

STUDIES ON THE METABOLISM OF SOME ORGANOPHOSPHORUS
INSECTICIDES IN SUSCEPTIBLE AND RESISTANT STRAINS
OF HOUSEFLY (MUSCA DOMESTICA L.)

A thesis submitted for the degree of Doctor of Philosophy,
in the Faculty of Science, University of London

by

JONATHAN BRIAN LEWIS, B.Sc. (Leeds)

September, 1970

Department of Insecticides & Fungicides,
Rothamsted Experimental Station,
Harpenden, Hertfordshire

ABSTRACT

The metabolism of diazinon, diazoxon and parathion was studied, both in vitro and in vivo, in six strains of housefly (Musca domestica L.). Two strains of housefly (ocra SRS and ac;ar;bwb;ocra SRS) were fully susceptible, and one strain (SKA) was highly resistant, to these three organophosphorus insecticides. The other three strains studied had either a resistance factor (gene a) located on chromosome II derived from the SKA strain (strains 29 and 393) or a resistance factor on chromosome V derived from the SKA strain (strain 466.500). Strains 29 and 393 were resistant to all three organophosphorus insecticides, whilst strain 466.500 was resistant to diazinon and diazoxon, but not to parathion.

The strains with gene a had a glutathione-requiring, anaerobic system, located in the 100,000 g supernatant fraction, which metabolised diazinon, diazoxon and parathion principally to their desethyl derivatives. The synergist S,S,S-tributylphosphorotriethionate (TBTP) inhibited this system but sesamex, another synergist, did not. Preliminary experiments were carried out on the enzymology of this system.

The strains with the resistance factor on chromosome V had an NADPH and oxygen-requiring system, located in the microsomes, which metabolised diazoxon and, possibly diazinon, to at least three unidentified metabolites, but did not metabolise parathion.

Sesamex inhibited this system and TBTP did not.

In addition, all six strains studied had an NADPH and oxygen-requiring microsomal system which cleaved diazinon and parathion to diethylphosphorothioic acid and diazoxon to diethylphosphoric acid, and converted the thionates to their more potent anti-cholinesterase oxygen analogues, diazoxon and paraoxon. Sesamex inhibited this system also, whilst TBTP did not. An explanation of the effects of sesamex and TBTP on the toxicity of diazinon, parathion and diazoxon to the various strains is suggested and the contribution of these metabolic pathways to housefly resistance to organophosphorus insecticides is discussed.

PUBLICATIONS

Preliminary accounts of some of this work have been published (Nature, 224, 917 (1969)) and presented at the 5th British Insecticide and Fungicide Conference (Proc. 5th Br. Insectic. & Fungic. Conf., Brighton, vol. 2, p. 465 (1969)).

Reprints of these two papers, and of a paper by Sawicki & Farnham (Bull. Ent. Res. 59, 409 (1969)) which is frequently referred to, are enclosed at the end of this thesis.

ACKNOWLEDGEMENTS

The author wishes to express his debt of gratitude to Drs D. H. Calam and R. M. Sawicki for the helpful supervision that they have so kindly provided and to Professor R. T. Williams, Director of Studies, for his interest in this research project.

Special thanks are due to Dr Sawicki for providing an abundant supply of houseflies and to Dr C. Potter, Head of the Department of Insecticides and Fungicides, for enabling the author to work in his department.

The author also wishes to thank the various members of his family for their interest and encouragement and Mrs J. Dixon for typing the manuscript.

CONTENTS

	Page
<u>TITLE</u>	1
<u>ABSTRACT</u>	2
<u>PUBLICATIONS</u>	4
<u>ACKNOWLEDGEMENTS</u>	5
<u>CONTENTS</u>	6
<u>GENERAL INTRODUCTION</u>	8
Resistance	9
Genetics of Resistance	10
Biochemistry of Resistance	12
Purpose of work	17
<u>MATERIALS AND METHODS</u>	20
A) BIOLOGICAL MATERIALS	21
(a) Strains of houseflies	21
(b) Rearing of houseflies	25
B) CHEMICALS	26
(a) Insecticides	26
(b) Metabolites	27
C) DOSING AND <u>IN VIVO</u> EXTRACTION TECHNIQUES	39
(a) Treatment of insects	39
(b) Extraction techniques	39
D) <u>IN VITRO</u> TECHNIQUES	41
(a) Subcellular fractions	41

	Page
(b) Partial purification of enzymes by gel chromatography	42
(c) Starch-gel electrophoresis	44
E) ANALYTICAL TECHNIQUES	47
(a) Paper chromatography	47
(b) Thin-layer chromatography	48
(c) Gas-liquid chromatography	48
(d) Autoradiography	48
(e) Spectrometry	49
<u>RESULTS AND DISCUSSION</u>	51
Introduction	52
A) <u>IN VITRO</u> METABOLISM	53
(a) Preliminary experiments	54
(b) Metabolism by the microsomal fraction	71
(c) Metabolism by the soluble fraction	102
B) <u>IN VIVO</u> METABOLISM	117
C) DISCUSSION	130
<u>CONCLUSIONS</u>	142
<u>REFERENCES</u>	145
<u>APPENDIX</u>	158
<u>In vitro</u> metabolism of ethyl chlorthion	159

GENERAL

INTRODUCTION

Resistance

The resistance of insects to insecticides, which has been defined by the World Health Organisation (W.H.O., 1957) as "the development of an ability in a strain of insects to tolerate doses of toxicants which would prove lethal to the majority of individuals in a normal population of the same species", has assumed considerable medical and agricultural importance this century and, with no immediate alternatives to chemical methods of pest control available, the problem is likely to grow.

The first occurrence of resistance to insecticides was found in 1908, when the San Jose scale became resistant to lime-sulphur sprays. Three species of scale insect had acquired resistance to cyanide in California by 1925 and the codling moth became resistant to lead arsenate in Colorado in 1928 (Brown, 1957). With the advent of the organic insecticides, the occurrence of resistance increased greatly and at least 224 species of insects or acarines are now resistant to one or more insecticides (Anon., 1969). In addition, the phenomenon of resistance to toxic chemicals, as well as occurring in insects, has also been found in rats (Cuthbert, 1963), bacteria (Moyed, 1964) and fungi (Harding, 1964).

The first instance of the resistance of houseflies (Musca domestica L.) to insecticides was found in Sweden in 1946 (reported by Weissman, 1947) and shortly afterwards Sacca (1947)

reported another case in Italy; both of these field strains were resistant to the organochlorine insecticide, DDT. Keiding (1956) then found field strains of housefly that were resistant to the organophosphorus insecticides, diazinon and parathion, in Denmark in 1955 and houseflies have now become resistant to a wide spectrum of insecticides (Georghiou, 1965).

Resistance is not inducible during the lifetime of an insect but is inherited (Bruce & Decker, 1950). It arises from the maintenance of insecticidal selection pressure, with the insecticide acting as a "powerful sieve for concentrating resistant mutants that were present in low frequencies in the original populations" (Crow, 1957) and, thus, resistance to insecticides is an example of accelerated microevolution (Brown, 1957; Georghiou, 1965).

Genetics of resistance

After Bruce & Decker (1950) had showed that housefly resistance to DDT was inherited, much work was done on the genetics of insecticide resistance (reviewed by Milani, 1967). Initially, it was thought (Nguy & Busvine, 1960; Oppenoorth, 1959a) that resistance to organophosphorus insecticides was determined in houseflies by one incompletely dominant gene. Oppenoorth & van Asperen (1960) reported that this gene, which they called gene a, was associated with low aliesterase activity and Franco & Oppenoorth

(1962) then showed that gene a was located on chromosome II (nomenclature of Wagoner, 1967). Oppenoorth (1959b) had suggested that one of his strains (strain F) might have a further resistance factor and later both Tsukamoto & Suzuki (1966), in the Hokota strain, and Sawicki & Farnham (1969), in the SKA strain, discovered three genes for resistance to diazinon and these genes were located on chromosomes II, III and V. The gene on chromosome V which, unlike gene a, is independent of aliesterase concentration, is possibly the same gene as that reported by Oppenoorth (1959b) in strain F, which was derived from the same parents as the SKA strain.

Dr R. M. Sawicki, of Rothamsted, has isolated strains of housefly which each have one chromosome derived from the SKA strain (Sawicki & Farnham, 1967b and 1969). The SKA strain, of wild phenotype, was crossed with two multi-marker susceptible strains using the methods of Tsukamoto (1964). One susceptible strain had visible recessive mutant markers (Milani, 1967) on four chromosomes:-

I - ac; curved wings

II - ar; aristapedia substituted by
tarsal segments

III - bwb; brown body

IV - unmarked

V - ocra; yellow eyes

The other susceptible strain had only the visible

recessive mutant markers ar; ocra. The strains with one chromosome derived from the SKA strain were obtained by substituting one of the marked chromosomes of the susceptible parent with an unmarked chromosome of the SKA strain. Hence strain 393, which has the markers ac; bwb; ocra and is the offspring of the quadruple-marked susceptible strain, lacks the marker ar and therefore has chromosome II derived from the SKA strain. Toxicity studies (Sawicki & Farnham, 1969) showed that factors on chromosomes II, III and V conferred resistance to diazinon and diazoxon; the factors on chromosomes II and, possibly, III gave resistance to parathion (Table 2, page 23).

Biochemistry of Resistance

i) Enzymology

Organophosphorus compounds react with enzymes by covalently binding their phosphoryl moiety to the hydroxyl group of a serine residue in the enzyme (Mahler & Cordes, 1966). The reaction occurs in two stages: firstly, the organophosphorus compound phosphorylates the enzyme with the concomitant hydrolysis of the phosphorus ester; secondly, the phosphoryl moiety is hydrolysed from the enzyme. The rate of this second reaction determines whether the organophosphorus compound is a substrate for or inhibitor of the enzyme (Dixon & Webb, 1964). Hence, organophosphorus compounds with diethyl phosphoric acid esters are more potent anticholinesterase agents than the dimethyl analogues, because they are hydrolysed from the enzyme more slowly than the dimethyl analogues (Aldridge, 1953c).

The toxicity of the organophosphorus compounds is due to their inhibition of cholinesterase enzymes (Spencer & O'Brien, 1957). The cholinesterases, which degrade acetylcholine the mediator in transmission of nerve impulses (Augustinsson, 1950), are a sub-group of the esterases, enzymes which hydrolyse a great variety of organic esters. Organophosphorus compounds are both substrates and inhibitors of the esterase family of enzymes (Aldridge, 1953a, b).

Much of the early work on the resistance of insects to organophosphorus insecticides involved a search for differences in the activities and inhibition characteristics of the various esterases of resistant and susceptible strains. No differences in housefly cholinesterases were found (Oppenoorth, 1958; Mengle & Casida, 1960; Bigley & Plapp, 1961), but differences were observed in the level of aliesterase activity, as measured by in vitro hydrolysis of methylbutyrate; extracts from resistant houseflies showed less aliesterase, and more organophosphorus-metabolising, activity than extracts from susceptible flies (van Asperen & Oppenoorth, 1959) and this led these workers to postulate their "mutant aliesterase" theory, in which a particulate (van Asperen & van Mazijk, 1965) aliesterase enzyme is changed, as the result of a mutation on chromosome II, into an organophosphorus-metabolising "phosphatase" enzyme (Oppenoorth, 1965).

Other workers (Ogita, 1962; Menzel, et al., 1963; Ogita & Kasai, 1965; van Asperen, et al., 1965; Collins & Forgash,

1968) studied the esterase and phosphatase patterns of resistant and susceptible strains of houseflies by starch- and agar-gel electrophoresis. Although differences were observed in the patterns, no definite correlation with resistance could be made.

ii) Metabolism

Diggle & Gage (1951) observed that parathion was highly toxic in vivo but not in vitro, and this led them to suggest that parathion was activated by living organisms. Gage (1953) later identified the more potent anti-cholinesterase metabolite of parathion as its oxygen analogue, paraoxon. Cockroach nerve cord preparations required aerobic conditions for this activation process which was stopped by heating (Metcalf & March, 1953) suggesting that it was an enzymic process and Davison (1954) then showed that parathion activation occurred in rat liver microsomes fortified with pyridine nucleotide cofactor.

Metcalf (1967) has pointed out that, because the phosphorus atom is more electrophilic in the phosphate configuration than in the phosphorothionate, the phosphate esters are about 1,000 times more reactive with cholinesterase than the phosphorothionates, and 10-100 times less stable to hydrolysis.

A possible cause of resistance to organophosphorus insecticides is, therefore, a reduction in the quantity of the phosphate analogues accumulating in the insect. This may occur either by lowering the rate of oxidation of the thionate, or by

increasing the rate of detoxification of the phosphate, or a combination of both. Organophosphorus compounds are readily detoxified by hydrolysis because the relatively strong acids which are produced as a result of hydrolysis are poor cholinesterase inhibitors due to their strong negative charge and the reduced electrophilicity of the phosphorus atom (Metcalf, 1967). The increased polarity of the products of hydrolysis would also facilitate their excretion (Parke, 1968).

In practice, the results reported for organophosphorus insecticide metabolism by resistant and susceptible strains of housefly have not been conclusive. Whilst some in vitro studies have suggested that resistant flies do detoxify more insecticide (Oppenoorth, 1958; El Bashir & Oppenoorth, 1969), in vivo studies have not (Mengle & Casida, 1960; Kreuger, et al., 1960; Forgash, et al., 1962), with the exception of Matsumura & Hogendijk (1964) who reported greater detoxification of diazinon and parathion by resistant flies both in vivo and in vitro.

The metabolism of organophosphorus insecticides by insects is carried out in vitro by two subcellular fractions: the microsomal (Nakatsugawa & Dahm, 1965) and the soluble fraction (Fukami & Shishido, 1966). In houseflies, the microsomes, which require oxygen and NADPH for maximal activity, metabolise methylcarbamates (Tsukamoto & Casida, 1967a), DDT (Oppenoorth & Houx, 1968) and aldrin (Ray, 1967) as well as organophosphorus insecticides

(Nakatsugawa, et al., 1969a). The principal microsomal metabolites of the phosphorothionate compounds were diethyl phosphorothioic acid and the oxygen analogues (Nakatsugawa, et al., 1969a).

The soluble fraction contains an enzyme system which hydrolyses the alkyl-phosphate bond of organophosphorus compounds in the presence of reduced glutathione. This system, which is found predominantly in mammals, metabolises the dimethyl, rather than the diethyl, organophosphorus insecticides to their monomethyl (desmethyl) derivatives, with the concomitant formation of S-methylglutathione (Holdingworth, 1969; Novello et al., 1968; Hutson, et al., 1968). It is less important in insects and has not been reported in houseflies, although reduced glutathione is required for housefly metabolism of DDT (Lipke & Kearns, 1959) and hexachlorocyclohexanes (Clark, et al., 1969).

iii) Synergists

Insecticide synergists are compounds which, although relatively non-toxic themselves, greatly increase the toxicity of insecticides. Hence they are of importance in improving the economics of pest control, increasing the spectrum of activity of an insecticide and restoring the activity of an insecticide to which insects have become resistant (Metcalf, 1968).

Sesamex, a methylenedioxyphenyl synergist, increases the toxicity of many insecticides to houseflies but decreases the toxicity of ("antagonises") parathion (Sun & Johnson, 1960).

Nakatsugawa & Dahm (1965) showed that sesamex inhibited cockroach microsomal metabolism of parathion and Esaac & Casida (1969) and Wilkinson & Hicks (1969) suggested that the methylenedioxyphenyl compounds act as synergists by competing as substrates for the microsomal mixed-function oxygenases. Sesamex has little effect on the toxicity of diazinon and parathion in the SKA strain, but antagonises diazinon and parathion in the strains with only the resistance factor on chromosome II and synergises diazinon in the strain with only the resistance factor on chromosome V (Sawicki & Farnham, 1967 and 1969).

Plapp, et al. (1963) found that S,S,S-tributylphosphorotriethionate (TBTP) acted as a synergist of malathion by inhibiting aliesterase enzymes; this compound synergises diazinon and parathion in the two strains with only the resistance factor on chromosome II (Sawicki & Farnham, 1969).

Purpose of Work

Other workers (Mengle & Casida, 1960; Kreuger, et al., 1960; Forgash, et al., 1962; Farnham, et al., 1965) have shown that delayed penetration of insecticides contributes to resistance but that there is usually residual resistance even to injected insecticides. Sawicki & Farnham (1969) found that, alone, the resistance factor on chromosome III of the SKA strain delayed knockdown by some organophosphorus insecticides by reducing the rate of penetration of the insecticide, but it did not affect kill.

Preliminary results, not reported in this thesis, confirmed that the strain with only chromosome III behaved metabolically like the susceptible strains and that its slight resistance was due to the delayed penetration of insecticide.

The purpose of the work reported in this thesis was, therefore, to elucidate the biochemical modes of action of the genes for resistance located on chromosomes II and V of the SKA strain. Both of these genes confer resistance to diazinon and, as this was one of the insecticides to which resistance originally arose in the field and with which the SKA strain was selected, it was used as the main insecticide for this investigation. The metabolism of parathion, another insecticide to which resistance originally arose in the field, was also studied because most of the resistance of the SKA strain to this compound is conferred by gene a on chromosome II, rather than by the gene on chromosome V. Parathion thus acted as a cross-check on the diazinon results.

The modes of action of the genes on chromosome II and V were elucidated by examining the esterase patterns obtained by electrophoresis and by examining the metabolism of diazinon, diazoxon and parathion in vitro (by the microsomal and soluble fractions) and in vivo in two susceptible strains, in the SKA strain, in two strains with chromosome II derived from the SKA strain and in one strain with chromosome V derived from the SKA strain. The results obtained are related to the genetics of resistance of the SKA strain. The effect of the two synergists, sesamex and TBTP, on the pathways of

metabolism of diazinon, diazoxon and parathion was also studied and the results related to the effects of these synergists on the toxicity of these insecticides to the six strains of housefly investigated.

MATERIALS
AND
METHODS

A) BIOLOGICAL MATERIALS

(a) Strains of houseflies

Six strains of housefly (Musca domestica L.) were studied. Table 1 summarises their character and some relevant toxicity data. The houseflies were bred by Dr R. M. Sawicki of Rothamsted.

The designation of the physiological mutants follows Georgiou (1965) and the numbering of the chromosomes is that of Wagoner (1967).

(i) The SKA strain

This strain, of wild phenotype, resulted from a cross between two diazinon-resistant strains (203a, Danish and Latina, Italian) and from continual selection with diazinon (Sawicki & Farnham, 1964). It is very resistant to diazinon (x 300), diazoxon (x 160), parathion (x 70) and several other insecticides (El Bashir, 1966) and is almost immune to DDT (Sawicki & Farnham, 1967a).

Table 2 gives the location of the resistance factors of the SKA strain and the degree of resistance conferred in strains with the isolated chromosome.

(ii) Strain ac;ar;bwb;ocra SRS

This strain, with visible recessive mutant markers on chromosomes I, II, III and V, is closely related to the W.H.O.

Table 1

Toxicity of diazinon, diazoxon and parathion to females of six strains of housefly,
with and without pretreatment with sesamex and TBTP (from Sawicki & Farnham, 1967b, 1969)

Strain	Type of resistance	Located on Chromosome No:	Diazinon LD50 (µg/fly)			Diazoxon LD50 (µg/fly)			Parathion LD50 (µg/fly)		
			Alone	+ Sesamex	+ TBTP	Alone	+ Sesamex	+ TBTP	Alone	+ Sesamex	+ TBTP
<u>ac;ar;bwb;ocra</u> SRS	None (Susceptible)	-	0.040	0.020	0.025	0.026	0.006	0.012	0.026	0.016	0.015
<u>ocra</u> SRS	None (Susceptible)	-	0.059	0.029	0.030 (approx.)	0.020	0.008	0.007	0.02	0.01 (approx.)	0.01 (approx.)
29	gene <u>a</u>	II	0.76	1.35	0.36	0.20	0.09	0.12	-	NOT AVAILABLE	-
393	gene <u>a</u>	II	0.50	0.96	0.23	0.20	0.08	0.08	0.41	0.80	0.095
466.500	Sesamex-inhibited	V	0.37	0.025	0.27	0.12	0.007	0.06	0.032	0.015	0.019
SKA	gene <u>a</u> , Penetration and Sesamex-inhibited	II III V	13.0	6.2	10.0	4.2	1.7	4.2	1.8	3.0	1.8

Table 2

Location of the resistance factors in the SKA strain and the degree of
resistance conferred in strains with the isolated chromosomes (from
Sawicki & Farnham, 1969)

Chromosome No.				
I	II gene <u>a</u>	III pen	IV dld	V Ses
Chlorthion chloroxon c. x 2	diazinon x 13 diazoxon x 8 parathion x 15 paraaxon x 3.6 methyl-parathion x 7 methyl-paraaxon x 2.4 ethyl-malathion x 4 ethyl-malaaxon x 6.5 malathion x 1.4 malaaxon x 6 ethyl-chlorthion x 48 ethyl-chloroxon x 14 chlorthion x 14 chloroxon x 12	diazinon x 2* chlorthion x 2* dielldrin x 2* DDT x 2* tributyl tin acetate x 12 spectran x ? parathion x ?	dielldrin x 700	diazinon x 9 diazoxon x 4.6 ethyl-malaaxon x 3.3 DDT x 10 methoxychlor x 20
	<u>Deh</u> DDT x 1000			

*measure of delayed penetration, not resistance to kill

Standard Reference Strain and is fully susceptible to organophosphorus insecticides (Sawicki & Farnham, 1969).

(iii) Strain ocra SRS

This strain, with the recessive visible mutant marker ocra on chromosome V, is also related to the W.H.O. Standard Reference Strain and is fully susceptible to organophosphorus insecticides (Sawicki & Farnham, 1969).

(iv) Strain 29

This strain, marked with ocra, was obtained from crossing the SKA strain with a double-marker susceptible strain, ac;ocra SRS. Chromosome II is derived from the SKA strain and gene a, located on this chromosome, confers resistance to many organophosphorus insecticides (Table 2) and is associated with low aliesterase activity (Oppenoorth, 1959b).

(v) Strain 393

This strain, which is triple-marked ac;bwb;ocra, was obtained by crossing the SKA strain with strain ac;ar;bwb;ocra SRS. Strain 393, like strain 29, has chromosome II and hence gene a derived from the SKA strain (Sawicki & Farnham, 1969).

(vi) Strain 466.500

This strain, triple-marked ac;ar;bwb, was also obtained by crossing the SKA strain with strain ac;ar;bwb;ocra SRS.

Chromosome V is derived from the SKA strain and a factor Ses, located on this chromosome confers resistance to a few organophosphorus and organochlorine insecticides (Table 2) and is inhibited by sesamex (Sawicki & Farnham, 1969).

(b) Rearing of houseflies

The rearing and handling of houseflies was described in detail by Sawicki et al., (1966). Houseflies were used seven days after emergence.

B) CHEMICALS

(a) Insecticides, additives and cofactors

The following insecticides were used:

- (i) diazinon - O,O-diethyl, O-2-isopropyl-6-methyl-4-pyrimidinyl phosphorothionate - 99.5% pure^(a)
- (ii) diazoxon - O,O-diethyl, O-2-isopropyl-6-methyl-4-pyrimidinyl phosphate - 97% pure^(a)
- (iii) parathion - O,O-diethyl, O-p-nitrophenyl phosphorothionate - 99% pure^(b)
- (iv) paraoxon - O,O-diethyl, O-p-nitrophenyl phosphate - purity unknown^(b)

The following labelled insecticides were used:-

diazinon (specific activity = 1.0 mCi/mmole)^(a) and parathion (specific activity = 15.1 mCi/mmole)^(c) were labelled with Carbon-14, in the ethyl groups. Diazoxon (specific activity = 4.1 mCi/mmole)^(c) was labelled with phosphorus-32.

Also used were the following additives:-

sesamex (2-(3,4-methylenedioxyphenyl)3,6,9-trioxaundecane)^(d) and TBTP (S,S,S-tributylphosphorotrithionate)^(e).

-
- (a) given by Messrs J. R. Geigy, S.A., Basle
 - (b) given by Bayer G.m.b.H., Leverkusen
 - (c) bought from the Radiochemical Centre, Amersham
 - (d) given by Messrs Mitchell Cotts, London
 - (e) bought from Eastman Kodak Ltd, Liverpool

The cofactors, NADPH (reduced nicotinamide-adenine dinucleotide phosphate) and GSH (reduced glutathione) were bought from the Boehringer Corporation (London) Ltd, London.

(b) Metabolites

Except for O,O-diethyl phosphoric acid (DEPA), which was bought, ^(e) all the metabolites were synthesised. The structure and identity of the metabolites was confirmed by either NMR or Mass Spectrometry (see section E.(e)).

(i) O,O-diethyl phosphoric acid (DEPA) was prepared by alkaline hydrolysis of diazinon (Plapp & Casida, 1958a).

Diazinon (1 mmole) and potassium hydroxide (4 mmoles), each dissolved in 95% ethanol (5 ml), were mixed and incubated at 32.5°C for 16 hours. The solution was neutralised (3N hydrochloric acid), concentrated to 2.0 ml and chromatographed on Whatman No. 1 paper with propan-2-ol/ammonia (75:25, v/v) to give a single spot at $R_f = 0.76$. Diethyl phosphoric acid (DEPA) had an $R_f = 0.63$ in this system. The R_f values are similar to those published by Plapp & Casida (1958b): DEPTA, $R_f = 0.77$; DEPA, $R_f = 0.61$.

Gas chromatography (section E.c) indicated a single component, the mass spectrum of which, obtained by combined gas chromatography-mass spectrometry, gave the required molecular ion at m/e 170.

(e) bought from Eastman Kodak Ltd, Liverpool

[^{14}C -ethoxy] DEPTA was similarly prepared from [^{14}C -ethoxy] diazinon.

(ii) Desethyl diazoxon (O-ethyl, O-2-isopropyl-6-methyl-4-pyrimidinyl hydrogen phosphate) was prepared by the method used by Hollingworth et al. (1967) to synthesise the desmethyl derivatives of Sumioxon and methyl-paraoxon.

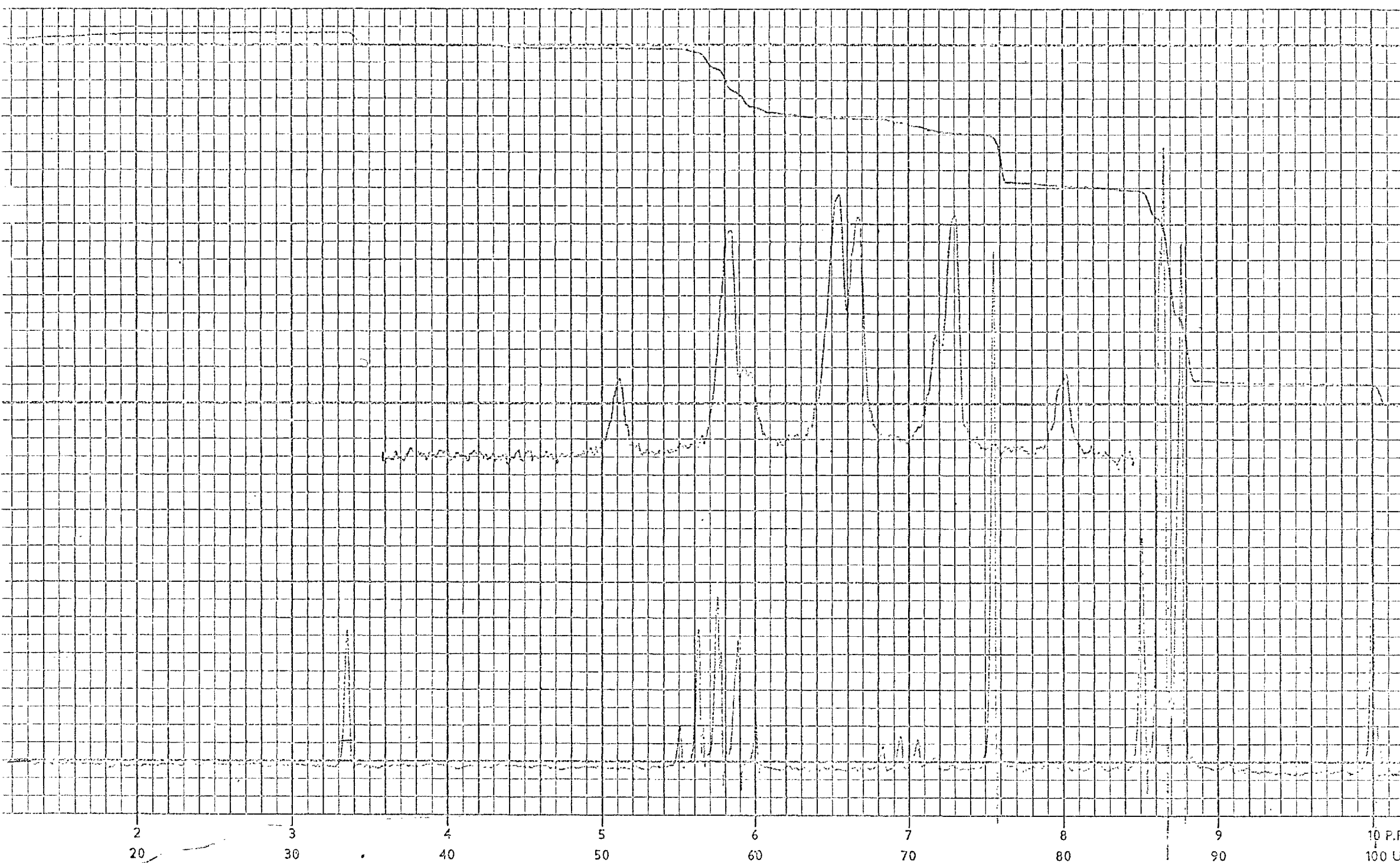
Equimolar quantities of diazoxon and anhydrous sodium iodide were refluxed in dry acetone (30 mls) for 36 hours, the acetone was evaporated and the residue, dissolved in chloroform (5 ml), was eluted from a column (1.0 cm x 15 cm) of "Florisol" (Hopkin & Williams Ltd) with petroleum ether (40-60°), cyclohexane, diethyl ether, acetone and methanol (20 ml each), successively.

Desethyl diazoxon was eluted in the methanol fraction which was then slightly acidified (3N hydrochloric acid), dried over anhydrous sodium sulphate, evaporated and the residue washed with chloroform.

The NMR spectrum of diazoxon (Figure 1a) in CCl_4 , with peaks at τ 3.35 (singlet, 1H; heterocyclic H), 5.75 (double quartet, 4H; $2 \times \text{O}-\text{CH}_2$, $J = 8, 7$ c/s), 6.9 (septet, 1H; $\text{CH}(\text{CH}_3)_2$, $J = 7$ c/s), 7.6 (singlet, 3H; heterocyclic CH_3), 8.6 (triplet, 6H; $2 \times \text{CH}_2\text{CH}_3$, $J = 7$ c/s) and 8.7 (doublet, 6H; $\text{CH}(\text{CH}_3)_2$, $J = 7$ c/s) is similar to that recorded for diazinon (Babad, et al., 1968); the main difference is a change in J_{PH} from 11 to 8 c/s.

The sample of desethyl diazoxon, produced as described

IFT 8	REF. STD. TMS	R.F. FIELD 1mV	R.F. GAIN 1/2	REMARKS J = 8 c/s.	SENSITIVITY NORM: 1/2 INT: 1	SWEEP RATE. 32 128 UNITS PER MIN.	TIME CONST. 0.05 SECS. 0.15 M. SECS.	SCALE FACTOR 6 C/S PER UNIT	SPECTRUM No. 831
----------	------------------	-------------------	------------------	-----------------------	------------------------------------	---	--	--------------------------------	---------------------



NUCLEUS
SUBMITTED
BY
COMPOUND
FIG. 1A.
DIAZOXON
(NMR SPECTRUM)
SOLVENT
CONC.
OPERATOR

above, had an NMR spectrum measured in D_2O , which differed from the spectrum of diazoxon in that the triplet and double quartet from the $O.CH_2.CH_3$ group were halved in intensity relative to the other peaks. An extra peak occurred due to a small amount of methanol (Figure 1b).

(iii) Desethyl diazinon (O-ethyl, O-2-isopropyl-6-methyl-4-pyrimidinyl hydrogen phosphorothionate) was prepared by reacting diazinon with sodium thiophenoxide, as described by Miller (1962), because refluxing thionates with sodium iodide yielded S-methyl phosphoric acid derivatives (Hollingworth, et al., 1967). This is achieved by using a nucleophilic reagent which does not react with the thiolate anion.

