvent system A. The zone corresponding to 1-(p-nitrophenyl)ethanol was eluted with methanol and this was assayed by the same method as described for p-nitrobenzoic acid. Colour development was allowed to proceed for 3 hrs. before the measurement and calculation was made by reference to a calibration curve prepared from known amounts of 1-(p-nitrophenyl)ethanol. Recoveries of known amounts of material added to 5 ml. of incubation mixture were 71 S.E.M. $\pm$ 3.2 (6).

Table 2. R_f Values of some aromatic nitro and amino compounds.

Chromatograms were run on Whatman No.4 paper until solvent fronts had moved 12 inches. Solvent systems : A, n-hexane/di-isopropyl ether (5:1, v/v) run on paper treated with formamide-saturated ether and dried; B, butan-2-one/2N NH_3 (1:1, v/v); C, butan-1-ol/ammonia (sp. gr. 0.88)/water (4:1:5 v/v); D, ethanol/water (7:3, v/v) on paper treated with 5% (v/v) olive oil in ether and dried; E, n-hexane/di-isopropyl ether (5:1, v/v) on paper treated with a saturated solution of carbowax 4000 in ether-ethanol (50:1) and dried; F, benzene/butan-1-ol/ammonia, sp.gr.0.88 (2:5:2, v/v), upper phase; G, butan-1-ol saturated with water.

Compound	A	B	C	D	E	F	G
1-(p-Nitrophenyl)ethanol	0.20	0.92	0.93	0.92	0.30		
2-(p-Nitrophenyl)ethanol	0.15	0.91	0.90	0.90	0.25		
p-Nitrophenylacetic acid	0.02	0.60	0.42	0.81	0.34		
p-Nitroethylbenzene	0.91	0.92	0.91	0.70	0.90		
p-Nitrobenzoic acid	0.01	0.71	0.73	0.81	0.32	0.31	0.83
p-Nitrohippuric acid		0.58	0.29			0.23	0.24
p-Aminobenzoic acid		0.49	0.07			0.05	0.80
p-Aminohippuric acid						0.05	0.10
p-Nitrobenzyl alcohol		0.95	0.68				
p-Nitrotoluene	0.92	0.93	0.90	0.72	0.91		

Fig. 2 . Time curves for colour development by coupling diazotized p-aminobenzyl alcohol at various concentrations with N-(1-naphthyl)ethylenediamine.

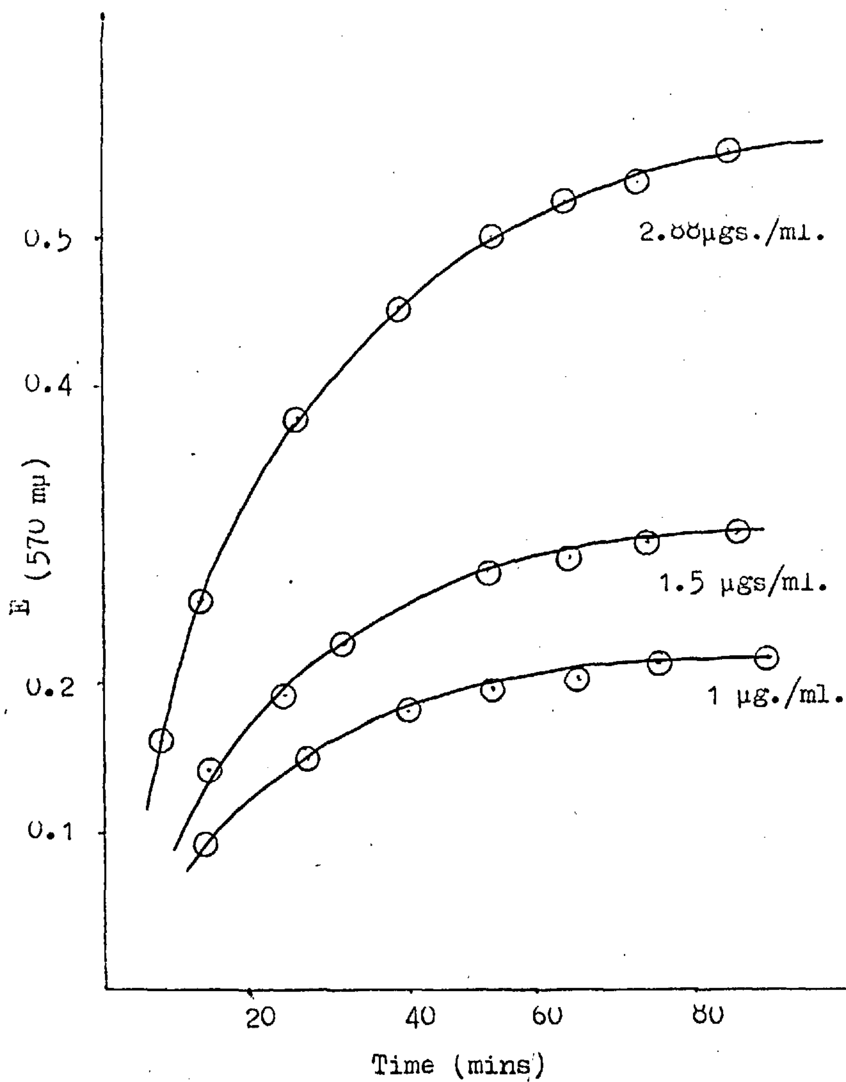


Fig. 3. Absorption spectra of the dye formed by coupling diazotized p-aminobenzyl alcohol with N-(1-naphthyl)ethylenediamine.

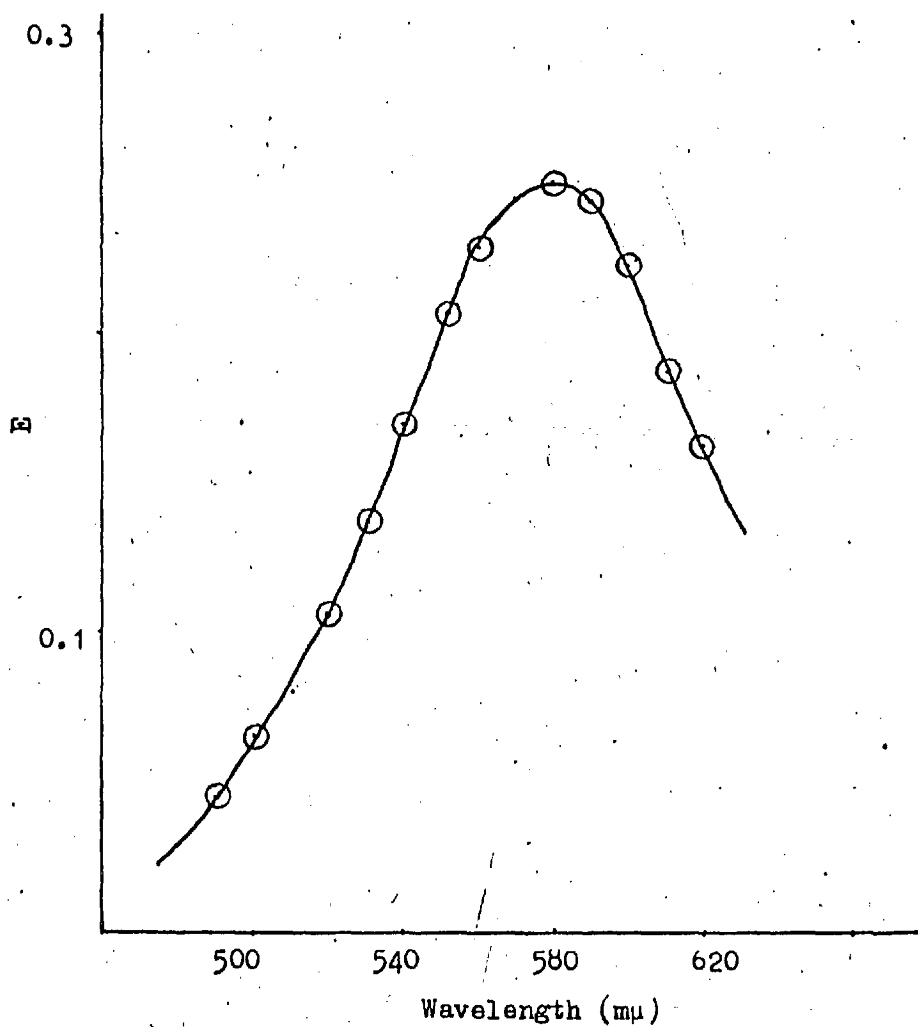
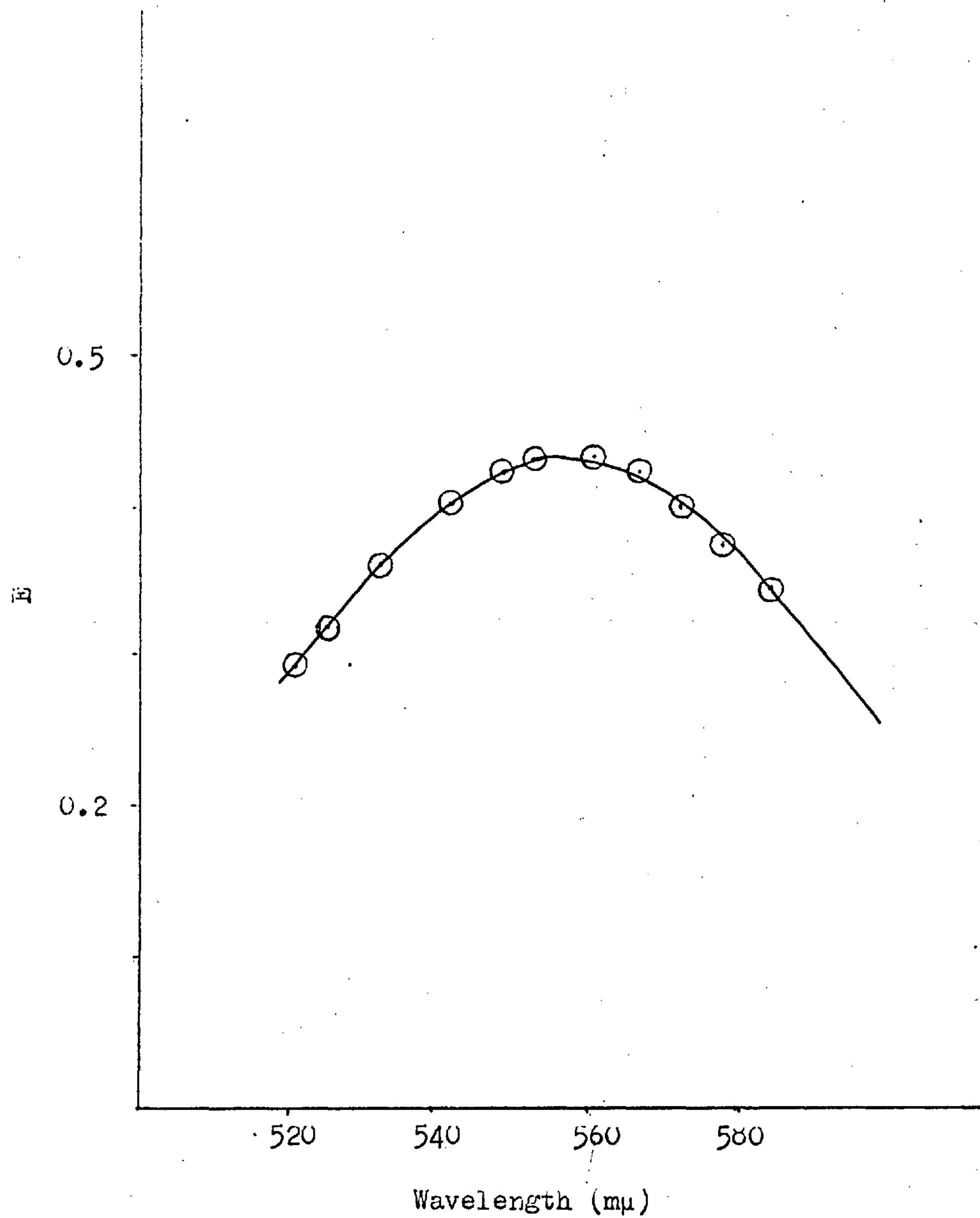


Fig. 4 . Absorption maxima of the dye formed by coupling diazotized p-aminobenzoic acid with N-(1-naphthyl)ethylenediamine.



CHAPTER II

Section C. Metabolism of p-nitrotoluene in mammals and insects in vivo.

Identification of the metabolites of p-nitrotoluene :- The metabolites were identified as described under methods. Insects were used 16 hrs. after dosing, while in the case of rat and rabbit 24 hr. urine samples were examined. p-Nitrobenzoic acid was found to be the major metabolite in every species of insects and mammals studied. In some cases p-nitrohippuric acid (locust, rat & rabbit) and p-aminobenzoic acid (rat, rabbit & cockroach) were found as minor metabolites. The results are given in Table 3.

Quantitative assay of p-nitrotoluene oxidation :- Quantitative assays were made on four large insects or 1g. batches of small insects at different time interval after dosing. A linear relationship was noticed between time and amount of p-nitrobenzoic acid formed. In the case of the rat and rabbit, urine samples were collected every 24 hrs. for 4 days after dosing. 5 Ml. of 0.2M acetate buffer, pH 4.6 was added to 5 ml. of concentrated urine and the mixture was incubated with 1 ml. of β-glucuronidase solution (5000 units/ml. ; Ketodase, W.R.Warner & Co.Ltd) overnight at 38°. The hydrolysate was concentrated and

chromatographed. Details of the procedure followed for the estimation of the metabolites, p-nitrobenzoic acid and p-nitrohippuric acid are given under methods (P.54). These compounds were still present in 5th and 6th day urine but only in trace amounts. The results are given in Tables 4,5 & 6.

Table 3. Qualitative examination of *p*-nitrotoluene metabolites in insects and mammals.

Below are given the metabolites identified in rat and rabbit urine and in the whole insects dosed with *p*-nitrotoluene (200 μg./kg.). The method used for identification has been described in Section B.

Species	PNBA	PNHA	PABA
Rat	+	+	+
Rabbit	+	+	+
Locust(*Schistocarca*)	+	+	–
Cockroach(*Peripleneta*)	+	–	+
Cockroach(*Blatta*)	+	–	+
Cricket(*Gryllus*)	+	–	–
Flour Beetle(*Tenebrio*)	+	–	–
Mustard Beetle (*Phaedon*)	+	–	–
Housefly(*Musca*)	+	–	–
Cotton stainer (*Dysdercus*)	+	–	–
Caterpillar(*Pieris*)	+	–	–

The abbreviations used are : PNBA, *p*-nitrobenzoic acid ; PNHA, *p*-nitrohippuric acid ; PABA, *p*-aminobenzoic acid.

+ and – indicate the presence and absence respectively of any metabolite.

Table 4. Metabolism of p-nitrotoluene in insects *in vivo* (Dose : 200 µg./g.).

The methods used for measuring p-nitrobenzoic acid has been described in Section B.

Species	Formation of p-nitro-benzoic acid. (µg./g. insect/hr.)	$t_{\frac{1}{2}}$ (hrs.)
Locust (*Schistocerca*)	10	13
Cockroach (*Peripleneta*)	9	15
Cockroach (*Blatta*)	12	11
Cricket (*Gryllus*)	12	12
Flour beetle (*Tenebrio*)	8	19
Mustard beetle (*Phaedon*)	20	6
Housefly (*Musca*)	25	2
Cotton stainer (*Dysdercus*)	5	30
Caterpillar (*Pieris*)	8	25

The half-life values ($t_{\frac{1}{2}}$) were calculated by plotting the log. values of residual p-nitrotoluene in the body at various time intervals after dosing against time.

Table 5 . Metabolism of p-nitrotoluene by female albino rats. Dose 200 mg./kg.

The animals were dosed orally and the urinary metabolites p-nitrobenzoic acid and p-nitrohippuric acid assayed as described in Section B. The results represent averages of three animals.

Day	% Dose excreted as		
	p-Nitrobenzoic acid (total)	p-Nitrohippuric acid	Total
1	29.5	5.5	35
2	17.5	4.5	22
3	8	3	11
4	2.5	*	2.5
	57.5	13	70.5

* less than 0.5% of the dose.

Table 6 . Metabolism of p-nitrotoluene by female New Zealand white rabbits. Dose 200 mg./kg. .

The animals were dosed orally and the urinary metabolites p-nitrobenzoic acid and p-nitrohippuric acid were measured as described under methods (Section B). The results represent averages of two animals.

Day	% Dose excreted as		
	p-Nitrobenzoic acid(total)	p-Nitrohippuric acid	Total
1	40	11	51
2	12.5	2	14.5
3	6	1	7
4	7	0.8	7.8
	65.5	14.8	80.3

CHAPTER II

Section D. Metabolism of *p*-nitrotoluene and *p*-nitroethylbenzene in mammals and insects *in vitro*.

Metabolism of *p*-nitrotoluene in intact tissues of locust and cockroach :- In each experiment tissues from six insects were added to 2 ml. of saline together with 7.3 µmoles of *p*-nitrotoluene in 0.02 ml. of acetone. They were incubated in air in a thermostatically controlled shaking water bath (Mickle Incubation Shaker) at 37° for 30 mins. (Cohen & Smith 1964). The mixture was then ground in a Potter-Elvehjem homogenizer and *p*-nitrobenzoic acid was estimated by the standard method. All the locust tissues examined produced *p*-nitrobenzoic acid as the major metabolite; *p*-nitrohippuric acid was also found in trace amounts. The results given in Table 7 are expressed as the amount of *p*-nitrobenzoic acid formed per locust per hour. The cockroach tissues were found to be devoid of any *p*-nitrotoluene oxidizing activity.

Metabolism of *p*-nitrotoluene by insect homogenates :- In locust, the fat body, gastric cecae and gut are the most effective in oxidizing *p*-nitrotoluene *in vitro* (Table 7). Homogenates of these tissues, prepared as previously described (Sec.B) were

examined for *p*-nitrotoluene oxidase activity alone and also in the presence of various cofactors. Similar investigations were also carried out with homogenates of whole houseflies (*Musca*), cotton stainers (*Dysdercus*), caterpillars (*Pieris*), and beetles (*Tenebrio* & *Phaedon*). Homogenates used in each incubation mixture corresponded to 3g. of insects. A typical incubation mixture contained 4 ml. of homogenate preparation, 0.5 ml. of 0.1*M* Tris buffer, pH 7.5, 7.3 μmoles of *p*-nitrotoluene in 0.02 ml. of acetone. The final volume was made up to 5 mls. . The added cofactors common to all incubations were NAD, 0.3 μmols, glucose-6-phosphate 19 μmoles, nicotinamide, 40 μmoles, $MgCl_2$, 30 μmoles. In addition to these, some mixtures contained 0.27 μmole of $NADPH_2$ (cofactor mixture 1) and some 0.3 μmole of $NADH_2$ (cofactor mixture 2). All incubations were carried out at 37° for 30 mins. . Qualitative and quantitative examination of the mixtures showed that the only preparation which had *p*-nitrotoluene oxidizing activity was the locust fat body homogenate. Even with this tissue, the amount of *p*-nitrobenzoic acid formed is only just enough to be seen on paper chromatograms. Addition of cofactors was found to have no effect on the enzyme activity of the preparations (Table 8).

Examinations of the p-nitrotoluene oxidizing activity of locust cell fractions :- Since only one of the homogenates used showed p-nitrotoluene oxidizing activity, the possibility of the presence of endogenous inhibitors were considered. 10,000g. Supernatant fractions from various homogenates were assayed for p-nitrotoluene oxidizing activity. The supernatant preparations were made as described under methods (Sec.B). Each preparation was incubated both alone and supplemented with the various cofactors, described for whole homogenate incubations. All incubations were carried out at 37° for 1hr. in air. The results are given in Table 8. This shows that with the exception of locust fat body 10,000g. supernatant, all other insect preparations were inactive. Centrifugation of locust fat body homogenate yielded an active enzyme in the 10,000g. supernatant. The enzyme activity of this fraction does not increase by the addition of any cofactor (Table 10).

Properties of the locust fat body p-nitrotoluene oxidizing enzyme system :- Various properties of the enzyme system were studied. In all experiments the standard incubation mixture contained 4 ml. of enzyme preparation, 0.5 ml. of 0.1M tris buffer,pH 7.4 and 7.3 µmoles of the substrate in 0.05 ml. of acetone. The final volume was made up to 5 ml. and the reaction was carried out in air for 30 mins. at 37°. The standard method was used for the p-nitrobenzoic acid assays. The effect of added cofactors was tested in a number of experiments

with locusts from different batches but no increase in enzyme activity was produced in the standard incubation mixture by the addition of NAD, NADP, $NADH_2$ or $NADPH_2$ (NAD & $NADH_2$ 0.3 μmole each ; NADP & $NADPH_2$ 0.27 μmole each) , 49 μmoles of nicotinamide and 30 μmoles of $MgCl_2$. The results are given in Table 10. The locust fat body enzyme is similar to that of the vertebrate liver in that, centrifugation at approximately 10,000g. for 10 mins. yields an active enzyme system in the supernatant. Centrifugation of this supernatant at 90,000g. for 1hr. yields a sediment which is inactive unless combined with 90,000g. supernatant (Table 10). Preparations of fat body homogenate were centrifuged at various speeds for 10 mins. and the activities of the supernatants produced were graphed (Fig. 6). The graph showed a broad maximum in the region 10-12,000g. . All samples were centrifuged at 10,000g. for 10 mins. thereafter. The activity of the enzyme preparations were found to be linear with time for periods up to 1hr. and normally reactions were carried out for 30 mins. (Table 11). The optimum substrate concentration was found to be 1.75 mM and a double-reciprocal plot of substrate concentration and reaction velocity gave an apparent K_m value 6.3×10^{-4} (Figs. 8 & 10). The pH of the reaction mixture was varied by addition of 0.5 ml. of 0.1M Tris buffer to the standard reaction mixture. The final volume was made up to 5 ml. and the reaction velocity was measured between 6.5 and 8.5 (Fig. 7). A narrow pH-activity curve was obtained peaking at 7.4 .

The stability of the enzyme preparation was examined by storing it under different conditions (Table 13). It was found that about 20% of the activity was lost when stored at 0° for 16 hrs. or if left at room temperature for three hours. When the homogenate was left at room temperature for three hours before centrifugation, no activity was found in the 10,000g. supernatant. Experiments were carried out to determine the effect of homogenizing fat body for different periods on the p-nitrotoluene oxidizing activity of the 10,000g. supernatant. The results in Table 12 show that homogenization of the tissue for more than 2 mins. destroys the enzyme.

Metabolism of p-nitroethylbenzene in the 10,000g. supernatant of locust fat body :- The enzyme preparation from 25 locusts was incubated for 1hr. with 36.5 μmoles of p-nitroethylbenzene at 37° in air. After acidification with 3 ml. of 10N HCl and extraction with ether, the ether extract was examined by paper chromatography and ionophoresis. The major metabolite found was 1-(p-nitrophenyl)-ethanol together with a trace of p-nitrophenylacetic acid. In quantitative assays the rate of formation of the alcohol was found to be 2 μg./locust/hr. .

The effect of locust fat body homogenate on p-nitrotoluene oxidation by rabbit liver :- Locust fat body 10,000g. sup. enzyme was found to be inhibited by the sedimented fractions and this inhibiting effect was not altered by boiling the sediment for 15mins.(Table 14).

The nature of this inhibition was investigated using rabbit liver enzyme prepared as described previously, with inhibitor concentrations equivalent to the sediments of 0.5-1.25 locusts' fat body over a range of substrate concentrations . The incubations were carried out at pH 7.4 , fortified with various cofactors (Table 16). The results at each substrate concentration indicated an irreversible inhibition in which inhibitor from 1.5 locusts was sufficient to completely inhibit the 10,000g. sup. enzyme from 0.75g. rabbit liver in an incubate containing the standard cofactors (Fig. 11). Attempts were made to measure the rate of inhibition by leaving resuspended locust fat body 10.000g. sediment in contact with the rabbit enzyme at 0° for varying periods, centrifuging off the inhibitor sediment and assaying the remaining supernatant. A small reduction of activity was found after leaving the fat body sediment in contact with rabbit enzyme, but most of the inhibitory activity could be centrifuged away (Table 18). The sediment did not appear to interfere with the determination of p-nitrobenzoic acid (Tables 18). As prolonged homogenisation (2 min.) gave a progressively less active enzyme (Table 12), the possibility of an inhibitor being released on protracted

grinding was considered. A sample of 10,000g. sediment from a preparation that had been homogenised for 30 sec. was resuspended in sucrose and ground at 0° for various times to examine whether its inhibitory power increased. This quantity of locust inhibitor reduced the rate of oxidation of a rabbit enzyme preparation from 144 mμmoles to 100 mμmoles p-nitrobenzoic acid/min. but no increased inhibition was found if the homogenisation was prolonged and no water soluble inhibitor was released (Table 20). Attempts to prepare an oxidizing enzyme from locust gut were not successful though in vitro this tissue forms more p-nitrobenzoic acid than the fat body (Table 7). Assays were therefore made using rabbit enzyme with the equivalent of homogenised parts of the gut of one locust added. Whole homogenate of mid gut was markedly inhibitory (Table 15).

Inhibition of vertebrate enzyme by other insects :- The 10,000g. supernatant equivalent to 4g. of flies from homogenates made in either 0.25 M sucrose or 0.15 M KCl had no oxidizing activity with p-nitrotoluene as substrate even when fortified with the amounts of NADP, NAD, $MgCl_2$ and nicotinamide used with rabbit enzyme. The 90,000g. sediment

fraction of the fly preparation (4 g.) remained inactive when 90,000g. supernatant from 0.5g. rabbit liver was added with the cofactors used in the rabbit assay system. Fly homogenates were also effective inhibitors of the rabbit oxidizing enzyme (Table 17), and the inhibition by homogenate of 0.05 or 0.075g. of flies was only partly offset by the addition of very large amounts of $NADPH_2$ or by the use of a regenerating system for $NADPH_2$ (Table 17). Cockroach fat body, mustard beetles and caterpillars (Table 18) contained much less inhibitor than flies but nevertheless their 10,000g. supernatants were inactive when tested for *p*-nitrotoluene oxidizing power, either alone or with added cofactors.

The stability of $NADPH_2$ in various insect preparations :-
The inhibition of rabbit liver *p*-nitrotoluene oxidizing enzyme by 10,000g. supernatants from various insects could have been due to their rapidly destroying the cofactors necessary for the proper functioning of the rabbit enzyme system. This might also explain the failure to obtain any any *p*-nitrotoluene oxidation in insect preparations other than that from locust fat body. Another factor which was

considered was the possible presence of some inhibitor(s) which interferes with the regeneration of $NADPH_2$. Experiments were carried out to find out the stability of $NADPH_2$ in insect preparations both alone and in the presence of added nicotinamide. The effect of addition of glucose-6-phosphate on the level of $NADPH_2$ in the systems was also examined in order to determine whether there was enough glucose-6-phosphate dehydrogenase in there for the regeneration of $NADPH_2$. The level of $NADPH_2$ in any sample was followed by a direct spectrophotometric measurement at 340 mμ. The results show (Fig. 12) that $NADPH_2$ is stable in locust fat body 10,000g. supernatant and addition of G-6-phosphate and nicotinamide has no effect. Small amounts of $NADPH_2$ were oxidized in caterpillar and beetle preparation but most of it was regenerated by the addition of G-6-phosphate (Figs. 12 & 14). Whole fly 10,000g. sup. rapidly oxidized most of the added $NADPH_2$ in about 10 min. but significant amount of it was regenerated by the addition of G-6-phosphate . The presence of nicotinamide apparently has no effect (Fig. 15). Fly abdomen preparation behaved rather differently from the whole fly 10,000g. sup. . $NADPH_2$ disappeared much more slowly in this preparation than observed in whole fly 10,000g sup. .

Metabolism of p-nitrotoluene in housefly abdomen preparation :- 10,000g. Supernatant preparations from fly abdomen were found to be capable of oxidizing p-nitrotoluene to p-nitrobenzoic acid. No other metabolite was produced. Attempts were made to fortify the enzyme system by adding various cofactors and maximum activity was obtained with NADP and NAD (Table 19). Preliminary examinations indicated that both microsomal fraction and 90,000g. supernatant are required for the enzyme activity. The oxidation of p-nitrobenzyl alcohol by fly enzyme was measured under the conditions used for the oxidation of p-nitrotoluene and over the concentration range 0.2-1mM. The double reciprocal plot of these results was linear and gave a value of $K_m = 2.8 \times 10^{-3}$. The rate of oxidation of p-nitrobenzyl alcohol at low concentrations (3 µg./g. of flies/hr. at 0.2mM) was considerably lower than the rate of formation of p-nitrobenzoic acid from p-nitrotoluene in the routine assay.

Table 7 . Rate of formation of p-nitrobenzoic acid from p-nitrotoluene by locust organs *in vitro*.

Organs from six hoppers were used in each experiment . The incubation was done in saline to which 7.3 µmoles of the substrate in 0.02 ml. of acetone had been added. After the incubation was completed the mixture was ground and p-nitrobenzoic acid was estimated as described in the text.

Organ	Formation of p-nitrobenzoic acid, mµmoles / locust / hr.		
	Males	Females	Mixed sexes
Fat body	30.5	37.1	47.3
Gastric cecae	16.8	28.7	24
Foregut	4.19	5.98	10.8
Midgut	4.19	5.98	11.4
Hindgut	1.8	1.8	4.8
Malpighian tubes	1.8	3.6	11.4
Total	59.3	83.2	109.6

Table 8 . p-Nitrotoluene oxidizing activity in insect homogenates and 10,000g. supernatants.

4 Ml. of a preparation equivalent to 3g. of insects was incubated with 0.5 ml. of 0.1M Tris buffer, pH 7.5 and 7.3 µmoles of p-nitrotoluene for 30 min. at 37° . The cofactor mixtures used have been described in the text. Methods used for qualitative examination have been described in Sec. B.

Homogenate	p-Nitrotoluene oxidizing activity in the presence of		
	No cofactor	Cof. mix. 1	Cof. mix 2
Locust fat body	+	+	+
Gastric cecae	-	-	-
Gut	-	-	-
Housefly (Musca)	-	-	-
Cotton stainer(Dysdercus)	-	-	-
Caterpillar (Pieris)	±	-	-
Mustard beetle(Phaedon)	-	-	-

+ , Indicates the presence and -, the absence of of enzyme activity.

Table 9 . Effect of speed of centrifugation of the locust fat body homogenate on the p-nitrotoluene oxidizing activity of the supernatant.

Aliquots of a fat body homogenate preparation were centrifuged at different speed and the supernatant fractions were assayed for p-nitrotoluene oxidizing activity. The incubation mixture contained 4 ml. of enzyme, 0.5 ml of 0.1 M tris buffer, pH 7.4 and 7.3 μmoles of substrate in a total volume of 5 ml. . The reaction was carried out for 30 min. at 37° . p-Nitrobenzoic acid was measured by the standard method.

Speed of centrifugation	p-Nitrobenzoic acid formed, mμmoles / locust / hr.
600 g.	9.7
1700 g.	14
10,000 g.	32
12,500 g	26
18,000 g.	13

Table 10 . Cofactor requirements and sub-cellular distribution of the p-nitrotoluene oxidizing enzyme system of locust fat body.

