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CONJUGATIONS OF CARBARYL IN INSECTS

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440782

TO MY WIFE AND PARENTS

### Abstract

The conjugation of carbaryl and its initial breakdown products in insects has been examined.

Houseflies, blowflies and grass grubs were dosed with [<sup>3</sup>H]carbaryl and the water-soluble metabolites examined by a combination of paper chromatographic and ionophoretic techniques. These revealed the presence of 1-naphthyl dihydrogen phosphate, 1-naphthyl hydrogen sulphate and 1-naphthyl  $\beta$ -D-glucoside in the extracts, as well as at least seven other unidentified substances, probably including the phosphate, sulphate and glucoside conjugates of oxidation products of carbaryl.

The conjugation of 1-naphthol, one of the primary metabolites of carbaryl, was examined in greater detail in flies and grass grubs. Isotope dilution and paper chromatographic analyses of extracts of insects dosed with [<sup>14</sup>C]1-naphthol revealed the presence of the phosphomonoester, sulphate, and glucoside conjugates of 1-naphthol, but phosphodiester and glucosiduronic acid conjugates could not be detected. A new metabolite of 1-naphthol was present in extracts of dosed flies.

This new metabolite, and also the corresponding

p-nitrophenol metabolite, was isolated from extracts of flies fed with the parent phenols and characterised as a new conjugate, the  $\beta$ -D-glucoside 6-(dihydrogen phosphate). Some of the properties of this new conjugate were determined.

1-Naphthyl  $\beta$ -D-glucoside 6-phosphate probably accounted for one of the unidentified carbaryl conjugates.



Acknowledgements

I am greatly indebted to my supervisor, Professor J.N. Smith, for his inspiring encouragement and the unfailing interest he has shown, and I am grateful for the valuable experience I have gained in working under him.

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## INTRODUCTION

## Insecticidal Properties of Carbaryl

### General and Historical

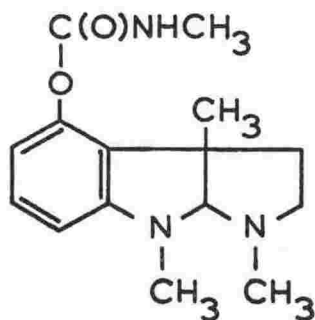
Carbaryl, (Sevin, 1-naphthyl N-methylcarbamate) is a contact insecticide with weak systemic activity, which has a relatively low mammalian toxicity (oral LD<sub>50</sub> in rats is 0.5 g./kg., dermal LD<sub>50</sub> in rabbits is 2 g./kg.) (Parke, 1968). It is one of a number of derivatives of carbamic acid which exhibit pronounced physiological action (Metcalf, 1955); the basis of this action being an inhibition of acetylcholinesterase.

The history of the toxic carbamate esters began in 1925 with Stedman's successful elucidation of the structure of the principle alkaloid eserine from Physostigma venenosum (Stedman & Barger, 1925). This substance, also known as physostigmine, is an ester of N-methylcarbamic acid (Table 1). Following this, Stedman (1926) investigated many synthetic analogues of eserine and found that m-dimethylaminophenyl N-methylcarbamate methiodide was a potent cholinergic drug. The corresponding N,N-dimethyl carbamate ester which is more stable was subsequently introduced as the well known prostigmine (Table 1).

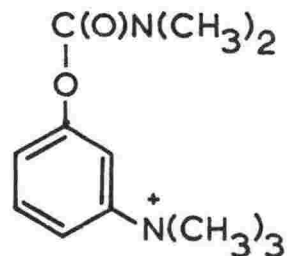
These and other synthetic carbamate drugs are ionic water-soluble compounds, and as such have no insecticidal activity,

although they are good inhibitors of insect cholinesterase in vitro (Metcalf & March, 1953). The first insecticidal carbamates were non-ionic N,N-dimethyl carbamate esters introduced by the Geigy Company after 1947 (Metcalf, 1955). These included Isolan, Demiton, and Pyrolan (Table 2).

Table 1. Medicinal carbamic acid esters.



Eserine



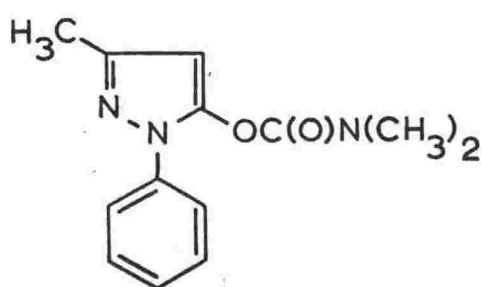
Prostigmine

These compounds have a rapid knock-down action on flies, are potent contact poisons to aphids, thrips, weevils and some other small insects, and also have some systemic activity.

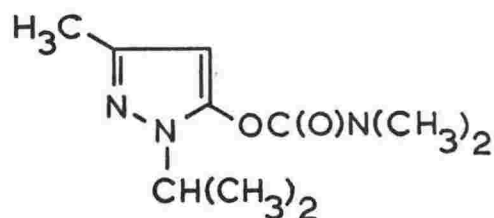
Carbaryl was the first of the insecticidal N-methyl carbamates to be introduced. Virtually all carbamates subsequently introduced

have been N-methylcarbamates, these being generally more effective than N,N-dimethylcarbamate esters (Metcalf & Fukuto, 1965; O'Brien, Hilton & Gilmour, 1966).

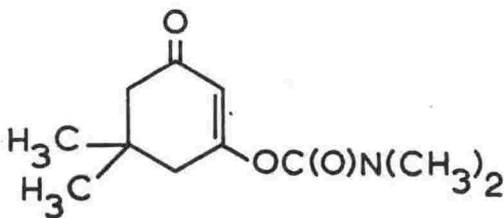
Table 2. Some N,N-Dimethylcarbamates.



Pyrolan



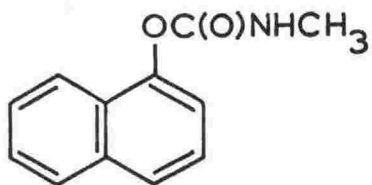
Isolan



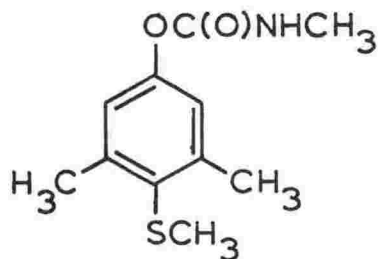
Dimetan

Such carbamates are (Table 3): Zectran, Bayer 39007, Mesurol, Hercules AC 5727, Furadan, and an oxime carbamate, Temik.

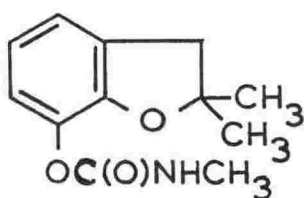


Table 3. N-methylcarbamates.

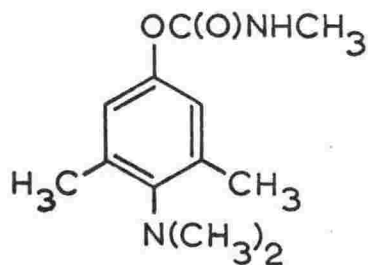
Carbaryl



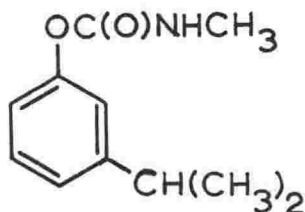
Mesurol



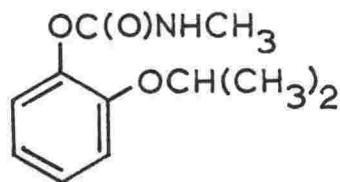
Furadan



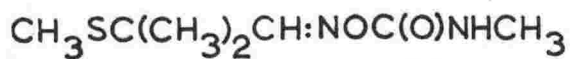
Zectran



Hercules AC5727



Bayer 39007



Temik

### Mode of Action

In mammals, carbamates act as autonomic drugs stimulating structures innervated by the cholinergic nerves. Action results from inhibition of cholinesterase, allowing local accumulation of acetylcholine at the synapses with consequent excessive activity and finally, blockade (Casida, 1963). The insecticidal activity of carbamates appears to result primarily from similar action. Thus eserine effects ventral nerve cord transmission in cockroaches in this manner (Tobias, Kollros & Savit, 1946). Toxic carbamates are usually potent inhibitors of cholinesterases in vitro (Casida, Augustinsson & Jonsson, 1960). Ionic pharmacological carbamate esters are non-toxic to insects (Metcalf & March, 1953). It is now realised that the site of action of cholinergic agents in insects, the insect ganglion, possesses a barrier slowing down the penetration of ionic substances. Unlike the vertebrates, the insects do not have a peripheral, ion-sensitive cholinesterase (O'Brien, 1959b).

Insecticidal carbamates can also inhibit insect aliesterases in vivo and in vitro (Plapp & Bigley, 1961), differing from eserine, but it is considered that this is not crucial, compared with cholinesterase inhibition. Not all good non-ionic anticholinesterases exhibit toxicity, but inactivity of such compounds can probably be explained on the basis of rapid removal due to metabolic breakdown.

### Mechanism of Inhibition

The reaction of carbamates with acetylcholinesterase takes

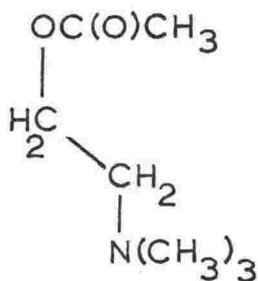
place in three stages. First there is a binding of the carbamate to the active site to form a reversible complex. This is followed by a slow, irreversible carbamylation of the enzyme. Finally, an even slower, but still significant, hydrolysis of the inactivated enzyme occurs, resulting in decarbamylation and regeneration of the original enzyme. This reaction is analogous to those of organophosphates and acetylcholine. However, organophosphates phosphorylate the enzyme rapidly with only very slow dephosphorylation occurring and in the case of acetylcholine, both acetylation and deacetylation occur very rapidly.

The overall action of carbamates is that of a competitive substrate with very low turnover (Fukuto, Fahmy & Metcalf, 1967). In practical conditions of inhibition small levels of reversible enzyme complex exist along with larger levels of carbamylated enzyme.

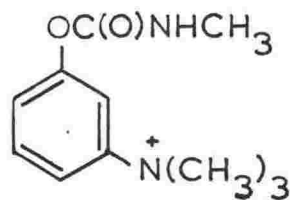
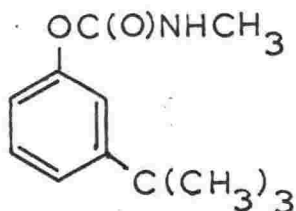
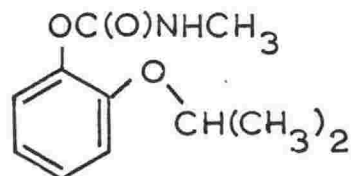
#### Relationship between Structure and Activity

Steric factors predominate in the requirements for carbamates to have good anticholinesterase activity, and the most potent compounds are those that conform closely to the structure of acetylcholine, such as m-dimethylaminophenyl N-methylcarbamate methiodide (Table 4).

Highly active non-ionic carbamates have a relatively bulky group having a large van der Waal's energy of interaction with the anionic site of cholinesterase, about 5Å from the carbamyl group. Thus tertbutylphenyl N-methylcarbamate and o-isopropoxyphenyl N-methylcarbamate (Table 4) are potent anticholinesterases (Metcalf &

Table 4. Acetylcholine and Structural Analogues

Acetylcholine

m-Dimethylaminophenyl  
N-methylcarbamatem-tert-Butylphenyl  
N-methylcarbamateo-Isopropoxyphenyl  
N-methylcarbamate

Fukuto, 1965) in spite of the absence of ionic binding possibilities at the anionic site. The tertbutylphenyl compound is twenty times and two hundred times more toxic than the o- and p-isomers respectively, and the o-isopropoxyphenyl compound is fifteen and one hundred and

fifty times more toxic than its m- and p-isomers respectively (Metcalf & Fukuto, 1965). Carbaryl also conforms reasonably to this structural requirement, (Table 3). Its inhibition constant ( $K_i$  ( $\text{min}^{-1} \text{ moles}^{-1}$ ) is  $1.3 \times 10^5$ ) of bovine erythrocyte cholinesterase is comparable with that of o-isopropoxyphenyl N-methylcarbamate ( $K_i$  is  $1.1 \times 10^5$ ) (O'Brien et al., 1966).

#### Correlation between Activity and Toxicity

The weak insecticidal activity of medicinal carbamates has been commented on, this non-toxicity being due to an inability to penetrate to the sites of action.

Even with non-ionized carbamates, good correlation between anticholinesterase activity and toxicity to insects seldom exists. Thus, although there is fair correlation for thrips (Kolbezen, Metcalf & Fukuto, 1954), little correlation is apparent for houseflies (Kolbezen et al., 1954; Casida et al., 1960). However, if metabolic breakdown of carbamates is decreased by administration of microsomal enzyme inhibitors, such as piperonyl butoxide, correlation is markedly improved (Metcalf & Fukuto, 1965; Metcalf, Fukuto & Winton, 1960). In such a situation the toxicity of a normally rapidly metabolised carbamate such as carbaryl is substantially increased.

Since metabolism evidently plays an important part in determining the toxicity of a carbamate, a knowledge of the metabolic processes involved is an essential part of the understanding of the action of a carbamate.

## Metabolism of Carbaryl

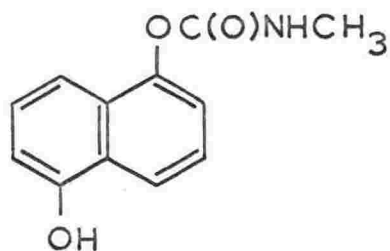
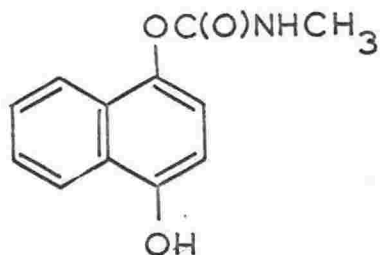
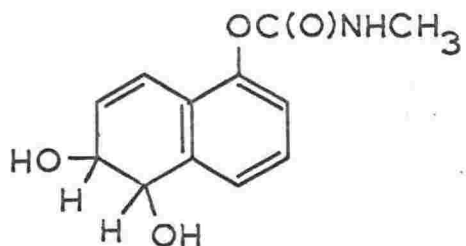
### General Mechanisms

Initial studies on the metabolism of carbaryl were carried out by Eldefrawi & Hoskins on houseflies, large milkweed bugs, and German cockroaches (1961). All species metabolised carbaryl and resistant flies did so at a faster rate than susceptible flies. Polar metabolites were formed in each case, although there was considerable species difference in the number formed. However, the same series of polar products from each insects were produced by the metabolism of 1-naphthol, and 1-naphthol itself was formed from carbaryl by the German cockroach. Hence it was concluded that a hydrolysis at the ester bond was the first step in carbaryl metabolism.

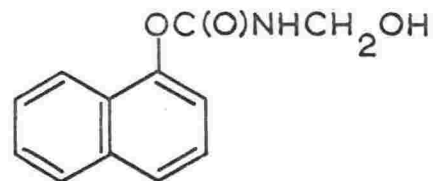
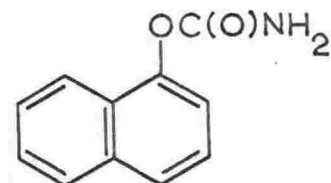
More recently, extensive studies on the metabolism of carbaryl in both whole animals and in isolated enzyme systems have been carried out, using carbaryl labelled with  $^{14}\text{C}$  on the aromatic ring, carbonyl and N-methyl groups. These have indicated that hydroxylations, rather than hydrolysis, are important in initial steps of carbaryl metabolism. It is now clear that three routes of metabolism are operative on carbaryl, these being: (a) hydroxylation of the N-methyl side-chain, (b) hydroxylation of the aromatic ring, and (c) hydrolysis of the ester bond (Table 5). The initial products can then be conjugated with endogenous substances to form water-soluble products. These reactions are typical of the so-called detoxication reactions by which foreign compounds in general are metabolised.

Table 5. Products of carbaryl metabolism.

## RING HYDROXYLATION

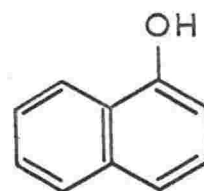
5-Hydroxy-1-naphthyl  
N-methylcarbamate4-Hydroxy-1-naphthyl  
N-methylcarbamate5,6-Dihydro-5,6-dihydroxy-  
1-naphthyl N-methylcarbamate

## SIDE-CHAIN HYDROXYLATION

1-Naphthyl N-hydroxymethyl-  
carbamate

1-Naphthyl carbamate

## HYDROLYSIS



1-Naphthol

## The Detoxication Reactions

### The fate of foreign compounds

All substances encountered by an organism which are neither utilised for energy production nor for building of tissue components, are known as foreign compounds (Parke, 1968) or xenobiotics (Mason *et al.*, 1965). When a foreign compound enters the tissue of a living organism it is obviously in the interests of the organism to get rid of the compound as rapidly and as efficiently as possible, since at best it can be of no use and the worst can be lethal. Thus it has been found (Williams, 1959) that most xenobiotics undergo chemical alteration in organisms ultimately resulting in the excretion of specific metabolites.

The metabolism of xenobiotics involves two phases of reaction (Williams, 1967). In the first phase, metabolic transformation, an alteration of the molecule generally involving oxidation, reduction or hydrolysis occurs. These transformations often result in the rendering of a toxic compound very much less toxic, and hence the description, "detoxication reactions" (Williams, 1959). However, since in many cases non-toxic or slightly toxic compounds are rendered considerably more toxic the terms "inactivation reaction" and "activation reaction" are more useful (Smith, 1968). Second phase reactions, the conjugations, are syntheses in which a foreign compound bearing a suitable functional group or any of its first phase metabolites is combined with an endogenous substrate (Williams, 1959). Conjugations nearly always result in molecules which are more polar



and less lipid-soluble, and readily excreted. Also conjugations nearly always result in a detoxification of toxic compounds, and hence may be classed as true detoxication reactions.

### Metabolic Transformations

#### (1) Oxidations: the microsomal enzymes

The most important of the first phase reactions is oxidation. Many oxidations take place in the endoplasmic reticulum of vertebrate liver cells, or in analogous tissues of other cells (Brodie, Gillette & La Du, 1958). The enzymes responsible appear to be closely associated with the lipoprotein membrane of the endoplasmic reticulum. When tissues are homogenised, the endoplasmic reticulum breaks up into small vesicles forming the so-called 'microsomes'. These can be sedimented by centrifugation at about 100,000g for one hour after prior removal of cell fragments, nuclei and mitochondria by medium-speed centrifugation. The supernatant remaining after sedimentation of the microsomes is the 'soluble fraction'.

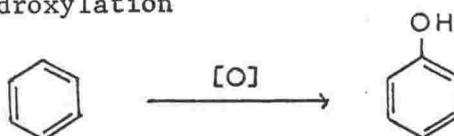
The wide range of oxidations occurring in the microsomal enzyme systems are catalysed by mixed function oxidases (Mason, North & Vanneste, 1965). Thus all reactions require reduced coenzyme,  $\text{NADPH}_2$ , and molecular oxygen. Oxygen introduced into the substrate is derived from molecular oxygen, and not water.

Microsomes of many species are markedly activated by administration of certain foreign compounds, including drugs, pesticides and polycyclic hydrocarbons. This effect has been observed in many

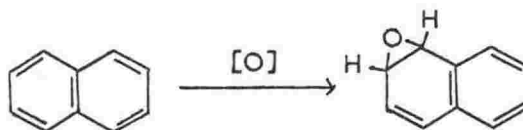
mammals, but evidence for induction of insect microsomes is uncertain (Smith, 1968). The stimulatory effect is apparently effected by an increase in the amounts of the microsomal enzymes (Ernster & Orrenius, 1965; Remmer & Merker, 1965). Microsomal enzymes are also inhibited by certain specific compounds, resulting in decreased rates of metabolism (Brodie, 1956). These compounds include SKF 525A (diethylaminoethyl diphenyl-n-propylacetate), Lilly 18947, (2,4-dichloro-6-phenylphenoxyethyl diethylamine) and piperonyl butoxide -[2-(2-butoxyethoxy)ethoxy]-4,5-(methylenedioxy)-2-propyl toluene.

Oxidations carried out by the microsome enzymes are (Williams, 1959; Parke, 1968):

(a) Aromatic hydroxylation

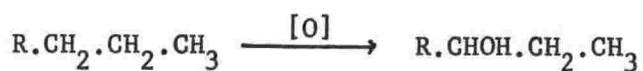
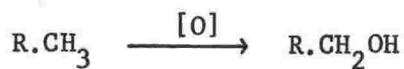
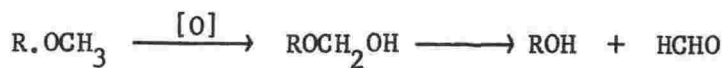
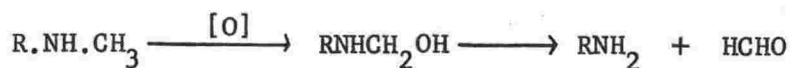


(b) Epoxidation

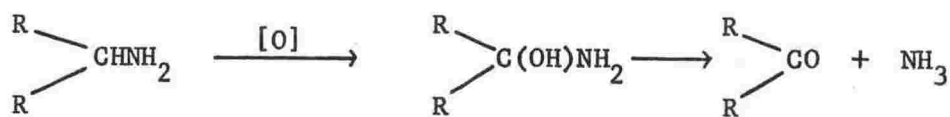


The epoxides formed are usually reactive and undergo further reactions by reduction or with water or conjugation enzyme systems to form dihydro-mono-ols, dihydro-diols, and their conjugates (Gillette, 1967). A few compounds, such as the chlorinated hydrocarbon aldrin, form stable epoxides (Bann, 1956; Giannotti, Metcalf & March, 1956).

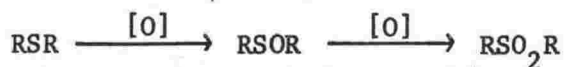
## (c) Aliphatic oxidation

(d) O-Dealkylation(e) N-Dealkylation

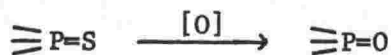
## (f) Deamination



## (g) Sulphur oxidation



## (h) Desulphuration



In addition to the microsomal oxidation systems, oxidases and dehydrogenases present in soluble fractions and mitochondria carry out a number of oxidations of foreign compounds. These include oxidation of alcohols and aldehydes to carboxylic acids, oxidative deaminations, and aromatisation of alicyclic compounds (Williams, 1959).

## (2) Reductions

A variety of aromatic nitro compounds are reduced to the corresponding amines by microsomal and soluble nitro reductases. Azo compounds are reduced in two stages to yield two molecules of aromatic amines, via the hydrazo compound (Williams, 1959).

Other reductions observed are (Parke, 1968):

- (1) Conversion of ketones to secondary alcohols.
- (2) Reduction of some aldehydes to alcohols.
- (3) Saturation of double bonds.
- (4) Formation of amides from hydroxamic acids.
- (5) Reductions of disulphides and sulfoxides.
- (6) Dehydroxylations.

## (3) Hydrolysis

A number of esterases are present in liver, blood plasma and insect tissues (Williams, 1959) capable of hydrolysing a wide variety of ester linkages in foreign compounds. Esters, amides, phosphate esters, hydroxamic acids, carbamate esters and nitriles all can undergo hydrolysis to a certain extent (Parke, 1968).

#### (4) Miscellaneous

A few other metabolic transformations not included above which take place are: ring scissions of certain heterocyclic and aromatic compounds (Williams, 1959), cyclisations (Parke, 1968), dehydrochlorinations, for instance the formation of DDE from DDT (Williams, 1959), and dehalogenations.

#### Conjugations

Conjugations are biosyntheses in which a foreign compound or any of its metabolites bearing a suitable functional group, combine with readily available endogenous substrates to form conjugates. In most cases conjugation requires an active intermediate, usually a nucleotide, and a transferring enzyme. Conjugation reactions can be divided into two types (Williams, 1967).

- (1) Those in which the conjugating agent forms part of the activated intermediate.
- (2) Those in which the compound being conjugated first forms part of the activated intermediate.

Type 1 conjugations include (a) glycosylations in which glycoside moiety is transferred from a uridine diphosphate coenzyme to form  $\beta$ -glycosides. Substrates include hydroxy compounds, aromatic carboxylic acids and substituted carboxylic acids which do not undergo  $\beta$ -oxidation, and certain amines, amides and thiols (Williams, 1963).

(b) Ethereal sulphate formation in which 3'-phosphoadenosine-5'-phosphosulphate acts as sulphate donor to phenols and certain aliphatic alcohols, amines and thiols.

(c) Methylation in which the methyl group is transferred from S-adenosyl methionine to phenolic acids, polyhydric phenols, aromatic amines and N-heterocycles.

(d) Aromatic amines are acetylated to some extent by acetyl coenzyme A.

Type 2 conjugation. Coenzyme A derivatives of aromatic and heterocyclic carboxylic acids, and some substituted aliphatic acids react with amino acids to form peptide conjugates. Glycine, glutamine, ornithine, arginine and to a minor extent, aspartic acid, serine and taurine are used in peptide conjugations (Williams, 1967).

#### Others

(a) Glutathione forms S-alkyl or S-aryl derivatives with arene-1,2-epoxides, alkyl halides, halogenated aromatic hydrocarbons and nitrobenzenes (Williams, 1967). The glutathione conjugate formed often undergoes further reaction in tissues, forming cysteine and N-acetyl cysteine (mercapturic acid) conjugates. The glutathione conjugation is unique, in that a preliminary activation of either substrate is not necessary. The enzyme catalyses a reaction which is thermodynamically favourable and which sometimes proceeds at a measurable non-enzymic rate (Smith, 1968).

(b) Hydrogen cyanide is converted to thiocyanate in many animal species (Smith, 1964). The source of sulphur is probably thiosulphate.

## Oxidation and Hydrolysis of Carbaryl

### In mammals

Mammalian liver microsome preparations fortified with  $\text{NADPH}_2$ , and incubated with [naphthyl- $^{14}\text{C}$ ]carbaryl have been shown to yield 1-naphthyl N-hydroxymethylcarbamate, 4-hydroxy-1-naphthyl N-methylcarbamate, 5,6-dihydro-5,6-dihydroxy-1-naphthyl N-methylcarbamate, 5-hydroxy-1-naphthyl N-methylcarbamate, 1-hydroxy-5,6-dihydro-5,6-dihydroxynaphthalene and 1-naphthol (Leeling & Casida, 1966; Knaak, Tallant & Bartley, 1965; Dorrough & Casida, 1964).

Casida found that the major products from [naphthyl- $^{14}\text{C}$ ]-carbaryl in a  $\text{NADPH}_2$ -fortified rat liver microsome preparation were the N-hydroxymethyl compound, 7%; the dihydro-diol, 4%; the 4-hydroxycarbaryl, 5%; and 1-naphthol, 9% of administered dose. However 60% of the activity appeared as unidentified water-soluble fraction. When this microsomal preparation was fortified with the soluble portion of the liver homogenate, its activity was increased, with unchanged carbaryl decreasing from 12% to 3% and and the aqueous fraction increasing to 86%. However there was a decrease in the organosoluble fraction, indicating that conjugation of initial metabolites was probably taking place. Incubation of recovered ether-soluble metabolites with glutathione, glucuronide and sulphate conjugating systems suggested that glucuronide and sulphate conjugates, but not glutathione conjugates

could have been formed. Participation of each of these systems was judged by the disappearance of labelled metabolites from the ether fraction.

Agents which are inhibitors of microsomal oxidations are fairly effective inhibitors of carbaryl metabolism by liver microsomes.

Metabolism in whole animals is more complex. Ether extracts of excreta from rats or rabbits dosed with labelled carbaryl, contain most of the compounds identified in the microsomal systems, as well as several more unidentified compounds (Leeling & Casida, 1966). However, ether-solubles form only a minority of excreted products; about 10% of [naphthyl-<sup>14</sup>C]carbaryl label, the rest appearing as water solubles (Knaak et al., 1965).