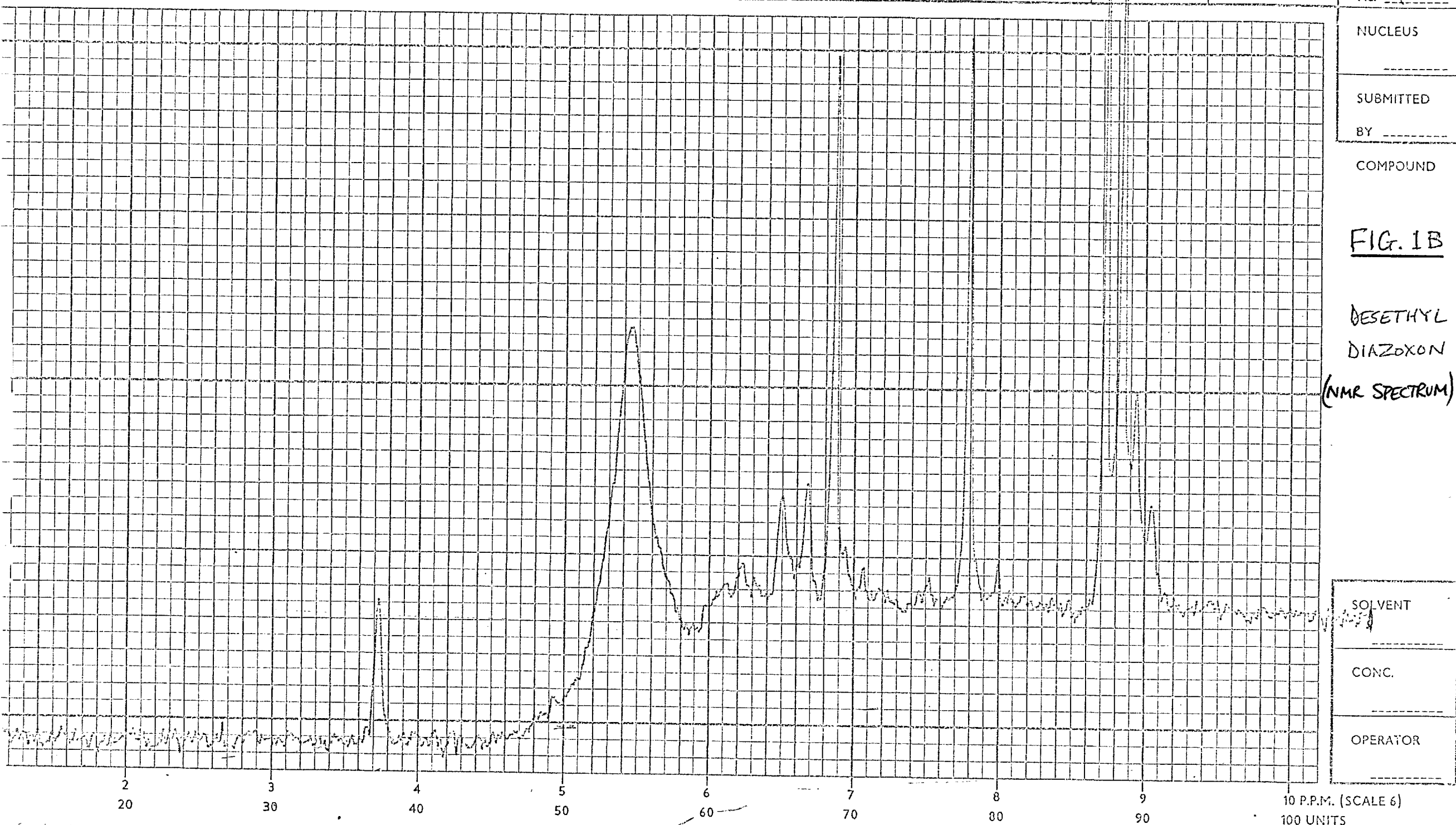
Equimolar quantities of sodium hydroxide, thiophenol and diazinon were dissolved, in that order, in 95% ethanol, and refluxed for 18 hours. The ethanol was then evaporated and the product purified on "Florisil" (eluted in acetone), as for desethyl diazoxon, and identified by NMR.

The NMR spectrum of diazinonⁱⁿ CCl_4 (Figure 2a) was the same as that recorded (Babad, et al., 1968). The spectrum of desethyl diazinon in D_2O showed the same features but with the intensities for the $O.CH_2.CH_3$ group halved (Figure 2b).

(iv) Desethyl parathion (O-ethyl, O-p-nitrophenyl hydrogen phosphorothionate) was prepared like desethyl diazinon.

The NMR spectrum of parathion (Figure 3a) in CCl_4 was

IFT 2	REF. STD. —	R.F. FIELD 1 mV	R.F. GAIN 112	REMARKS	SENSITIVITY NORM: 2 INT :	SWEEP RATE. 32 UNITS PER MIN.	TIME CONST. 0.1 SECS. 0.15 M. SECS.	SCALE FACTOR 6 C/S PER UNIT	SPECTRUM No. 825
----------	----------------	--------------------	------------------	---------	---------------------------------	-------------------------------------	---	-----------------------------------	---------------------



NUCLEUS

SUBMITTED

BY

COMPOUND

FIG. 1B

DIETHYL
DIAZOXON

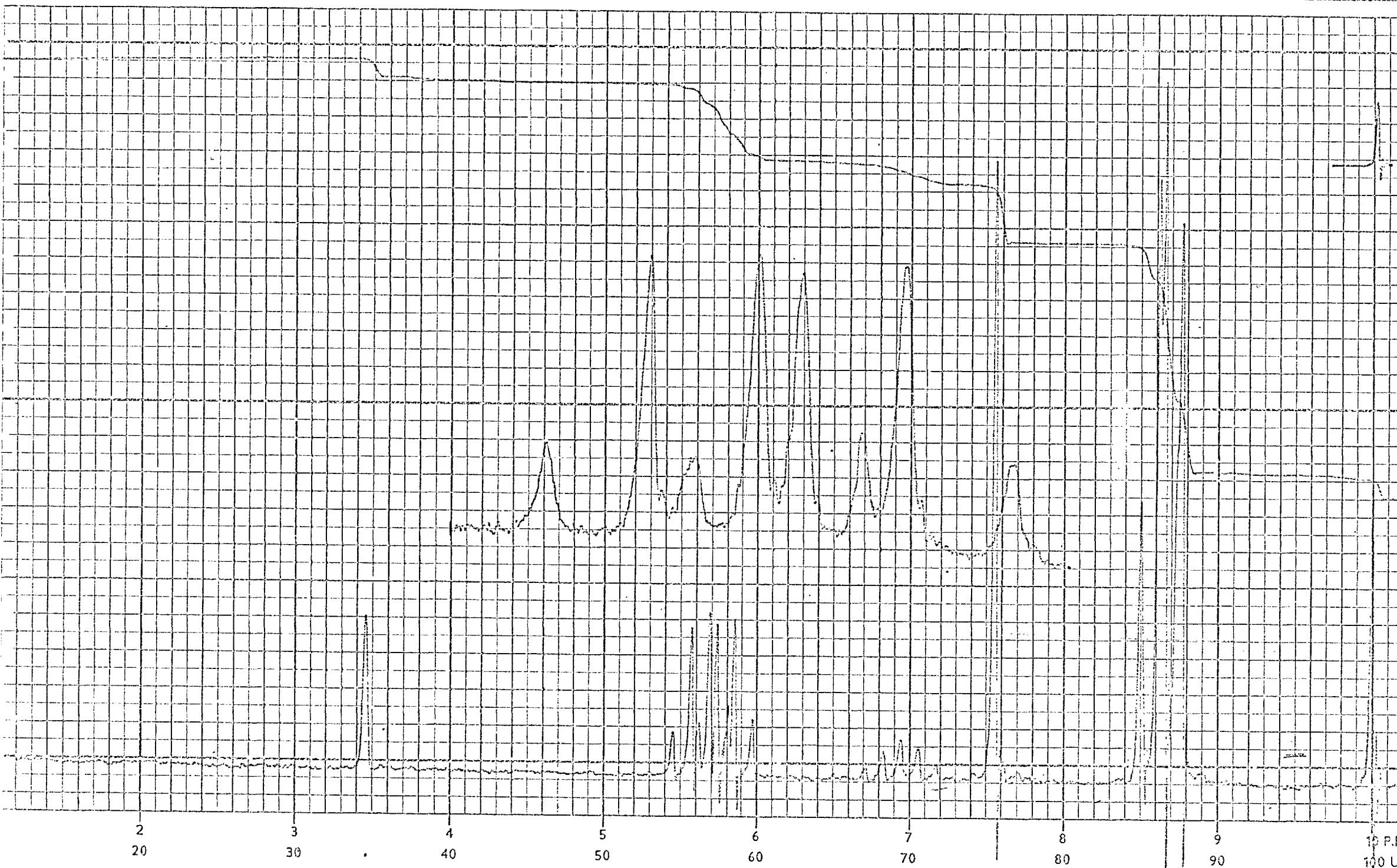
(NMR SPECTRUM)

SOLVENT

CONC.

OPERATOR

SHIFT	REF. STD. TMS	R.F. FIELD 1mV	R.F. GAIN 1/2	REMARKS J=10c/s.	SENSITIVITY NORM: 1/2 INT: 6	SWEEP RATE. 32 128 UNITS PER MIN.	TIME CONST. 0.05 SECS. 0.15 M. SECS.	SCALE FACTOR 6 C/S PER UNIT	SPECTRUM No. 830
-------	------------------	-------------------	------------------	---------------------	------------------------------------	---	--	--------------------------------	---------------------



NUCLEUS

SUBMITTED
BY

COMPOUND

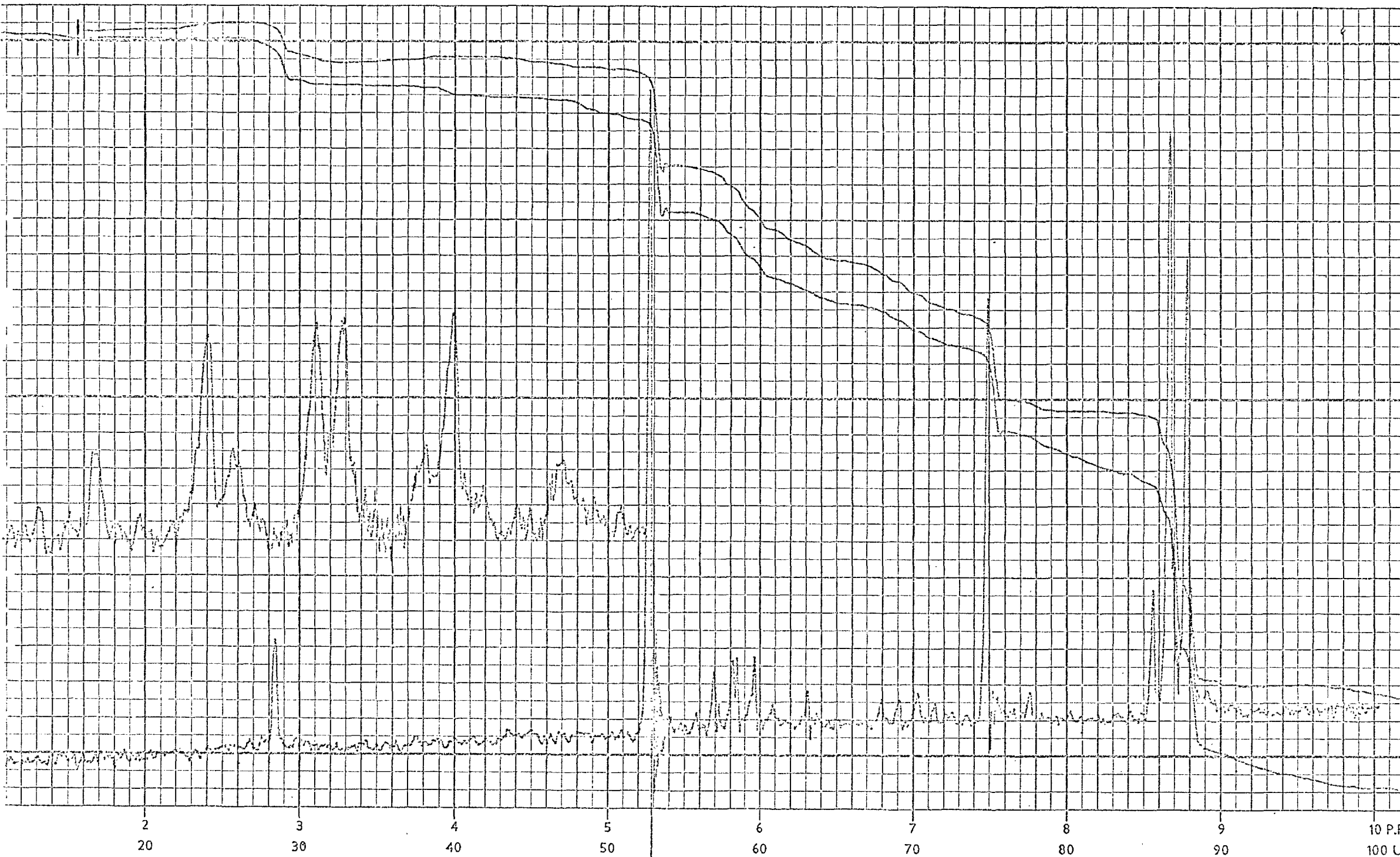
FIG. 2A
DIAZINON
(NMR SPECTRUM)

SOLVENT

CONC.

OPERATOR

HIFT 8	REF. STD. —	R.F. FIELD 125 V	R.F. GAIN 1/2	REMARKS	SENSITIVITY NORM: 1 INT: 4	SWEEP RATE. 32 128 UNITS PER MIN.	TIME CONST. 0.05 SECS. 0.15 M. SECS.	SCALE FACTOR 60 C/S PER UNIT	SPECTRUM No. 887
-----------	----------------	---------------------	------------------	---------	----------------------------------	---	--	---------------------------------	---------------------



NUCLEUS
SUBMITTED
BY
COMPOUND

FIG. 2B
DESETHYL
DIAZINON
(NMR SPECTRUM)

SOLVENT
CONC.
OPERATOR

the same as that recorded (Babad, et al., 1968) and the spectrum of desethyl parathion, in D_2O , differed, as with desethyl diazinon only in the intensities of the peaks from the ethoxy group and in the expected small shift due to the change of solvent (Figure 3b).

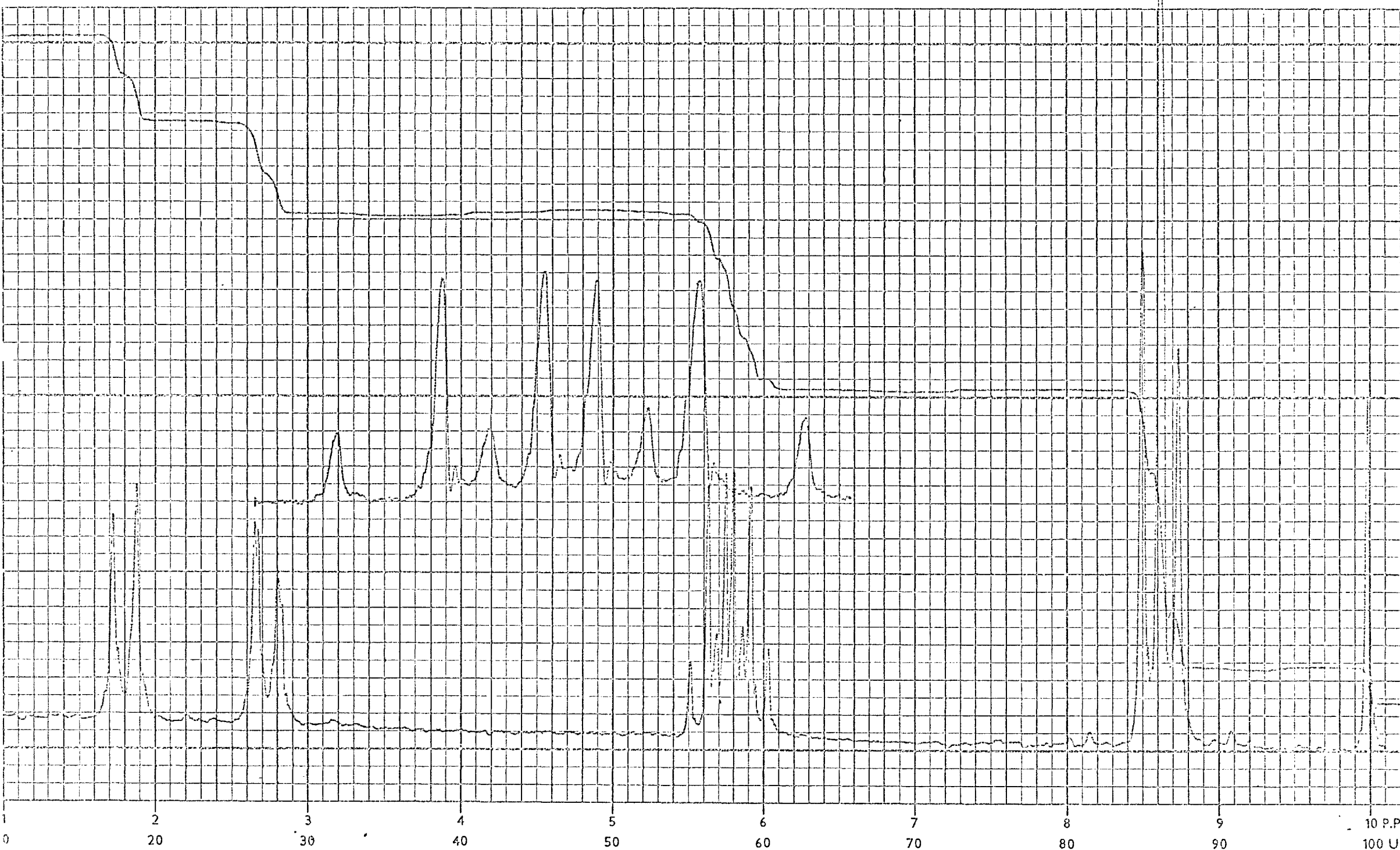
(v) Monoethyl phosphoric acid (MEPA: O-ethyl phosphoric acid) and (vi) Monoethyl phosphorothioic acid (MEPTA: O-ethyl phosphorothioic acid) were prepared by aqueous hydrolysis of desethyl diazoxon and desethyl diazinon, respectively.

Alkaline hydrolysis of desethyl diazinon appeared to give both diethyl phosphorothioic acid and phosphorothioic acid instead of MEPTA. The identity of MEPTA was not confirmed.

(vii) S-Ethyl glutathione was synthesised by alkylating reduced glutathione with iodoethane in alkaline solution and precipitating unreacted material as the silver salt (Foxwell, 1965).

The product, recrystallised from water/acetone (50:300, v/v), had m.p. 199-200°C, decomp. (Foxwell [1965] gives m.p. 194°C, decomp.). The yield was 40%. Comparison of the NMR spectrum of this material in D_2O /potassium hydroxide with that of GSH in D_2O /potassium hydroxide showed new peaks at τ 7.4 (quartet, 5H; $S\text{-}\underline{CH}_2\text{-CH}_3$) and τ 8.8 (triplet, 5H; $S\text{-}\underline{CH}_2\text{-}\underline{CH}_3$) (Figure 4a, b). No unchanged GSH was detected in the product.

SHIFT 08	REF. STD. TMS	R.F. FIELD 100V	R.F. GAIN 1/2	REMARKS	SENSITIVITY NORM: 1/2 INT: 1	SWEEP RATE. 32 UNITS 128 PER MIN.	TIME CONST. 0.25 SECS. 0.15 M. SECS	SCALE FACTOR 6(1) C/S PER UNIT	SPECTRUM No. 931
-------------	------------------	--------------------	------------------	---------	------------------------------------	---	---	--------------------------------------	---------------------



NUCLEUS

SUBMITTED

BY

COMPOUND

FIG. 3A

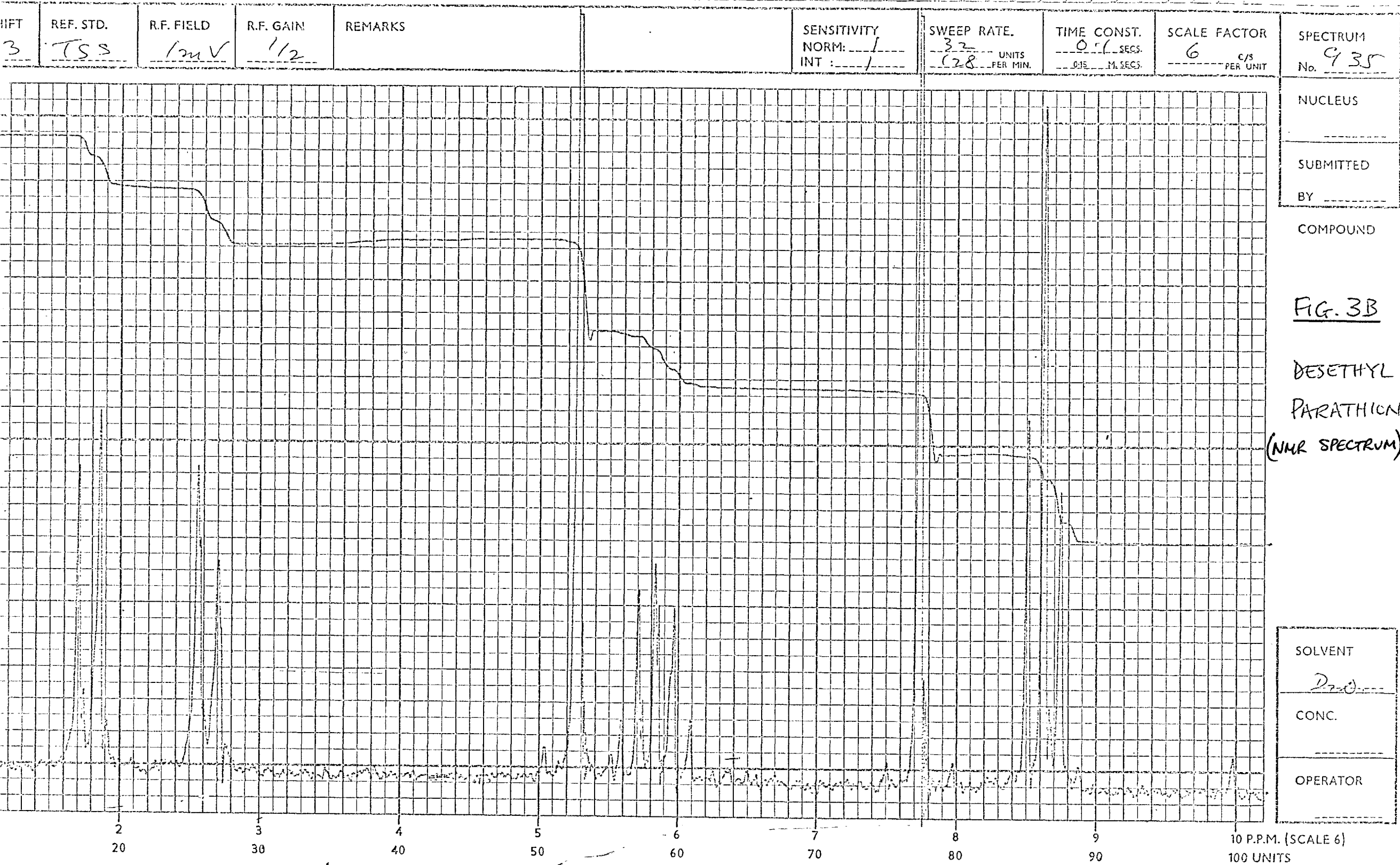
PARATHION
(NMR SPECTRUM)

SOLVENT

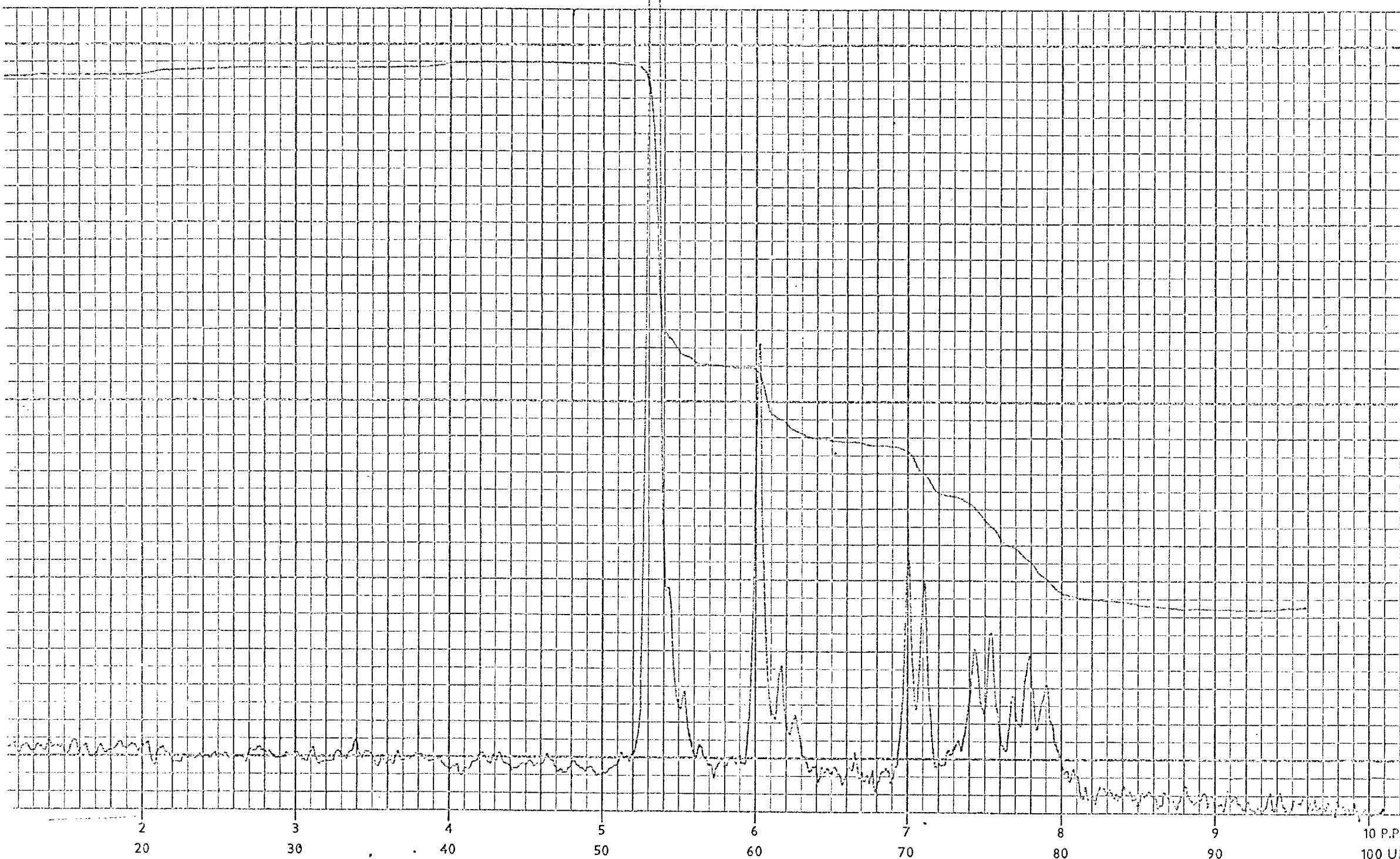
CCl₄

CONC.

OPERATOR



IFT 5	REF. STD.	R.F. FIELD 1mV	R.F. GAIN 1/2	REMARKS	SENSITIVITY NORM: 2 INT: 2	SWEEP RATE. 32 UNITS PER MIN. 128	TIME CONST. 0.25 SECS. 0.15 M. SECS.	SCALE FACTOR 6 C/S PER UNIT	SPECTRUM No. 430
----------	-----------	-------------------	------------------	---------	----------------------------------	---	--	--------------------------------	---------------------

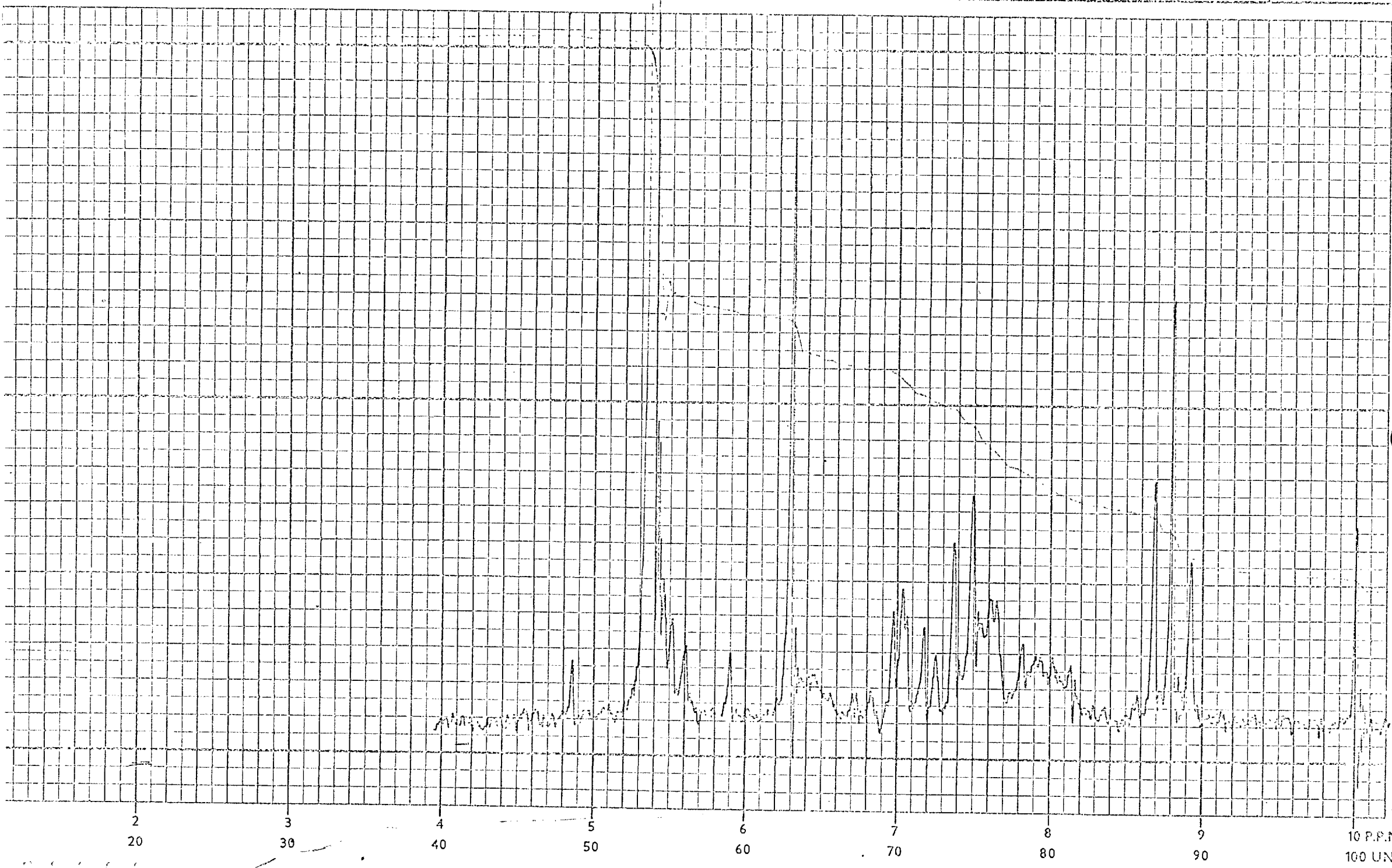


NUCLEUS
SUBMITTED
BY
COMPOUND

FIG. 4A
REDUCED
GLUTATHIONE
(NMR SPECTRUM)

SOLVENT
CONC.
OPERATOR

IFT 4	REF. STD. TSS	R.F. FIELD 1mV	R.F. GAIN 1/2	REMARKS	SENSITIVITY NORM: 1 INT: 1	SWEEP RATE 3.2 12.8 UNITS PER MIN.	TIME CONST. 0.05 SECS. 0.15 M. SECS.	SCALE FACTOR 6 C/S PER UNIT	SPECTRUM No. 948
----------	------------------	-------------------	------------------	---------	----------------------------------	--	--	--------------------------------	---------------------



NUCLEUS
SUBMITTED BY
COMPOUND
FIG. 4B
S-ETHYL GLUTATHIONE (NMR SPECTRUM)
SOLVENT D ₂ O/KOH
CONC.
OPERATOR

C) DOSING AND IN VIVO EXTRACTION TECHNIQUES

(a) Treatment of insects:

Insecticide was topically applied to seven-day old female houseflies using an Arnold Hand Micro-applicator (Burkard Scientific Ltd, Rickmansworth) fitted with an "Agla" all-glass syringe (Burroughs Wellcome). Each fly was dosed on the ventral side of the thorax with 1 µg of insecticide in 1 µl acetone. The synergists, sesamex (2 µg/fly) and TBTP (1 µg/fly), were topically applied in 1 µl acetone to the ventral side of the abdomens of the houseflies 1-4 hours before the insecticide.

The treated flies were incubated at room temperature for various periods and killed by immersing in petroleum ether (6 ml of 40-60°) for 1 min, which also washed off unpenetrated insecticide.

Controls were either untreated or dosed after immersing in a boiling water-bath for 5 mins.

(b) Extraction techniques:

Three extraction procedures were developed to study insecticide metabolism in living flies:- two were used to detect metabolites by gas-liquid chromatography, and a third was used to detect metabolites of radioactive insecticides.

(i) Extractions with organic solvents for G.L.C.

(a) This procedure was a modification of the method of Blinn & Gunther (1955). Batches of 120 houseflies, after washing

in petroleum ether, were homogenised in more petroleum ether (20 ml of 40-60°) and the homogenate extracted with acetonitrile (4 x 7.0 ml). The combined acetonitrile extractions were dried (Molecular Sieve 4A); evaporated to dryness, and the residue dissolved in acetone (0.2 ml). The washings were reduced to less than 5 µl and injected into the gas-liquid chromatograph (G.L.C.).

(b) With the method of Gwiazda & Lord (1967) the houseflies were homogenised in TRIS buffer (20 ml of 0.05M, pH 7.0) after washing in petroleum ether. The homogenate was centrifuged (2,000 g for 10 min) and the supernatant extracted with cyclohexane (2 x 20 ml), acidified (3N hydrochloric acid) and re-extracted with diethyl ether (3 x 20 ml). The combined cyclohexane extracts and the ether extracts were separately dried (Molecular Sieve 4A), evaporated to less than 5 µl and injected into the G.L.C.

(ii) Aqueous extraction for radioisotope experiments:

Batches of 60 houseflies, after immersing in petroleum ether, were homogenised in water (3.0 ml); the homogenate was centrifuged (13,000 g for 10 min) to remove insect debris and ice-cold acetone (3.0 ml) was added to the supernatant. The mixture was left overnight at -10°C, then centrifuged (1,000 g for 5 mins) to precipitate the protein, and an aliquot (0.35 ml or 0.5 ml) of the supernatant was examined by paper chromatography.