A typical incubation mixture contained the enzyme preparation (4 ml.), the cofactors quoted, 0.5 ml. of 0.1M Tris buffer, pH 7.4, and 7.3 µmoles of the substrate in a total volume of 5 ml. . The reactions were carried out for 30 min. at 37° and the enzyme assayed as described in the text.

Incubations	p-Nitrobenzoic acid formed, mµmoles / locust / hr.
10,000g. sup. alone	37
+ NADP + NAD + G-6-phosphate + nicotinamide	32
+ $NADPH_2$ + NAD + nicotinamide	42
+ $NADH_2$ + nicotinamide	30
Microsome	nil
90,000g. supernatant	4.2
Microsome + 90,000g. supernatant	32

Table 11. Rate of formation of p-nitrobenzoic acid from p-nitrotoluene in locust fat body 10,000g. supernatant.

The standard incubation mixture contained 4 ml. enzyme, 0.5 ml. tris buffer, pH 7.4 and 7.3 μmoles of p-nitrotoluene. The reactions were carried out for different lengths of time and p-nitrobenzoic acid was measured by the standard method.

Incubation period in minutes.	p-Nitrobenzoic acid formed mμmoles / locust
10	5.4
20	11.4
30	14
45	20

Table 12 : p-Nitrotoluene oxidizing activity of 10,000 g. supernatant fractions of locust fat body preparation homogenized for various lengths of time.

A fat body preparation was homogenized and aliquots were taken out at different intervals, centrifuged and the 10,000 g. supernatant fractions were assayed for p-nitrotoluene oxidizing activity.

Period of homogenization in min.	p-Nitrobenzoic acid produced, mμmoles / locust / hr.
2	30.8
4	30.8
6	24.8
8	13.6
12	nil

Table 13. Activity of the p-nitrotoluene oxidizing system in locust fat body preparations stored under various conditions.

Enzyme preparations used for the assays given below were obtained from the same stock fat body homogenate. 4.5 ml. of enzyme was incubated with 0.5 ml. of tris buffer and 7.3 µmoles of p-nitrotoluene for 30 min. . p-Nitrobenzoic acid was measured by the standard method.

Sample	% Loss of activity
10,00g. sup. stored a 0° for 16 hr.	20.5
10,000g. sup. stored at room temperature for 3 hr.	20
10,000g. sup. stored at 0° for 49 hr.	68.5
10,000g. sup. from homogenate left at room temperature for 3 hr. before centrifugation.	100

Table 14. Inhibition of locust fat body enzyme by the sedimented fraction.

10,000g. supernatant (4.5 ml.) was incubated with 7.3 µmoles of p-nitrotoluene at 37° in a total volume of 5 ml. in air at pH 7.4 . Sediments equivalent to three locusts' fat bodies were used as inhibitor.

Incubation	p-Nitrobenzoic acid formed mµmoles / locust / hr.		
	Expt. 1	Expt. 2	Expt.3
No inhibitors	71.9	61.9	24
10,000g. sediment added after incubation	-	-	24
1000g. sediment	12.7	-	-
10,000g. sediment	3	20.6	5.7
10,000g. sediment heated 15 min. at 100	-	20.6	9.7
Washingd of 10,000g. sediment after heating for 15 min. at 100° .	-	-	20.6

- , Means not determined .

Table 15 . Inhibition of rabbit liver *p*-nitrotoluene oxidation enzyme system by homogenates of locust organs.

Whole homogenates equivalent to organs of one locust in 0.25*M* sucrose were incorporated in the standard rabbit enzyme mixture and the oxidation of *p*-nitrotoluene measured by the standard method.

Homogenate added to rabbit enzyme	% Inhibition
Mid-gut	46
Fat body 10,000g sediment	32.4
Fore-gut	15
Hind-gut	0
Gastric cecae	0
Malpighian tubes	0

Table 16 . Inhibition of rabbit enzyme by locust fat body 10,000g. sediment.

Each incubation mixture contained 10,000g. supernatant from a homogenate of 0.75g. rabbit liver with the addition of 10,000g. sediment from locust fat body homogenate equivalent to stated no. of locusts in a total vol. of 5 ml. containing 0.675 µmole $NADPH_2$, 0.75 µmole NAD, 10 µmoles $MgCl_2$, 24 µmoles nicotinamide and 0.5 ml. of 0.1 M Tris buffer at pH 7.4. Incubations were carried out at 37° in air, with shaking for 0.5 hr. . p-Nitrobenzoic acid was measured as described in the text.

Inhibitor concentration (no. of locusts)	Substrate concentration		
	M/2720	M/1360	M/680
	Amount of p-nitrobenzoic acid formed, µmoles / g. of liver / hr.		
0.5	1.06	1.3	2.18
0.75	0.99	1.07	1.73
1	0.6	0.69	1.27
1.25	0.27	0.29	0.67

Table 17 . Inhibition of rabbit oxidation enzyme by housefly preparations.

Fly preparations were incorporated in the standard rabbit assay system along with the cofactors quoted and the mixture assayed with p-nitrotoluene as described in the text.

Additions to rabbit system.	% Inhibition
No additions	0
Fly homogenate(0.25g. of fly)	100
90,000g. sup.(0.25g. of fly)	44.4
Homogenate (0.1g. of fly)	100
Homogenate (0.075g. of fly)	31
Homogenate (0.01g. of fly)	0
Homogenate(0.075g. of fly) + 10.7 µmoles of $NADPH_2$	19.4

Table 18 . Inhibition of rabbit microsomal oxidation by insect preparations.

Rabbit enzyme and cofactors were used as described in the text. Inhibitors were added at 0° and centrifuged off at 10,000g. after stated contact times. p-Nitrobenzoic acid produced was estimated by the standard method. Inhibitors were 0.25M sucrose homogenates of :- A, 0.5g. cockroach fat body ; B, 1g. cockroach fat body ; C, D, 10,000g, sediment from locust fat body ; E, 0.25g. of mustard beetles ; F, 0.5g. of mustard beetles ; G, 0.5g. cabbage caterpillar.

	Contact times in min.	p-Nitrobenzoic acid formed, µmoles / g. of liver / hr.						
		A	B	C	D	E	F	G
No inhibitors		1.94	1.94	1.89	1.15	1.94	1.94	2.75
Inhibitor added after incubation		2.1			1.1	1.94		
Inhibitor not removed before incubation		1			0	1.05		2.61
Inhibitor alone		0	0	0	0	0	0	0
Inhibitor centriguged off before assay	4	1.94	2.39	1.39	0.67	1.82	1.53	
	14	1.92	2.39	1.2	0.81	1.75	1.53	
	24	1.8	2.39	1.2	0.74	1.68	1.58	
	34			1.2				

Table 19 . Cofactor requirements of the p-nitrotoluene oxidizing enzyme system in fly.

The 10,000g. supernatant (4 ml.) from fly abdomen homogenate equivalent to 1 g. of flies was incubated with 7.3 μmoles of p-nitrotoluene , 0.5 ml. of Tris buffer, pH 7.5, and various cofactors quoted in a total volume of 5 ml. . The incubations were done for 1 hr. at 37° in air. p-Nitrobenzoic acid was measured by the standard method.

Additions	Yield of p-nitrobenzoic acid, mμmoles / g. of flies/hr.
10,000g. supernatant	trace
+ NADP + NAD + G-6-P + $MgCl_2$ + Nic.	77.8
+ NADP + G-6-P + $MgCl_2$ + Nic.	65.9
+ NAD + G-6-P + $MgCl_2$ + Nic.	35.9

The abbreviations used are : G-6-P, glucose-6-phosphate; Nic. , nicotinamide.

Table 20 . Effect of prolonged homogenization on the inhibitory activity of the locust fat body 10,000g. sediment.

Rabbit liver enzyme (0.5g.) was incubated with the inhibitor (0.5 locust) , 0.68 µmole $NADPH_2$, 0.75 µmole NAD, 10 µmoles of $MgCl_2$, 24 µmoles of nicotinamide, 7.3 µmoles of p-nitrotoluene, and 0.5 ml. of 0.1M Tris buffer at pH 7.4 , in a total volume of 5 ml. . The reactions were carried out at 37° in air for 30 mins. and p-nitrobenzoic acid formed was measured by the standard method.

Homogenization period (of the resuspended 10,000g. sediment)	% Inhibition
0	30
2	30
4	27.4
6	33
6 (10,000g. supernatant)	4
6 (10,000g. sediment)	34

Fig. 5. Progress curve of the p-nitrotoluene oxidising enzyme system in locust fat body 10,000g. sup. .

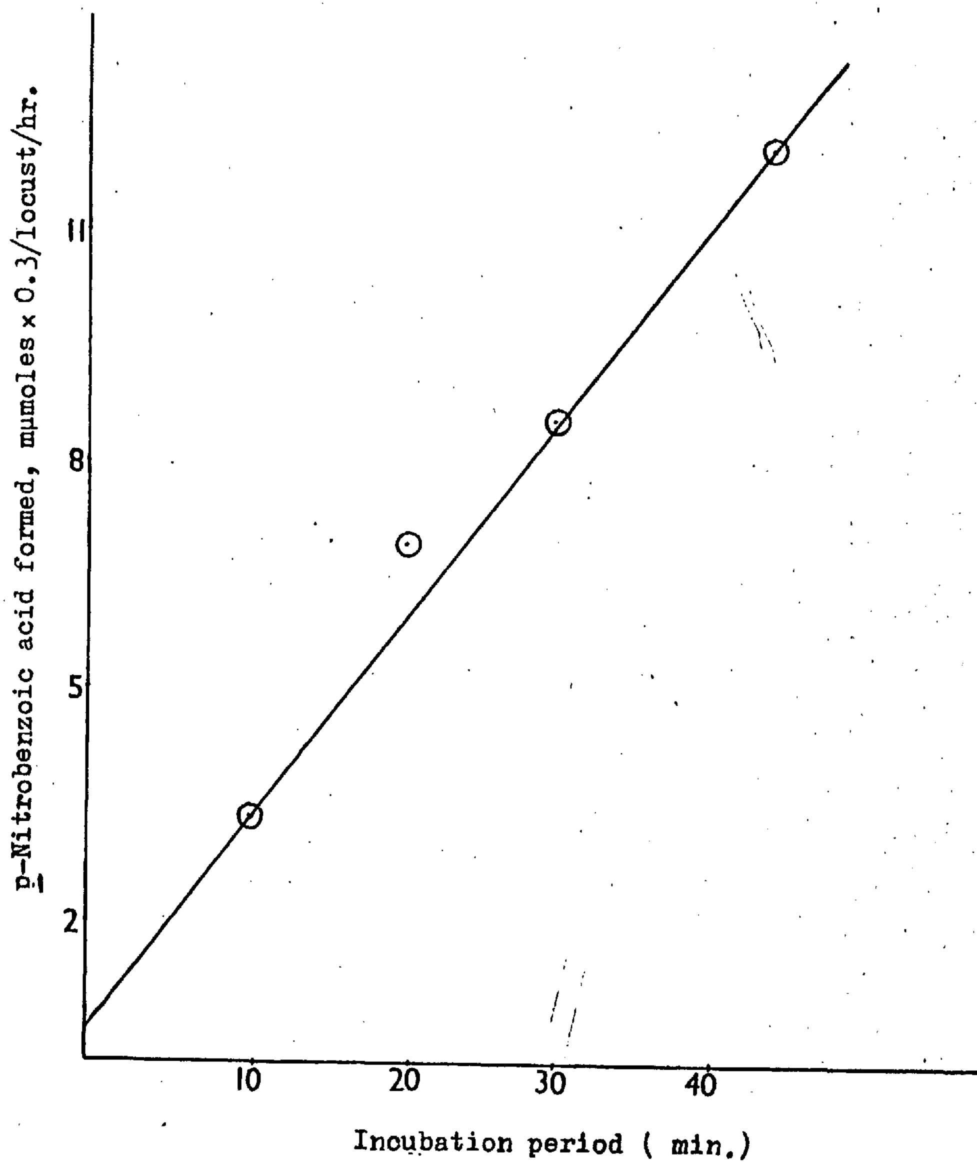


Fig. 6. Effect of speed of centrifugation on the p-nitrotoluene oxidizing activity of the locust fat body enzyme.

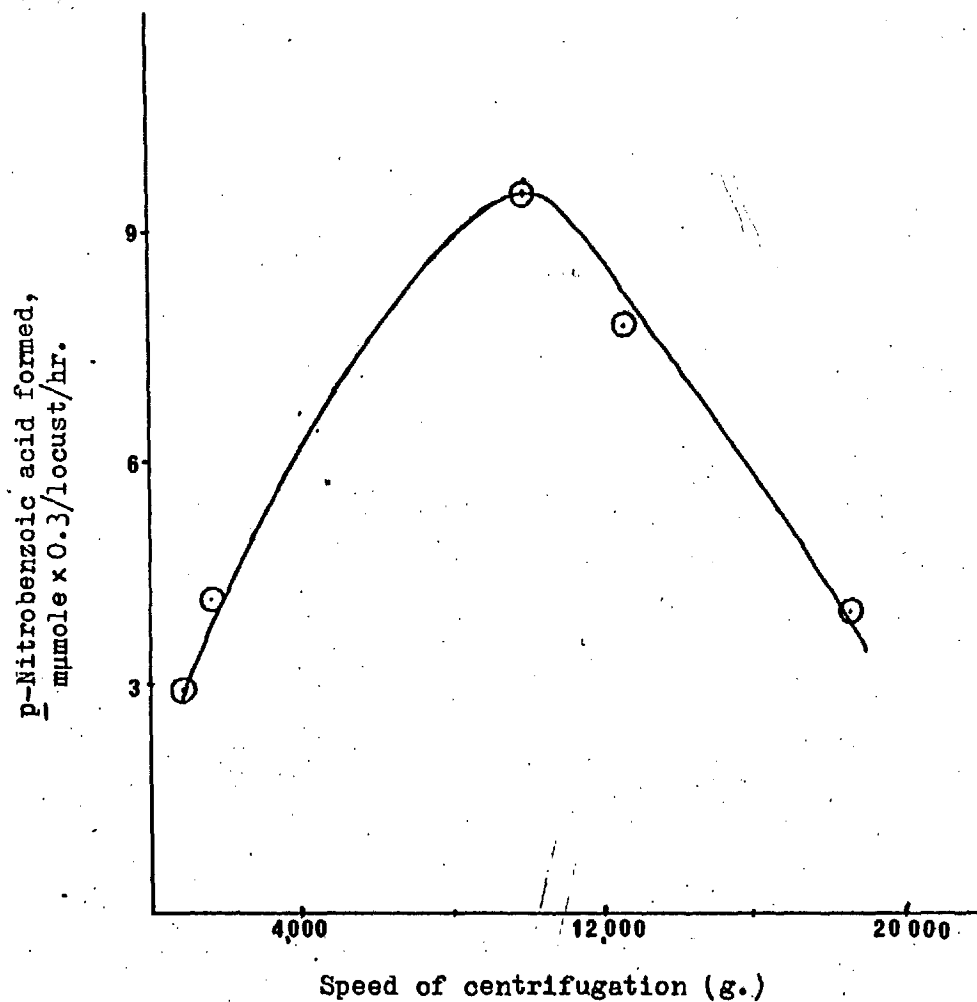


Fig. 7. Effect of pH on the rate of oxidation of p-nitrotoluene by locust fat body enzyme system.

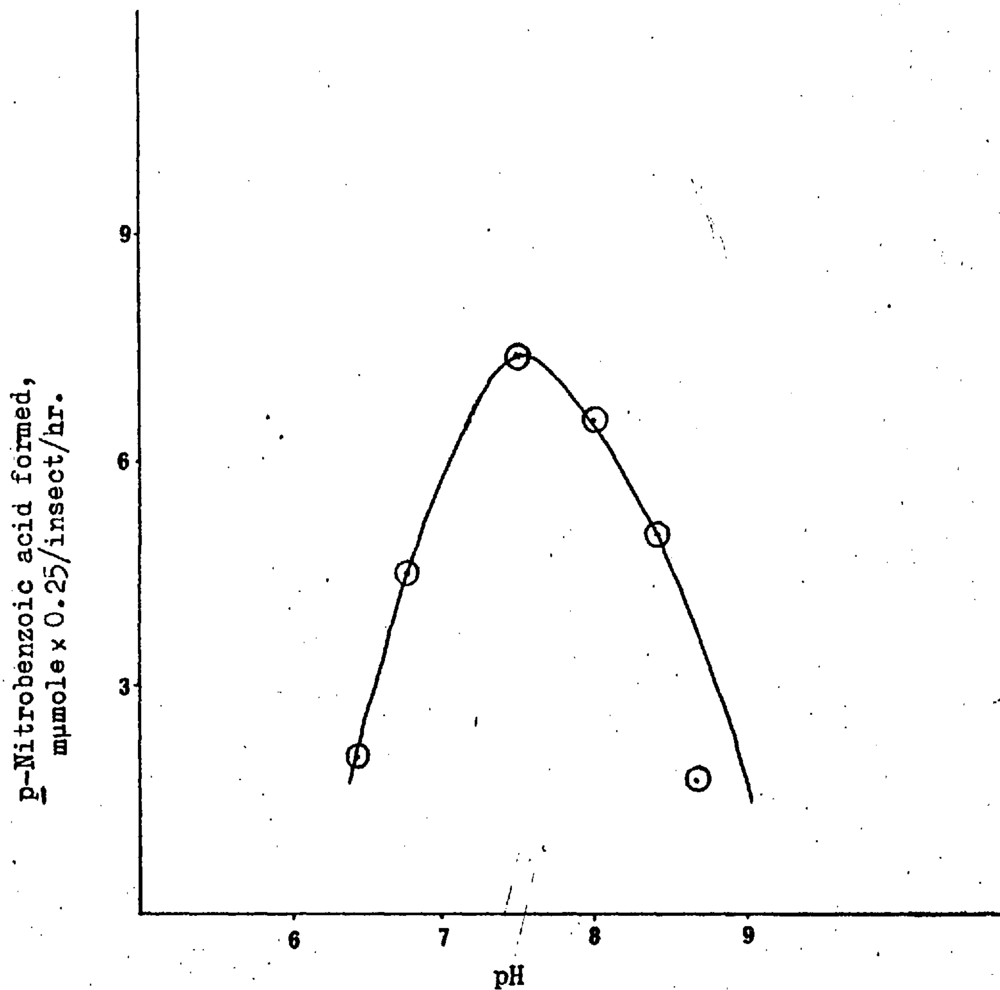
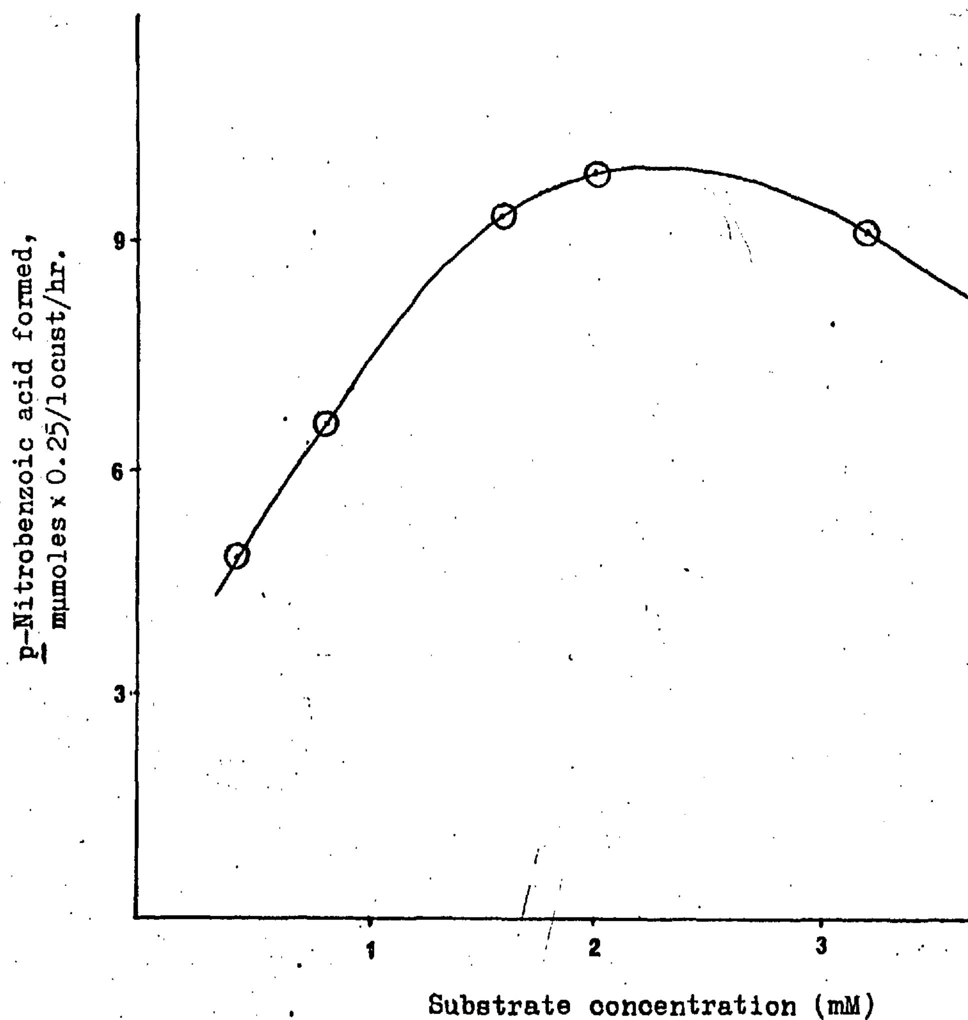


Fig. 8. Effect of substrate concentration on the p-nitrotoluene oxidizing activity of locust fat body 10,000g. supernatant.



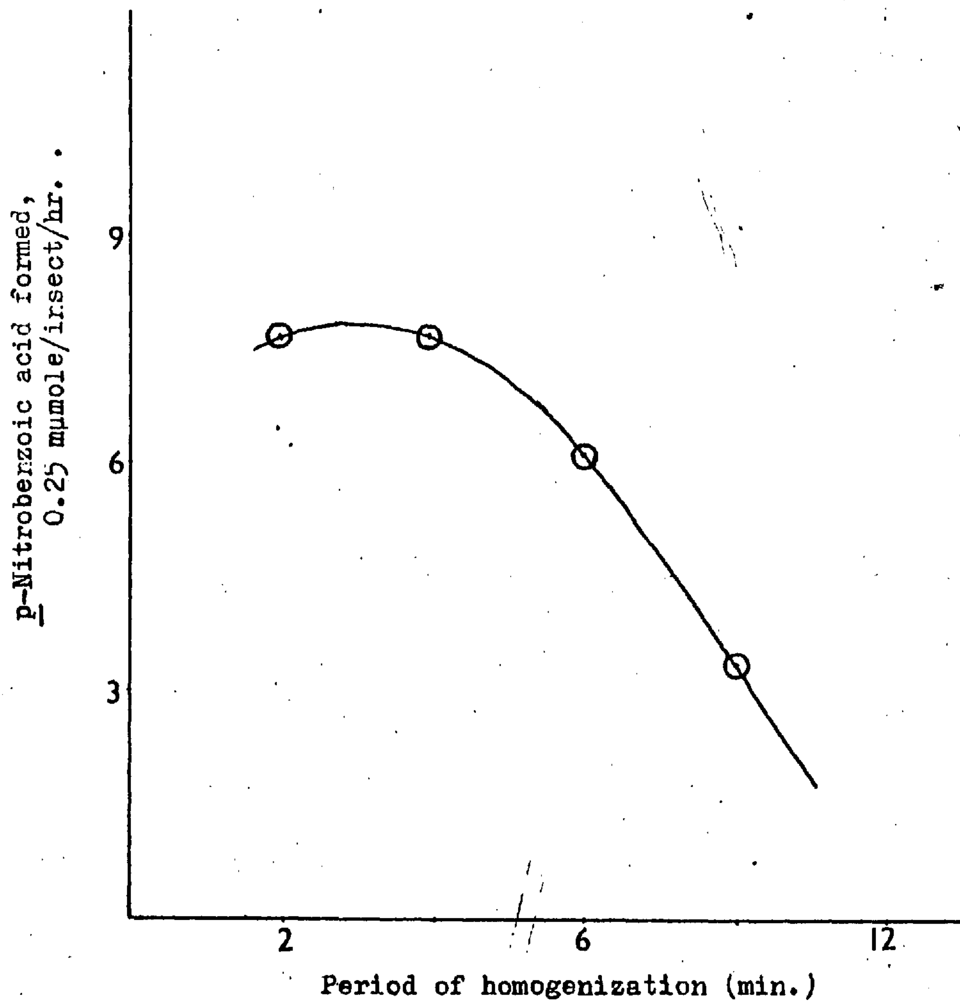


Fig. 9. Effect of prolonged homogenization on the p-nitrotoluene oxidizing activity of locust fat body enzyme.

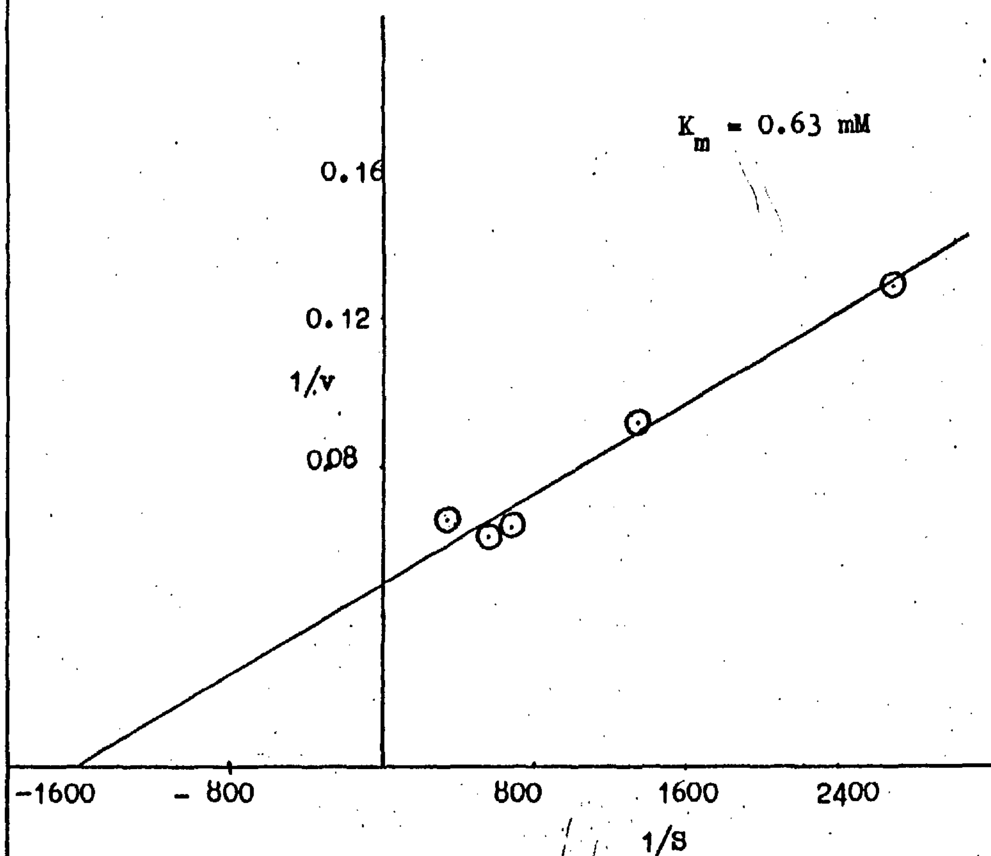


Fig. 10. Lineweaver-Burk plot of 1/v against 1/S for locust fat body p-nitrotoluene oxidizing enzyme system.

Fig. 11. Inhibition of rabbit liver p-nitrotoluene oxidizing enzyme system by locust fat body 10,000g. sediment at various substrate concentrations.