#### In insects

The metabolism of carbaryl in insects is remarkably similar to that in mammals, and considerable evidence has accumulated suggesting that with insects also, hydroxylation is as important as hydrolytic degradation.

Metabolism in the American cockroach involves hydroxylation of the N-methyl group and at several positions in the aromatic ring, 4-hydroxy and 5,6-dihydro-5,6-dihydroxy derivatives being identified (Dorough & Casida, 1964). These three compounds and also 1-hydroxy-5,6-dihydro-5,6-dihydroxynaphthalene and 1-naphthol have been identified in ether extracts of carbaryl-dosed boll weevil and boll worm adults (Andrawes & Dorough, 1967). These compounds each made



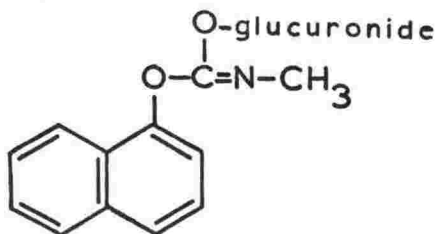
up from 3% to 7% of the total dose whereas unidentified water solubles constituted 50% of the total.

Fly microsome preparations readily break down carbaryl forming water solubles and ether-soluble hydroxylated derivatives (Price & Kuhr, 1968; Tsukamoto & Casida, 1968). The hydroxylation is inhibited by 1,3-benzodioxole compounds, and also by carbon monoxide which combines with the cytochrome P450 implicated in microsomal oxidations (Price & Kuhr, 1968). Hydroxylation is stimulated by  $\text{NADPH}_2$  (Tsukamoto & Casida, 1968). Marked synergism of carbaryl with piperonyl butoxide occurs in houseflies. Thus in the insects, systems analogous to those in mammals are operating to hydroxylate carbaryl.

## Conjugations of Carbaryl

### In mammals

Conjugates occurring in urines of intact animals dosed with labelled carbaryl have been determined (Knaak et al., 1965). In the rat and guinea pig a major product is a tentatively identified direct conjugate between carbaryl and glucuronic acid, 1-naphthyl N-methylimidoglucuronide constituting about 12-20% of administered dose.



1-Naphthyl N-methylimidoglucuronide

Both naphthyl glucuronide and sulphate were present, each about 20% of dose, and also conjugates of 4-hydroxycarbaryl, 4-(methylcarbamoyloxy)-1-naphthyl glucuronide; 10-15%; and 4-(methylcarbamoyloxy)-1-naphthyl sulphate, being 8% of administered dose.

These conjugates are in somewhat different proportions than what might be expected from the proportions of microsomal products

formed. Notably absent are conjugates of N-hydroxymethyl and dihydro-diol metabolites. The N-hydroxymethyl compound possibly undergoes N-dealkylation since 11% of [N-methyl- $^{14}\text{C}$ ]carbaryl label administered to rats is excreted as  $^{14}\text{CO}_2$  (Krishna & Casida, 1966). However, two to three minor unidentified products are usually present.

The elimination of conjugates of carbaryl metabolism by man and sheep compares with that of guinea pig and rat. However, the monkey and pig differ in that conjugates of 1-naphthol are virtually absent in excreta, the carbaryl glucuronide and the glucuronide or sulphate conjugates of 4-hydroxycarbaryl being prominent (Knaak, Tallant, Kozbelt & Sullivan, 1968). The dog is even more different as it liberates neither 1-naphthyl nor 4-hydroxy-carbaryl conjugates, the only identified conjugate being the carbaryl-glucuronide (Knaak & Sullivan, 1967). However, this does not mean as the author claims, that the dog is unable to hydroxylate carbaryl. Extensive N-demethylation and possibly decarbamylation occurred, as while 80% of [naphthyl- $^{14}\text{C}$ ]carbaryl label was recovered from excreta, only 35% of [N-methyl- $^{14}\text{C}$ ]carbaryl label was so recovered. At least five unidentified metabolites bearing naphthyl- $^{14}\text{C}$  label but not N-methyl- $^{14}\text{C}$  label were present in urine and faeces of dogs dosed with labelled carbaryl. Determination of  $^{14}\text{CO}_2$  recoveries from dogs dosed with [carbonyl- $^{14}\text{C}$ ]-carbaryl would help here.

### In insects

Little work on the identification of conjugates formed by insects from carbaryl and its metabolites has been done, compared with that done on mammalian conjugation.

Eldefrawi & Hoskins (1961) suggested that carbaryl formed the same series of products in insects as 1-naphthol, but these were not identified. German cockroaches also degraded carbaryl to 1-naphthol and its conjugates although once again these were not identified (Ku & Bishop, 1967). The formation of conjugates is evidently important, since in one quantitative study of the degradation of carbaryl by boll weevil and boll worm adults (Andrawes & Dorough, 1967), water soluble products constituted over 50% of applied dose.

By analogy with the conjugations of carbaryl in vertebrates, conjugates of 1-naphthol and hydroxylation products of carbaryl should be products of carbaryl detoxication in insects. The detoxication of phenolic substances, including 1-naphthol, in vertebrates and non-vertebrates has been extensively investigated, and it has been found that they typically form several conjugates, with some differences between vertebrates and non-vertebrates. It can be suggested that conjugates of carbaryl and its derivatives in insects will be those of phenol conjugations typical of insects. The conjugations of phenols are considered in detail overleaf.

## The Metabolism of Phenols: Conjugation Reactions

Phenols sometimes undergo hydroxylation to a minor extent in mammals. For instance, phenol itself is hydroxylated to quinol and catechol in the rabbit to an extent of about 10% (Parke & Williams, 1953). The main reaction of phenols, however, is conjugation.

### Glycoside Conjugations

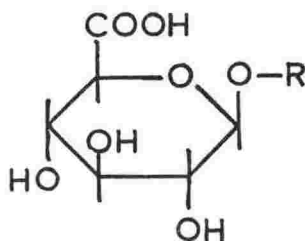
#### (1) Glucuronide formation

Phenols, as well as other foreign organic compounds containing a suitable functional group, are conjugated in vertebrates to form  $\beta$ -D-glucosiduronic acids, simply referred to as glucuronides.

The formation of glucuronides requires the prior formation of the coenzyme UDPGA, uridine diphosphate glucuronic acid (Storey & Dutton, 1955). The glucuronyl moiety is then transferred to the aglycone by UDP glucuronyltransferases, forming the conjugate (Dutton, 1961). Glucuronic acid is not a source of the glucuronide moiety, instead UDPGA is formed from uridine diphosphate glucose, UDPG, by oxidation by uridine diphosphate glucose dehydrogenase and NAD (Dutton, 1961). Phenyl glucuronides are not formed in vivo by oxidation of preformed phenyl glucosides. Glucuronide formation occurs mainly in the liver, but also to some extent in the kidney, gastro-intestinal mucosa, and in the skin (Dutton & Stevenson, 1959).

The UDPGA synthesising enzymes are located in the soluble fractions of tissues but the glucuronyltransferases are located in microsomal fractions.

Glucuronides have the  $\beta$ -D-pyranoside structure:



Phenols form ether-type glucuronides (Williams, 1959).

Glucuronide formation occurs in most mammals examined (Williams, 1959) and is usually the major conjugation route for phenols. Cats are an exception among mammals in having only a limited facility for glucuronidation, apparently owing to a deficiency of the glucuronyltransferase enzymes (Brodie, 1961). UDPGA occurs in both cat liver and kidney (Dutton & Grieg, 1957; Dutton, 1959). All birds so far examined form glucuronides. Reptiles have been less studied, but formation of glucuronides has been observed in the tortoise, grass snake, green lizard and alligator (Smith, 1964; Brodie & Maikel, 1962). Amphibia (salamanders, frog and the toad) form glucuronides (Brodie & Maikel, 1962). Formation in the frog is dependent on the nutritional status, as well fed

frogs excrete phenols mainly as the glucuronide, but if starved, phenols are excreted mainly as the sulphate conjugate (Brodie et al., 1958).

Fresh water fish have difficulty in forming glucuronides, and are sensitive to small amounts of phenol in the water. Apparently the mechanism for UDPGA synthesis is lacking (Brodie & Maikel, 1962). Marine fish, on the other hand, are probably able to form phenyl glucuronides, since extensive conjugation of aminobenzoic acids with glucuronide occurs in flounder, goosfish and dogfish (Huang & Collins, 1962) and marine fish are able to synthesise UDPGA (Dutton & Montgomery, 1958).

Glucuronides are hydrolysed by  $\beta$ -glucuronidase to the aglycone and free glucuronic acid.  $\beta$ -Glucuronidase is present in most animal tissues particularly the liver, kidney and intestinal tract. While glucuronide conjugates of foreign compounds are probably not hydrolysed to any extent by tissue  $\beta$ -glucuronidases, glucuronides excreted into the bile may be hydrolysed by intestinal  $\beta$ -glucuronidase and the resultant aglycones be reabsorbed resulting in an enterohepatic circulation of the compounds (Williams, Millburn & Smith, 1965).  $\beta$ -Glucuronidase can catalyse synthesis of glucuronides in vitro by glucuronyl transfer from other glucuronides (Fishman & Green, 1957), but since a relatively high concentration of acceptor is required, and transfer occurs only to aliphatic alcohols and not to phenols (Tsukamoto, Kato, Yoshida & Tatsumi, 1964), it cannot be

expected that formation of glucuronides by this route in vivo is important.

## (2) Glucoside formation

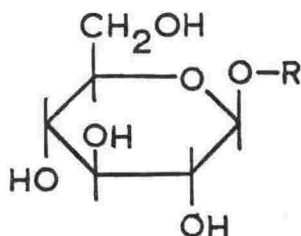
Invertebrates apparently do not form glucuronides, and insects instead form  $\beta$ -glucoside conjugates from phenols (Smith, 1955), carboxylic acids (Smith, 1964) and simple thiols (Gessner & Acara, 1966). Many natural metabolites are also excreted as glucosides by insects (Smith, 1964). Glycoside conjugation in other invertebrates has not been greatly studied but molluscs are probably able to form glucosides since formation of o-aminophenol glucoside has been demonstrated in gut and stomach preparations of snails and slugs (Dutton, 1965). Glucuronide was not formed. Detection of glycoside conjugates in intact animals is made difficult by the presence of high levels of glycosidases found in these animals. Formation of glycosides from phenol could not be detected in scorpions, spiders or ticks (Smith, 1968).

Although glucuronide conjugation is best known since it occurs in common laboratory animals and mammals in general,  $\beta$ -glucoside conjugation is probably a more widespread phenomenon since the number of species involved is much larger than those involved in glucuronide conjugation.

The synthesis of glucosides appears to be similar to glucuronide synthesis in vertebrates; the glucose moiety is



is transferred to the aglycone from a uridine coenzyme, uridine diphosphate glucose, UDPG (Trivellani, 1960; Dutton & Duncan, 1960). Formation has been shown to take place in the locust fat body (Trivellani, 1960) and in cockroach and fly abdomen fillets, in presence of added UDPG (Dutton & Ko, 1964). Glucosides, like glucuronides, have the  $\beta$ -D-pyranoside structure:



In plants, glycosides are readily formed with many naturally occurring substrates such as alkaloids, flavanoids, etc. (Harbourne, 1964). Although many glycosides of natural products are formed, foreign compounds are usually detoxified by formation of glucosides, or sometimes gentiobiosides, formed from glucosides by transfer of an additional glucose moiety from UDPG.

Although it has been found that glucosides are normal conjugates of phenols in insects, a few reports have suggested that glucuronides may also be synthesised. Silkworms apparently formed glucuronides of 3-hydroxykynurenine and 3-hydroxyanthranilic acid as products of tryptophan metabolism (Inagami, 1955) although the silkworm also forms 3-hydroxykynurenine glucoside (Linzen &

Ishiguro, 1966) and both the silkworm and locust form 3-hydroxy-anthranilic acid glucoside (Ishiguro & Linzen, 1966). Terriere, Boose & Roubal (1961) have reported the detection of 1-naphthyl glucuronide and also glucuronides of 1,2-dihydro-1-naphthol and 1,2-dihydro-1,2-dihydroxynaphthalene as products of naphthalene and 1-naphthol metabolism in flies, by paper chromatographic techniques and hydrolysis by a glucuronidase preparation. Zayed, Hassan & Hussein (1965) and Hassan, Zayed & Abdel-Hamid (1965) found metabolites of Dipterex (dimethyl 2,2,2-trichloro-1-hydroxyethylphosphonate) and its methyl ether in the cotton leaf worm Prodenia litura which were claimed to be the ester glucuronides of the demethylated compounds. However, Smith & Turbert (1964) were unable to detect 1-naphthyl glucuronide as a conjugate of 1-naphthol in the housefly, although the glucoside conjugate was readily isolated. Dutton & Ko (1964) were unable to detect the presence of glucuronyltransferase in housefly abdomens, although glucosyltransferase enzymes could be readily demonstrated.

### (3) Rare glycoside conjugates

A few other sugars may possibly participate in conjugation reactions. Imidazole-4-acetic acid, itself a product of histamine metabolism, is converted into a ribose derivative, 1-ribosylimidazole-4-acetic acid, in rats, mice, rabbits, and to some extent in cats (Schayer, 1958). This reaction could possibly be an extension of

normal nucleoside metabolism (Williams, 1959). A single incidence of N-acetylglucosamine conjugation has been recorded. 17 $\beta$ -Estradiol administered to male or normal or ovariectomised-hysterectomised female rabbits is excreted as a double conjugate of 17 $\alpha$ -estradiol with glucuronic acid and N-acetylglucosamine, (estra-1,3,5(10)-trien-17 $\alpha$ -yl-2'-acetamido-2'-deoxy- $\beta$ -D-glucopyranosid-3-yl- $\beta$ -D-glucopyranosiduronic acid) (Layne & Sheth, 1964; Collins, Williams & Layne, 1967). The 17 $\alpha$ -estradiol is inactive towards UDP-N-acetylglucosamine, but the 3-glucuronide of 17 $\alpha$ -estradiol apparently can accept N-acetylglucosamine from UDP-N-acetylglucosamine (Jirku & Layne, 1965).

#### Sulphate Conjugation

Phenols are conjugated with sulphate to form ethereal sulphates which are acid esters of sulphuric acid, RO.SO<sub>2</sub>.OH. Sulphuric acid can also form diesters, but these have not been found as conjugates (Williams, 1967). N-Sulphates or phenylsulphamic acids have also been recorded, as conjugates of p-aminobenzoic acid in the rabbit and rat (Williams, 1963), birds (Smith, 1964), and spiders (Hitchcock & Smith, 1964). Small amounts of sulphate esters of simple alcohols have been detected in vertebrate liver preparations by the use of <sup>35</sup>SO<sub>4</sub> (Bostrom & Vestermark, 1961).

The sulphate conjugation of phenols has been found to occur in all mammals examined, including the whale, although the pig is apparently less efficient than other mammals (Williams, 1963).

Other vertebrates, including the hen and crow (Smith, 1964), the alligator and snake, and the amphibians the frog, toad and salamander, but excluding fish and tadpoles, can form sulphate conjugates of phenols (Brodie & Maikel, 1962). However, marine fish may have some capacity for sulphate formation since scymol sulphate has been found in elasmobranch bile and cyprinol sulphate in carp bile (Haslewood, 1955).

All insects examined use this conjugation; for example, m-aminophenol is excreted partly as the O-sulphate by locusts, the stick insects, moth larvae and other insects (Smith, 1955), and 1-naphthyl sulphate is a conjugate of 1-naphthol in flies (Smith & Turbert, 1964). Sulphate conjugation has also been observed in molluscs, and possibly crustacea and echinoderms (Smith, 1968).

Enzymic synthesis of sulphates takes place by transfer of sulphate from adenosine-3'-phosphate-5'-phosphosulphate (PAPS) to a phenol by sulphokinases. PAPS is formed from ATP and inorganic sulphate in the liver and other tissues (Robbins & Lipmann, 1957). Several sulphokinases are known. Phenol sulphokinase, which transfers sulphate from PAPS to phenols, is found in soluble fractions of mammalian liver, kidney and intestinal mucosa.

When sulphate conjugation occurs in conjunction with glycoside conjugation, it is usually less important than the glycoside conjugation. This is possibly due to a limited availability of inorganic sulphate. If rabbits are given small doses

of a phenol, the sulphate forms a substantial part of the excreted products. With larger doses however, the amount of sulphate reaches a limiting value and glucuronide predominates (Smith, 1964). 1-Naphthyl glucoside predominates over the corresponding sulphate conjugate in excreta of flies dosed with 1-naphthol (Smith & Turbert, 1964).

#### Phosphate Conjugation

Inorganic phosphate is more abundant than inorganic sulphate in tissues, and organic phosphates are widely encountered in biochemistry, yet phosphate conjugates of phenols have only rarely been encountered (Parke, 1968). The hallucinogen, psilocin (2-(dimethylaminoethyl)-4-hydroxyindole) occurs in certain species of fungi *Conocybe* and *Psilocybe* as its O-phosphate ester (Downing, 1962). Phosphate esters of phenols are not normally found in mammals, but a phosphate ester of 1,4-dihydroxy-2-methylnaphthalene is found in the excreta of rats after feeding menadione (Vitamin K<sub>3</sub>, 2-methyl-1,4-naphthoquinone) (Jaques, Millar & Spinks, 1955). Phosphorylated intermediates of the K vitamins have been implicated in oxidative phosphorylation (Hatefi, 1963). A diaryl phosphate ester, di-(2-amino-1-naphthyl)hydrogen phosphate is excreted by the dog and man, but not the rat or rabbit, after feeding 2-naphthylamine (Boyland, Kinder & Manson, 1961). This is the only recorded instance of a diaryl phosphate ester occurring as a detoxication product.

Recently the formation of phosphate esters as detoxication

products has been reported in the housefly, which excreted a compound with paper chromatographic and ionophoretic properties of 1-naphthyl-dihydrogen phosphate after feeding of 1-naphthol (Smith & Turbert, 1964). Distribution of this new conjugation mechanism amongst invertebrates seems erratic. The onychophoran, *Peripatus*, forms phosphate esters from phenols and the scorpion *Palamnaeus* also forms traces, although other arachnids apparently do not (Smith, 1968). Tyrosine O-phosphate occurs naturally in third instar larvae of *Drosophila* during the rapid growth period following moulting but disappears on puparium formation (Mitchell & Lunan, 1964).

Although not a phenol conjugation, phosphorylation as a detoxication mechanism has been noted in a certain *E. coli* strain resistant to the amino-sugar antibiotics kanamycin and paromamine. Enzyme solutions from *E. coli* carrying R factor phosphorylated the 3-hydroxyl group of the 6-amino-6-deoxy-D-glucose moiety of kanamycin and the 3-hydroxyl group of the glucosamine moiety of paramamine to produce inactive products. ATP was an absolute requirement for the inactivation. Antibiotic activity could be restored by the action of phosphatase on the inactivated products (Umezawa *et al.*, 1967).

### Methylation

Methylation of amines, phenols and sulphydryl groups by transfer of methyl groups from S-adenosylmethionine is an important

reaction of biochemistry (Greenburg, 1963). Methylation of foreign compounds, however, is limited to substituted catechols. An enzyme, catechol-O-methyltransferase, present in the soluble fraction of several mammalian tissues, methylates foreign catechols, which are excreted largely as their monomethyl derivatives, as well as the endogenous catecholamine hormones. S-Adenosylmethionine acts as methyl donor (Williams, 1963, 1964). A microsomal catechol-O-methyltransferase also occurs in rat and rabbit liver. The catechol-O-methyltransferases do not methylate monohydric phenols, and apart from one compound, 3,5-diiodo-4-hydroxybenzoic acid, the excretion of methylated monohydric phenols by animals has not been observed to occur. 3,5-Diiodo-4-hydroxybenzoic acid is methylated by a soluble iodophenol O-methyltransferase which can also methylate a number of related iodophenols, for example, diiodotyrosine (Williams, 1963). Recently an enzyme capable of methylating a number of alkyl, methoxy and halophenols in vitro has been described (Axelrod & Daly, 1968). This enzyme is located in microsomal fractions of mammalian liver and lung tissue, and has no action on catechols or 3,5-diiodo-4-hydroxybenzoic acid. The physiological significance of this enzyme is not clear, since the excretion of simple phenols as methyl esters has not been observed. Also many of the ethers that could be formed by this enzyme would be immediately susceptible to demethylation again by the microsomal oxidases.

### The Functions of the Xenobiotic Metabolising Systems

The routes by which carbaryl and foreign compounds or xenobiotics in general are detoxified, particularly with regard to the conjugation reactions, have already been discussed. Several theories have been put forward to explain why foreign compounds should be metabolised by these routes, and how detoxication is effected.

### The chemical defence of hypothesis

The transformations of xenobiotics initially observed were reactions that resulted in a reduction of the toxicity of the compound being metabolised. Thus phenyl sulphate excreted by organisms administered with phenol (Baumann, 1876) was shown to be considerably less toxic than the parent phenol. Hence, these reactions came to be regarded as detoxication reactions and it was supposed that these were special chemical defences utilised by the body to guard against the effects of toxic foreign compounds (Sherwin, 1922). Sherwin considered that this chemical defence mechanism worked in two stages. First the body tried to eliminate the foreign compound by complete oxidation. If this foreign compound was able to withstand oxidation or to yield to it only partly, a second action was brought into play, a synthetic type reaction in which the foreign compound was combined with an endogenous molecule or radicle. This resulted in a reduction of toxicity and also an



increase in water-solubility. These two steps correspond to the phase I and phase II reactions as proposed by Williams (1959).

The hypothesis of a chemical defence based on detoxication implies that the metabolism of a foreign compound should always result in a reduction of toxicity or pharmacological activity. It is now known that as far as phase I reactions are concerned, this is not so, and there are many instances in which metabolism of a compound results in a retention or increase of toxicity or pharmacological activity. Notable examples are: the activation of phosphorothionate insecticides (O'Brien, 1959a) such as the conversion of parathion (O,O-diethyl O-p-nitrophenyl phosphorothionate) into the toxic oxo analogue paraoxon (O,O-diethyl O-p-nitrophenyl phosphate) (Gage, 1953), the epoxidation of heptachlor and aldrin to heptachlor epoxide (Perry, Mattson & Buckner, 1958), and dieldrin (Perry, Pearce & Buckner, 1964; Brooks & Harrison, 1964), and the hydroxylation and rearrangement of tri-O-cresyl phosphate to form the anticholinesterase cyclic saligenin phosphate derivatives (Eto, Casida & Eto, 1962). Microsomal activation of drugs is encountered with the hydroxylation of acetanilide to form N-acetyl-p-aminophenol (Brodie & Axelrod, 1948; Mitoma, Posner, Reitz & Udenfriend, 1956), O-demethylation of codeine to morphine (Adler & Latham, 1950), and the dealkylation of phenacetin to N-acetyl-p-aminophenol (Brodie & Axelrod, 1949) as a few examples. In the

case of carbaryl, hydroxylation of the naphthalene ring does not substantially reduce its anticholinesterase activity in vitro (Oonnithan & Casida, 1968).

The phase II reactions, the conjugations, do seem to result always in a reduction of toxicity, especially the widely encountered glycoside, sulphate and amino acid conjugations. Hence the conjugations may be regarded as true detoxication reactions. Moreover, many of the activated products produced by phase I reactions are subsequently detoxified by conjugation, such as the conjugation of N-acetyl-p-aminophenol with sulphate and glucuronate, resulting in a net detoxication by the combination of phase I and phase II reactions. Conjugation is a likely reason for the observed non-toxicity in vivo in insects of the hydroxylated derivatives of carbaryl (Leeling & Casida, 1966).

However, as has been recently emphasised (Smith, 1968), non-toxic foreign compounds may be metabolised as readily or more readily than toxic ones, hence it does not seem that toxicity of a xenobiotic is a direct basis for metabolism or conjugation.

Many of the transformations of foreign compounds of the phase I type appear to be carried out by enzymes of normal metabolic pathways, such as the oxidation of alcohols and aldehydes to carboxylic acids, hydrolysis of ester and amide bonds, and  $\beta$ -oxidation of  $\omega$ -substituted aliphatic acids (Williams, 1959; Parke, 1968). Hence it does not seem strictly correct to suppose

that a special defence mechanism exists to deal with foreign compounds. The idea that such a separate system does exist has been revived by the discovery of the microsomal oxidase enzymes (Brodie et al., 1955). These enzymes have the capacity to metabolise a wide variety of generally lipid-soluble foreign compounds, but not their natural endogenous counterparts (Brodie, 1956). Hence Brodie has suggested that the role of these enzymes is the metabolism of foreign compounds and not compounds of normal metabolism. In support of this it was noted that inhibitors of these enzymes such as SKF 525A and Lilly 18947 do not inhibit enzymes which catalyse reactions of similar natural compounds (Brodie et al., 1958). Also aquatic animals, such as the frog and fish, which apparently could readily excrete lipid-soluble compounds did not seem to possess the microsomal enzymes. It is now known though, that many of the normal steroid reactions, such as the biosynthesis of cholesterol (Scallen, Dean & Schuster, 1968), and the various hydroxylations of the steroid nucleus (Hayano et al., 1955), are carried out by microsomal enzymes. Also, microsomal oxidation systems have recently been demonstrated in frog and trout liver (Creaven, Parke & Williams, 1965), suggesting that the microsomes have normal biochemical functions.

The conjugation reactions, apart from methylation and acetylation, probably come closest to being special 'detoxication' reactions. They are normally responsible for inactivation of the

steroid hormones and natural compounds such as bilirubin (Schmid, Hammaker & Axelrod, 1957) and naturally occurring toxic substances such as phenol, cresol and aromatic acids which are absorbed from the gastro-intestinal tract from ingested food or as products of microfloral action (Williams, 1959).

#### Physico-chemical action hypotheses

The toxicity of a compound is partly dependent on properties which determine its rate of penetration to and concentration at the site of action. Berczeller (1917) suggested that the effects of conjugation could be explained in terms of surface tension changes. He observed that whereas saturated solutions of foreign compounds such as certain phenols or aromatic carboxylic acids have a considerably lower surface tension than that of water, the solutions of sulphate, glucuronide or sulphate conjugates of these compounds generally have an only slightly lower surface tension than that of water alone. According to his hypothesis, a substance which lowered the surface tension of water considerably would tend to accumulate at cell surfaces, tending to reach a toxic concentration. Conversion into a compound which did not markedly lower the surface tension of water would result in a reduction of toxicity, since the product would no longer tend to accumulate at cell surfaces but would be dispersed in solution.

However, some strongly ionised acids such as o-nitrobenzoic acid are excreted unchanged, or only metabolised slightly, such as

o-chlorobenzoic and salicylic acids (Williams, 1959), even though their solutions possess rather low surface tensions (Rose & Sherwin, 1926). The concentration at cell surfaces can hardly be a crucial aspect of activity of most toxic foreign compounds and hence the concept has not proved very versatile (Williams, 1959).

Probably the increase of water solubility that occurs with the conjugation of a foreign compound is more important than the reduction of surface tension effects. The conversion of a foreign compound into a strong electrolyte results in a reduction of the capacity to penetrate lipid cellular membranes (Hober, 1947). According to Brodie (1958), most conversions of drugs result in an increased polar nature, preventing them from passing cellular barriers and reaching sites of action. This effect is important when compounds effecting nerve function are considered. O'Brien (1959b) has shown that ionic compounds are ineffective as inhibitors of nerve function in vivo in insects. The lipid solubility of barbiturate drugs has an important bearing on the time of onset of anaesthesia in mammals (Brodie & Hogben, 1957).

The formation of glucosides by plants may also be of use, in that the conjugated compounds are concentrated in the watery vacuoles of the leaves or in the bark, and hence are removed from sites of metabolic activity.

#### Increased acidity hypothesis and the excretion of strong electrolytes

A clearer insight into the function of a detoxication

system can be gained from a consideration of the excretory mechanisms of animals. Excretion of a xenobiotic in some form or another must be its ultimate fate, since there is little means or room for storage of large amounts of foreign compounds in animal tissues.