D) IN VITRO TECHNIQUES

(a) Subcellular fractions:

Subcellular fractions of houseflies were prepared to investigate the nature of the enzymes responsible for the metabolism of organophosphorus insecticides. These enzymes were partially purified by gel chromatography and electrophoresis. Three subcellular preparations of the housefly were studied: microsomes, the soluble fraction and a mixture of the two (the 13,000 g supernatant). The methods of preparation and incubation were modified from Tsukamoto & Casida (1967a) and Neal (1967a).

(i) Preparations:

Seven-day old female houseflies were homogenised in phosphate buffer (20 flies/ml of 0.15M KH_2PO_4 -NaOH buffer, pH 7.3) containing bovine albumin (2% w/v; British Drug Houses) in an ice-bath. The homogenate was centrifuged (13,000 g for 10 min) and the resulting supernatant was recentrifuged (100,000 g for 60 min) to give a soluble fraction (fluid) and a microsomal fraction (precipitate). The microsomal pellet was resuspended in a volume of phosphate buffer equal to the volume of the soluble fraction.

(ii) Incubation conditions:

A typical reaction mixture had material from 10 flies (i.e. 0.5 ml of either the 13,000 g supernatant, the microsomal fraction or the soluble fraction) in a total volume of 2.0 ml

phosphate buffer which contained NADPH (2 mg; 1.3 mM final concentration), GSH (4 mg; 6.5 mM) and radioactive insecticide (10 μ g in 10 μ l acetone). Sesamex (20 μ g) and TBTP (10 μ g) were added, when required, in acetone (10 μ l). In some experiments, GSH was replaced by iodoacetate (3 mM), p-chloromercuribenzoate (0.05 mM), ethylenediamine tetracetic acid (EDTA: 3 mM), dithiothreitol (1.0 mM) or L-cysteine (1.0 mM) dissolved in phosphate buffer.

The mixture was shaken in a water-bath (30°C), in air or nitrogen, usually for 2 hours, and the reaction terminated by adding ice-cold acetone (3.0 ml). The mixture was left at -10°C overnight to allow the precipitate to settle and aliquots (0.5 ml) of the resulting clear supernatant were then examined by paper chromatography.

An enzymic preparation immersed in a boiling water-bath for 5 mins was used as a control.

(b) Partial purification of enzymes by gel chromatography:

To define more clearly the enzymes involved in insecticide metabolism in resistant and susceptible houseflies, an attempt was made to partially purify both the microsomal and soluble fractions by Sephadex chromatography.

A Pharmacia K 15/30 column (i.d. 1.5 cm x 30 cm) was packed to a height of 27.5 cm in each experiment. Non-specific phosphatase activity in the eluates was assayed with a solution of 1-naphthyl disodium orthophosphate (0.04%, w/v) (British Drug Houses)

and "Fast-Blue RR" (0.1%, w/v) (G. T. Gurr Ltd, London) in phosphate buffer (pH 7.4). Phosphatases liberate free naphthol which reacts with the diazo dye to give a dark brown-black colour.

(i) CM-Sephadex C-25:

Female houseflies were homogenised in phosphate buffer (20 flies/ml of 0.05M KH_2PO_4 - K_2HPO_4 ; pH 7.4), centrifuged (100,000 g for 60 mins) and the supernatant passed down the column with an elution gradient of phosphate buffer of 0.05M (50 ml) to 0.2M (50 ml). All the protein (detected by the Biuret test: Dawson, et al., 1969) and phosphatase activity was eluted immediately after the void volume (V_0 = 15 ml). An aliquot of this fraction was incubated with [^{32}P] diazoxon and GSH (see: D (a) ii).

(ii) Sephadex G-200:

This was used to try and separate the proteins not retained on CM-Sephadex C-25. Female houseflies were homogenised in phosphate buffer (20 flies/ml of 0.15M KH_2PO_4 -NaOH; pH 7.3), the homogenate was centrifuged (100,000 g for 60 min) and the supernatant (5.0 ml) passed down the column. The eluant was phosphate buffer (0.1M KH_2PO_4 - K_2HPO_4 ; pH 7.4). The eluate was assayed for protein and phosphate activity. The fraction with phosphatase activity was incubated with radioactive insecticides (see: D (a) ii), with and without NADPH and GSH.

(iii) Sephadex G-25:

The microsomal fraction was desalted to provide a system in which the cofactor requirements of the enzymes could be studied.

The microsomal fraction from female houseflies was suspended in distilled water (20 fly equivalents/ml) and passed down a column with distilled water as eluant.

Protein and phosphatase activity was eluted immediately after the void volume. This fraction was incubated with [^{14}C] diazinon, with and without NADPH and GSH (see: D (a) ii).

(c) Starch-gel electrophoresis:

Possible interstrain differences in phosphatase enzymes were investigated by the method of Sargent (undated).

Hydrolysed starch (15 gm; British Drug Houses) was dissolved in borate buffer (100 ml of $0.025\text{M H}_2\text{BO}_3$ - 0.01M NaOH : pH 8.5) and the solution heated gently to 95°C , with vigorous swirling. The solution became viscous at about 65°C , but the viscosity diminished at the higher temperature. The flask was briefly evacuated, to remove air bubbles, and the starch solution was then poured into a Perspex mould (0.6 cm x 10 cm x 18.5 cm), lightly smeared with paraffin oil. Excess starch was immediately scraped off and a slot-former placed 6.0 cm from one end. The cooled gel was covered and stored for 6-7 hours at 5°C .

Female houseflies, homogenised in TRIS buffer (50 flies/ml of 0.2M TRIS: pH 7.0) in an ice-bath, were centrifuged (13,000 g for 20 mins) and aliquots (10 μ l) of the supernatant placed in the slots. The gel, in its mould, was placed in the buffer chamber of the Shandon Universal electrophoresis apparatus (Shandon Scientific Ltd, London) and was connected to the borate bridge buffer (0.3M HBO_3 -0.06M NaOH: pH 8.5) by two wicks of Whatman 3MM paper. The buffer chamber was maintained at 5°C. A constant voltage (63 Volts; 3.5 volts/cm) was applied along the gel for 16 hours after which the gel was removed from the mould and immersed for 60 mins in a solution of 1-naphthyl disodium orthophosphate and Fast Blue RR in TRIS buffer (0.05M; pH 7.0). The stained gel was washed in methanol/glacial acetic acid/water (50:1:50, by vol.) and replaced in the mould for photography.

Gels were also examined for protein distribution by immersing in Nigrosine dye solution (0.2% w/v in 40% ethanol and 2.5% trichloroacetic acid). Proteins stained black.

Insecticide hydrolysing activity was investigated by soaking filter-paper in a parathion solution and placing the paper flat on the gel. After incubation in a saturated chamber for 3 hours, the paper and gel were sprayed with N-sodium hydroxide and examined for the yellow colour of p-nitrophenol (Robinson, et al., 1951).

Eluates from the Sephadex G-200 column were examined by electrophoresis. The eluates were reduced in volume by adding dry

Sephadex G-25 and a little of this Sephadex was then placed in the gel slots.

E) ANALYTICAL TECHNIQUES

Insecticide metabolites were examined by paper, thin-layer and gas-liquid chromatography. Gas-liquid chromatography was used with non-radioactive metabolites and paper-chromatography was used principally for labelled insecticide metabolites.

(a) Paper chromatography

Two Whatman papers, No. 1 and silica-impregnated SG 81 (Reeve Angel & Co., London), were used to separate radioactive metabolites by ascending chromatography.

Four solvent systems were used (Table 3); acetonitrile/water/ammonia (40:9:1, by vol.) (Gwiazda & Lord, 1967); propan-2-ol/3N ammonia (3:1, v/v) (Plapp & Casida, 1958b); butan-1-ol/glacial acetic acid/water (11:4:5 by vol.) (Morello, et al., 1968); hexane/acetone (4:1, v/v). The last solvent was used only with Whatman SG 81 paper.

Metabolites were identified by two-dimensional co-chromatography with reference compounds, and autoradiography. Unlabelled reference compounds were detected colorimetrically by spraying:

(i) after exposure to bromine vapour, the chromatograms were sprayed with ferric chloride (1 g FeCl_3 dissolved in 100 ml 1% hydrochloric acid and diluted to 400 ml with ethanol) followed immediately by sulphosalicylic acid (1% w/v in 80% ethanol). The chromatograms were dried (2 min at 100°C) and then lightly sprayed

with water. Phosphorus compounds showed as white spots against a pink-brown background.

(ii) Sulphur-containing compounds were detected by spraying the chromatograms with 2,6-dichloro-p-benzoquinone-4-chlorimine (1% w/v, in acetone) followed by 2,6-dibromoquinone-4-chlorimide (0.5% w/v in acetone). Sulphur compounds appeared brick-red against a pale pink background.

Table 3

Rf values of insecticides and metabolites in paper chromatography
with four solvent systems

	Rf value in solvent systems			
	acetonitrile water ammonia	propan-2-ol 3N ammonia	butan-1-ol acetic acid water	hexane acetone
diazinon	1.00	1.00	1.00	1.00
diazoxon	1.00	1.00	1.00	0.53
parathion	1.00	1.00	1.00	1.00
paraoxon	1.00	1.00	1.00	0.51
desethyl diazinon	0.85	0.95	0.84	0
desethyl diazoxon	0.60	0.80	-	0
desethyl parathion	0.90	0.95	-	0
DEPTA	0.65	0.76	0.71	0
DEPA	0.40	0.63	0.69	0
MEPTA	NOT	ASCERTAINED	-	-
MEPA	0.18	0.54	-	0
S-ethyl glutathione	0.09	0.26	0.42	0

(b) Thin-layer chromatography

Thin-layer chromatography (with alumina, silica gel and cellulose supports) was tried to separate metabolites. It was abandoned when it showed no advantages over paper chromatography.

(c) Gas-liquid chromatography

A Varian Aerograph 1520 gas chromatograph fitted with a phosphorus detector (flame ionisation), used under the following conditions: Injector, 167°C; Column (2 metre, 20% diethylene glycol succinate on Chromosorb W), 130°C; nitrogen flow, 30 ml/min; detector (hydrogen 18 ml/min; air, 240 ml/min), 218°C. This separated DEPA (retention time of 8 min) and DEPTA (retention time 20 min) from diazinon and diazoxon which had very long retention times under these conditions.

(d) Autoradiography

The radioactive insecticides and their metabolites were detected by autoradiography of the paper chromatograms. Films (Ilford Industrial "G" X-ray) were clamped to the chromatograms by sheets of window glass for about 10 days and then developed (Ilford Idex developer and Ilfofix fixer).

Radioactive areas of the chromatograms were dissected and counted on a Panax XAA-04 Autoscaler Analyser with SC-LP2 Scintillation Counter (Panax Equipment Ltd, Redhill, Surrey), by pressing against a solid scintillator (Naton 136), protected from

contamination by a thin film of Melinex.

(e) Spectrometry

Spectra were obtained with a Hitachi Perkin-Elmer RMU 6E mass spectrometer (with the help of Dr D. H. Calam) and a 60 MHz Perkin-Elmer R10 Nuclear Magnetic Resonance Spectrometer (with the help of Dr N. F. Jones).

RESULTS

AND

DISCUSSION

Introduction

The microsomal fractions of mammalian livers (Davison, 1954) and insect tissues (Fenwick, 1958; Fukami & Shishido, 1963) metabolise organophosphorus insecticides, in the presence of pyridine nucleotides and oxygen, to their more potent anti-cholinesterase oxygen analogues. Microsomal preparations from mammals (Nakatsugawa, et al., 1969b) and insects (Nakatsugawa, et al., 1969a; Shishido & Fukami, 1963) also detoxify organophosphorus insecticides. In addition, the soluble fraction, especially in mammals, degrades organophosphorus insecticides and reduced glutathione is involved in this reaction (Fukami & Shishido, 1966; Hollingworth, et al., 1967).

Siekevitz (1965) defined microsomes as "the high speed pellet (from 100,000 g to 250,000 g for 60 to 120 mins) resulting when the supernatant fluid from the mitochondrial fraction is sedimented". This definition is employed here. The soluble fraction is regarded as the supernatant fluid resulting from this high speed centrifugation.

I have investigated the metabolism of diazinon, diazoxon and parathion in the six strains of housefly, both in vivo and in vitro, and I have correlated the results obtained from subcellular preparations (in vitro) with those obtained from whole flies (in vivo).

A) IN VITRO METABOLISM

All the studies on in vitro metabolism were carried out with radioactive insecticides and the metabolites were separated by paper chromatography and detected by autoradiography.

(a) PRELIMINARY EXPERIMENTS:

Preliminary experiments established the conditions for optimal activity of the subcellular preparations.

(i) Activity of various preparations

There are conflicting reports on the best housefly material for metabolism studies. Tsukamoto & Casida (1967a) and Nakatsugawa, et al. (1968) used preparations from abdomens instead of whole flies, although Nakatsugawa, et al. (1969a) later found no differences in metabolism between headless and whole flies. Hook, et al. (1968) found that centrifuged abdomen preparations had no more metabolising activity than uncentrifuged abdomen homogenates.

To clarify these various reports, the diazinon-metabolising activity of uncentrifuged abdomen homogenates, centrifuged (13,000 g for 10 min) abdomen homogenates and centrifuged (13,000 g for 10 min) whole fly homogenates, all in phosphate buffer (0.15M KH_2PO_4 -NaOH, pH 7.3), was investigated (Table 4).

Table 4 shows that centrifuged, whole fly preparations metabolised diazinon significantly faster than abdomen preparations and, in contrast to Hook, et al. (1968), centrifuged abdomens were more

Table 4

Diazinon metabolism by subcellular preparations of strain 29.F71

Incubation	Metabolites (μ g) formed from diazinon		
	DEPTA	DEPA	TOTAL
uncentrifuged abdomen	1.6	0.7	2.3
centrifuged abdomen	2.4	0.8	3.2
centrifuged whole-fly	3.0	1.0	4.0

active than uncentrifuged. In all subsequent experiments, centrifuged homogenates of whole flies were used because there was no advantage in using abdomens.

The removal of housefly abdomens is a lengthy and laborious procedure and some loss of metabolising activity, as compared to whole-fly preparations, probably occurred during this stage. The uncentrifuged abdomen homogenates possibly contained endogenous inhibitors which sedimented on centrifuging at 13,000 g.

(ii) Optimum buffer

A variety of buffers have been used for studying housefly subcellular metabolism of insecticides and so, to find the optimal buffer, homogenates of houseflies in three buffers (0.15M KCl, Ray, 1967; 0.15M KH_2PO_4 -NaOH, pH 7.3, Schonbrod & Terriere, 1966; 0.15M KH_2PO_4 -NaOH plus 0.25M sucrose, pH 7.3,

Tsukamoto & Casida, 1967a) were prepared, centrifuged (13,000 g for 10 min) and incubated with diazinon for 60 min.

The formation of DEPTA was highest in sucrose-phosphate (2.2 μ g) and lowest in chloride (1.3 μ g). However, the sugar caused severe streaking of metabolites in paper chromatography and so phosphate (1.6 μ g DEPTA formed) was subsequently used; Schonbrod & Terriere (1966) also found higher metabolism with preparations in phosphate than in chloride buffer.

(iii) Optimum pH

The optimum pH of phosphate buffer for organophosphorus insecticide metabolism was investigated by incubating diazoxon and parathion in reaction mixtures of pH 6.9, 7.3 and 7.6 (Table 5a, b). These pH values were obtained by adding 1.0 ml of phosphate buffer of pH 5.8, 7.3 and 8.0 respectively to each reaction mixture which contained enzyme and cofactors in 1.0 ml phosphate buffer, pH 7.3.

Table 5a

Desethylation of diazoxon by the soluble fraction of strain

SKA.A.F70 with GSH, incubated at different pH

Incubation	Metabolites (μ g) formed from diazoxon Desethyl diazoxon
pH 6.9	1.5
pH 7.3	4.0
pH 7.6	3.3

Table 5b

Metabolism of parathion by 13,000 g supernatant preparations
of strain 29.F106, incubated at different pH

Incubation	Metabolites (μ g) formed from parathion			
	DEPTA	DEPA	Ethyl glutathione	Paraoxon
pH 6.9	0.4	0.2	0.2	none
pH 7.3	0.7	0.2	0.3	0.2
pH 7.6	0.8	0.2	0.3	0.3

Tables 5a, b show that the optimum pH for desethylation of both diazoxon and parathion was about 7.3, but the optimum pH for the metabolism of parathion to DEPTA and paraoxon was slightly higher. Because no clear pH optimum emerged, the phosphate buffer was standardised at pH 7.3 in subsequent experiments.

(iv) Effect of bovine albumin

The effect of bovine albumin on the metabolism of diazinon by both microsomal and soluble preparations was examined (Table 6), because of reports that this protein enhanced housefly microsomal metabolism of aldrin (Ray, 1967) and Baygon (Tsukamoto & Casida, 1967b) and retarded the loss of microsomal activity with ageing (Tsukamoto & Casida, 1967b; Hook, et al., 1968).

Table 6

Diazinon metabolism by 13,000 g supernatant preparation of strain SKA.B.F62, with and without bovine albumin

Incubation	Metabolites (μ g) formed from diazinon			
	DEPTA	DEPA	Desethyldiazinon	Ethylglutathione
Without albumin:				
+ NADPH	2.2	1.0	none	none
+ NADPH + GSH	4.2	1.0	0.7	1.0
With 2% w/v albumin:				
+ NADPH	2.5	1.0	none	none
+ NADPH + GSH	4.5	1.0	1.2	1.8

Table 6 shows that 2% w/v bovine albumin marginally increases the formation of DEPTA from diazinon (mainly microsomal metabolism) and significantly raised the quantity of glutathione-dependent desethylation (in the soluble fraction); 2% w/v albumin was therefore included in later experiments.

It has been suggested (Tsukamoto & Casida, 1967b; Hook, et al., 1968) that endogenous inhibitors bind with albumin in preference to the microsomal enzymes. It appears from Table 6 that, in diazinon metabolism, albumin is even more effective in removing inhibitors of the soluble enzyme system.

(v) Effect of sex on metabolism

Female houseflies are more resistant to organophosphorus

insecticides than males (Sawicki & Green, 1965) and so possible differences in rates of metabolism of diazinon were studied in male and female flies (Table 7).

Table 7

Diazinon metabolism by 13,000 g supernatant preparations of males and females of strain SKA.B.F62

Incubation	Metabolites (µg) formed from diazinon			
	DEPTA	DEPA	Desethyldiazinon	Ethylglutathione
Males:				
+ NADPH	1.1	0.8	-	-
+ NADPH + GSH	2.7	0.8	trace	1.0
Females:				
+ NADPH	2.5	1.0	-	-
+ NADPH + GSH	4.5	1.0	1.2	1.8

Table 7 shows that female houseflies metabolise diazinon twice as fast as males when the rates of metabolism of the two sexes are compared on a fly-to-fly basis. Even on a weight-for-weight comparison of the sexes the females, which are 50% heavier than males, still detoxify diazinon at about 150% of the rate of the males and this probably explains the increased resistance of the females.

(vi) Optimum concentrations of enzyme and substrate

The optimum concentration of housefly subcellular preparation was examined by incubating 5, 10 and 20 fly-equivalents (i.e. 0.25, 0.50 and 1.0 ml centrifuged homogenate) with diazinon (Table 8).

Table 8

Diazinon metabolism by various concentrations of 13,000 g supernatant preparation of strain SKA.A.F52, with NADPH

Incubation	Metabolites (μ g) formed from diazinon		
	DEPTA	DEPA	TOTAL
0.25 ml	2.0	0.4	2.4
0.50 ml	2.9	0.6	3.5
1.00 ml	2.8	0.6	3.4

Table 8 shows that the optimum concentration of enzyme was 0.50 ml (i.e. 10 fly-equivalents) per reaction mixture. The lack of increased activity with the higher concentration of enzyme was probably due to rate limiting concentrations of either substrate or cofactors, and so the effect of varying the concentration of diazinon was studied with 10 fly-equivalents per reaction mixture (Table 9 and Figure 5).

Table 9

Metabolism of varying concentrations of diazinon by 13,000
supernatant preparation of strain SKA.A.F62

Incubation	Metabolites (μ g) formed from diazinon				TOTAL
	DEPTA	DEPA	Desethyldiazinon*	Ethylglutathione*	
10 μ g diazinon	3.0	1.6	trace	1.5	6.1
20 μ g diazinon	6.2	2.2	2.2	2.8	13.4
30 μ g diazinon	7.8	3.0	3.6	5.6	20.0

*In desethylation, the ethyl group is transferred to glutathione and the resulting S-ethylglutathione gives a more discrete spot for counting than does desethyl diazinon. The value for S-ethylglutathione therefore reflects more accurately the quantity of desethylation.

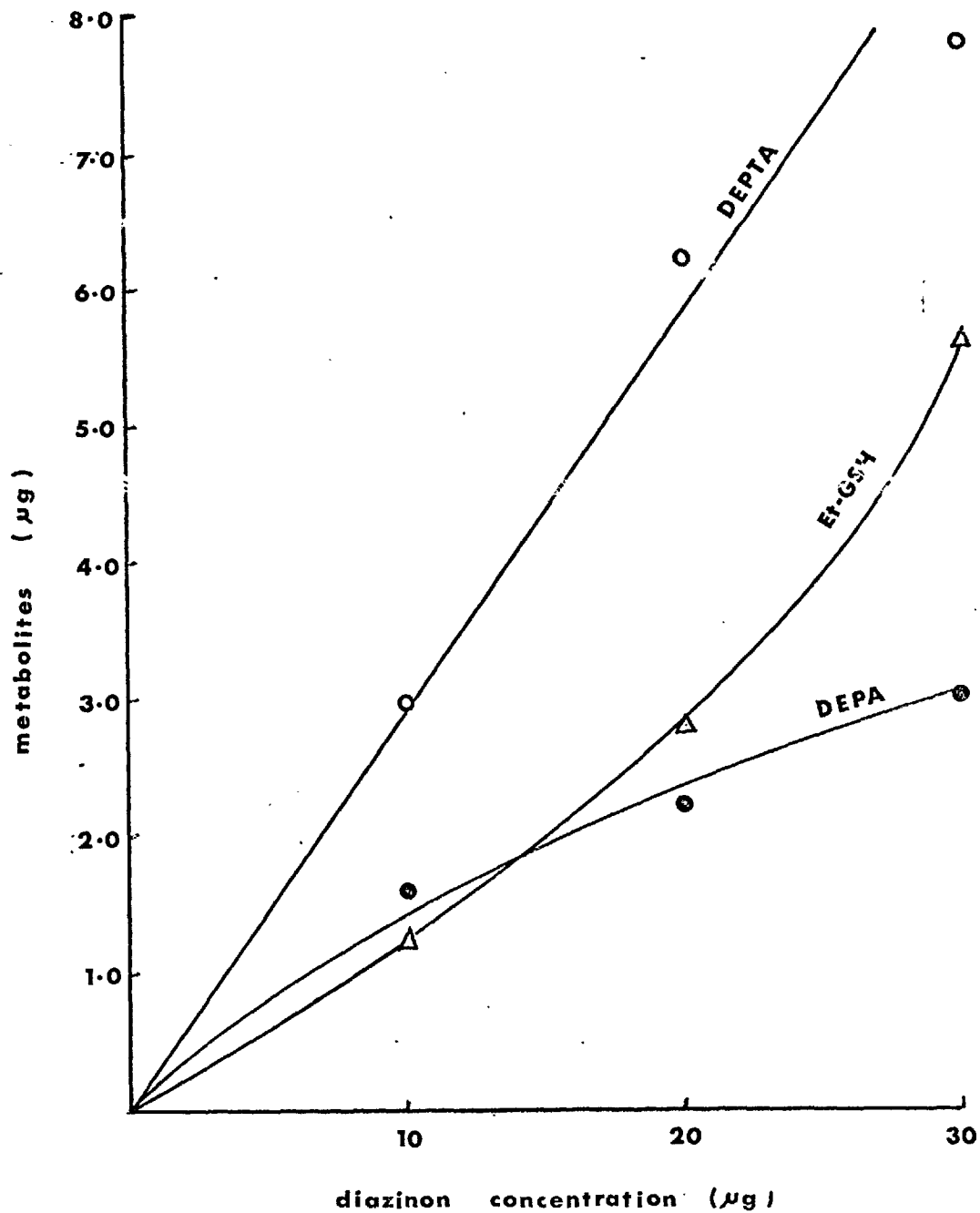


Figure 5 Metabolism of diazinon at varying concentrations
(Data from Table 9)

The linearity of the results in Table 9 and Figure 5 confirms that the limiting factor in the rate of diazinon metabolism is substrate concentration, not enzyme concentration. Although more metabolism occurred with higher doses of diazinon, the pattern of formation was not significantly different from that obtained with 10 μ g (about two-thirds of the dose being metabolised at each concentration).

Therefore, for reasons of economy, 10 μ g was used as the standard dose of insecticide in subsequent experiments.

The standard reaction mixture (see Materials and Methods Section, page 4) evolved from the above preliminary experiments.

It became apparent, during preliminary experiments, that two subcellular fractions, the microsomes and the soluble (100,000 g supernatant), each contained insecticide metabolising activity (Table 10a, b and Plates 1 and 2) and each fraction had different cofactor requirements; the microsomal fraction contained the oxygen and NADPH-requiring mixed-function oxygenase system and the soluble fraction had a glutathione-S-transferase system.

The pattern of subcellular location of parathion metabolism in the SKA strain was almost identical with that of diazinon (Plate 3).

Table 10a

Diazinon metabolism by subcellular fractions of strain SKA.A.F54
and SKA.A.F60 (mean values of two experiments)

Incubation	Metabolites (µg) formed from diazinon			
	DEPTA	DEPA	Desethyldiazinon	Ethylglutathione
13,000 g supernatant:				
+ NADPH	1.7	0.5	-	-
+ NADPH + GSH	3.6	0.5	0.9	1.9
microsomes:				
+ NADPH	1.8	0.5	-	-
+ NADPH + GSH	3.2	0.5	-	-
soluble fraction:				
+ NADPH	0.1	trace	-	-
+ NADPH + GSH	0.5	trace	0.3	2.0

Table 10b

Diazoxon metabolism by subcellular fractions of strain SKA.B.F57
and SKA.B.F58 (mean values of two experiments)

Incubation	Metabolites (µg) formed from diazoxon	
	DEPA	Desethyldiazoxon
13,000 g supernatant:		
+ NADPH	0.5	0.2*
+ NADPH + GSH	0.5	1.5*
microsomes:		
+ NADPH	0.5	- *
+ NADPH + GSH	1.0	- *
soluble fraction:		
+ NADPH	0.1	0.3
+ NADPH + GSH	0.25	3.7

*Two other metabolites detected



STRAIN - SKA B F-8
SUBSTRATE - ³²P-DIAZOXON

+	+	+	+	+	+	+	+
CONTROL	STD.	+GSH	STD.	+GSH	STD.	+GSH	
	<u>10.000 mg</u>		<u>100.000 mg</u>		<u>100.000 mg</u>		
	SUPERNATANT		PPT.		SUPERNATANT		

Plate 1

Autoradiogram of diazoxon metabolism by subcellular preparations of the SKA strain, showing (from bottom to top) the formation of phosphoric acid, (MEPA?), DEPA, one unknown, desethyl diazoxon, and another unknown. Solvent front is unchanged diazoxon.

STRAIN: SKA.A.F54



+	+	+	+	+	+	+	+
CONTROL	STD	+GSH	STD	+GSH	STD	+GSH	+GSH
	<u>100.000 ng</u>		<u>100.000 ng</u>		<u>100.000 ng</u>		
	SUPERNATANT		ppt		SUPERNATANT		

Plate 2

Autoradiogram of diazinon metabolism by subcellular preparations of the SKA strain, showing (from bottom to top) the formation of S-ethylglutathione, DEPA, DEPTA and desethyl diazinon. Solvent front consists of diazoxon and unchanged diazinon.



Plate 3

Autoradiogram of parathion metabolism by subcellular preparations of the SKA strain, showing (from bottom to top) the formation of S-ethylglutathione, unknown (MEPTA?), DEPA, DEPTA and desethyl parathion (near solvent front). Solvent front consists of paraoxon and unchanged parathion.

The metabolising activity of the two subcellular fractions was examined in greater detail and is described separately. The results reported in the following sections indicate the metabolic pathways for diazinon, diazoxon and parathion shown in Figure 6a, b.

Figure 6a Pathways of metabolism of diazinon and diazoxon

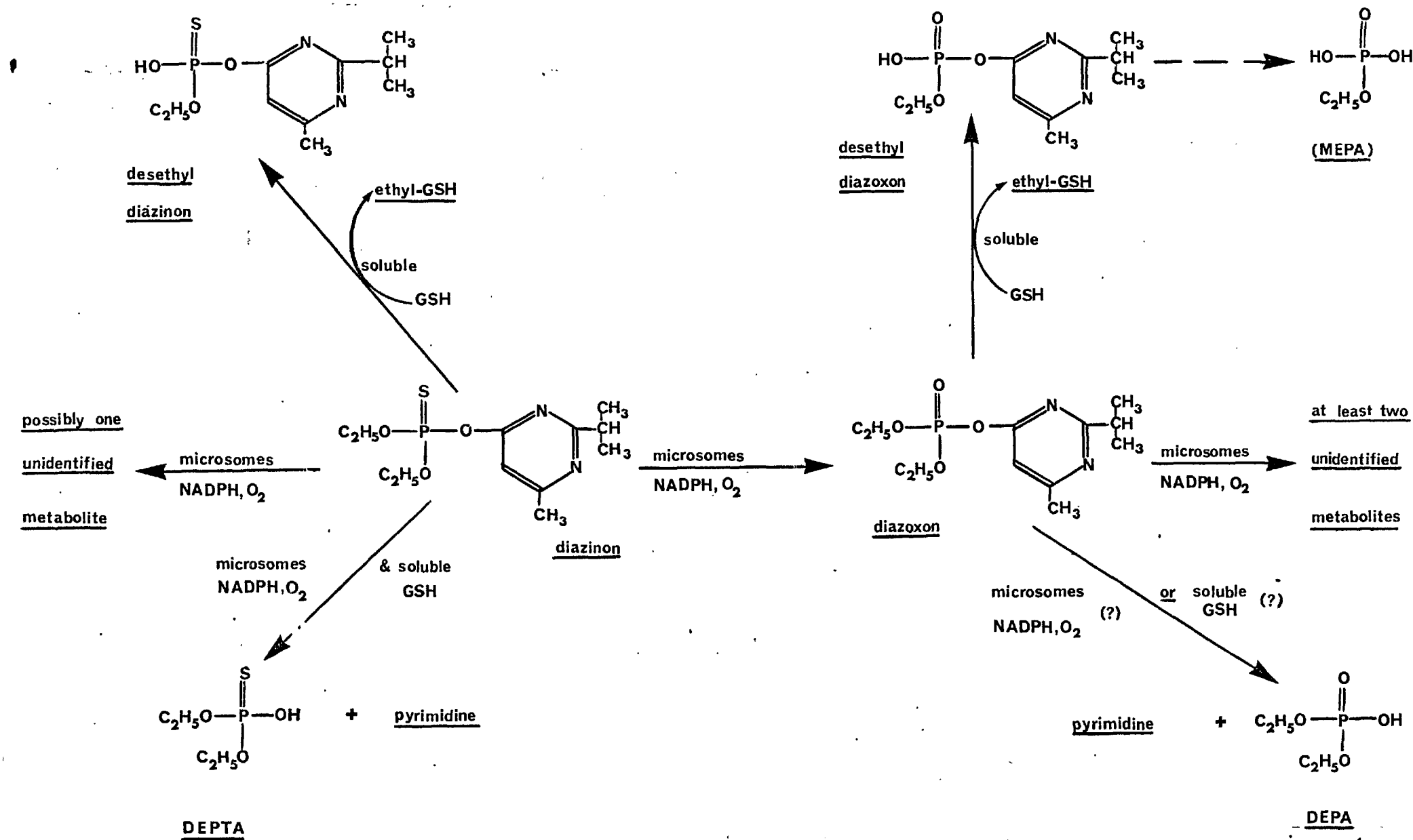
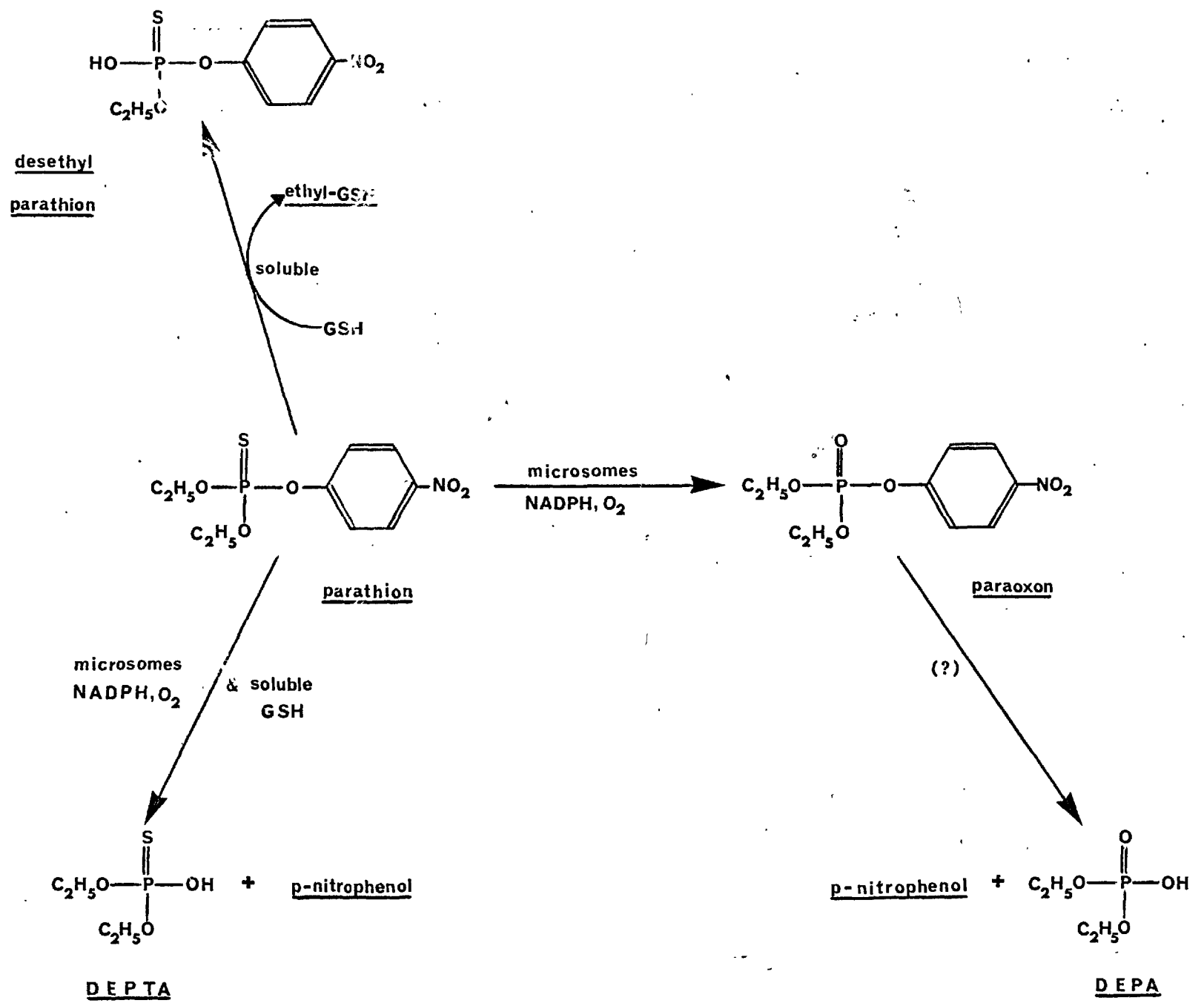


Figure 6b. Pathways of metabolism of parathion



(b) METABOLISM BY THE MICROSOMAL FRACTION

Table 10a, b shows that there is a microsomal system which cleaves diazinon to diethylphosphorothioic acid (DEPTA) and diazoxon to diethylphosphoric acid (DEPA). This system also cleaves parathion to DEPTA, converts parathion and diazinon to their oxygen analogues, paraoxon and diazoxon, and in certain strains metabolises diazinon and possibly diazinon to at least three other compounds.