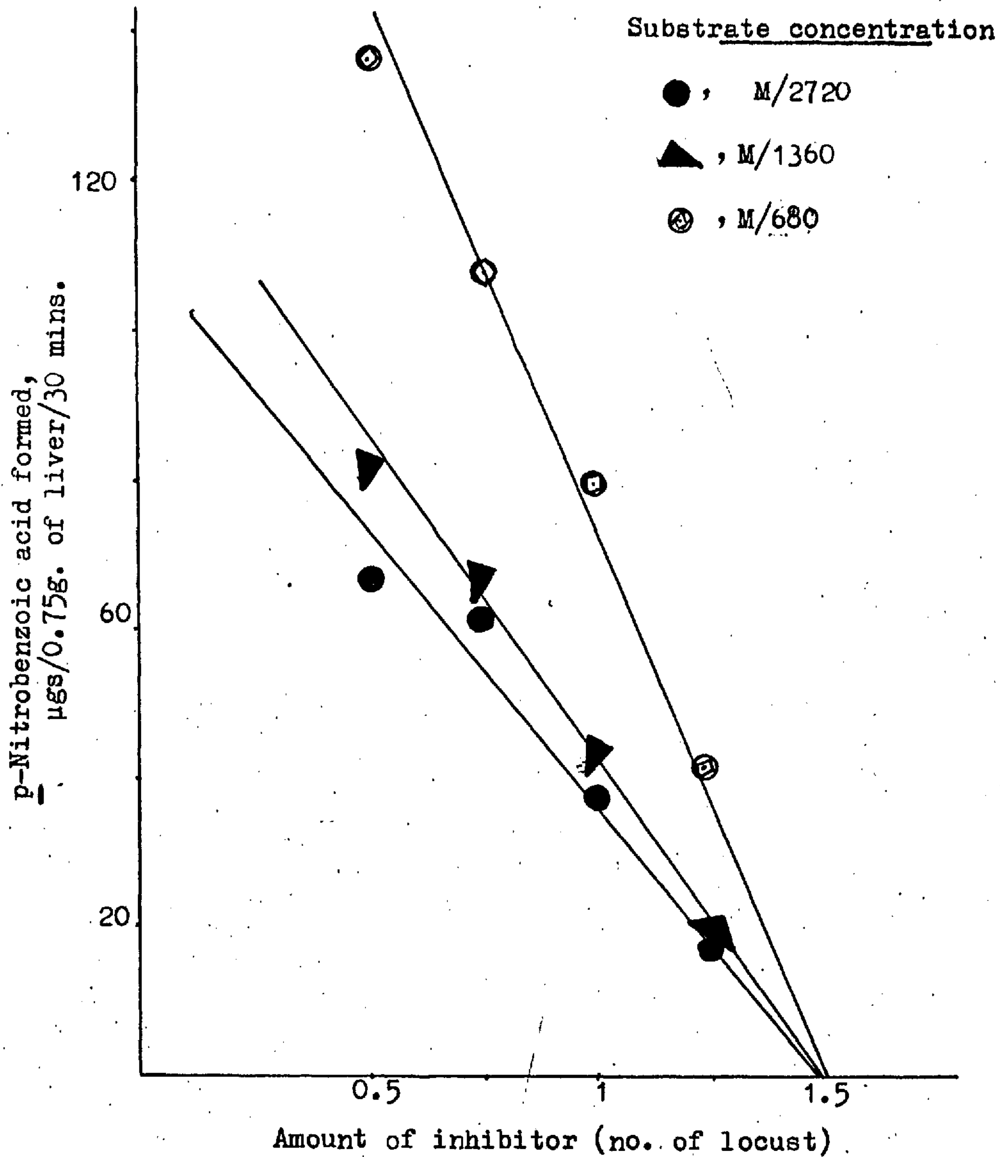
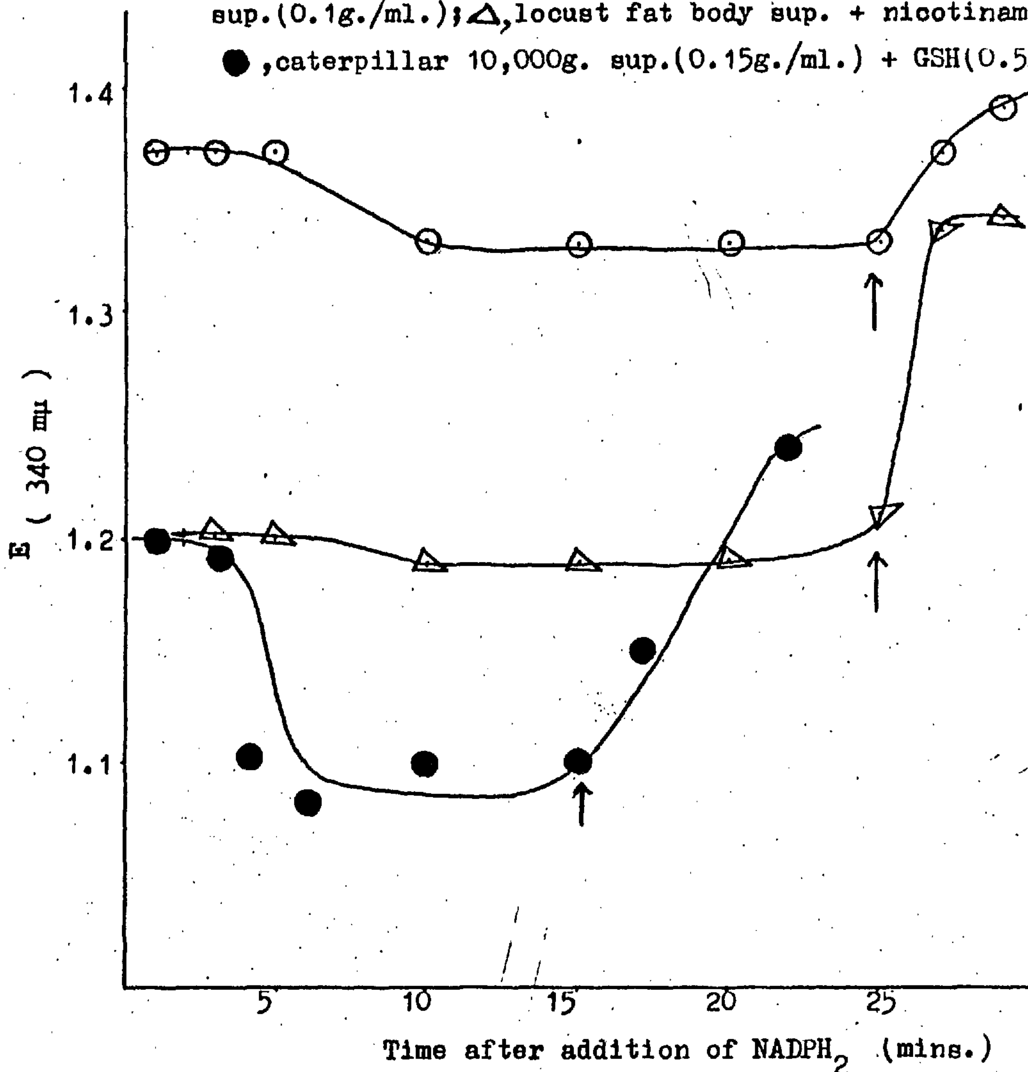


Fig. 12. Stability of $NADPH_2$ in various insect 10,000g. supernatant preparations.

$NADPH_2$ (1.35 µmoles) was added at zero time to the the following preparations: ○, locust fat body sup. (0.1g./ml.); △, locust fat body sup. + nicotinamide; ●, caterpillar 10,000g. sup. (0.15g./ml.) + GSH (0.5mg./ml.)



Arrows indicate the points at which G-6-Phosphate was added.

Fig. 13. Destruction of $NADPH_2$ by fly abdomen 10,000g. supernatant.

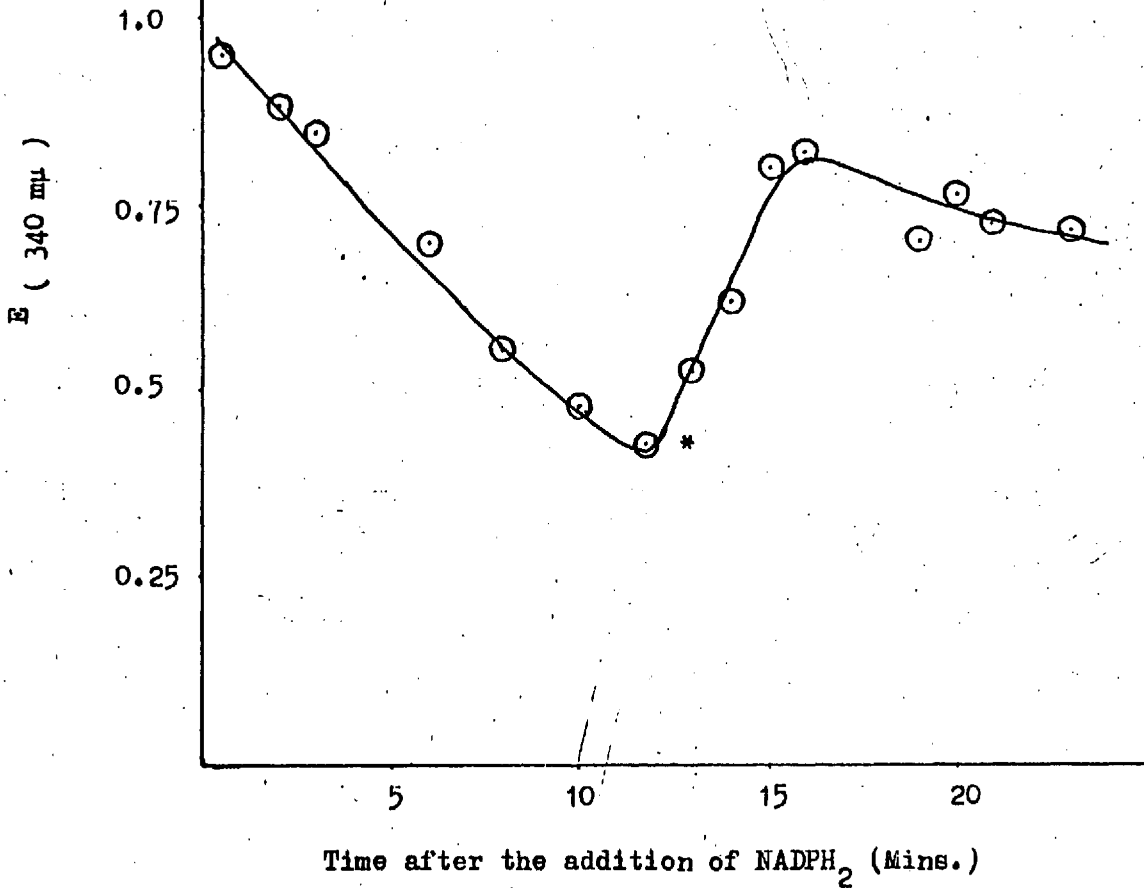


Fig. 14. Destruction of $NADPH_2$ by mustard beetle 10,000g. supernatant preparation.

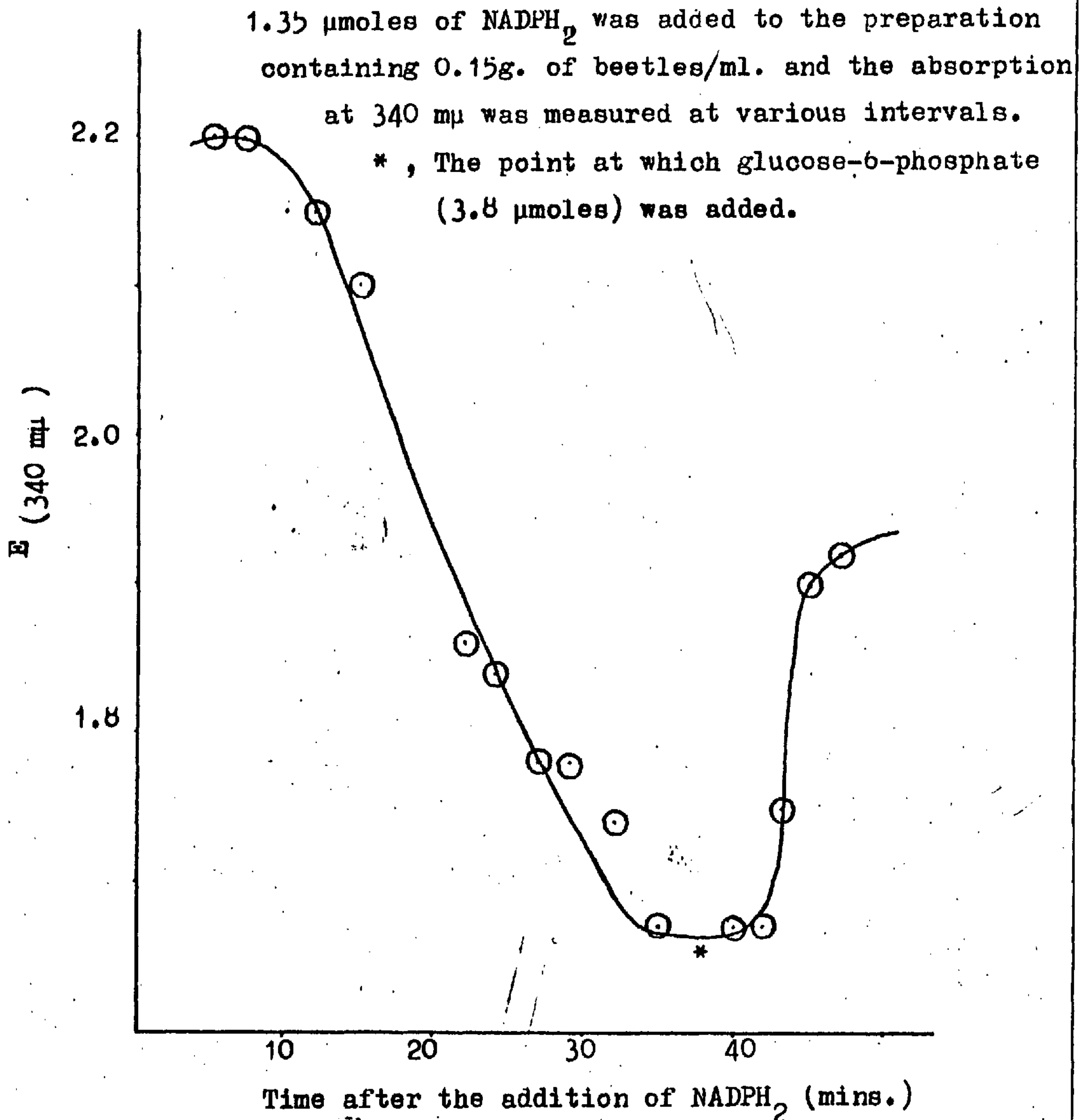
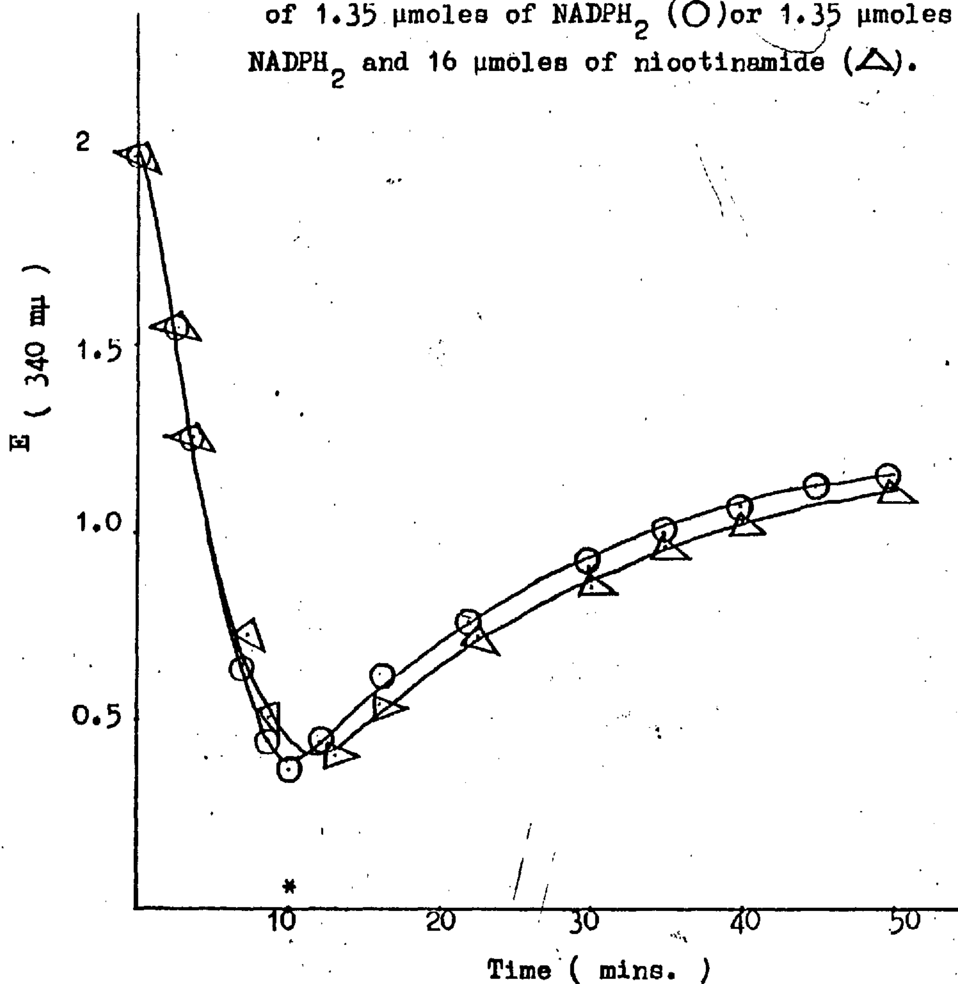


Fig. 15. Destruction of $NADPH_2$ by whole fly 10,000g. supernatant preparations.

The preparations used contained 3 flies/ml. . The levels of $NADPH_2$ were followed spectrophotometrically at various times after the addition of 1.35 µmoles of $NADPH_2$ (○) or 1.35 µmoles of $NADPH_2$ and 16 µmoles of nicotinamide (△).



*, Time when glucose-6-phosphate(3.8 µmoles) was added.

Results and discussion

The oxidation by an insect of an insectioide is often of primary importance in deciding its effectiveness , since this reaction can lead to either activation of the molecule or deactivation. In practical insecticides the more active conjugation mechanisms such as glucosidation usually act only after the oxidation enzymes have introduced a centre for conjugation. Results in Table 3 show that *in vivo* *p*-nitrotoluene undergoes one main reaction in all species studied- oxidation of the methyl group to carboxyl group to form *p*-nitrobenzoic acid. In addition to this reaction, in rat, rabbit and locust, oxidation is followed by glycine conjugation forming *p*-nitrohippuric acid. In rat and rabbit 70 and 80% respectively of administered *p*-nitrotoluene was excreted as *p*-nitrobenzoic acid in free and conjugated form in four days (Tables 5& 6). Table 4 shows that the rates of *p*-nitrotoluene oxidation *in vivo* in various insect species are in the order, housefly $>$ mustard beetle $>$ cricket = cockroach(Blatta) $>$ locust $>$ cockroach(Peripleneta) $>$ flour beetle = caterpillar cotton stainer . Housefly oxidizes

p-nitrotoluene 5 times as fast as cotton stainer. Half life of *p*-nitrotoluene in the intact body was determined by measuring the amounts of *p*-nitrobenzoic acid formed in various time intervals and plotting the log.values of the residual *p*-nitrotoluene against time (Table 4). The half life of *p*-nitrotoluene in flies was 2 hr. against 30 hr. in cotton stainers. It should be mentioned here that flies and mustard beetles were dosed topically, whereas the others were by injection(i.p). *In vitro* studies showed that in locust,fat body is the most active of all organs in oxidizing *p*-nitrotoluene (Table 7). Females appeared to be more efficient than males.

The study of the feeble oxidation systems in insects is difficult, particularly when low doses of toxic compounds are used and the use of microsomal enzyme preparations rather than whole insects is more convenient and can ease the problem of separating metabolites from irrelevant tissue material. Insect microsomal preparations are usually made from homogenates of whole insects and are therefore more complex than vertebrate liver preparation. The diversity of tissues in the homogenate may introduce a variety of

endogenous inhibitors. Previous attempts to locate the main site of phosphorothionate and phosphoroamidate oxidizing enzymes in insects were not successful. Inspite of the fact that a number of intact tissue homogenates oxidized these compounds and the enzymes concerned were found to be similar to those in mammalian liver in their cofactor requirements, no single tissue was the principal site of metabolic oxidations as is the liver in mammals (O'Brien 1960). The inconclusiveness of such metabolic studies in insects might be due to two reasons; first, use of tissue homogenates, and second, the differences in the relative proportion of oxidases and phosphatases in various tissues. As only one reason is mainly involved in the metabolism of p-nitrotoluene in insects (Table 3), it is a more suitable substrate than any of the compounds mentioned above for *in vitro* metabolic studies in insects. The preparations examined for p-nitrotoluene oxidizing activity were made from intact tissues (locust & cockroach), homogenates of tissues (locust & cockroach) and whole insect homogenates (caterpillar, mustard beetle, flour beetle & fly). 10,000g. supernatants of all homogenate preparations were also assayed for p-nitrotoluene oxidizing enzyme activity. With all insect

preparations, incubations were done alone and in the presence of various cofactors(Table 8). Satisfactory enzyme extracts were obtained only from two preparations, locust fat body and fly abdomen. In both cases, centrifugation at approx. 10,000g. for 10 min. yielded an active enzyme in the supernatant and the microsomal sediment of this supernatant at 90,000g. for one hour was inactive unless combined with the 90,000g. supernatant. The enzymes converted *p*-nitrotoluene to *p*-nitrobenzoic acid and no alcoholic metabolite was detected. The locust fat body enzyme oxidized *p*-nitroethylbenzene mainly to 1-(*p*-nitrophenyl)ethanol together with a trace of *p*-nitrophenylacetic acid. No 2-(*p*-nitrophenyl)ethanol was found. The fly enzyme needed NAD and $NADPH_2$-generating system(Table 19), but the locust enzyme could not be fortified by added cofactors(Table 10). This may be due to the presence of sufficient endogeneous cofactors in the locust fat body (Fenwick 1958). The activity of this preparation using *p*-nitrotoluene as the substrate ranged from 5-10 μgrams/locust/hr. The enzyme reaction is linear with time up to one hour and gives maximum activity at pH 7.4 (Figs. 5 & 7). It lost about a fifth of its activity when stored at 0° for 16

hours (Table 13). Assays of the oxidizing activity of the 10,000g. supernatant from the fat body were made at intervals on a large group of locusts. Rates of 3.6, 4.5, 4.5, and 6.4 µg/hr./locust were obtained 2, 4, 7 and 11 days after collection. Moulting started in the group at 11 days and enzyme from adults assayed on the fourteenth and eighteenth days had rates of 1.5 and 2 µg. of p-nitrobenzoic acid/hr./locust. Similar locust and fly tissue preparations have been reported to oxidize Shradan (octamethyl pyrophosphoramide) and Sevin (1-naphthyl N-methylcarbamate) respectively(Fenwick 1958; Casida 1963).

The reason for the failure to get any enzyme activity in cockroach and whole fly 10,000g supernatant could not be understood as these preparations have been reported to be capable of hydroxylating DDT and naphthalene (Agosin et al 1961 ; Terriere et al 1962). Investigations carried out by incubating various insect preparations with rabbit liver enzyme and assaying the p-nitrobenzoic acid produced by the later showed that locust fat body 10,000g. sediment and homogenates of locust gut, cockroach , mustard beetles, flies and caterpillars inhibited p-nitrotoluene oxidizing enzyme system (Tables 15, 17 & 18). Cockroach fat body , mustard beetles, and caterpillars contained much less inhibitor than

flies but nevertheless their 10,000g. supernatant were inactive when assayed for p-nitrotoluene oxidizing power. The locust fat body 10,000g sediment inhibitor was heat stable (Table 14) and appeared to be irreversible in nature (Fig. 11). Though prolonged homogenisation of the fat body gave a progressively less active enzyme (Table 12), this was not due to release of excess inhibitor from the nuclei or mitochondria (Table 20). Attempts were made to measure the rate of the inhibition by leaving resuspended locust fat body 10,000g. sediment in contact with rabbit enzyme at 0° for varying periods., centrifuging off the inhibitor sediment and assaying the remaining supernatant. A small reduction of activity was found after leaving fat body sediment in contact with rabbit enzyme, but most of the inhibitory activity could be centrifuged away (Table 18).

The stability of $NADPH_2$ in some diluted insect preparations was measured (Figs. 12, 13, 14 & 15). The results show that loss of $NADPH_2$ is more rapid in whole fly 10,000g. sup. than in others. However, the 340 mμ absorption could be restored significantly by adding glucose-6-phosphate (Fig. 15). This suggests that the

nucleotide structure is not attacked in these preparations by any nucleosidase catalysing the hydrolysis of nucleotides. It was also noticed that inhibition of rabbit enzyme by fly preparation was supressed only very slightly by using 16-fold excess of $NADPH_2$ (Table 17). Further investigations on the whole fly 10,000g. supernatant using p-nitrobenzyl alcohol as substrate showed that the alcohol and aldehyde dehydrogenases was present there in sufficient amounts. So, it is likely that the inhibitor(s) interferes with the first hydroxylation step of the reaction i.e. formation of p-nitrobenzyl alcohol from p-nitrotoluene.

CHAPTER III.

METABOLISM OF SOME ALKYLBENZENES IN MAMMALS AND INSECTS.

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Section A. Chemistry, metabolism and toxicity of alkylbenzenes.

Physical properties :- The alkylbenzenes are highly refractive colourless substances very sparingly soluble in water, having a low viscosity and surface tension. Alkylation of the benzene ring diminishes the solubility in water. The water solubility of mono-alkylbenzenes decreases with the length of the side chain. The boiling point increases with increasing molecular weight. A progressive decrease in volatility accompanies the lengthening of the side chain. The intensity of their odour depends on the number of alkyl groups, their length and the degree of saturation and the branching of the side chain in the molecule. In general, lengthening of the side chain diminishes the perceptible odour of the compound since the vapour pressure decreases with increasing molecular weight.

Chemical oxidation :- The homologues of benzene undergo oxidation with a wide variety of oxidizing agents including permanganate, dichromate, dil. hydrochloric acid, sulphuric acid and chromic acid. The benzene ring is not usually attacked but in most cases side chain règardless of its length is converted to a carboxyl group. α-Methylenic

groups are often preferential points of free radical attack resulting in hydrogen abstraction, the removal of these atoms being facilitated by the low dissociation energies of the corresponding carbon-hydrogen bonds ; these are associated with the appreciable resonce energies of the benzyl-type radicals formed. These reactions are of primary importance in the oxidation of alkylbenzenes. Kooyman (1951) studied the reactivity of aromatic hydrocarbons towards the trichloromethyl radical , using their ability to retard the addition of carbon tetrachloride to cetene as the criterion.

$$X\bullet + Ph-CH_2-R \longrightarrow XH + Ph-\dot{C}H-R$$

where X• denotes an active radical , capable of continuing the chain reaction , e.g. by abstracting a chlorine atom from carbon tetrachloride to produce a new $\dot{C}Cl_3$ radical. Compounds capable of giving stable radicals by reactions analogous to the one above exert a marked retarding influence on the carbon tetrachloride addition reaction. Kooyman showed that : (i) little or no retardation occurs by compounds not containing α-methylenic hydrogen atoms (ii) α-methylenic activity is increased by substitution at

the α-methylenic groups. The effect of various substituents on reactivity as expressed by Kooyman seem to be analogous to the influence on bond strengths. The following table illustrates the influences of substitution on C-H bond energies in some simple hydrocarbons (Robert and Skinner 1949).

Table 21 . Dissociation energies of C—H bonds.

Compound	D_{C-H} (Kcal)	Difference with respect to D_{C-H} in methane (Kcal)
CH_4	102	0
CH_3-CH_3	97.5	4.5
$CH_3-CH_2-CH_3$	90.8	11.2
$(CH_3)_3-CH$	86.5	15.5
$C_6H_5-CH_3$	77.5	24.5
$CH_2=CH-CH_3$	78	24

The table on the next page shows the relative reactivities of some alkylbenzenes towards various free radicals (Kooyman 1951 ; Russell 1956).

Table 22 . Relative reactivities of aryl alkanes toward some free radicals.

Compound	Peroxy radical	Trichloromethyl radical
Cumene	1	1
Ethylbenzene	0.59	0.37
Toluene	0.075	0.08
p-Nitrotoluene	0.025	
p-Nitrocumene	0.53	
p-Xylene	0.12	

The figures represent relative reactivities per α-hydrogen atom taking cumene as the standard.

The rate of reaction of a peroxy radical with various substituted hydrocarbons shows a pronounced dependence on polarity. Electron- withdrawing groups tend to decrease and elctron- donating groups increase the reactivity of the α-hydrogen atoms of compounds toward a free radical. The reactivities of the various toluenes and cumenes examined cannot alone be explained from a consideration of the resonance energy of the aralkyl radical formed. *p*-Chloro , *p*-nitro , *p*-cyano or *p*-bromo groups increase the resonance stabilization of benzyl radicals, whereas these groups decrease the reactivity

of toluene toward a peroxy radical (Szwarc $1948_{a,b}$; $1951_{a,b}$). It was also pointed out that the bond strength observed for substituted benzyl bromides is apparently not connected with the Hammett σ-value of the substituent since p-methyl and p-nitro group have nearly the same effect on the carbon-bromine bond dissociation energy. The capacity of aryl compounds with substituted side chains to auto-oxidize has been studied (Hock and Lang 1943). Ethylbenzene was found to be more readily attacked than toluene , xylenes and cymenes in agreement with Kooyman's results. tert-Butylbenzene was unaffected. In permanganate oxidation of alkylbenzenes complete oxidation of the alkyl group to the carboxylic acid occurs in most cases. Some compounds containing a tertiary hydrogen can be oxidized to the corresponding alcohol in excellent yield (Stewart 1965). The autoxidation of aryl alkanes at benzylic positions can be initiated by permanganate . There seems little doubt that resonance-stabilized benzyl radicals are intermediates in these reactions (Waters 1946_{b}). Oxidation of alkylbenzenes by chromic acid mainly occurs on the α-carbon atom. Regardless of its length , the alkyl group is finally oxidized to a carboxyl group (Fittig et al 1869 ; Friedel and Balsohn 1879;

Abell 1951). Ethylbenzene gives benzoic acid along with some acetophenone using chromic acid in acetic acid. iso-Propylbenzene gives some 2-phenyl-2-propanol along with acetophenone (Boedtker 1901 ; Meyer and Bernhauer 1929). tert-Butylbenzene having no α-hydrogen is very resistant to oxidation. n-Propylbenzene gave only benzoic acid under the same conditions. In aqueous acidic chromic acid solution , besides benzoic acid n-propylbenzene gives acetic acid ; n-butylbenzene gives propionic and acetic acids and iso-propylbenzene produces only benzoic acid (Jurecek et al 1959). The oxidation using aqueous sodium dichromate occurs principally at the end of the alkyl side chain rather than at the α-position (Reitsema and Allphin 1962). Ethylbenzene gives phenylacetic acid in 96% yield. Vigorous conditions lead to the degradation of the acid to benzoic acid. n-Propylbenzene , iso-propylbenzene and n-butylbenzene give 3-phenylpropionic acid , 2-phenylpropionic acid and 4-phenylbutyric acid respectively. Chromyl chloride oxidation of alkylbenzenes occur preferentially at the carbon-one removed from the aromatic ring. Thus ethylbenzene gives phenylacetaldehyde as the major product. Similar results have been obtained with cumene and

n-propylbenzene (Wieberg 1965). As a result of a number of investigations , there is a considerable body of data concerning the Cr(VI) oxidations of aryl alkanes. Mares and Rocek (1961) found that in 99% acetic acid, the relative rates of oxidation of toluene , ethylbenzene , iso-propylbenzene and tert-butylbenzene are 1: 7.2 :71.1:0.019. According to Wieberg and Evans (1960) the relative rates of oxidation of hydrocarbons tend to parallel their rates of reactions with hydrogen-atom abstractors. One possible mechanism for carbon-hydrogen bond cleavage which is in accord with the experimental observations is the following:-

$$R_3 CH + Cr(VI) \longrightarrow R_3 C\bullet + Cr(V)$$

The radical is probably oxidized by one of the chromium species , possibly forming an ester which is then hydrolysed giving the final product. Dimroth and Schweizer (1923) observed that lead tetraacetate converts toluene to benzyl acetate. Ethylbenzene under same conditions gives acetate of methylphenyl carbinol (Detilleux and Jadot 1955). Dewar (1949) proposed that in these reactions a radical chain reaction is involved. This is supported by the observation that oxidation of ethylbenzene by lead tetraacetate is accelerated by the addition of 5% of dibenzoyl peroxide.

Metabolism :- The earlier work of Theirfelder and Klenk (1924) on the metabolism of *n*-alkylbenzenes higher in the series than toluene showed that most of these compounds underwent ω-oxidation to phenyl fatty acids which were then converted by β-oxidation to either benzoic or phenylacetic acid according to the number of carbon atoms in the side chain. More recently the metabolism of these compounds in rabbits *in vivo* was studied (Smith *et al* 1954 a, b ; Robinson and Williams 1955 ; El Masri *et al* 1956). It was found that ω-oxidation only accounts for a portion of the alkylbenzene , a substantial part of the compound being metabolized by oxidations involving carbon atoms in the alkyl chain other than the ω-atom . From these findings the metabolism of ethylbenzene , *n*-propylbenzene , *iso*-propylbenzene , *n*-butylbenzene and *tert*-butylbenzene in the rabbit can be summarized as follows :-

(i) Ethylbenzene :- The main oxidation of ethylbenzene occurs at the activated α-methylenic group to give methylphenylcarbinol which is the precursor of hippuric acid and mandelic acid. Methylphenylcarbinol is excreted in the urine as its glucuronide . ω-Oxidation also takes place as 15-20 % of the dose is excreted as phenaceturic acid .

(ii) n-Propylbenzene :- In the rabbit , the major metabolites of n-propylbenzene are the glucuronides of ethylphenylcarbinol and benzylmethylcarbinol. These compounds account for over half the dose fed . About 15 % of the dose is converted to hippuric acid which probably arises from oxidation of the carbinols or of β-phenylpropionic acid.