A volatile foreign compound or metabolite such as ether or benzene may be expelled in expired air. Foreign compounds may also appear in secretions such as saliva, sweat or milk, apparently by passive diffusion across lipoid membranes (Sisodia & Stowe, 1964). However, the main route of elimination in vertebrates is in the urine or bile, and in insects through the Malpighian tubules.

Basically, the excretion in vertebrates and insects involves a filtration of proteins and cellular components from blood followed by an active resorption of water and salts required by the organism. The modified filtrate is then eliminated. In this system, non-polar, fat-soluble substances are reabsorbed through the lipoid membranes of the resorption structures and are retained along with the reabsorbed water. Water-soluble or highly ionised substances that are not actively reabsorbed, cannot pass through the lipoid barriers and hence are excreted.

In the vertebrate kidney the ultrafiltrate is produced by the glomerulus or Bowman's capsule, and passes through the proximal and distal convoluted renal tubules before discharge. The

glomerular fluid contains all the non-protein components of the plasma in the same concentration as in the plasma. In the proximal tubule mechanisms exist for the active secretion of ionised organic acids and bases. Compounds secreted by this means can be transferred into the tubular urine against high concentration gradients, thus increasing clearance beyond that obtained by glomerular filtration alone (Weiner & Mudge, 1964). Readsorption of water and essential salts and organic compounds by active transport occurs in the distal tubule. Lipid-soluble, non-polar compounds present in the glomerular filtrate are also readsorbed at this stage, by passive diffusion, whereas ionised or polar compounds are not readsorbed. Excretion of weak acids or bases is dependent on the pH of the urine; excretion is increased by a change of pH favouring ionisation of the compound.

Biliary excretion occurs by the secretion into the bile of ionic substances by active transport processes from the hepatic parenchymal cells. This active secretion occurs with compounds that have a molecular weight greater than 300 (Abou-El-Makarem, Millburn, Smith & Williams, 1967). Thus biliary excretion increases with increases of molecular weight above 300, accompanied by a parallel decrease in urinary excretion (Webb, Fonda & Brouwer, 1962). Conjugates excreted into the bile are liable to hydrolysis by hydrolytic enzymes present in the bile or intestinal secretions.

Hydrolysis products, if non-polar, may be readsorbed through the gut and recirculated back to the liver for rejugation and resecretion into the bile. Such an enterohepatic circulation has the effect of prolonging the retention of certain foreign compounds (Williams et al., 1965).

In insects the protein-free filtrate is produced by the Malpighian tubules, which are free in the haemocoel and connect with the alimentary canal at the junction of the mid gut and hind gut (Stobbart & Shaw, 1964). The filtrate or urine is apparently produced by diffusion of water, salts and organic compounds into the tubule and is isoosmotic with the haemolymph. The flow of water is stimulated by active secretion of potassium ions (Ramsay, 1953, 1954, 1955). The urine is discharged into the gut and excreted. Absorption of much of the water and essential salts and organic compounds occurs in the rectum. Readsorption of lipid-soluble material probably takes place here also although the situation is complicated by the presence of the cuticular lining of the rectum, which apparently behaves as a molecular sieve, with a low permeability to molecules the size of trehalose or larger (Stobbart & Shaw, 1964). Before excretion in the faeces partial hydrolysis of conjugates by gut hydrolases is possible (Robinson, Smith & Williams, 1953a; Robinson, 1957; Newcomer, 1954) depending on several factors such as the mixing of gut contents and Malpighian tubule fluid concentration in the rectum and frequency of elimination.



Contamination of the tubule fluid with midgut contents may be small, especially when fluid excretion is high (Shaw & Stobbart, 1963). If hydrolysis could occur and the compound released could be readsorbed in the rectum, a circulation analogous to the vertebrate enterohepatic circulation could occur, delaying the elimination of foreign compounds.

Excretion of a compound then in vertebrates and insects requires that it be ionised or polar. The most useful conjugates encountered in vertebrates, the sulphate, glucuronide and amino acid conjugates are all strong or moderately strong acids, being completely ionised at physiological pH values. These compounds are readily excreted by the kidney tubules whereas their precursors are not (Williams, 1959) for the reasons outlined above. As far back as 1932 Quick put forward the hypothesis that conjugation is a mechanism for converting foreign compounds into acidic compounds which can be readily excreted, rather than being a direct detoxication mechanism in the sense of rendering toxic molecules inert. More recently this concept of function has been extended to include the microsomal oxidations (Brodie et al., 1958).

Compounds which are strong acids such as the sulphonic acids, or strong carboxylic acids such as salicylic, o-chlorobenzoic, and o-nitrobenzoic acids are excreted mostly unchanged (Williams, 1959), lack of conjugation being explained by their ready excretion due to their acidic nature, and inability to penetrate effectively to sites of conjugation. Methylation does not occur as a conjugation to any

great extent apart from the catechol methylations, which seems reasonable since acidic or polar compounds would not normally be formed by methylation. The only exception is the formation of the N-methyl derivatives of pyridines and quinolines in vivo, and in this case methylation results in the formation of strong bases which are very much stronger than the precursors and are readily excreted, being completely ionised at physiological pH values (Robinson, Smith & Williams, 1953b). Glucosides formed by insects are not acidic; however this is not important since glucosides are polar, water-soluble and lipid-insoluble, and thus would be readily excreted by the insect excretory system. It has been shown that non-essential sugars are not readily reabsorbed by the insect rectum (Stobbart & Shaw, 1964).

The reabsorption of water by specific structures of the excretory systems with the production of concentrated urines is essential to animals living on dry land or in a hyperosmotic medium such as sea water to avoid large water loss and dessication. The fresh water fish, however, ingests large amounts of water from its environment and excretes large amounts of dilute urine. The large volume of urine would be sufficient to flush out lipid-soluble foreign compounds without the aid of conjugation. It is found that fresh water fish in fact lack a complete glucuronide synthesising system, apparently without any disadvantage (Brodie & Maikel, 1962).

In summary it can be said that the metabolism of a foreign

compound is not dependent on its toxicity but on its chemical structure. If a suitable functional group is present the compound will probably be conjugated. The glycoside, sulphate and peptide conjugation enzymes are of wide utility with low substrate specificity which can deal with a wide variety of compounds provided a hydroxyl or carboxyl group is present or can be introduced by oxidation or hydrolysis. These systems also conjugate normal compounds such as the steroid hormones. The main function of a conjugation reaction appears to be to convert a relatively lipid-soluble non-polar compound into an ionic or highly polar water-soluble compound that can be readily excreted. In some cases the conversion into a substance which can no longer penetrate cell barriers or concentrate in lipid structures will also be important, in plants as well as in animals.

The metabolism of foreign compounds by the phase I reactions (Williams, 1959) is largely by enzymes of normal metabolic pathways and depends on resemblance to natural substrates. The microsomal enzymes, however, have a well developed capacity for metabolising general lipid-soluble non-polar compounds and can metabolise a wide range of xenobiotics, especially by oxidation. Oxidation of analogous but slightly more polar normal compounds is apparently hindered by lipo-protein barriers (Brodie et al., 1958). The microsomal enzymes probably function to render molecules less lipid-soluble or to introduce a functional group for the action of

the conjugating enzymes.

Because the physico-chemical properties of a conjugate will be largely dominated by the hydrophilic portion of the molecule, a conjugate will almost always be non-toxic, regardless of the toxicity of the original molecule. However, the phase I reactions do not in most cases result in large changes of solubility, and the masking, unmasking, creation or elimination of a functional group can have an indifferent, inactivating or activating effect on the toxicity or pharmacological activity of a foreign compound. Consequently the phase I reactions can have an important bearing on the action of a pesticide or drug.

The detoxication mechanisms probably serve to protect organisms from the toxic effects of accumulated natural xenobiotics occurring in the diet and normal active compounds. The synthetic xenobiotics encountered by organisms today as pesticides, food additives, drug and industrial chemicals are an extra burden on the existing enzyme and transport systems. It is possible that a synthetic compound can be encountered against which an organism has little defence. The compound may be resistant to transformation by any of the enzyme systems present or, on the other hand, may so resemble a normal substrate that normal functioning will be seriously disrupted. Examples of the former case are the chlorinated hydrocarbons, many of which are only very slowly metabolised, and, being lipid-soluble and therefore not able to be excreted, tend to

accumulate in fatty tissues and can reach lethal concentrations. An example of the latter case is fluoroacetate, only very rarely encountered naturally, which mimics acetate and enters the citric acid cycle, eventually forming fluorocitric acid which inhibits aconitase, thus blocking the tricarboxylic acid cycle and disrupting energy production (Peters, 1963).

## EXPERIMENTAL

## Conjugation of Carbaryl and 1-Naphthol in Insects

### Materials and Methods

#### Reference compounds and diluents

1-Naphthyl  $\beta$ -D-glucoside was prepared by the general method of Glazer & Wulwek (1924). Tetra-O-acetyl- $\alpha$ -D-glucosyl bromide, (Barczai-Martos & Korosy, 1950) in acetone (15%), was mixed with a 1.5 molar excess of sodium naphthoxide in water (10%) and left at room temperature for four hours with gentle stirring. The bulk of the acetone was removed under vacuum and the remaining oily suspension extracted with chloroform. The aqueous layer was discarded and the chloroform solution washed once quickly with 0.5 N-NaOH, and then washed three times with water and dried with anhydrous sodium sulphate. The chloroform was then evaporated at 50° under vacuum and the remaining oil dissolved in two volumes of ethanol. On cooling, crystals of 1-naphthyl glucoside tetra-acetate formed, and were recrystallised from ethanol ethyl acetate (5:1 v/v) m.p. 174°; yield 25% recrystallised. The tetra-acetate was deacetylated in absolute ethanol with a catalytic amount of sodium ethoxide. On concentrating the solution and adding benzene, 1-naphthyl  $\beta$ -D-glucoside crystallised out, and was recrystallised from

water, m.p.  $170^{\circ}$ .

(1-Naphthyl glucosid)uronic acid was prepared by the procedure of Marsh & Levvy (1958) by oxidation of the glucoside with oxygen in presence of Adam's platinum catalyst. An aqueous solution of 1-naphthyl  $\beta$ -D-glucoside and freshly prepared catalyst were placed in a tall narrow cylindrical vessel with a coarse sintered glass at its base, through which oxygen was forced into the mixture. The vessel was placed in a water bath maintained at  $85^{\circ}$ . The pH of the reaction mixture was maintained at 8 during the reaction by addition of 0.5 M- $\text{NaHCO}_3$ . After the calculated amount of bicarbonate had been taken up and the solution filtered, the product was isolated as its sparingly soluble *p*-toluidine salt by addition of *p*-toluidine hydrochloride, yield 40%, m.p.  $153-5^{\circ}$  (Berenbom & Young, 1953). The *p*-toluidine salt was converted to the free acid by the method of Berenbom & Young (1951). Sodium (1-naphthyl glucosid)uronate was prepared by neutralisation of the free acid and crystallised from ethanol-water. (Found: Na, 6.1;  $\text{H}_2\text{O}$ , 9.8;  $\text{C}_{16}\text{H}_{15}\text{NaO}_7 \cdot 2\text{H}_2\text{O}$  requires Na, 6.1,  $\text{H}_2\text{O}$ , 9.5%).

1-Naphthyl potassium sulphate was prepared by the action of chlorosulphonic acid on 1-naphthol in pyridine, followed by neutralisation with saturated aq. KOH, according to Burkhardt & Lapworth (1926). The *p*-toluidine salt was prepared by the



method of Barton & Young (1943) by dissolving separately equimolar amounts of the potassium salt and *p*-toluidine hydrochloride in minimal amounts of water at 60°, mixing, and cooling rapidly. The *p*-toluidine salt crystallised out immediately. (Found: S, 9.9;  $C_{17}H_{17}NO_4S$  requires S, 9.7%). The 5-aminoacridine salt of 1-naphthyl hydrogen sulphate was obtained as the very sparingly soluble crystalline precipitate by mixing aqueous solutions of the *p*-toluidine salt and 5-aminoacridine hydrochloride. (Found: S, 7.8;  $C_{23}H_{18}N_2O_4S$  requires S, 7.7%). Di-1-naphthyl hydrogen phosphate, m.p. 137°, was prepared by the method of Friedman & Seligman (1950). The cyclohexylamine salt of di-1-naphthyl hydrogen phosphate was very sparingly soluble in water and sparingly soluble in ethanol, m.p. 216°. (Found: equiv. wt. 445;  $C_{26}H_{28}NO_4P$  requires, equiv. wt. 449).

Di-sodium 1-naphthyl phosphate was a commercial sample that was shown to be pure by paper chromatography. The monocyclohexylammonium salt of 1-naphthyl dihydrogen phosphate was sparingly soluble in water and was obtained by the addition of one equivalent of cyclohexylammonium hydrochloride to a moderately acidified (pH 3-4) solution of the disodium salt; m.p. 193°, (Found: equiv. wt. 338;  $C_{16}H_{22}NO_4P$  requires equiv. wt. 323).

Sulphur in sulphate esters was estimated by weighing as barium sulphate after hydrolysis to inorganic sulphate in dilute hydrochloric acid and precipitation with barium chloride. Equivalent weights were determined by titration in glacial acetic acid against 0.1 N acetous perchloric acid.

#### Labelled Compounds

[<sup>14</sup>C]1-naphthol (1 mc/m-mole) was obtained from the Radiochemical Centre, Amersham, Bucks., England. Solutions were kept in the dark and the purity was checked by dilution analysis. After a few months the stock solution showed a slight decomposition and was repurified by thin layer chromatography using the solvent system ether-isooctane (3:7 v/v).

[<sup>3</sup>H]carbaryl was prepared from [<sup>3</sup>H]1-naphthol and methyl isocyanate. 1-Naphthol was tritiated by the method of Yavorsky & Gorin (1962) adapted for use on a small scale by Hilton & O'Brien (1964). The tritiating reagent was prepared from 180 mg. of phosphorus pentoxide quickly weighed into a small vial with a plastic cap and 0.65 µl. THO (approx. 4 c/ml.). Instead of using a 3 mm. magnetic follower to stir the mixture, two glass beads were added to the vial and the mixture stirred by rotating and shaking. Then boron trifluoride gas, generated by heating boron trifluoride ammoniate with conc. H<sub>2</sub>SO<sub>4</sub> in an

oil bath at 200<sup>0</sup>, was passed into the vial slowly for 7 min. with shaking. 200 mg. of 1-naphthol in 10 ml. of cyclohexane was then added, the cap replaced, and shaking continued for 20 hr. The contents were poured into a separating funnel and the vial rinsed with cyclohexane, water, and then chloroform. The mixture contained much polymeric material insoluble in cyclohexane but soluble in chloroform. The organic fraction, after washing with water three times and drying with anhydrous sodium sulphate formed, on removal of solvent under vacuum, a sticky gum. Thin layer chromatography of a portion of this using solvent system E revealed on examination under u.v. the presence of at least three substances besides 1-naphthol. Hence the gum was taken up into ether and purified by extraction into dilute alkali and re-extraction into ether following acidification. The final ether extract was applied as a wide band on a thin layer plate and chromatographed with solvent system E. A major band of  $R_F$  0.53 corresponding to 1-naphthol was present along with another minor fluorescent band of  $R_F$  0.30. The yield of 1-naphthol was apparently very low so no attempt was made to recover it from the chromatogram before conversion into carbaryl.

Silica gel containing the [<sup>3</sup>H]1-naphthol was scraped into a test-tube, covered with ether, excess methyl isocyanate and a trace of triethylamine as catalyst were added, and the tube was

kept securely stoppered. After 24 hr. the ether was decanted off and the silica gel washed several times with ether, and the combined ether extracts were evaporated to small volume and chromatographed on thin layer as above in solvent system E. A strip of the chromatogram was sectioned and counted in the scintillation spectrometer. Most of the activity was present as a peak corresponding to the carbaryl band (quenching under u.v.;  $R_F$ , 0.25) and a small peak (<5%) corresponding to the 1-naphthol band.

[ $^3\text{H}$ ]Carbaryl was eluted off the silica gel with ethanol and made up to 10 ml. Carbaryl in this solution was estimated by u.v. absorption ( $\lambda$  max 278,  $\epsilon$  max  $6.09 \times 10^3$ ) and radioactivity was assayed in the scintillation spectrometer; yield; 3 mg., sp. act. 41 mc/m-mole. The ethanolic solution was evaporated to dryness and sufficient dry acetone added to give a concentration of 0.5  $\mu\text{g.}/\mu\text{l.}$ , for dosing. Assay by thin layer chromatography in solvent system E showed that 99.9% of the activity chromatographed as carbaryl.

#### Insects and dosing

Houseflies, Musca domestica, were an insecticide-susceptible strain obtained from the Wallaceville Animal Research Station, New Zealand or a DDT-resistant but carbamate-susceptible strain supplied by Dr. D. Spiller, Auckland. Larvae were reared

in the laboratory on an agar gel containing 100 g. of yeast, 100 g. of full cream milk powder, and 20 g. agar/l. Adults were kept in wire mesh cages and had access to sucrose, or sucrose and milk powder mixture, and water. Eggs were laid on milk-soaked cotton rolls. Insecticide-susceptible blowflies, Lucilia sericata, were reared in the same way except that adults had access to fresh liver for egg laying. Grass grubs, Costelytra zealandica (White), were collected in the field by the Entomology Division of the D.S.I.R. at Nelson and sent packed in individual compartments in ice-cube trays packed with moist soil. The grubs were stored in the trays at 4° for 2-3 weeks until required.

[<sup>3</sup>H]Carbaryl was administered to the insects by topical application at a dose of 0.5 ug. in 1 µl. acetone/insect.

[<sup>14</sup>C]1-Naphthol was dissolved in aq. (50/50) acetone (5 mg./ml.) and administered topically to the thorax of flies and by injection into the haemocoel of grass grubs at a dosage of 0.25 µg./mg. of insect. Average weight of the houseflies was 20 mg., of blowflies 50 mg., and of grass grubs 100 mg./insect.

### Ionophoresis

Ionophoresis was carried out on Whatman's No.1 paper clamped between glass plates, 40 cm. x 9 cm. Paper strips, 46 cm. x 9 cm., were cut with the length running across the machine-run direction of the paper, to minimise diffusion effects along the

direction of migration. The ends of the paper dipped into reservoirs of electrolyte containing the electrodes. A potential difference of 400 v. was applied across the ends of the paper giving a field strength of 10 v./cm. For buffers of pH values 2 to 11 Britton & Robinson's straight-line buffer mixture (B.D.H. Ltd.), was used, and outside this range appropriate strengths of hydrochloric acid or sodium hydroxide were used.

Compounds were applied as small diameter spots on a line 18 cm. from the cathode end of the paper. Buffer was applied by dipping the ends into buffer solution and allowing it to soak just up to the starting line, from each direction. Excess buffer was then blotted off. For identification, reference compounds were run alongside the unknowns. Compounds were located under u.v., and sections containing radioactive compounds were cut out and assayed in the scintillation spectrometer.

No significance could be attached to absolute migration rates since no allowance was made for electro-osmotic effects. All migrations were measured relative to reference glucoside to show the degree of separations achieved.

#### Chromatography

Paper chromatography was carried out on Whatman's Nos. 1, 4, 31 or 3 MM papers, using the descending technique. Solvent fronts were usually allowed to run 40 cm. from the origin.

Solvent systems used were: A, butan-1-ol-ethanol water, (17:3:20, by vol.); B, butan-1-ol-aq.  $\text{NH}_3$  (sp. gr. 0.88)-water, (4:1:5, by vol.); C, propan-1-ol-aq.  $\text{NH}_3$  (sp. gr. 0.88)-water, (7:3 v/v); and D, butan-2-one-water-2N-acetic acid, (80:20:1, by vol.). Upper layers of two-phase systems were used.

Thin layer plates, 0.3 mm. thickness were made up using Merck Silica Gel G.F. Solvent fronts were allowed to run 10 cm. from the origin. Solvents used were: E, di-isopropyl ether-isooctane (1.5:7.5, v/v); and F, di-ethyl ether-isooctane (3:7 v/v).

#### Enzyme preparations

The following hydrolases were used in the identification of conjugates: alkaline phosphatase (purified, Sigma Chemical Co., St. Louis, Mo., U.S.A.),  $\beta$ -glucosidase (emulsin, L. Light & Co. Ltd., Colnbrook, Bucks.) (possessing slight phosphatase activity), and a 'bovine ileum phosphatase' which possessed slight  $\beta$ -glucosidase activity (British Drug Houses Ltd., London).

#### Measurement of Radioactivity

Samples in dilution analyses were counted in a Geiger-Muller end window counting system. The samples were counted at infinite thickness ( $> 60 \text{ mg./cm}^2$  for  $^{14}\text{C}$ ), in aluminium planchets positioned reproducibly in a mounting under the counting tube.

The counting tube and source mounting were housed in a lead castle.

Solid samples were applied on to the planchets as pastes with alcohol or n-heptane, and carefully dried under an infra-red lamp. With care, an even layer was obtained free from cracks or shrinkage. [ $^{14}\text{C}$ ]1-Naphthol administered to insects was measured by adding a dose to a weighed amount of phenoxyethanol and counting as an infinitely thick liquid layer, of the same geometry as the solid samples. Homogenates were assayed as aqueous suspensions.

The apparatus was calibrated with a standard [ $^{14}\text{C}$ ]polystyrene source (1  $\mu\text{C/g.}$ ) mounted in a planchet and having the same geometry with respect to the counter as the unknown sources. Under the set conditions this standard source gave a count rate of 2180 c.p.m. During assays, sufficient counts were allowed to accumulate to give a standard deviation of 2%, except that counts were not continued for more than 1 hr.

A Packard 4312 scintillation spectrometer was used for tritium counting and quantitative  $^{14}\text{C}$  counting. Tritium was counted with discriminator settings set at 50-1000, with 56% gain, and  $^{14}\text{C}$  counted with the discriminator settings at 50-500 with 5% gain. Efficiency of counting in homogenous systems was monitored with the automatic external standardisation (A.E.S.) facility. Under set conditions counts in the A.E.S. channel



were compared with an efficiency vs. A.E.S. counts correlation curve, set up using standard samples. For tritium monitoring discriminator settings used in the A.E.S. channel were 1000- $\infty$  at 3% gain, and for  $^{14}\text{C}$ , 300-700 at 2% gain.

A solution consisting of 5 g. 3,5-diphenyloxazole and 0.3 g. 1,4-bis(4-methyl-5-phenoxazol-2-yl) benzene/l. of toluene was used as the scintillation fluid for all counting. In unquenched systems toluene-soluble substances could be counted with efficiencies of 38% for tritium and 78% for  $^{14}\text{C}$ , at the discriminator settings used above.

Paper chromatograms on 2 cm. wide strips of Whatman No.4 paper were cut into 1 cm. sections and counted in glass vials containing 5 ml. of scintillator. Counting efficiency of sodium [ $^{14}\text{C}$ ]benzoate on Whatman No.1 or 4 paper was 52% at the discriminator settings used. This value was used to calculate the recovery of 1-naphthol metabolites on paper chromatograms. Quenching by material on the chromatograms was shown to be negligible by comparing the count rate of 0.001  $\mu\text{C}$  of sodium [ $^{14}\text{C}$ ]benzoate on 1 cm. sections of similar chromatograms prepared from undosed insects with that on 1 cm. x 2 cm. sections of clean chromatography paper. Efficiency was also unaffected by the position of the paper in the vial with regard to the photo-multiplier tubes, and by varying the weight of paper in the vial.

Interference by phosphorescent materials on the chromatograms was absent. Assay of sections of chromatograms from undosed insects showed that only background levels of activity were present.

The counting efficiency of tritium on paper was not assessed, although other workers have reported efficiencies of 5-10% (Gill, 1964), being somewhat dependent on presence of quenching material, and to geometrical factors.

Thin layer chromatograms were marked out into 1 cm. or 0.5 cm. sections and the silica gel scraped into vials containing 5 ml. toluene scintillator for counting. When toluene-soluble compounds were present it was assumed that these were totally eluted off the gel into the scintillator and counted at maximum efficiencies. The presence of silica gel at the bottom of the vials did not affect the efficiency of tritium or  $^{14}\text{C}$  counting in toluene scintillator.

## Results 1. Metabolism of Carbaryl

### Paper Chromatography

Twenty houseflies, 10 blowflies, or 10 grass grubs were dosed as described with [ $^3\text{H}$ ]carbaryl and kept 24 hr. in conical flasks loosely plugged with cotton wool. The insects were transferred to Potter-Elvehjem homogeniser tubes and the flasks and cotton carefully rinsed with several small portions of acetone and water alternately, the washings being added to the insects in the tubes for homogenisation. The homogenates were diluted with sufficient acetone to give a final water-acetone ratio of 1:5, total volume 10 ml., and refluxed gently in the tubes at 60 $^{\circ}$  for 4 hr., along with 1 mg. of the non-radioactive reference compounds 1-naphthyl phosphate, sulphate, and glucoside, and 0.5 g. of non-radioactive 1-naphthol and carbaryl. Preliminary experiments had shown that refluxing was necessary to solubilise activity absorbed on to solid residues.

The extracts were centrifuged to remove solid debris, the debris being re-extracted once with acetone-water. The combined extracts were evaporated to small volume, made up to 5 ml. with water, and extracted with 4 x 5 ml. benzene. The addition of the non-radioactive 1-naphthol and carbaryl was

necessary to ensure complete extraction of the corresponding radioactive compounds into the benzene fractions, as otherwise they interfered with the chromatography of the aqueous fraction.

Benzene extracts were pooled and made up to 50 ml. with benzene. Portions of 0.5 ml. were transferred to scintillation vials containing 19.5 ml. toluene scintillator, and counted in the scintillation spectrometer with quench determination. This was used to calculate the recovery of active toluene-soluble compounds from the insects, and hence, by difference, the % of the injected dose converted into water-soluble metabolites (Table 6). Recovery of toluene solubles from control groups of insects that were homogenised immediately after dosing was greater than 95% of the injected dose.

The aqueous fractions were concentrated again and transferred on to Whatman No.4 paper as bands 10 cm. across. The papers were cut into 2 cm. wide strips and chromatographed with solvent system A. Strips were then cut into 1 cm. sections and counted in the scintillation spectrometer.