(i) Requirement for added NADPH

The effect of NADPH on the cleavage of diazinon, diazoxon and parathion was investigated (Table 11a, b, c) because microsomal preparations from both mammalian livers (Davison, 1954) and insects (Nakatsugawa & Dahm, 1965) are reported to require reduced pyridine nucleotide cofactors for maximal organophosphorus insecticide metabolising activity; NADPH is more effective than NADH in insect preparations (Nakatsugawa & Dahm, 1965; Tsukamoto & Casida, 1967a).

Table 11a

DEPTA formation from diazinon by 13,000 g supernatant preparations of several strains of housefly, with and without added NADPH

Strain	DEPTA (μ g) formed from diazinon	
	+NADPH	-NADPH
SKA.B.F52	2.4	0.4
SKA.A.F63	2.0	0.6
SKA.B.F63	2.7	0.0
29.F77	1.5	0.6
29.F85	0.9	0.4
393.F40	0.8	0.1
466.500.B.F30	0.8	0.0
466.500.A.F30	1.9	0.7
ocra SRS.F104	0.3	0.0

Table 11b

DEPA formation from diazoxon by 13,000 g supernatant preparations
of several strains, with and without added NADPH

Strain	DEPA (μg) formed from diazoxon	
	+NADPH	-NADPH
SKA.B.F58	0.36	0.36
29.F80	0.20	0.17
393.F45	0.33	0.24
466.500.A.F32	0.07	0.03
466.500.B.F35	0.18	0.14
<u>ac;ar;bwb;ocra</u> SRS.F55	0.07	0.05

Table 11c

DEPTA formation from parathion by 13,000 g supernatant preparations
of two strains, with and without added NADPH

Strain	DEPTA (μg) formed from parathion	
	+NADPH	-NADPH
SKA.A.F72	0.7	0.3
29.F106	0.7	0.2

Tables 11a, b, c show that, in several strains of flies, added NADPH significantly increased the cleavage of diazinon and parathion to DEPTA, although some residual cleavage occurred without added NADPH. This residual cleavage could be due either to endogenous NADPH in the preparation or to the action of non-specific phosphatases or to the residual activity of the GSH-dependent soluble mechanism.

In contrast, added NADPH only marginally increased the cleavage of diazoxon to DEPA. This may be due to the presence of a microsomal enzyme different to that which cleaves diazinon; Fouts & Gram (1969) have postulated the existence of a number of different microsomal mixed-function oxygenases. An alternative explanation is that diazoxon cleavage activity is equal to or less than the residual level of diazinon cleavage in the absence of added NADPH. Thus any endogenous NADPH in the preparation sufficient to give the slight residual diazinon cleavage would also be sufficient to give all the diazoxon cleavage.

Cleavage of diazoxon does not decrease in the absence of the soluble fraction (see Table 10b) and, therefore, residual diazoxon cleavage in the absence of NADPH is not caused by the GSH-dependent soluble mechanism.

A fuller explanation of diazoxon cleavage in the absence of added NADPH might result if any NADPH present in the microsomal preparation was blocked or removed.

Table 10a, b also shows that GSH, in the presence of NADPH, significantly increases microsomal cleavage of diazinon and diazoxon. Further studies on the respective roles of these two cofactors are described in Results Section (b) iii.

(ii) Effect of incubation with oxygen

Because Neal (1967a) reported that mammalian liver

microsomes metabolise parathion fastest in an atmosphere of air and that metabolism was inhibited by nitrogen, the effect of oxygen on housefly microsomal metabolism of diazinon and diazoxon was compared (Table 12) by incubation under nitrogen or air. A nitrogen atmosphere was obtained by perfusing the reaction mixture with nitrogen for 5 min in a test tube, and then stoppering the tube during incubation.

Table 12

Effect of oxygen on diazinon and diazoxon metabolism by 13,000 g supernatant preparations with NADPH

Strain	DEPTA (µg) formed from diazinon		DEPA (µg) formed from diazoxon	
	+ NITROGEN	+ AIR	+ NITROGEN	+ AIR
29.F77	0.9	1.5	-	-
393.F41	0.2	1.1	-	-
<u>ocra</u> SRS.F105	0.2	0.7	-	-
393.F44	-	-	0.24	0.35
466.500.B.F34	-	-	0.21	0.23
SKA.A.F57	-	-	0.5	0.4

The requirements for oxygen and for NADPH (previous section (b) i) are similar. Both NADPH and air greatly increase the amount of DEPTA produced from diazinon but have little effect on the cleavage of diazoxon to DEPA.

The formation of DEPA and of residual DEPTA under nitrogen may be due either to oxygen dissolved in the buffer or bound

to the microsomes, or to the action of phosphatases, including the GSH-dependent enzyme. Later results will show that the GSH-dependent enzyme is as efficient under nitrogen as under air.

These results indicate that diazinon, and parathion, are metabolised by the NADPH- and oxygen-requiring microsomal mixed-function oxygenase system in houseflies, but do not indicate whether diazoxon is metabolised by the same system as the thionates.

Nakatsugawa, et al. (1969b) expressed doubts about the nature of the diazoxon cleavage mechanism in rat subcellular preparations.

(iii) Effect of compounds with reduced sulphur groups

Compounds containing reduced sulphur groups increase the overall metabolism of parathion by rat liver microsomes (Neal, 1967b). Because Table 10 a, b showed that GSH, in the presence of NADPH, significantly increased microsomal cleavage of diazinon and diazoxon, the effect of compounds containing reduced sulphur (GSH and dithiothreitol, Cleland's Reagent) was further investigated (Table 13a, b).

Table 13a

Diazinon metabolism in 13,000 g supernatant preparations from several strains, with and without GSH and dithiothreitol, with NADPH

Strain	Incubation	Metabolites (µg) formed from diazinon		
		DEPTA	DEPA	desethyl diazinon
466.500.A.F30	standard	3.1	trace	none
	+ GSH	4.7	trace	trace
	+ dithiothreitol	4.9	trace	none
29.F77	standard	1.5	0.4	none
	+ GSH	2.1	0.3	0.8
	+ dithiothreitol	1.5	0.2	none
SKA.A.F52	standard	2.9	0.6	none
	+ GSH	4.3	0.7	1.6
	+ dithiothreitol	4.1	0.8	none

Table 13b

Diazoxon metabolism in 13,000 g supernatant preparations of
several strains, with and without GSH and dithiothreitol

Strain	Incubation	Metabolites (µg) formed from diazoxon	
		DEPA	desethyl diazoxon
466.500.A.F33	standard	0.22	none *
	+ GSH	0.35	0.5 *
	+ dithiothreitol	0.35	none *
466.500.B.F34	standard	0.23	0.06 *
	+ GSH	0.34	0.5 *
	+ dithiothreitol	0.28	none *
393.F44	standard	0.35	0.2
	+ GSH	0.50	1.8
	+ dithiothreitol	0.50	0.1
29.F81	standard	0.42	0.16
	+ GSH	0.53	1.60
	+ dithiothreitol	0.60	0.09
SKA.B.F56	standard	0.54	0.3 *
	+ GSH	0.45	3.50 *
	+ dithiothreitol	0.54	none *
SKA.A.F57	standard	0.38	0.25 *
	+ GSH	0.75	5.00 *
	+ dithiothreitol	0.35	0.13 *

* traces of two unidentified metabolites

L-cysteine behaved similarly to dithiothreitol.

Table 13a, b shows that GSH increases the metabolism of diazinon and diazoxon in some strains of houseflies. Dithiothreitol generally slightly increased the formation of DEPTA and DEPA, but did not stimulate desethylation. The increase in metabolism caused

by the reduced sulphur groups cannot be attributed definitely to the microsomes or to the soluble fraction in Table 13 because 13,000 g supernatant preparations were used. However, desethylation occurs entirely in the soluble fraction and DEPTA and DEPA formation occurs mainly in the microsomes (see Table 10a, b); it is probable therefore that, whereas only GSH affects the soluble desethylation mechanism, all the compounds with reduced sulphur groups increase metabolism by the microsomes.

Pinto & Bartley (1969), working with rat liver homogenates, postulated that GSH supplied the "reducing power" for metabolising peroxides to alcohols. GSH was regenerated from oxidised glutathione by the oxidation of NADPH (Rall & Lehninger, 1952) or by the action of thiol compounds. This scheme bears out the suggestions of Neal (1967b) that stimulation of rat liver microsomal mixed-function oxygenase activity by reduced sulphur compounds is due to these compounds either maintaining essential thiol groups (e.g. GSH, in this scheme) in a reduced state or acting as redox couples in microsomal electron transfer.

The role of the reduced sulphur compounds in housefly microsomal metabolism of organophosphorus insecticides was further investigated by using sulphur-inhibitors (described in a later section) and by desalting the microsomes (Table 14).

A microsomal preparation was passed down a column of Sephadex G-25, to remove any essential cofactors, metal ions, etc., of molecular weight less than 5,000. A lack of activity in the

eluate would indicate that cofactors are required and subsequent retrieval of activity on the addition of cofactors should show what these essential cofactors are. The fraction containing material of molecular weight greater than 5,000 (i.e. microsomes) was eluted immediately after the void volume and aliquots of this fraction were incubated with diazinon, with and without added NADPH and GSH cofactors.

Table 14

Metabolism of 20 µg diazinon by microsomal preparations of strain SKA.B.F63, before and after desalting, incubated with and without cofactors

Incubation	Metabolites (µg) formed from diazinon		
	DEPTA	DEPA	TOTAL
Before desalting:			
NO COFACTORS	none	none	none
+ GSH alone	0.4	0.3	0.7
+ NADPH alone	2.7	1.5	4.2
+ GSH + NADPH	5.8	1.7	7.5
After desalting:			
NO COFACTORS	none	none	none
+ GSH alone	0.3	0.8	1.1
+ NADPH alone	3.6	1.5	5.1
+ GSH + NADPH	4.5	2.8	7.3

The results in Table 14 confirm that microsomal metabolism of diazinon requires NADPH (section b.i.) as the essential cofactor. As the results before and after desalting are almost identical, the desalting procedure did not remove any other important cofactors of

microsomal metabolism. Table 14 also shows that, whilst GSH significantly increases diazinon metabolism in the presence of NADPH, GSH alone has only a slight effect on housefly microsomal metabolism of diazinon. It is unlikely, therefore, that GSH is the direct source of "reducing power" for diazinon metabolism by microsomes. The possibility remains that GSH, di-thiothreitol and cysteine stimulate microsomal metabolism by maintaining some essential microsomal thiol groups in a reduced state or by acting as redox couples in microsomal electron transfer, as suggested by Neal (1967b).

(iv) Effect of possible inhibitors

The stimulatory effect of compounds containing reduced sulphur groups on diazinon metabolism was studied further by attempting to block endogenous reduced sulphur groups with iodoacetate, which alkylates thiol groups, and p-chloromercuribenzoate (pCMB) which forms mercaptides with thiols (Dixon & Webb, 1964) (Table 15).

Table 15

Diazinon metabolism by 13,000 g supernatant preparations of three strains, with and without thiol-inhibitors

Strain	Incubation	Metabolites (µg) formed from diazinon			% inhibition
		DEPTA	DEPA	TOTAL	
466.500.A.F30	+ NADPH	3.1	trace	3.1	-
	+ NADPH + pCMB	1.9	trace	1.9	39
29.F77	+ NADPH	1.5	0.4	1.9	-
	+ NADPH + IODOACETATE	0.9	0.2	1.1	42
	+ NADPH + pCMB	1.1	0.2	1.3	31
SKA.A.F52	+ NADPH	2.9	0.6	3.5	-
	+ NADPH + IODOACETATE	1.8	0.4	2.2	37
	+ NADPH + pCMB	2.4	0.4	2.8	20

Table 15 shows that the thiol-blocking reagents inhibit metabolism of diazinon and these results, which agree with those of Neal (1967b) for rat liver microsomal metabolism of parathion, confirm that thiol groups function in the metabolism of organophosphorus insecticides.

Another common inhibitor of metabolism, ethylenediaminetetracetic acid (EDTA), gave inconclusive results. Combined with the complete recovery of microsomal activity in the presence of NADPH and GSH after desalting, this suggests that divalent metal ions, which are chelated by EDTA, are not particularly important in microsomal metabolism of organophosphorus insecticides.

(v) Effect of synergists

The effect of sesamex, a microsomal inhibitor (Sun & Johnson, 1960; Nakatsugawa & Dahm, 1965) and S,S,S-tributylphosphorotrithionate (TBTP), an aliesterase inhibitor (Plapp, et al., 1963), on the cleavage of diazinon, parathion and diazoxon in several strains of housefly was also studied (Tables 16a, b, c).

Table 16a

Diazinon metabolism by 13,000 g supernatant preparations of
various strains with NADPH, with and without synergists

Strain	Incubation	Metabolites (μg) formed from diazinon			% inhibition
		DEPTA	DEPA	TOTAL	
<u>ocra</u> SRS.F102	standard	0.8	none	0.8	-
	+ SESAMEX	0.3	none	0.3	62
	+ TBTP	0.8	none	0.8	0
<u>ac;ar;bwb;ocra</u> SRS.F51	standard	1.2	none	1.2	-
	+ SESAMEX	0.3	none	0.3	75
	+ TBTP	1.2	none	1.2	0
<u>ac;ar;bwb;ocra</u> SRS.F86	standard	0.9	none	0.9	-
	+ SESAMEX	0.7	none	0.7	22
466.500.F30	standard	0.8	trace	0.8	-
	+ SESAMEX	0.5	trace	0.5	37
	+ TBTP	0.8	trace	0.8	0
466.500.A.F30	standard	3.1	trace	3.1	-
	+ SESAMEX	1.3	trace	1.3	58
	+ TBTP	3.2	trace	3.2	-
466.500.A.F50	standard	1.8	none	1.8	-
	+ SESAMEX	0.3	none	0.3	83
29.F76	standard	1.5	0.2	1.7	-
	+ SESAMEX	0.9	0.3	1.2	29
	+ TBTP	1.8	0.4	2.2	*
29.F85	standard	0.9	0.2	1.1	-
	+ SESAMEX	0.4	0.2	0.6	45
393.F38	standard	1.2	0.2	1.4	-
	+ SESAMEX	0.6	0.2	0.8	43
	+ TBTP	1.2	0.2	1.4	0
393.F41	standard	1.1	0.2	1.3	-
	+ SESAMEX	0.4	trace	0.4	69
SKA.B.F51	standard	2.6	0.4	3.0	-
	+ SESAMEX	1.4	0.5	1.9	37
	+ TBTP	2.4	0.5	2.9	3
SKA.A.F63	standard	2.0	1.5	3.5	-
	+ SESAMEX	0.8	trace	0.8	77
SKA.A.F74	standard	2.9	0.4	3.3	-
	+ SESAMEX	1.3	0.3	1.6	52

Table 16b

Parathion metabolism by 13,000 g supernatant preparations of
various strains with NADPH, with and without sesamex

Strain	Incubation	Metabolites (µg) formed from parathion			% inhibition
		DEPTA	DEPA	TOTAL	
<u>ocra</u> SRS.F25	standard	0.3	none	0.3	-
	+ SESAMEX	0.1	none	0.1	66
<u>ac;ar;bwb;ocra</u> SRS.F86	standard	0.25	none	0.25	-
	+ SESAMEX	0.2	none	0.2	20
466.500.A.F50	standard	0.2	none	0.2	-
	+ SESAMEX	0.15	none	0.15	25
29.F98	standard	0.4	0.3	0.7	-
	+ SESAMEX	0.2	0.2	0.4	43
SKA.B.F71	standard	0.6	0.3	0.9	-
	+ SESAMEX	0.5	0.3	0.8	11
SKA.A.F74	standard	0.25	0.1	0.35	-
	+ SESAMEX	0.2	0.05	0.25	29

Table 16c

Diazoxon metabolism by 13,000 g supernatant preparations of various
strains with NADPH, with and without synergists

Table 16a shows that sesamex significantly inhibited the cleavage of diazinon to DEPTA, whereas TBTP did not. This confirms that diazinon is cleaved to DEPTA principally by the microsomal mixed-function oxygenase system. Sesamex also inhibited parathion cleavage (Table 16b), but to a lesser extent than diazinon.

Table 16c shows that sesamex inhibits diazoxon cleavage much less than diazinon cleavage and TBTP gave almost the same level of inhibition. In conjunction with the lack of variation in diazoxon cleavage found in the experiments on the requirements for oxygen and NADPH, these results question whether diazoxon cleavage is microsomal. Table 10b showed that DEPA formation from diazoxon occurs mainly in the microsomal preparation, but it is possible that the enzyme involved in DEPA formation is not a mixed-function oxygenase. Nakatsugawa et al. (1969b) also doubted that diazoxon cleavage was microsomal.

Because the mode of action of sesamex as an inhibitor of microsomal cleavage of diazinon was not clear, the kinetics of this inhibition were studied (Table 17; Figures 7 and 8) by the graphic methods described by Dixon & Webb (1964).

Table 17

DEPTA formation from diazinon by 13,000 g supernatant preparation
of strain SKA.B.F54, at various concentrations of substrate and
sesamex, incubated with NADPH for 60 mins

Diazinon dose (μg)	Sesamex dose (μg)	DEPTA (μg) formation
5.0	0	1.15
	10	0.85
	20	0.65
10.0	0	1.50
	10	1.05
	20	0.90
20.0	0	2.10
	10	1.60
	20	1.25

Figure 7 shows the Lineweaver-Burke double reciprocal plot, of $1/v$ versus $1/s$, which suggests that sesamex inhibits diazinon cleavage to DEPTA non-competitively (Dixon & Webb, 1964). From this graph, the rate of the cleavage reaction (V) is 1.0×10^{-2} $\mu\text{moles/hour}$ and the Michaelis constant (K_m) is 3.0×10^{-2} μM for this reaction.

However, the plot of $1/v$ versus i (inhibitor concentration) (Figure 8) shows the characteristics of competitive inhibition (Dixon & Webb, 1964). This suggests that diazinon (for cleavage to DEPTA) and sesamex are bound to the same microsomal enzyme (hence competitive inhibition) but at different, but close, active

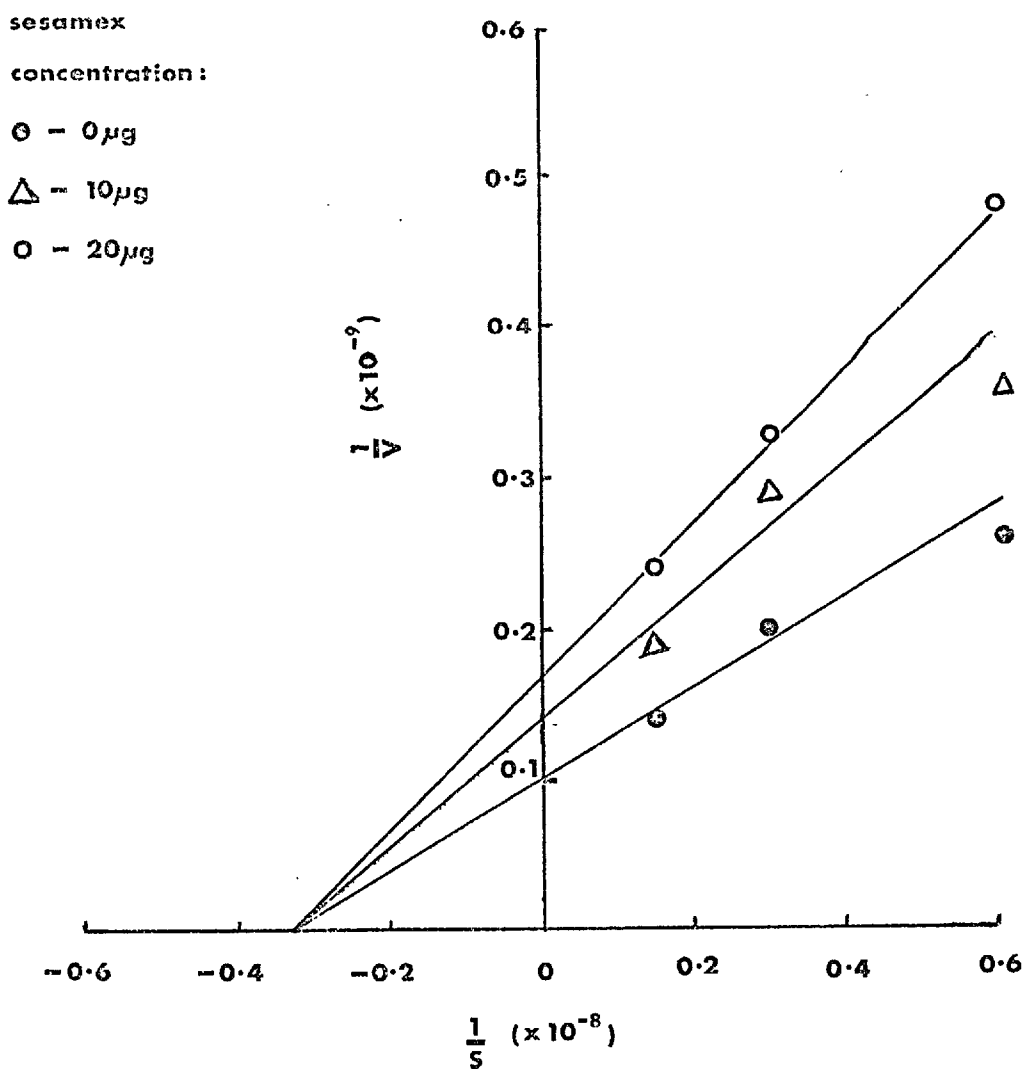


Figure 7 Plot of $1/v$ vs $1/s$ for diazinon at varying concentrations of sesamex

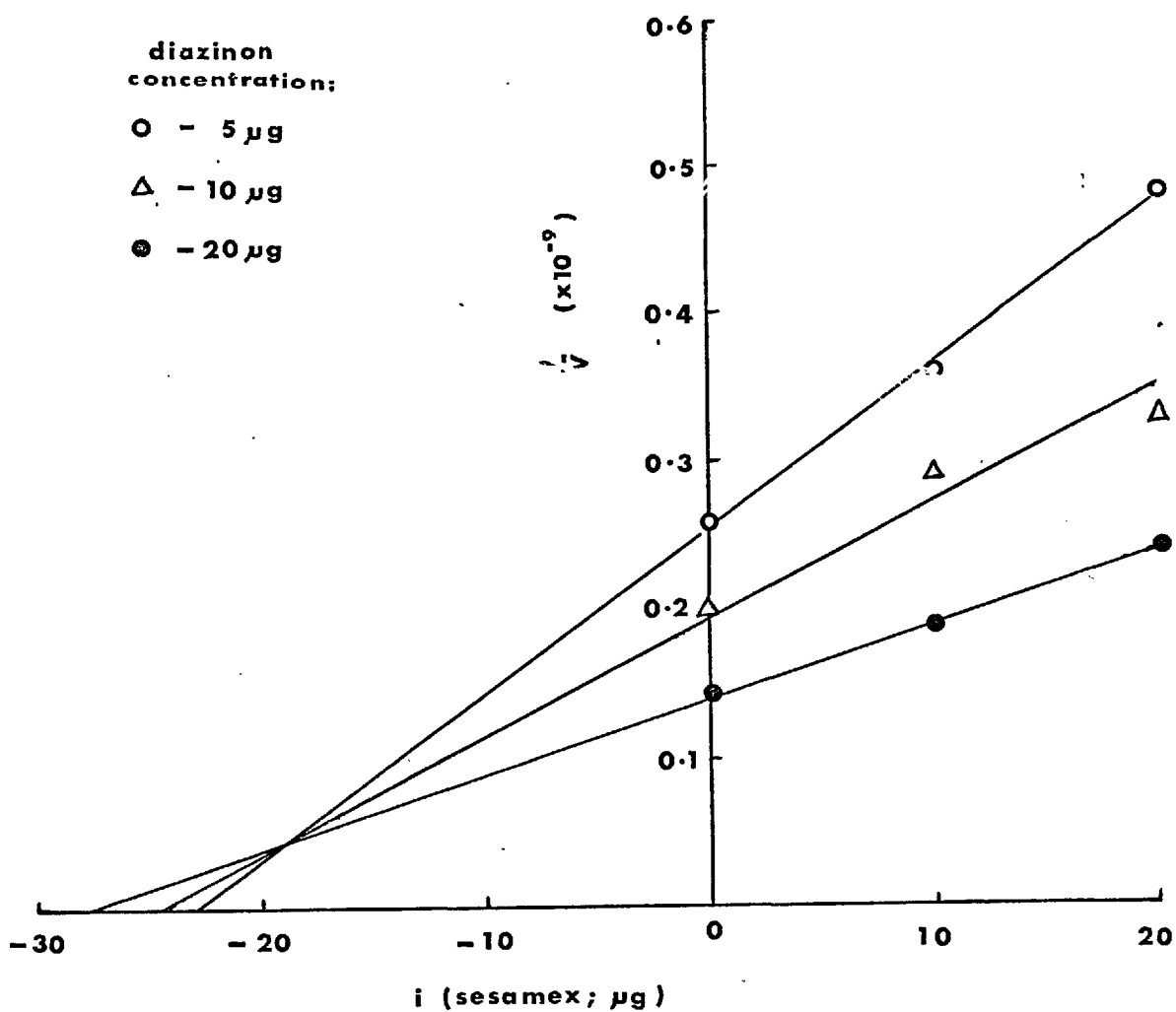


Figure 8 Plot of $1/v$ vs i for diazinon, with sesamex as inhibitor

sites (non-competitive inhibition).

Full elucidation of the mechanism of action of sesamex as a synergist and microsomal inhibitor will depend on more detailed studies on the kinetics, not only of diazinon cleavage but also of the formation and detoxification of diazoxon.

Since this work was done, Isaac & Casida (1969) and Wilkinson & Hicks (1969) reported that the methylenedioxyphenyl compounds are metabolised by the microsomes and they have suggested that these compounds act as synergists by competing with the insecticides as substrates for the microsomal mixed-function oxygenases.

(vi) Formation of metabolites

Figures 6a, b (pages 69, 70) show the pattern of metabolism of diazinon, diazoxon and parathion.

1) Formation of DEPTA and DEPA

13,000 g supernatant preparations from all strains cleaved diazinon and parathion to DEPTA and diazoxon to DEPA (Table 18) and Table 10a, b (page 64) showed that most of this activity is located in the microsomes.

Table 18

Cleavage products of diazinon, diazoxon and parathion from 13,000 g supernatant preparations, incubated with NADPH

(all values mean of determinations on at least three different generations)

Strain	Metabolites (μg) from:				diazoxon DEPA
	diazinon		parathion		
	DEPTA	DEPA	DEPTA	DEPA	
<u>ocra</u> SRS	0.7	none	0.2	none	0.1
<u>ac;ar;bwb;ocra</u> SRS	1.3	none	0.4	none	0.1
466.500	1.4	none	0.3	none	0.1
29	1.5	0.4	0.5	0.3	0.3
393	1.8	0.2	0.4	0.2	0.2
SKA	2.7	0.6	0.4	0.3	0.4

Table 18 shows that no marked interstrain differences were detected in the quantities of DEPTA formed from diazinon and parathion, except from diazinon in the SKA strain and strain ocra SRS. However, strains with gene a (strains 29, 393 and SKA) produced significantly more DEPA, when incubated both with diazoxon and with the thionates, than the susceptible strains and strain 466.500. Because no DEPA was obtained when 13,000 g supernatant fractions were incubated with [14 C-ethoxy] DEPTA, all DEPA produced must have resulted from cleavage of the oxygen analogues (i.e. diazoxon and paraoxon). This agrees with the results of Matsumura & Hogendijk (1964) and Nakatsugawa, et al. (1969b), who also found no conversion of DEPTA to DEPA.

Therefore, resistance caused by gene a appears to increase detoxification of the oxygen analogues by cleaving them to DEPA.

2) Formation of unknown metabolites

Strains 466.500 and SKA, both of which have the chromosome V resistance factor, metabolised diazinon and/or diazoxon, but not parathion, to at least three unidentified compounds. Two of these metabolites were derived from diazoxon, not diazinon, and were detected on Whatman No. 1 paper with acetonitrile/water/ammonia ($R_f = 0.53$ and 0.69) (Plate 1); the parent compound of the other metabolite ($R_f = 0.59$, on Whatman SG 81 paper with hexane/acetone) is not known (Plates 4A and 4B show this metabolite and diazoxon).

These three metabolites, which have not been detected in any of the other strains, are produced only in the microsomal fraction of the two strains with the chromosome V resistance factor. Their formation requires NADPH and oxygen, increases with GSH and dithiothreitol and is inhibited by sesamex. Microsomal preparations of the SKA strain produced 1-2 μ g of these unidentified metabolites from 10 μ g diazoxon doses.

Strain 466.500 is not resistant to either parathion or paraoxon and this susceptibility can be explained by the total lack of formation of the parathion/paraoxon analogues of these three metabolites in this strain. Comparison of the structures of parathion and diazinon (Figures 6a, b; pages 69,70) suggests that a possible

SUBSTRATE: ^{14}C -DIAZINON, ON WHATMAN SG81 PAPER.

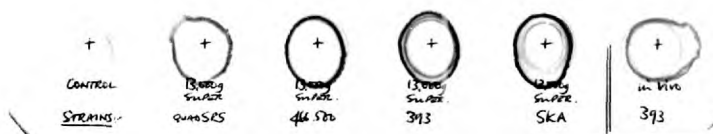


Plate 4A

Autoradiogram of diazinon metabolism by 13,000 g supernatant preparations showing formation of unidentified metabolite (and diazoxon) in strains with resistance factor on chromosome V.

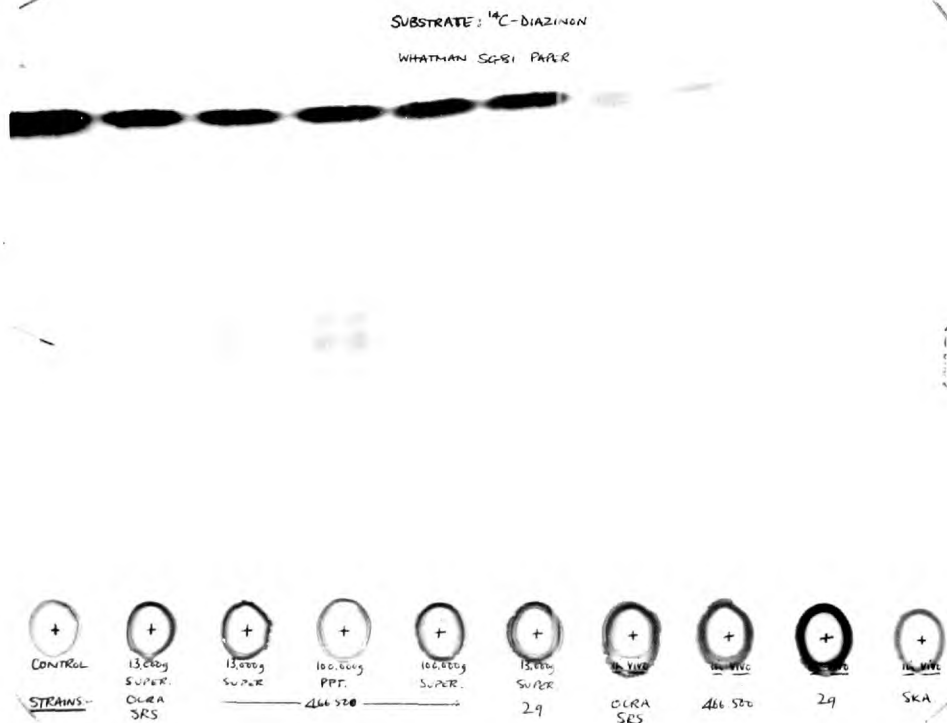


Plate 4B. Autoradiogram of diazinon metabolism showing formation of diazoxon and unidentified metabolite by microsomal (100,000 g precipitate) fraction of strain 466.500, and formation of diazoxon in strain ocra SRS.

mechanism of action of the chromosome V microsomal resistance factor is oxidation of either the methyl or isopropyl groups of the pyrimidinyl moiety of diazinon/diazoxon, because these groups are absent from parathion/paraoxon.