(iii) iso-Propylbenzene :- Rabbits largely convert iso-propylbenzene to carbinols up to 70 % which are excreted conjugated with glucuronic acid . About 40 % of the dose is oxidized to 2-phenylpropan-2-ol. Hydratropyl alcohol and hydratropic acid which are formed by oxidation of one of the β-carbons of the side chain are produced in roughly equal amounts and account for about half of the dose . Small amounts of ethereal sulphate and mercapturic acid have been reported as metabolites of iso-propylbenzene , but nothing is known of their nature.

(iv) n-Butylbenzene :- The butyl chain is oxidized by rabbits at the α- and (ω-1)- positions . The glucuronides of methylphenylethyl carbinol and phenyl-n-propyl carbinol are the major metabolites in the urine . From 15-20 % of the dose is excreted as phenaceturic acid. This may be the further oxidation product of methylphenylethyl carbinol or 4-phenylbutyric acid , the ω-oxidation product of n-butylbenzene .

tert-Butylbenzene :- The metabolic fate of this compound in the rabbit appears to be simple . The compound is almost completely oxidized at one of the β-carbon atoms to 2,2-dimethyl-2-phenylethanol which is excreted as its glucuronide . Traces of α,α-dimethylphenylacetic acid were also detected in the urine.

Toxicity :- The aromatic hydrocarbons have a particular affinity for nerve tissue because of its high lipid content . Their presence in the cells of the brain interferes with normal metabolic processes , resulting in signs of central nervous system depression : sluggishness , stupor , anaesthesia , narcosis and coma . The narcotic potency depends on chain length , the extent of branching and the number of alkyl groups attached to the benzene ring . The effect decreases markedly with chain length , falling off at four carbon atoms and diminishing steadily . When given orally , toluene and ethylbenzene are 'fast acting' by comparison with *n*-propyl and *n*-butylbenzene , their rate of action depending on their rate of absorption into the blood and transport to the brain . The effect is probably due to the fact that the rate of absorption of a compound into the blood stream from the gut depends partly on its water

solubility . Since the water solubility of the hydrocarbons diminishes rapidly with chain length , the short chained hydrocarbons are better absorbed and therefore show a more rapid toxic action. However , the duration of the nervous system depressant effect increases with the length and branching of the side chain . Thus for example cumene and n-butylbenzene are 'long acting' as compared to toluene and ethylbenzene . From a systematic study of the toxicity of a number of alkylbenzenes it has been possible to draw certain conclusions as to the relationship between chemical constitution and toxicity for this family of compounds (Gerade 1960) . The percentage mortality in rats receiving a single oral dose of the hydrocarbons (5mg / Kg). are shown in the following table . For compounds with single unbranched side chains the toxicity is maximal with the two carbons in the side chain and as the chain lengthens , the toxicity decreases . Branching of the side chain tends to increase the toxicity .

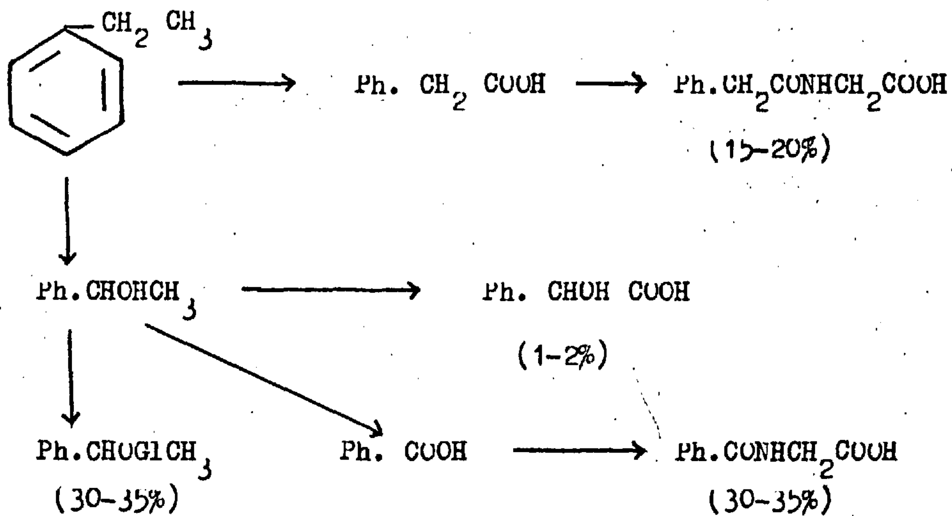
Table 23 . Toxicity of some mono-substituted derivatives of benzene in male albino rats . Dose 5 mg/Kg , given orally . (Gerade 1960).

Compound	% Mortality *
Toluene	30
Ethylbenzene	70
n-Propylbenzene	20
iso-Propylbenzene	60
n-Butylbenzene	20

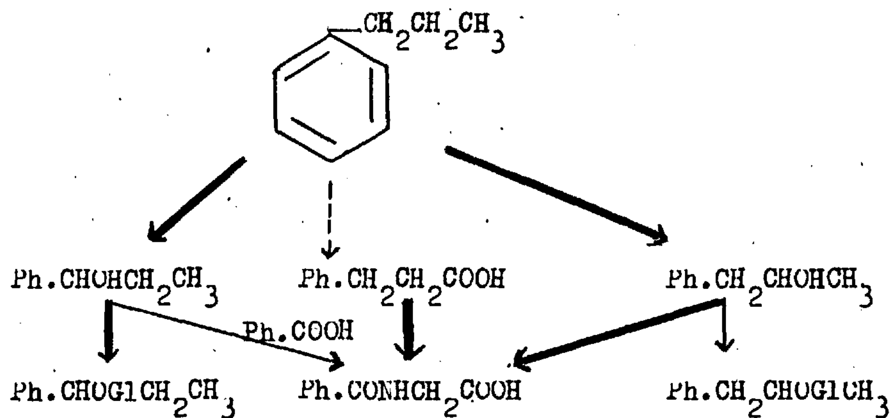
* In each experiment 10 rats were used .

Fig. 16 Metabolism of ethylbenzene and n-propylbenzene in the rabbit (Williams 1959).

Ethylbenzene

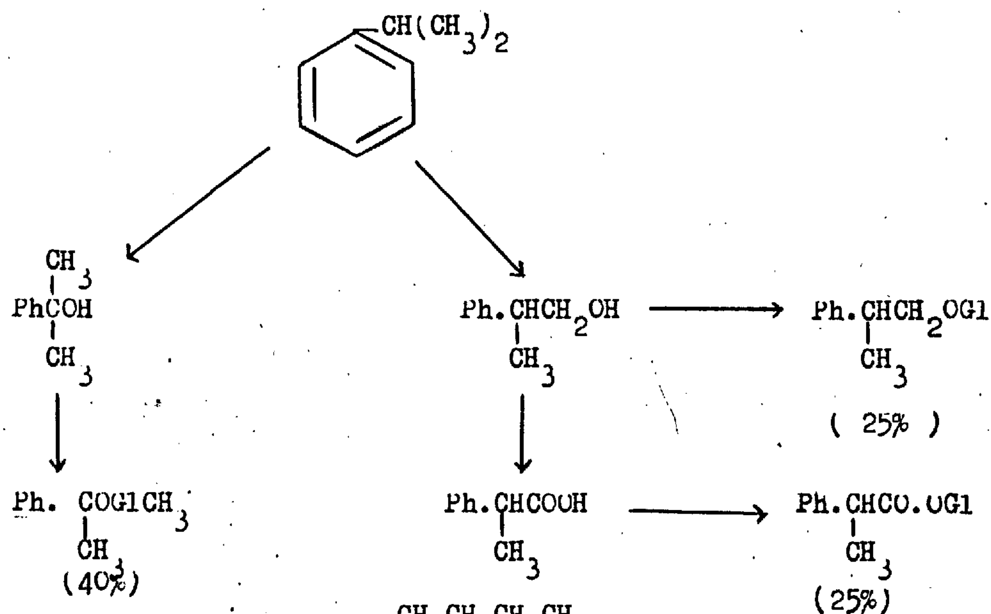


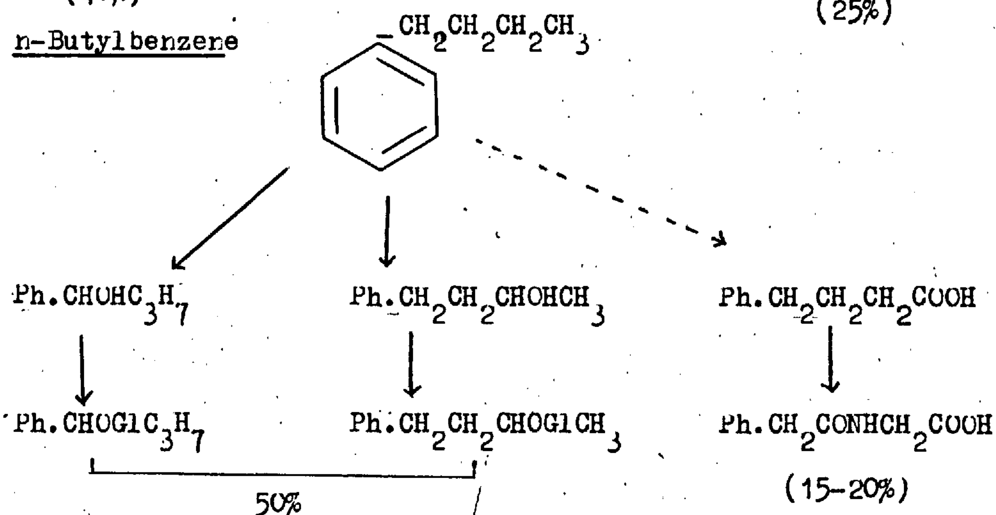
n-Propylbenzene*



* In the scheme for n-propylbenzene the major routes are given in thick arrows, the minor routes in thin arrows and the hypothetical routes in dotted arrows .

Fig. 17 . Metabolism of iso-propylbenzene and n-butylbenzene in the rabbit (Williams 1959).





The hypothetical route is shown in dotted arrow .

CHAPTER III

Section B . Gas-Liquid Chromatography

Gas chromatography is a special form of general chromatography in which a gas is the mobile phase, the solute travelling through the column as a plug of gas or vapour where it may become partly dissolved in or adsorbed on the stationary phase. The suggestion that a gas might be used as the mobile phase was first made by Martin & Synge in 1941 but it was not implemented until the work of James et al (1952) and Cremer et al (1951 a,b, c). In recent years the technique has penetrated almost every area of analytical and biochemical research. Gas chromatography can be accomplished using either a liquid on an inert particulate support (GLC or gas liquid chromatography) or an active solid as the stationary phase (Gas solid chromatography or GSC). Alternatively , the liquid can be coated on the internal surface of a long capillary tube with very small bore. The columns most widely used in gas liquid chromatography are of glass or metal , packed uniformly with a finely divided free-flowing powder prepared by impregnating an inert solid with a liquid of low volatility . The liquid must not be eluted from the column at the

operating temperature employed. The injection block is heated electrically to vapourise the sample which is then carried down the column by the carrier gas. Ideally the constituents of the sample move at different rates and emerge from the column at different times. Their presence in the carrier gas can be detected by various means and the response of the detector is usually registered on a strip chart recorder. The data are recorded as a series of peaks spread out along a longitudinal time axis as shown in the figures in the following section. Each peak represents a chemical compound or a mixture of compounds with identical partition coefficients. The time required for each component to emerge from the column at a given temperature is characteristic of the compound and is known as its retention time. The area under the peak is proportional to its concentration in the sample.

Principle of separation :- Each compound has a characteristic partition coefficient, which is given by the following equation :

$$K = \frac{\text{weight of solute/ml. of stationary phase}}{\text{weight of solute/ml. of mobile (gas) phase}}$$

Thus if the partition coefficient of a compound 'A' is small it

will pass through the column rapidly, since it will not be retarded by the stationary liquid. Another compound with a larger partition coefficient will pass through the column slowly and register a peak on the recorder chart at a later time than the compound 'A'. It must be remembered that the solute present in the gas phase is in dynamic equilibrium with the same solute in the liquid phase at all times.

Column :- The column is the key element in the separation process. Most columns used vary from 3 to 6 mm in internal diameter and 4 to 12 feet in length. They may be of glass, copper, stainless steel or aluminium. The requirements for the solid support used in columns are that it should have a low adsorptive capacity in addition to a large surface and that the individual particles should be of fairly uniform size. The supporting material commonly used include celite, ground firebrick and glass beads. In order to attain high column efficiency, the supporting material must be closely graded by careful sieving or other means. The stationary phase should normally be chemically similar to the compounds to be separated. However, an important factor which limits the choice is that the liquid phase must not be highly viscous at the operating temperature, nor must it have an appreciable vapour pressure. If the boiling point of the

/-

component mixture is very different, it is probable that an adequate separation will be obtained on a non-polar liquid phase such as squalene or silicone oil or a slightly polar compound such as phthalate ester. Amounts of liquid phase used in column packings have varied from 0.01 per cent (W/W) to about 50 per cent (W/W). In loading a column, even packing is desirable so that the gas flow does not vary either across the column or irregularly along its length.

Operational temperature :- The effect of temperature on column efficiency is complex. In general, increases of temperature lowers column efficiency and it is therefore usually desirable to work at room temperatures. However, the longer retention times of components of low vapour pressure encountered at low temperature with a consequent diffusion of peaks may be a disadvantage. This may be partly countered by using a lower liquid phase to support ratio. A recent development has been the use of programmed heating in which the column is subjected to an exactly controlled temperature rise, which reduces the retention times of the less volatile components enabling a mixture of compounds with a wide range of volatility to be analysed more rapidly.

Column efficiency :- The efficiency of a column is

/-

determined by the number of theoretical plates present.
The number of theoretical plates can be determined from the peak width and the retention volume by means of the following expression.

$$n = 16 \left[\frac{\text{Retention volume}}{\text{Peak width}} \right]^2$$

n Represents the number of theoretical plates.
The number varies with the nature of the solute involved as well as with the column characteristics. In practice, a theoretical plate efficiency of about 5,000-6,000 , when coupled with the use of appropriate liquid phases or preparation of appropriate derivatives is generally satisfactory. The following factors give the most efficient columns :

1. Low ratio of inlet pressure to outlet pressure.
2. Optimum overall gas velocity.
3. The use of suitable carrier gas.
4. A small column diameter.
5. A small proportion of stationary phase.
6. A small sample size.
7. A small range of particle sizes of the support.

<u>Detectors</u> :- Several properties of chemical substances have

been used as the basis for detection and a wide choice of types is available. Those of importance are :

1. Katharometers
2. Gas-density meters
3. Flamedetectors
4. Ionization detectors

Of these, the latter is the most recent inovation and has largely superseded the others. They have the advantage that they are much more sensitive, responding to as little as 10^{-10} to 10^{-15} mole of a solute in the carrier gas, and are more stable with respect to changes in operating parameters. A number of ionization detectors have been described in the literature (Lipsky et al 1960, Lovelock 1961) but only a few have found wide spread use as yet. These include the argon ionization, the flame ionization, the radio-frequency and the electron capture detector. Basically, most of these ionization detectors operate on the same principle; an organic compound is ionized, and the ions (or electrons) formed are used to carry an electric current. The magnitude of the signal is then recorded with or without intermediate amplification. The detector used for the work presented in this thesis was of flame ionization type. Its operating principle is described below.

Flame ionization detectors :- In the flame ionization detector, carrier gas from the column is mixed with a hydrogen/air mixture and is burnt in the detector at a jet above in which a collector electrode is placed (Fig. 18). The rate of ion production in the flame is measured by applying a potential of 100-300 volts between the jet and the collector electrode. This is sufficient to give a saturation current. When an organic substance is added to the flame the ionization increases greatly, the ionization efficiency per carbon atom being about 1 in 10^5 (as only about one hydrogen molecule in 10^{12} produces an ion). The reason for the high ionization efficiency of carbon is not yet fully understood. According to one view the ions derive from minute carbon particles formed in the flame, solid carbon having a much lower ionization potential than organic molecules. According to another view, the ionization results from pooling of energy stored in excited molecules, radicals and free atoms. The flame detector is not adversely affected by water and therefore has a great advantage over other detectors in the analysis of aqueous solutions. The response to organic compounds is roughly proportional to the amount of carbon they contain but this does not hold for oxygenated and nitrogen containing compounds which give a

lower response than expected from their carbon content.

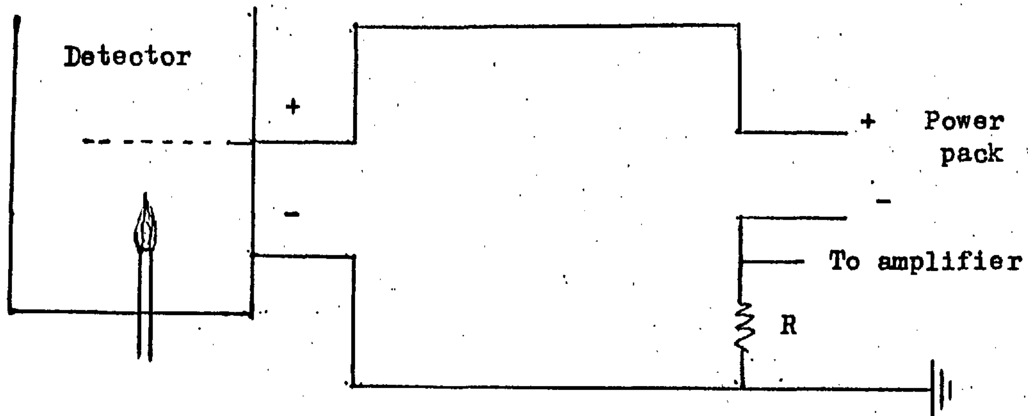
Gas chromatography of aromatic alcohols and acids :-

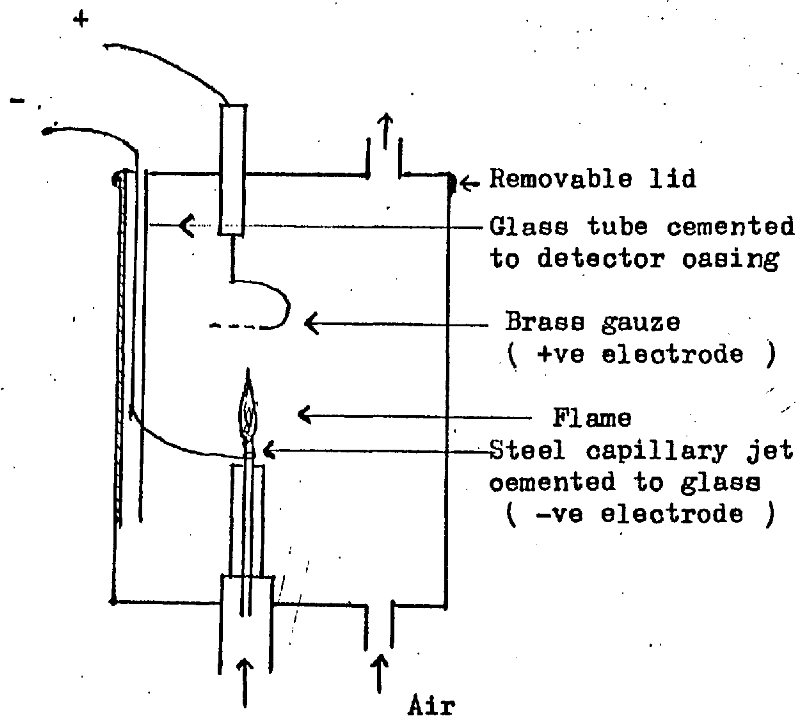
Characterization and estimation of alcohols and acids at low concentration levels was an extremely difficult task before the introduction of gas chromatography. The earlier methods used were not sensitive enough to measure trace amounts of alcohols or acids. Moreover, they were mostly based on non-specific chemical reactions. The development of GLC procedures has provided the first effective method for the qualitative and quantitative study of mixtures of acids and of alcohols at microgram or submicrogram level. Another advantage of the method is that samples of less than analytical purity can be used since the amount of impurities is readily measured. For GLC analysis of acids it is necessary to convert them to more volatile derivatives, the methyl ester being at present the derivative of choice. In recent years gas chromatography has been successfully used for the examination of a number of aromatic acids (Sweeley & Williams 1961, Williams 1962, Horning & Vandenheuvel 1963). It has also been found invaluable for the analysis of aliphatic alcohols (Storr 1962, Horning & Vandenheuvel 1963). The main initial problem associated with the direct GLC separation of alcohols lies in tailing effects and

irreversible adsorption resulting from the use of supports which are not completely inert. The various stationary phases which have been found satisfactory include glycerol, phthalate, adipate and sebacate esters, diglycerol, tetraethyleneglycol dimethylether, polypropylene and polyethylene glycols of various molecular weights (Storrs 1962 ; Horning & Vandenheuvel 1963). The work presented in this thesis shows that gas-chromatography can also be satisfactorily used for the analysis of aromatic alcohols.

It has been described in the previous section that, in mammals, the alkylbenzenes are metabolized mainly to the corresponding alcohols and excreted as their glucuronides. In the past, the metabolites have been identified and estimated by isolating the glucuronides which were produced in vivo in large amounts, when high doses of the hydrocarbons were given. Metabolic studies of these compounds in vitro, however, requires far more sensitive methods in order to estimate the alcohols and acids which are their expected metabolites. Gas chromatography was found suitable for this purpose and the details of the methods developed for the analysis of relevant aromatic alcohols and acids are described in the following section.

Fig. 18. Flame ionization detector and its basic measuring circuit.





<u>Experimental</u> :- The instrument employed was made by Gas Chromatography Ltd . It comprised an electrically heated oven (13″×10.5″×13.5″) accomodating the spiral-shaped chromatographic columns and surmounted by the detector . Signals from the detector was fed via an amplifier (Gas Chromatography Ltd , IE 11) to a 11″ chart width 1 mv FSD recorder (Honeywell Model 143-18). The amplifier was fitted with variable input impedances . The input impedance was selected by using the appropriate input position on the selector switch and the corresponding plug at the rear of the amplifier . A metal flame ionisation detector (IE 201) was used throughout the work . Detector voltage available ranged from 100V to 1400V . A variable output selector switch gave sensitivity changes from 1-100 . It was found that sensitivities higher than 25 could not be used for quantitative work due to the high noise level . For routine run the chart speed of the recorder was set at 12″/hr. .

<u>Column preparation</u> :- Copper tubing (I.D. 1/8″) was used for making the columns . The stationary phase was dissolved in Analar ethyl acetate and the required amount of the supporting medium (GLC grade celite) was added to the

solution . The organic solvent was removed on a hot water bath , stirring the mixture throughout the evaporation . The celite was spread on a tray , dried for three hours at 150^0 in an oven , and 80-100 mesh fraction collected . Filling the copper tubing was done by putting in small amount of packing material at a time and tapping the column repeatedly in order to achieve uniform packing . Each new column was conditioned in the chromatography oven with a small flow of nitrogen passing through it for at least 24 hr. before use .

<u>Temperature and carrier gas</u> :- The oven was always left at 50^0 . The thermostat of the oven was set at the desired operational temperature for at least 2 hr. before running any sample through . Most of the work presented here was done between 100^0 and 130^0 . Oxygen free nitrogen was used as the carrier gas (20 lbs./in^2) .

<u>Materials</u> :- Celite (G-cel, acid washed) , polyethylene glycol adipate and polyethylene glycol (Carbowax 4000) used for making columns were obtained from Griffin and George Ltd . Alkylbenzenes were commercial samples , which contained trace amounts of alcohols and ketones.

They were distilled and left over sodium for a week and then redistilled using a fractionating column . Aromatic alcohols and acids were redistilled or recrystallized commercial samples or were prepared by published methods (Smith, Smithies & Williams 1954c ; Robinson, Smith & Williams 1954 ; Robinson & Williams 1955 ; El Masry, Smith & Williams 1956) and were homogeneous by gas chromatography . Some physical constants of the aromatic hydrocarbons, alcohols and acids used are given in the following table .

Table 24 .

Compound	B.P (°C)	M.P (°C)
Ethylbenzene (L.Light & Co Ltd)	135	
1-Phenylethanol (B.D.H. Ltd)	205	
2-Phenylethanol	220	
2-Phenylacetic acid (B.D.H. Ltd)		76-78
n-Propylbenzene (Eastman org. Chemicals)	159	
1-Phenylpropan-1-ol	107/15 mm.	
1-Phenylpropan-2-ol	105/15 mm.	

Table 24 . Contd.

Compound	B.P (°C)	M.P (°C)
1-Phenylpropan-3-ol (L.Light & Co Ltd.)	235	
3-Phenylpropionic acid (B.D.H. Ltd)		48-50
iso-Propylbenzene	152	
2-Phenylpropan-2-ol (L.Light & Co Ltd.)	202	
2-Phenylpropan-1-ol L.Light & Co Ltd.)	114/14 mm.	
Hydratropic acid	265	
n-Butylbenzene	182	
1-Phenylbutan-1-ol	169/100 mm.	
1-Phenylbutan-2-ol	121/20 mm.	
1-Phenylbutan-3-ol	160/20 mm.	
1-Phenylbutan-4-ol	140/14 mm.	
4-Phenylbutyric acid		52

<u>Identification and assay of alcohols and acids (as their methyl esters) isolated from various tissues</u> :- Identification of the alcohols and acids in the incubation mixtures was done on various columns (Table 25) , by comparison of their retention times with those of reference compounds and by demonstration of their identity of retention times in mixed chromatograms (i.e. co-chromatography) . Phenols had high retention times on the columns used and no peaks were found in the experimental extracts in this region. With the apparatus used , the minimum detectable quantity of an alcohol or ester was 0.01 μg and normally 2μl volumes of n-hexane solutions were used in injections . Preliminary investigations on the effect of oven temperature and carrier gas flow rate on the detector response and separation of the compounds concerned showed that the conditions mentioned in Table 26 were most suitable .Quantitative measurements were made by comparison with the height or peak area (for compounds of retention times more than ten minutes) from an injection of a standard solution of the compound being measured and the value corrected for losses on extraction by reference to the appropriate correlation curve.

Chapter 111

METABOLISM OF SOME ALKYLBENZENES IN INSECTS AND MAMMALS

Section C :- Incubation , enzyme assay, results and discusstion.

Incubations:- Enzymes from vertebrate liver and insects were prepared as described previously. The standard incubation mixture contained 4 ml. of supernatant fraction (1 g.) , 0.68 µmole NADP, 0.75 µmole NAD, 7.7 µmole glucose-6-phosphate, 10 µmole $MgCl_2$, 24 µmole nicotinamide and 0.5 ml. of 0.1 M tris buffer, pH 7.4 . Substrates, 1 mg in 0.05 ml. acetone , were added to test and enzyme-free control mixtures to start the reaction. Controls were also prepared from separately incubated enzymes which were mixed with the other components immediately before assay . Incubations were carried out for 1hr. in 50 ml. beakers in a shaking water bath (Mickle Incubation Shaker) at 37° in air. Other incubations requiring 25-30 ml. volumes were carried out in 250 ml. beakers. The oxidation rate , per gm. liver or insect was the same whether measured in a 5 ml. or 25 ml. incubation mixture and oxidation rates were linear during the period used for routine incubations.

<u>Extraction of metabolites of alkylbenzenes from the incubation mixtures and preparation of the methyl esters</u> :- Incubation mixtures (5-25 ml.) were acidified with 10N H_2SO_4 (1.5 ml.) and extracted with ether (3 x 7 ml.). After drying over anhydrous sodium sulphate the ether was evaporated in a stream of N_2 . The residue dissolved in 100 µl. of hexane and 2-5 µl. portions used for analysis. In the determination of acidic metabolites the incubation mixtures were extracted with ether as above and acidic compounds transferred into 5 ml. of 0.5N sodium hydroxide which was then acidified with 2N HCl and extracted into ether (2 x 5 ml.). The ether was dried, evaporated in N_2 as before and treated with a freshly distilled ethereal solution of diazomethane , until a permanent yellow colour was obtained. After 1 hr. this solution was evaporated in a stream of N_2 and the residue was dissolved in 100 µl. of n-hexane for gas chromatography. Recovery experiments in which 5-50 µgs. of the different alcohols and acids studied were added to different volumes of 10,000g. supernatant from liver, locust fat body or housefly preparations showed that a constant loss of 5-7 µg. of alcohol and acid occured. This loss occured during the evaporation of the ether extract

and was more significant at higher temparatures . The volumes of ether were therefore standardised as above and evaporation carried out at 0° . Under these conditions linear correlation curves relating alcohol or acid added and amounts recovered were obtained .

Table 25. Gas-liquid chromatography of metabolites of alkylbenzenes.

Retention times are given in minutes on A: 3% (w/w) polyethylene glycol adipate on Celite, 6ft., 100°; B: 3% (w/w) Carbowax 4000 on Celite, 6ft., 125°; C: 2% (w/w) Carbowax 4000 on Celite, 6ft., 120°; D: 1.5% (w/w) Carbowax 4000 on Celite, 6ft., 90°. The flow rate of N_2 was 100ml./min. .

	A	B	C	D
Benzyl alcohol	-	-	4.5	-
Methyl benzoate	2.0	-	1.5	-
Acetophenone	-	-	2.0	-
1-Phenylethanol	2.5	3.0	3.5	-
2-Phenylethanol	5.0	4.5	6.0	-
Methyl phenylacetate	3.5	-	3.0	-
p-Ethylphenol	-	-	20.0	-
1-Phenylpropan-1-ol	4.5	5.0	-	5.5
1-Phenylpropan-2-ol	2.0	3.8	-	5.5
1-Phenylpropan-3-ol	9.0	9.5	-	11.0
2-Phenylpropan-1-ol	5.0	5.5	-	7.5
2-Phenylpropan-2-ol	2.5	2.3	-	3.0
Methyl phenylpropionate	6.0	-	-	-
Methyl 2-phenylpropionate	3.0	-	-	-
1-Phenylbutan-1-ol	6.5	6.8	-	10.5
1-Phenylbutan-2-ol	5.0	5.0	-	8.5
1-Phenylbutan-3-ol	7.5	7.8	-	10.5
1-Phenylbutan-4-ol	13.5	11.5	-	-
Methyl phenylbutyrate	9.5	-	-	-
2,2-Dimethylphenylethanol	-	7.8	-	-

Table 26 . Oxidation of alkylbenzenes by fly and locust enzyme.

Enzymes were prepared from flies and locusts and incubations were carried out as described in the text. The results with n-propylbenzene and iso-propylbenzene represent mean values of two determinations.

Substrates and products	Relative rates of formation of metabolites, mμmoles / hr./ g. of whole insect	
	Locust	Fly
Toluene		
Benzoic acid	24, 18, 66, 54	N/d
p-Nitrotoluene		
p-nitrobenzoic acid	30	144, 102, 240, 90
n-Propylbenzene		
1-Phenylpropan-1-ol	3.6	7.2
1-Phenylpropan-2-ol	$<$0.6	$<$0.6
1-Phenylpropan-3-ol	$<$0.6	$<$0.6
3-Phenylpropionic acid	$<$0.6	$<$0.6
iso-Propylbenzene		
2-Phenylpropan-1-ol	$<$ 0.6	$<$ 1.2
2-Phenylpropan-2-ol	3	16.2
2-Phenylpropionic acid	$<$ 0.6	$<$ 1.2

N/d = not determined

Table 27 . Oxidation of alkylbenzenes by rabbit liver enzymes.