Histograms prepared from these scans of each of the insects (Fig.1) showed major radioactive peaks corresponding to reference 1-naphthyl phosphate, sulphate, and glucoside. A major peak was also present near the origin, and two smaller peaks, one just after the origin peak, and the other just before

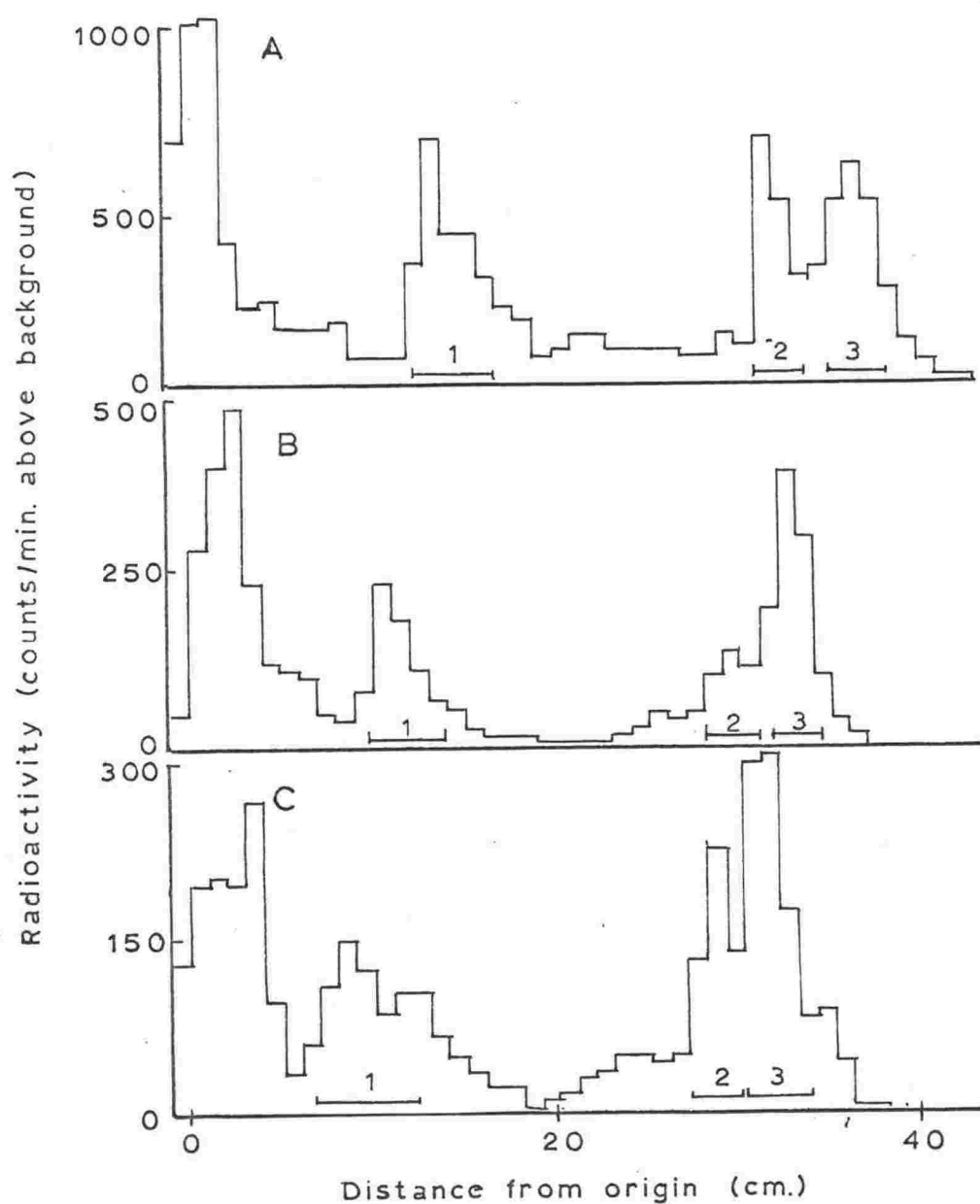


Fig.1. Chromatography of water-soluble extracts from A, houseflies; B, blowflies; C, and grass grubs, dosed with [ $^3\text{H}$ ]carbaryl. The extracts were prepared and chromatographed as described in the text, and 1 cm. sections of the chromatograms counted in the scintillation spectrometer. Bars indicate positions of co-chromatographed non-radioactive conjugates of 1-naphthol with 1, phosphate; 2, sulphate; and 3, glucoside.

Table 6. Formation of water-soluble metabolites  
of [ $^3\text{H}$ ]carbaryl by insects

Insects were dosed with 0.5  $\mu\text{g}$ . [ $^3\text{H}$ ]carbaryl, and after 24 hr. the total of water-soluble metabolites formed was determined as described in the text.

Water-soluble metabolites, as % of dose

Houseflies (groups of 20)	66	70	
Blowflies (groups of 10)	62	65	
Grass grubs (groups of 10)	49	55	52

the sulphate peak. Control chromatograms prepared from insects homogenised immediately after dosing, showed only slight activity above background at the origin, and no significant activity in any of the other regions.

#### Enzymic hydrolysis of metabolites

Metabolites were further identified by treatment with phosphatase or  $\beta$ -glucosidase, and counting of toluene-soluble radioactive material released by hydrolysis.

Chromatograms of water-soluble metabolites from carbaryl-dosed insects were prepared and counted in the scintillation spectrometer as previously. After counting, the paper sections

were removed from the vials and carefully set aside, and the vials were counted again to confirm that no activity had been eluted off the papers into the toluene scintillator.

The sections of paper were washed with toluene to remove traces of scintillation fluid, dried, set out on glass plates and soaked with 0.05 ml. of alkaline phosphatase solution (B.D.H., bovine ileum phosphatase) or  $\beta$ -glucosidase solution (L. Light & Co. Ltd.). The papers were set aside overnight in a warm humid place, and then dried, examined under u.v., and replaced in vials containing 5 ml. scintillator, and the vials counted; first with the papers in, and then with the papers removed. Counts recorded in vials after paper had been removed represented toluene-soluble material eluted off the papers into the scintillator. As it was initially shown that no toluene-soluble material was present, this must have arisen from action of the enzyme solution.

Phosphatase-treated chromatograms had strong blue fluorescent regions near the origin and at the zone corresponding to reference 1-naphthyl phosphate, both coinciding with the radioactive peaks previously detected. Prior to phosphatase treatment these regions had deep purple fluorescence. Histograms of phosphatase-treated chromatograms of water-soluble metabolites of [ $^3\text{H}$ ]carbaryl in the housefly, blowfly and grass grub (Fig.2) showed peaks of toluene-soluble activity corresponding

to the origin peak and phosphate peak of untreated chromatograms. No activity was released from under the sulphate peak or the minor one preceding it, but slight activity was eluted from the glucoside region, possibly owing to the slight  $\beta$ -glucosidase activity of the phosphatase preparation used.

Glucosidase-treated chromatograms had the strong blue fluorescence characteristic of 1-naphthol and derivatives in regions corresponding to all four of the major radioactive peaks noted previously. Histograms of glucosidase-treated chromatograms (Fig.3), prepared as above, showed that toluene-soluble activity was released from regions corresponding to all four of the major water-soluble radioactive peaks. The glucosidase used had weak phosphatase activity which could explain the hydrolysis of the origin and phosphate peaks, although the presence of glucosides in these regions cannot be ruled out. However, glucosides would not be expected to have such low  $R_F$  values with the solvents used for chromatography, unless possessing a rather polar aglycone. Release of activity from under the 'sulphate' peaks suggests that some of the material was of glucoside nature. Release of activity from under the 'glucoside' peaks was less than expected, since the glucosidase preparation had strong activity; possibly some glucosidase-resistant material was present.



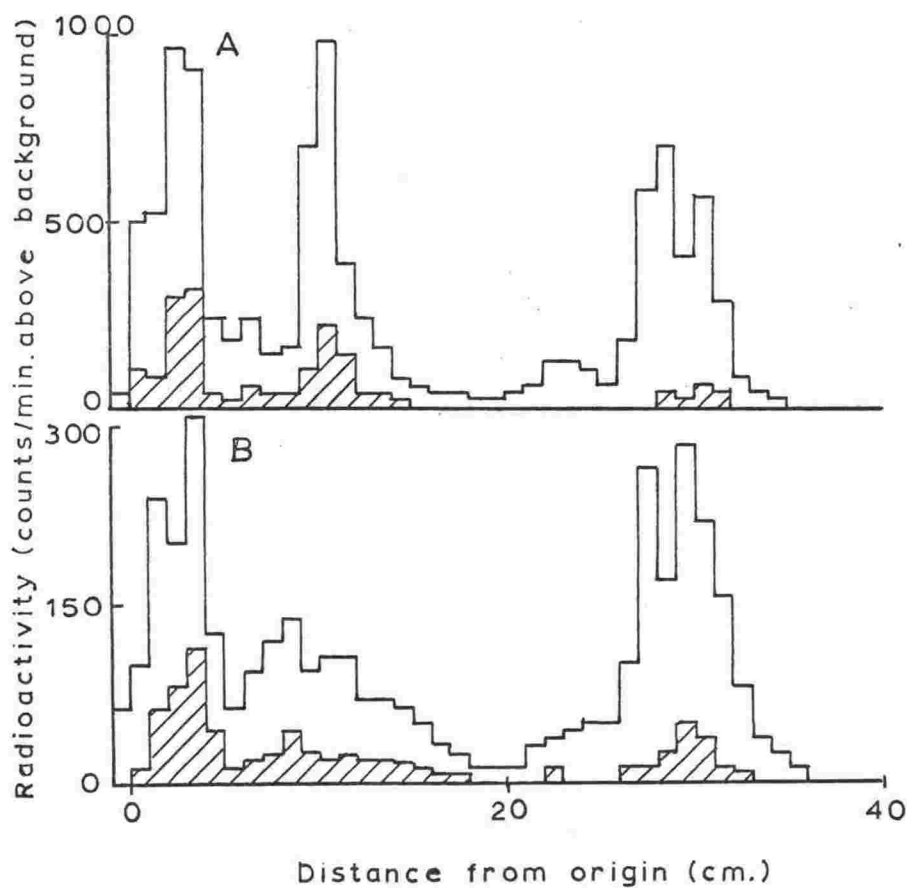


Fig.2. Action of phosphatase on water-soluble metabolites of [ $^3\text{H}$ ]carbaryl in A, the housefly; and B, grass grub. Open areas indicate counts on untreated chromatograms of aqueous extracts, and the shaded areas, toluene-soluble activity released by treatment with phosphatase, as described in the text.

### Ionophoresis of water-soluble metabolites

The substances giving rise to the four major peaks on the paper chromatograms described previously, designated 'origin', 'phosphate', 'sulphate', and 'glucoside' peaks, were further examined by ionophoresis.

Chromatograms, 10 cm. wide, of aqueous extracts of 20 houseflies or 10 blowflies or grass grubs dosed with [ $^3\text{H}$ ]carbaryl were prepared as previously. The four main sections of radioactivity, located by assay of 2 cm. wide vertical strips in the scintillation spectrometer, were cut out and eluted with 0.1 N- $\text{NH}_3$ . These eluates were concentrated and portions subjected to ionophoresis at pH values of 2.5, 8 and 12.5 (0.05 N-NaOH).

These pH values allowed ready separation of phosphomonoesters, sulphate esters, glucosiduronic acids, glucosides and free phenols, and other compounds with free phenolic groups. Relative migrations of 1-naphthol and its conjugates are quoted in Table 7 to indicate degree of separations obtainable.

Each radioactive eluant was applied as a spot on the centre of the starting line, and reference 1-naphthyl phosphate and glucoside were applied as spots on either side. After ionophoresis was completed the papers were dried and the reference compounds located under u.v. Also visible if present were the reference compounds added to the initial homogenates and eluted off the

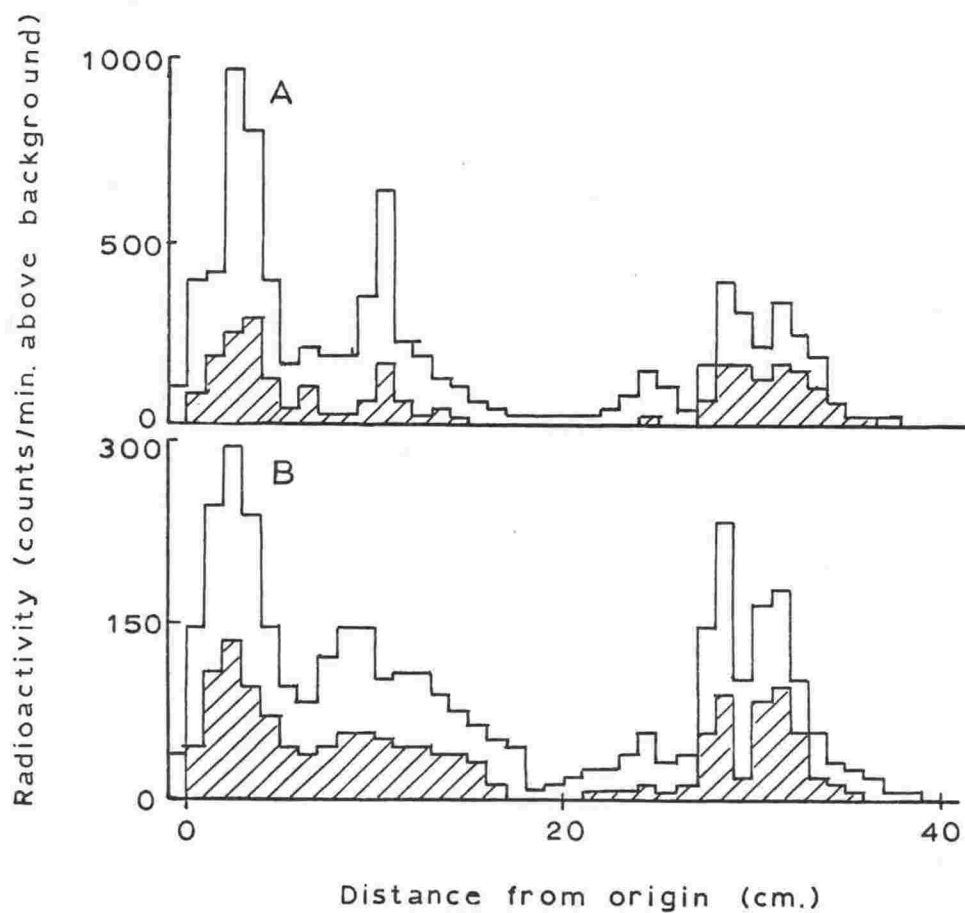


Fig.3. Action of  $\beta$ -glucosidase on water-soluble metabolites of [ $^3\text{H}$ ]carbaryl in the housefly, A, and grass grub, B. Open areas indicate counts on untreated chromatograms, and shaded areas the toluene-soluble activity released by  $\beta$ -glucosidase treatment.

chromatogram and ionophoresed along with the radioactive material. Median strips containing radioactive material were cut out for a distance of 15 cm. towards the anode from 1 cm. on the cathode side of the starting line, cut into 1 cm. sections and counted in the scintillation spectrometer.

A complex picture was revealed by ionophoresis, all the four major radioactive peaks from the chromatograms being resolved into two or more components. Housefly and blowfly histograms were similar, while grass grub histograms differed quantitatively in some respects, and were less complicated.

Housefly and blowfly. The 'origin' eluate had at least three components, a, b and c (Fig.4). Two of these, b and c, had phosphate-type migrations at pH values 2.5 and 8, although not coinciding exactly with reference 1-naphthyl phosphate. Component 'a' did not migrate at pH values 2.5 or 8, but all components apparently had larger migrations at pH 12.5 indicating presence of free phenolic hydroxyl groups.

The 'phosphate' eluate also had three components, d, e and f. Component d did not migrate, but was rather smaller in the pH 8 and 12.5 runs than in the pH 2.5 run, and at this pH could possibly be due to activity bound on to residues on the starting line since the peak did not coincide with the reference glucoside. Components e and f had phosphate-type migrations, e being much

Table 7. Ionophoresis of 1-naphthol and conjugates

Figures represent migration, relative to glucoside towards the anode after ionophoresis for 2 hr. at 10 v./cm., in Britton & Robinson buffer, pH 2.5 and 8, or 0.05 N-NaOH, pH 12.5.

	pH 2.5	pH 8	pH 12.5
1-naphthol	0	0.5	4
phosphate	5.5	8.5	9
sulphate	5	5	6
glucuronide	0	4	4.5
glucoside	0.0	0.0	0.0

larger than f. Component f coincided exactly with reference and co-ionophoresed 1-naphthyl phosphate.

The sulphate eluate resolved into two components, g and h. Component g did not migrate, and h coincided with 1-naphthyl sulphate. At pH 12.5 some of the activity in g seemed to move with h, indicative of the presence of phenolic compounds.

The 'glucoside' peak eluate had two components, i and j; component i coinciding with reference glucoside and j having a sulphate-type migration. Again at pH 12.5 migrations were

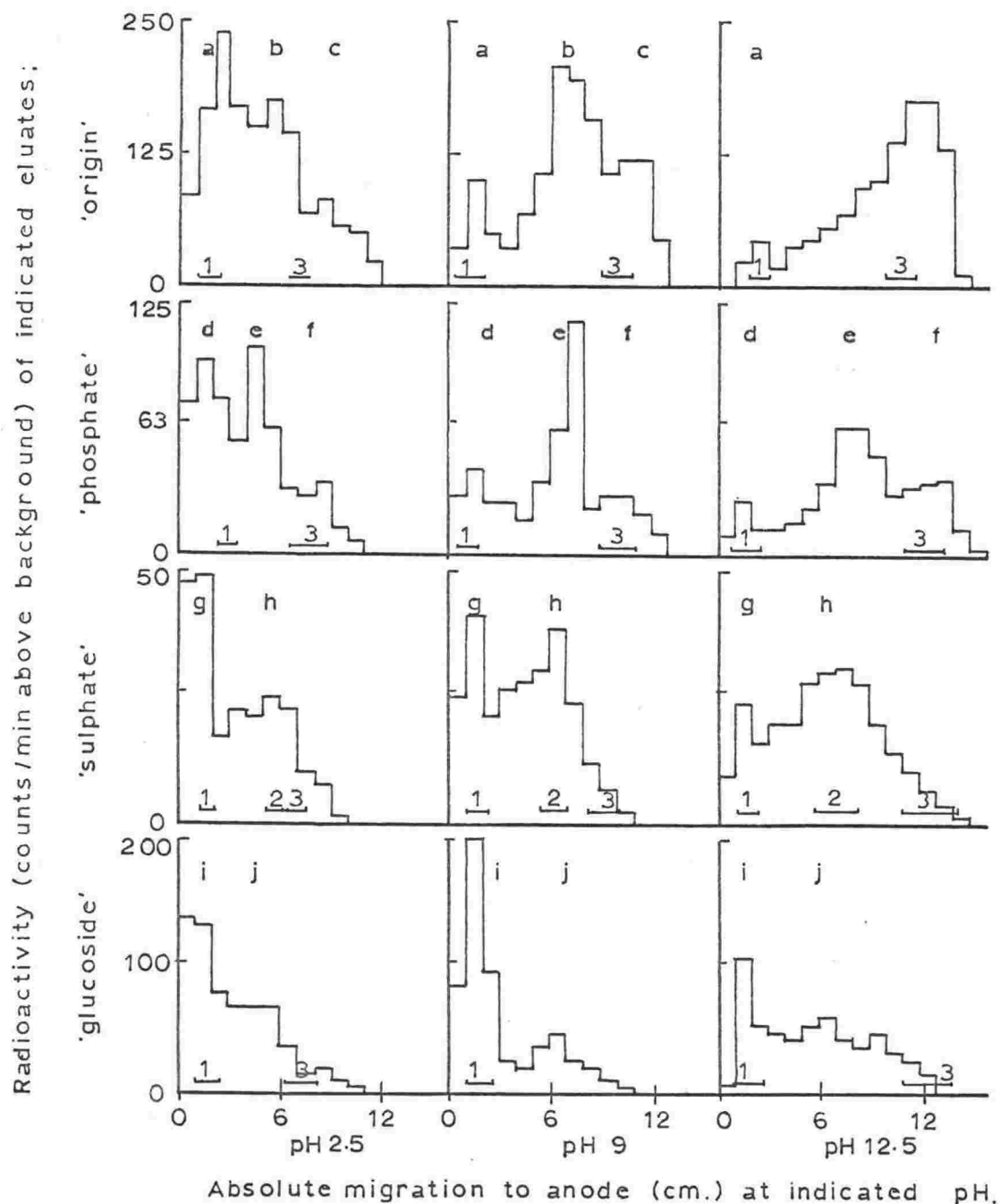


Fig.4. Ionophoresis of major peaks from chromatograms of water-soluble [ $^3\text{H}$ ]carbaryl metabolites from the blowfly. Bars indicate positions of reference compounds: 1, 1-naphthyl glucoside; 2, 1-naphthyl sulphate; and 3, 1-naphthyl phosphate. Lettered peaks are referred to in the text.

somewhat increased, with a new component appearing ahead of j.

Grass grub (Fig.5). The 'origin' eluate had three components, with similar migrations as the corresponding housefly and blowfly components, except that the neutral peak was more dominant and did not move even at pH 12.5.

The 'phosphate' eluate also had three components, d, e and f similar to the corresponding housefly and blowfly eluate. However, component d was very minor, and component f, coinciding with 1-naphthyl phosphate was as prominent as e.

The 'sulphate' eluate migrated as a very sharp peak coinciding with 1-naphthyl sulphate, with only minor other peaks being present.

The 'glucoside' eluate behaved similarly to the corresponding housefly and blowfly eluate.

## Results 2. Conjugation of 1-Naphthol.

### Dilution analyses

Ten grass grubs were each injected with 20  $\mu\text{g}$ . (0.5  $\mu\text{c}$ .) of [ $^{14}\text{C}$ ]1-naphthol and left for 8 hr. The grubs were then homogenised in ice-water in a Potter-Elvehjem homogeniser, and the homogenate diluted to 10 ml. with ice-water. Portions of the homogenate ( 1 ml.) containing the equivalent of one grass grub were diluted to 5 ml. with acetone and refluxed gently for

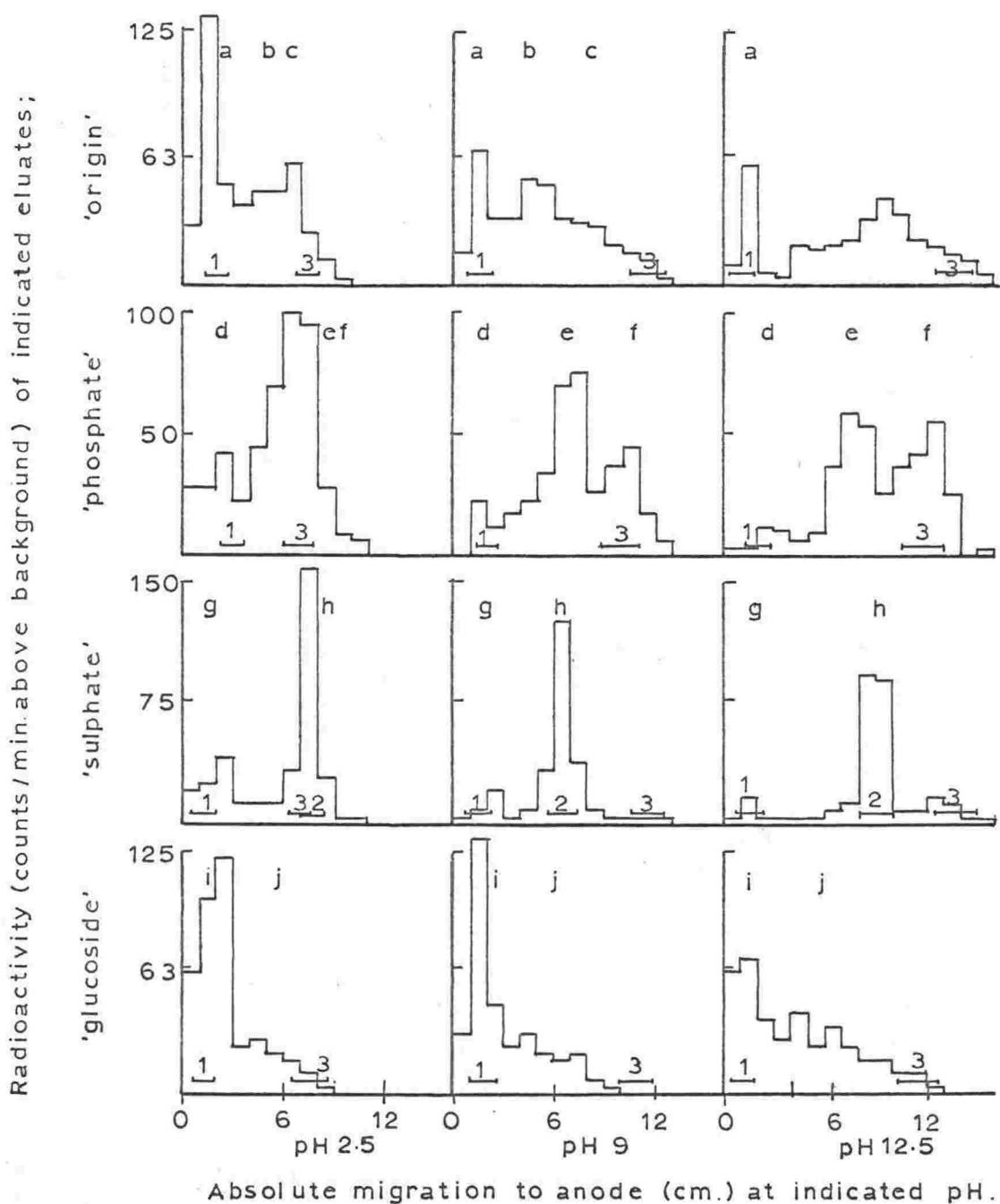


Fig.5. Ionophoresis of major peaks from chromatograms of water-soluble [ $^3\text{H}$ ]carbaryl metabolites from the grass grub. For explanations, see Fig.14.



4 hr. along with 300 mg. of one of the dilution compounds. Portions of the homogenate were also withdrawn before, during and after sampling for dilution analyses, and the specific activity assayed directly to check on the injected dose, and to ensure that the homogenate remained homogenous during sampling. Diluents used were: 1-naphthol, 1-naphthyl potassium sulphate, 1-naphthyl disodium phosphate, di-1-naphthyl hydrogen phosphate, 1-naphthyl  $\beta$ -D-glucoside, and (1-naphthyl glucosid)uronic acid.

The extracts were centrifuged to remove solid residues, the residues being re-extracted once briefly with acetone-water, and the combined extracts evaporated to small volume on a rotary evaporator. Preliminary experiments in which the residues were assayed showed that the 4 hr. refluxing was necessary to solubilise absorbed activity.

1-Naphthol was recovered from the concentrated residue by extraction with ether, which was evaporated to dryness, and the residual 1-naphthol crystallised from n-heptane to constant specific activity.

The concentrated eluates containing the other diluents were transferred to large sheets of Whatman No.31 or 3 MM paper and chromatographed in solvent system C, except in the case of 1-naphthyl glucoside which was chromatographed in solvent system A.

The zone of diluent in each case was located on the chromatogram by its fluorescence under u.v., cut from the chromatogram, and eluted from the paper with 0.1 N-NH<sub>3</sub>.

The eluates were concentrated to small volume under vacuum and the diluents crystallised to constant specific activity either directly or as suitable derivatives. The chromatographic procedure resulted in considerable clean-up and except with the di-1-naphthyl phosphate and 1-naphthyl glucuronide diluents, constant specific activity was attained after 3 to 4 crystallisations, and the products, on conversion into second derivatives, had equivalent specific activities.

1-Naphthyl dihydrogen phosphate was crystallised from its concentrated eluate, weakly acidified with acetic acid, as its monocyclohexylammonium salt; m.p. 193°, and recrystallised from water. It was then converted to the free acid; m.p. 155°, which crystallised from water.

1-Naphthyl hydrogen sulphate was isolated as its *p*-toluidine salt, crystallised from water, and then converted to its 5-aminoacridine salt.

1-Naphthyl β-D-glucoside, m.p. 170°, obtained as a residue by evaporating the eluate to dryness, was crystallised from benzene-ethanol and then converted to the tetra-acetate, m.p. 174° with pyridine-acetic anhydride, and crystallised from ethanol.

Results from the dilution analyses overleaf are summarised in Table 8.

Di-1-naphthyl hydrogen phosphate was isolated as its cyclohexylamine salt, m.p.  $216^{\circ}$  (decomp.), which crystallised from ethanol. The specific activity still decreased after three crystallisations and on conversion to the free acid, the equivalent specific activity decreased still further.

Ten mg. of the free acid was chromatographed on 8 cm. wide strips of Whatman No.4 paper in solvent D, and the paper cut into 2 cm. sections and assayed in the scintillation spectrometer. Most of the residual radioactivity separated from the di-1-naphthyl phosphate zone, located under u.v., and only a background level of counts were recorded in this zone.

Activity associated with this zone was 42 disintegrations/min. which meant that the maximum amount of di-1-naphthyl phosphate formed could not have exceeded 0.1% of the dose of 1-naphthol.

(1-Naphthylglucosid)uronic acid was isolated as its sodium salt and crystallised from ethanol-water. In this case also, the specific activity still decreased after three crystallisations and again on conversion into the free acid. Therefore 10 mg. of the free acid was chromatographed similarly to the di-1-naphthyl phosphate, but in solvent A. Maximum activity in the glucuronide zone was 70 disintegrations/min. limiting the

Table 8. Formation of [ $^{14}\text{C}$ ]1-naphthol conjugates by grass grubs

Groups of 10 grass grubs were dosed at room temperature and 8 hr. later metabolites were analysed by dilution analysis as described in the text.