Attempts were made to form the 2:4-dinitrophenylhydrazine derivative of possible aldehyde or carbonyl metabolites by chromatographing microsomal reaction mixtures of the strains with the chromosome V resistance factor, incubated with diazoxon, on Whatman No. 1 paper in acetonitrile/water/ammonia and then spraying lightly with 2:4-dinitrophenylhydrazine in HCl (Dawson, et al., 1969). When chromatographed at right angles in the same solvent, the unidentified metabolites appeared to be slightly retarded by the spray ($R_f = 0.45$ for both) but there was insufficient material to show as the yellow 2:4-dinitrophenylhydrazine derivative and the results rather depended on the degree of spraying; diazoxon and DEPA were also retarded if the chromatograms were sprayed too heavily.

Recently, Mucke, et al. (1970) have reported isolating the primary and tertiary alcohols formed by oxidation of the isopropyl group of the pyrimidinyl moiety. These metabolites were found in the urine of rats dosed with diazinon; diazinon was first cleaved to DEPTA and the pyrimidinyl moiety, and the pyrimidinyl moiety was then hydroxylated at the isopropyl group. It is possible that the unidentified metabolites found in strains with the chromosome V factor could be similar derivatives of diazoxon and/or diazinon.

All strains also metabolised diazinon, diazoxon and

parathion to metabolites with an $R_f = 0.2$ (acetonitrile/water/ammonia system). The diazoxon metabolite occurred entirely in the microsomes (Plate 1; page 65) and was probably MEPA. The parathion metabolite occurred in both the microsomal and soluble fractions (Plate 3, page 67) but only in the presence of GSH; it has not been identified. Only minute traces of this metabolite were found in diazinon reactions. This metabolite is possibly MEPTA.

Formation of diazoxon and paraoxon

All six strains metabolise diazinon and parathion to their oxygen analogues, diazoxon and paraoxon, respectively. These reactions occur almost entirely in the microsomes (Plate 5) and require NADPH and oxygen. GSH stimulates the reaction, which is inhibited by sesamex (Plate 6); the formation of the oxygen analogues is therefore probably carried out by the mixed-function oxygenase system. Because diazoxon and paraoxon are further metabolised in the resistant strains, quantitative differences between the strains are difficult to ascertain. However, significant interstrain differences in the formation of the oxygen analogues do appear to occur (Table 19).

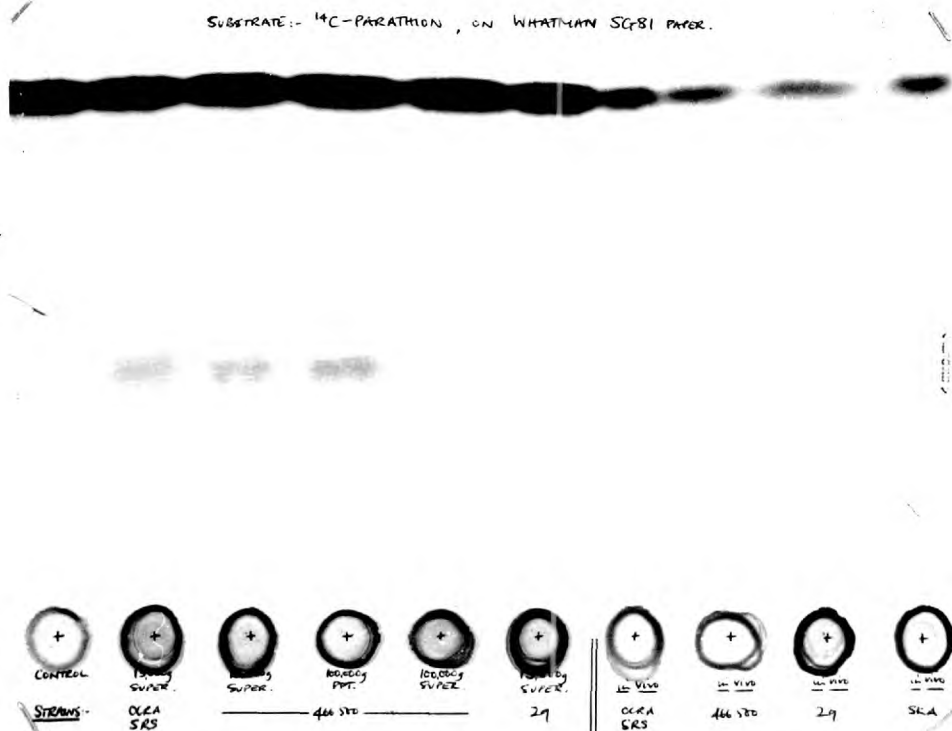


Plate 5

Autoradiogram of parathion metabolism, showing formation of paraoxon in the microsomal (100,000 g precipitate) fraction in vitro; and showing paraoxon formation in susceptible but not resistant strains, in vivo.

SUBSTRATE: ^{14}C -DIAZINON, ON WHATMAN SG81 PAPER.

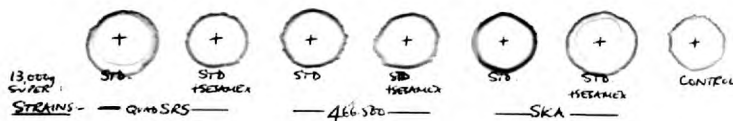


Plate 6

Autoradiogram of diazinon metabolism by 13,000 g supernatant preparations, showing inhibition of formation of both diazoxon and unidentified metabolite by sesamex.

Table 19

Diazoxon, paraoxon and DEPA formation by 13,000 g supernatant preparations of various strains⁺, incubated with NADPH

Strain	Metabs. (µg) of diazinon		Metabs. (µg) of parathion	
	Diazoxon	DEPA	Paraoxon	DEPA
<u>ocra</u> SRS	0.40	none	0.13	none
<u>ac;ar;bwb;ocra</u> SRS	0.20	none	0.14	none
466.500	0.80	none	0.10	none
29	0.15	0.3	0.07	0.3
393	0.15	0.3	0.02	0.2
SKA	1.20	0.5	0.14	0.2

⁺ mean values of determinations on at least three generations

Table 19 shows the susceptible strains (ocra SRS and ac;ar;bwb;ocra SRS with diazinon; ocra SRS, ac;ar;bwb;ocra SRS and 466.500 with parathion) produce the least amount of oxygen analogue (sum of oxygen analogue and DEPA) but they do not detoxify any, and so these strains have the highest accumulated concentration of oxygen analogues, with the exception of the SKA strain. The strains with gene a produce more diazoxon and paraoxon than the susceptibles but they detoxify these oxygen analogues to DEPA, so that the concentration of paraoxon and diazoxon that accumulates is small.

The SKA strain detoxifies much diazoxon and paraoxon, but produces even more and so the accumulated concentration of these compounds is high. This strain generally metabolises significantly

more insecticide, especially diazinon, than any of the other strains, in vitro. This will be further discussed later.

It is noteworthy that the quantity of DEPA produced from a diazoxon dose is almost identical to the amount produced from a diazinon dose, although in the latter case the diazoxon concentration is much lower (Table 18). This suggests that the mechanism(s) of cleavage of diazoxon is rapidly saturated and raising the concentration of diazoxon does not significantly alter its rate of detoxification. This could be verified by experiments on the kinetics of diazoxon metabolism.

(vii) Effect of time of incubation on metabolite formation

Table 20 and Figure 9 show that formation of DEPTA and DEPA from diazinon was initially rapid, but then levelled off.

Table 20

DEPTA and DEPA formed from diazinon by 13,000 g supernatant preparations of the SKA strain, incubated for varying times

Incubation Time	Metabolites (μ g) formed from diazinon	
	DEPTA	DEPA
15 min	1.8	trace
30 min	2.1	0.2
60 min	2.4	0.4
90 min	2.6	0.5
120 min	2.6	0.5

Nakatsugawa, et al. (1969c) obtained similar results in parathion metabolism by rat liver microsomes and they suggested that

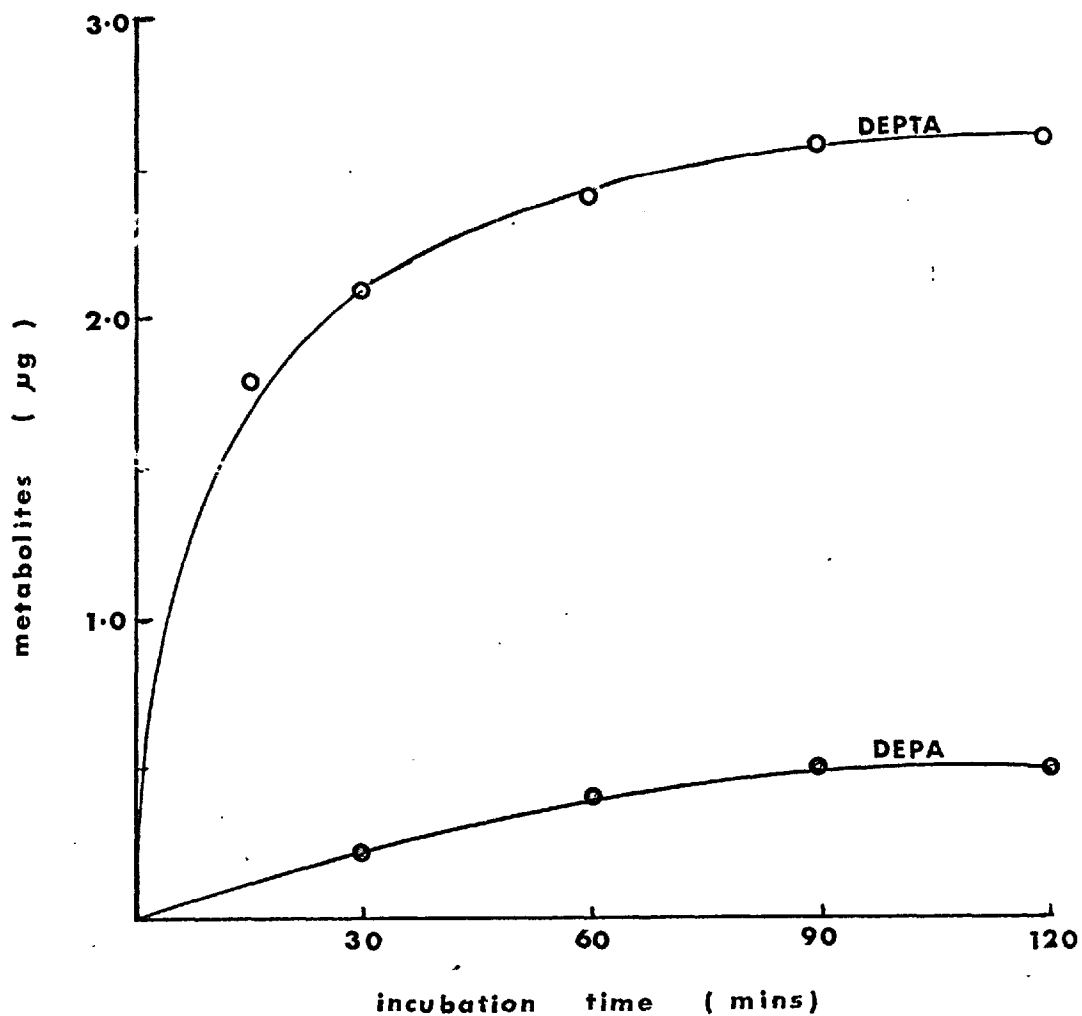


Figure 9 Metabolism of diazinon with time (Data from Table 20)

the levelling off was due either to gradual inactivation of the microsomal enzymes or to exhaustion of the NADPH supply.

(viii) Electrophoresis of 13,000 g supernatant preparations

13,000 g supernatant preparations of all the six strains of housefly were studied by starch gel electrophoresis. Six distinct bands were visible when the gels were incubated in a solution of 1-naphthyl phosphate and "Fast Blue RR". Five of these bands were anionic (Plate 7) and later work (see: Metabolism by soluble fraction, section vii) showed that the cationic band was probably the microsomes. Intra- and interstrain variations in the phosphatase patterns were observed, but these differences could not be related to resistance.

No p-nitrophenol formation was detected when the gels were incubated with parathion, probably due to the deficiencies of the detection methods; p-nitrophenol would be produced only in minute quantities by the concentration of enzyme (less than one fly-equivalent) present in the gels. The gels were also stained with Nigrosine in an attempt to detect interstrain patterns of protein distribution. However, only heavy long black streaks were produced and these obscured any possible interstrain differences.

Ogita (1962), Collins & Forgash (1968) and Menzel, et al. (1963) studied the esterases of resistant and susceptible strains of housefly by acrylamide and starch gel electrophoresis. All these

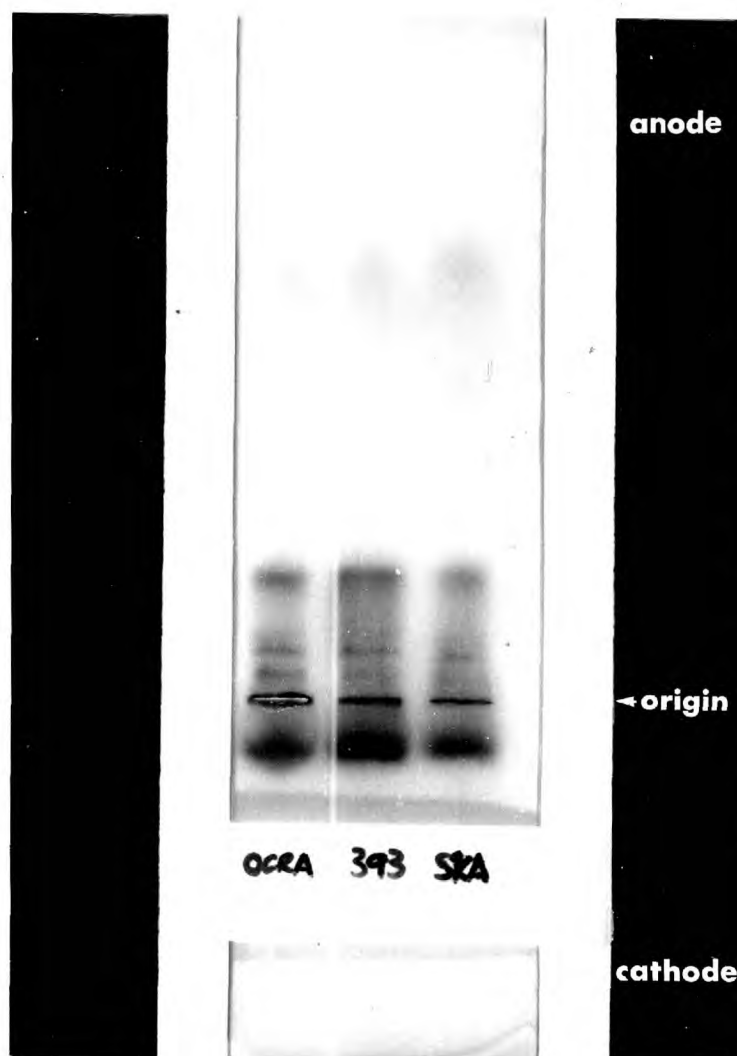


Plate 7

Photograph of starch-gel after staining with 1-naphthyl phosphate and Fast Blue RR, showing one cathodic (at bottom) and four anodic bands.

workers found considerable variation in the patterns of esterases obtained by incubating the gels with 1-naphthyl acetate or 1-naphthyl phosphate, but they could not correlate these differences with resistance.

(c) METABOLISM BY THE SOLUBLE FRACTION

The results in Table 10a, b (page 64) showed that the SKA strain has a further mechanism for metabolising some organophosphorus insecticides. This mechanism, which cleaves diazinon and parathion to DEPTA and diazoxon to DEPA in small quantities, primarily desethylates these insecticides (Figure 6a, b; pages 69,70) in the presence of reduced glutathione (GSH) and is located in the soluble fraction of subcellular preparations of the strains with gene a (chromosome II).

Fukami & Shikado (1966) reported a glutathione-dependent enzyme, present in the 100,000 g supernatant (soluble) fractions of rat liver and cockroach mid-gut, which degraded methyl parathion to the monomethyl (desmethyl) derivative, but only cleaved diazinon and diazoxon to DEPTA and DEPA, respectively.

(i) Requirement for GSH and added NADPH

Tables 13a, b (page 75) showed that 13,000 g supernatant preparations, particularly of strains with gene a, metabolise diazinon and diazoxon to their desethyl derivatives in significant quantities in the presence of GSH, but not with dithiothreitol. The desethyl derivatives were also not formed with L-cysteine.

Parathion metabolism was similar to that of diazinon.

Tables 21a, b show that NADPH is not required for the GSH-dependent desethylation mechanism and that this mechanism is

possibly slightly more active in the absence of NADPH.

Table 21a

Diazinon metabolism by 13,000 g supernatant preparations of
several strains, with GSH, with and without NADPH

Strain	Incubation	Metabolites (µg) formed from diazinon		
		DEPTA	DEPA	Ethylglutathione
<u>ocra</u> SRS.F104	+ NADPH	1.0	none	0.3
	- NADPH	0.6	none	0.2
466.500.F30	+ NADPH	1.6	none	trace
	- NADPH	1.4	none	trace
393.F40	+NADPH	1.3	0.2	0.6
	- NADPH	0.6	trace	0.8
29.F77	+ NADPH	2.1	0.3	0.8
	- NADPH	1.2	0.1	0.8
SKA.A.F53	+ NADPH	5.3	0.6	0.8
	- NADPH	1.3	0.2	1.0
SKA.B.F52	+ NADPH	3.6	0.6	1.3
	- NADPH	1.8	0.2	1.3

Table 21b

Diazoxon metabolism by 13,000 g supernatant preparations of
three strains with GSH, with and without NADPH

Strain	Incubation	Metabolites (µg) formed from diazinon	
		DEPA	Desethyldiazoxon
466.500.A.F52	+ NADPH	0.2	0.3
	- NADPH	0.1	0.3
29.F80	+ NADPH	0.2	0.9
	- NADPH	0.2	1.1
SKA.B.F58	+ NADPH	0.45	1.5
	- NADPH	1.0	3.9

The residual cleavage activity (i.e. DEPTA and DEPA formation) in the absence of NADPH is largely due to a soluble GSH-dependent system (c.f. Table 10a, b).

The metabolising system present in the soluble fraction, unlike the microsomal system, has an absolute requirement for GSH but does not require NADPH.

(ii) Effect of incubation under air or nitrogen

Tables 22a, b show that the GSH-dependent mechanism of metabolism of diazinon and diazoxon is not inhibited by lack of oxygen.

Table 22a

Diazinon metabolism by 13,000 g supernatant preparation of strain 393.F41 with GSH and NADPH incubated under air or nitrogen

Incubation	Metabolites (μ g) formed from diazinon		
	DEPTA	DEPA	Ethylglutathione
air	1.6	0.2	1.0
nitrogen	1.5	0.2	0.8

Table 22b

Diazoxon metabolism by 13,000 g supernatant preparations of three strains with GSH and NADPH, incubated under air or nitrogen

Strain	Incubation	Metabolites (μ g) from diazoxon	
		DEPA	Desethyldiazoxon
466.500.B.F34	air	0.34	0.5
	nitrogen	0.12	0.1
393.F44	air	0.30	1.6
	nitrogen	0.35	1.7
SKA.A.F57	air	0.75	5.0
	nitrogen	0.74	5.6

The GSH-dependent soluble mechanism therefore appears to be totally distinct from the microsomal mechanisms because NADPH and oxygen are not required for maximal activity and only reduced glutathione is.

(iii) Effect of synergists and inhibitors

Because the metabolites formed by the soluble fraction were produced only in the presence of GSH, it was not possible to study the effect of the usual thiol inhibitors, which would have immediately combined with any added GSH. However, iodoethane (at 10^{-4} M final concentration), which alkylates GSH, inhibited 70% of diazinon and parathion metabolism by the soluble fraction of strains SKA and 29, in the presence of GSH.

The effect of the two insecticide synergist, sesamex and TBTP, on the GSH-dependent mechanism was examined (Tables 23a, b, c).

Table 23a

Diazinon metabolism by 13,000 g supernatant preparations of several strains with GSH and NADPH, with and without sesamex and TBTP

Strain	Incubation	Metabolites (µg) formed from diazoxon			% inhibition of ethylglutathione
		DEPTA	DEPA	Ethylglutathione	
393.F51	standard	2.8	0.2	1.2	-
	+ sesamex	1.7	0.2	1.4	*
SKA.A.F63	standard	4.0	0.5	1.8	-
	+ sesamex	3.5	0.4	1.6	11
SKA.B.F62	standard	4.5	1.0	1.8	-
	+ TBTP	3.4	1.0	1.0	48
SKA.B.F61	standard	-	-	2.8	-
	+ TBTP	-	-	1.9	32
29.F108	standard	1.8	0.5	1.1	-
	+ sesamex	1.3	0.2	1.1	0
	+ TBTP	1.9	0.6	0.7	36

* stimulation

Table 23b

Diazoxon metabolism by 13,000 g supernatant and soluble preparations
of various strains with GSH and NADPH, with and without sesamex and TBTP

Strain	Incubation 13,000 g supernatants	Metabolites (µg) formed from diazoxon		% inhibition of desethyl- diazoxon
		DEPA	Desethyldiazoxon	
<u>ac;ar;ocra;bwb</u> SRS.F58	standard + TBTP	0.4 0.3	0.8 0.6	- 25
29.F82	standard + TBTP	0.9 0.5	2.6 1.3	- 50
29.F83	standard + TBTP	0.2 0.36	2.3 1.5	- 35
SKA.B.F58	standard + TBTP	0.45 0.49	1.5 1.3	- 13
SKA.B.F57	standard + TBTP + sesamex	0.5 0.6 0.3	2.7 2.2 3.1	- 19 *
466.500.A.F34	standard + TBTP + sesamex	0.4 0.3 0.4	0.8 0.6 0.8	- 25 0
	Soluble fraction			
SKA.B.F57	standard + sesamex + TBTP	0.22 0.16 0.25	3.8 3.6 3.3	- 5 13
SKA.B.F58	standard + TBTP	0.24 0.30	4.1 2.2	- 46

Table 23c

Parathion metabolism by 13,000 g supernatant preparations of several strains with GSH and NADPH, with and without sesamex and TBTP

Strain	Incubation	Metabolites (µg) formed from parathion			% inhibition of ethyl-glutathione
		DEPTA	DEPA	Ethylglutathione	
SKA.B.F69	standard	0.7	0.4	0.40	-
	+ TBTP	0.7	0.4	0.30	25
SKA.B.F71	standard	0.7	0.3	0.40	-
	+ TBTP	0.8	0.3	0.40	0
29.F108	standard	0.5	0.1	0.25	-
	+ sesamex	0.4	0.07	0.25	0
	+ TBTP	0.5	0.1	0.15	40

Table 23 shows that sesamex does not affect the desethylation of diazinon, diazoxon and parathion; lower DEPTA and DEPA formation is probably due to inhibition of the microsomal cleavage mechanisms present in the 13,000 g supernatant preparations.

Table 23a, b, c also shows that TBTP inhibits the GSH-dependent desethylation of diazinon, diazoxon and, less conclusively, parathion.

Sesamex antagonises, and TBTP slightly synergises, diazinon and parathion in the strains with gene a (Table 1; page 22). The lack of effect of sesamex on the desethylation mechanism explains why this compound does not synergise the strains with gene a. Conversely, TBTP inhibits the desethylation mechanism, reducing the detoxifying capability of strains with gene a

and thus causing synergism of these insecticides in these strains.

(iv) Formation of metabolites

Figures 6a, b (page 697^o) show the pathways of metabolism of diazinon, diazoxon and parathion. The principal metabolites of the soluble fraction, in the presence of GSH, were the desethyl derivatives of diazinon, diazoxon (Table 10a, b; page 64) and parathion. Table 24 indicates that these metabolites were found primarily in the strains with gene a.

Table 24

Desethylation of diazinon, parathion and diazoxon by 13,000 g supernatant preparations of various strains, with GSH

Strain	Metabolism (µg) of:		
	diazinon + Ethylglutathione	parathion* Ethylglutathione	diazoxon* Desethyldiazoxon
<u>ocra</u> SRS	0.6	0.2	0.5
<u>ac;ar;ocra;bwb</u> SRS	0.5	0.15	0.5
466.500	0.3	0.15	0.4
29	1.3	0.35	1.9
393	1.6	0.35	1.7
SKA	1.9	0.35	2.1

+ mean value of determinations on at least four different generations

* mean value of determinations on at least three different generations

* mean value of determinations on at least two different generations

In desethylation, the [¹⁴C-ethyl] moiety of diazinon and parathion was transferred to the thiol group of GSH, to give S-ethylglutathione. S-Ethylglutathione could not be detected from

desethylation of diazoxon because of the position of the label. The quantity of desethylation of parathion and diazinon is given by the sum of the activities of the desethyl derivatives and ethylglutathione, or twice the activity of ethylglutathione, that was found, because both ethyl groups were labelled. Thus, from Table 24, the SKA strain desethylated 38% of diazinon doses, 7% of parathion doses and 21% of diazoxon doses.

Desethyl/diazoxon was detected as a metabolite when diazinon was incubated with 13,000 g supernatant preparations of the SKA strain in the presence of GSH.

The soluble fraction also cleaved diazinon and parathion to DEPTA, and diazoxon to DEPA, in small quantities in the presence of GSH (Table 10a, b; page 64). Data on the interstrain differences in the cleavage of diazoxon to DEPA are not available. However, Table 25 shows that whilst there are almost no interstrain differences in the cleavage of diazinon to DEPTA, a strain with gene a (strain 29) cleaves significantly more parathion to DEPTA than two strains which are susceptible to parathion.

Table 25

DEPTA formation from diazinon and parathion by soluble fractions with GSH. Values mean of determinations on three different generations

Strain	Metab. (µg) of diazinon DEPTA	Metab. (µg) of parathion DEPTA
<u>ocra</u> SRS	1.3	0.03
466.500	1.3	0.03
29	1.2	0.4

The unidentified parathion metabolite ($R_f = 0.2$ in acetonitrile/water/ammonia) was formed by the soluble, as well as the microsomal, fraction (see Plate 3; page 67).

(v) Formation of metabolites with time

Table 26 and Figure 10 show the effect of varying the time of incubation on the metabolism of diazinon and parathion by the soluble fraction.

Table 26

Ethylglutathione formation from diazinon and parathion by 13,000 g supernatant preparations of strain SKA, with GSH, with varying length of incubation

Incubation Time	Metab. (μ g) from diazinon Ethylglutathione	Metab. (μ g) from parathion Ethylglutathione
30 min	1.4	0.2
60 min	2.0	0.3
90 min	2.6	0.4
120 min	3.1	0.4

The metabolism of diazinon and parathion by the soluble fraction continues for at least two hours, in contrast to the rapid levelling-off effect observed with the microsomes. This agrees with the results of Nakatsugawa, et al. (1969c) for parathion metabolism in the rat.

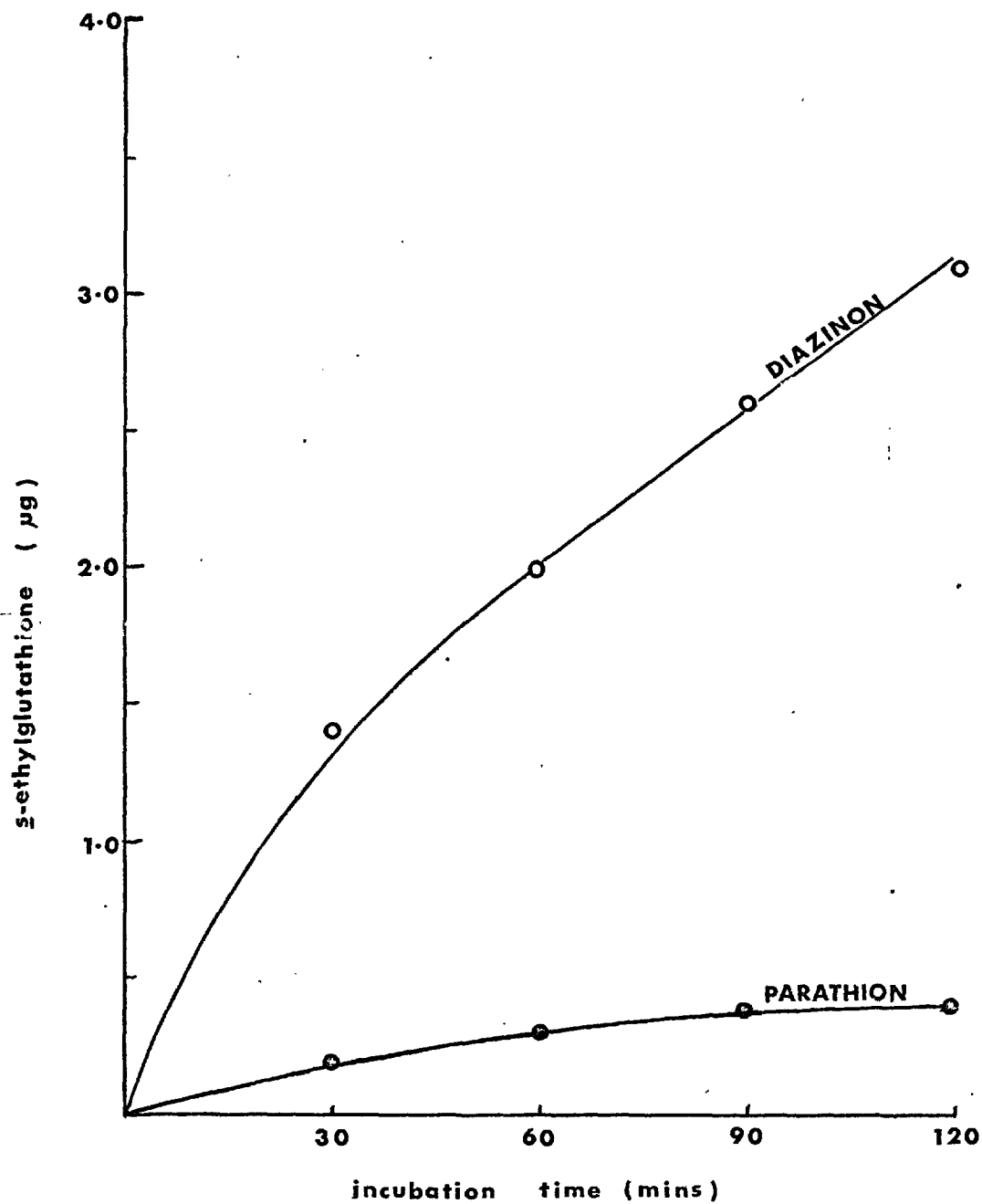


Figure 10 Formation of S-ethylglutathione from diazinon and
parathion, with time (Data from Table 26)

(vi) Investigation of the soluble fraction enzyme

Some work on the enzymology of the soluble enzyme system was begun. Table 10a, b (page 64) showed that a soluble, GSH-dependent system both desethylated and cleaved diazinon, diazoxon and parathion. It was not clear whether these two reactions were carried out by one or two enzymes.

The mutation giving rise to the resistance gene a most probably altered an enzyme already present in susceptible flies. Partial purification of the soluble enzyme(s) was therefore undertaken in an attempt (a) to clarify the constituents of the soluble, GSH-dependent mechanism present in strains with gene a and (b) to detect differences between the soluble enzymes of a susceptible strain (ocra SRS) and the strains with gene a.

Soluble fraction preparations were passed down Sephadex columns and aliquots of the eluates examined for phosphatase activity by incubation with 1-naphthyl phosphate and "Fast Blue RR". Where phosphatase activity was present, further aliquots were incubated with GSH and insecticide. Initially, preparations of the SKA strain were passed down a column of CM-Sephadex C-25, which separates solutes of molecular weight less than 10,000 on the basis of their ionic charges. Diazoxon was desethylated by the fraction eluted immediately after the void volume and this fraction also contained all the phosphatase activity.

Columns of Sephadex G-200, which separates solutes of molecular weight 5,000-800,000 on the basis of their molecular size,

were then tried (Table 27).

Table 27

Diazinon metabolising activity of soluble fraction of SKA strain
eluted from Sephadex G-200

Tube No	Elution Volume	Metabolites (μ g) Formed from diazinon		
		DEPTA	DEFA	Ethylglutathione
4	15-20 ml	none	none	none
5	20-25 ml	0.5	none	1.8
6	25-30 ml	1.5	none	4.0
7	30-35 ml	1.2	none	2.6
8	35-40 ml	none	none	none

Table 27 shows that most of the diazinon-metabolising activity was eluted around tube 6 (25-30 ml eluate). This fraction, which also showed most phosphatase activity, both desethylated and cleaved diazinon, parathion and diazoxon (Plate 8). Added GSH considerably increased both reactions (Plate 8), and NADPH had no effect. These results suggest that, in the strains with gene a, the same soluble enzyme(s) metabolises all three insecticides.