The incubations and enzyme assays were carried out as described in the text. The results are expressed as mean ± standard deviation. Figures in parantheses give the number of animals used.

Substrates and products	Rate of formation of products, mμmoles / hr. / g. of whole rabbit.		
p-Nitrotoluene			
p-Nitrobenzoic acid	41.7	+ 6.0	(3)
Ethylbenzene			
1-Phenylethanol	15.6		(1)
2-Phenylethanol	<0.6		(1)
Phenylacetic acid	1.8		(1)
n-Propylbenzene			
1-Phenylpropan-1-ol	15.2	+ 3.0	(3)
1-Phenylpropan-2-ol	5.4	+ 1.5	(3)
1-Phenylpropan-3-ol	<0.6		(3)
3-Phenylpropionic acid	3	+ 0.6	(3)

Table 28 . Oxidation of alkylbenzenes by rabbit liver enzymes.

Incubations and enzyme assays were carried out as described in the text. The results are expressed as mean ± standard deviation. The figures in parantheses give the number of animals used.

Substrates and products	Rate of formation of products, mμmoles / hr. / g. of whole rabbit.		
iso-Propylbenzene			
2-Phenylpropan-1-ol	3	± 1.0	(3)
2-Phenylpropan-2-ol	14.9	± 4.5	(3)
2-Phenylpropionic acid	2.4	± 0.6	(3)
n-Butylbenzene			
1-Phenylbutan-1-ol	7	± 1.8	(3)
1-Phenylbutan-2-ol	<0.6		(3)
1-Phenylbutan-3-ol	4	± 1.5	(3)
1-Phenylbutan-4-ol	<0.6		(3)
4-Phenylbutyric acid	2.4	±	(3)
tert-Butylbenzene			
2,2-Dimethylphenylethanol	5	± 1.3	(3)
2,2-Dimethylphenylacetic acid	<0.6		(3)

Table 29 :- Cofactor requirement of the rabbit liver enzyme responsible for the conversion of iso-propylbenzene to 2-phenylpropan-2-ol .

Incubations contained the supernatant fraction equivalent to 1 g. of liver in a total volume of 5 ml. , containing 0.5 ml. of 0.1 M tris buffer, pH 7.4 and cofactors as shown below. The amounts of various cofactors used have been given in the text. Substrates, 8.3 µmoles in 0.5 ml. acetone were added to the mixtures and the reactions were carried out for 1 hr. at 37° in air.

Additions	Yield of 2-phenypropan-2-ol µmole / g. of liver / hr.
NADP + NAD + G-6-Phosphate	0.64
NADP + G-6-Phosphate	0.72
NAD + G-6-Phosphate	0.29
NAD + NADP	0.59

Table 30. Cofactor requirement and subcellular distribution of enzyme activity for oxidation of iso-pripylbenzene to 2-phenylpropan-2-ol by rabbit liver.

Subcellular fractions (4ml.) equivalent to 1 g. of liver were incubated with 10 µmoles of $MgCl_2$, 24 µmoles of nicotinamide, 8.3 µmoles of substrate, various cofactors and 0.5 ml. of 0.1M tris buffer, pH 7.4 at 37° for 1 hr. 0.675 µMoles of NADP, 0.675 µmole of $NADPH_2$ 7.7 µmoles of glucose-6-phosphate, and 50 units of glucose-6-phosphate dehydrogenase were added as indicated.

Tissue fraction	NADP	$NADPH_2$	G-6-P	G-6-P. dehyd.	Yield of 2-Phenylpropan-2-ol, µmole/g./hr.
10,000g. sup.	-	-	-	-	0.1
10,000g. sup.	+	-	-	-	0.81
Microsome + sup.	+	-	-	-	0.71
Microsome	+	-	-	-	0.24
Microsome	+	-	+	+	0.65
Microsome	-	+	-	-	0.59

Table 31 . Species difference in the oxidation of p-nitrotoluene and iso-propylbenzene in flies and vertebrates.

Rabbit liver and fly abdomen enzymes were prepared and incubated as described in the text and assays performed for p-nitrobenzoic acid or 2-phenylpropan-2-ol. The results are presented as mean value ± standard deviation. The figures in the parantheses give the number of animals used.

Species	Rate of oxidation, µmoles /g. of liver or 10 g. whole insect / hr.	
	p-Nitrotoluene	iso-Propylbenzene
Coypu	1.71 ± 0.25 (3)	0.66 ± 0.04 (3)
Chinchilla rabbit	1.38 ± 0.15 (3)	0.45 ± 0.05 (3)
Hamster@	0.96	0.55
Guinea pig	0.61 ± 0.1 (3)	0.37 ± 0.1 (2)
Cat	0.49 ± 0.06 (3)	0.42 ± 0.05 (2)
Rat	0.37 ± 0.06 (3)	0.24@
Flies normal	0.84	0.22
FC(carbamate)*	1.2	0.37
SKA(Diazonin & DDT)*	0.48	0.29
P(pyrethrins)*	1.14	0.29
A(organophosphates)*	0.72	0.29
R(reverted strain)*	0.72	0.29

@ livers from two animals were pooled.

* Resistant to insecticides in parantheses.

Fig. 19. pH- Activity curve of iso-propylbenzene oxidation by rabbit-liver enzyme.

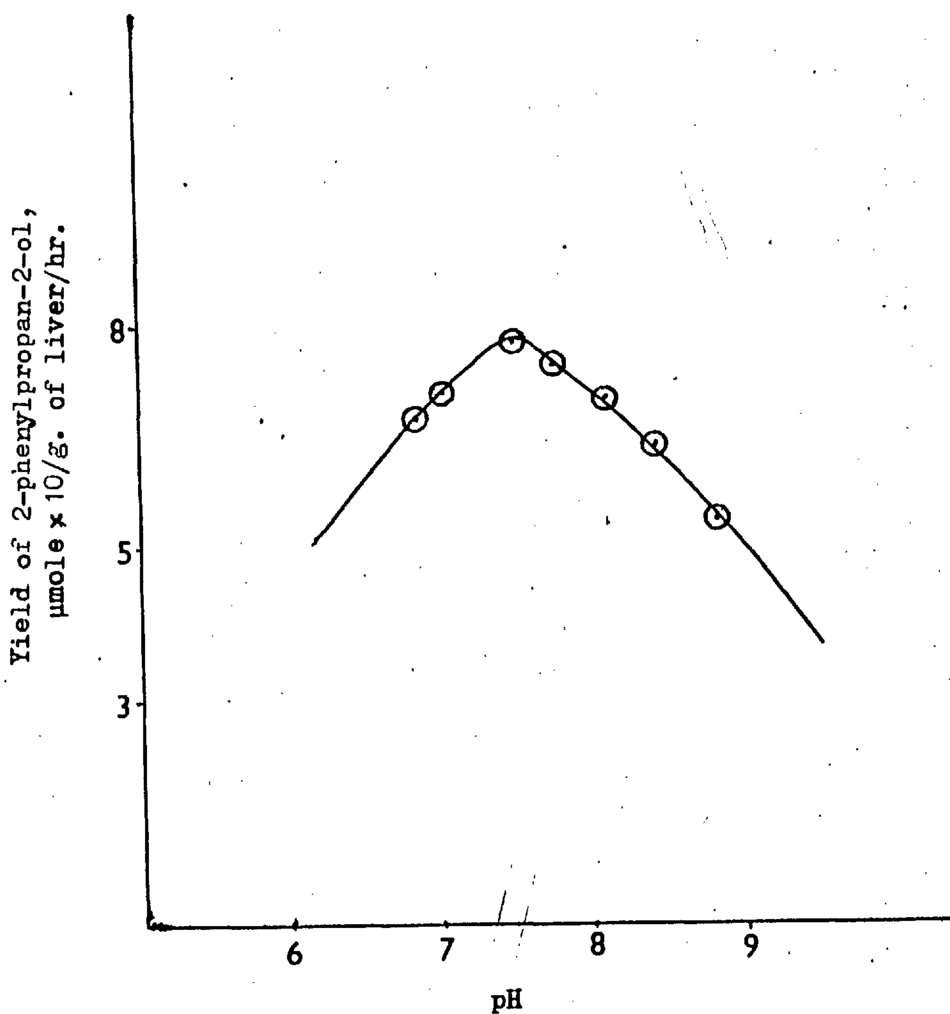


Fig. 20 . Gas chromatographic separation of the alcoholic metabolites of *iso*-propylbenzene in the rabbit *in vitro*.

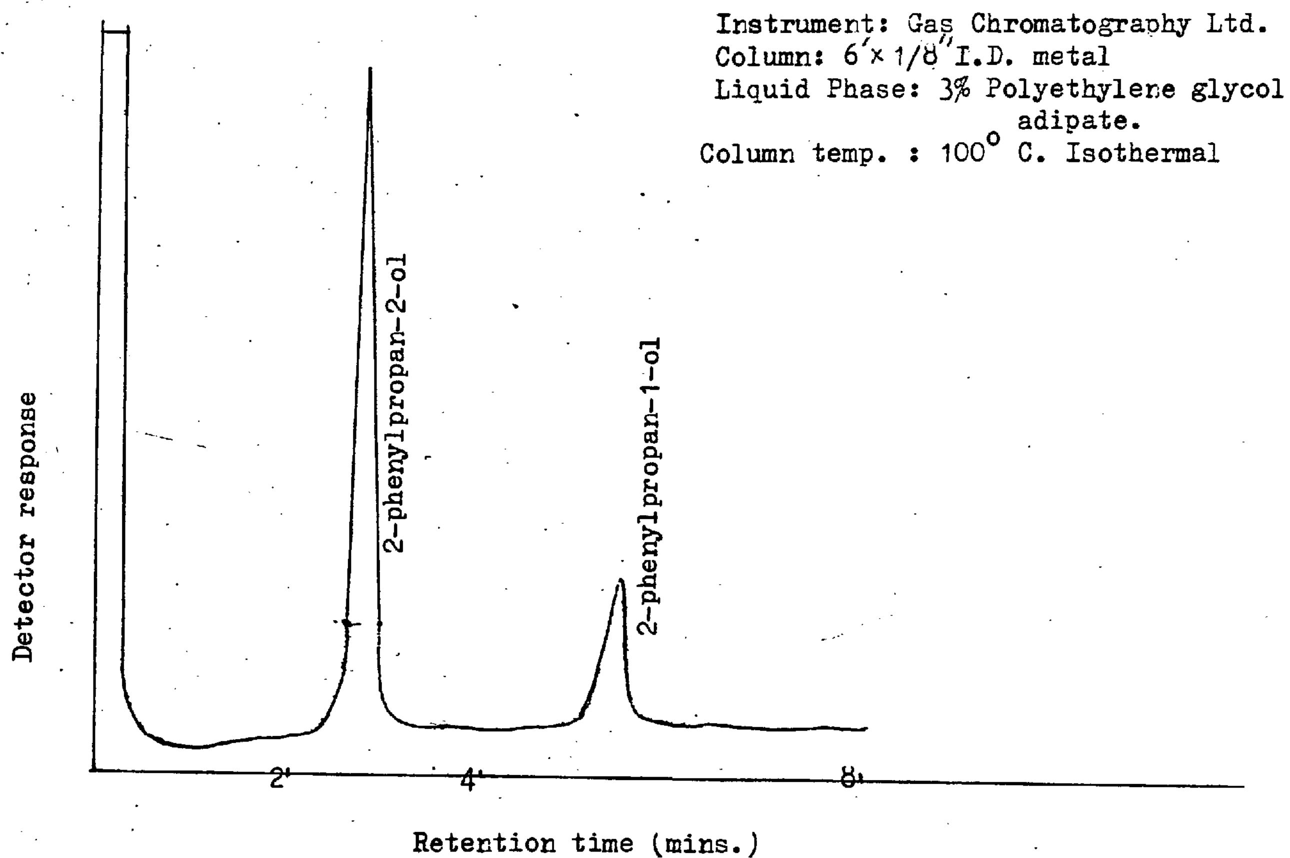


Fig.21 . Gas chromatogram of the methyl esters of some aromatic acids.

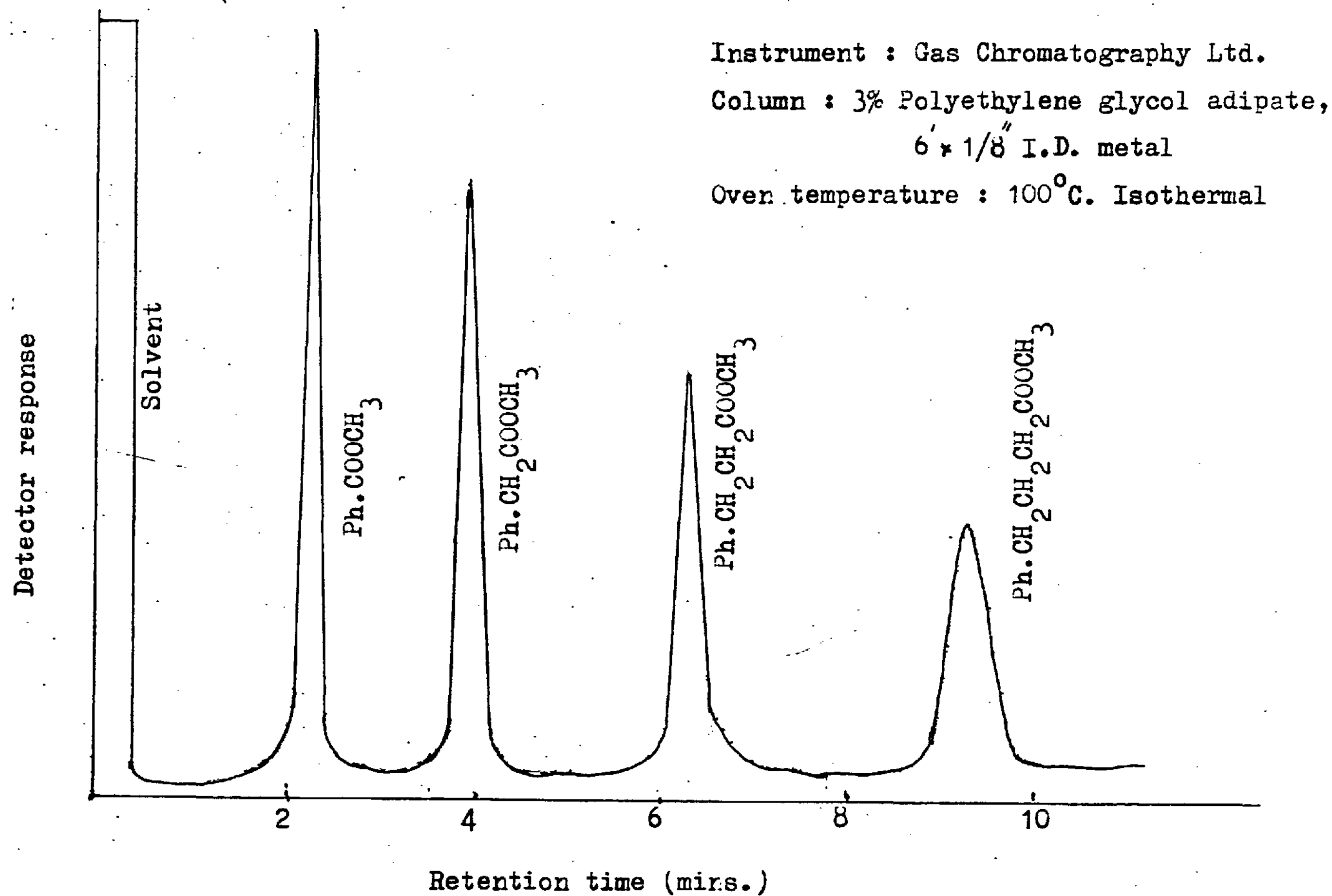
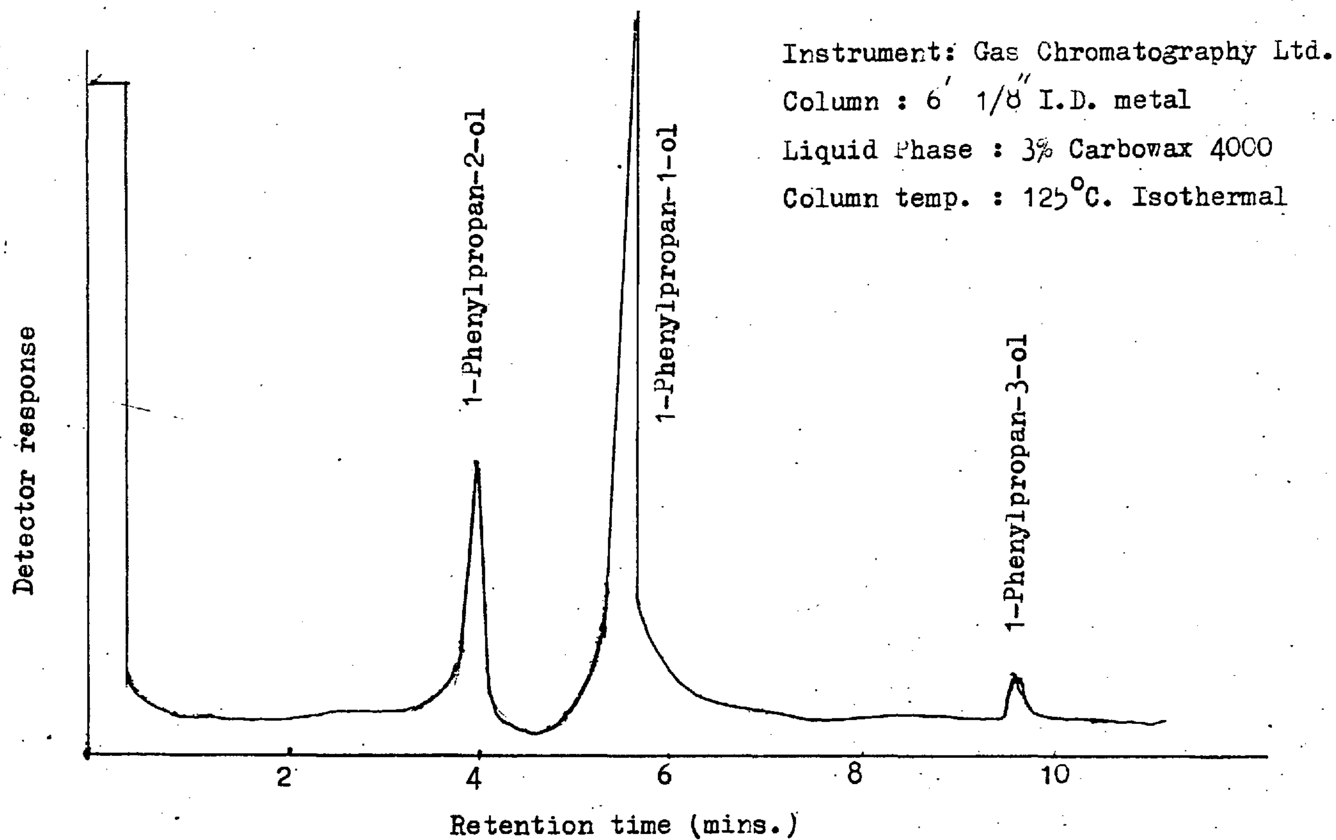


Fig. 22. Gas chromatogram of the alcoholic metabolites of n-propylbenzene in the rabbit *in vitro*.



Results and discussion

The metabolism of a number of alkylbenzenes by vertebrate liver, housefly abdomen and locust fat body preparations was studied. The metabolic studies with *p*-nitrotoluene showed that (Chapter II), the locust fat body and fly abdomen have similar enzymic characteristics to the mammalian liver. So, for further metabolic studies in insects these two tissue preparations were used. In experiments with insect tissues where the enzyme was derived from large numbers of insects(30-40 locusts or up to 1000 flies), the preparations were assayed with *p*-nitrotoluene as substrate to confirm the activity of the enzyme. The other two compounds included in the metabolic studies in both insects and mammals were *n*-propylbenzene and *iso*-propylbenzene. The results (Tables 26, 27 & 28) show that the routes of metabolism of each of these compounds in locust, fly and rabbit are identical. The only difference is in rate. Oxidation of the methyl group to carboxyl group was the only reaction observed with *p*-nitrotoluene in all species studied. The major metabolites of *n*-propylbenzene were the corresponding

secondary alcohols, 1-phenylpropan-1-ol and 1-phenylpropan-2-ol. The other metabolite formed by the rabbit liver was the ω-oxidation product 3-phenylpropionic acid. Insect enzyme preparations oxidized n-propylbenzene to 1-phenylpropan-1-ol (Tables 26 & 27). Both in insects and mammals , α-carbon atom was the main site of oxidation in iso-propylbenzene, producing 2-phenylpropan-2-ol. Oxidation of one of the β-carbon atoms also occured, producing hydrotropyl alcohol and hydrotropic acid as minor metabolites. Locusts oxidize toluene more rapidly than p-nitrotoluene. The difference in the rate of oxidation of p-nitrotoluene in locusts and flies *in vitro* is in agreement with the observations *in vivo* (Tables 4 & 26). Flies are more effective than other insects in oxidizing alkyl side chain. With p-nitrotoluene, 0.03 and 0.11 µmole of the substrate is oxidized / g. of whole insect / hr. in locust and fly respectively. The rate of formation of p-nitrobenzoic acid (µmoles / hr. / g. of whole insect or animal) from p-nitrotoluene in locust fat body, fly abdomen and rabbit liver preparation has the order ; fly > rabbit > locust. With the increase in size of the alkyl side chain from methyl to n-propyl and iso-propyl , the order changes to - rabbit > fly > locust. It is noticeable that in rabbit, the

rate of oxidation of n-propylbenzene and iso-propylbenzene is approximately half the rate observed with p-nitrotoluene, whereas in locust and fly the drop in enzyme activity was found to be markedly higher, approx. 90%. Of course, it should be taken into account that the figures quoted for the insects are probably minimal since the oxidation of p-nitrotoluene occurs in other organs of the locust and the fat body accounts for only about half of the oxidizing capacity of the insect (Table 7). Similar consideration probably apply to the fly abdomen preparation used here. n-Butylbenzene is oxidized 0.015 µmole / hr. / g. of whole animal , compared to 0.024 µmole / hr. / g. with n-propylbenzene. Attempts to identify the metabolites of n-butylbenzene in insects were not successful. Probably the rate of reaction is too small to to detect. The results obtained in vitro studies of ethylbenzene, n-propylbenzene, iso-propylbenzene, n-butylbenzene & tert-butylbenzene confirm the earlier in vivo studies of these compounds on the rabbit where the metabolites were isolated from urine (Williams 1959). It is clear that the metabolism of alkylbenzenes essentially involve the oxidations of their side chains producing the corresponding alcohols and

acids which in the living animal are excreted as various conjugates. The major oxidation product of the alkylbenzenes, where this is possible, was the secondary alcohol formed by hydroxylation at the α-methylene group of the side chain. The oxidation of the penultimate (ω-1) methylene group of straight chains, e.g. in n-butylbenzene was somewhat slower and occured to the same extent as the oxidation of terminal methyl group. The ω-oxidation product was found to be the corresponding acid. Where no α- or penultimate methylene group was available, the oxidation of the molecule was much slower and tert-butylbenzene was oxidized nearly ten times slower than toluene or p-nitrotoluene by the same preparation. No phenolic metabolite was identified in any case. Ethylbenzene was mainly oxidized at the α-methylene group yielding 1-phenylethanol. Phenylacetic acid was the minor metabolite. In n-propylbenzene , more oxidation took place at the α-methylene group (0.015 μmole/hr./g. of whole rabbit) than at the (ω-1) carbon atom (0.005 μmole/hr./g.). ω-Oxidation product 3-phenylpropionic acid was also found in small amounts (0.003 μmole/hr./g. of whole rabbit). The oxidation of iso-propylbenzene took place almost exclusively at the α-methylene group yielding 2-phenylpropan-2-ol (0.015 μmole/hr./g. of whole rabbit). The other metabolites were the oxidation products of one of the β-carbon atoms, 2-phenylpropan-1-ol and 2-phenylpropionic acid (0.003 μmole & 0.002 μmole/

hr./g. of whole rabbit respectively). Like others, preferential oxidation of the methylene group nearest to the benzene ring was also observed in _n_-butylbenzene (0.007 μmole/hr./g. of whole animal). The oxidation of the penultimate methylene group was somewhat slower and occured to about the same extent as the oxidation of terminal methyl group. The ω-oxidation product 4-phenylbutyric acid was not found in the urine of rabbit dosed with _n_-butylbenzene (Fig. 17). It is likely that _in vivo_ this compound is β-oxidized to phenylacetic acid which is excreted as its glycine conjugate. This conjugate was found in the urine and accounted for 15-20% of the dose. _tert_-Butylbenzene was oxidized at one of the β-carbon atoms producing 2,2-dimethyl-phenylcarbinol (0.005 μmole/hr./g. of whole rabbit). The results (Tables 27 & 28) show that in the same species the total rate of oxidation varies with substrates. Rabbit oxidizes _p_-nitrotoluene 1.7 and 2.9 times as fast as _n_-propylbenzene and _n_-butylbenzene respectively. Ethylbenzene appears to be less readily oxidized than _n_-propylbenzene. This needs further examination as the ethylbenzene oxidation rates quoted in Table 27 were obtained from one animal. Species differences in mammals in the rate of oxidation of alkylbenzenes were studied by using _p_-nitrotoluene and _iso_-propylbenzene as substrates. These compounds are ideal for such

investigations as metabolic reactions of each of them in all species used are restricted almost exclusively to one pathway. The liver 10,000g. supernatant preparations oxidized *p*-nitrotoluene to *p*-nitrobenzoic acid and *iso*-propylbenzene to 2-phenylpropan-2-ol. The *p*-nitrotoluene oxidizing activity was found to be in the order rabbit > coypu > hamster > guinea pig > cat > rat and rabbit liver was 3.7 times more active than rat liver (Table 31). The *iso*-propylbenzene oxidase actvity was in the order coypu > rabbit > hamster > guinea pig > cat > rat , coypu liver being 1.9 times more active than rat liver (Table 31). The pattern is identical with the species differences in the coumarin-7-hydroxylase activity of liver microsomal enzyme (Williams 1963). The only difference is that rats can oxidize alkyl side chains of *p*-nitrotoluene and *iso*-propylbenzene whereas no 7-hydroxylation was obtained in rat with coumarin.Same order of differences in the rabbit, guinea pig and rat was observed in the oxidative metabolism of aniline, hexabarbitone and antipyrene (Brodie 1964 ; Quinn et al 1958). This indicate that one common factor, for example, the formation of "hydroxylating intermediate" is probably involved in species differences in the metabolic oxidations of various types.

According to Terriere et al (1962 & 1965a) resistant strains of houseflies have greater microsomal hydroxylating activity than normal strain. No such differences were observed in the rate of

oxidation of p-nitrotoluene and iso-propylbenzene in the several strains used here (Table 31).

The optimum pH, cofactor requirements and sub-cellular distribution of the rabbit liver alkyl side chain hydroxylase was determined. The enzyme was assayed with iso-propylbenzene as the substrate. The optimum pH was found to be 7.4 (Fig. 19). The enzyme was located in the microsomal fraction and needed a $NADPH_2$-generating system and oxygen for its activity (Tables 29 & 30). These results and previous investigations by Gillette(1959) indicate that the alkyl side chain hydroxylase belong to the community of microsomal oxidative enzymes in the liver (Brodie 1956 ; Brodie et al 1958). Solubilization and fractionation of the enzyme are needed to find out whether more than one enzyme are involved in the metabolic attack on different carbon atoms in the side chain of alkylbenzenes containing more than one carbon atom in the alkyl group.

CHAPTER IV.

INDUCTION AND INHIBITION OF p-NITROTOLUENE AND ISO-PROPYLBENZENE OXIDIZING ENZYME SYSTEMS IN MAMMALS AND INSECTS.

Chapter IV

INDUCTION AND INHIBITION OF p-NITROTOLUENE AND iso-PROPYLBENZENE OXIDIZING ENZYME SYSTEMS IN MAMMALS AND INSECTS.

Section A :- Introduction : Stimulation and inhibition of metabolic oxidation in mammals and insects .

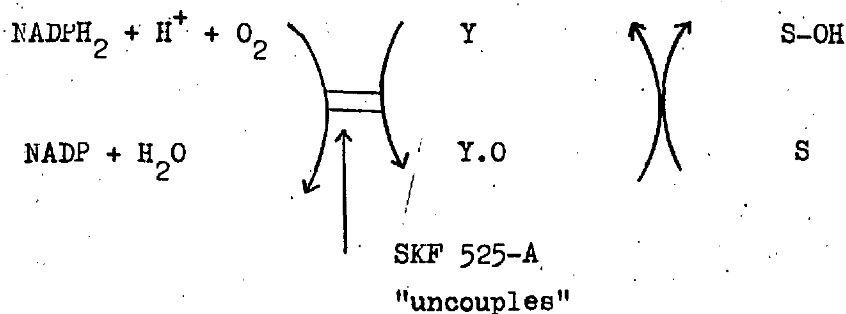
Stimulation and inhibition of oxidation in mammals :- It is now known that pretreatment of animals with a wide variety of compounds increases the activity of the enzyme systems that catalyze a number of metabolic reactions , particularly oxidations (Remmer 1962 , 1964) . 3-Methylcholanthrene and a number of other polycyclic hydrocarbons , such as 3,4-benzpyrene stimulate the rat liver microsomal enzymes that oxidize 2-acetylaminofluorene , 3,4-benzpyrene and zoxazolamine (Gillette 1963) . These effects have also been demonstrated in living animals . Barbiturates stimulate oxidation by microsomal enzymes non-specifically (Remmer 1958 , 1959). It has been shown that barbital and phenobarbital given to rats increases the activity of the microsomal enzymes which metabolize zoxazolamine , phenylbutazone , 3,4-benzpyrene , 3-methylaminoazobenzene , meprobamate , pethidine and phenobarbital . Some compounds including phenobarbital , tolbutamide , phenylbutazone , aminopyrine and meprobamate activate their own metabolism . Fouts (1963 $_{a,b}$) and Koransky et al (1964) showed that DDT , Chlordan and

mary other insecticides are strong enzyme inducers . After an injection of hexachlorcyclohexane or DDT , the stimulation of microsomal enzymes in the rat lasts four weeks whereas with phenobarbital pretreatment the increase in the enzyme activity reaches a maximum on the second or third day , declining to normal values after 5-7 days (Remmer 1962) .There is evidence suggesting that foreign compounds enhance the activity of the microsomal enzymes in rat liver through a number of mechanisms (Gillette 1963) . According to Remmer (1964) the stimulatory effect of a compound on microsomal enzyme activity is caused by an increased synthesis of enzyme protein .