Diluent	Specific activity of derivatives $\mu\text{c./g.}$		Total activity $\mu\text{c.}$		% activity of total dose
	1st	2nd	1st	2nd	
Naphthyl dihydrogen phosphate	a, 0.081 b, 0.155	0.098 0.186	26 50	26 52	7.2 14.5
Naphthyl hydrogen sulphate	a, 0.76 b, 0.77	0.59	26	25	7.0 6.0
Naphthyl glucoside	a, 0.77 b, 0.73	0.39	22	21	5.0 3.5
1-Naphthol (b)	1.16				78

Activity of total dose: a, 0.54  $\mu\text{c.}$ ; b, 0.63  $\mu\text{c.}$

amount of glucuronide that could have been formed by the grass grub to 0.2% of the dose. The carrier glucuronide was carefully eluted from the paper after counting and electrophoresed at pH 4. Activity associated with the glucuronide zone was now approximately 4 disintegrations/min., limiting the maximum amount of glucuronide present to 0.01% of the dose of 1-naphthol.

#### Quantitative paper chromatography

Insects were dosed with [ $^{14}\text{C}$ ]1-naphthol, and extracts were prepared as for the carbaryl metabolism studies, except that (1-naphthylglucosid)uronic acid was used as an additional reference compound, and 4 g. of non-radioactive 1-naphthol was added to the acetone extracts, in place of the 0.5 g. carbaryl and 0.5 g. 1-naphthol used previously. Benzene extracts were combined and the  $^{14}\text{C}$  activity assayed by counting 1 ml. in vials containing 10 ml. scintillator in the scintillation spectrometer with quench determination. The activity was taken to be due to 1-naphthol since other non-aqueous soluble metabolites were not expected in any quantity, and this was used to calculate the amount of 1-naphthol recovered from the homogenates. Aqueous extracts were chromatographed on 2 cm. wide strips of Whatman No.4 paper in solvent system A and counted in the scintillation spectrometer as for the carbaryl metabolism studies, except that the spectrometer was set up for  $^{14}\text{C}$  counting.

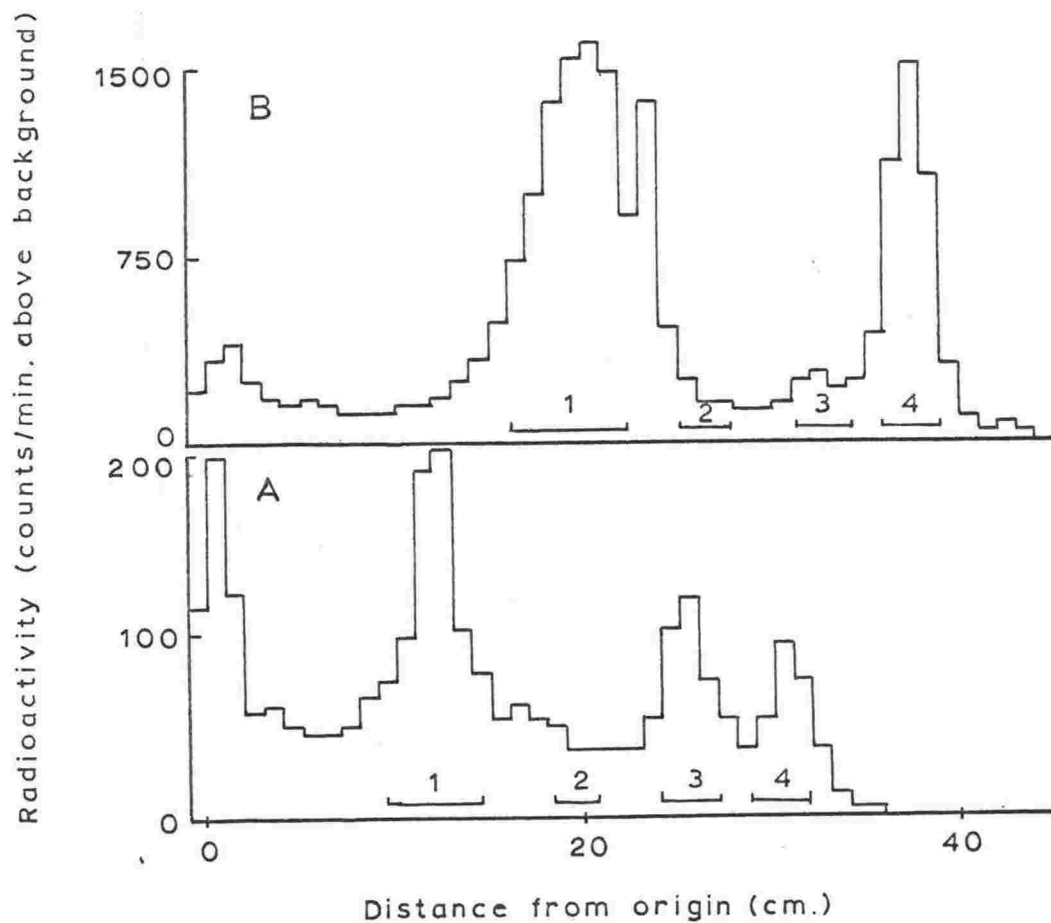


Fig.6. Chromatography of [ $^{14}\text{C}$ ]1-naphthol conjugates from A, the grass grub; and B, blowfly. Extracts of dosed insects were prepared and chromatographed in solvent A as described in the text, and the chromatograms sectioned and counted in the scintillation spectrometer. Bars indicate the positions of co-chromatographed non-radioactive reference compound; 1, 1-naphthyl phosphate; 2, 1-naphthyl glucuronide; 3, 1-naphthyl sulphate; 4, 1-naphthyl glucoside.

Histograms of chromatograms of grass grub extracts (Fig.6) showed peak radioactivity corresponding to the reference 1-naphthyl phosphate, 1-naphthyl sulphate, and 1-naphthyl glucoside, together with a small amount of activity near the origin, but no peak of activity coinciding with the reference 1-naphthyl glucuronide zone. Extracts made from insects homogenised immediately after dosing contained only 1-naphthol, and histograms of aqueous extracts had no significant activity in any of the above zones, except for a small amount at the origin.

Histograms of chromatograms of fly extracts (Fig.6) had similar peaks of activity, but the activity in the region of reference naphthyl phosphate sometimes partially resolved into two peaks, the new peak lying between the reference phosphate and glucuronide. These two peaks could be completely separated by chromatography in solvent B. Fly extracts prepared as before were chromatographed on 2 cm. wide strips of Whatman No.4 paper in solvent B overnight, the solvent being allowed to drip from the end of the papers. The new product (metabolite X) chromatographed as a sharp peak ahead of the 1-naphthyl phosphate (Fig.7). Grass grub extracts did not contain this new product, which often contained more activity than the phosphate conjugate on chromatograms of fly extracts.

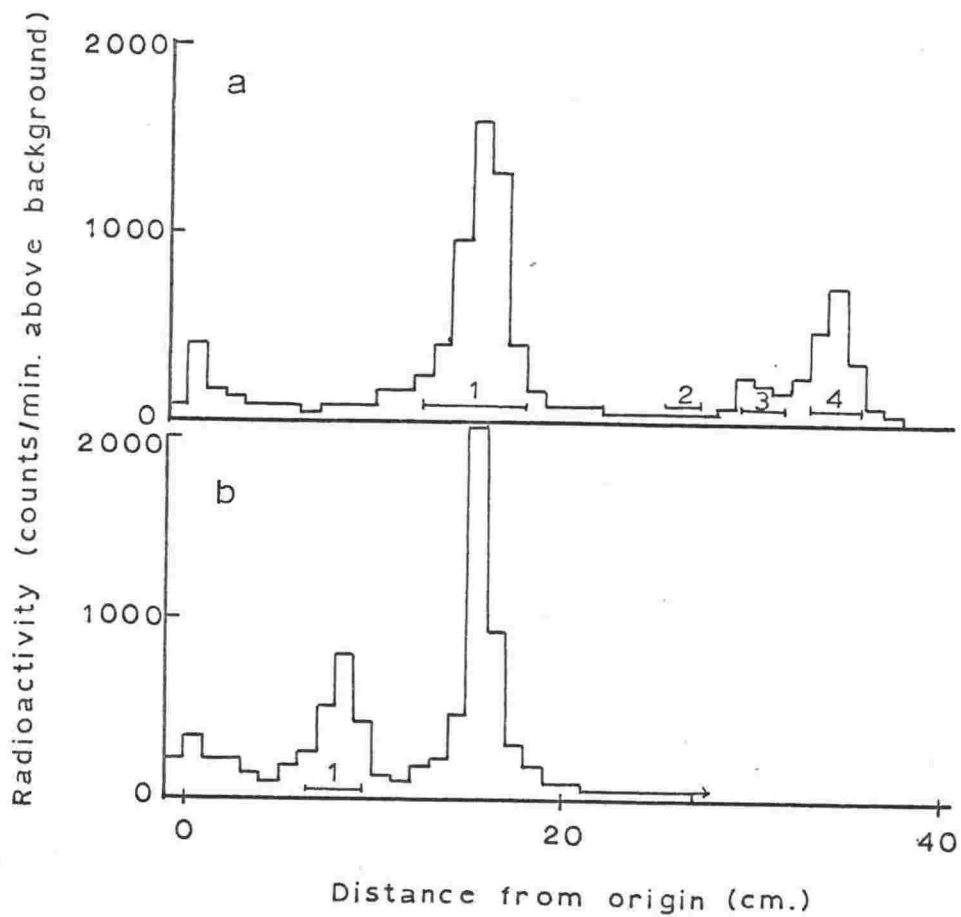


Fig.7. Chromatography of [ $^{14}\text{C}$ ]1-naphthol metabolites from the housefly in solvent A (a) and solvent B (b). Preparation of extracts and chromatography were as described in the text. Bars indicate positions of co-chromatographed reference conjugates as in Fig.6.



The amount of, each conjugate formed by the grass grub, expressed as a percentage of the total injected activity, was calculated by totalling the activity, corrected to 100% efficiency, under the respective peaks on chromatograms run in solvent A. Measurements of sulphate and glucoside conjugates present in fly extracts were also taken from chromatograms run in solvent A, whereas measurements of the phosphate conjugate and metabolite X were taken from chromatograms run in solvent B. The results from 12 fly experiments and 8 grass grub experiments are summarised in Table 9.

#### Enzymic hydrolysis of conjugates

The conjugates of [ $^{14}\text{C}$ ]1-naphthol identified on the chromatograms were further characterised by their sensitivity to hydrolysis by enzymes.

Chromatograms of [ $^{14}\text{C}$ ]1-naphthol conjugates from the housefly, run in solvent system A, were treated with alkaline phosphatase ('purified', Sigma Co.), or Light's emulsin and assayed for released toluene-soluble radioactivity as for the chromatograms of [ $^3\text{H}$ ]carbaryl metabolites.

Histograms of the treated chromatograms (Fig.8) showed that the phosphatase treatment released considerable activity from the peak in the reference phosphate region, but little from the other peaks, including the 'metabolite X' peak.

Table 9. Formation of [ $^{14}\text{C}$ ]1-naphthol metabolites by insects

Groups of 5 insects were dosed at room temperature and after indicated time were analysed for metabolites as described in the text.

		Dose recovered, (moles %) as:					
		1-Naphthyl dihydrogen phosphate	1-Naphthyl hydrogen sulphate	1-Naphthyl glucoside	Metabolite X	Toluene- soluble (1-naphthol)	
<hr/>							
Houseflies							
2 hr.	10.4, 4.5	1.0, 0.5	3.2, 5.0	5.3, 11.5	79, 68		
4 hr.	19.6, 6.7	1.7, 1.0	5.7, 5.5	4.7, 17.0	65, 62		
6 hr.	29.0, 7.2	1.8, 1.5	3.7, 6.2	4.4, 22.0	56, 65		
<hr/>							
Blowflies							
2 hr.	4.3, 1.1	2.9, 0.6	4.5, 4.9	17.1, 4.0	63, 88		
4 hr.	4.0, 1.6	7.5, 0.8	9.5, 4.2	16.0, 7.5	50, 84		
6 hr.	6.0, 3.5	3.9, 1.6	6.5, 5.4	25.0, 13.0	46, 78		
<hr/>							
Grass Grubs							
8 hr.	8.0 (4.0-13.0) [9]	5.0 (3.0-8.0) [8]	3.5 (2.0-6.0) [8]	-	-	78 (72-83) [7]	
8 hr. (at 5°)	4.1 4.9	2.5, 2.5	2.0, 2.0	-	-	87, 88	

Values for grass grubs show means and ranges of the number of experiments shown in the square brackets.

In contrast to the carbaryl studies, no activity was released from the origin peak, indicating a lack of phosphatase-sensitive substances in this region. The emulsin, which had a weak phosphatase activity as well as  $\beta$ -glucosidase activity, released considerable toluene-soluble radioactivity from the 'glucoside' peak, and to a lesser extent from both the 'phosphate' and 'metabolite X' peaks. No hydrolysis occurred in the sulphate or origin regions.

#### Formation of glucuronide conjugates in flies

No radioactivity due to glucuronide formation in flies dosed with [ $^{14}\text{C}$ ]1-naphthol could be detected on chromatograms of extracts of dosed flies, but the sensitivity was limited, owing to the presence of residual radioactivity in the reference glucuronide zones from slight overlapping of adjacent peaks (Fig.6). Therefore chromatograms of fly extracts prepared as before from flies dosed with [ $^{14}\text{C}$ ]1-naphthol and containing reference 1-naphthyl glucuronide, were run in solvent B, and the glucuronide zone eluted with 0.1 N-ammonia and further purified by paper ionophoresis at pH 12.5. The papers were cut into 1 cm. sections and counted in the scintillation spectrometer in the usual manner.

On ionophoresis, most of the residual radioactivity moved ahead of the glucuronide zone towards the anode. Activity in the

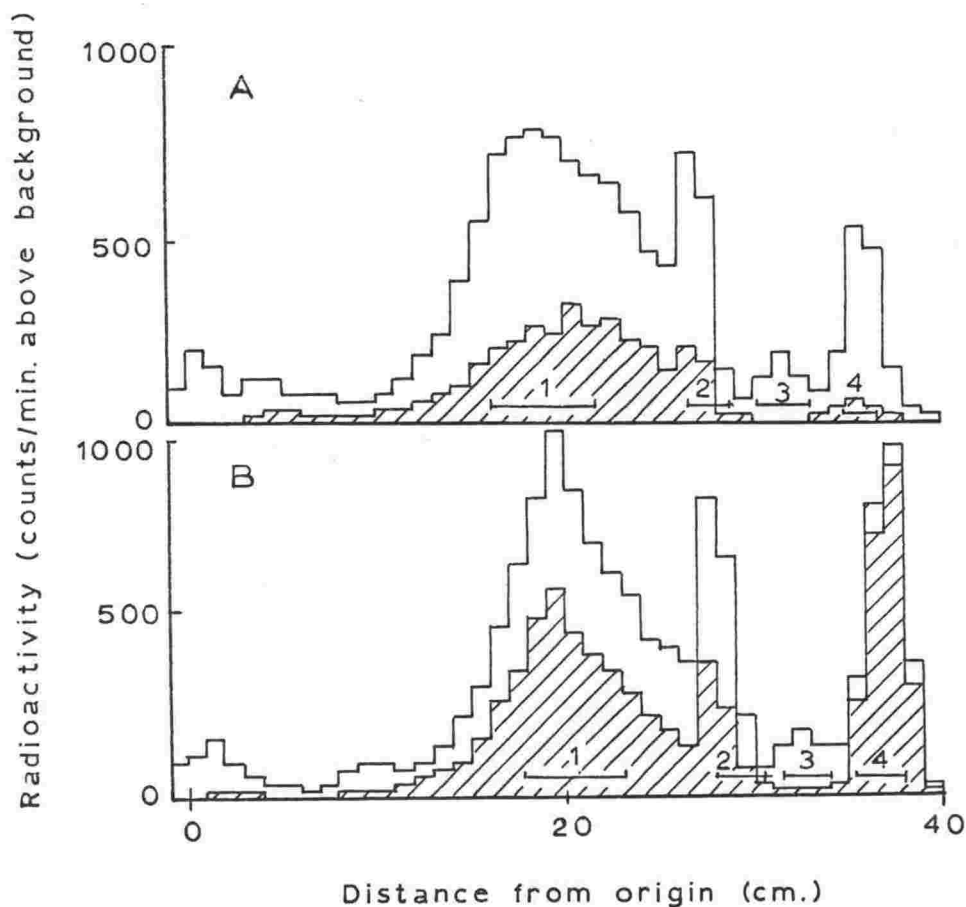


Fig.8. Action of A, phosphatase; and B, emulsin on chromatograms of [ $^{14}\text{C}$ ]1-naphthol conjugates from the housefly. Open area: counts on untreated chromatograms; shaded area: counts on treated chromatograms. Bars indicate positions of reference conjugates as in Fig.6.

glucuronide zones from an extract of 3 houseflies each dosed with 0.2  $\mu$ c. [ $^{14}$ C]1-naphthol and from an extract of 2 blowflies each dosed with 0.4  $\mu$ c. amounted to 45 and 80 disintegrations/min. respectively, indicating a glucuronide formation of less than 0.01% in each case.

The presence of reference glucuronide in the zones located by u.v. fluorescence was checked by the naphthoresorcinol reaction. After scintillation counting the paper strips thought to contain glucuronide were removed from the vials, rinsed briefly with toluene, dried, and eluted with a few drops of 0.1 N-NH<sub>3</sub>. The eluate was boiled with an equal volume of conc. HCl and a speck of naphthoresorcinol, then cooled and shaken with ether. The presence of glucuronide was confirmed by the formation of an intense purple colour in the ether layer.

### Structure of the New Phenol Conjugate

The unknown metabolite of 1-naphthol, metabolite X, was often the most prominent metabolite of [ $^{14}\text{C}$ ]1-naphthol in flies. Evidence is presented here to show that this metabolite is a new conjugate of 1-naphthol, the 6-(dihydrogen phosphate) ester of 1-naphthyl  $\beta$ -D-glucoside. The corresponding conjugate of *p*-nitrophenol has also been examined, since *p*-nitrophenol and its conjugates offer advantages of ready detection on paper and in solution, and the conjugates are more readily hydrolysed to their components than most phenol conjugates, facilitating structural studies.

### Materials and Methods

#### Reference compounds

Disodium  $\alpha$ - and  $\beta$ -glycerophosphate, disodium *p*-nitrophenyl phosphate (B.D.H. Ltd.), dAMP disodium salt, dGMP di-ammonium salt, and sodium di-*p*-nitrophenyl phosphate (Calbiochem Corp., Calif.), were commercial samples, homogenous by paper chromatography. Glucose 6-(barium phosphate) was prepared from the potassium salt (B.D.H. Ltd.) by passage through a cation exchange resin in hydrogen form, and neutralisation of the acid effluent with aq.- $\text{Ba}(\text{OH})_2$ . The hexahydrate crystallised out on standing.

Di-m-nitrophenyl hydrogen phosphate and m-nitrophenyl dihydrogen phosphate were prepared as their cyclohexylamine salts by neutralisation with cyclohexylamine solution of di-m-nitrophenyl phosphorochloridate and m-nitrophenyl phosphorodichloridate, prepared from m-nitrophenyl and phosphorus oxychloride by the general method of Friedman & Seligman (1950). The cyclohexylamine salt of di-m-nitrophenyl hydrogen phosphate crystallised from ethanol, m.p.  $136^{\circ}$ , (Found: C, 49.5; H, 5.2; N, 9.5;  $C_{18}H_{22}N_3O_8P$  requires C, 49.2; H, 5.0; N, 9.6%).

The dicyclohexylamine salt of m-nitrophenyl dihydrogen phosphate crystallised from ethanol-water, m.p.  $178-80^{\circ}$ , (Found: C, 51.7; H, 7.7; N, 10.0;  $C_{18}H_{32}N_3O_6P$  requires C, 51.6; H, 7.7; N, 10.1%).

(p-Nitrophenyl glucosid)uronic acid was prepared similarly to the 1-naphthol compound by the catalytic oxidation of p-nitrophenyl glucoside, prepared from p-nitrophenol by the same route as 1-naphthyl glucoside. The compound was isolated from the acidified reaction mixture as its sparingly soluble p-toluidine salt. The p-toluidine salt of (p-nitrophenyl  $\beta$ -D-glucosid)uronic acid crystallised from water in plates, m.p.  $176^{\circ}$ ,  $[\alpha]_D^{20} -77^{\circ}$  (C, 1.0 in 0.1 N-HCl) (Found: C, 54.2; H, 5.6; N, 7.1;  $C_{19}H_{22}N_2O_9$  requires C, 54.0; H, 5.3; N, 6.6%).

[<sup>14</sup>C]1-naphthol metabolite X was eluted from chromatograms in solvent system B of extracts of flies dosed with [<sup>14</sup>C]1-naphthol, prepared as described previously.

#### Chromatography

Paper chromatography was carried out as already described, and, in addition to the solvents used previously, the following solvent systems were used for paper chromatography:

G, butan-1-ol-acetic acid-water, (4:1:5, by vol.);

H, 2-methylpropan-2-ol-aq. NH<sub>3</sub> (sp. gr. 0.88)-water, (6:3:1, by vol.); and I, benzene-acetic acid-water, (1:1:2, by vol.).

Paper ionophoresis carried out as already described.

#### Absorption spectra

Ultra-violet spectra, and all measurements of extinction were performed on the Perkin-Elmer u.v.-cord, unless otherwise stated. Infra-red spectra were recorded on the Unicam SP-200 infra-red spectrophotometer as nujol mulls. Spectra were calibrated by comparison with a reference spectrum of a polystyrene standard recorded immediately afterwards on the same machine.

#### Preparation of extracts

Groups of 2,000-10,000 houseflies, kept from time of emergence from pupae in large glass containers, flushed



continuously with compressed air to control humidity, were allowed to feed on a 1% solution of p-nitrophenol or 1-naphthol in milk and sugar for one week, or until all were dead. Flies were discarded and the excreta was washed off the insides of the containers with 80% ethanol. The washings were centrifuged to remove insoluble material and concentrated to small volume for paper chromatography.

## Results

### Paper chromatography of conjugates

p-Nitrophenol. Chromatograms of excreta extracts from flies on Whatman No.1 or 3 MM paper in solvent system G showed the presence of five u.v. quenching zones of  $R_F$  values 0.45, 0.55, 0.65, 0.75 and 0.95. The last four zones co-chromatographed with reference spots of p-nitrophenyl phosphate, sulphate and glucoside, and free p-nitrophenol, respectively. The  $R_F$  0.55 zone gave a positive Hanes & Isherwood (1949) test for phosphate, and rapidly yielded free p-nitrophenol when treated with Sigma purified alkaline phosphatase. The zones corresponding to reference sulphate and glucoside conjugates yielded p-nitrophenol when treated with dilute acid (0.1 N-HCl) at 100°, and  $\beta$ -glucosidase, respectively.

The zone of  $R_F$  0.45 did not correspond to any of the reference compounds, and was not hydrolysed by either of the

enzymes overleaf or by dilute acid. It did react with titanous chloride followed by Ehrlich's reagent (p-N,N-dimethylamino-benzaldehyde) to give a yellow colour, as did the other zones, indicating the presence of an intact aromatic nitro group. It also gave a positive phosphate test with the molybdate reagent. This zone, designated p-nitrophenol metabolite X, was cut from chromatograms prepared as above from extracts of 2000 flies, eluted with 0.1 N-NH<sub>3</sub>, and rechromatographed twice, in solvent system B, and then G again.

The p-nitrophenol metabolite X on the final chromatogram, free of any other p-nitrophenol conjugates, was either tested directly on the paper, or eluted off for further tests, as below.

1-Naphthol. Chromatography of extracts in solvent system G showed the presence of three purple fluorescent zones, of R<sub>F</sub> values 0.5, 0.75 and 0.85, as well as a bright blue fluorescent zone of 1-naphthol at the solvent front. The 0.75 and 0.85 zones were shown to be 1-naphthyl sulphate and glucoside respectively, as for the p-nitrophenol conjugates.

The R<sub>F</sub> 0.5 zone, which co-chromatographed with both reference 1-naphthyl phosphate and [<sup>14</sup>C]1-naphthol metabolite X, was eluted from the chromatograms and rechromatographed with solvent system B, the solvent being allowed to run off the edge of the paper. Two purple fluorescent zones were now evident, a slow band corresponding to 1-naphthyl phosphate; and a faster

band corresponding to [ $^{14}\text{C}$ ]1-naphthol metabolite X. This metabolite X zone was eluted off and purified by rechromatography with solvent H, for characterisation as for the *p*-nitrophenol metabolite X.

#### Reactions on paper

The metabolite from either phenol did not readily decolourise alkaline permanganate, indicating absence of reducing properties.

No hydrolysis to the free phenol could be detected after treatment with glucosidase, alkaline phosphatase, or 0.1 N-HCl at 100° for 1 hr., or by a combination of enzyme hydrolysis followed by treatment with 0.1 N-HCl at 100°. However, slight hydrolysis was detected after treatment first with glucosidase, and then phosphatase. Free 1-naphthol was detected by the blue colour with Gibbs' reagent (2,6-dichloro-*p*-benzoquinone chloroimide), and *p*-nitrophenol by the yellow colour in ammonia fumes.

No reaction was obtained with ninhydrin either before or after treatment on the paper with 0.1 N-HCl at 100° for 1 hr., indicating the absence of amino acid conjugates or residues.

Tests for the presence of reducing sugars with ammoniacal silver nitrate were negative, although on one occasion slight colour was noticed with the 1-naphthol compound which first had been heated at 100° in presence of pH 4.5 buffer for 1 hr.

The *p*-nitrophenol metabolite gave a positive test for phosphate with the Hanes-Isherwood molybdate reagent (Hanes & Isherwood, 1949) but the 1-naphthol compound did not react. However, both compounds gave a positive test for phosphate ester with dilute ferric chloride spray followed by sulphosalicylic acid spray (Wade & Morgan, 1953). The compounds appeared as white spots on a buff background.

#### Ultra-violet spectra

The u.v. absorption spectrum of each metabolite purified by chromatography three times is shown in Fig.9. Eluates were diluted with 0.1 N-HCl or 0.1 N-NaOH until a solution with a maximum extinction of about 0.7 was obtained. The spectrum of each metabolite was very similar to spectra of conjugates of the corresponding phenol, Table 10. The spectra were substantially the same in acid or alkaline conditions.