However, starch-gel electrophoresis of both a soluble preparation and the eluate in tube 6 gave two of the anionic phosphatase bands, (~~bands c and e, reported previously~~). These results do not therefore clarify whether the two reactions, desethylation and cleavage, are carried out by one or two enzymes. The absence of the cationic band from electrophoresis of soluble preparations



Plate 8

Autoradiogram of parathion, diazinon and diazoxon metabolism by fraction 6 eluted from Sephadex G-200 column, showing desethylation activity of this fraction.

confirms that this band is microsomal.

When the soluble fraction of strain ocra SRS was passed down the column of Sephadex G-200, the eluate in tube 6 showed much phosphatase activity but almost no desethylating activity. This suggests that the mutation which gave rise to gene a altered a phosphatase enzyme to give the GSH-dependent, desethylating enzyme. The resulting desethylation enzyme is insufficiently different, however, to be separated by the technique employed.

This result also explains why no significant interstrain differences were detected by starch-gel electrophoresis, as the enzymes apparently cleave 1-naphthyl phosphate regardless of their activity towards organophosphorus insecticides.

The enzymes of the soluble fraction can only be studied in more detail when they have been purified further than was achieved in the above experiments.

B) IN VIVO METABOLISM

Diazinon and diazoxon metabolism by living houseflies was studied both by gas-liquid chromatography of unlabelled material and by paper chromatography and autoradiography with radioactively labelled insecticide. Parathion metabolism was studied by paper chromatography and autoradiography of labelled material only.

Gas-liquid chromatography provided limited information because it was more complicated and less accurate, quantitatively and qualitatively, than paper chromatography combined with autoradiography. Paper chromatography of unlabelled compounds was unsuccessful because the spray reagents used failed to detect the small quantities of metabolites formed in vivo from unlabelled material. The bulk of the results described here were, therefore, obtained with labelled insecticides.

(i) Metabolite analysis by gas-liquid chromatography

The extraction procedure which involved homogenising whole flies in petroleum ether (Materials and Methods, page 39) was reasonably efficient in extracting DEPTA, but no other metabolites were detected by gas-liquid chromatography of these extracts. The values obtained for DEPTA formation from diazinon by the various strains generally agreed with the results from the carbon-14 experiments (reported later, Table 30a; page 125). A notable difference was that the quantity of DEPTA detected decreased after 40 min incubation. This was possibly because the DEPTA was too tightly bound to cellular constituents to be removed by petroleum ether and this solvent was also insufficiently polar to extract other metabolites.

The other procedure used (organic extraction of aqueous homogenates; Materials and Methods, page 40) extracted both DEPTA and DEPA. However, no other metabolites were detected by this method which was only about 25% efficient for DEPTA and DEPA extraction. Figure 11 shows a representative gas-liquid chromatograph trace for samples extracted by this method.

(ii) Effect of synergists

The effects of the two synergists, TBTP and sesamex, on the metabolism of diazinon and parathion was examined in an attempt to confirm the in vitro effects of these compounds and to explain the toxicity data shown in Table 1 (page 22).

Pretreatment of houseflies with TBTP had no detectable effect on their metabolism of diazinon and parathion. This was expected because TBTP had little effect on the in vitro metabolism of parathion and diazinon to DEPTA and DEPA, the two principal metabolites found in vivo, and the desethyl derivatives were found in such small quantities in vivo that no significant variation in their formation was detected.

Tables 28a, b show the effect of pretreatment with sesamex on the metabolism of diazinon and parathion in strain 466.500 and in two strains with gene a.

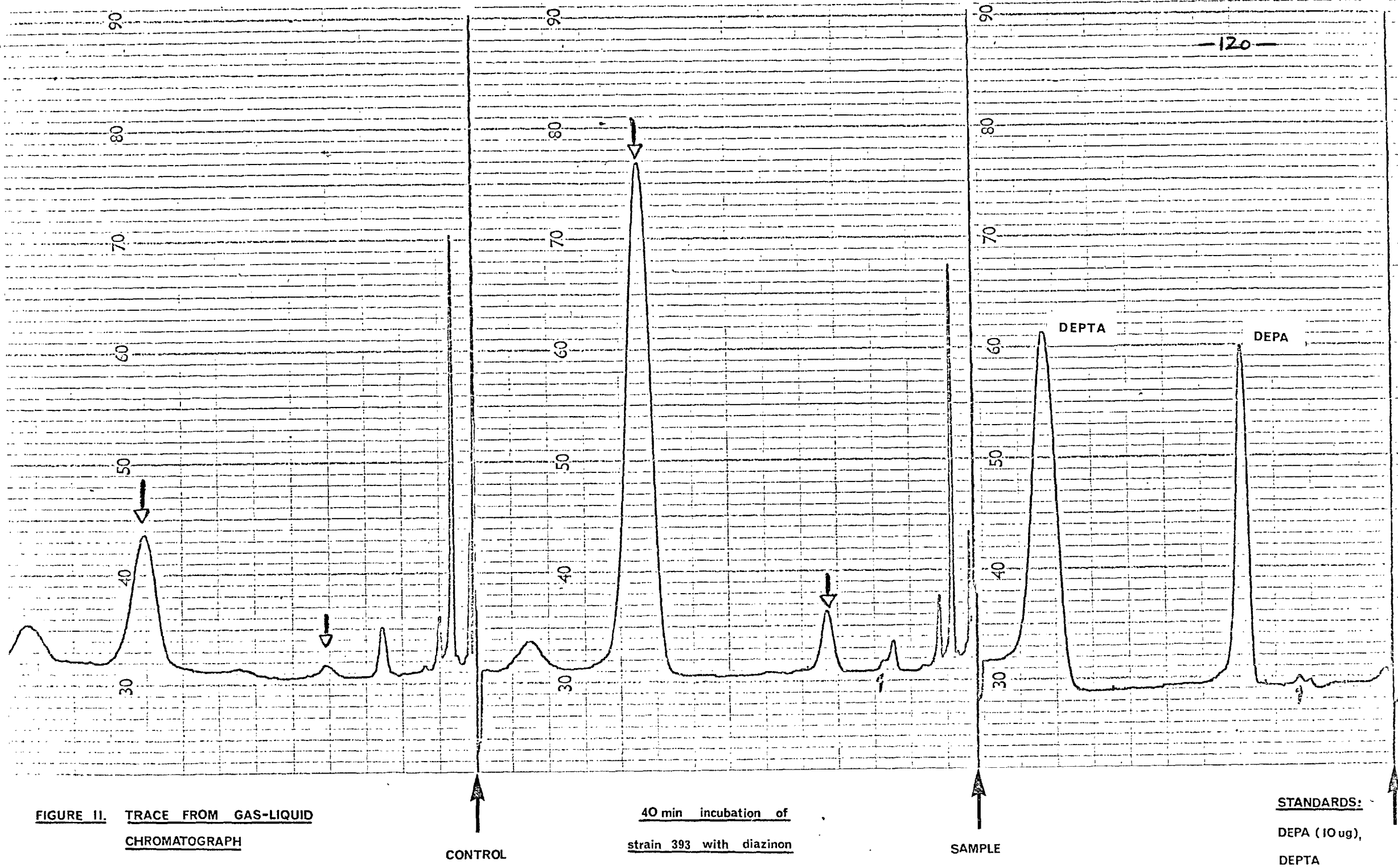


Table 28a

Effect of sesamex on diazinon metabolism by three strains,
incubated for 60 mins

Strain	Incubation	Metabolites (% of dose) of diazinon				
		DEPTA	DEPA	diazoxon	Desethyl- diazinon	Unidentified metabolites
466.500.B.F53	- sesamex	5.2	2.1	trace	-	0.2
	+ sesamex	3.2	1.4	trace	-	0.2
466.500.A.F59	- sesamex	4.6	2.2	-	-	-
	+ sesamex	1.1	-	-	-	-
29.F102	- sesamex	2.0	2.0	-	0.3	-
	+ sesamex	5.1	1.6	-	-	-
393.F69	- sesamex	6.0	2.4	-	-	-
	+ sesamex	1.3	-	-	-	-

Table 28b

Effect of sesamex on parathion metabolism by three strains,
incubated for 60 mins

Strain	Incubation	Metabolites (% of dose) of parathion			
		DEPTA	DEPA	Paraoxon	Desethyl-parathion
466.500.B.F14	- sesamex	1.6	trace	0.3	-
	+ sesamex	0.2	--	-	-
466.500.A.F59	- sesamex	1.1	0.1	0.2	-
	+ sesamex	0.1	-	-	-
29.F108	- sesamex	2.4	0.6	-	0.2
	+ sesamex	0.3	trace	-	trace
393.F69	- sesamex	1.4	0.8	-	0.3
	+ sesamex	0.4	trace	-	0.4

Tables 28a, b show that sesamex reduces all parathion and diazinon metabolism in strains 29, 393 and 466.500. These results contrast with those obtained in vitro and they do not explain why sesamex synergises parathion and diazinon in the strain with the resistance factor on chromosome V and antagonises these insecticides in the strains with gene a (Table 1; page 24), except in Table 28b where sesamex increases the formation of desethylparathion by strain 393. More detailed studies on the effect of sesamex on the rates of formation of the various metabolites are required to explain the mode of action of this synergist, both in vivo and in vitro.

(iii) Formation of metabolites with time

Table 29a and Figure 12 show the effect of varying the time of incubation on the metabolism of parathion by strains 29 and 466.500.

Table 29a

Parathion metabolism in strains 29 and 466.500 with varying time of incubation. Values means of determinations on two different generations

Strain	Incubation Time	Metabs. (% of dose) of parathion		
		DEPTA	DEPA	Paraoxon
466.500	30 min	0.4	none	none
	60 min	1.1	none	0.11
	120 min	2.2	none	0.22
29	30 min	0.8	0.3	none
	60 min	1.4	0.5	none
	120 min	3.5	1.1	none

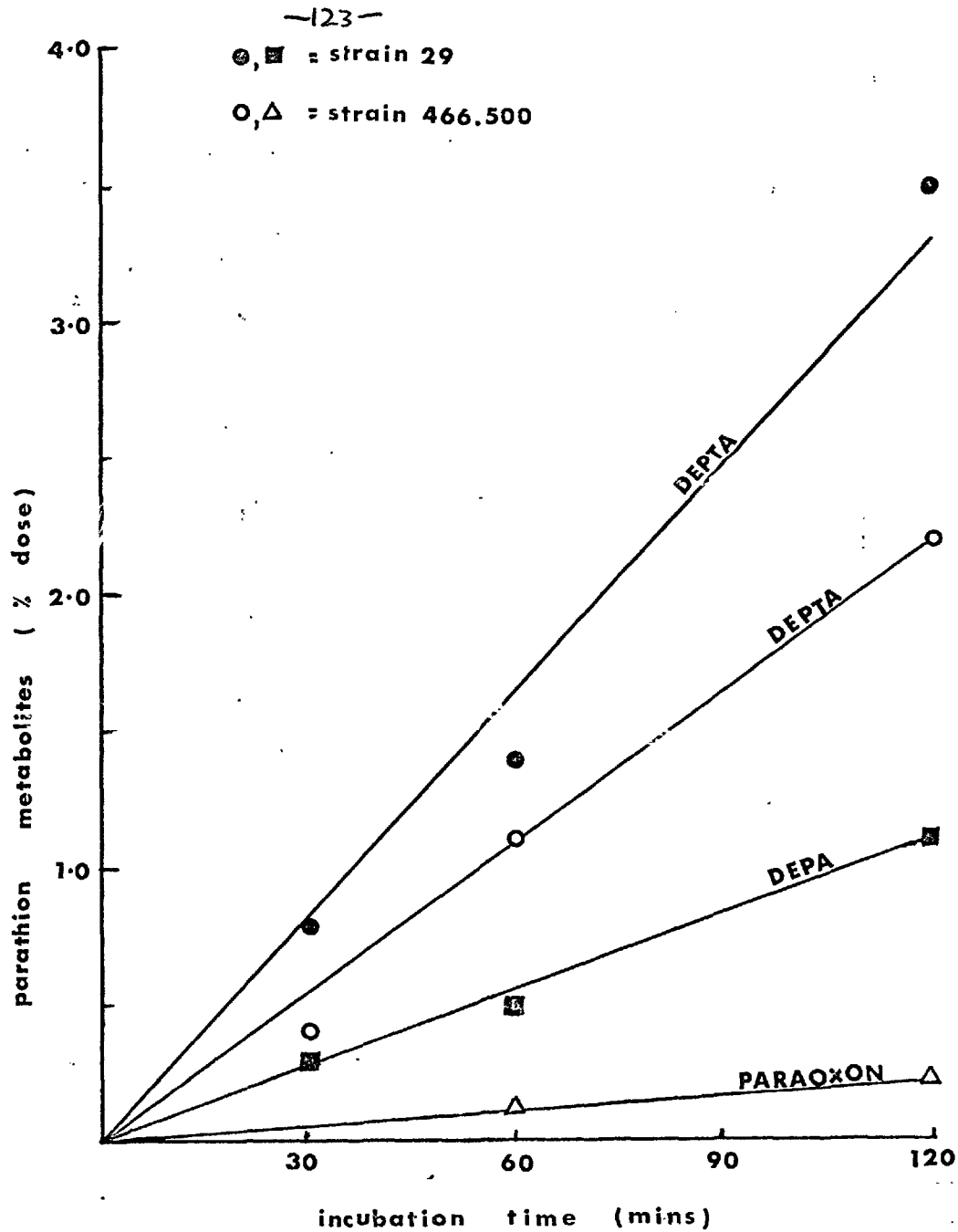


Figure 12 Formation of parathion metabolites, in vivo, by strains 29 and 466.500 (Data from Table 29a)

Strains 466.500 and 29 metabolised parathion at different but almost constant rates up to two hours. The linear rate of formation of DEPTA and DEPA contrasts with the result in vitro in which the rate of production of these metabolites by the microsomal fraction rapidly levelled off (Results and Discussion, page 97). Furthermore, it is noteworthy that since all the flies were knocked down within two hours of dosing with parathion at 1 µg/fly, knockdown does not slow the rate of metabolism.

Extensive data on the effect of time of incubation on diazinon metabolism were not obtained, but Table 29b gives the results of one generation of strains 29 and 466.500 incubated for 60 and 120 minutes.

Table 29b

Diazinon metabolism in strains 29.F101 and 466.500.B.F52 incubated for 60 and 120 mins

Strain	Incubation Time	Metabolites (% of dose) of diazinon*			
		DEPTA	DEPA	Desethyl-diazinon	Unidentified metabolites
29.F101	60 min	5.8	0.9	0.1	-
	120 min	8.8	1.5	0.6	-
466.500.B.F52	60 min	3.6	0.5	-	0.3
	120 min	5.0	0.8	-	0.7

* Diazoxon was not detected in this experiment; both strains are resistant to diazinon

Table 29b shows that the pattern of metabolism of diazinon with time is similar to that of parathion.

(iv) Formation of metabolites

All the metabolites of diazinon, parathion and diazoxon that were found in the in vitro studies (Figures 6a, b; page 697^o) were also detected in vivo, in the appropriate stains (Tables 30a, b).

Table 30a

Metabolism of ^{14}C -diazinon, by four strains, incubated for 60 mins.

Values mean of determinations on at least two different generations

Strain	Metabolites (% of dose) formed from diazinon			
	DEPTA	DEPA	Diazoxon	Desethyl diazinon
<u>ocra</u> SRS	4.4	0.4	0.5	none
466.500	3.6	0.6	none	none*
29	5.8	0.8	none	0.3
SKA	2.6	0.6	none	none

* Unidentified metabolite (Rf = 0.59, in hexane/acetone) = 0.6% of dose

Table 30b

Metabolism of ^{14}C -parathion by four strains, incubated for 60 mins

Strain	Metabolites (% of dose) formed from parathion			
	DEPTA	DEPA	Paraaxon	Desethylparathion
<u>ocra</u> SRS*	0.6	none	0.06	none
466.500 ⁺	1.1	none	0.15	none
29 ⁺	1.6	0.4	none	0.15
SKA*	0.6	0.2	none	none

* Value for determination on one generation

⁺ Mean value for determination on four different generations

Table 30a, b confirms the in vitro result that diazinon is metabolised faster than parathion by all strains and shows that DEPTA, which was detectable within minutes of dosing, was the principal metabolite of both of the thionate insecticides. DEPTA was produced in greatest quantity by strain 29 and it is noteworthy that the strains with gene a produced more DEPA than the other strains, especially after dosing with parathion (Table 30b and Plate 9). The cleavage mechanism forming both DEPTA and DEPA appears, therefore, to be an important component of the detoxication system of strains with gene a.

Table 30a, b also shows that the potent anticholinesterase oxygen analogues, diazoxon and paraoxon, were detected only in the strains that are susceptible to the parent insecticides. Thus strain 466.500 accumulates the oxygen analogue of parathion, to which it is susceptible, but does not accumulate the oxygen analogue of diazinon, to which it is resistant. Diazoxon and paraoxon are prevented from reaching a toxic concentration in the various resistant strains by their rapid detoxication to DEPA, to their desethyl derivatives or to the unidentified metabolites of diazoxon.

The results in Table 30a, b for the SKA strain contrast with those obtained in vitro, in that the level of insecticide metabolism is lower in the SKA strain than in the other strains. This is due to the presence in the SKA strain of the resistance factor

Substrate: ^{14}C -PARATHION, in vivo

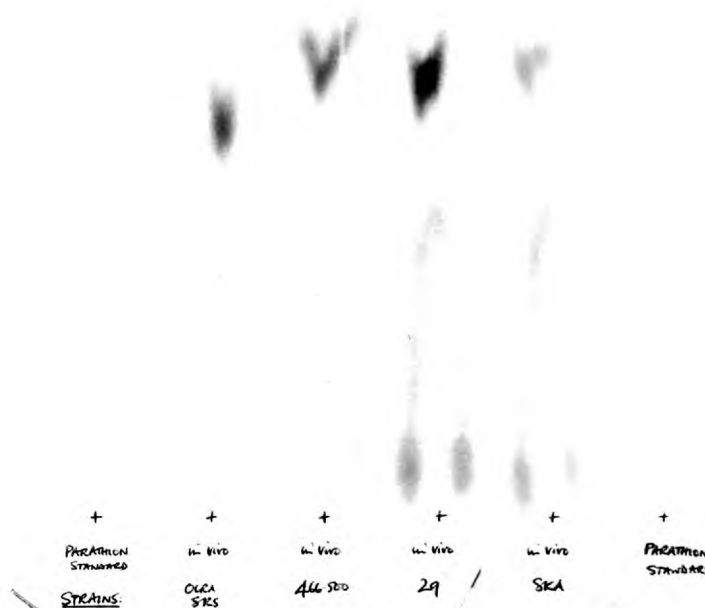


Plate 9

Autoradiogram of parathion metabolism in vivo, showing interstrain pattern of formation of DEPA.

on chromosome III, which reduces the rate of penetration of some insecticides (Sawicki & Farnham, 1969; Sawicki, 1970). Table 31 and Plate 9 show that the spot at the solvent front, after paper chromatography of extracts in acetonitrile/water/ammonia, was much smaller in the SKA strain than in the other strains.

Table 31

Size of the solvent front spots after dosing various strains with diazinon or diazoxon

Strain	Incubation Time	Solvent Front (% of dose)
a) <u>with diazoxon:</u>		
466.500.B.F45	30 min	6.9
SKA.A.F69	30 min	3.3
b) <u>with diazinon:</u>		
ocra SRS.F130	60 min	9.0
466.500.B.F54	60 min	8.1
29.F106	60 min	8.4
SKA.B.F77	60 min	6.6

As the spot at the solvent front consists mainly of unchanged insecticide, the results in Table 31 in conjunction with those in Table 30 confirm that less diazinon penetrates into flies of the SKA strain. Despite the smaller amount of insecticide and DEPTA recovered from the SKA strain, a relatively higher proportion of the diazinon which penetrates is finally metabolised to DEPA in the SKA strain (Tables 30a and 31) than in the other strains; this rapid detoxication of diazoxon might explain the high resistance of the SKA strain. Table 30b and Plate 9 suggest that the same effect

obtains for the high resistance ~~to~~ of the SKA strain to parathion.

Metabolism of diazoxon

Detailed data on the in vivo metabolism of diazoxon were not obtained due to the limited availability of [^{32}P] labelled diazoxon. However, all strains produced DEPA within 10 mins of topical dosing with diazoxon. In vivo, as well as in vitro, the amount of DEPA formed from diazoxon was similar to the quantity formed when diazinon was applied (i.e. 0.3-0.8% of the insecticide dosed).

The interstrain pattern of diazoxon metabolism was similar to that found in vitro. Strains with gene a produced much desethyldiazoxon, which was detected 10 mins after dosing; the SKA strain desethylated 3.6% of a diazoxon dose (1 $\mu\text{g}/\text{fly}$) within 60 mins.

The two strains with the resistance factor on chromosome V metabolised diazoxon to the two unidentified metabolites ($R_f = 0.53$ and 0.69 in acetonitrile/water/ammonia). Again, these metabolites were detected 10 mins after dosing and the SKA strain metabolised 1.0% of a diazoxon dose (1 $\mu\text{g}/\text{fly}$) to these two metabolites within 30 mins.

C) DISCUSSION

The results described for the metabolism of diazinon, diazoxon and parathion in the six strains of housefly studied indicate that, although some metabolic pathways are common to all strains, others occur only in resistant strains and the combination of these pathways in the SKA strain accounts for its high resistance to these organophosphorus insecticides. All the metabolic pathways found in vitro were also detected in vivo in the appropriate strains of housefly, which confirms that they were not artefacts of the subcellular preparations (Hook, et al., 1968). No significant interstrain variation, which could be related to resistance, was detected by starch-gel electrophoresis and this confirms the findings of others (Ogita, 1962; Menzel, et al., 1963; Ogita & Kasai, 1965; van Asperen et al., 1965; Collins & Forgash, 1968).

All six strains of housefly have a microsomal system capable of metabolising diazinon and parathion to DEPTA and of converting the phosphorothionate insecticides to their more potent anti-cholinesterase oxygen analogues, paraoxon and diazoxon (Figure 6a, b; pages 69,70). This microsomal system, which requires NADPH and oxygen for maximal activity, is stimulated by some compounds with reduced sulphur groups and is inhibited by the methylenedioxyphenyl compound sesamex. It therefore appears to be similar to the microsomal mixed-function oxygenase systems reported by others to metabolise insecticides in mammals (Davison, 1954; Shishido & Fukami, 1963; Neal, 1967a, b) and insects (Ray, 1967; Tsukamoto & Casida, 1967a;

Oppenoorth & Houx, 1968; Nakatsugawa & Dahm, 1965; Nakatsugawa et al., 1969a; Hook, et al., 1968). This system, however, is probably not a resistance mechanism as there was no significant interstrain difference in the quantity of diazinon and parathion metabolised to DEPTA by the microsomes, with the exception of the SKA strain, which metabolised more diazinon, and ocra SPS which metabolised less (Table 18, page 88). The reason for the higher in vitro metabolism by the SKA strain is not clear, but may be due to a factor lost in the breeding of the sub-strains. However, resistant strains do convert slightly more of the thionates to their oxygen analogues and cleave these faster to DEPA (Table 19; page 96) and this agrees with the findings of El Bashir & Oppenoorth (1969). It is not clear whether diazoxon is cleaved to DEPA by the microsomal mixed-function oxygenase system because NADPH and oxygen are not required and the mechanism is not significantly inhibited by sesamex. Nakatsugawa, et al. (1969b) reported similar results for diazoxon metabolism by rat liver microsomes.

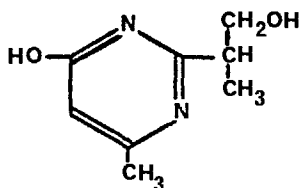
Diazoxon and paraoxon were detected in vivo in susceptible but not in resistant strains of housefly and therefore resistance appears to result from the more efficient prevention of the accumulation of the oxygen analogues. The resistance mechanisms of detoxification of diazinon, diazoxon and parathion will be discussed separately.

Microsomal resistance mechanism

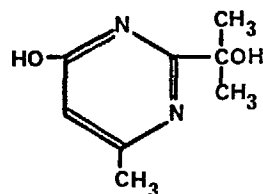
The two strains with the gene, located on chromosome V, which confers resistance to diazinon and diazoxon but not parathion

(strains 466.500 and SKA) (Sawicki & Farnham, 1969) have a microsomal system which metabolises diazoxon, and possibly diazinon, to at least three unidentified metabolites. None of these metabolites were detected in any other strains and neither were analogous metabolites of parathion or paraoxon detected. This microsomal system also requires NADPH and oxygen for maximal activity, is stimulated by some compounds with thiol groups and is inhibited by sesamex (Plate 6). Strains with the gene on chromosome V therefore probably achieve their resistance by rapidly metabolising diazoxon to these unidentified metabolites, thus preventing its accumulation.

Although these three metabolites have not been identified, it is thought that they may be the result of oxidation of the methyl or isopropyl groups of the pyrimidinyl ring, because parathion and diazinon differ principally in the presence or absence of these groups in the ring moieties (see Figure 6a, b; pages 69,70) and the gene on chromosome V confers resistance to diazinon but not parathion (Sawicki & Farnham, 1969). Mucke, et al. (1970) have shown in rats that diazinon is cleaved to DEPTA and the pyrimidinyl moiety, and the isopropyl group of the pyrimidine is then hydroxylated to give both the primary and tertiary alcohol derivatives:



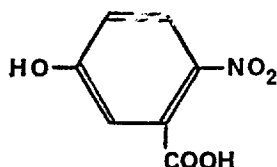
PRIMARY ALCOHOL DERIVATIVE



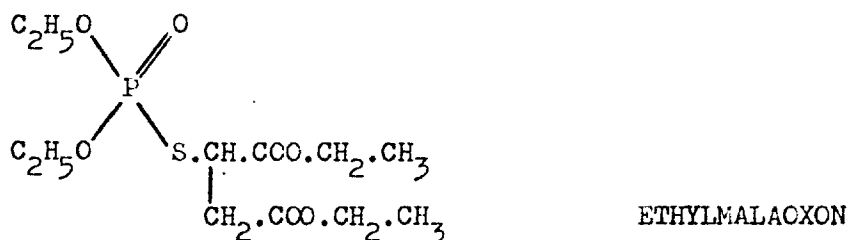
TERTIARY ALCOHOL DERIVATIVE

Hook, et al. (1968) reported a similar example in several mammals in which the methyl group of fenitrothion (folithion; dimethyl,

4-nitro-3-methylphenyl phosphorothionate) is first cleaved to dimethyl phosphorothioic acid and 4-nitro-3-methylphenol and the methyl group of the phenol moiety is then oxidised to carboxylic acid, giving 2-nitro-5-hydroxybenzoic acid:-



Strain 466.500 is also very slightly resistant to ethylmalaoxon (Table 2; page 23) (Sawicki & Farnham, 1969) and this compound also has free alkyl groups remote from the phosphoryl moiety:



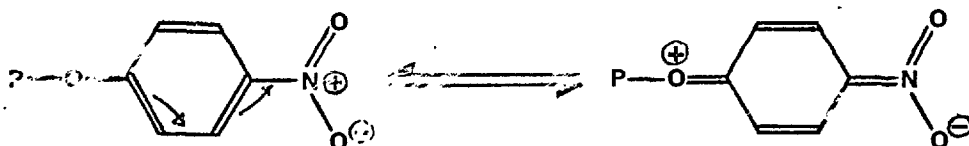
Soluble resistance mechanism

The three strains with gene a, located on chromosome II, (strains 29, 393 and SKA) are resistant to diazinon, diazoxon, parathion and several other organophosphorus insecticides (Table 2; page 23) (Sawicki & Farnham, 1969). These strains have a soluble, glutathione-requiring enzyme system which desethylates diazinon, diazoxon and parathion with the concomitant formation of S-ethylglutathione

(Figure 6a, b; page 6970). This enzyme, which has not previously been reported in houseflies, is similar to that found in mammals and other insects (Hollingworth, 1969; Morello, et al., 1968; Hutson, et al., 1968; Fukami & Shishido, 1966). It is noteworthy that in all these reports the enzyme desmethylated the dimethyl insecticides. Casida (1958) reported that the dimethyl insecticides were desalkylated in preference to the diethyl and that desalkylation was more common in mammals than in insects. It is of interest that, although the diethyl organophosphorus insecticides are more potent anti-cholinesterase agents than the dimethyl (Aldridge, 1953c), strains with gene a are more resistant to diethyl organophosphorus insecticides than dimethyl (Table 2; page 23) (Oppenorth, 1959a; Sawicki & Farnham, 1966). This increased resistance maybe due to the greater specificity of the desalkylation system of these strains for the diethyl compounds rather than the dimethyl. Unfortunately no radioactively labelled dimethyl analogues of diazinon, diazoxon or ~~paraaxon~~^{thion} were available for comparison of the rates of desalkylation by strains with gene a. This enzyme is probably responsible for the considerable cross-resistance of strains with gene a to the dialkyl phosphorothionate insecticides (Table 2; page 23).

The soluble, glutathione-requiring system of the strains with gene a also cleaves diazinon and parathion to DEPTA and diazoxon to DEPA. It is not clear whether these reactions are carried out by one enzyme or two. Both involve hydrolysis of the phosphorus-oxygen bond but, whereas the ethyl group is transferred to the sulphydryl group of glutathione in desethylation, the role of glutathione in cleavage of the ~~pyrimidinyl~~^{aryl}-oxygen-phosphorus bonds is not clear. Diazinon and diazoxon are cleaved predominantly at the ethyl-oxygen-phosphorus position, but both positions are broken to about the same extent in

parathion (Tables 24 and 25; page 109, 110). This may be explained by comparing the structures of diazinon and parathion (Figure 6a, b; page 69, 70). Parathion has an electron withdrawing group ($-\text{NO}_2$) on the ring, which diazinon does not, and thus electrons would be pulled towards the $-\text{NO}_2$ group in parathion (and paraoxon):



resulting in a slight positive charge on the oxygen of the nitro-phenyl-oxygen-phosphorus bond which would increase the likelihood of nucleophilic attack (by $-\text{OH}^-$ groups) at this position, and hence favour the formation of DEPTA (and DEPA) rather than the desethyl derivative.

Table 18 (page 88) shows that no DEPA is found when the 13,000 g supernatant preparations of susceptible strains and strain 466.500 are incubated with diazinon or parathion, but it is found in all strains when diazoxon is the substrate. DEPA was produced only from cleavage of the phosphates and not from DEPTA which confirms the results of Matsumura & Hogendijk (1964) and Nakatsugawa *et al.*, (1969b). It is possible that the DEPA produced on incubation of diazinon and parathion with subcellular preparations of the strains with gene a arises from the soluble, glutathione-dependent cleavage of the oxygen analogues (see Table 10b, page 64 for diazoxon cleavage). These oxygen analogues are produced from the thionates in very small amounts and the quantities present might be below the threshold

concentration required for cleavage by the microsomes, but not below the threshold for the soluble, GSH-dependent mechanism. The data for diazoxon cleavage in Table 19 (page 96) were obtained with a relatively high (10 µg) concentration of diazoxon; interstrain differences might be more pronounced at lower concentrations of diazoxon.

The strains with gene a therefore prevent the accumulation of diazoxon and paraoxon by rapidly metabolising them both to DEPA and to their desethyl derivatives.

It seems probable that the "mutant aliesterase" governed by gene a, (proposed by Oppenoorth and van Asperen (1960)), is in fact the glutathione-requiring desethylation enzyme. Although van Asperen and van Mazijk (1965) reported that the "mutant aliesterase" was a particulate enzyme and the desethylation enzyme is soluble, the soluble enzyme obeys the criteria to be expected of the "mutant aliesterase". It is present in trace quantities in susceptible strains but has greatly increased activity in strains with gene a, (Table 24; page 109) suggesting modification of an enzyme already present in susceptible strains; this increased activity is only found in strains with gene a; and the enzyme is inhibited by TBTP, reputedly an aliesterase inhibitor (Plapp, et al., 1963), whereas the microsomal (particulate) enzymes are not inhibited by TBTP. The low level of phosphatase activity reported by van Asperen & Oppenoorth (1960) would be expected of this system in the absence of glutathione. It was not possible to differentiate between the naphthyl phosphate-hydrolysing fraction of

a susceptible strain and the desethylating fraction of a strain with gene a when these fractions were eluted from Sephadex G-200 columns, which suggests that the enzymes involved are of similar size; this would be expected with enzymes derived from alleles of the same gene (Oppenoorth & van Asperen, 1960). It is not clear whether the greatly increased desethylation activity of the strains with gene a is due to an increase in the activity or in the concentration of the desethylation enzyme.

Action of Synergists

The two synergists examined had opposite effects on the metabolism of diazinon and parathion by the two resistance mechanisms. Sesamex inhibited the microsomal system (Plate 6; page 95) but not the soluble and TBTP inhibited the soluble (Table 23; page 106) but not the microsomal system.

Thus sesamex synergises diazinon and parathion in strains ocra SRS, ac;ar;bwb;ocra SRS and strain 466.500 because these strains have only microsomal detoxification mechanisms. However, as sesamex also inhibits the microsomal activation of diazinon and parathion (Plate 6), it is necessary to postulate a differential inhibition of the microsomal enzymes by sesamex to favour activation. There is not only evidence for different microsomal enzymes for the activation and detoxification processes (Neal, 1967b) but also of differential inhibition of microsomal enzymes by methylenedioxyphenyl synergists (Jaffe, et al., 1969).

Sesamex antagonises diazinon and parathion

(Table 1; page 22) in the strains with gene a alone (strains 29 and 393) by inhibiting the microsomal activation mechanism but not inhibiting the powerful soluble desethylation mechanism. Thus, after pretreatment with sesamex, strains with gene a form less of the oxygen analogues whilst their detoxification capacity is hardly impaired. TBTP slightly synergises diazinon, diazoxon and parathion in both the strains with gene a alone (Table 1; page 22) by inhibiting the soluble mechanism and thus reducing the detoxification capability of these strains. The slight synergism of these insecticides by TBTP in the other strains reflects the low level of desethylation activity that is present in these strains.