In the past few years a number of compounds have been shown to inhibit metabolic oxidations . The best known of this inhibitors is the diethylaminoethanol ester of diphenylpropylacetic acid (SKF 525 A). This compound prolongs the action of a variety of drugs by inhibiting their metabolism *in vivo* (Axelrod *et al* 1954 ; Brodie 1956 ; Cook *et al* 1957 ; Kato *et al* 1962). This is explained by the finding that the inhibitor in concentrations as low as 10^{-4} M blocks the *in vitro* oxidation of many compounds

including barbiturates , codeine and aminopyrine . Some compounds like diphenylpropylacetate (SKF acid) , diphenypropylethanol , diphenylpropylethylamine and triparanol are potent inhibitors of microsomal enzymes *in vitro* , but have very little activity *in vivo* . Although no relationship between the structure and the potency of the inhibitors has been observed , minor changes in the chemical structure of a particular series of compounds can result in remarkable changes in activity (La Du et al 1954 ; Neubert and Herkin 1955). A number of monoamine oxidase inhibitors , such as iproniazid and β-phenylisopropylhydrazine (JB 516) inhibit hexabarbital metabolism both *in vivo* and *in vitro* ; however it was found that the activity of these compounds as monoamine oxidase inhibitors is not related to their ability to inhibit the metabolism of foreign compounds (La Roche and Brodie 1960) . Many other compounds have been reported to prolong the action of drugs by inhibiting metabolic oxidations. They include β-diethylaminoethylphenyldiallyl acetate (CFT 1201) , diphenylcarbamate , diphenylacetate , chlorcyclizine , chloramphenicol , a number of the *N*-ethyl piperidyl derivatives of benzylate and the methylenedioxyphenyl compounds .

(Gillette 1963 ; Fine and Molloy 1964) . The methylenedioxyphenyl compounds and SKF 525A were reported to inhibit *in vitro* the *N*-methylhydroxylation of a number of carbamate insecticides by rat liver microsome—$NADPH_2$ — molecular oxygen system (Casida 1963 ; Hodgson *et al* 1960 , 1961). The mode of action of inhibitors still remains obscure. Most of the work concerned has been done with SKF 525A. This compound apparently does not alter the generation of $NADPH_2$ in the soluble fraction of liver nor the electron transport system in microsomes , for it does not inhibit glucose-6-phosphate dehydrogenase (Gillette 1963). Since it affects various types of oxidation it has been suggested that this inhibition is at a step common to the metabolism of all of this compounds . Netter (1962) postulated that SKF 525A uncouples the oxidation of $NADPH_2$ from the reaction forming the hydroxylating intermediate (s) as shown below.



Y.O represents the hydroxylating intermediate and S is the compound oxidized.

Induction and inhibition of metabolic oxidations in insects :- In recent years attempts have been made to utilize enzyme induction and inhibition studies for evaluating the role of metabolism in relation to resistance phenomenon in insects . Resistance has been defined by the W . H . O . expert committee on insecticides (1957) as " the development of an ability in a strain of insects to tolerate doses of toxicants which would prove lethal to the majority of individuals in a normal population of the same species " . Growing evidence indicates that resistance may largely be attributable to a more rapid detoxication by the resistant than the susceptible strains , rather than a change in absorption rate or other factors (Sternburg et al 1954 ; Metcalf 1955 ; O'Brien 1960 ; Smith 1962 ; Cassida 1963 ; Fine 1963 ; Terriere 1965a) . Morello (1964) showed that the degree of tolerance to DDT [2,2-bis (p-chlorophenyl)-1,1,1-trichloroethane] by resistant strain of *T. infestans* can be modified by the specific *in vivo* inhibition or stimulation of the DDT-hydroxylating enzyme system . SKF 525A and iproniazid inhibit the hydroxylating enzyme and thereby potentiate the effect of DDT . On the other hand , the mortality of 3,methylcholanthrene pretreated insects

decreased due to the induction of enzymic hydroxylation. Terriere *et al* (1965 a) found that housefly strain , selected for resistance to naphthalene has a correspondingly high capacity to hydroxylate *in vitro*. The effect appears to be similar to those of phenylbutazone , meprobamate and other compounds which stimulate their own metabolism in mammals.

It has been found that pyrethrin synergists, a group of compounds containing the methylenedioxyphenyl group effectively synergize the insecticidal action of a number of compounds against various species of insects including resistant strains (Metcalf 1955 ; O'Brien 1960 ; Sun & Johnson 1960 ; Casida 1963 ; Brooks *et al* 1963 ; Hadaway *et al* 1963). Resistant insects appear generally to revert to nearly the same degree of susceptibility as non-resistant strains when they are subjected to an appropriate synergist. It was suggested that they interfere with metabolic oxidations. This is supported by the finding of Hewlett *et al* (1961) , who showed that SKF 525-A effects the toxicity of pyrethrins and malathion, a phosphorous insecticide in a similar manner to the methylenedioxyphenyl compounds. Terriere *et al* (1965 b) reported that several synergists which were effective in increasing the susceptibility of houseflies to naphthalene also inhibit hydroxylation *in vitro*.

Chapter IV

INDUCTION AND INHIBITION OF p-NITROTOLUENE AND iso-PROPYLBENZENE OXIDIZING SYSTEM IN MAMMALS AND INSECTS .

Section B :- Materials , methods , results and discussion.

Materials :- Piperonylbutoxide (3,4-methylenedioxy-6-propylbenzyl-n-butyldiethyleneglycol ether) , sulfoxide [1-methyl-2 (3,4-methylenedioxyphenyl)-ethyl-octylsulfoxide], n-propylisome (di-n-propyl 6,7-methylenedioxy -3-methyl-1,2,3,4-tetrahydronaphthalene 1,2-dicarboxylate) , sesamex [2-(3,4-methylenedioxyphenoxy) 3,6,9-trioxaundecane] and DMC (4,4'-dichloro-α-methylbenzhydrol) were the gift of Dr. B. C. Fine of London School Of Hygiene and Tropical Medicine . SKF 525A (diethylaminoethyl diphenyl-n-propylacetate hydrochloride) was provided by Professor R. T. Williams. m-Isopropyl phenyl N-methylcarbamate (IPMC) was obtained from Union Carbide and recrystallized from benzene m.p. , 70-72°.

Pretreatment with phenobarbitone and 3,4-benzpyrene :- Male rats weighing 50g. were injected intraperitoneally with aqueous sodium phenobarbitone (35mg / Kg) twice daily for four days . Control animals were similarly dosed with water. A second group were given a single dose of 3,4-benzpyrene(25mg/Kg.)

in arachis oil with corresponding controls . On the day following the final dose , liver homogenate 10,000g. supernatant was prepared and its oxidizing ability assayed using p-nitrotoluene and iso-propylbenzene as described previously. Locust hoppers were given the same dose level of phenobarbitone and benzpyrene for three days and one day respectively before preparation of fat body 10,000g. supernatant. Appropriate controls which had received solvent injections were also used . Assays were carried out with p-nitrotoluene and iso-propylbenzene .

LD_{50} determination in locusts :- The LD_{50} of m-isopropyl-phenyl-N-methyl carbamate was determined on normal 5th instar (4 day old) locusts and those which had been given pheno-barbitone for three days . The compound was applied dissolved in kerosine extract bottom (KEB) , an aromatic petroleum extract of low viscosity . In each experiment (50 insects) , the carbamate solution was applied by means of a micro-drop syringe or a micro-capillary tube (described in " A-L.R.C. -F.A.O Insecticide testing kit for locusts" published by Anti-Locust Research Centre , London) to the intersegmented region between first and second abdominal sternites (MacCuaig 1958 ; MacCuaig et al 1961). Before dosing each locust was weighed and the

volume of carbamate solution adjusted to give a pre-selected amount of the compound per g. of body weight. For applying volumes less than 1 µl , the special micro-capillary tube mentioned above and obtained from Anti-Locust Research Centre , London was used . The capillary tube was 1 µl in total volume and had a scale attached to it which was divided into tenths . For use , the free end of the tube was inserted into a rubber cap fitted at the end of a 1/4" glass tubing . When the tip of the capillary was touched against the surface of a liquid , the liquid filled it . By applying a cotton wool plug moistened with the liquid to the tip of the capillary the liquid could be slowly drawn out again until the desired volume remained in the capillary . For dosing a finger was placed over the hole in the rubber bulb and the bulb was gently compressed meanwhile holding the free end of the capillary against the insect . The insects were recorded as dead , when they could not right themselves after being turned upside-down . The mortalities were noted 24hrs. after dosing . In all cases , the toxicities were estimated from probit regression lines.

Selection of houseflies with p-nitrotoluene :- The strain of houseflies used in this experiment was obtained from the London School of Hygiene and Tropical Medicine . This strain on selection with DDT for 6-8 generations was found to develop 10-20 fold resistance to DDT (Fine 1963). Attempts were made to determine whether selection of these flies with p-nitrotoluene has any effect on their p-nitrotoluene oxidizing activity . The selection was continued for six generations and the enzyme activity was assayed at each stage . The following method was used for selection and rearing of the flies (Potter 1963) . One day old flies were dipped for 3 min. in a 30 % aqueous acetone solution of p-nitrotoluene (0.1%) . The solution was filtered off in a Buchner funnel and the flies , dried for 3 min. by the suction of air through the funnel , were transferred into glass containers , checked for kill 24 hrs. after treatment , transferred into other containers and allowed to mate . The eggs were collected from the mated survivors .The larval medium used consisted of dried yeast (50 g.) , milk powder (50 g.) and agar (10 g.) in water (500 ml.) . The mixture was boiled for 5 min. and then poured into jam jars (6) and covered with a thin layer of sawdust . The larvae pupaeted

in the sawdust layer .After emergence the flies were fed on sucrose and water . They were normally used for enzyme preparation the day after emergence .

Incubation and enzyme assay :- The preparation of the enzyme and the constitution of the standard incubation mixture have been previously .In experiments with *iso*-propyl benzene , instead of using the standard cofactor mixture 0.68 μmole of NADP , and 7,7 μmole of glucose-6-phosphate were used . All inhibitors were taken up in acetone solution and 0.05 ml was added to a mixture when wanted . Methods for the estimation of *p*-nitrobenzoic acid and 2-phenypropan-2-ol have been described in chapter II and chapter III respectively .

Table 32. Effect of pretreatment of rats and locusts with phenobarbitone and 3,4-benzpyrene on the *in vitro* oxidation of *p*-nitrotoluene.

Rat liver or locust fat body enzyme was prepared and incubated at 37° in air with added cofactors as described in the text. Animals were dosed with phenobarbitone for 4 days or with 3,4-benzpyrene for one day before preparation of the enzymes or measurement of the toxicity of *m*-isopropyl *N*-methyl carbamate (IPMC). The results are expressed as mean ± standard deviation. Figures in parenthesis give the number of determinations.

Pretreatment	Rate of formation of *p*-nitrobenzoic acid µmole / g. of liver or locust / hr.		LD_{50} of IPMC µg./locust
	Rat	Locust	
Water control	0.41 + 0.02 (2)	0.038 + 0.006 (3)	12
Arachis oil control	0.41 + 0.08 (2)	n/d	n/d
Phenobarbitone	0.84 + 0.1 (3)	0.029 + 0.004 (3)	10
Benzpyrene	0.68 + 0.15 (3)	0.022 + 0.004 (3)	n/d

n/d = Not determined

Table 33 . Effect on isopropylbenzene oxidase of pretreatment of female rats with phenobarbitone and 3,4-benzpyrene.

Rat liver enzyme was prepared and incubated at 37° in air with the addition of cofactors as described in the text. Animals were dosed with phenobarbitone for 4 days or with benzpyrene for one day before preparation of the enzyme. The results represent averages of two animals.

Pretreatment	Rate of formation of 2-phenylpropan-2-ol, µmole / g. of liver / hr.
Water control	0.26
Arachis oil control	0.26
Phenobarbitone	0.51
3,4-Benzpyrene	0.4

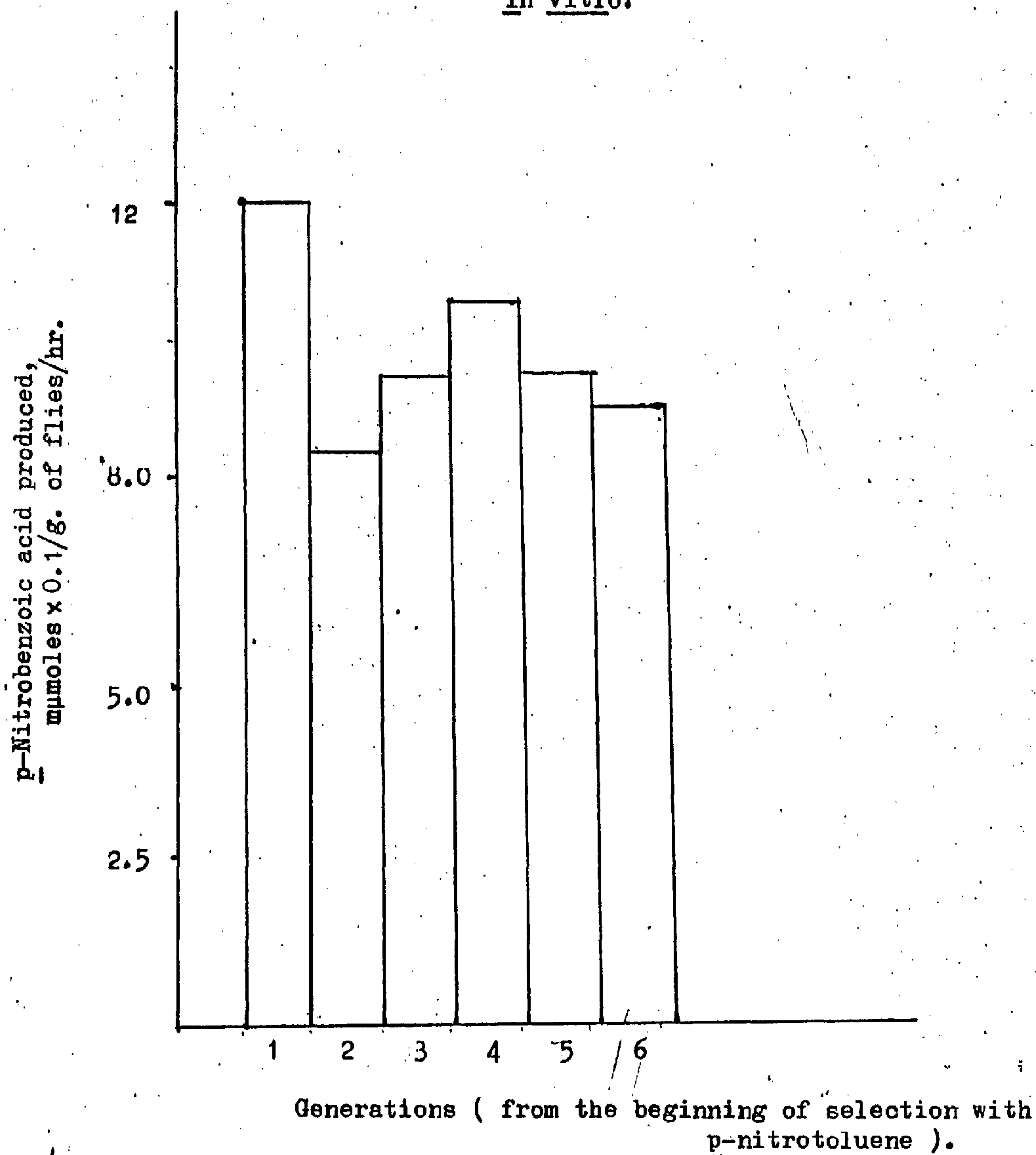
Table 34 . Inhibition of p-nitrotoluene oxidation by insecticide synergists.

The 10,000 g. supernatant enzyme from rabbit or rat liver or from fly abdomen homogenates was incubated with p-nitrotoluene under conditions described in the text, and p-nitrobenzoic acid formed in 0.5 hr. compared in normal preparations with those containing inhibitors.

Inhibitor concentration / Inhibitor	% inhibition of enzyme from: Rabbits		Rats	Flies	
	0.5 mM	0.1 mM	0.5 mM	0.1 mM	0.01 mM
Piperonyl butoxide	27	6	22	100	51
Sulfoxide	44	18	33	100	48
n-propylisome	22	7	19	100	39
Sesamex	44	20	27	n/d	n/d
D.MC.	8	n/d	3	n/d	11
SKF 525-A	32	11	38	n/d	26

n/d = not determined

Fig. 23. Effect of selection with p-nitrotoluene on the p-nitrotoluene oxidizing activity of housefly *in vitro*.



Results and discussion

Methylenedioxyphenyl synergists such as piperonyl butoxide, sulfoxide, *n*-propylisome and sesamex are often included in various insecticide formulations. Several types of metabolic oxidations which are thought to be inhibited or blocked by these compounds include, conversion of P=S to P=O , formation of amine oxides, epoxidation and aromatic hydroxylation (Sun & Johnson 1960 ; Lichtenstein *et al* 1963 ; Terriere 1965_b). Contamination of foodstuff by these synergists can be dangerous as they may interfere with the metabolism and thereby the elimination from the body of toxic foreign compounds including pesticide residues. Results in Table 34 show that the above mentioned four compounds inhibit the *p*-nitrotoluene oxidizing enzyme system in rabbit, rat and fly. The fly enzyme inhibition is higher than those observed with rat and rabbit enzyme. Between the four compounds, sulfoxide is the most effective inhibitor in rat and rabbit, whereas in flies it is piperonyl butoxide. Though at 0.5m*M* concentration SKF 525-A and piperonyl butoxide were almost equally effective in inhibiting *p*-nitrotoluene oxidation by rabbit liver, at 0.01m*M* concentration, the later compound was found to be more active when used with fly enzyme. At this

concentration piperonyl butoxide and SKF 525-A inhibit fly enzyme preparation 51 and 26% respectively (Table 34). DMC 1,1-bis-(p-chlorophenyl)ethanol which is an effective synergist for DDT (Summerford et al 1951 a,b ; Speroni 1952 ; March et al 1952) was found to be a poor inhibitor in all species studied. This and the finding that some of the methylenedioxyphenyl compounds tried as DDT synergists are ineffective (Hoskins & Perry 1951 ; March et al 1952) indicate that it is metabolic dehydrochlorination and not oxidation which determines the toxicity of DDT. It was reported that DMC acts as a competitive inhibitor for DDT dehydrochlorinase (Perry et al 1953). The p-nitrotoluene oxidation inhibitory power of the synergists in flies ranked in the following order piperonyl butoxide > sulfoxide > n-propylisome. This order agrees with the previous findings on the in vivo synergistic action and in vitro inhibition of naphthalene hydroxylation by these compounds (Sun & Johnson 1960 ; Terriere et al 1965b). The similar relative reactivities of these compounds in various metabolic oxidations indicate that the inhibition involves a step common to these reactions.

Results in Tables 32 & 33 show that phenobarbitone and 3,4-benzpyrene both induce the the alkyl side chain oxidation in rat

liver. The enzyme was assayed by using two substrates, _p_-nitrotoluene and _iso_-propylbenzene. In each case phenobarbitone was the better enzyme inducer of the two. Similar treatment of locusts did not result in any enzyme stimulation (Table 32). In contrary, little decrease in the enzyme activity was noticed. LD_{50} determinations of IPMC (_m_-isopropylphenyl-_N_-methylcarbamate) in normal and phenobarbitone pretreated locusts did not show any change in their tolerance to IPMC. Oxidation of the alkyl group is an important feature of the metabolism of alkylphenyl _N_-methylcarbamates (Casida 1963 ; Hook & Smith 1967). So induction of the non-specific enzyme system in locusts would increase the LD_{50} of IPMC. Fig. 23 shows that selection of a strain of flies with _p_-nitrotoluene for 6 generations does not have any effect on its _in vitro_ _p_-nitrotoluene oxidizing activity. On selection with DDT for this length of period, this strain was found to build up 10-20 fold resistance (Fine 1963). In a similar attempt Terriere et al (1965a) succeeded in selecting a fly strain for resistance to naphthalene. This strain after selection was reported to have correspondingly high capacity to hydroxylate naphthalene _in vitro_. Inspite of the fact that the importance of metabolism in the survival of resistant insects is now

widely accepted, further evidence is needed to establish a clear relationship in a strain of insects under selection, between the increasing resistance *in vivo* to the compound concerned and stimulation of its rate of metabolism at various stages of the selection period. The dipping technique used here for selection of flies with *p*-nitrotoluene was found satisfactory for various insecticides (Potter 1963). So, the results obtained indicate that either the ability of developing resistance and enzyme induction are not common to all compounds or the effects of selection was too small to be detected due to the use of adult flies for selection. Bruce & Decker (1950) reported that, a strain of flies bred in a media of DDT has a resistance of more than 300 times normal after 10 generations as compared to a resistance of about 5 times normal when the adults alone were selected.

GENERAL DISCUSSION.

General discussion

The metabolism of *p*-nitrotoluene is similar in insects and mammals (Table 3), the major product being *p*-nitrobenzoic acid. Besides *p*-nitrobenzoic acid, the other metabolites identified are *p*-nitrohippuric acid and *p*-aminobenzoic acid. 70-84% of the dose is oxidized and excreted in free and conjugated form following oral administration (200 mg./Kg.) of *p*-nitrotoluene to rat and rabbit (Tables 5 & 6). Rates of oxidation of *p*-nitrotoluene in insects *in vivo* were determined by assaying *p*-nitrobenzoic acid at different time intervals after dosing. The experiments, though not comparable to the excretion studies in mammals, were the only way of following the *in vivo* metabolism rate in insects. The results (Table 4) show the species difference in the rate of oxidation of *p*-nitrotoluene in insects. It has the order fly $>$ mustard beetle $>$ cricket = cockroach (Blatta) $>$ locust $>$ cockroach (Peripleneta) $>$ flour beetle $>$ cotton stainer = caterpillar. The rates of oxidation appear to be considerably lower than those described for other detoxication reactions such as *O*-conjugation (Smith & Turbert 1964) or glutathione conjugations (Cohen *et al* 1964). Since oxidation is frequently the first biochemical attack made

on a foreign molecule ; small differences in the oxidative detoxication is likely to produce significant differences in the biological activity of a molecule having an alkylaryl grouping. With the high doses used in the present work the rates of reaction probably show the maximum oxidative capacity of the insects for aliphatic centres. Although the various insects used would not necessarily show the same relationships at the much lower doses used for routine insecticide treatment, it would appear that different rates of oxidation could be an important factor governing the differential toxicity in these species of insecticides having aliphatic side chain.

Review of literature shows that in the last decade *in vivo* metabolic studies in insects have not been very fruitful. The main reason is, with low doses of toxic compounds that can be given to insects, seperation of the extremely small amounts of metabolites produced, from voluminous tissue material is hardly possible. During the same period impressive advances have been made in the metabolic studies of foreign compounds in mammals by using liver enzyme preparations. The work presented here shows that a similar,

non-specific, foreign-compound-metabolizing, enzyme system, also occurs in insects and it works *in vitro*, though the activity is considerably lower than the mammalian liver enzyme. Locust fat body and fly abdomen 10,000g. supernatant preparations are capable of oxidizing *p*-nitrotoluene to *p*-nitrobenzoic acid. No alcohol or phenolic metabolites were detected. Various characteristics of the locust fat body enzyme were studied using *p*-nitrotoluene as substrate. Both microsomal fraction and the 90,000g. sup. were necessary for the enzyme activity. The optimum substrate concentration was 1.75 m*M* and optimum pH 7.4 (Figs. 7 & 8). Attempts to determine the cofactor requirements of the enzyme were not successful, as storing the preparation under the conditions necessary for dialysis decreased the enzyme activity to a great extent (Table 13). The effect of adding cofactors was tested at different times on the preparations from a number of batches of locusts but no increase in activity was produced (Table 10). The fat body enzyme activity varied with the age of the locust; the late-fifth instar locusts being most active. A similar pattern of changes in the phosphoramidate oxidizing enzyme activity in locust fat body during the life

cycle was observed by Fenwick (1958). In a series of more than 50 experiments the rate of formation of p-nitrobenzoic acid in 5th instar locust fat body preparation ranged from 5-10 μgs./insect/hr. . No oxidation occured if the reaction was carried out in nitrogen atmosphere. Prolonged homogenization gave progressively less active enzyme (Table 12), which was not due to the release of inhibitor(s) from the cells on protracted grinding (Table 20). The 10,000g. sup. enzyme from the locust fat body was inhibited by sedimented fractions made from homogenate and the inhibitory power of the 10,000g. sediment was not destroyed by heating for 15 mins. at 100° (Table 14). The nature of this inhibitor(s) was studied by using rabbit enzyme at a range of substrate concentrations. The results at each substrate concentration indicated an irreversible inhibition in which inhibitor from 1.5 locusts was sufficient to completely inhibit the 10,000g. supernatant enzyme from 0.75g. of rabbit liver in an incubate containing the standard cofactors (Table 16; Fig. 11). A small reduction of activity was found after leaving fat body sediment in contact with rabbit enzyme, but most of the inhibitory activity could be centrifuged away (Table 18) .

Fly abdomen enzyme gives maximum activity when NADP and NAD are incorporated in the incubation mixture (Table 19). The rate of oxidation of p-nitrotoluene by locust fat body and fly abdomen preparations *in vitro* has the same order as observed *in vivo* (Table 4). Fly enzyme is 3-4 times more active than locust enzyme. The oxidation of p-nitrobenzyl alcohol by fly enzyme was measured under the conditions used for the oxidation of p-nitrotoluene and over the concentration range 0.2-1 mM . The rate of oxidation of p-nitrobenzyl alcohol at low concentrations (3μg./g. of flies/hr. at 0.2mM) was considerably lower than the rate of formation of p-nitrobenzoic acid from p-nitrotoluene in the routine assay. The terminal oxidation of alkyl groups to primary alcohols and thence to carboxylic acids has been reported to involve the soluble alcohol dehydrogenase (Gillette 1959) in vertebrate liver but this seems unlikely to have occured in the insect enzymes. The very low concentrations of p-nitrobenzyl alcohol which would have been produced in the oxidation of p-nitrotoluene by fly enzyme would have been oxidised too slowly to account for the measured yields of p-nitrobenzoic acid. Moreover at these slow rates of oxidation some unchanged alcohol would have been expected to occur in the final assays, whereas no

product with the slow colour development characteristic of *p*-nitrobenzyl alcohol (Fig. 2) was detected. It seems more likely that the oxidation of a methyl group to carboxyl in the insect microsomal enzyme preparation was achieved without any intermediate stage appearing free in solution and without the aid of a soluble dehydrogenase.

The insect species which did not give active *p*-nitrotoluene enzyme preparations include cricket, flour beetle, mustard beetle, cotton stainer, caterpillar and houseflies (when whole insects were used). In each case whole insects were used for homogenate preparation. This is likely to introduce a variety of endogenous inhibitors. Homogenates of locust gut and gastric cecae which as intact tissues oxidize *p*-nitrotoluene (Table 7), contained significant amounts of material which inhibited other microsomal oxidation systems (Table 15). Stronger inhibition of the rabbit enzyme was observed with locust fat body 10,000g. sediment and fly homogenates (Tables 16 & 17), which could not be reversed to any significant extent by incorporating a large amount of $NADPH_2$ in the incubation mixture. A variety of natural inhibitors of microsomal oxidations have been reported in different liver preparations (Gillette 1963) including enzymes

hydrolysing the cofactors. The hydrolysis of cofactors by pyridine nucleosidases can probably be excluded as possible inhibitors in locust or fly preparations since the addition of nicotinamide has little effect on the oxidizing activity of the locust or fly enzyme system. Similar observations have been reported by Schonbrod et al (1965). Fig. 15 shows that in whole fly 10,000g. supernatant the nucleotide structure of $NADPH_2$ remains intact and there is adequate glucose-6-phosphate dehydrogenase level in the preparation. It is therefore possible to restore the level of $NADPH_2$ to nearly its initial value by adding glucose-6-phosphate. Similar observations were made with mustard beetle and caterpillar preparations (10,000g.supernatant) (Figs. 12 & 14). Fly abdomen 10,000g. supernatant behaves somewhat differently than whole insect preparation in that it oxidizes $NADPH_2$ more slowly (Fig. 13). Preliminary investigations indicated that fly preparation does not inhibit the oxidation of p-nitrobenzyl alcohol to p-nitrobenzoic acid by rabbit liver enzyme. It appears from these findings that the inhibition of rabbit enzyme p-nitrotoluene oxidizing activity by insect preparations is not due to destruction of $NADPH_2$. The inhibitor(s) probably uncouples the formation of hydroxylating intermediate from $NADPH_2$ oxidation.

The oxidation of p-nitrotoluene by insect fat body preparation supports the analogy which has repeatedly been drawn between the insect fat body and mammalian liver. The biochemical processes which occur in these two tissues are often very similar, and it is not easy to find important differences (Kilby 1963). The other metabolic reactions in locusts which mainly occur in the fat body include glucoside formation (Trivelloni 1960 ; Smith & Turbert 1961) and phosphoramidate oxidation (Fenwick 1958).

The pattern of oxidation of the alkylbenzenes in vitro is qualitatively similar in both rabbit and the two insect species used. The in vitro metabolism in rabbit was found to be similar to earlier in vivo findings (Williams 1959). The major oxidation product of the alkylbenzenes (where this is possible), was the secondary alcohol formed by hydroxylation of the methylene group of the side chain. Preferential oxidation of the methylene group nearest to the benzene ring has also been observed in p-nitroethylbenzene (Chapter II, Sec.D). The oxidation of the penultimate methylene group of a straight chain, e.g. n-butylbenzene, was somewhat slower and occured to about the same extent as the oxidation of the terminal

methyl group to the carboxyl. The results obtained with _n_-butylbenzene show that oxidation is not restricted to α-methylene and ω-1 carbon atoms. The ω-carbon atom is also involved in the metabolism of _n_-butylbenzene (Table 28), the product being 4-phenylbutyric acid. This metabolite was not found in the urine of rabbit dosed with _n_-butylbenzene. It is likely that _in vivo_ 4-phenylbutyric acid is β-oxidized to phenylacetic acid and excreted as its glycine conjugate which accounted for 15-20% of the dose (El Masri _et al_ 1956). Where no α- or penultimate methylene group was available the oxidation of the molecule was much slower and _tert_-butylbenzene was oxidized nearly ten times more slowly than was toluene or _p_-nitrotoluene (Tables 27 & 28) by the same enzyme preparation. The various properties of the rabbit liver alkyl side chain hydroxylase were studied by using _iso_-propylbenzene as the substrate. The results show that it belongs to the group of $NADPH_2$-O_2 dependant microsomal oxidative enzyme system (Table 29 & 30).

The relative rates of metabolic oxidations of alkylbenzenes (Tables 27 & 28) appears to be significantly different from the known pattern of their chemical oxidation. The rates of chemical oxidation of these compounds were satisfactorily explained in terms of the relative reactivities

of the α-methylene group towards free radicals (Kooyman 1951 ; Russel 1956). The reactivities of aromatic hydrocarbons containing an activated methylene group are best explained by considering them to be derivatives of toluene. Substitution of an α-hydrogen atom of toluene by a methyl group considerably increases the reactivity of the other hydrogen atom. Here, the polar effect of the side chain substituent and increased resonance stabilization compliment each other, and this makes the α-methylene group more susceptible to free radical attack. Substitution of a second methyl group to give cumene increases the reactivity of the remaining hydrogen atom still further. On this basis, a free radical (•OH or $\dot{O}_2H$) should oxidize cumene more readily than ethylbenzene and p-nitrotoluene. Also, the rate of oxidation of p-nitrotoluene should be slower than toluene as p-nitro group decreases the reactivity of toluene molecule towards free radicals (Russel 1956). The results show that rabbit enzyme oxidizes p-nitrotoluene more easily than ethylbenzene or cumene (Tables 27 & 28).