#### Acid dissociation constants

(a) Ionophoresis. [ $^{14}\text{C}$ ] Labelled metabolite X from flies dosed with [ $^{14}\text{C}$ ]1-naphthol, and *p*-nitrophenol metabolite X eluted from chromatograms of excreta of *p*-nitrophenol-fed flies were subject to paper ionophoresis at various pH values, along with reference phosphate, and sulphate or glucuronide conjugates. The [ $^{14}\text{C}$ ]1-naphthol compound was located on the papers by assay

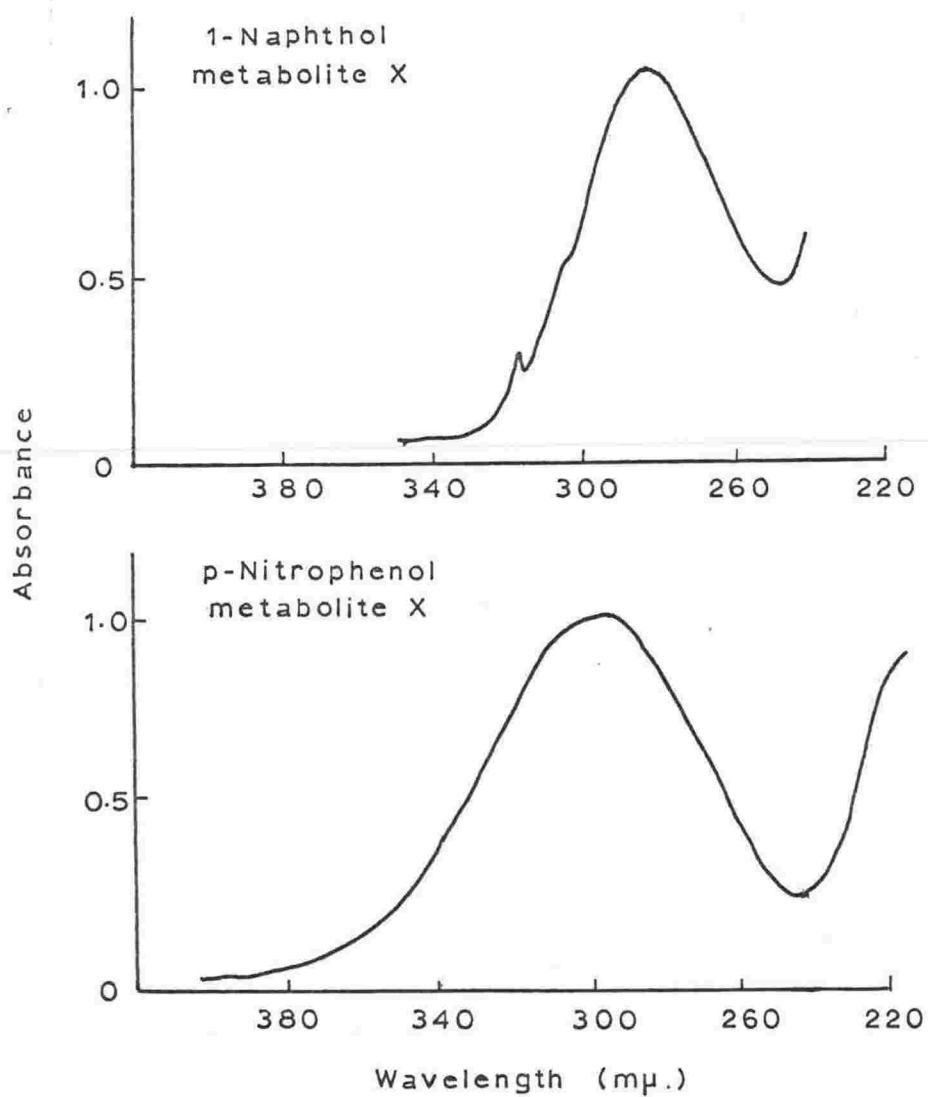


Fig.9. U.v. spectra of 1-naphthol metabolite X and p-nitrophenol metabolite X.

Table 10. Ultra-violet absorption maxima of 1-naphthol and p-nitrophenol metabolite X and related compounds

	$\lambda$ max.	$\epsilon$ (cm.)
1-naphthol metabolite X	319	1.6
in 0.1 N-HCl and 0.1 N-NaOH	280	7.5
1-naphthyl phosphate		
in 0.1 N-NaOH	285	( $\epsilon$ max., 4,500)
p-nitrophenol metabolite X	298	5
in 0.1 N-HCl and 0.1 N-NaOH	215	5
p-nitrophenol sulphate		
in 0.1 N-NaOH	280	( $\epsilon$ max., 8,900)

in the scintillation spectrometer, and the others by quenching or fluorescence under u.v.

Both compounds behaved as dibasic acids, with pKa values of approximately 1 and 6 (Fig.10). The migration pattern of each metabolite was similar to that of the corresponding phosphate conjugate, except that in each case the migration rate was somewhat less than that of the corresponding phosphate at the same pH. The migration rate of the singly ionised p-nitrophenol metabolite X and ionised p-nitrophenyl glucuronide (a monobasic acid) were similar.

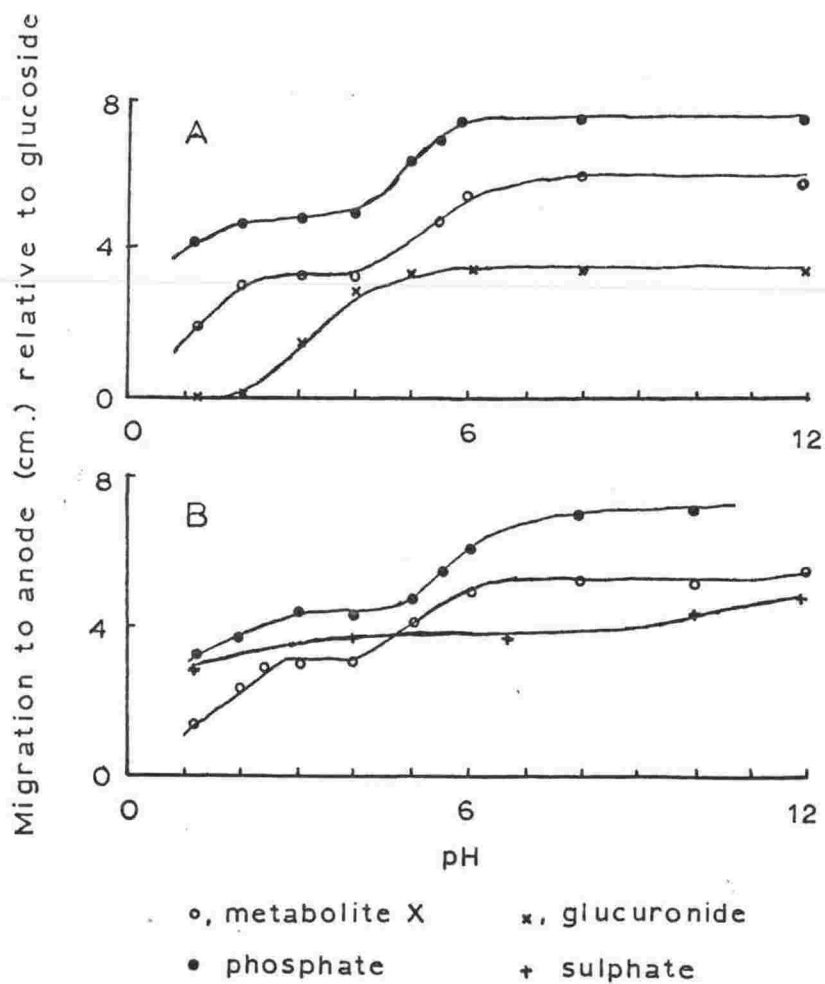


Fig.10. Ionophoresis of a, p-nitrophenol conjugates, and b, 1-naphthol conjugates, at various pH values. Ionophoresis was carried out at 40 v/cm. for 1.5 hr.

(b) Titration curves. Three times chromatographed metabolites were used. Eluates from the final chromatograms were evaporated to small volume and converted to free acid by passage through a column of Amberlite IR 105 cation exchanger in hydrogen form. The solution was then evaporated to just dryness twice at reduced temperature under vacuum to remove volatile acids present (for instance, residual acetic acid from the chromatography).

The free acid was then taken up in 2.0 ml. of  $\text{CO}_2$ -free distilled water and titrated with 0.12 N-NaOH ( $\text{CO}_2$ -free), using the Radiometer automatic titration assembly and recorder.

The titration curves (Fig.11) for both metabolites had two inflections indicating two end points. However, volume of titrant required to reach the first end point was about three times that further required to reach the second end point. Possibly the eluates contained chloride or sulphate ions that would have been converted to free acid on passage through the cation exchange column.

From the titration curves, after correcting for effects of dilution and limiting pH of titrant, it was calculated that the second dissociation constants,  $\text{pK}_2$ , for the 1-naphthol and p-nitrophenol metabolites were 6.1 and 6.0 respectively. It was not possible to calculate the first dissociation constants,



as the solutions were too dilute (approximately 0.05 M), and were possibly also contaminated with strong acid.

#### Calculation of $\epsilon$ max. by titration

After the titrations above the solutions were diluted suitably and the extinction measured at the wavelengths of peak absorption determined earlier. The number of equivalents in each solution could be calculated from the amount of alkali required for titration between the first and second end points and hence the  $\epsilon$  max. could be calculated (Table 11). These compared well with values for conjugates of the corresponding phenols.

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Table 11.  $\epsilon$  max. of unknown metabolite, and related compounds

Values were determined by titration as in the text.

	$\lambda$ max. (m $\mu$ )	log $\epsilon$ max.
p-nitrophenol metabolite X	298	3.84
sulphate	280	3.93
1-naphthol metabolite X	280	3.6
phosphate	285	3.65

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#### Acid and alkaline hydrolysis

The 1-naphthol metabolite was very resistant to acid or alkaline hydrolysis. After heating in 5 N-NaOH or 5 N-HCl at

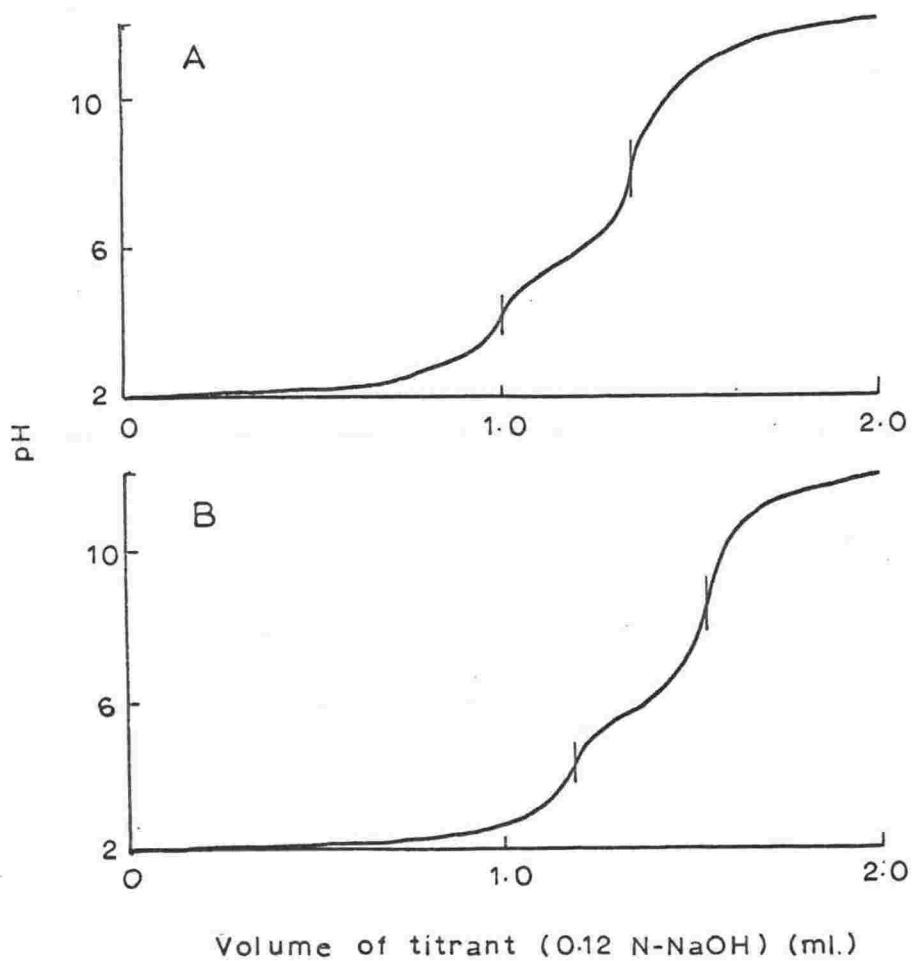


Fig.11. Titration of A, *p*-nitrophenol metabolite X, and B, 1-naphthol metabolite X, against 0.12 N-NaOH. Points of inflection, marked by vertical lines, indicate end points.

100° for 1 hr. u.v. spectra showed little change from those of control samples. However, after 5 hr. heating the peak at 280 mμ had diminished and absorption peaks of 1-naphthol appeared at 292 mμ in acid medium and 323 mμ in alkaline medium, but hydrolysis was not complete. A small amount of inorganic phosphate was also liberated by prolonged heating in the strong acid or alkali, as shown by the formation of a yellow crystalline precipitate with ammonium molybdate and concentrated nitric acid.

The p-nitrophenol metabolite was hydrolysed in 5 N-HCl at 100° with release of free p-nitrophenol and inorganic phosphate. After 1 hr. in 5 N-HCl at 100° the u.v. spectrum of the metabolite had changed completely to exhibit a new peak at 318 mμ, of approximately the same absorption as the 298 mμ peak before hydrolysis, and an inflection at 220 mμ in acid medium. In alkaline medium the new substance exhibited a single peak at 400 mμ with a doubling of optical density. These new spectra were exactly identical to spectra of p-nitrophenol in acid and alkaline medium, determined on the same machine (Beckman DB u.v. spectrophotometer).

The identity of the p-nitrophenol released on hydrolysis was confirmed by extracting the acid hydrolysate with ether, and chromatographing the ether extract on paper in solvent systems B and I. The extract gave a single spot on each chromatogram with

Table 12. Chromatography of hydrolysate extract and  
p-nitrophenol

p-Nitrophenol metabolite X was hydrolysed as in the text and an ether extract of the hydrolysate chromatographed in the solvents below.

	R <sub>F</sub> in solvents		Colour reactions with:		
	B (Butanol- ammonia- water)	I (Benzene- acetic- water)	U.V.	Ehrlich's reagent & TiCl <sub>3</sub>	Ammonia fumes
Extract	.62	.64	quenching	yellow	yellow
p-nitrophenol	.61	.64	quenching	yellow	yellow

u.v. quenching and colour reactions of p-nitrophenol. The R<sub>F</sub> values were identical to those of reference p-nitrophenol (Table 12).

ε Max. of p-nitrophenol metabolite X. Measurement of the extinction at wavelengths of maximum absorption of the metabolite and the p-nitrophenol liberated on hydrolysis as above enabled a calculation of ε max. for the metabolite to be made, since that for p-nitrophenol is known. Found; log ε max. (298 mμ) = 3.93. This compared well

with the figure obtained by titration, ( $\log \epsilon \text{ max.} = 3.84$ , Table 11).

The presence of inorganic phosphate in the acid hydrolysate was demonstrated by the formation of a copious yellow precipitate with nitric acid, ammonium molybdate and 1-2 drops concentrated ammonia. Inorganic phosphate was not present before hydrolysis. Inorganic sulphate was not present, neither before nor after hydrolysis, as shown by testing with dilute barium chloride. Formation of a precipitate of barium sulphate could not be detected.

Further tests were carried out on the p-nitrophenol metabolite X as this was more readily hydrolysed than the corresponding 1-naphthol metabolite.

Rate of release of inorganic phosphate and p-nitrophenol versus pH

From 1000 flies fed p-nitrophenol, a solution of the unknown metabolite equivalent to 28 mg. of p-nitrophenol was obtained, in a volume of 65 ml. This solution was neutralised with a trace of concentrated hydrochloric acid and used in the next and subsequent experiments.

Portions of the eluate, (2.5 ml.) were heated at  $80^{\circ}$  with 10 ml. of buffer. At set time intervals p-nitrophenol released was estimated by withdrawing 0.3 ml. fractions, diluting to 3 ml. with 0.2 N-NaOH, and reading the extinction at 400 m $\mu$ . Absorption due to unhydrolysed metabolite did not interfere at this

wavelength. Inorganic phosphate released was determined by the method of Fiske & Subba Row (1925) on 2 ml. portions. The blue colour of the reduced phosphomolybdate complex was measured at 700 m $\mu$ . Buffers used were: citrate, (pH values 2, 3, 4, 5, and 6); barbitone, (pH values 7, 8 and 9); and borate, (pH values 10 and 11).

In the time of the experiment p-nitrophenol was released only at low or high pH (Table 13). Phosphate was not released at any pH from 0 to 11 but hydrolysis occurred at pH 13 (0.1 N-NaOH).

Table 13. Release of p-nitrophenol from the unknown metabolite at various pH

A solution of the metabolite was heated at 100°C in buffer for the times indicated. p-Nitrophenol was estimated as described in the text. Figures represent mg.

Time (hr.)	pH					
	5N-HCl	0	1-9	10	11	13
0.17	0.008	-	-	-	-	0.008
1.0	0.024	0.004	-	-	-	0.022
4.5	0.030	0.015	-	0.003	0.004	0.030

- indicates negligible amount released. Hydrolysis was complete at 0.030 mg.

Relative rates of release of phosphate and p-nitrophenol  
in 1 N-HCl and 0.1 N-NaOH.

Eluate was heated at pH values 0 and 13 and aliquots withdrawn at set intervals and analysed for organic phosphate and p-nitrophenol as described overleaf. Graphs were plotted showing the relative rates of release of inorganic phosphate and p-nitrophenol from p-nitrophenol metabolite X at the pH and temperature indicated (Fig.12). It can be seen from the graphs that the rate of release of p-nitrophenol was faster than that of inorganic phosphate in both acid and alkali, and especially so in acid. After 2 hr. in 1 N-HCl at 100° release of p-nitrophenol was 85% complete whereas the release of phosphate was only 25% complete. At completion of hydrolysis at pH 0 the molar ratio of p-nitrophenol to phosphate released was approximately 1:1.

Chromatography of acid hydrolysate

A sample of the unknown p-nitrophenol metabolite equivalent to 2 mg. of p-nitrophenol was partially hydrolysed in 1 N-HCl at 100° for 2 hr., and the acid removed by evaporation to dryness under vacuum at below 50°C. The residue was taken up in a little water and chromatographed on Whatman No.1 paper in the solvent systems G and H along with reference and mixed spots of glucose glucose-6-phosphate and p-nitrophenol. Carbohydrates

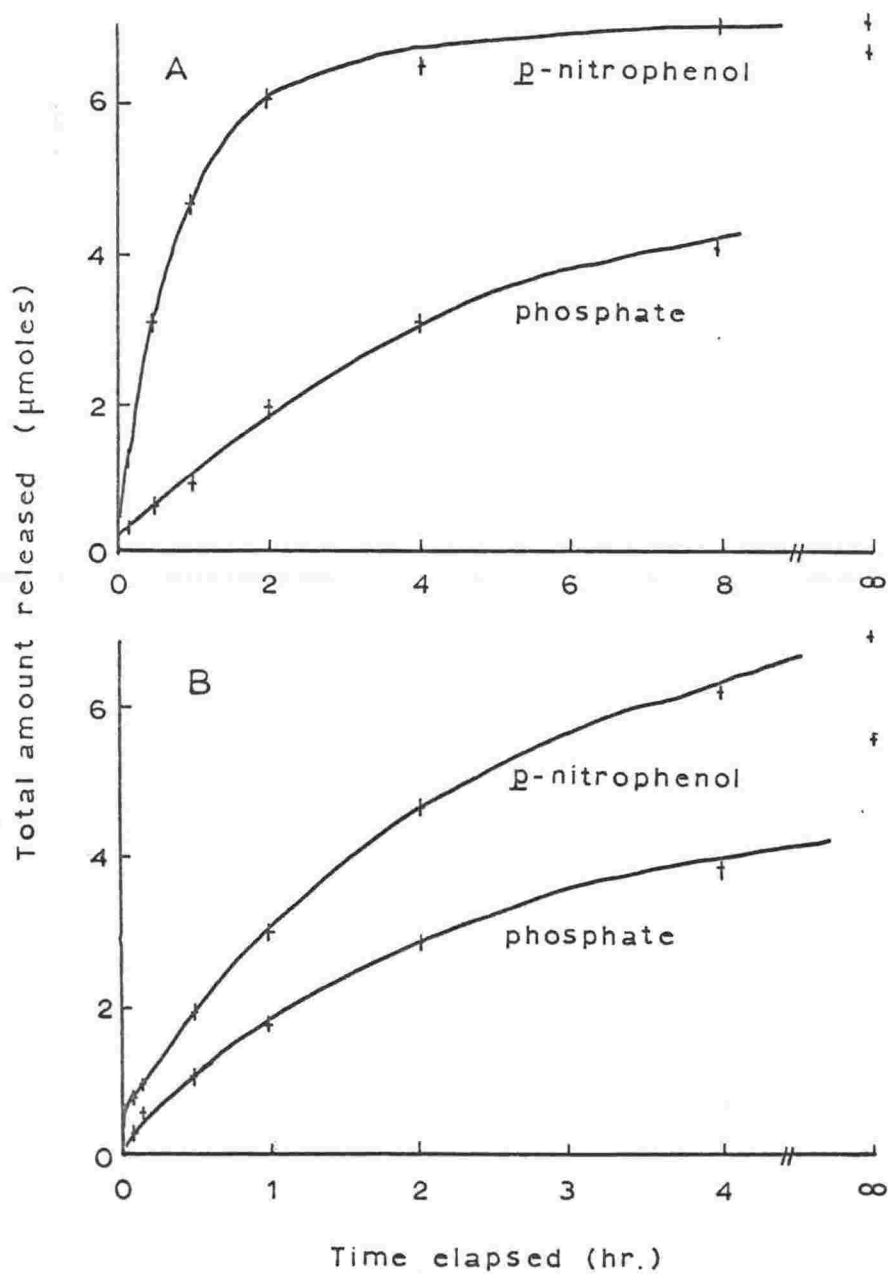


Fig.12. Relative rates of release of inorganic phosphate and p-nitrophenol on hydrolysis of p-nitrophenol metabolite X at A, pH 0 and 100°, B, pH 13 and 80°.



Table 14. Paper chromatography of a partial hydrolysate of p-nitrophenol metabolite X and related compounds

	R <sub>F</sub> value in solvent		Colour reactions with:			
	G	H	aniline phthalate	m-phenylene diamine	FeCl <sub>3</sub> -sulpho-salicylic	U.V.
glucose	0.20	0.56	brown	yell.-brown		
glucose 6-phosphate	0.10	0.17	brown	faint yell.	white	
p-nitrophenol	0.95	0.89				quench
metabolite X	0.40	0.65	brown		white	quench
hydrolysate (1)	0.40	0.65	brown		white	quench
(2)	0.95	0.89				quench
(3)	0.20	0.56	faint brown	yellow brown		
(4)	0.10	0.17	brown	faint yellow	white	

were detected with aniline phthalate spray and m-phenylene diamine. According to the chromatograms, the hydrolysate contained a little starting material, glucose 6-phosphate, a little glucose, and considerable p-nitrophenol (Table 14).

Isolation of unknown metabolites as cyclohexylamine salts; infra-red spectra

Eluate containing three times chromatographed p-nitrophenol metabolite obtained from 20,000 flies, was converted to free acid by passage through a cation exchange column. The pH of the solution was then brought back to 8.5 by the addition of cyclohexylamine, and evaporated down to small volume, 0.1 ml., and diluted with 5 volumes of acetone. White crystals gradually separated out in the cold, yield; 20-50 mg.

The p-nitrophenyl glucoside 6-(dicyclohexylammonium phosphate) was recrystallised from the minimum quantity of hot water, diluted with acetone, in presence of a trace of cyclohexylamine; m.p.  $159^{\circ}$ ;  $[\alpha]_D^{25} -68^{\circ}$ ; (Found; C, 47.0; H, 7.70;  $C_{24}H_{42}N_3O_{11}P \cdot 2H_2O$  requires C, 46.8; H, 7.53%).

The infra-red spectrum of this metabolite (Fig.13) had absorption bands characteristic of p-substituted nitrobenzene compounds at  $1600\text{ cm}^{-1}$  and  $1590\text{ cm}^{-1}$  (aromatic),  $1510\text{ cm}^{-1}$  and  $1350\text{ cm}^{-1}$  (aromatic nitro group),  $1260\text{ cm}^{-1}$  (aromatic C-O-),  $855\text{ cm}^{-1}$  (C-H out-of-plane deformation in p-disubstituted benzene

ring) and  $755\text{ cm}^{-1}$  (Bellamy, 1954; Colthup, Daly & Wiberley, 1964). These absorptions were also present in infra-red spectra of *p*-nitrophenyl glucoside and *p*-nitrophenyl disodium phosphate (Fig.13). The presence of a carbohydrate moiety was indicated by the group of overlapping bands associated with ring and -C-O stretching at  $1100\text{ cm}^{-1}$ ,  $1075\text{ cm}^{-1}$  and  $1035\text{ cm}^{-1}$  also present in the spectrum of *p*-nitrophenyl glucoside. The weak absorption present at  $900\text{ cm}^{-1}$ , and also on the *p*-nitrophenyl  $\beta$ -D-glucoside spectrum, is a characteristic of pyranose  $\beta$ -D-glucosides, and not pyranose  $\alpha$ -D-glycosides (Barker, 1956). The very weak  $925\text{ cm}^{-1}$  could be due to a glycopyranose structure, since  $\alpha$ -D- and  $\beta$ -D-glycopyranoses have a ring vibration of moderate intensity in this region (Barker, Bourne & Whiffen, 1956).

The presence of the ionised phosphomonoester moiety was established by the strong  $970\text{ cm}^{-1}$ . This band ( $\text{PO}_3^{2-}$  symmetric stretching) is a characteristic of fully ionised phosphomonoesters, and can be used to distinguish them from phosphodiester which have a strong,  $=\text{PO}_2^-$  symmetric stretching band at  $1080\text{ cm}^{-1}$  (Shimanouchi, Tsuboi & Kyoguku, 1964). It was always present in the spectra of several phosphomonoesters run as reference spectra, but not in spectra of diesters (Fig.14). Another band, due to  $-\text{PO}_3^{2-}$  degenerate stretching, would be expected at  $1100\text{ cm}^{-1}$ , but this could not be distinguished from the other absorptions in this

region. Ionised phosphodiester have a strong, antisymmetric stretching band at  $1230\text{ cm}^{-1}$  (Shimanouchi, *et al.*, 1964).

The  $1635\text{ cm}^{-1}$  absorption possibly indicated the presence of water of crystallisation (Barker *et al.*, 1956), and the  $1545\text{ cm}^{-1}$  could be attributed to the cyclohexylammonium ion (Colthup *et al.*, 1964). Multiple absorptions in the region  $3500\text{--}3340\text{ cm}^{-1}$  were evident, attributable to NH and OH stretching vibrations.

The 1-naphthyl metabolite X was isolated as its cyclohexylamine salt similarly to the *p*-nitrophenol metabolite. The white crystalline solid, obtained by precipitation with acetone, did not recrystallise readily from acetone-water, but crystallised from a minimum quantity of hot ethanol on dilution with an equal volume of ether, m.p.  $156^{\circ}$  ( $[\alpha]_D^{20} -49^{\circ} \pm 3^{\circ}$ ). However, on subsequent crystallisations from ethanol the m.p. decreased and finally an oil was obtained which would not crystallise. Chromatography of this in propan-1-ol-ammonia (solvent system C) showed the presence of a new component moving ahead of the metabolite X component. Possibly esterification with ethanol was taking place.