The kinetics of TBTP inhibition were not studied, but those of sesamex were. The results obtained suggested that sesamex inhibited diazinon metabolism by the microsomes both competitively and non-competitively and this agrees with the reports of Wilkinson (1968).

The SKA strain

The foregoing Results indicate some reasons why the SKA strain has such a high level of resistance to some organophosphorus insecticides (Table 1, page 22; and El Bashir, 1966). This strain has not only the common microsomal detoxification mechanism but also the two resistance mechanisms located on chromosomes II and V. Together, these mechanisms confer much of the SKA strain's resistance to diazinon (x 80), diazoxon (x 40) and parathion (x 25) (Sawicki, to be published). In addition, the SKA strain has the factor on chromosome

III which reduces the rate of penetration of some insecticides (Sawicki & Farnham, 1969; Sawicki, 1970). This factor probably acts by limiting the quantity of insecticide entering the insect to a level at which the microsomal and soluble systems can detoxify most of the phosphorothionates (to DEPTA, their desethyl derivatives and, with diazinon, to one of the unidentified metabolites). This in turn reduces the quantity of phosphorothionate available for activation to the phosphate and the resulting concentration of phosphate is low enough for rapid and total detoxification (to DEPA, the desethyl derivatives and, with diazoxon, to the two unidentified metabolites). Thus, the detoxification mechanisms, combined with the penetration factor, prevent accumulation of the active anti-cholinesterase agents.

Of the synergists, sesamex slightly synergises diazinon and diazoxon but antagonises parathion, and TBTP has little effect on the SKA strain (Table 1; page 22). Antagonism of parathion by sesamex is explicable because, like strains 29 and 393, the SKA strain has only the soluble detoxication mechanism for resistance to parathion. With diazinon and diazoxon however, the response of the microsomal and soluble mechanisms to sesamex (i.e. synergism and antagonism) almost cancel each other, the balance being slightly towards synergism. Inhibition of one of the mechanisms by one of the synergists would be expected to reduce the level of resistance in a strain with the two detoxification resistance mechanisms, to that of a strain with the unaffected resistance mechanism. That this does not occur in the SKA strain is a measure of the effect of the

penetration factor combined with one detoxification resistance mechanism alone.

CONCLUSIONS

The metabolism of diazinon, diazoxon and parathion by the SKA strain of housefly was compared with that of two susceptible strains and three strains with one gene for resistance inherited from the SKA strain. No diazoxon or paraoxon, the active anti-cholinesterase metabolites of diazinon and parathion, were detected in the resistant strains in vivo or in vitro, in contrast to the susceptible strains.

In the strains of housefly with the resistance gene a located on chromosome II, diazoxon and paraoxon are prevented from accumulating by rapid metabolism, in the presence of reduced glutathione, to diethyl phosphoric acid and to their desethyl derivatives.

In the strains of housefly with the gene for resistance on chromosome V, diazoxon is prevented from accumulating by its rapid metabolism to diethyl phosphoric acid (to a lesser extent than in strains with gene a) and to at least two other unidentified metabolites. Parathion analogues of these unidentified metabolites were not detected so that paraoxon accumulated in strain 466.500, thus explaining the susceptibility of this strain to parathion but not to diazinon or diazoxon.

The SKA strain has both of the detoxification mechanisms associated with chromosomes II and V, as well as the common microsomal mechanism found in all strains. These three detoxification mechanisms confer much of the resistance of the SKA strain and this resistance is

raised still further by the combination of these mechanisms with the gene on chromosome III which lowers the rate of penetration of insecticides and thus reduces the quantity of insecticide inside the insect to a level at which the detoxification mechanisms can rapidly and completely prevent the accumulation of the toxic oxygen analogues.

The effect of the two synergists, sesamex and TBTP, on the toxicity of diazinon, diazoxon and parathion to the six strains of housefly has been discussed.

REFERENCES

- Aldridge, W. N. (1953a) - Serum esterases - Biochem. J., 53, 110
- (1953b) - Serum esterases - Biochem. J., 53, 117
- (1953c) - The inhibition of erythrocyte cholinesterase
by tri-esters of phosphoric acid - Biochem. J., 54, 442
- Anon (1969) - "Principles of plant and insect pest control" - Vol 3,
p. 495, Nat'l. Acad. of Sci., Washington, D.C.
- Asperen, K. van & Mazijk, M. E. van (1965) - Agar gel-electrophoretic
esterase patterns in houseflies - Nature, 205, 1291
- Asperen, K. van, Mazijk, M. E. van & Oppenoorth, F. J. (1965) -
Relation between electrophoretic esterase patterns and
organophosphate resistance in Musca domestica - Ent. exp.
appl., 8, 163
- Asperen, K. van & Oppenoorth, F. J. (1959) - Organophosphate resistance
and esterase activity in houseflies - Ent. exp. appl.,
2, 48
- (1960) - The interaction between
organophosphorus insecticides and esterases in homogenates
of organophosphate susceptible and resistant houseflies -
Ent. exp. appl., 3, 68
- Augustinsson, K. B. (1950) - Acetylcholine esterase and cholinesterase -
in "The Enzymes" (Ed. Summer & Myrback), 1st Edition,
Vol. 1, p. 443 (Academic, New York)
- Babad, H., Herbert, W. & Goldberg, M. C. (1968) - Nuclear Magnetic
Resonance studies of phosphorus pesticides - Anal. Chim.
Acta, 41, 259

- Bashir, E. S. El (1966) - "The effects of chemical selection of resistance patterns and the causes of resistance to insecticides" - Ph.D. Thesis, London University
- Bashir, E. S. El & Oppenoorth, F. J. (1969) - Microsomal oxidations of organophosphate insecticides in some resistant strains of houseflies - *Nature*, 223, 210
- Bigley, W. S. & Plapp, F. W. (1961) - Esterase activity and susceptibility of parathion at different stages in the life cycle of organophosphorus resistant and susceptible house flies - *J. Econ. Ent.*, 54, 904
- Blinn, R. C. & Gunther, F. A. (1955) - Determination of residues of O,O-diethyl-O-(2-isopropyl-6-methyl-4-pyrimidinyl) phosphorothionate in milk - *J. Agric. Fd Chem.*, 3, 1013
- Brown, A. W. A. (1957) - Insecticide-resistance and Darwinism - *Botyu-Kagaku*, 22, 277
- Bruce, W. N. & Decker, G. C. (1950) - Housefly tolerance for insecticides - *Soap*, 26, 122
- Casida, J. E. (1958) - The metabolism of insecticides by insects - *Proc. 4th International Cong. Biochem.* XII, p. 216
- Clark, A. G., Murphy, S. & Smith, J. N. (1969) - The metabolism of hexachlorocyclohexanes and pentachlorocyclohexanes in flies and grass grubs - *Biochem. J.*, 113, 89
- Collins, W. J. & Forgash, A. J. (1968) - Acrylamide gel electrophoresis of housefly esterases - *J. Insect Physiol.*, 14, 1515

- Crow, J. F. (1957) - Genetics of insect resistance to chemicals -
Ann. Rev. Ent., 2, 227
- Cuthbert, J. H. (1963) - Further evidence of resistance to warfarin
in the rat - Nature, 198, 807
- Davison, A. N. (1954) - Conversion of schradan and parathion by an
enzyme system of rat liver - Nature, 174, 1056
- Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1969) -
"Data for Biochemical Research" - 2nd Ed., O.U.P.
- Diggle, W. M. & Gage, J. C. (1951) - Cholinesterase inhibition by
parathion in vivo - Nature, 168, 990
- Dixon, M. & Webb, E. C. (1964) - "Enzymes", 2nd Ed., Longmans, London
- Esaac, E. G. & Casida, J. E. (1969) - Metabolism in relation to mode
of action of methylenedioxyphenyl synergists in houseflies -
J. Agric. Fd Chem., 17, 539
- Farnham, A. W., Lord, K. A. & Sawicki, R. M. (1965) - Study of some
of the mechanisms connected with resistance to diazinon
and diazoxon in a diazinon-resistant strain of houseflies -
J. Insect Physiol., 11, 1475
- Fenwick, M. L. (1958) - Intracellular particles of locust fat body -
Nature, 182, 607
- Fouts, J. R. & Gram, T. E. (1969) - The metabolism of drugs by
subfractions of hepatic microsomes: the case for
microsomal heterogeneity - in "Microsomes and Drug
Oxidations" p. 81, Academic Press

- Forgash, A. J., Cook, B. J. & Riley, R. C. (1962) - Mechanisms of resistance in diazinon-selected multi-resistant Musca domestica - J. econ. Ent., 55, 544
- Foxwell, C. J. (1965) - "The metabolism of S-alkylglutathiones" - Ph.D. Thesis, London University
- Franco, M. G. & Oppenoorth, T. J. (1962) - Genetical experiments on the gene for low aliesterase activity and organo-phosphate resistance in Musca domestica L. - Ent. exp. appl., 2, 119
- Fukami, J. & Shishido, T. (1963) - Studies on the selective toxicities of organic phosphorus insecticides (I) Activation of ethyl parathion in mammal and insect - Botyu-Kagaku, 28, 63
- Fukami, J. & Shishido, T. (1966) - Nature of a soluble glutathione-dependent enzyme system active in cleav^{ing} of methyl parathion to desmethyl parathion - J. econ. Ent., 59, 1338
- Gage, J. C. (1953) - A cholinesterase inhibitor derived from O,O-diethyl O-p-nitrophenyl thiophosphate, in vivo - Biochem. J., 54, 426
- Georghiou, G. P. (1965) - Genetic studies on insecticide resistance - Adv. Pest Control Res., 6, 171
- Gwiazda, M. & Lord, K. A. (1967) - Factors affecting the toxicity of diazinon to Musca domestica L. - Ann. appl. Biol., 59, 221

- Harding, P. J. (1964) - Assaying for biphenyl resistance in Penicillium digitatum in California lemon packing houses - Plant Disease Repr. 48, 43
- Hollingworth, R. M. (1969) - Dealkylation of organophosphorus esters by mouse liver enzymes in vivo and in vitro - J. Agric. Fd Chem., 17, 987
- Hollingworth, R. M., Metcalf, R. L. & Fukuto, T. R. (1967) - Selectivity of sumithion compared with methyl parathion. Metabolism in the white mouse - J. Agric. Fd Chem., 15, 242
- Hook, G. E. k., Jordan, T. W. & Smith, J. N. (1968) - Factors affecting insect microsomal oxidations - in "Enzymatic Oxidations of Toxicants", N. Carolina State University, p. 27
- Hutson, D. H., Pickering, B. A. & Donniger, C. (1968) - Phosphoric acid triester: Glutathione alkyl transferase - Biochem. J., 106, 20P
- Jaffe, H., Fujii, K., Guerin, H., Sengupta, M. & Epstein, S. S. (1969) - Bi-modal effect of piperonyl butoxide on the o- and p-hydroxylations of biphenyl by mouse liver microsomes - Biochem. Pharmacol., 18, 1045
- Keiding, J. (1956) - Resistance to organic phosphorus insecticides of the housefly - Science, 123, 1173
- Kreuger, H. R., O'Brien, R. D. & Dauterman, W. C. (1960) - Relationship between metabolism and differential toxicity in insects and mice of diazinon, dimethoate, parathion and

- acethion - J. econ. Ent., 53, 25
- Lipke, H. & Kearns, C. W. (1959) - DDT dehydrochlorinase - J. Biol. Chem., 234, 2129
- Mahler, H. R. & Cordes, E. H. (1966) - "Biological Chemistry", Harper & Row, New York
- Matsumura, F. & Hogendijk, C. J. (1964) - The enzymatic degradation of parathion in organophosphate - susceptible and resistant houseflies - J. Agric. Fd Chem., 12, 447
- Mengle, D. C. & Casida, J. E. (1960) - Biochemical factors in the acquired resistance of houseflies to organophosphorus insecticides - J. Agric. Fd Chem., 8, 431
- Menzel, D. B., Craig, R. & Hoskins, W. M. (1963) - Electrophoretic properties of esterases from susceptible and resistant strains of the housefly, Musca domestica L. - J. Insect Physiol., 9, 479
- Metcalf, R. L. (1967) - Mode of action of insecticide - synergists - Ann. Rev. Ent., 12, 229
- Metcalf, R. L. (1968) - The role of oxidative reactions in the mode of action of insecticides - in "Enzymatic oxidations of Toxicants" - N. Carolina State Univ., p. 151
- Metcalf, R. L. & March, R. B. (1953) - Further studies on the mode of action of organic thiophosphate insecticides - Ann. Ent. Soc. Amer., 46, 63
- Milani, R. (1967) - "Genetics of insect vectors of disease", Ed. J. W. Wright & R. Pal, Elsevier, p. 315

- Miller, B. (1962) - Ease of displacement of thiol and oxide anions from methyl groups: carbon basicities of anions of oxygen and sulphur - Proc. Chem. Soc., p. 303
- Morello, A., Vardanis, A. & Spencer, E. Y. (1968) - Mechanism of detoxication of some organophosphorus compounds: the role of glutathione-dependent demethylation - Canad. J. Biochem., 46, 885
- Moyed, H. S. (1964) - Biochemical mechanisms of drug resistance - Ann. Rev. Microbiol., 18, 347
- Mucke, W., Alt, K. O. & Esser, H. O. (1970) - Degradation of ¹⁴C-labelled diazinon in the rat - J. Agric. Fd Chem., 18, 208
- Nakatsugawa, T. & Dahm, P. A. (1965) - Parathion activation enzymes in the fat body microsomes of the American cockroach - J. econ. Ent., 58, 500
- Nakatsugawa, T., Tolman, N. M. & Dahm, P. A. (1968) - Degradation and activation of parathion analogs by microsomal enzymes - Biochem. Pharmacol., 17, 1517
-
- (1969a) - Metabolism of S³⁵-parathion in the housefly - J. econ. Ent., 62, 408
-
- (1969b) - Oxidative degradation of diazinon by rat liver microsomes - Biochem. Pharmacol., 18, 685
-
- (1969c) - Degradation of parathion in the rat - Biochem. Pharmacol., 18, 1103

- Neal, R. A. (1967a) - Studies on the metabolism of diethyl-4-nitro-phenyl phosphorothionate (parathion) in vitro - Biochem. J., 103, 183
- (1967b) - Studies on the enzymic mechanism of the metabolism of diethyl 4-nitrophenyl phosphorothionate (parathion) by rat liver microsomes - Biochem. J., 105, 289
- Nguy, V. D. & Busvine, J. R. (1960) - Studies on the genetics of resistance to parathion and malathion in the housefly - Bull. Wld Hlth. Org., 22, 531
- Ogita, Z. (1962) - Genetico-biochemical analysis on the enzyme-activities in the housefly by agar-gel electrophoresis - Jap. J. Genetics, 37, 518
- Ogita, Z. & Kasai, T. (1965) - Genetico-biochemical analysis of specific esterases in Musca domestica - Jap. J. Genetics, 40, 173
- Oppenoorth, F. J. (1958) - A mechanism of resistance to parathion in Musca domestica - Nature, 181, 425
- (1959a) - Resistance patterns of various organo-phosphorus insecticides in some strains of houseflies - Ent. exp. appl., 2, 216
- (1959b) - Genetics of resistance to organophosphorus compounds and low aliesterase activity in the housefly - Ent. exp. appl., 2, 304
- (1965) - Biochemical genetics of insecticide resistance - Ann. Rev. Ent., 10, 185

- Oppenoorth, F. J. & Asperen, K. van (1960) - Allelic genes in the housefly producing modified enzymes that cause organophosphate resistance - Science, 132, 298
- Oppenoorth, F. J. & Houx, N. W. H. (1968) - DDT resistance in the housefly caused by microsomal degradation - Ent. exp. appl., 11, 81
- Parke, D. V. (1968) - "The biochemistry of foreign compounds", Pergamon
- Pinto, R. E. & Bartley, W. (1969) - The effect of age and sex on glutathione reductase and glutathione peroxidase activities and on aerobic glutathione oxidation in rat liver homogenates - Biochem. J., 112, 109
- Plapp, F. W., Bigley, W. S., Chapman, G. A. & Eddy, G. W. (1963) - Synergism of malathion against resistant houseflies and mosquitoes - J. econ. Ent., 56, 643
- Plapp, F. W. & Casida, J. E. (1958a) - Bovine metabolism of organophosphorus insecticides - J. Agric. Fd Chem., 6, 662
- _____ (1958b) - Ion exchange chromatography for hydrolysis products of organophosphate insecticides - Anal. Chem., 30, 1622
- Rall, T. W. & Lehninger, A. L. (1952) - Glutathione reductase of animal tissues - J. Biol. Chem., 194, 119
- Ray, J. W. (1967) - The epoxidation of aldrin by housefly microsomes and its inhibition by carbon monoxide - Biochem. Pharmacol., 16, 99

- Robinson, D., Smith, J. N. & Williams, R. T. (1951) - Studies in detoxication - Biochem. J., 50, 221
- Sacca, G. (1947) - Sull'esistenza di mosche domestiche resistenti al DDT - Riv. di Parassitol., 8, 127
- Sargent, J. R. (undated) - "Methods in Zone Electrophoresis", B.D.H. Publications, Poole
- Sawicki, R. M. (1970) - Interaction between the factor delaying penetration of insecticides and the desethylation mechanism of resistance in organophosphorus-resistant houseflies - Pestiv. Sci., 1, 84
- Sawicki, R. M. & Farnham, A. W. (1964) - A dipping technique for selecting houseflies, Musca domestica L., for resistance to insecticides - Bull. ent. Res., 55, 541
-
- (1967a) - Genetics of resistance to insecticides of the SKA strain of Musca domestica
- I. Location of the main factors responsible for the maintenance of high DDT-resistance in diazinon-selected SKA flies - Ent. exp. appl., 10, 253
-
- (1967b) - Idem II. Isolation of the dominant factors of resistance to diazinon - Ent. exp. appl., 10, 363
-
- (1969) - Examination of the isolated autosomes of the SKA strain of houseflies (Musca domestica L.) for resistance to several insecticides with and without pretreatment with sesamex and TBTP - Bull. Ent. Res., 59, 409

- Sawicki, R. M. & Green (1965) - Changes in the susceptibility of normal and resistant houseflies (Musca domestica L.) to diazinon with age - Bull. Ent. Res., 55, 715
- Sawicki, R. M., Franco, M. G. & Milani, R. (1966) - Genetic analysis of non-recessive factors of resistance to diazinon in the SMA strain of the housefly (Musca domestica L.) - Bull. Wld Hlth Org., 35, 893
- Schonbrod, R. D. & Terriere, L. C. (1966) - Improvements in the methods of preparation and storage of housefly microsomes - J. econ. Ent., 59, 1411
- Shishido, T. & Fukami, J. (1963) - Studies on the selective toxicities of organic phosphorus insecticides (II) The degradation of ethyl parathion, methyl parathion, methyl paraoxon and sumithion in mammal, insect and plant - Botyu-Kagaku, 28, 69
- Siekevitz, P. (1965) - Origin and functional nature of microsomes - Fed. Proc., 24, 1153
- Spencer, E. Y. & O'Brien, R. D. (1957) - Chemistry and mode of action of organophosphorus insecticides - Ann. Rev. Ent., 2, 261
- Sun, Y. P. & Johnson, E. R. (1960) - Synergistic and antagonistic actions of insecticide-synergist combinations and their mode of action - J. Agric. Fd Chem., 8, 261
- Tsukamoto, M. (1964) - Methods for the linkage-group determination of insecticide-resistance factors in the housefly - Botyu-Kagaku, 29, 51

- Tsukamoto, M. & Suzuki, R. (1966) - Genetic analyses of diazinon-resistance in the housefly - Botyu-Kagaku, 31, 1
- Tsukamoto, M. & Casida, J. E. (1967a) - Metabolism of methylcarbamate insecticides by the NADPH₂ requiring enzyme system of houseflies - Nature, 213, 49
- _____ (1967b) - Albumin enhancement of oxidative metabolism of methylcarbamate insecticide chemicals by the housefly microsome - NADPH₂ system - J. econ. Ent., 60, 617
- Wagoner, D. E. (1967) - Linkage group-karyotype correlation in the housefly determined by cytological analysis of X-ray induced translocations - Genetics, Princeton, 57, 729
- Weissman, R. (1947) - Untersuchungen uber das physiologische Verhalten von Musca domestica L. verschiedenen Provenienzen - Mitt. schweiz. entomol. Ges., 20, 484
- W.H.O. (1957) - W.H.O. Expert Committee on Insecticides - W.H.O. Tech. Rept. Ser. 7th Rept., 125
- Wilkinson, C. F. (1968) - Detoxification of pesticides and the mechanism of synergism - in "Enzymatic oxidation of toxicants", N. Carolina State Univ., p. 113
- Wilkinson, C. F. & Hicks, L. J. (1969) - Microsomal metabolism of the 1,3-benzodioxole ring and its possible significance in synergistic action - J. Agric. Fd Chem., 17, 829

APPENDIX

IN VITRO METABOLISM OF ETHYLCHLORTHION

Introduction

The metabolism of ethyl chlorthion was studied in vitro in five of the strains of housefly (Table A1) described previously. This work was done in collaboration with Dr K. A. Lord, although the results described here were those of the author alone. The SKA strain is highly resistant (c. x 500) to ethyl chlorthion and this resistance is conferred by gene a on chromosome II and the penetration factor on chromosome III (Sawicki & Farnham, 1969; Sawicki, 1970). The metabolism of ethyl chlorthion was examined in an attempt to confirm the mode of action of gene a; not only does gene a confer much of the SKA resistance to ethyl chlorthion but this insecticide is also greatly antagonised by sesamex and synergised by TBTP (Table A1), a response that is similar to, but more pronounced than, that found with diazinon and parathion in strains with gene a (see page 22).

Table A1

The toxicity of ethyl chlorthion to five strains of housefly and the effects of synergists (Sawicki & Farnham, 1969)

Strain	Type of Resistance	LD50 (µg/fly)		
		Alone	+ sesamex	+ TBTP
<u>ocra</u> SRS	none (susceptible)	0.067	0.16	0.023
<u>ac;ar;bwb;ocra</u> SRS	none (susceptible)	0.095	0.22	0.
393	gene <u>a</u>	4.6	*	0.28
466.500	sesamex-inhibited	0.081	0.20	0.049
SKA	gene <u>a</u> + penetration + sesamex-inhibited	42.0	*	15.5

* = excessive antagonism

Materials and Methods

[¹⁴C-ethoxy]ethyl chlorthion (0,0-diethyl, 0-3-chloro-4-nitrophenyl phosphorothionate) was synthesised by Dr Lord, using a method adapted from Hilton & O'Brien (1965). [¹⁴C]-ethanol was bought from the Radiochemical Centre, Amersham.

The preparation and incubation of the housefly subcellular fractions and the methods of detection of metabolites were as described previously.

Results

Strains with gene a metabolised ethyl chlorthion to at least six metabolites. Two of these metabolites, DEPTA and DEPA, were produced by the microsomal NADPH and oxygen-requiring system.

DEPTA, S-ethylglutathione and three unidentified metabolites ($R_f = 0.15, 0.2$ and 0.35 in acetonitrile/water/ammonia) were produced by the soluble, GSH-requiring system.

The two susceptible strains and strain 466.500 metabolised ethylchlorthion to DEPTA and DEPA only.

All the metabolites produced by the soluble, GSH-requiring system from strains with gene a were also produced by the same fraction, eluted from the Sephadex G-200 column, which desethylated diazinon, diazoxon and parathion as described previously, TBTP inhibited their formation. However, no desethyl ethyl chlorthion was detected, although the unidentified metabolite with $R_f = 0.2$ could be MEPTA, resulting from the further degradation of desethyl ethyl chlorthion.

The metabolite with $R_f = 0.35$ was distinct from DEPA and was produced in considerable quantity ($3.0 \mu\text{g}$ from a $10 \mu\text{g}$ dose) by the soluble fraction system from strains with gene a. This metabolite was always found associated with traces of ninhydrin-staining material on the chromatograms.

It is noteworthy that ethyl chlorthion, which differs from parathion only in having a chlorine atom attached to the nitro-phenol ring, has a much higher LD_{50} ($42 \mu\text{g}/\text{fly}$) in the SKA strain than parathion ($4.2 \mu\text{g}/\text{fly}$) and this is reflected in the rate of metabolism of the insecticides. A 13,000 g supernatant preparation of the SKA strain metabolised $5.4 \mu\text{g}$ of a $10 \mu\text{g}$ ethyl chlorthion dose in two hours and $1.1 \mu\text{g}$ of a $10 \mu\text{g}$ parathion dose. The values for diazinon

were intermediate (LD50 = 13 µg/fly; 4.1 µg metabolised).

Discussion and Conclusions

The resistance to ethyl chlorthion of the strains with gene a appears to be due to the considerable metabolism of this insecticide by the soluble, GSH-requiring system, which desethylates diazinon, diazoxon and parathion and which is not present in the other strains. TBTP synergises ethyl chlorthion by inhibiting this detoxication mechanism. Sesamex does not inhibit this mechanism and so antagonises ethyl chlorthion by inhibiting its probable activation to ethyl chloroxon, whilst not decreasing its detoxification.

The principal unidentified metabolite of ethyl chlorthion (Rf = 0.35), which is found in conjunction with ninhydrin-staining material, may be a conjugate of glutathione of the type reported by Clark, et al. (1969) for hexachlorocyclohexanes. Attempts to synthesise a conjugate of ethyl chlorthion and glutathione were unsuccessful and the quantities produced enzymically were insufficient for identification.

References

- Clark, A. G., Murphy, S. & Smith, J. N. (1969) - The metabolism of hexachlorocyclohexanes and pentachlorocyclohexanes in flies and grass grubs - Biochem. J., 113, 89
- Hilton, B. D. & O'Brien, R. D. (1965) - Synthesis of H³-ethyl parathion - J. econ. Ent., 58, 1025

Sawicki, R. M. (1970) - Interaction between the factor delaying penetration of insecticides and the desethylation mechanism of resistance in organophosphorus-resistant houseflies - Pestic. Sci., 1, 84

Sawicki, R. M. & Farnham, A. W. (1969) - Examination of the isolated autosomes of the SKA strain of houseflies (Musca domestica L.) for resistance to several insecticides with and without pretreatment with sesamex and TBTP - Bull. Ent. Res., 59, 409

Detoxification of Diazinon by Subcellular Fractions of Diazinon-resistant and Susceptible Houseflies

HOUSEFLIES metabolize dialkyl phosphorothionate insecticides, such as diazinon and parathion, to the corresponding dialkyl phosphorothioic acids^{1,2}. This reaction is thought to be controlled by gene *a*, on chromosome II (ref. 3), associated with low aliesterase activity⁴. The cleavage mechanism is reported to be in the microsomes^{2,5,6}. I have found three detoxification mechanisms in subcellular fractions of six strains (Table 1) of housefly and related these to the genetics of diazinon-resistance^{7,8} of the flies.

Table 1. FACTORS OF DIAZINON-RESISTANCE IN STRAINS OF HOUSEFLY⁷

Strain*	Degree of resistance	Type of resistance	Located on chromosome (ref. 3)	Detoxification mechanism
<i>ocra</i> SRS	1	None (susceptible)	—	A
<i>ac</i> ; <i>ar</i> ; <i>bwb</i> ; <i>ocra</i> SRS	1	None (susceptible)	—	A
29	10-15	Gene <i>a</i> (low aliesterase)	II	A + B
393	10-15	Gene <i>a</i> (low aliesterase)	II	A + B
466-500	10	Sesamex-inhibited	V	A + C
SKA	400	Gene <i>a</i>	II	A + B + C
		and penetration factor	III	
		and sesamex-inhibited	V	

* Strains 29, 393 and 466-500 were derived from the SKA strain.

The subcellular fractions were prepared by differential centrifugation of whole-fly homogenates in 0.15 M phosphate buffer (pH 7.3). The supernatant fluid from centrifuging at 10,000*g* for 10 min was recentrifuged at 100,000*g* for 60 min, to give a soluble fraction (fluid) and a microsomal fraction (precipitate). Material from ten flies was incubated with insecticide, and any other reactants, in 2 ml. buffer and shaken in air or nitrogen at 30° C. Decomposition products of [¹⁴C-ethoxy]diazinon and [³²P]diazoxon in the reaction mixture were identified by paper chromatography, using two solvent systems: (I) acetonitrile/water/ammonia (40:9:1), and (II) propan-2-ol/ammonia (75:25). Autoradiography was also used. The products were measured by solid scintillation counting of the dissected areas of the chromatograms⁹. Desethyl diazinon (*R_F* 0.85 and 0.95 in solvent systems I and II respectively) and desethyl diazoxon (*R_F* 0.60 and 0.80 in systems I and II respectively) were identified by co-chromatography with materials synthesized by methods described for the preparation of analogous derivatives of other insecticides¹⁰.

The first mechanism (*A*), cleavage of diazinon and diazoxon to diethyl phosphorothioic acid (DEPTA) and diethyl phosphoric acid (DEPA), respectively, occurred entirely in the microsomal fraction and required oxygen. NADPH and reduced glutathione (GSH) separately increased this metabolism five-fold and, together, ten-fold. Sesamex, a methylene dioxyphenoxy type of synergist, inhibited this mechanism, but S,S,S-tributylphosphorothionate (TBTP), a synergist that is an aliesterase inhibitor, did not. All strains used this degradative pathway, which seems to be a common mode of detoxification. Five of the strains metabolized 20 per cent of a 10 μ g diazinon dose to DEPTA, and 3 per cent of a 10 μ g diazoxon dose to DEPA within 60 min. The diazinon selected SKA strain degraded significantly more diazinon and diazoxon than the other strains, by this mechanism. These results contrast with others¹¹ which showed greater quantitative differences between resistant and susceptible strains.

The second mechanism (*B*), desethylation of diazinon and diazoxon, occurred entirely in the soluble fraction of strains with gene *a* and required GSH as cofactor. Monoethyl phosphorothionate and monoethyl phosphate were also detected, indicating further decomposition of the desethyl derivatives of diazinon and diazoxon. NADPH and oxygen were not required for desethylation which was slightly greater without them. Desethylation was unaffected by sesamex but was inhibited by TBTP. Strains with gene *a* desethylated up to 40 per cent of a 10 μ g dose of either diazinon or diazoxon within 2 h in the presence of GSH, whereas only trace quantities of desethyl derivatives were detected with the other strains, indicating that the desethylating mechanism is associated with gene *a*.

The third mechanism (*C*) occurred in the microsomal fraction of the strains with the gene for resistance on chromosome V. Diazoxon, but not diazinon, was degraded to yield two unidentified metabolites and NADPH and oxygen were required. The mechanism was inhibited by sesamex and was unaffected by TBTP.

Strain 466-500 and strain Fc (ref. 12) may have a common resistance mechanism, because both have a gene for resistance on chromosome V, and strain F was a parent of the SKA strain. Strain 466-500 is, however, homozygous, while strain Fc is not (N. W. Houx, personal communication). Both strains are resistant to diazinon, but only strain Fc is resistant to parathion and paraoxon^{7,13}.

It is noteworthy that GSH increased metabolism by the two microsomal pathways (*A* and *C*), as well as being essential for desethylation (*B*).

These results explain some of the effects of sesamex and TBTP on the toxicity of diazinon and diazoxon to different strains of housefly. Detoxification mechanism *A* is inhibited by sesamex and therefore this synergist should increase toxicity for all strains in which mechanism

A is dominant. This is observed with the strain⁷ with the gene for resistance on chromosome V, which has mechanisms *A* and *C*, and with the susceptible strains^{7,8} which have mechanism *A* only.

Sesamex, however, decreases the toxicity of diazinon and diazoxon to strains 29 and 393 (refs. 7 and 8). These strains have gene *a* and use desethylation (mechanism *B*), which is unaffected by sesamex, as a major degradative pathway. TBTP, which inhibits mechanism *B*, slightly increases toxicity, as would be expected, in strains 29 and 393 (refs. 7 and 8). Neither synergist influences the toxicity of diazinon to the polyfactorial SKA strain.

I thank Drs D. H. Calam, K. A. Lord and R. M. Sawicki for their help.

J. B. LEWIS

Rothamsted Experimental Station,
Harpenden,
Hertfordshire.

Received July 28, 1969.

¹ Matsumura, F., and Hogendijk, C. J., *J. Agric. Food Chem.*, **12**, 447 (1964).

² Nakatsugawa, T., Tolman, N. M., and Dahm, P. A., *J. Econ. Ent.*, **62**, 408 (1969).

³ Wagoner, D. E., *Genetics (Princeton)*, **57**, 729 (1967).

⁴ Oppenoorth, F. J., *Ent. Exp. Appl.*, **2**, 304 (1959).

⁵ Nakatsugawa, T., and Dahm, P. A., *Biochem. Pharmacol.*, **17**, 1577 (1968).

⁶ Fukami, J., and Shishido, T., *J. Econ. Ent.*, **59**, 1338 (1966).

⁷ Sawicki, R. M., and Farnham, A. W., *Bull. Ent. Res.* (in the press).

⁸ Sawicki, R. M., and Farnham, A. W., *Ent. Exp. Appl.*, **10**, 363 (1967).

⁹ Gwiazda, M., and Lord, K. A., *Ann. Appl. Biol.*, **59**, 221 (1967).

¹⁰ Hollingworth, R. M., Metcalf, R. L., and Fukuto, T. R., *J. Agric. Fd. Chem.*, **15**, 242 (1967).

¹¹ ElBasheir, S., and Oppenoorth, F. J., *Nature*, **223**, 210 (1969).