The figures quoted for microsomal oxidation of rabbit liver may be taken as a fair guide to the behaviour of these compounds in the intact organism, since the liver is virtually the only organ carrying out these metabolic reactions. The figures quoted for the insects, on the other hand , are probably minimal since the oxidation of p-nitrotoluene occurs

in other organs of the locust and the fat body accounts for only about half of its oxidizing capacity (Table 7). Similar considerations probably apply to the abdomen preparation used. The results in Tables 26, 27 & 28 suggest that there is no great difference between the oxidation rates of alkylbenzenes in whole insects and whole vertebrates if the results are expressed in terms of activity/g. of whole animal. Compared on the basis of activity/g. of tissue, rabbit liver is more efficient than the insect tissues. On the other hand, the difference in oxidizing activity between individual insect or vertebrate species may be considerable (Table 31) and it may be possible to take advantage of this in modifying a toxic molecule to increase its selective action by increasing its susceptibility to oxidation. The differences in the rate of oxidation of the methyl group in insects (Table 4) indioate that there is a possibility of achieving species-selectivity of an insecticide by inserting a small alkyl group in the molecule. On the other hand, it appears from the metabolic studies of higher alkylbenzenes (Tables 26, 27 & 28) that incorporation of a large alkyl group, e.g. n-butyl or tert-butyl group in a molecule may increase the insect to mammalian toxicity ratio as, unlike insects, mammals can still oxidize such groups and thus introduce centres for

more active conjugation reactions. Species differences observed in the alkyl side chain oxidation(Table 31) has the same pattern as was found in aromatic hydroxylation (Williams 1963). This suggests that one common rate limiting step is involved in all hydroxlations involving aromatic ring or alkyl side chain.

The LD_{50} of m-iso-propylphenyl-N-methylcarbamate in the locusts used in this work was about 10 μg. (Table 32) whereas in flies the LD_{50} is 90 μg./gm. (Metcalf & Fukuto 1965). Oxidation of the alkyl groups in alkylphenyl-N-methylcarbamates is a feature of their metabolism along with the oxidation of the N-methyl group (Casida 1963; Hook & Smith 1967). The introduction of a hydroxyl group in the alkyl group would enable conjugation and elimination to take place and would be expected to result in a non-toxic product. It is significant that the enzyme preparations from several strains of fly studied here were about three times more active in oxidizing iso-propylbenzene than those from locusts(Table 26 & 31). The induction of microsomal oxidation enzymes in vertebrates by a variety of compounds is now well known (Remmer 1962 ; Conney & Burns 1962) and evidence of a similar induction in insects has been reported (Morello 1964). Another type

of enzyme induction has been reported by Terriere *et al* (1965a). They showed that treatment of flies with naphthalene for a few generations increase their capacity to hydroxylate naphthalene *in vitro*. No such effect was observed in flies with *p*-nitrotoluene in six generations (Fig. 20). Pretreatment of rats with phenobarbitone or benzpyrene stimulated the rate of oxidation of *p*-nitrotoluene and *iso*-propylbenzene by the liver 10,000g. supernatant but neither compound had any effect on the locust enzyme (Tables 32 & 33). Neither did pretreatment with phenobarbitone have any effect on the toxicity of *m*-isopropylphenyl-*N*-methylcarbamate towards locusts (Table 32). The results in Table 34 show that the increasingly popular practice of using synergists in insecticide formulations (to overcome resistance of insects to insecticides) needs careful examination considering their inhibition of the mammalian liver enzyme system. Contamination of foodstuff by these synergists is likely to inhibit the metabolism and thereby elimination of foreign toxic compounds from the body. Due to the similarities of foreign compound metabolizing enzymes in mammals and insects it is unlikely that any compound or group of compounds will inhibit any particular enzyme in insects but not in mammals. So, the only rational basis of designing safe insecticides still remains in the exploitation of enzymic

differences in insects and mammals than anything else. The most important prerequisite for this principle to be of practical use is better understanding of the fate of foreign compounds in insects.

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Comparative Detoxication

12. THE OXIDATION OF *p*-NITROTOLUENE AND *p*-NITROETHYLBENZENE IN INSECTS*

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Oxidation of a foreign molecule in the body usually yields a more polar metabolite which is more easily excreted and frequently lacks the biological activity of the non-polar precursor (Williams, 1959, pp. 717–739). In vertebrates, when compounds susceptible to oxidation either in an aromatic ring or in an alkyl side chain are metabolized, reaction occurs almost exclusively in the alkyl side chain (Williams, 1959, pp. 197–204). Insects can oxidize the aromatic ring (Smith, 1962) but little information is available on the relative ease of metabolism of alkyl side chains in these invertebrates.

The metabolism of the alkyl side chains is of interest since these are structural features of several useful insecticides such as Diazinon [*OO*-diethyl-*O*-(2-isopropyl-4-methylpyrimidin-5-yl)phosphorothionate], Ruelene [*O*-(4-*tert*.-butyl-2-chlorophenyl)-*N*-methyl-*O*-methylphosphoramidate] and a series of alkylaryl *N*-methylcarbamates (Metcalf, 1955).

We wished therefore to assess the ability of several insect species to oxidize aliphatic groups, and chose *p*-nitrotoluene for initial study as it carried the simplest aliphatic side chain and the oxidation product, *p*-nitrobenzoic acid, is capable of easy detection and estimation. The metabolism of this compound has been well studied in vertebrates (Williams, 1959, pp. 417, 418) and it has recently been used as a substrate for the microsomal oxidation system of rabbit liver (Gillette, 1959).

EXPERIMENTAL

Compounds. *p*-Nitrotoluene, m.p. 48°, *p*-nitrobenzoic acid, m.p. 240°, *p*-nitroethylbenzene, b.p. 245°/760 mm. Hg, n_D^{20} 1·5458, and *p*-nitrophenylacetic acid, m.p. 153°, were commercial samples and free of trace impurities by paper chromatography. 1-(*p*-Nitrophenyl)ethanol, b.p. 162°/16 mm. Hg, and 2-(*p*-nitrophenyl)ethanol, m.p. 56°, were prepared by sodium borohydride reduction of *p*-nitroacetophenone and methyl *p*-nitrophenylacetate respectively.

Insects and dosing. Fifth-instar locust hoppers were obtained from the Anti-Locust Research Centre and kept and dosed as previously described (e.g. Cohen & Smith, 1964; Myers & Smith, 1953). Houseflies were a normal strain obtained from the Rothamsted Experimental Station as pupae and were used within 3 days of emergence. Other insects were also obtained from Rothamsted and dosed immediately.

Compounds were administered at 200 μg./g. by injection in 0·01–0·1 ml. of acetone, except to flies and mustard beetles which were dosed topically at 100 and 200 μg./g. respectively.

Paper chromatography and ionophoresis. Acidic metabolites were identified by ionophoresis as described by Smith (1958). Paper chromatography was carried out by downward displacement and approximate R_F values are quoted in Table 1. Separation of 1- and 2-(*p*-nitrophenyl)-ethanol was achieved by allowing solvent to run off the bottom of the chromatogram in solvent system A or E. Compounds were detected on paper as described by Cohen & Smith (1964).

Estimation of p-*nitrobenzoic acid.* Single large insects or groups weighing approx. 1 g. were homogenized in 5 ml. of water containing 0·5 ml. of 0·5N-NaOH. Protein was removed by addition of 0·5 ml. of 10% (w/v) $ZnSO_4$, and 10 ml. of CCl_4 was added to extract unchanged *p*-nitrotoluene. After shaking and centrifugation, 3 ml. of the supernatant was acidified with 0·2 ml. of 2N-HCl and reduced by shaking for 0·5 min. with 1 ml. of 1% (w/v) zinc amalgam. The *p*-aminobenzoic acid formed was then determined according to the Bratton & Marshall (1939) procedure. In some insects, particularly flies, large blank values were obtained from untreated flies but these were minimized by extracting the azo dye into 5 ml. of amyl alcohol in which the interfering azo-colour was not soluble.

Recovery of *p*-nitrobenzoic acid from aqueous solution was quantitative and recoveries of *p*-nitrobenzoic acid added to 1 g. of locust homogenate were 83·2 S.E.M. ± 1·9 (10).

Estimation of 1-(p-*nitrophenyl)ethanol in enzyme mixtures.* The reaction mixture (5 ml.) was acidified with 3 ml. of 10N-HCl and extracted with 3 × 5 ml. of ether. The ether layer was evaporated to small bulk and transferred to a paper chromatogram and run in solvent system A. The zone corresponding to 1-(*p*-nitrophenyl)ethanol was eluted with methanol and this was determined by the procedure described above for *p*-nitrobenzoic acid. Colour development was allowed to proceed for 3 hr. before measurement and calculation was made by reference to a calibration curve prepared from known amounts of a methanol solution of 1-(*p*-nitrophenyl)ethanol. Recoveries of known amounts

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Table 1. R_F *values of some nitro compounds*

Chromatograms were run on Whatman no. 4 paper until solvent fronts had moved 12 in. Solvents: A, *n*-hexane–di-isopropyl ether (5:1, v/v) run on paper treated with formamide-saturated ether and dried; B, butan-2-one–2N-NH_3 (1:1, v/v); C, butan-1-ol–ammonia (sp.gr. 0·88)–water (4:1:5, by vol.); D, ethanol–water (7:3, v/v) on paper treated with 5% (v/v) olive oil in ether and dried; E, *n*-hexane–di-isopropyl ether (5:1, v/v) on paper treated with a saturated solution of Carbowax 4000 in ether–ethanol (50:1) and dried.

Solvent	A	B	C	D	E
1-(*p*-Nitrophenyl)ethanol	0·20	0·92	0·93	0·92	0·30
2-(*p*-Nitrophenyl)ethanol	0·15	0·91	0·90	0·90	0·25
p-Nitrobenzoic acid	0·01	0·71	0·43	0·81	0·32
p-Nitrophenylacetic acid	0·02	0·60	0·42	0·81	0·34
p-Nitrotoluene	0·92	0·93	0·90	0·72	0·91
p-Nitroethylbenzene	0·91	0·92	0·91	0·70	0·90

of material added to 5 ml. of incubation mixture were 71 S.E.M. ± 3·2 (6).

Tissue incubations. Organs were removed from locusts and incubated in saline as described by Cohen & Smith (1964) at 37° in air. Organs from six hoppers were used in each experiment in 2 ml. of saline to which 1·0 mg. of *p*-nitrotoluene in 0·02 ml. of acetone had been added. After a suitable time, usually 30 min., the mixture was ground in a Potter–Elvehjem homogenizer and the *p*-nitrobenzoic acid estimated as above.

Centrifuged fractions were prepared from 25 locust fat bodies by homogenizing in 20 ml. of 0·25M-sucrose for 1 min. at 0–3°. The homogenate was then centrifuged at 10000*g* for 10 min. at 3° and the supernatant used for enzymic experiments.

RESULTS

Metabolism of p-*nitrobenzoic acid*

Insects (1–5 g.) were dosed as described above and homogenized and deproteinized after 5 hr. The supernatant was concentrated to small bulk *in vacuo* and examined by paper chromatography and ionophoresis. Only *p*-nitrobenzoic acid was detected as a metabolite, except in *Periplaneta* where traces of *p*-aminobenzoic acid were found.

Quantitative assays made on four large insects or 1 g. batches of small insects at intervals of 5 hr. after dosing showed a linear relationship between time and amount of *p*-nitrobenzoic acid formed (Table 2).

Oxidation also occurred in intact isolated tissues of the locust in saline (Table 3), but homogenization of any of these tissues for 1 min. in a Potter–Elvehjem homogenizer gave a preparation devoid of oxidizing power. Active preparations could be made from locust fat-body homogenate, but not from other insects or organs, by centrifugation at 10000*g* for 10 min. at 0–3°.

The 10000*g* supernatant of five locusts in 5 ml. of 0·25M-sucrose was incubated at 37° in air with 1 mg. of *p*-nitrotoluene. Examination of the mixture after deproteinization showed *p*-nitrobenzoic acid to be the only metabolite and quantitative assays showed the rate of formation to be

Table 2. *Rates of formation of* p-*nitrobenzoic acid from insects dosed with* 200 μg. *of* p-*nitrotoluene/g.*

	Formation of *p*-nitrobenzoic acid (μg./g. of insect/hr.)
Locust (*Schistocerca*)	10
Cockroach (*Periplaneta*)	9
Cockroach (*Blatta*)	12
Cricket (*Gryllus*)	12
Flour beetle (*Tenebrio*)	8
Mustard beetle (*Phaedon*)	20
Housefly (*Musca*)	25
Cotton stainer (*Dysdercus*)	5
Caterpillar (*Pieris*)	8

Table 3. *Rates of formation of* p-*nitrobenzoic acid from* p-*nitrotoluene by locust organs* in vitro

For conditions see the text.

	Formation of *p*-nitrobenzoic acid (μg./locust/hr.)		
	Males	Females	Mixed sexes
Fat body	5·1	6·2	7·9
Gastric cecae	2·8	4·8	4·0
Foregut	0·7	1·0	1·8
Midgut	0·7	1·0	1·9
Hindgut	0·3	0·3	0·8
Malpighian tubes	0·3	0·6	1·9
Total	9·9	13·9	18·3

linear up to 90 min. In a series of more than 50 experiments the rate of formation of *p*-nitrobenzoic acid in this type of preparation ranged from 5 to 10 μg./hr./locust and was not affected by addition of NADPH, NADP, NAD, NADH, Mg^{2+} ions, nicotinamide or glucose 6-phosphate.

Assays of the oxidizing activity of the 10000*g* supernatant from the fat body were made at intervals on a large group of fifth-instar hoppers. Rates of 3·6, 4·5, 4·5 and 6·4 μg./hr./locust were obtained 2, 4, 7 and 11 days after collection. Moulting started in the group at 11 days and

enzyme from adults assayed on the fourteenth and eighteenth days had rates of 1·5 and 2 μg. of *p*-nitrobenzoic acid/hr./locust.

Oxidation of p-*nitroethylbenzene*

The 10 000*g* supernatant from 25 locusts was incubated for 60 min. with 5 mg. of *p*-nitroethylbenzene at 37° in air. After acidification with 3 ml. of 10 N-hydrochloric acid and extraction with ether, the ether extract was examined by paper chromatography and ionophoresis. The major metabolite found was 1-(*p*-nitrophenyl)ethanol together with a trace of *p*-nitrophenylacetic acid. No 2-(*p*-nitrophenyl)ethanol was found.

In quantitative assays with 10 000*g* supernatant from five locusts the rate of formation of the alcohol was 2 μg./locust/hr.

DISCUSSION

The oxidative metabolism of *p*-nitrotoluene is similar in insects and vertebrates, the major product being *p*-nitrobenzoic acid. Oxidation of the ethyl side chain in *p*-nitroethylbenzene occurred mainly at the α-methylenic group, and similarly methylphenylcarbinol is the main oxidation product of ethylbenzene in rabbits (Smith, Smithies & Williams, 1954). The locust oxidizing system is also similar to that of rabbit in that it can be located in the 10 000*g* supernatant of fat body or liver. Some activity was also found in the gut of the insect and good agreement was found between rates of oxidation in whole insect, isolated organ and 10 000*g* supernatant from the fat body.

With the high doses used in the present work the rate of oxidation of *p*-nitrotoluene was constant over the experimental period and the values quoted in Table 2 probably show the maximum oxidative capacity of the insects for aliphatic centres. These would not necessarily show the same ratios with the much lower doses absorbed if insecticides were being oxidized, but they suggest that different rates of oxidation could be a factor in different rates of detoxication of insecticides having aliphatic side chains.

The rates of oxidation found are considerably lower than has been found for other detoxication reactions such as *O*-conjugation (Smith & Turbert, 1964) or glutathione conjugations (Cohen, Smith & Turbert, 1964), but oxidation is frequently the first biochemical attack made on a foreign molecule, and in molecules having an alkylaryl grouping small differences in the oxidative detoxication could be the basis of differential toxicity.

Oxidation of a methylene group adjacent to an aromatic ring is a feature of the metabolism of DDT [2,2,2-trichloro-1,1-di-(*p*-chlorophenyl)ethane] in some insects. This α-methylenic oxidation yields Kelthane [2,2,2-trichloro-1,1-di-(*p*-chlorophenyl)ethanol] as the metabolite and an intensification of this reaction under DDT pressure can give rise to insecticide-resistant strains (Agosin, Michaeli Miskus, Nagasawa & Hoskins, 1961; Tsukamoto, 1959). The present results suggest that it would be worth while to investigate the possibility of alkyl-chain oxidation in other insecticides containing alkylaryl structures.

SUMMARY

1. *p*-Nitrotoluene was converted into *p*-nitrobenzoic acid in nine species of insects.

2. The most rapid oxidation occurred in houseflies.

3. In isolated locust organs, most oxidation occurred in fat body and gut.

4. *p*-Nitroethylbenzene was converted into 1-(*p*-nitrophenyl)ethanol by locust fat-body homogenates; traces of *p*-nitrophenylacetic acid were also formed.

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Inhibitors of Microsomal Oxidations in Insect Homogenates

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1. Homogenates of insect tissues were assayed for enzymes capable of oxidizing *p*-nitrotoluene to *p*-nitrobenzoic acid. 2. Locust fat-body homogenate 10000g supernatant was an effective enzyme and required no added cofactors. 3. Homogenates of other insects or locust organs and 10000g sediment from locust fat-body were not active and inhibited microsomal oxidations carried out by locust fat-body or rabbit liver enzyme. 4. Inhibitory power was high in homogenates of whole flies and of fly heads or thoraces. 5. Inhibition appeared to involve both irreversible inactivation of enzyme and the removal of essential cofactors.

In vertebrates the methyl groups in toluene and substituted toluenes are readily oxidized to the corresponding aromatic acids and the enzymology of this process has been studied by Gillette (1959) with liver microsomal enzyme. Alkyl side chains of barbiturates and other drugs are also oxidized by liver microsomal enzymes (Cooper & Brodie, 1957). Some preliminary enzymic studies with locust fat-body homogenate as the source of the enzyme were made (Chakraborty & Smith, 1964) and similar centrifugal preparations have been used in the study of the metabolism of naphthalene and a variety of insecticides (Arias & Terriere, 1962; Agosin, Michaeli, Miskus, Nagasawa & Hoskins, 1961; Fenwick, 1958; Nagatsugawa & Dahm, 1962, 1965). In this work the activity of the insect enzymes has usually been weaker than similar preparations from rat or rabbit liver and we have therefore examined some insect preparations for endogenous inhibitors that might account for the low activity.

EXPERIMENTAL

Insects. Fifth-instar locusts (*Schistocerca gregaria*) were from the Anti-Locust Research Centre, London, and enzymes made from mid-instar specimens. Other insects from previously used strains (Chakraborty & Smith, 1964) were obtained from the Insecticide Department, Rothamsted, Herts. Flies, unless otherwise stated, were used within 2 days of emergence as adults. At a later stage of the work an insecticide-sensitive strain of housefly was obtained from Wallaceville Animal Research Station, New Zealand, and reared in the laboratory.

Estimation of p-*nitrobenzoic acid.* This was carried out by the method used by Chakraborty & Smith (1964).

Estimation of p-*acetamidobenzoic acid.* This was carried out by the technique described by Hook & Smith (1967).

Preparation of vertebrate enzymes. Liver from rabbit or mouse was homogenized in 4vol. of 0·25M-sucrose with a Teflon pestle in a glass Potter–Elvehjem type grinder. The homogenate was centrifuged at 10000g for 10min. and the supernatant ('10000g supernatant') used as enzyme. All operations were carried out below 3°. Incubations were in 50ml. beakers in a shaking water bath in air at 37° and in a total volume of 5ml. containing, in most experiments, enzyme equivalent to 0·5g. of rabbit liver or 0·25g. of mouse liver. Rabbit liver incubations were fortified with 0·5mg. each of $NADPH_2$ and NAD and 6mg. each of $MgCl_2$ and nicotinamide. Mouse enzyme was fortified with 0·2mg. each of NADP and NAD, 5mg. of glucose 6-phosphate, 1mg. of $MgCl_2$ and 6mg. of nicotinamide. Each 5ml. of incubation mixture was at pH 7·4 and contained 1ml. of 0·05M-tris buffer, pH 7·4.

p-Nitrotoluene (1mg.) or *p*-acetamidotoluene (0·5mg.) was added to the incubation mixtures in 0·05ml. of acetone to start the reaction. Reaction rates were linear with time for up to 1hr. and in inhibition experiments incubations were stopped after 0·5hr.

Insect homogenates. These were normally prepared in 0·25M-sucrose, as described above, except in the few cases noted below where 0·15M-KCl was used. Where centrifugal fractions were used the whole homogenate was centrifuged at 3° at 10000g for 10min. to give a '10000g supernatant' and '10000g sediment' fraction.

Clear-cut separation of a 'nuclei and debris' fraction by centrifugation at 900g was difficult because of the nature of the sediment and in most cases the 10000g sediment contained this debris as well as the 'mitochondrial fraction'. 'Microsomal' pellets were prepared by sedimentation of the 10000g supernatant at 90000g for 1hr. ('90000g sediment') for some experiments.

Conditions for the use of locust preparations as an oxidizing enzyme were those used by Chakraborty & Smith (1964) in which *p*-nitrotoluene was used as substrate.

Oxidation of $NADPH_2$. This was followed by direct spectrophotometric measurement at 340mμ in either the Beckman DB spectrophotometer or the recording Unicam SP.700. Concentrated fly homogenates rapidly became

anaerobic below the surface layer so that the amount of homogenate had to be restricted to maintain the aerobic conditions in the cuvette. Normally a concentration equivalent to 2 flies/ml. was suitable. The reaction was started by adding 0·5mg. of $NADPH_2$ in 0·01ml. of water to the 0·5cm. cuvette that contained the enzyme diluted into 0·05M-tris buffer, pH 7·4, to give a total volume of 1·5ml.

RESULTS

Preparation and properties of the locust fat-body enzyme. Homogenates of locust fat body usually showed a low capacity to convert *p*-nitrotoluene into *p*-nitrobenzoic acid, which was not increased by fortification with 0·2mg. of oxidized or reduced NAD or NADP or by addition of 6mg. of nicotinamide or magnesium chloride to the standard 5ml. of reaction mixture.

The locust homogenate was similar to vertebrate liver enzyme (Gillette, 1959) in that centrifugation at approx. 10000*g* for 10min. yielded an active enzyme in the supernatant and the 'microsomal' sediment of this supernatant at 90000*g* for 1hr. was inactive unless combined with 90000*g* supernatant. Samples of fat-body homogenate prepared as described above were centrifuged at various *g* values for 10min. and activities of the supernatants plotted graphically. The curve showed a broad maximum at the region 10000–12000*g* and 10000*g* for 10min. was used as a routine thereafter.

The effect of adding cofactors was tested at different times on a variety of batches of locusts but no increase in activity was produced in the locust 10000*g* supernatants by the addition of 0·2mg. of NAD, $NADH_2$ or $NADPH_2$ or 6mg. of either nicotinamide or magnesium chloride. The activity of this preparation with *p*-nitrotoluene as substrate was in the range 5–10mμmoles of *p*-nitrobenzoic acid/min./locust (Chakraborty & Smith, 1964).

The enzymic reaction of preparations containing enzyme from five locusts' fat-bodies in 5ml. of incubation mixture was linear with time for 1hr. and measurements were therefore carried out for 0·5hr.

The optimum substrate concentration for *p*-nitrotoluene was 1·5mM and a double-reciprocal plot of substrate concentration and reaction velocity gave an apparent K_m value 6×10^{-4}M.

When the pH was varied in tris buffer and reaction velocity measured between pH 6·5 and 8·5, a narrow pH–activity curve was obtained with a maximum at pH 7·4.

The enzyme lost about a fifth of its activity when stored at 0° for 16hr. or if left at room temperature for 3hr. When the homogenate was left at room temperature for 3hr. before centrifugation no activity was found in the 10000*g* supernatant.

No oxidation occurred if the reaction was carried out in a nitrogen atmosphere and incorporation of mM-piperonylbutoxide or SKF 525A (diethylaminoethyldiphenylpropyl acetate) into the standard reaction mixture decreased the activity by 40%.

Inhibition by sedimentable fractions of locust fat-body. The 10000*g* supernatant enzyme from locust fat-body was inhibited by sedimented fractions made from crude homogenate and the inhibitory power of the 10000*g* sediment was not destroyed by heating in 0·25M-sucrose solution for 15min. at 100° (Table 1).

The nature of the inhibition by fat-body 10000*g* sediment, resuspended in 0·05M-sucrose, was tested with rabbit enzyme prepared as described above and inhibitor equivalent to sediment from 0·5–1·25 locusts' fat-bodies at a range of substrate concentrations. The results at each substrate concentration indicated an irreversible inhibition in which inhibitor from 1·5 locusts was sufficient to completely inhibit the 10000*g* supernatant enzyme from 0·75g. of rabbit liver in an incubation mixture containing the standard cofactors (Fig. 1).

Attempts were made to measure the rate of the inhibition by leaving resuspended locust fat-body 10000*g* sediment in contact with rabbit enzyme at 0° for various periods, centrifuging off the inhibitor sediment and assaying the remaining supernatant. A small decrease in activity was found after leaving fat-body sediment in contact with rabbit enzyme,

Table 1. *Inhibition of locust fat-body enzyme by sedimented fractions*

Supernatant (10000*g* for 10min.) equivalent to fat bodies of six locusts was incubated with 1mg. of *p*-nitrotoluene at 37° in a total volume of 5ml. in air at pH 7·4. Sediments equivalent to three locusts' fat bodies were used as inhibitors.

Inhibitor added	*p*-Nitrobenzoic acid formed in 0·5hr. (μg.)			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
No inhibitor	36	31	12	36
10000*g* sediment added after incubation	—	—	12	—
1000*g* sediment	6·4	—	—	0
10000*g* sediment	1·5	10·4	2·8	0
10000*g* sediment heated for 15min. at 100°	—	10·4	4·8	—
Washings of 10000*g* sediment after heating for 15min. at 100°	—	—	10·4	—

but most of the inhibitory activity could be centrifuged away (Table 2). The sediment did not appear to interfere with the determination of *p*-nitrobenzoic acid (Tables 1 and 2).

Prolonged homogenization of fat bodies (2min.) gave a progressively less active enzyme, activity being zero after 6min., and the possibility of an inhibitor being released from the cells on protracted grinding was therefore considered. A sample of 10000*g* sediment equivalent to 0·5 locust fat body from a preparation that had been homogenized for 0·5min. was resuspended in sucrose and ground at 0° for various times. This quantity of locust inhibitor decreased the rate of oxidation of a rabbit enzyme preparation from 144 mμmoles to 100 mμ-moles of *p*-nitrobenzoic acid/min., but no increased inhibition was found if the homogenization was prolonged to 6min. and no water-soluble inhibitor was released.

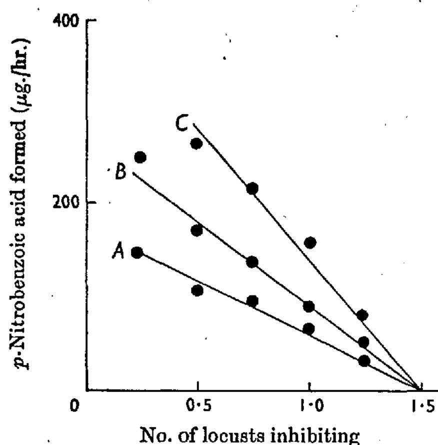

Fig. 1. Inhibition of rabbit enzyme by locust fat-body 10000*g* sediment. Each incubation mixture contained 10000*g* supernatant from a homogenate of 0·75g. of rabbit liver with the addition of 10000*g* sediment from locust fat-body homogenate equivalent to the stated number of locusts in a total volume of 5ml. containing 0·5mg. of $NADPH_2$, 0·5mg. of NAD, 2mg. of $MgCl_2$, 3mg. of nicotinamide and 0·5ml. of 0·1M-tris buffer, pH 7·4. Incubations were carried out at 37° in air, with shaking for 0·5hr. Curves *A*, *B* and *C* had 0·25, 0·5 and 1·0mg. respectively of *p*-nitrotoluene added to 5ml. of incubation mixture and oxidation of *p*-nitrotoluene to *p*-nitrobenzoic acid was measured as described in the text.

In experiments of this type and in those shown in Table 2 the locust sediment rendered only a portion of the rabbit activity ineffective and could be centrifuged away, but if assays were carried out while the suspended sediment was present, along with the added cofactors, no oxidation occurred.

Attempts to prepare an oxidizing enzyme from locust gut were not successful though *in vitro* locust gut forms more *p*-nitrobenzoic acid from *p*-nitrotoluene than the fat body (Chakraborty & Smith, 1964). Assays were therefore made with rabbit enzyme with the equivalent of homogenized parts of the gut of one locust added. Whole homogenate of mid-gut was markedly inhibitory (Table 3).

Inhibition of vertebrate enzyme by other insects. The 10000*g* supernatant equivalent to 4g. of flies from homogenates made in either 0·25M-sucrose or 0·15M-potassium chloride had no oxidizing activity with *p*-nitro- or *p*-acetamido-toluene as substrate when fortified with the amounts of NADP, NAD, magnesium chloride and nicotinamide used with rabbit enzyme. Fly microsomes (90000*g* sediment) from 4g. of flies were also inactive when 90000*g* supernatant from 0·5g. of rabbit liver was added with the cofactors used in the rabbit assay system.

Table 2. *Inhibition of rabbit microsomal oxidation by insect preparations*

Inhibitors were added to the enzyme at 0° and centrifuged off at 10000*g* after the stated contact times. Cofactors were then added and assays carried out at 37° for 0·5hr. as described in the text with *p*-nitrotoluene as substrate. Inhibitors were 0·25M-sucrose homogenates of: A, fat body from 0·5g. of cockroach; B, fat body from 1g. of cockroach; C and D, 10000*g* sediment from fat body of 0·5g. of locust; E, 0·25g. of mustard beetles; F, 0·5g. of mustard beetles; G, 0·5g. of cabbage caterpillar.

	Contact time (min.)	*p*-Nitrobenzoic acid formed in 0·5hr. (μg.)						
	Expt. ...	A	B	C	D	E	F	G
No inhibitors	—	81	81	79	48	81	81	115
Inhibitor added after incubation	—	88	—	—	46	81	—	—
Inhibitor not removed before incubation	—	42	—	—	0	44	—	109
Inhibitor alone	—	0	0	0	0	0	0	0
Inhibitor centrifuged off before assay	4	81	100	58	28	76	64	—
	14	80	102	51	34	73	64	—
	24	75	100	51	31	70	66	—
	34	—	—	51	—	—	—	—

Whole-fly homogenates were also effective inhibitors of the rabbit oxidizing enzyme (Table 4), and the inhibition by homogenate of 0·05g. or 0·075g. of flies was only offset by the addition of very large amounts of $NADPH_2$ or by the use of a regenerating system for $NADPH_2$.

Cockroach fat body, mustard beetles and caterpillars (Table 2) contained much less inhibitor than flies but nevertheless their 10000*g* supernatants were inactive when tested for *p*-nitrotoluene-oxidizing power.