The i.r. spectrum of the once recrystallised 1-naphthol metabolite (Fig.15) had absorption bands characteristic of 1-naphthol derivatives, at  $1600\text{ cm}^{-1}$ ,  $1585\text{ cm}^{-1}$ ,  $1270\text{ cm}^{-1}$  and  $1240\text{ cm}^{-1}$  (aromatic ring),  $800\text{ cm}^{-1}$  and  $775\text{ cm}^{-1}$  (C-H out-of-plane

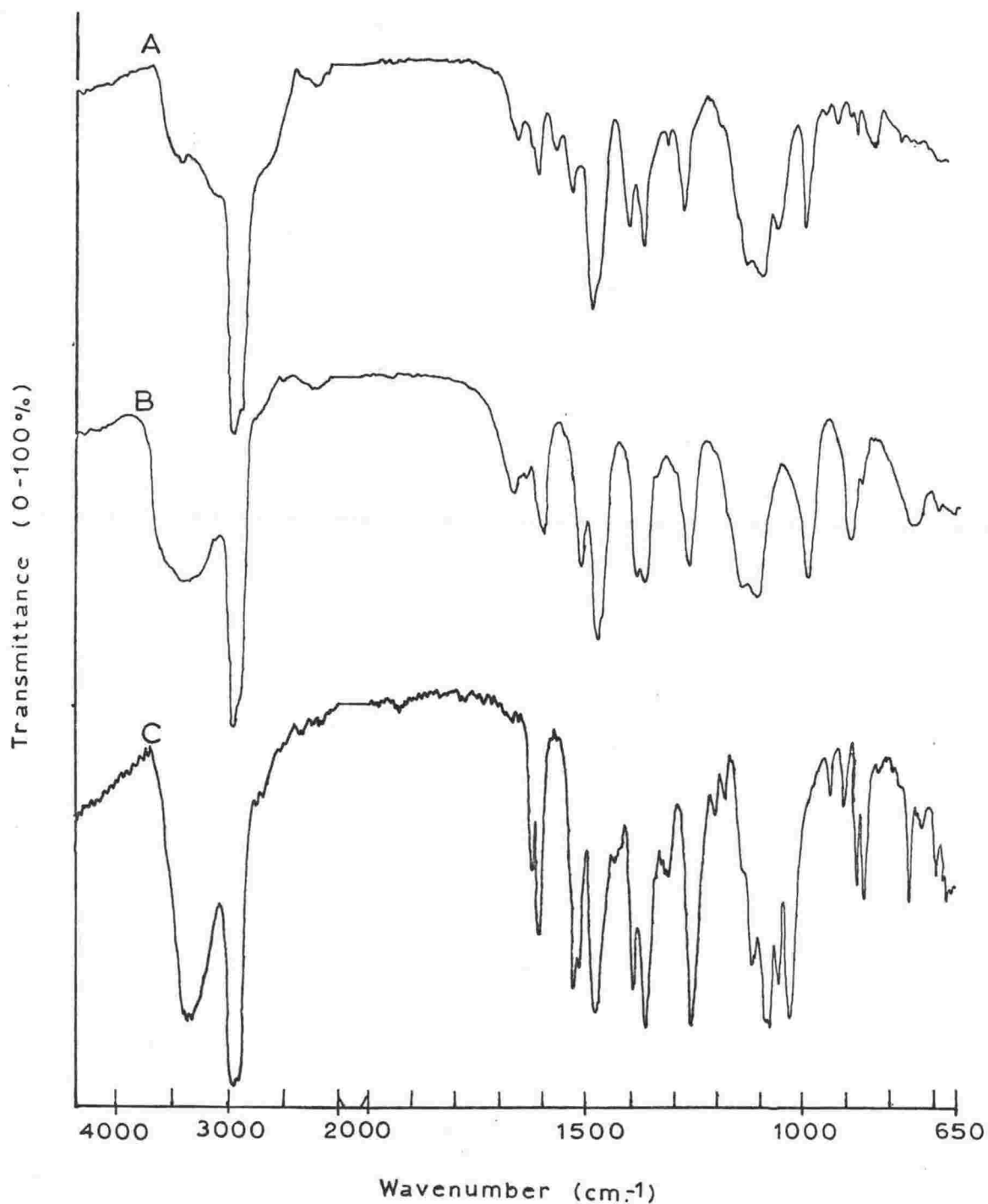


Fig.13. Infra-red spectra for A, p-nitrophenol metabolite X, isolated from fly faeces as the cyclohexylamine salt, B, p-nitrophenyl disodium phosphate and C, p-nitrophenyl  $\beta$ -D-glucoside, as nujol mulls.

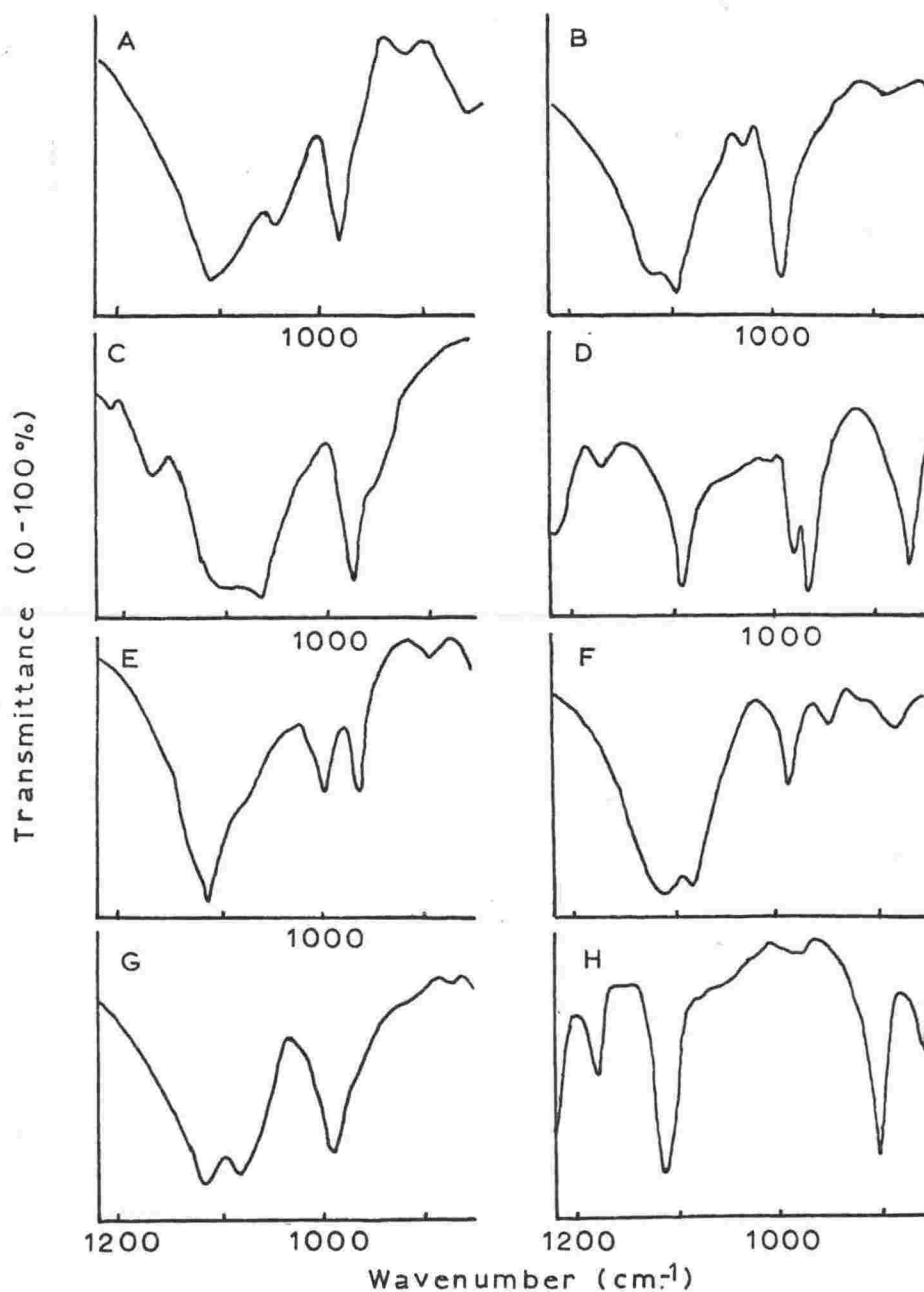


Fig.14. Infra-red spectra of some phosphate esters:  
 A, glucose 6-phosphate, barium salt; B, dAMP, disodium salt;  
 C, dGMP, ammonium salt; D, di-m-nitrophenyl cyclohexylammonium phosphate;  
 E, m-nitrophenyl dicyclohexylammonium phosphate;  
 F, disodium  $\alpha$ -glycerophosphate; G, disodium  $\beta$ -glycerophosphate;  
 H, di-p-nitrophenyl sodium phosphate. Compounds were examined as nujol mulls.

bending of the 1-substituted naphthalene nucleus) (Colthup et al., 1956). These were also present in spectra of 1-naphthyl glucoside and phosphate. Overlapping absorptions of ring and -C-O stretching of carbohydrate were present at  $1090\text{ cm}^{-1}$ ,  $1060\text{ cm}^{-1}$ ,  $1035\text{ cm}^{-1}$ ,  $1010\text{ cm}^{-1}$ . A weak absorption was present at  $905\text{ cm}^{-1}$  and was possibly due to a  $\beta$ -D-glucopyranose structure as this was also present on the spectrum for 1-naphthol  $\beta$ -D-glucoside and on the spectra of the corresponding *p*-nitrophenyl compounds. A strong band attributable to the  $\text{PO}_3^{2-}$  symmetric stretching frequency was present at  $970\text{ cm}^{-1}$ . Again as on the spectrum for the *p*-nitrophenol metabolite X multiple absorptions in the region  $3500\text{ cm}^{-1}$  -  $3340\text{ cm}^{-1}$  were evident, attributable to the N-H and O-H stretching vibrations.

#### Synthesis of glucoside 6-phosphates and derivatives

Reaction of phosphorus oxychloride on *p*-nitrophenyl glucoside in pyridine at low temperature (Fischer, 1914).

Dry *p*-nitrophenyl  $\beta$ -glucoside, (1 g.) was dissolved in 3.25 ml. dry pyridine, then cooled to  $-25^\circ$  and mixed with 0.3 ml. phosphorus oxychloride in 1.1 ml. pyridine also at  $-25^\circ$ , and left for 1 hr. Water (1.1 ml.) was then added with shaking, and the mixture allowed to warm slowly to room temperature. A further 11 ml. water was then added and the solution evaporated down to small volume under vacuum at  $40^\circ$  to remove most of the pyridine. The residue was taken up in water and passed through a cation

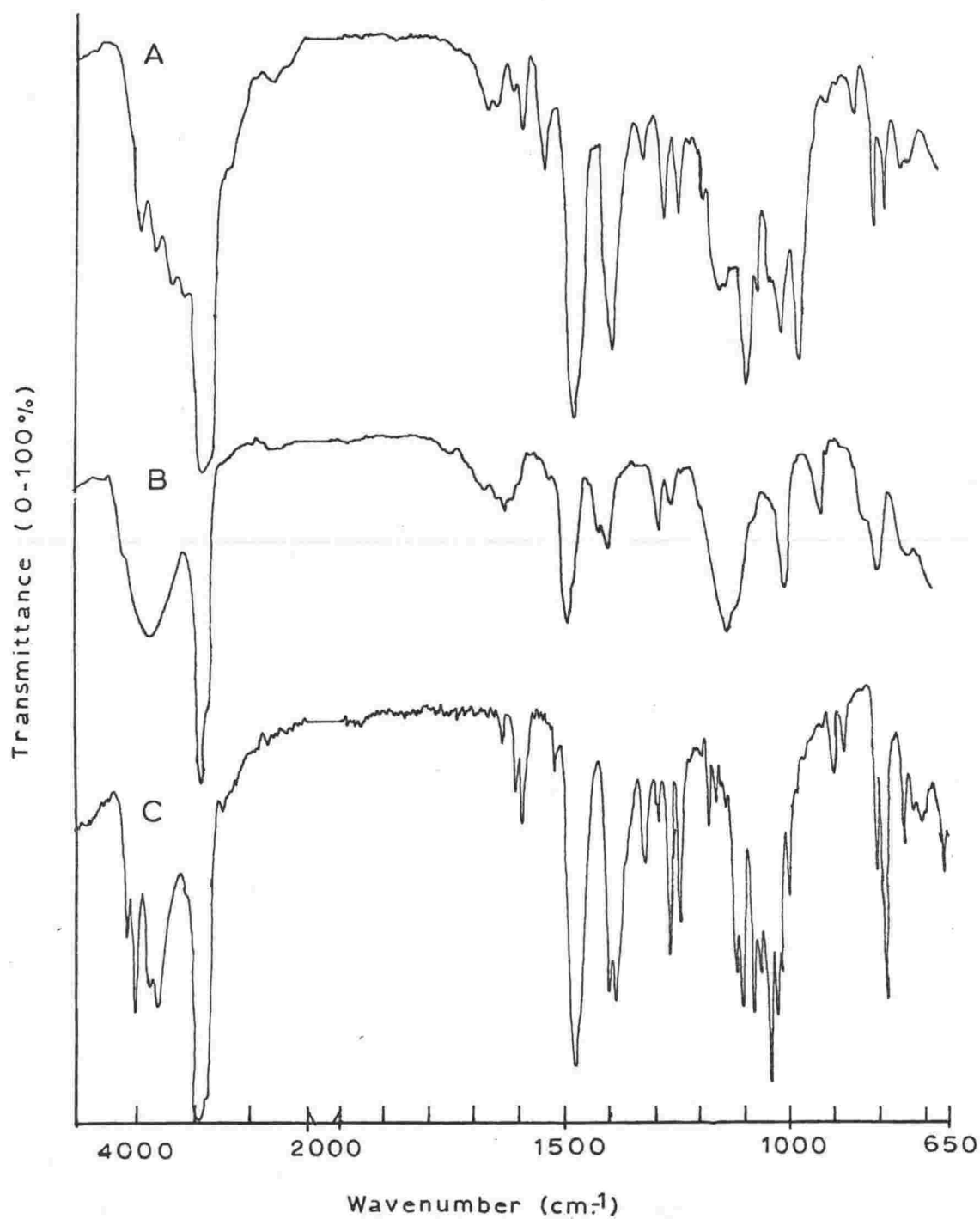


Fig.15. Infra-red spectra for A, 1-naphthol metabolite X isolated from fly faeces as the cyclohexylamine salt, and B, 1-naphthyl disodium phosphate, and C, 1-naphthyl  $\beta$ -D-glucoside, as nujol mulls.



exchange column in acid form to remove the last traces of pyridine. The resulting solution was again carefully evaporated to dryness at reduced temperature to remove hydrochloric acid. This residue was taken up in 2 ml. water and brought to pH 8.5 by addition of cyclohexylamine. On addition of two volumes of acetone a white crystalline precipitate settled out which was recrystallised from ethanol-ether, m.p.  $219^{\circ}$  (decomp.) ( $[\alpha]_D^{20} - 76^{\circ}$ ).

This substance did not appear to be the desired p-nitrophenyl glucoside 6-phosphate but instead had properties of the cyclohexylamine salt of p-nitrophenyl  $\beta$ -D-glucoside 4,6-(hydrogen phosphate). (Found: C, 47.3; H, 6.4; N, 5.8;  $C_{18}H_{27}N_2O_{10}P$  requires C, 46.7; H, 5.9; N, 6.05%).

The infra-red spectrum of this substance (Fig.16) was very similar to that of the p-nitrophenyl metabolite X, (Fig.13) except that the absorption at  $970\text{ cm}^{-1}$  attributed to  $PO_3^{2-}$  symetric stretching was absent, and new absorptions,  $1210\text{ cm}^{-1}$  and  $1230\text{ cm}^{-1}$  attributable to  $PO_2^-$  antisymetric stretching were present. The symetric stretching frequency of ionised phosphodiester of  $1080\text{ cm}^{-1}$  may also have been present but could not be distinguished from the C-O stretching frequencies.

A sample of the substance co-chromatographed on paper with the p-nitrophenyl metabolite in an acidic solvent, but in alkaline solvent had a larger  $R_F$  than the metabolite (Table 15).

Table 15. Chromatography of synthetic product and  
metabolite X

Substance	R <sub>F</sub> in solvent;	
	C (propanol-ammonia)	G (butanol-acetic)
synthetic product	.66	.39
mixture	.38, .67	.38
metabolite	.37	.39

The product on ionophoresis behaved as a strong monobasic acid (Table 16). Thus up to pH 4 it had a migration rate similar to that of the ~~acetylacetonol~~ metabolite X, but this migration did

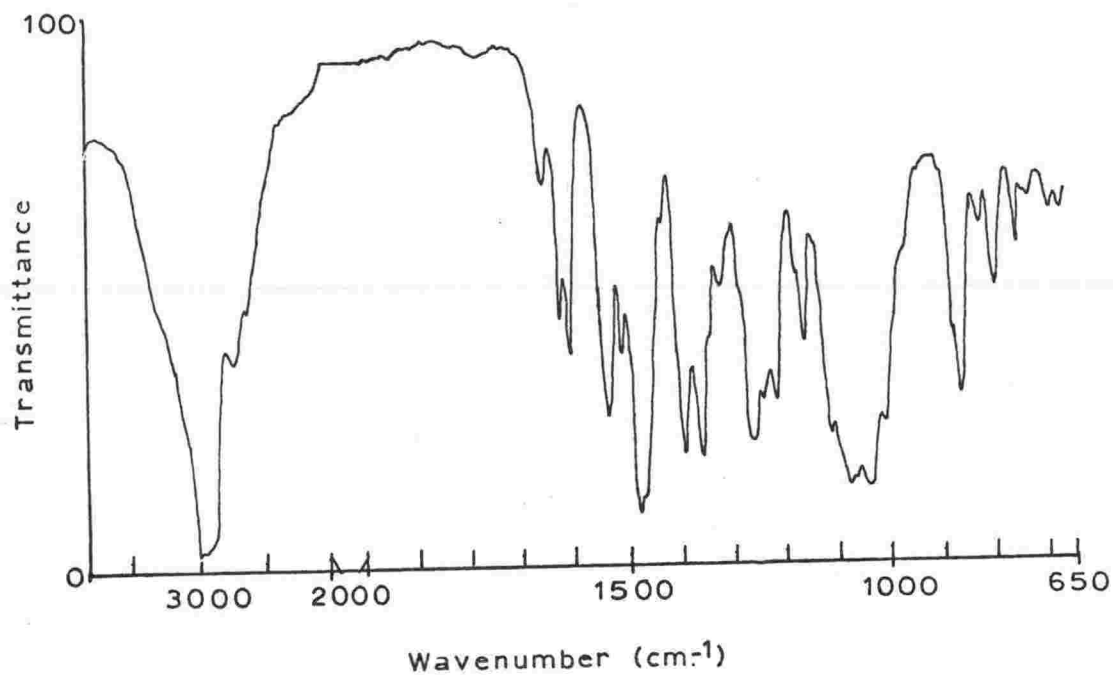


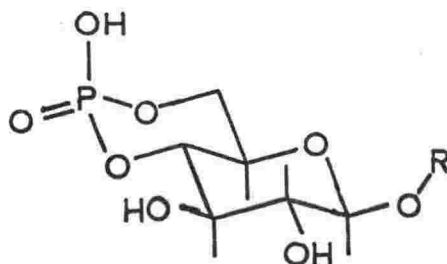
Fig.16. Infra-red spectrum (nujol mull) of the product obtained by reaction of phosphorus oxychloride on *p*-nitrophenyl glucoside in pyridine, as described in the text.

Table 16. Ionophoresis of a synthetic product and  
p-nitrophenyl metabolite X

Figures are migration to the anode in centimeters  
after ionophoresis at 10 v/cm., for 1.5 hr.

pH	3	4	6	7	8	9
synthetic product	3.0	3.0	3.0	2.75	3.0	2.5
metabolite X	3.0	3.0	5.0	5.25	5.25	5.0
p-nitrophenyl phosphate	4.75	5.25	7.25	7.25	7.25	7.25

phosphate) derivatives. Participation by the 4-hydroxyl to form the cyclic phosphates is apparently favoured by steric factors. The preferred configuration of  $\beta$ -glucosides is the C1 configuration (Reeves, 1950). This brings the 4-hydroxyl and the 6-hydroxyl into position such that on cyclic phosphate formation a double chair configuration is formed, with all the bulky groups equatorial to the plane of the molecule (Baddiley et al., 1954):



Configuration of  $\beta$ -glucoside 4:6-phosphates.

Thus it seems probable that a similar cyclisation reaction was taking place in the reaction of phosphorus oxychloride with *p*-nitrophenyl  $\beta$ -D-glucoside in

pyridine. Attempts to suppress the participation by the 4-hydroxyl by reaction at lower temperature ( $-30^{\circ}$  -  $-35^{\circ}$ ), with shorter reaction times (30 min.), and ensuring that hydrolysis of the phosphoryl chloride intermediates was complete before warming, were unsuccessful, only the cyclic phosphate again being isolated.

The reaction was repeated using 0.80 g. dry 1-naphthyl- $\beta$ -D-glucoside. During reaction of the glucoside and phosphorus oxychloride a white precipitate of pyridine hydrochloride was formed which redissolved on the addition of water. During evaporation off of pyridine a gummy precipitate formed which redissolved in water on addition of cyclohexylamine to pH 8. This solution was evaporated to small volume but yielded only an oily product and no crystalline material. This oil was taken up in water again and converted to free acid by passage through the cation exchange column. The eluate was cloudy and soon formed a gummy precipitate. This substance was suspended in water and redissolved by the addition of cyclohexylamine. Concentration of this solution and dilution with acetone once again produced a gummy precipitate but no crystalline material.

This material consisted chiefly of one component, which had chromatographic properties corresponding to those of the p-nitrophenyl glucoside 4:6-cyclic phosphate. Thus chromatography on paper in solvent G (butanol-acetic acid-water) yielded a

purple fluorescing spot of similar  $R_F$  to the 1-naphthol metabolite X, whereas in alkaline solvent (B, butanol-ammonia-water) a rather diffuse spot of  $R_F$  0.45 moving ahead of the 1-naphthol metabolite X spot,  $R_F$  0.18, was produced.

Action of phosphorus oxychloride on glucosides in an aqueous baryta suspension

A method used by Fischer (1914) for the synthesis of methyl  $\alpha$ -D-glucoside 6-phosphate was used.

p-Nitrophenyl  $\beta$ -D-glucoside 6-phosphate. A suspension of 0.13 g. powdered p-nitrophenyl  $\beta$ -glucoside and 0.6 g. powdered barium hydroxide octahydrate in 1.0 ml. of water was stirred at 0° with 0.072 ml. phosphorus oxychloride in 0.2 ml. ether, for 1 hr. Unconsumed barium hydroxide was then neutralised by passing in CO<sub>2</sub>, and the suspension was filtered.

Electrophoresis of a portion of the filtrate at pH values 4 and 9 indicated the presence of a large amount of p-nitrophenyl glucoside, a trace of p-nitrophenol, but no trace of p-nitrophenyl glucoside phosphates. A portion of the residue was brought into solution by shaking with cation exchange resin in hydrogen form. Electrophoresis of this solution at pH values 4 and 8 showed the presence of a small amount of a substance with the same ionophoretic properties as the unknown p-nitrophenol metabolite along with some unchanged glucoside (Table 17). This substance co-chromatographed

with the unknown *p*-nitrophenol metabolite in the solvent systems C, G and H (Table 17).

Attempts to isolate the synthetic glucoside phosphate from the large amounts of barium phosphates and carbonate by extraction of the residue were unsuccessful.

1-Naphthyl  $\beta$ -D-glucoside 6-phosphate. The preparation above was repeated using 0.15 g. 1-naphthyl glucoside and 0.075 ml. phosphorus oxychloride. Electrophoresis indicated the presence of only 1-naphthyl glucoside in the filtrate obtained after neutralisation with CO<sub>2</sub> but the residue contained a trace of substance with ionophoretic properties similar to those of the unknown 1-naphthol metabolite. This substance had similar R<sub>F</sub> values to the unknown metabolite in the solvent systems B, G and H on paper chromatography (Table 17). The yield was too small for successful isolation by extraction from the residue with hot water and conversion to the cyclohexylammonium salt.

Preparation of glucoside 6-(dibenzyl phosphate)s.

Successful synthesis of phosphomonoesters of biological interest is greatly facilitated by the use of protected phosphorylating agents and reagents (Khorana, 1961). The use of dibenzylphosphorochloridate as a monofunctional phosphorylating agent has been developed by Todd and co-workers (Atherton, Howard & Todd, 1948; Atherton, Openshaw & Todd, 1945). Reaction of dibenzyl



Table 17. Paper chromatography and ionophoresis of the unknown conjugates of p-nitrophenol and 1-naphthol and the synthetic glucoside 6-phosphates

	$R_F$ in solvent				Migration to anode in			
	B	C	G	H	cm. at 10 v/cm. for 2 hr.			
					pH 3	pH 4	pH 8	pH 9
p-nitrophenol metabolite X		0.14	0.40	0.65	2.5	2.5	5.5	5.5
synthetic p-nitrophenol glucoside 6-phosphate		0.14	0.40	0.65	2.5	2.5	5.5	5.5
1-naphthol metabolite X	0.13		0.50	0.62	2.75	3	5	5
synthetic 1-naphthol glucoside 6-phosphate	0.12		0.48	0.60	3	3	5	5

phosphorochloridate with an alcohol in the presence of a base such as pyridine yields a phosphotriester. The benzyl groups are removed under mild conditions by catalytic hydrogenation using a palladium catalyst to produce the desired alkyl phosphomonoester.

Accordingly, the preparation of 1-naphthyl  $\beta$ -D-glucoside 6-phosphate was attempted by reaction of dibenzyl phosphorochloridate and 1-naphthyl  $\beta$ -D-glucose 2,3,4-triacetate to give 1-naphthyl  $\beta$ -D-glucoside 2,3,4-triacetate 6-(dibenzyl phosphate). Removal of the dibenzyl groups by hydrogenation and of the acetyl groups with a catalytic amount of sodium methoxide in methanol (Lardy & Fischer, 1952) offered a route to the desired 1-naphthyl glucoside 6-phosphate. Although the presence of the readily reducible nitro group precluded the preparation of the p-nitrophenyl glucoside by catalytic hydrogenation of the corresponding p-nitrophenyl glucoside 6-(dibenzyl phosphate), preparation of this dibenzyl phosphotriester was also attempted, in order to compare its properties with those of the 1-naphthol compound and other p-nitrophenyl glucoside derivatives.

Glucoside triacetates with free primary hydroxyl were prepared by selective tritylation of 1-naphthyl and p-nitrophenyl glucosides in the C-6 position, followed by acetylation of the secondary hydroxyls and subsequent detritylation according to the general procedure of Reynolds & Evans (1942).

1-Naphthyl and p-nitrophenyl 6-O-trityl-β-D-glucoside  
2,3,4-triacetate

Dry p-nitrophenyl glucoside (8.41 g.) or dry 1-naphthyl β-D-glucopyranoside (8.6 g.) and 7.86 g. trityl chloride were dissolved in 28 ml. dry pyridine and left in a tightly stoppered flask for 20 hr. Then 28 ml. acetic anhydride and a further 28 ml. pyridine were added and the mixture set aside for another 20 hr. The mixture was then poured slowly into 3 l. of water with rapid stirring, the stirring being continued for 2 hr. The tritylated sugars obtained in almost quantitative yield formed white granular amorphous precipitates and were collected by filtration and washed well with water. p-Nitrophenyl 2,3,4-tri-O-acetyl-6-O-trityl-β-D-glucopyranoside crystallised from alcohol; m.p. 110-111°,  $[\alpha]_D^{20} + 6.6 \pm 5^\circ$ , (C, 1.0 in EtOH), (Found: C, 66.7; H, 5.4; N, 1.8;  $C_{37}H_{35}NO_{11}$  requires C, 67.5; H, 5.27; N, 2.09%).

1-Naphthyl 2,3,4-tri-O-acetyl-6-O-trityl-β-D-glucopyranoside crystallised from alcohol (needles); m.p. 169-70°  $[\alpha]_D^{20} -14.5 \pm 5^\circ$  (C, 1.0 in  $CHCl_3$ ); (Found: C, 72.8; H, 5.9;  $C_{41}H_{38}O_9$  requires C, 73.0; H, 5.68%).

1-Naphthyl and p-nitrophenyl 2,3,4-triacetyl glucosides

The above trityl compounds (15 g.) were powdered and suspended in 50 ml. glacial acetic acid and a slight excess of 38% HBr in glacial acetic acid was added rapidly with shaking. The

sugars rapidly dissolved and almost immediately trityl bromide began to crystallise out. After 5 min. the trityl bromide was filtered off and the filtrates were slowly poured into 2 l. of rapidly stirred water to precipitate the triacetates. Stirring was continued until the precipitated triacetates had become hard and crumbly. These were filtered off, washed with water, dried, and recrystallised from ethanol-di-isopropyl ether or toluene.

p-Nitrophenyl 2,3,4-tri-O-acetyl- $\beta$ -D-glucopyranoside crystallised from toluene with solvent of crystallisation, m.p.  $124^{\circ}$  with partial resolidification and remelting at  $135-145^{\circ}$ ;  $[\alpha]_D^{20}$ ,  $-21^{\circ} \pm 5^{\circ}$  (C, 1.0 in EtOH), (Found: C, 54.2; H, 5.32; N, 2.90; O, 37.2; loss on heating at  $120^{\circ}$  9.5;  $C_{18}H_{21}NO_{11}$   $1/2$   $C_7H_8$  requires C, 54.5; H, 5.32; N, 2.96; O, 37.2; loss on heating, 9.73%).