The inhibition by fly preparations of mouse liver oxidation of 2mM-*p*-acetamidotoluene was measured with both whole-fly homogenate and fly 10000*g* supernatant. The results followed the pattern of the locust inhibition (Fig. 1) with a negative linear relation between amount of inhibitor and reaction velocity, and in three experiments with whole-fly homogenates complete inhibition of the standard mouse liver preparation was reached with 10 flies. When 10000*g* supernatant from a 0·15M-potassium chloride homogenate of houseflies was used as source of inhibitor in eight similar experiments, complete inhibition of the mouse enzyme was reached with material from 40–50 flies per incubation. Distribution of inhibitory activity in flies is shown in Table 5.

The stability of $NADPH_2$ in some diluted fly homogenates was measured (Tables 6 and 7). Loss of $NADPH_2$ was rapid in homogenates containing fly heads and the loss was stimulated by addition of menadione or catechol. However, when glucose 6-phosphate (1mg./ml. of reaction mixture) was added the extinction at 340mμ was restored to its initial level. In every experiment the rate of recovery of the extinction was higher than its fall had been and it was concluded that this amount of glucose 6-phosphate was adequate to maintain the $NADPH_2$ in its reduced form even in the presence of polyphenols or quinones.

DISCUSSION

The oxidation by an insect of an insecticide is often of primary importance in deciding its effectiveness since this reaction can lead to either activation

Table 3. *Inhibition of rabbit oxidation system by homogenates of locust organs*

Whole homogenates equivalent to organs of one locust in 0·25M-sucrose were incorporated in the standard rabbit enzyme mixture and the oxidation of *p*-nitrotoluene was measured as described in the text.

Addition to rabbit enzyme	*p*-Nitrobenzoic acid formed in 0·5hr. (μg.)
No addition	123
Fat-body 10000*g* sediment	83
Mid-gut homogenate	67
Hind-gut homogenate	125
Fore-gut homogenate	105
Gastric-cecae homogenate	147
Malpighian-tube homogenate	127

Table 5. *Inhibition of mouse liver enzyme by housefly homogenates*

Mouse liver enzyme and cofactors were incubated for 0·5hr. as described in the text with *p*-acetamidotoluene as substrate. The 10000*g* supernatant of fly organs (equivalent to 20 flies) was incorporated in the standard mouse assay mixture.

	p-Acetamidobenzoic acid formed (μg.)	
Age of flies ...	2 days	8 days
Additions to mouse enzyme		
No addition	136	136
Fly-head 10000*g* supernatant	98	103
Fly-thorax 10000*g* supernatant	68	40
Fly-abdomen 10000*g* supernatant	94	65

Table 4. *Inhibition of rabbit oxidation enzyme by housefly preparations*

Fly homogenates were incorporated in the standard rabbit assay preparation along with the extra cofactors quoted and the mixtures assayed with *p*-nitrotoluene as described in the text.

Addition to rabbit system	*p*-Nitrobenzoic acid formed in 0·5hr. (μg.)			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
No addition	133	115	103	103
Fly homogenate (0·25g. of fly)	0	—	—	—
Fly 90000*g* supernatant (0·25g. of fly)	74	—	—	—
Fly homogenate (0·1g. of fly)	—	0	—	—
Fly homogenate (0·075g. of fly)	—	—	71	76
Fly homogenate (0·075g. of fly)+$NADPH_2$ (8mg.)	—	—	83	74
Fly homogenate (0·05g. of fly)	—	62	—	—
Fly homogenate (0·05g. of fly)+NADP (0·2mg.)+glucose 6-phosphate (5mg.)	—	81	—	—
Fly homogenate (0·01g. of fly)	—	117	—	—

Table 6. *Oxidation of $NADPH_2$ in housefly homogenates*

Cuvettes contained 10000*g* supernatant equivalent to 2 flies/ml. in 0·05M-tris buffer, pH 7·4. Reactions were started by adding 0·5mg. of $NADPH_2$/ml. to the test cuvette. Whole homogenate was used in Expts. 2, 5 and 7 and 10000*g* supernatant in others.

			$10^3 \times \Delta E_{340}^{1\,cm.}$ in 10min.		
Expt. no.	Age of flies (days after emergence)	Homogenate ...	Fly heads	Fly thoraces	Fly abdomens
1	—		123	18	10
2	12		112	36	17
3	2		310	0	0
4	3		113	0	0
				Fly thoraces + abdomens	
5	1		93	7	
6	4		93	0	
7	1		98	0	

Table 7. *Stimulation of $NADPH_2$ oxidation in fly homogenates*

The 12000*g* supernatant of whole flies used contained the equivalent of 2 flies/ml. in 0·05M-tris buffer, pH 7·4.

Addition to cuvette	$10^3 \times \Delta E_{340}^{1\,cm.}$/10min.
None	26, 25, 27·6
Cytochrome *c* (50μM)	95, 93·5, 95
Haemoglobin (50μM)	22, 20, 25
Menadione (1mM)	877, 877
Catechol (1mM)	750, 700, 730

or deactivation of the molecule. In practical insecticides the more active detoxication mechanisms such as glucosidation can usually act only after the oxidation enzymes have introduced a suitable functional group capable of conjugation.

The study of the feeble oxidation systems in insects is difficult, particularly when low doses of toxic compounds are used, and the use of 'microsomal' enzyme preparations rather than whole insects could ease the problem of separating metabolites from irrelevant tissue material. Insect 'microsomal' preparations are usually made from homogenates of whole insects and are therefore more complex than vertebrate liver preparations. The diversity of tissues in the homogenate may introduce a variety of endogenous inhibitors and it is significant that, of nine species used to investigate the oxidation of *p*-nitrotoluene *in vivo*, a satisfactory enzyme extract was only obtained from locusts, where it was possible to work with a fat-body homogenate rather than one made from whole insects (Chakraborty & Smith, 1964). Homogenates of locust gut, which oxidizes *p*-nitrotoluene as actively *in vitro* as the fat body, were inactive and contained significant amounts of material that inhibited other microsomal oxidation systems.

A variety of natural inhibitors of microsomal oxidations have been reported in different liver preparations (Gillette, 1963) including enzymes hydrolysing the cofactors, but the nicotinamide nucleosidases can probably be excluded as possible inhibitors in locust or fly preparations since the addition of nicotinamide has little effect on the oxidizing activity of locust or fly microsomal enzymes (Chakraborty & Smith, 1964; Schonbrod, Philleo & Terriere, 1965). In the $NADPH_2$ oxidation experiments described above it was possible to restore the extinction at 340mμ to its initial value by adding glucose 6-phosphate 1hr. after it had fallen to zero. This also suggests that the cofactor had not been attacked by any nucleosidase.

At least two inhibitory mechanisms appear to be acting in insect 10000*g* sediment. A heat-stable factor irreversibly inactivated a proportion of rabbit enzyme activity, and a second insoluble factor that further decreased the activity if left in the assay mixture could be removed by centrifugation (Tables 1 and 2). It is possible that the second mechanism removed some cofactor from the assay system.

The removal of $NADPH_2$ by oxidation is a factor in this facet of the fly inhibition and this oxidation was especially active in fly heads. The $NADPH_2$ oxidase was stimulated by cytochrome *c* and menadione and the presence of quinone-linked $NADPH_2$ oxidation is therefore a possible cause of loss of activity in insect preparations that may contain polyphenols and their oxidases. However, fly homogenates have adequate glucose 6-phosphate-dehydrogenase activities and the incorporation of glucose 6-phosphate into the enzyme mixture maintained the $NADPH_2$ in the reduced form even when high menadione-linked oxidation was present.

Fly head and thorax contained substantial amounts of inhibitor (Table 5) and the fat body,

which is a probable source of *p*-nitrotoluene-oxidizing enzyme in flies, was mainly in the abdomen. Homogenates prepared from fly abdomens had therefore a more favourable ratio of enzyme to inhibitor. For this reason in subsequent work with flies the oxidation enzyme has been prepared from abdomen homogenates and an $NADPH_2$-regenerating system has been used in preference to the addition of $NADPH_2$ as cofactor (Chakraborty & Smith, 1967).

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Enzymic Oxidation of some Alkylbenzenes in Insects and Vertebrates

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1. Oxidation rates of alkylbenzenes have been measured in 10 000*g* supernatants of vertebrate livers, locust fat bodies and housefly abdomens. 2. Activity per g. of insect was greater in fly than locust preparations but both were of the same order as a range of vertebrate species. 3. Methyl groups of toluene and *p*-nitrotoluene were oxidized more rapidly than the side chains of higher homologues. 4. In the higher homologues hydroxylation occurred most readily at the α-methylene group and less readily at penultimate methylene and terminal methyl groups. 5. Oxidations in both vertebrates and insects were inhibited by piperonylbutoxide and similar synergists. 6. Oxidation activity was stimulated by pretreatment of rats, but not locusts, with phenobarbitone or 3,4-benzopyrene.

Selectivity of action is a desirable property of insecticides and the selective destruction of the insecticide by detoxication mechanisms in different species is one way of achieving this effect. The best established examples of this are found among those organophosphates that contain carboxy ester or amide groups (Kreuger & O'Brien, 1959; Uchida, Dauterman & O'Brien, 1964; Smith, 1964) where low mammalian toxicity results from the more extensive hydrolysis of ester or amide links in these species. The term 'selectophore' has been applied to those organic functional groups that offer a point of selective attack for a detoxication mechanism and a knowledge of such groups would be of value in the design of insecticides. So far, little attempt has been made to find selectophoric groups that depend for their action on metabolic processes other than hydrolysis.

Some vertebrates can oxidize the alkyl chain in alkylbenzenes with great ease (El Masry, Smith & Williams, 1956) and it was shown that a range of insects varied considerably in their ability to oxidize the methyl group in nitrotoluene (Chakraborty & Smith, 1964). This suggested to us that methyl and other alkyl groups might function as selectophores, and since little is known of their fate in insects we have studied the metabolism of a series of alkylbenzenes in insects and enzyme preparations and have compared these results with those from similar experiments with vertebrate material.

EXPERIMENTAL

Reference compounds. Alkylbenzenes were commercial samples that contained variable amounts of alcohols and ketones. They were distilled and left over powdered sodium for a week and then redistilled through an efficient fractionating column with a variable reflux head. Before use in an incubation mixture they were checked by gas–liquid chromatography for absence of alcohols or ketones.

Aromatic alcohols and acids were redistilled commercial samples used previously or were prepared by methods then described (El Masry *et al.* 1956; Smith, Smithies & Williams, 1954; Robinson, Smith & Williams, 1955; Robinson & Williams, 1955) and were homogeneous by gas–liquid chromatography.

Piperonylbutoxide (3,4-methylenedioxy-6-propylbenzyl-*n*-butyl diethylene glycol ether), sulphoxide [1-methyl-2-(3,4-methylenedioxyphenyl)ethyloctyl sulphoxide], *n*-propylisome (di-*n*-propyl 6,7-methylenedioxy-3-methyl-1,2,3,4-tetrahydronaphthalene-1,2-dicarboxylate), sesamex [2-(3,4-methylenedioxyphenoxy)-3,6,9-trioxaundecane] and D.M.C. (4,4′-dichloro-α-methylbenzhydrol) were a gift from Dr B. C. Fine; SKF525A (diethylaminoethyldiphenyl-*n*-propyl acetate hydrochloride) was a sample provided by Professor R. T. Williams.

Insects. Locusts (*Schistocerca gregaria*) were obtained as fifth-instar hoppers from the Anti-Locust Research Centre and enzymes were prepared from mid-instar specimens. Resistant strains of houseflies were obtained from the London School of Hygiene and Tropical Medicine and a non-resistant strain from the Rothamsted Experimental Station. Houseflies were obtained as pupae and after emergence the adults were fed on sucrose and water for periods of up to a week. They were normally used for enzyme preparation the day after emergence.

Vertebrate species. These were obtained from the same sources as those described by Creaven, Parke & Williams (1965).

Preparation of enzymes. Fat bodies from fifth-instar locusts were homogenized and centrifuged to give a 10000*g* supernatant as previously described (Chakraborty & Smith, 1964). When *p*-nitrotoluene or toluene was substrate, enzyme from 5–10 locusts was used in a total

volume of 5ml. of incubation mixture. For higher alkylbenzenes, fat-body enzyme from 30–50 locusts was necessary and a total incubation solution of 20–25ml. was used.

Abdomens of adult houseflies were similarly homogenized in a glass Potter–Elvehjem homogenizer with Teflon pestle and centrifuged at 0–3° for 10min. at 10000*g*. When *p*-nitrotoluene was used as substrate, the supernatant fraction from 2g. of flies in 5ml. of incubation mixture was used, and when higher alkylbenzenes were substrates, abdomens from 5–15g. of flies were used in an incubation volume of 20–25ml.

In fly enzyme experiments each 5ml. of incubation mixture contained 0·5mg. of NADP, 0·5mg. of NAD, 2mg. of glucose 6-phosphate, 0·5mg. of $MgCl_2$, 1mg. of nicotinamide and 0·5ml. of 0·1M-tris buffer, pH7·4.

Vertebrate livers were homogenized in 5vol. of 0·25M-sucrose at 0° and centrifuged for 10min. at 10000*g*. Incubations contained the supernatant fraction equivalent to 1g. of liver in a total volume of 5ml. containing 0·5mg. of NADP, 0·5mg. of NAD, 2mg. of glucose 6-phosphate, 2mg. of $MgCl_2$, 3mg. of nicotinamide and 0·5ml. of 0·1M-tris buffer, pH7·4.

Substrates, 1mg. in 0·05ml. of acetone, were added to test and enzyme-free control mixtures to start the reaction. Controls were also prepared from separately incubated enzymes that were mixed with the other components immediately before assay. Incubations of 5ml. samples were for 1hr. in 50ml. beakers in a shaking water bath at 37° in air. Other incubations requiring 25–30ml. volumes were in 250ml. beakers. The oxidation rate, per g. of liver or per insect, of the standard substrate, *p*-nitrotoluene, was the same whether measured in a 5ml. or 25ml. incubation mixture, and oxidation rates were linear with time for at least 60min. but routine assays were normally carried out for 30min.

Extraction of metabolites of alkylbenzenes from incubation mixtures. Incubation mixtures, 5–25ml., were acidified with 1·5ml. of 10N-H_2SO_4 to each 5ml. and extracted with 3×7ml. of ether. After drying over Na_2SO_4 the ether was evaporated at 0° in a stream of N_2. The residue was dissolved in 100μl. of *n*-hexane and 2–5μl. portions were used for gas–liquid chromatography (see below).

In the determination of acidic metabolites the incubation mixture was extracted with ether as above and acidic compounds were transferred into 5ml. of 0·5N-NaOH, which was acidified with 2N-HCl and extracted with 2×5ml. of ether. The ether was dried and evaporated as above and treated with a freshly distilled ethereal solution of diazomethane, to give a permanent yellow colour (approx. 1ml.). After 1hr. this solution was evaporated in a stream of N_2 at 0° and the residue dissolved in 100μl. of *n*-hexane for gas–liquid chromatography.

Recovery experiments in which 5–50μg. of the different alcohols or acids studied were added to 15–25ml. volumes of 10000*g* supernatants from liver, locust fat body or houseflies, showed a constant loss of 5–7μg. from each amount of alcohol or methyl ester added. This loss occurred during the evaporation of the ether extract and was more significant at higher temperatures. The volume of ether were therefore standardized as above and evaporation was carried out at 0°. Under these conditions linear correlation curves relating alcohol or acid added and amounts recovered were obtained. By using these calibrations and standardized extraction conditions the recovery of known quantities of alcohols or acids from incubation mixtures were 100S.E.M. ±1·5 (30) over the range 10–50μg. Quantities of methyl esters or alcohols smaller than 5μg. were not detectable.

Gas–liquid chromatography. Copper tubes, 6ft. in length and ⅛in. internal diam., packed with stationary phases on 100-mesh Celite were used. Nitrogen was used as carrier with flow rate of 100ml./min. and compounds were detected by flame ionization. Stationary phases and temperatures of operation are shown along with retention times in Table 1. With the apparatus used, the minimum detectable quantity of an alcohol or ester was 0·01μg. and normally 2μl. volumes of *n*-hexane solutions were used in injections.

Quantitative measurements were made by comparison with the peak height from an injection of a standard solution of the compound being measured, and the values were corrected for losses on extraction by reference to the appropriate correlation curve.

Identification of metabolites was made on the columns (Table 1) by comparison of the retention times of metabolites and reference compounds and by demonstration of identity of retention times in mixed chromatograms. Phenols had high retention times on the columns used and no peaks were found in the experimental extracts in this region.

Determination of p-*nitrobenzoic acid.* The method previously described was used (Chakraborty & Smith, 1964). In experiments where *p*-nitrobenzyl alcohol was the substrate, ether was used instead of CCl_4 to extract excess of substrate and the diazo colours were measured within 10min. of coupling. *p*-Nitrobenzyl alcohol gave the same intensity of diazo colour in the procedure for assaying *p*-nitrobenzoic acid but the speed of azo coupling was

Table 1. *Gas–liquid chromatography of metabolites of alkylbenzenes*

Retention times are given in minutes on A: 3% (w/w) polyethylene glycol adipate on Celite, 6ft., 100°; B: 3% (w/w) Carbowax 4000 on Celite, 6ft., 125°; C: 2% (w/w) Carbowax 4000 on Celite, 6ft., 120°; D: 1·5% (w/w) Carbowax 4000 on Celite, 6ft., 90°. The flow rate of N_2 was 100ml./min.

	A	B	C	D
Benzyl alcohol	—	—	4·5	—
Methyl benzoate	2·0	—	1·5	—
Acetophenone	—	—	2·0	—
1-Phenylethanol	2·5	3·0	3·5	—
2-Phenylethanol	5·0	4·5	6·0	—
Methyl phenylacetate	3·5	—	3·0	—
p-Ethylphenol	—	—	20·0	—
1-Phenylpropan-1-ol	4·5	5·0	—	5·5
1-Phenylpropan-2-ol	2·0	3·8	—	5·5
1-Phenylpropan-3-ol	9·0	9·5	—	11·0
2-Phenylpropan-1-ol	5·0	5·5	—	7·5
2-Phenylpropan-2-ol	2·5	2·3	—	3·0
Methyl phenylpropionate	6·0	—	—	—
Methyl 2-phenylpropionate	3·0	—	—	—
1-Phenylbutan-1-ol	6·5	6·8	—	10·5
1-Phenylbutan-2-ol	5·0	5·0	—	8·5
1-Phenylbutan-3-ol	7·5	7·8	—	10·5
1-Phenylbutan-4-ol	13·5	11·5	—	—
Methyl phenylbutyrate	9·5	—	—	—
2,2-Dimethylphenylethanol	—	7·8	—	—

considerably slower, the intensity reaching its maximum only after several hours.

RESULTS

With normal animals. Incubation mixtures were set up as described above with vertebrate livers, housefly abdomens or locust fat bodies as sources of enzymes and alkylbenzenes as substrates. In all insect experiments, where the enzyme was derived from large numbers of insects (30–50 locusts or up to 1000 flies), the preparations were assayed with *p*-nitrotoluene as substrate to confirm the activity of the enzyme.

The oxidation of *p*-nitrobenzyl alcohol by fly enzyme was measured under the conditions used for the oxidation of *p*-nitrotoluene and over the concentration range 0·2–1 mM. The double-reciprocal plot of these results was linear and gave a K_m value $2 \cdot 8 \times 10^{-3}$ M. The rate of oxidation of *p*-nitrobenzyl alcohol at low concentrations (3 μg./g. of flies/hr. at 0·2 mM) was considerably lower than the rate of formation of *p*-nitrobenzoic acid from *p*-nitrotoluene in the routine assay.

The effect on the oxidation of *p*-nitrotoluene of some compounds used as insecticide synergists or drug extenders was investigated and inhibition was found with both types of compound (Table 2).

Oxidation rates of alkylbenzenes other than toluene were lower than that of *p*-nitrotoluene and large numbers of insects were needed to obtain sufficient activity to permit detection of the metabolites. Toluene and *p*-nitrotoluene were converted into benzoic acid or *p*-nitrobenzoic acid by enzymes from all sources and no alcoholic metabolites were detected, whereas with the other alkylbenzenes from ethylbenzene onwards alcohols were the major metabolites.

After extraction with ether to remove metabolites, as described, above the incubation mixtures were boiled with N-sulphuric acid for 10 min. to hydrolyse any conjugates of the alcohols. No further metabolites were extracted from these solutions and it was concluded that the low activity was not due to loss of alcoholic metabolites as glucosides or glucosiduronates. No phenolic metabolites or ketones were found in the extracts. In rabbit liver enzyme the optimum pH for the oxidation of isopropylbenzene to dimethylphenylcarbinol was at pH 7·4 and the cofactor requirements were as described above, both NAD and NADP being required for optimum yield in the presence of the $NADPH_2$-generating system.

Results of quantitative experiments are summarized in Tables 2, 3 and 4.

In some experiments with locusts and flies the enzyme was tested with lower substrate concentrations of *n*-propylbenzene and isopropylbenzene. The concentration used as a routine, 0·2 mg./ml. or about 2 mM, appeared to be optimum but lower concentrations considerably decreased the rates of oxidation.

With pretreated animals. Male rats weighing 50 g. were injected intraperitoneally with aqueous sodium phenobarbitone (35 mg./kg.) twice daily for 4 days. Control animals were similarly dosed with water. A second group were given a single dose of 3,4-benzopyrene (25 mg./kg.) in arachis oil with corresponding controls. On the day after the final dose, liver-homogenate 10000 *g* supernatant enzymes were prepared and the oxidizing enzyme was assayed as described above with *p*-nitrotoluene.

Female locust hoppers were given the same dose rate of phenobarbitone and benzopyrene for 3 days and 1 day respectively before preparations of fat-body enzyme. Fat-body 10000 *g*-supernatant was prepared from these and from the appropriate controls which had received solvent injections only.

The LD_{50} of *m*-isopropylphenyl *N*-methylcarbamate was also determined on normal locusts and

Table 2. *Inhibition of* p-*nitrotoluene oxidation by insecticide synergists*

The 10000 *g* supernatant enzyme from rabbit or rat liver or from fly abdomen homogenates was incubated with *p*-nitrotoluene under conditions described in the text and *p*-nitrobenzoic acid formed in 0·5 hr. compared in normal preparations with those containing inhibitors.

	Percentage inhibition of enzyme				
	From rabbits		From rats	From flies	
Inhibitor concn.	0·5 mM	0·1 mM	0·5 mM	0·1 mM	0·01 mM
Inhibitor					
Piperonylbutoxide	27	6	22	100	51
Sulphoxide	44	18	33	100	48
n-Propylisome	22	7	19	100	39
Sesamex	44	-20	27	—	—
D.M.C.	8	—	3	—	—
SKF 525A	32	11	38	—	—

Table 3. *Oxidation of alkylbenzenes by rabbit, fly and locust enzyme*

Enzymes from rabbit liver, fly abdomen and locust fat body were prepared and incubations carried out as described in the text.

Substrate	Products	Rate of formation of products (mμmoles/min./g. of whole): Rabbits 1	Rabbits 2	Rabbits 3	Rabbits 4	Locusts	Flies
Toluene	Benzoic acid	—	—	—	—	0·40, 0·30, 1·10, 0·90	—
p-Nitrotoluene	*p*-Nitrobenzoic acid	—	0·80	0·30	—	0·50	2·40, 1·70, 4·00, 1·50
Ethylbenzene	1-Phenylethanol	—	—	—	0·26	—	—
	2-Phenylethanol	—	—	—	<0·01	—	—
	Phenylacetic acid	—	—	—	0·03	—	—
n-Propylbenzene	1-Phenylpropan-1-ol	0·20	0·30	0·26	—	0·06	0·12, 0·22
	1-Phenylpropan-2-ol	0·10	0·11	0·06	—	<0·01	<0·01
	1-Phenylpropan-3-ol	<0·01	<0·01	<0·01	—	<0·01	<0·01
	3-Phenylpropionic acid	0·04	0·05	0·06	—	<0·01	<0·01
Isopropylbenzene	2-Phenylpropan-1-ol	0·04	0·04	0·07	—	<0·01	<0·02
	2-Phenylpropan-2-ol	0·17	0·25	0·22	0·35	0·05	0·27
	2-Phenylpropionic acid	0·04	0·03	0·05	—	<0·01	<0·02
n-Butylbenzene	1-Phenylbutan-1-ol	0·09	0·15	0·11	—	—	—
	1-Phenylbutan-2-ol	<0·01	<0·01	<0·01	—	—	—
	1-Phenylbutan-3-ol	0·04	0·09	0·07	—	—	—
	1-Phenylbutan-4-ol	<0·01	<0·01	<0·01	—	—	—
	4-Phenylbutyric acid	0·04	0·04	0·04	—	—	—
tert.-Butylbenzene	2,2-Dimethylphenylethanol	0·06	0·10	0·09	—	—	—
	2,2-Dimethylphenylacetic acid	<0·01	<0·01	<0·01	—	—	—

those which had been given phenobarbitone for 3 days.

The oxidizing power of rat liver enzyme was roughly doubled by pretreatment but no stimulation of locust enzyme or change in the sensitivity of locusts to *m*-isopropyl *N*-methylcarbamate was found (Table 5).

DISCUSSION

The pattern of oxidation of the alkylbenzenes is qualitatively similar in rabbit and the two insect species used and confirms earlier studies on the rabbit where the metabolites were isolated from urine (Robinson & Williams, 1955; Robinson *et al.* 1955; El Masry *et al.* 1956). The major oxidation product of the alkylbenzene, where this was possible, was the secondary alcohol formed by hydroxylation at the α-methylene group of the side chain. Preferential oxidation of the methylene group nearest to the benzene ring was observed in *p*-nitroethylbenzene (Chakraborty & Smith, 1964). The oxidation of the penultimate methylene group of straight chains, e.g. in *n*-butylbenzene, was somewhat slower and occurred to about the same extent as the oxidation of terminal methyl groups to carboxyl. Where no α- or penultimate methylene

Table 4. *Oxidation of* p-*nitrotoluene and isopropylbenzene in flies and vertebrates*

Liver enzyme or fly homogenates were prepared and incubated as described in the text and assays performed for *p*-nitrobenzoic acid or 2-phenylpropan-2-ol.

	Rate of oxidation (μg./g. of liver or whole fly/hr.): *p*-Nitrotoluene	*m*-Isopropylbenzene
Rabbit	200	56, 60, 70
Coypu	180	85, 94
Hamster	160	75
Guinea pig	120	41, 60
Cat	90	61, 52
Rat	50	32
Flies*		
Normal	14	3
FC	20	5
SKA	8	4
P	19	4
A	12	4
R	12	4

* Insecticide resistance of strains was: FC, 5× to carbamates; SKA, 220× to diazinon, 1100× to DDT; A, 44× to parathion, 14× to malathion; P, pyrethrin-resistant.

Table 5. *Effect on oxidation enzyme of pretreatment of rats and locusts with phenobarbitone and 3,4-benzopyrene*

Rat liver or locust fat-body enzymes were prepared and incubated at 37° in air with the addition of cofactors as described in the text. Animals were dosed with phenobarbitone for 4 days or with benzopyrene for 1 day before preparation of the enzymes or measurement of toxicity of *m*-isopropyl *N*-methylcarbamate (IPMC).

	Rate of oxidation (mμmoles/min./g. liver or locust)			
	Isopropylbenzene	*p*-Nitrotoluene		LD₅₀ of IPMC (μg./locust)
Pretreatment	By rat liver	By rat liver	By locusts	
Water control	4·4	6·5, 7·0	0·6	12
Arachis oil control	4·4	5·9, 7·8	—	—
Phenobarbitone	8·6	15·0, 15·0, 12·0	0·4	10
Benzopyrene	6·7	11·0, 14·0, 9·0	0·3	—

group was available the oxidation of the molecule was much slower and *tert.*-butylbenzene was oxidized nearly ten times slower than toluene or *p*-nitrotoluene by the same enzyme preparation.

The terminal oxidation of alkyl groups to primary alcohols and thence to carboxylic acids has been thought to involve the soluble alcohol dehydrogenase (Gillette, 1959) in vertebrate liver but this seems unlikely to have occurred in the insect enzymes. The very low concentrations of *p*-nitrobenzyl alcohol that would have been produced in the oxidation of *p*-nitrotoluene by fly enzyme would have been oxidized too slowly to account for the measured yields of *p*-nitrobenzoic acid. Moreover, at these low rates of oxidation some unchanged alcohol would have been expected to occur in the final assays, whereas no product with the slow colour development characteristic of *p*-nitrobenzyl alcohol was ever detected. It seems more likely therefore that the oxidation of a methyl group via the alcohol to a carboxyl group occurs in or on the microsomal particles in the 10000g supernatant without the intermediate stages appearing free in solution. In this way the soluble alcohol dehydrogenase would not be involved in the process.

The values quoted for microsomal oxidations of rabbit liver may be taken as a fair guide to the behaviour of these compounds in the intact organism since the liver is virtually the only organ carrying out this metabolic reaction. The values quoted for the insects, on the other hand, are probably minimal since the oxidation of *p*-nitrotoluene occurs in other organs of the locust and the fat body accounts for only about half of the oxidizing capacity of the insect (Chakraborty & Smith, 1964). Similar considerations probably apply to the fly abdomen preparation used in the present work, and the results quoted in Table 3 suggest that there is no great difference between the oxidation rates of alkylbenzenes in whole vertebrates and whole insects if the results are expressed in terms of activity/g. of animal. On the other hand, the difference in oxidizing activity between individual insect or vertebrate species may be considerable and it may be possible to take advantage of this in modifying a toxic molecule to increase its selective action by increasing its susceptibility to oxidation.

The induction of increased amounts of microsomal oxidation enzymes in vertebrates by a variety of compounds is now well-known (Remmer, 1962; Conney & Burns, 1962) and evidence of a similar induction in insects has been reported (Morello, 1964). Pretreatment of rats with phenobarbitone or benzopyrene roughly doubled the rate of oxidation of *p*-nitrotoluene by the 10000g supernatant enzyme but in the present work neither compound stimulated the oxidation by locusts and pretreatment with phenobarbitone had no effect on the toxicity of an insecticidal carbamate towards locusts.

Species differences in rates of oxidation may, however, affect the toxicity of some carbamates since the LD_{50} of *m*-isopropylphenyl *N*-methylcarbamate in the locusts used in this work was about 10μg./g. whereas in flies the LD_{50} is 90μg./g. (Metcalf & Fukuto, 1965). Oxidation of the carbamates is generally believed to result in their detoxication (Metcalf & Fukuto, 1965) and it is significant that the enzyme preparations from several strains of fly studied in the present work were about three times more active in oxidizing isopropylbenzene than those from locusts (Tables 3 and 4).

Oxidation of the alkyl groups in alkylphenyl *N*-methylcarbamates is a feature of their metabolism along with the oxidation of the *N*-methyl group (Hook & Smith, 1967). The introduction of a hydroxyl group in the alkyl side chain would enable conjugation and elimination to take place and would be expected to result in a non-toxic product. If such a process accounted for a significant amount of the metabolic degradation of a

carbamate, the present results obtained suggest that a methyl group in a carbamate molecule might give more rapid detoxication than, e.g., a *tert.*-butyl group.

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