1-Naphthyl 2,3,4-tri-O-acetyl- $\beta$ -D-glucopyranoside crystallised from ethanol-di-isopropyl ether (1:1 v/v) or toluene, m.p.  $164^{\circ}$ ;  $[\alpha]_D^{20}$   $-117 \pm 5^{\circ}$  (C, 1.0 in  $CHCl_3$ ) (Found: C, 61.1; H, 5.54;  $C_{22}H_{24}O_9$  requires C, 61.1; H, 5.59%).

The presence of a free hydroxyl group in these compounds was indicated by the presence of a single sharp moderate absorption at  $3560\text{ cm}^{-1}$  in the i.r. spectra, instead of the broad, strong absorptions at slightly lower frequency present in the spectra of the free glucosides. 1-Naphthyl and p-nitrophenyl glucoside-tetraacetates had no absorption in this region. The i.r. spectra of the

triacetates also had a very strong absorption at  $1750\text{ cm}^{-1}$ , due to the acetyl carbonyl, and at  $1220\text{-}1240\text{ cm}^{-1}$  due to acetyl C-O as well as the bands of the corresponding glucoside spectra.

p-Nitrophenyl 2,3,4-tri-O-acetyl- $\beta$ -D-glucopyranoside  
6-(dibenzyl phosphate)

Attempts to prepare the dibenzyl phosphate derivatives by phosphorylation of the triacetates by the methods of Atherton et al., (1945, 1948) by reaction of equimolar amounts of the triacetate and dibenzyl phosphorochloridate in pyridine at room temperature, and in 2,6-lutidine at both room temperature and at  $0^{\circ}\text{C}$ , for various reaction times (2-24 hr.) were unsuccessful. In every case, only the starting triacetates were recovered from the reaction mixtures, in yields of up to 90%, identified by mixed m.p. and comparison of i.r. spectra. However, the p-nitrophenol compound was successfully prepared by a method used by M. Smith (1961) for the preparation of uridine 5'-phosphate and by Turner & Khorana (1959) for the preparation of thymidine 3'-phosphate, involving the use of 3-5 molar excess of dibenzyl phosphorochloridate at low temperatures.

A solution of 640 mg. (1.5 m-mole) of p-nitrophenyl glucoside-triacetate in 5 ml. dry pyridine in a tightly stoppered flask was frozen in a solid  $\text{CO}_2$ -acetone freezing mixture. Dibenzyl phosphorochloridate prepared from 1.31 g. dibenzyl phosphite and

0.66 g. N-chlorosuccinimide (Kenner, Todd & Weymouth, 1952), was quickly added to the frozen pyridine solution as a solvent-free oil using a wide jet pipette, the stopper was quickly replaced, and the flask shaken until the mixture became homogenous. The flask was then replaced in the freezing mixture for 1 hr., and then placed in the deep freezer at  $-20^{\circ}$  over night (18 hr.). Then 5 ml. of water was added, and after 10 min. the contents were allowed to reach room temperature and the water and pyridine were removed by evaporation under vacuum at below  $40^{\circ}$ . The resulting oil was taken up in ether and extracted twice with dilute HCl, and then with sodium bicarbonate solution, and finally washed twice with water. The ether layer was dried with anhydrous sodium sulphate and evaporated under vacuum to yield an oil. This oil was taken up in a little toluene and on standing over night yielded fluffy white crystals of the dibenzyl phosphate triester. These were collected (yield 400 mg.) and recrystallised from toluene. p-Nitrophenyl 2,3,4-tri-O-acetyl- $\beta$ -D-glucopyranoside 6-(dibenzyl phosphate) had m.p.  $115^{\circ}$ ,  $[\alpha]_D^{20} -21^{\circ}$  (C, 1.0 in EtOH). (Found: C, 56.3; H, 5.2; N, 2.0; P, 4.7;  $C_{32}H_{34}NO_{14}P$  requires C, 55.9; H, 4.98; N, 2.04; P, 4.5%).

The i.r. spectrum, (Fig.17), in addition to the bands present in the spectrum of p-nitrophenyl glucoside-triacetate had bands at:  $1280\text{ cm}^{-1}$  attributable to the P=O bond,  $1005\text{ cm}^{-1}$  attributable to P-O-alkyl absorption, and increased absorptions

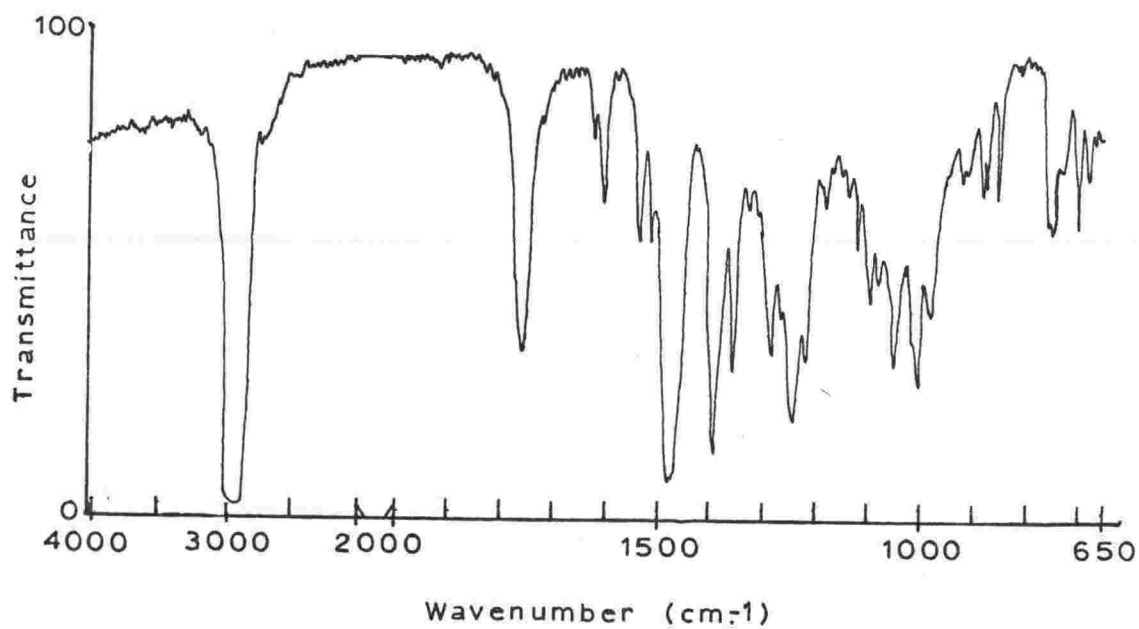


Fig.17. I.r. spectrum of synthetic *p*-nitrophenyl  
2,3,4-tri-*O*-acetyl- $\beta$ -D-glucoside 6-(dibenzyl phosphate).

at 750 and 700  $\text{cm}^{-1}$  attributable to out-of-plane C-H bending of the mono-substituted benzene ring, i.e. the benzyl group. Absorption in the region of 3570  $\text{cm}^{-1}$  due to the presence of free hydroxyl group, present in the i.r. spectrum of the triacetate, was absent.

Attempted dibenzyl phosphorylation of 1-naphthyl glucoside triacetate

The above preparation was repeated using 1.5 m-mole of 1-naphthyl glucoside-triacetate in place of *p*-nitrophenyl glucoside-triacetate. However, the oil finally obtained after washing with HCl,  $\text{NaHCO}_3$  and water as above could not be induced to crystallise. The i.r. spectrum of this oil had on top of the bands of the glucoside-triacetate spectrum strong bands at 750  $\text{cm}^{-1}$  and 700  $\text{cm}^{-1}$  indicating presence of benzyl groups. There were, however, only poorly resolved shoulders in the regions 1280  $\text{cm}^{-1}$  and 1005  $\text{cm}^{-1}$  where the P=O and P-O-alkyl bands were expected to appear. If 1-naphthyl glucoside triacetate 6-(dibenzyl phosphate) were present, it could only have been in low yield. The oil did not contain starting material as a toluene solution of the oil did not deposit crystals when seeded with a crystal of 1-naphthyl glucoside-triacetate.

The material resisted hydrogenation with palladium oxide catalyst. The oil was dissolved in ethanol and refluxed with Raney nickel for 30 min. Then the nickel was filtered off and



the ethanolic solution was shaken at room temperature with hydrogen at atmospheric pressure in presence of palladium oxide. After reduction of the palladium oxide no significant uptake of hydrogen occurred over 6 hr.

## DISCUSSION

### Metabolism of Carbaryl

The results show that carbaryl is excreted by insects as a complex mixture of water-soluble metabolites. Over 24 hr., these water-soluble products accounted for over 60% of the total administered dose for the housefly and blowfly, and 50% for the grass grub. The combination of the paper chromatography followed by ionophoresis of the peaks resolved, indicated the presence of ten or more conjugates in the mixture. These included 1-naphthyl phosphate, sulphate and glucoside, identified by co-chromatography and co-ionophoresis with reference compounds and on the basis of the formation of similar conjugates from 1-naphthol.

The work on 1-naphthol and *p*-nitrophenol metabolism showed that a new conjugate, glucoside 6-phosphate, is formed from phenols in flies, and probably 1-naphthyl glucoside 6-phosphate accounted for one of the remaining carbaryl conjugates. This chromatographed with the naphthyl phosphate on paper but was resolved as a slower moving peak by ionophoresis. The remaining compounds were also phosphate or glucoside 6-phosphate, sulphate and glucoside conjugates of undetermined compounds. From previous work it can be expected that these compounds were the various oxidation products of carbaryl. This is supported by the increased mobility exhibited by these compounds on

ionophoresis in alkaline medium, presumably due to the influence of phenolic group, which becomes ionised at high pH values.

#### Conjugations of 1-naphthol

Because of the complexity of the pattern of the water-soluble compounds resulting from metabolism of carbaryl by insects, and the apparent presence of phosphate-type conjugates, it was felt necessary to examine further the conjugations of 1-naphthol by insects.

The results from the dilution analyses and quantitative paper chromatography show that phosphate conjugates may be found in the extracts and excreta of insects dosed with phenols. In the experiments with the grass grub, housefly and blowfly, the phosphate conjugate was often a major constituent of the metabolised dose. This confirms a previous indication of the involvement of phosphate in detoxication reactions (Smith & Turbert, 1964) and establishes phosphate conjugation in the housefly and blowfly and the grass grub as being quantitatively as important as the sulphate and glucoside conjugation reactions.

Another conjugate was also present in extracts and excreta of flies dosed with 1-naphthol or *p*-nitrophenol. This has been identified as the corresponding aryl  $\beta$ -D-glucoside 6-phosphate (see overleaf). The quantitative paper chromatography

with labelled 1-naphthol showed that in the case of flies this conjugate also forms a prominent amount of the total conjugates of 1-naphthol. The grass grub seemed to form much less of the glucoside 6-phosphate conjugate. This could have possibly been partly due to the fact that the grass grubs were starved, since less glucoside and more sulphate conjugates were formed in the grass grubs compared to the housefly and blowfly.

The prominence of the phosphate and glucoside 6-phosphate conjugates encountered in this study raises the question of the apparent non-detection of these conjugates in previous studies of conjugations in insects. The phosphates and glucoside 6-phosphates have relatively low  $R_F$  values in the solvents usually used for paper chromatography of conjugates, particularly in alkaline, or non-polar solvents such as methyl ethyl ketone-water (Smith, 1955). Therefore it is possible that these could move with the large amounts of natural extractives that occur at low  $R_F$  values, and be overlooked. Identification of a conjugate is often based on a hydrolysis to the free components by treatment with acid or hydrolases, with subsequent identification of the unconjugated material. Since the phosphate (Brown, 1963) and glucoside 6-phosphate esters are resistant to acid hydrolysis, these could escape detection by this means. Also the identity of the sugar or anion portion of a conjugate is seldom checked and possibly conjugates presumed to have been

sulphates, glucosides or glucuronides may actually have been the phosphate or glucoside 6-phosphate conjugates.

Absence of glucuronide conjugates

The dilution analyses and quantitative paper chromatography studies showed that glucuronide conjugates were absent in extracts of insects dosed with [ $^{14}\text{C}$ ]1-naphthol, and could not have been present in amounts of more than 0.01% of the applied doses. This is in agreement with the work of Dutton & Ko (1964) on the absence of uridine diphosphate glucuronyl transferases in insect tissues, and of Smith & Turbert (1964). This poses the problem of the identity of compounds identified in insect tissues as glucuronic acid conjugates. It now seems possible that these compounds reported may actually have been phosphate or glucoside 6-phosphate conjugates.

The detection of 1-naphthyl glucuronide and other glucuronides as products of naphthalene and 1-naphthol metabolism in flies (Terriere, Boose & Roubal, 1961) was based on paper chromatographic work and hydrolysis to the free phenols by glucuronidase preparations. However, many glucuronidases obtainable, e.g. 'mollusc glucuronidases', contain other hydrolases including phosphatase, and especially since in the above work long incubation times of 24 hr. or more were employed,

it would be quite possible for phosphates and other conjugates to be hydrolysed by glucuronidase preparations.

Inagami's (1955) identification of (3-hydroxykynurenine glucosid)uronic acid in extracts of silkworms was based on a method used by Dalgleish (1952) to identify (3-hydroxykynurenine glucosid)uronic acid as a metabolite of tryptophan in vertebrates. Identification was based on its low  $R_F$  value, difficulty of hydrolysis, and behaviour as an acid on electroosmosis. A phosphate conjugate could equally have these properties.

However, the metabolites of Dipterex (dimethyl 2,2,2-trichloro-1-hydroxyethylphosphonate) and its methyl ether in Prodenia larvae identified as glucuronide conjugates (Zayed et al., 1965; Hassan et al., 1965) cannot be explained as phosphates. A product liberated by alkaline hydrolysis of the conjugates was extracted into ether and identified as glucuronic acid on the basis of paper chromatography, using ammoniacal silver nitrate and methyl orange as detection reagents. The ether extract contained material giving a positive Molisch reaction, but since neither glucuronic acid nor glucose 6-phosphoric acid are appreciably soluble in ether, the nature of the compound is not clear.

### Phosphodiester conjugates

The formation of a diaryl phosphate, di(2-amino-1-naphthyl) hydrogen phosphate as a metabolite of 2-naphthylamine in dogs and man (Boyland, Kinder & Manson, 1961) has been mentioned. In this study no evidence for the formation of di(1-naphthyl) hydrogen phosphate from 1-naphthol in flies or the grass grub could be found. This conjugate could not have exceeded 0.1% of the dose of 1-naphthol. Diaryl phosphates are generally stable to normal acids (Brown, 1963) hence it is unlikely that diaryl phosphates were being formed in vivo and then breaking up to form monoaryl phosphates on work-up of extracts or chromatography. The diaryl phosphate isolated by Boyland was rather easily hydrolysed to the monoaryl conjugate under acid but this was possibly a consequence of the presence in the compound of an o-amino group.

### Occurrence of phosphate esters

The rare occurrence of aryl phosphates in nature has been mentioned in the introduction. Glucoside 6-phosphates have not been reported as detoxication products before, and few instances of the occurrence of this type of compound have been recorded, although the closely related nucleotides, the riboside and deoxy-riboside phosphates, are of paramount importance in biochemistry.



An inorganic pyrophosphate-glucose phosphotransferase system has been characterised in vertebrate liver microsomes (Stetten, 1964) which appears to be identical with microsomal inorganic pyrophosphatase and glucose 6-phosphatase (Nordlie & Arion, 1965). A number of nucleoside di- and tri-phosphates can serve in place of inorganic pyrophosphate (Nordlie & Snoke, 1967) and a variety of sugars and derivatives, for example methyl  $\alpha$ - and  $\beta$ -glucosides, can substitute for glucose, resulting in the formation of glucoside 6-phosphates (Stetten, 1965).

Several microorganisms, for example E. coli and Staphylococcus aureus, accumulate sugars and glycosides as their 6-phosphate esters (Laue & MacDonald, 1967; Fox & Wilson, 1967; Kaback, 1968). The source of phosphate is phosphoenolpyruvate and is catalysed by a phosphoenolpyruvate-phosphotransferase system. The sugars and glycosides are transported through the cell membranes as their 6-phosphates and are hydrolysed inside the cells by phosphatases. Specific glycosidases also exist which hydrolyse the glycoside linkage of glycosides carrying a phosphate in the 6-position (Hengstenburg, Egan & Morse, 1967).

The source of the glucoside 6-phosphates detected as conjugates remains to be settled. The presence of both glucosides and glucoside 6-phosphates together raises the question whether

one of these may be a precursor of the other. The formation of glucosides by the UDP-glucosyl transferase system is well established, hence it is unlikely that a formation of glucosides by the splitting off of a phosphate from a preformed glucoside 6-phosphate will be of any importance. It is likely that the phosphate is formed by a secondary reaction on the glucoside, either in the insect tissues, or in the gut by the action of phosphatases, or possibly by the action of the gut microorganisms.

The action of phosphatases could also conceivably account for the formation of the aryl phosphates from the free phenols and inorganic phosphate, by a reversal of the hydrolysis reaction. However, the high concentrations of phosphate and phenol and low concentrations of water required for synthesis (Meyerhof & Green, 1949), especially in the case of the moderately acidic *p*-nitrophenol, make this unlikely. Non-specific phosphatases also have phosphotransferase activity (Morton, 1958), hence the phosphates encountered here could have been formed by transfer from some other preformed phosphate. However, transfer of phosphate by non-specific phosphatases has only been observed to occur from high energy phosphates such as phosphoenolpyruvate or phenyl phosphates to aliphatic alcohols (Meyerhof & Green, 1950). A high concentration of acceptor is required.

#### Significance of the new conjugations

The phosphate and glucoside 6-phosphates are two new

conjugates which occur in addition to the well established glucoside and sulphate conjugations in insects. It is not yet clear whether the phosphate and glucoside 6-phosphate conjugations have any advantage to the insect, over that of the glucoside and sulphate conjugations. The availability of a phosphate conjugating mechanism may be of advantage to an organism faced with a large load of, or a very toxic, foreign compound to be conjugated. The limited use of the sulphate conjugation in dealing with large loads of foreign compounds has been mentioned, being due to the limited availability of inorganic sulphate. The availability of an extra route such as the phosphate conjugation would increase the total conjugating capacity of an organism.

The phosphate conjugates of carbaryl were sometimes present in amount approaching that of the sulphate and glucoside conjugates combined. The non-toxicity of the oxidation products of carbaryl, such as the 4- and 5-hydroxy-1-naphthyl N-methylcarbamates, is probably due to a rapid conjugation with the hydroxyl groups, with detoxication. These compounds are as potent inhibitors of acetylcholinesterase in vivo as carbaryl itself (Oonithan & Casida, 1968). Since aryl phosphate conjugates of carbaryl form a substantial proportion of the total conjugates, they presumably assist in the rapid removal of the primary oxidation products of carbaryl.

Since the glucoside 6-phosphate conjugation requires a

source of glucose as well as of phosphate the availability of this system would not have the advantage of the aryl phosphate conjugation overleaf.

The occurrence of an active transport of ionic compounds in the Malpighian tubules, similar to that in the proximal tubules of the vertebrate kidney, has not been observed. Hence it does not seem that any advantage would be gained by the phosphate and glucoside 6-phosphate conjugates over glucoside in their excretion by the Malpighian tubules, if actually formed in the insect tissues. Since the glucoside 6-phosphates are resistant to  $\beta$ -glucosidase, these conjugates could be more resistant to gut hydrolases than the other conjugates, with a lessened chance of recirculation of the toxic aglycone.

#### Characterisation and properties of the glucoside 6-phosphates

The new metabolites of *p*-nitrophenol and 1-naphthol were established as glycoside phosphate conjugates on the basis of the i.r. and u.v. spectral, paper chromatographic and ionophoretic data, and analysis of a sample of the *p*-nitrophenyl conjugate, isolated at its cyclohexylamine salt. This glycoside phosphate was characterised as  $\beta$ -D-glucopyranoside 6-phosphoric acid on the basis of hydrolysis of the *p*-nitrophenol conjugate to glucose, glucose 6-phosphoric acid and free *p*-nitrophenol. The

$\beta$ -configuration of the glycosidic link was indicated by the negative specific rotations of the conjugates. These specific rotations, calculated for the free acids, (1-naphthyl  $\beta$ -D-glucoside 6-phosphoric acid,  $[\alpha]_D^{20} = -75^\circ$ ; *p*-nitrophenyl  $\beta$ -D-glucoside 6-phosphoric acid  $[\alpha]_D^{20} = -103^\circ$ ;) are similar to those of the corresponding glucoside and glucuronic acid conjugates (1-naphthyl  $\beta$ -D-glucoside,  $[\alpha]_D = -76^\circ$ , (water), (Smith & Turbert, 1964); (1-naphthyl  $\beta$ -D-glucosid)uronic acid,  $[\alpha]_D = -85^\circ$ , (ethanol), (Berenbom & Young, 1951); *p*-nitrophenyl  $\beta$ -D-glucoside,  $[\alpha]_D^{20} = -105^\circ$ , (water); (*p*-nitrophenyl  $\beta$ -D-glucosid)uronic acid,  $[\alpha]_D^{20} = -103^\circ$ , (0.1 N-HCl);). Thus the rule that  $\beta$ -D-glucosides and corresponding  $\beta$ -D-glucuronides have similar specific rotations (Bray, 1953) may be extendable to include glucoside 6-phosphates. Evidence for the  $\beta$ -configuration was also provided by the i.r. spectra.

The glycoside linkage in the conjugates was much more acid resistant to alkaline or acid hydrolysis than the corresponding  $\beta$ -D-glucosides. This is analogous to the situation with glucose 1,6-diphosphate in which the glycosidic linkage is hydrolysed much more slowly than in glucose 1-phosphate (Leloir & Cardini, 1963). Glycopyranosiduronic acids are also hydrolysed much more slowly in acid solution than the corresponding glycosides (Bemiller, 1967). The stabilising effect is apparently

due to steric effects in which a bulky group at C-5 of glycosides inhibits rearrangement to a transition state during hydrolysis. Inductive effects may also be involved in the case of polar groups such as the carboxylic acid group.

Glycosides are generally stable to alkaline hydrolysis. However, many phenyl glycosides, including aryl  $\beta$ -D-glucosides but not aryl  $\alpha$ -D-glucosides, are unstable to alkali (Ballou, 1954). Degradation of aryl  $\beta$ -D-glucosides in alkali results in the formation of 1,6-anhydro- $\beta$ -D-glucopyranose, so evidently the -OH group at C-6 participates in the hydrolysis, although a reaction route involving the formation of 1,2-anhydro- $\alpha$ -D-glycopyranose as an intermediate has been postulated (Ballou, 1954). The glycosidic link of *p*-nitrophenyl,  $\beta$ -D-glucoside 6-phosphate was readily broken in alkali, (50% degraded in 75 min. at pH 12 and 80°), as shown by the release of free *p*-nitrophenol, although at a much slower rate than that of *p*-nitrophenyl  $\beta$ -D-glucoside (50% degraded in 2 min. at pH 12, 80°, Piskiewicz & Bruice, 1967). The glycoside link in 1-naphthyl  $\beta$ -D-glucoside 6-phosphate was only very slowly degraded in alkali. Evidently the phosphate group at C-6 stabilised the glycosides to alkali. This is analogous to the situation with (aryl  $\beta$ -D-glucosid)uronic acids, which are not reducing the alkaline copper reagents unless first hydrolysed.

by acid (Bray, 1953).

Hydrolysis of glucose 6-phosphate in acid is very slow (Dawson, 1959) and likewise the hydrolysis of the phosphate ester link in glucoside 6-phosphates was slow in acid. Considerable amounts of glucose 6-phosphate were detected in acid hydrolysates of the p-nitrophenyl glucoside 6-phosphate.

Simple alkyl phosphomonoesters are stable in alkali, but sugar phosphates with a free reducing group, including glucose 6-phosphate, are rapidly degraded in alkali (Degani & Halmann, 1968). However, the release of phosphate from the glucoside 6-phosphates was very slow in alkali. In the case of the p-nitrophenol compound, release of inorganic phosphate at pH 12 and 80° was a little slower than release of p-nitrophenol. Probably hydrolysis of the glycoside link to form a free reducing group was necessary for the release of inorganic phosphate. This release of phosphate in alkali did not seem to be complete even after several half-lives. Degani & Halmann (1968) have found that glucose 6-phosphate is partially degraded in alkali to the relatively stable 6-phosphoglucometasaccharinic acid,

The glucose 6-phosphates appeared to be stable to  $\beta$ -glucosidase (emulsin). Specificity at C-6 is encountered with  $\beta$ -glucuronidases, which do not attack  $\beta$ -glucosides, and

$\beta$ -glucosidases which do not attack  $\beta$ -glucuronides. Also, a staphylococcal  $\beta$ -galactosidase has recently been described that cleaves the glycosidic bond of 6-phosphorylated  $\beta$ -D-galactosides but not the  $\beta$ -D-galactosides themselves (Hengstenberg, Egan & Morse, 1967). It is possible that emulsin could have had a weak action on the  $\beta$ -D-glucoside 6-phosphates that was not detectable on paper chromatograms.

#### Attempted synthesis of $\beta$ -D-glucoside 6-phosphates

The synthesis of the  $\beta$ -D-glucoside 6-phosphates by the action of phosphorus oxychloride in pyridine on the  $\beta$ -D-glucosides was frustrated by the ready participation of the C-4 hydroxyl in the phosphorylation reaction. A steric crowding of the C-5  $-\text{CH}_2\text{OPOCl}_2$  group by the aglycone of the  $\beta$ -D-glucosides, which have the C1 configuration (Reeves, 1950), may have assisted the cyclisation reaction by pushing the phosphoryl group closer to the C-4 hydroxyl. Fischer (1914) was able to successfully synthesise the 6-phosphate ester of methyl  $\alpha$ -D-glucoside, which would not have such a steric hinderance, but not of theophylline  $\beta$ -D-glucoside. Elimination of C-4 hydroxyl participation by carrying out the phosphorylation in aqueous medium resulted in a yield of phosphorylated product too low for isolation.

Such a steric effect may also have been partly responsible



for the very low reactivity of the C-6 hydroxyl group in the triacetylated sugars towards the dibenzyl phosphorochloridate. Such a lack of reactivity has been encountered by Smith, M. (1961) in phosphorylation of uridine at C-5'.

#### Significance of water-soluble detoxication products

In studies on the metabolism of carbaryl, emphasis has been on the organo-soluble extracts, both in excreta of intact organisms and as products of action of isolated enzyme systems such as the microsomal fractions. This is partly justified on the grounds that from a toxicological point of view the primary detoxication products are of more interest than the final conjugation products. Also, the analysis of water-soluble compounds is more difficult than that of those extracted by organic solvents due to difficulties of chromatography and separation from large amounts of natural extractives.

However, a knowledge of the water-soluble metabolites is desirable for several reasons. Use of isolated systems may give an untrue picture of the relative importance of competing pathways, since products which are rapidly conjugated in vivo may be further acted upon when formed in in vitro systems containing oxidising enzymes but lacking conjugation enzymes. Analysis of the conjugation products provides valuable clues as to the overall pattern of metabolism of a compound.

This study shows that future workers carrying out a detailed investigation of the metabolism of carbaryl through to its conjugation products will have to look for phosphate, glucoside 6-phosphate, sulphate and glucoside conjugates.

An important point to note is that both the phosphate and glucoside 6-phosphate conjugates are resistant to hydrolysis. If acid is used to hydrolyse conjugates, with consequent detection of the free phenols released, the phosphate and glucoside 6-phosphate conjugates could be overlooked unless treatment is sufficiently prolonged to ensure their breakdown. Care is also needed in application of enzymic hydrolysis, since the glucoside 6-phosphate conjugate requires first a phosphatase, and then a glucosidase, for the release of the free phenol.

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