¹² Oppenoorth, F. J., and van Asperen, K., *Ent. Exp. Appl.*, **4**, 311 (1961).

¹³ Bell, J. D., *Bull. Ent. Res.*, **58**, 137 (1968).

METABOLISM OF SOME ORGANOPHOSPHORUS INSECTICIDES BY STRAINS OF HOUSEFLY

J. B. Lewis and K. A. Lord
Rothamsted Experimental Station, Harpenden, Herts.

Summary Six strains of housefly were used in a study to relate the genetics of their resistance to organophosphorus compounds to their detoxification of these insecticides. Inconclusive results were obtained with whole flies, but subcellular fractions decomposed diazinon, diazoxon and ethyl chlorthion by several mechanisms. One mechanism, located in the microsomes, was common to all strains. Two other mechanisms, one soluble and another in the microsomes, were associated with resistance factors on chromosomes II and V, respectively. The effect of the synergists sesamex and S,S,S-tributylphosphorotriethionate on these mechanisms partly explains their effect on toxicity to the different strains.

Knowledge of how a substance affects a sensitive mechanism is not enough to predict its biological activity. Thus, organophosphorus insecticides kill by inhibiting cholinesterase but anti-cholinesterase activity is not a reliable guide to toxicity, because toxicity is much affected by other phenomena. These include entry of the poison into the insect (Farnham, A. W. *et al.*, 1965) and metabolism of the poison (Matsumura & Hogendijk, 1964; Nakatsugawa, T. *et al.*, 1969) to compounds of greater or lesser toxicity. When the dynamics of these processes follow the usual courses of biochemical reactions, these will depend on the concentrations of the insecticides and metabolites, which, in turn, are determined by the distribution of these compounds between the several phases of the insect, and some insecticides are strongly sorbed on insect solids, others are not (Gwiazda & Lord, 1967; Lord, 1968).

In addition, the size and shape (i.e. morphology) of the insect affects the dose required to kill, so toxicity can be described only in terms of numerous phenomena, each likely to influence, quantitatively if not qualitatively, others. At present few if any of these phenomena can be described precisely. In an attempt to control and minimise the effects of gross differences in morphology and chemical composition, we restricted our work to houseflies. Using strains of known genetic composition, which differ in their susceptibility to insecticides, we sought and examined differences of metabolism of poisons in attempts to relate the differences to susceptibility.

We are fortunate to have strains of houseflies in which resistance mechanisms have been genetically separated by Sawicki & Farnham (1967, 1969), who also supplied information about the toxicity of compounds and the effects of synergists on toxicity to these strains (Table 1). Diazinon has been used as the standard insecticide, because our first strains of flies were resistant to it. Ethyl chlorthion was used because some strains of flies resisted both it and diazinon, and others only diazinon (Sawicki & Farnham, 1969), suggesting different mechanisms of resistance, one effective against both and one against diazinon alone. This offered the possibility of identifying the mechanisms, in addition to separating them by genetic selection. We made preliminary studies of poisons applied to living flies, but because penetration is slow, only a small proportion

of the insecticide is metabolised. As penetration differences contribute to resistance, an uncertain proportion of the dose of insecticide can be metabolised, making it difficult to evaluate and compare metabolism in different strains. To study the metabolism of standardised amounts of insecticides, we therefore macerated houseflies and used subcellular fractions.

Table 1

The toxicity[†] of diazinon and ethyl chlorthion to strains of houseflies and the effects of synergists

Strain	LD50 µg/fly					
	Ethyl chlorthion			Diazinon		
	Alone	+TBTP	+sesamex	Alone	+TBTP	+sesamex
<u>ocra</u> SRS	0.067	0.023	0.16	0.029	0.014	0.012
<u>ac;ar;bwb;ocra</u> SRS	0.095	0.070	0.22	0.040	0.025	0.024
29	-	-	-	0.62	0.36	0.14
393	4.6	0.28	*	0.50	0.023	0.96
466.500	0.081	0.049	0.20	0.39	0.27	0.025
SKA	42.0	15.5	*	13.0	10.0	6.2

* excessive antagonism - LD50 not determined

[†] from Farnham & Sawicki (1967, 1969)

METHOD AND MATERIALS

Diazinon, diazoxon, and [¹⁴C-ethoxy] diazinon were kindly given by Messrs J. R. Geigy, and [³²P] diazoxon was bought from the Radiochemical Centre, Amersham, Bucks.

Desethyl diazinon and desethyl diazoxon were synthesised by methods described by Hollingworth, R. M. *et al.*, (1967) for the preparation of analogous derivatives of other insecticides, and the identity confirmed by NMR spectroscopy.

Ethyl chlorthion was given by Farbenfabriken Bayer. [¹⁴C-ethoxy] ethyl chlorthion was synthesised by a method adapted from Hilton & O'Brien (1965) using ¹⁴C ethanol purchased from the Radiochemical Centre, Amersham, Bucks. Its identity was confirmed by NMR spectroscopy and co-chromatography with authentic un-labelled material.

Desethyl ethyl chlorthion was prepared by a method analogous to desethyl diazinon, also by reacting one mole of ethanol with one mole of O-3-chloro-4-nitrophenyl dichlorophosphorothionate under conditions analogous to those used for the preparation of labelled ethyl chlorthion. The products co-chromatographed in system III and the results of NMR spectroscopy were consistent with the desired product. Monoethyl phosphorothioic acid (MEPTA) was prepared by condensing ethanol with thiophosphoryl chloride and separating the products on paper using system I. Diethyl phosphorothioic acid (DEPTA) was prepared in the same way as NEPTA, also by alkaline hydrolysis of parathion, and separated by chromatography. Diethyl phosphoric acid (DEPA) was bought (Eastman Kodak).

Sawicki & Farnham (1967, 1969) described the detailed genetics of the strains of houseflies studied (Table 2). The flies were kept at 20°C and fed on ad lib water, sugar and milk until used 4 to 7 days after emergence.

Table 2
Factors of diazinon-resistance in strains of housefly

Strain*	Type of resistance	Located on chromosome	Detoxification mechanism
<u>ocra</u> SRS	None (Susceptible)	-	C+D
<u>ac;ar;bwb;ocra</u> SRS	None (Susceptible)	-	C+D
29	gene <u>a</u> (low aliesterase)	II	A+C+D
393	gene <u>a</u> (low aliesterase)	II	A+C+D
466.500	sesamex-inhibited	V	B+C+D
SKA	gene <u>a</u> + penetration factor + sesamex-inhibited	II III V	A+B+C+D

* strains 29, 393 and 466.500 were derived from the SKA strain (Sawicki & Farnham, 1967, 1969)

1 µl of a solution of insecticide in acetone was applied to the ventral side of the thorax of individual flies, which were then kept at room temperature (20°C) for 1 or 2 hours as necessary and examined for decomposition products of the insecticides.

Batches of flies were ground in an all-glass macerator with hexane (10 flies/ml), to extract the bulk of unchanged insecticide. The solids were then reground with N HCl (4 flies/ml), centrifuged (1,000 g for 10 mins), washed with more N HCl (20 flies/ml) and re-centrifuged. The combined aqueous layers were extracted with 3 portions of 2 ml ether. The aqueous and ether layers were chromatographed and autoradiographed to separate and find the metabolites formed.

A second extraction procedure was also used for comparison. After extraction with hexane, the solids were reground with 80% aqueous acetone (4 flies/ml), centrifuged, washed with acetone (20 flies/ml) and re-centrifuged. The acetone extractions were combined and examined for metabolites.

Subcellular fractions were prepared by differential centrifugation of whole-fly homogenates in phosphate buffer (0.15 M, pH 7.3) containing bovine albumin (2% w/v; British Drug Houses). The supernatant fluid from centrifuging at 10,000 g for 10 mins was recentrifuged at 100,000 g for 60 mins to give a soluble fraction (fluid) and a microsomal fraction (precipitate). Typical reaction mixtures contained material from 10 flies, 2 mg NADPH (Boehringer Corp, London) and 4 mg reduced glutathione (Boehringer Corp, London) in 2 ml buffer and were shaken in air or nitrogen at 30°C. Sesamex (20 µgm), a methylene dioxiphenox type of synergist, and S,S,S-tributylphosphorotriethionate (TBTP) (10 µgm), a synergist that inhibits aliesterase, were added in 10 µl acetone. The reaction was ended by adding cold acetone (3.0 ml), and an aliquot of the supernatant fluid was chromatographed.

Chromatography

Four systems of chromatography were used to separate the insecticides and their metabolites. Two used Whatman No 1 paper with (I) acetonitrile:water: ammonia (40:9:1) and (II) propan2-ol:ammonia (75:25) as solvent. The third (III) used silica impregnated paper (Whatman SG81) and hexane:acetone (4:1) as solvent. The fourth (IV) used Whatman No 1 paper impregnated by dipping in 10% liquid paraffin with acetonitrile:water (3:7) as solvent.

Rf values of materials with solvent I were the same whether Whatman No 1 or SG81 paper was used, and solvent I was sometimes used after solvent III on SG81 paper in 2-way chromatography. Table 3 gives the Rf values of materials in these systems.

Table 3

Chromatography of diazinon, ethyl chlorthion and some possible metabolites

Compound	Rf values in solvent systems			
	I	II	III	IV
diazinon	1.0	1.0	-	0.05
diazoxon	1.0	1.0	-	0.8
desethyl diazinon	0.85	0.95	-	-
desethyl diazoxon	0.60	0.80	-	-
DEPTA	0.65	0.75	0	-
DEPA	0.40	0.70	0	-
ethyl chlorthion	1.0	1.0	0.87	-
ethyl chloroxon	1.0	1.0	0.30	-
desethyl ethyl chlorthion	0.95	0.95	0	-
MEPTA	0.06	-	0	-

A fifth system using Whatman No 1 paper with n-butanol:acetic acid: water (11:4:5) was used to try and separate amino acids and some radioactive products.

Radioactivity present in chromatograms was detected by autoradiography (Ilford Industrial "G" X-ray film) and assessed by solid scintillation counting (Naton 136) of dissected areas of chromatograms.

Phosphorus compounds were detected by exposing the chromatograms to bromine vapour and then spraying with 0.5% ferric chloride in 0.5% HCl in 80% ethanol. Phosphorus compounds showed as white areas against a "pink" background.

Insecticides were detected by spraying with 2% 4-(p-nitrobenzyl) pyridine in acetone, heating to 110°C for 10 mins and then spraying with 10% tetraethyl pentamine in acetone (Watts, 1965).

RESULTS

Metabolism by whole fly (in vivo)

Diazinon is metabolised to diethyl phosphorothioic acid (DEPTA) and diethyl phosphoric acid (DEPA). These metabolites are detected within 10 mins of dosing and their quantity increases with time of incubation.

Significant interstrain differences were not detected, and variation within the same strain was as great as between strains. Flies used more than 9 days after emergence metabolise diazinon less rapidly than younger flies and produce less DEPA.

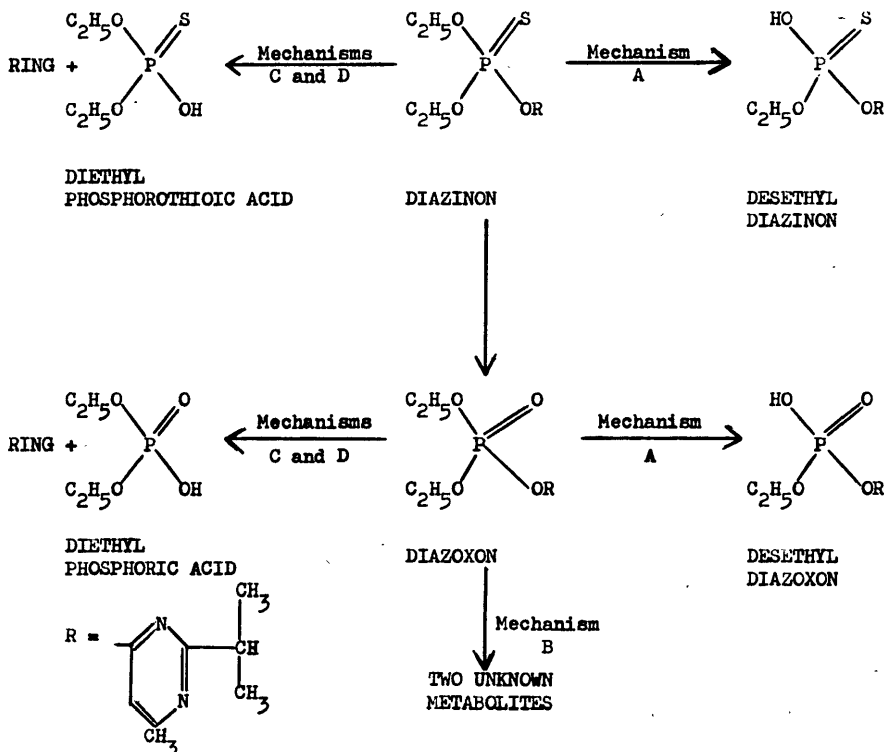
Ethyl chlorthion is metabolised by the SKA strain to several substances that resemble chromatographically those formed when ethyl chlorthion is incubated with insect subcellular fractions.

Metabolism by subcellular fractions (in vitro)

Three mechanisms concerned with detoxification of diazinon and diazoxon were described by Lewis (1969) (Table 2).

Figure 1

Metabolism of diazinon



Mechanism A, desethylation of diazinon and diazoxon, occurs entirely in the soluble fraction and requires reduced glutathione (GSH) as cofactor, but not NADPH or oxygen. This mechanism occurs only in strains with gene a (strains 29, 393 and SKA).

Mechanism B, degradation of diazoxon but not diazinon to two unknown metabolites, is microsomal and requires NADPH and oxygen. It was found only in strains with the sesamex-inhibited factor on chromosome V (strains 466.500 and SKA).

Mechanism C, the cleavage of diazinon and diazoxon to DEPTA and DEPA respectively, also occurs in the microsomes and requires NADPH and oxygen. It occurs in all strains and seems to be a common mode of detoxification. Significant interstrain differences (except for the SKA strain which metabolised slightly faster) were not found in this mechanism, in contrast to the results of El Basheir and Oppenorth (1969).

A fourth mechanism (D), cleavage of diazinon and diazoxon to DEPTA and DEPA respectively, by a soluble, GSH-requiring enzyme, was found. It also occurs in all strains, but is distinct from, and much less powerful than, mechanism C.

As mechanisms C and D occur in all strains, only A and B can be considered resistance mechanisms. Diazinon is activated to diazoxon by the microsomes, and this reaction requires NADPH and oxygen, in all strains.

GSH, in addition to being essential for mechanisms A and D, increases the activity of all the microsomal mechanisms (B, C and diazoxon formation). Two other reduced sulphur compounds, dithiothreitol and cysteine, also increase microsomal metabolism, but desethylation does not occur in their presence. Iodoacetate (3 mM) and p-chloromercuribenzoate (0.05 mM) inhibit microsomal metabolism, thus confirming a role for sulphur.

Microsomal metabolites were formed rapidly initially, but did not increase after 60 mins incubation, whereas the products from the soluble, GSH-requiring mechanisms increased steadily for at least 2 hours, confirming that these two pathways are distinct.

Sesamex inhibits all the microsomal mechanisms (B, C and diazoxon formation), whereas TBTP only inhibits the soluble mechanisms (A and D), which are unaffected by sesamex.

The metabolism of ethyl chlorthion is less clear. Mechanism C, the microsomal cleavage to DEPTA, takes place in quantities similar to that for diazinon.

However, desethylation (mechanism A) is less obvious than with diazinon, although toxicity studies with different strains (Sawicki & Farnham, 1969) suggest that this should be a major pathway of detoxification of ethyl chlorthion in the strains with gene a. Four metabolites are produced by the soluble fraction of gene a strains (29, 393 and SKA) only in the presence of GSH, which suggests a similarity to mechanism A (desethylation of diazinon). One of these metabolites ($R_f = 0.1$ in solvent I) is also found in diazinon metabolism and is probably S-ethyl glutathione. The other three ($R_f = 0.15, 0.2$ and 0.35 in solvent I) have not been identified. The metabolite with $R_f = 0.35$, produced in considerable quantity, is not DEPA, which also occurs. TBTP inhibits the formation of these four metabolites, but sesamex does not.

Preliminary tests suggest that the compound with $R_f = 0.35$ may be an amino acid derivative, because in chromatograms run in system I it is invariably associated with ninhydrin staining material. Two-way chromatograms, run first in system I then system V, did not disprove this hypothesis. It was thought that the compound might be a glutathione derivative analogous to that found with BHC (Clark, A. G. et al., 1969) but when the substance was dissected from a chromatogram and

hydrolysed overnight in 6N HCl at 110° in a sealed tube, chromatograms run in system V had mostly yellow ninhydrin-reacting substances, not corresponding to the constituent amino acids of glutathione.

DISCUSSION

Although our results are incomplete, they suffice to explain in terms of metabolism of poisons some of the toxic effects of diazinon and ethyl chlorthion observed by Sawicki & Farnham (1969). Consider first the toxicity and metabolism of diazinon and diazoxon in relation to the strains of housefly. The detoxication mechanism C is inhibited by sesamex, which increases toxicity not only to the susceptible strains in which this mechanism predominates, but also to the strain that has the sesamex-sensitive mechanism B. Sesamex does not increase the toxicity of diazinon and diazoxon to the strains 29 and 393 which have gene a as sole resistance factor and use desethylation (mechanism A) as a major degradative pathway. TBTP inhibits mechanism A and increases diazinon toxicity to the strains 29 and 393. For reasons not understood, neither synergist greatly affects the toxicity of diazinon to the polyfactorial strain SKA.

There is less information about the metabolism of ethyl chlorthion, but a soluble, glutathione-requiring mechanism (A) is probably common to both diazinon and ethyl chlorthion, although the products of decomposition are not strictly analogous, as relatively less desethyl chlorthion is formed and more of the unidentified metabolite ($R_f = 0.35$ in system II) suspected of being an amino acid derivative. The simplicity of this supposition is attractive, because TBTP increases the toxicity of both ethyl chlorthion and diazinon to the gene a strains (393 and 29).

The diminished toxicity of diazinon in gene a strains, and ethyl chlorthion in all strains, caused by sesamex possibly results from a partial inhibition of the microsomal oxidation to the analogous phosphates, rather than from a modification of detoxification mechanisms.

There remains one major anomaly in the toxicological results; it is the lack of synergism of both diazinon and ethyl chlorthion by either TBTP or sesamex in the polyfactorial SKA strain. This cannot be satisfactorily explained.

Acknowledgements

We thank M. Briley and Mrs R. Humphrey for their assistance.

References

- CLARK, A. G., MURPHY, S. and SMITH, J. N. (1969) *Biochem. J.* 113, 89.
EL BASHEIR, S. and OPPENOORTH, F. J. (1969) *Nature, Lond.* 223, 210.
FARNHAM, A. W., LORD, K. A. and SAWICKI, R. M. (1965) *J. Insect Physiol.* 11, 1475.
GWIAZDA, M. and LORD, K. A. (1967) *Ann. appl. Biol.* 59, 221.
HILLTON, B. D. and O'BRIEN, R. D. (1965) *J. econ. Ent.* 58, 1025.
HOLLINGWORTH, R. M., METCALF, R. L. and FUKUTO, T. R. (1967) *J. Agric. Fd Chem.*, 15, 242.
LEWIS, J. B. (1969) *Nature, Lond.* (in press) 224, 717.
LORD, K. A. (1968) *Soc. Chem. Ind. Monograph* 29, 35.
MATSUMURA, F. and HOGENDIJK, C. J. (1964) *J. Agric. Fd Chem.* 12, 447.
NAKATSUGAWA, T., TOLMAN, N. M. and DAHM, P. A. (1969) *J. econ. Ent.* 62, 408.
SAWICKI, R. M. and FARNHAM, A. W. (1967) *Ent. exp. appl.* 10, 363.
SAWICKI, R. M. and FARNHAM, A. W. (1969) *Bull. ent. Res.* 59, part 3 (in press).
WATTS, R. R. (1965) *J. Assoc. Off. Agric. Chem.* 48, 1161.

Examination of the isolated autosomes of the SKA strain of house-flies (*Musca domestica* L.) for resistance to several insecticides with and without pretreatment with sesamex and TBTP

R. M. SAWICKI and A. W. FARNHAM

Rothamsted Experimental Station, Harpenden, Herts.

Introduction

Resistance to many insecticides has been located in several resistant strains of house-flies (*Musca domestica* L.) (Franco & Oppenoorth, 1962; Hoyer & Plapp, 1966; Milani & Franco, 1959; Oppenoorth & Nasrat, 1966; Plapp & Hoyer, 1967; Ogita & Kasai, 1965; Sawicki *et al.*, 1966; Tsukamoto & Suzuki, 1964, 1966) using genetic marker techniques (Tsukamoto, 1964), but few of the factors of resistance have been isolated (Hoyer *et al.*, 1965; Lichtwardt, 1964; Milani & Franco, 1959; Oppenoorth & van Asperen, 1961; Sawicki & Farnham, 1967*b*, 1968) and fewer still examined to establish cross-tolerance patterns (Bell, 1968*b*; Hoyer & Plapp, 1968; Plapp & Hoyer, 1967, 1968).

This work continues the examination of multiple resistance in diazinon-selected SKA flies (Sawicki *et al.*, 1966; Sawicki & Farnham, 1967*a,b*, 1968). We have now isolated and examined each of the five autosomes of the SKA strain for resistance factors for DDT, methoxychlor, dieldrin and fourteen organophosphate insecticides and tested with the organophosphorus insecticides the effect of pretreatment with TBTP, an ali-esterase inhibitor (Plapp *et al.*, 1963), and sesamex, an inhibitor of microsomal activity (Nakatsugawa & Dahm, 1967).

Materials

House-flies

The strains of house-flies used were as follows.

- (i) SKA of wild phenotype, the result of a cross between two diazinon-resistant strains (203a, Danish × Latina, Italian) and of continuous selection with diazinon (Sawicki & Farnham, 1964).
- (ii) A multi-marker susceptible strain *ac;ar;bwb;ocra* SRS bred from single-marker strains sent by Professor Milani, University of Pavia. The *ar* mutant was reselected by us for susceptibility to DDT.
- (iii) Five triple-marker strains (Table I) derived from the cross SKA × *ac;ar;bwb;ocra* SRS. Each strain had one of its autosomes other than the IV derived from the

TABLE I. Strains of house-flies derived from the cross SKA × *ac;ar;bwb;ocra* SRS

Name or no. of strain	Mutant markers	Origin of autosomes		Resistance gene or factor
		Susceptible	SKA	
338	<i>ar; bwb; ocra</i>	II, III, (IV)*, V	I, (IV)	Slight chlordion resistance?
393	<i>ac; bwb; ocra</i>	I, III, (IV), V	II, (IV)	<i>a</i> (modified ali-esterase)
348	<i>ac; ar; ocra</i>	I, II, (IV), V	III, (IV)	<i>Pen</i> (delayed penetration factor)
DR	<i>ac; ar; bwb; ocra</i>	I, II, III, V	IV	<i>Dld</i> (dieldrin resistance)
466.500	<i>ac; ar; bwb</i>	I, II, III, (IV)	V, (IV)	<i>Ses</i> (sesamex inhibited factor)

*The brackets indicate that the IV linkage group could be derived from either parent or both.

(L 4217)

c

SKA parent. Three autosomes were derived from the susceptible multi-marker parent. The IV autosome was derived from either parent or both, except in the quadruple strain, where it was derived from the SKA strain. The strains retain the number of the single-pair progeny from which they were derived.

The resistance factors isolated were: gene *a*, intermediate for ali-esterase *a* (Oppenoorth, 1959); *Pen*, an incompletely recessive or intermediate gene, which delays the penetration of several insecticides into the insects, previously called *R2* (Sawicki & Farnham, 1968), probably identical to *organotin R* (Plapp & Hoyer, 1968); *Dld*, an incompletely dominant factor of resistance to dieldrin isolated from the SKA strain (Sawicki & Farnham, 1968); *Ses*, an incompletely dominant factor of resistance to DDT and diazinon, previously called *R3* (Sawicki & Farnham, 1967a) and similar in its response to sesamex to the gene *DDTmd* (Oppenoorth & Houx, 1968).

The designation of the physiological mutants follows Georgiou (1965). The numbering of the autosomes is according to Wagoner (1967).

Insecticides

The following insecticides were used:—

diazinon—O,O-diethyl O-2-isopropyl-4-methyl-6-pyrimidinyl phosphorothioate,¹
 diazoxon—diethyl 2-isopropyl-4-methyl-6-pyrimidinyl phosphate,¹
 parathion—O,O-diethyl O-p-nitrophenyl phosphorothioate,²
 paraoxon—diethyl p-nitrophenyl phosphate,²
 methyl-parathion—O,O-dimethyl O-p-nitrophenyl phosphorothioate,²
 methyl-paraoxon—dimethyl p-nitrophenyl phosphate,²
 ethyl-malathion—O,O-diethyl S-(1,2-di(ethoxycarbonyl)ethyl) phosphorodithioate,³
 ethyl-malaoxon—O,O-diethyl S-(1,2-di(ethoxycarbonyl)ethyl) phosphorothioate,³
 malathion—O,O-dimethyl S-(1,2-di(ethoxycarbonyl)ethyl) phosphorodithioate,³
 malaaxon—O,O-dimethyl S-(1,2-di(ethoxycarbonyl)ethyl) phosphorothioate,³
 ethyl-chlorthion—O,O-diethyl O-3-chloro-4-nitrophenyl phosphorothioate,²
 ethyl-chloroxon—diethyl 3-chloro-4-nitrophenyl phosphate,²
 chlorthion—O,O-dimethyl O-3-chloro-4-nitrophenyl phosphorothioate,²
 chloroxon—dimethyl 3-chloro-4-nitrophenyl phosphate,²
 DDT, methoxychlor, dieldrin and tri-butyl tin acetate (technical).

The additives were:—

sesamex—2-(3,4-methylenedioxyphenyl)3,6,9-trioxaundecane,
 TBTP—S,S,S-tributyl phosphorotrithioate.

Methods

The mass-rearing of the insects and the test methods are described elsewhere (Sawicki *et al.*, 1966). Virgin flies were paired when 3–4 days old in single-pair cages, *i.e.*, 140-ml disposable plastic containers with a one-inch circular hole in the bottom covered with gauze, and a gauze-covered top. The cage was placed inside an unmodified container and rested on the “food tray”, a flat inverted plastic cap containing three one-inch-long rolls of cotton wool soaked with milk. The flies fed from, and laid eggs into, the food tray through the gauze. The trays were changed daily, and those containing eggs were transferred into larval medium in plastic containers. More medium was added two days later. On emergence all adults were examined for phenotype, and sexed if required.

The females were treated when 3–7 days old by the topical application to the thorax of measured drops (1.0 μ l) of insecticide dissolved in acetone. The additives, sesamex (2.0 μ g/fly) and TBTP (1.0 μ g/fly), were applied on the tip of the abdomen

^{1,2,3} Kindly presented by J. R. Geigy S.A., Basle, Bayer G.m.b.H., Leverkusen, and Dr. J. E. Boyd, American Cyanamid, Princeton, respectively.

1–5 h before the insecticide. The dead insects were counted 24 h after treatment. Each insecticide was tested at least three times against each strain at four or five doses, with two replicates of 15 flies per dish. The insects pretreated with additives were treated with two doses of insecticide aimed to give 20 and 70% kill. The LD₅₀'s were estimated graphically. All doses are given in μg toxicant/fly. The activity of ali-esterase was determined spectrometrically (van Asperen, 1962) and the amount of insecticide retained in the external rinses of the insects four hours after treatment was determined by gas/liquid chromatography (Sawicki & Farnham, 1968).

Results

Isolation of the autosomes from the SKA strain

The autosomes of the SKA strain were isolated by substituting one of the marked autosomes of the susceptible multi-marker parent by the unmarked autosome of the SKA strain. This made selection with insecticides unnecessary.

The SKA parent, selected with diazinon, was crossed with the *ac;ar;bwb;ocra* SRS strain. Each of the four triple markers and the quadruple marker segregating in the test-cross progeny ♀ *ac;ar;bwb;ocra* SRS × ♂ F₁ (SKA × *ac;ar;bwb;ocra* SRS) was selfed. The four different combinations of triple markers segregating in F₃ were made up in single pairs (30 for each autosome), and only single-pair progenies breeding true for each of the triple-marker combinations were retained for setting up strains. Each of the triple-marker progenies had three marked autosomes derived only from the susceptible parent, the autosome without the fourth marker was derived only from the SKA flies, and the remaining autosome, the fourth unmarked in the susceptible strain, could be inherited from either parent or both. In the quadruple-marker progeny only the fourth autosome unmarked in the susceptible parent was derived from either parent or both. Selection with dieldrin ensured that the fourth autosome was from SKA flies (Sawicki & Farnham, 1968).

Table I shows the strains used for the tests. The second autosome of the SKA strain was isolated twice because the single-pair progeny isolated the first time was contaminated at F₂₁ by stray flies, and the fifth autosome was isolated differently because twice all the viable single-pair progenies were heterozygous for the *ocra* marker and *Ses*. Two of 62 single pairs set up from three fairly fertile heterozygous progenies bred during the second attempt (pairs 466 and 500), bred true for the absence of *ocra*, were identical in their response to diazinon and DDT and showed no loss or change in resistance during six generations. These strains were crossed to improve their poor fertility, and the resulting strain is called 466.500.

Fertility varied widely between the different series of single pairs; it was greatest in the *ar;bwb;ocra* and least in the *ac;ar;bwb* series. Sterile females laid either no eggs or a few batches of sterile eggs. A few F₂ flies were sterile (Table II). Heterozygotes were often more fertile than flies homozygous for the SKA strain's autosome, and in some single-pair progenies, especially those of the *ac;ar;bwb* series, quadruple markers were very rare in F₂ or appeared only in F₃.

TABLE II. *Analysis of the single pairs and their progenies from which the strains used in this work were derived*

Phenotype of single pairs	No. pairs	No. sterile	Heterozygous for fourth marker			Homozygous for SKA autosome	Autosome from SKA
			F ₁	F ₂	F ₃		
<i>ac; bwb; ocra</i>	30	1	19	7	0	3	I
<i>ac; bwb; ocra</i>	70	16	24	25	3	2	II
<i>ac; ar; ocra</i>	30	4	17	6	0	3	III
<i>ac; ar; bwb</i>	132	53	31	35	9*	2	V

*Two pairs sterile at F₂.

Location of the dominant factors of resistance to diazinon

The sixteen phenotypes segregating in the test-cross progeny ♀ *ac;ar;bwb;ocra* SRS × ♂ *F*₁ (*SKA* × *ac;ar;bwb;ocra* SRS) were tested with four doses of diazinon as described by Tsukamoto (1964). Dominant resistance factors were present only in flies unmarked on linkage groups II, III and V, indicating that *SKA* flies carry dominant resistance factors to diazinon on those linkage groups. The factors together gave different degrees of resistance, and the decreasing order of response occurred with flies heterozygous for the following linkage groups: II; III; V > II; V > II; III > III; V > II > V > III. Because the first linkage group carried no dominant factor of resistance, the seven possible combinations of resistance were duplicated and agreement within each pair of results was very good except for factor *Ses* (Table III). This disagreement is almost certainly an experimental error, because the replicates in male flies tested in the same way were very good.

TABLE III. *Response of females of sixteen phenotypes of the test-cross progeny*
♀ *ac;ar;bwb;ocra* SRS × ♂ *F*₁ (*SKA* × *ac;ar;bwb;ocra* SRS)

Phenotype	% insects killed at indicated doses (µg diazinon/♀)				Resistance factors	No. flies of given phenotype*
	2.0	1.0	0.5	0.25		
+++;	17	13	4	0	<i>a;Pen;Ses</i>	90
<i>ac</i> +++;	17	3	10	3	<i>a;Pen;Ses</i>	66
++ <i>ar</i> +++;	100	93	23	3	—; <i>Pen;Ses</i>	73
+++; <i>bwb</i> +	63	20	3	3	<i>a</i> —; <i>Ses</i>	59
+++; <i>ocra</i>	97	50	13	0	<i>a;Pen</i> —	57
<i>ac;ar</i> +++;	100	94	30	10	—; <i>Pen;Ses</i>	31
<i>ac</i> +++; <i>bwb</i> +	60	21	7	4	<i>a</i> —; <i>Ses</i>	27
<i>ac</i> +++; <i>ocra</i>	87	57	7	0	<i>a;Pen</i> —	28
++ <i>ar;bwb</i> +	100	100	48	27	—;—; <i>Ses</i>	24
++ <i>ar</i> +++; <i>ocra</i>	100	100	100	97	—; <i>Pen</i> —	35
+++; <i>bwb;ocra</i>	100	95	45	5	<i>a</i> —;—	29
<i>ac;ar;bwb</i> +	100	100	93	31	—;—; <i>Ses</i>	22
<i>ac;ar</i> +++; <i>ocra</i>	100	100	100	77	—; <i>Pen</i> —	15
<i>ac</i> +++; <i>bwb;ocra</i>	100	96	40	3	<i>a</i> —;—	14
++ <i>ar;bwb;ocra</i>	—	100	100	100	—;—;—	10
<i>ac;ar;bwb;ocra</i>	—	100	100	100	—;—;—	8

*Out of a sample of 568 flies.

Segregation departed very significantly from normality in the progeny of the test-cross ♀ *ac;ar;bwb;ocra* SRS × ♂ *F*₁ (*SKA* × *ac;ar;bwb;ocra* SRS) and flies with most mutant markers were fewest (Table III). The sex ratio was normal.

Response of the strains to insecticides

This section deals with the response of the individual autosomes of the *SKA* strain inbred into the SRS stock. Each of five strains with an *SKA* autosome was tested with several insecticides to locate and measure the resistance of any factor present on the autosome, and the response was compared with that of the susceptible *ac;ar;bwb;ocra* SRS and *SKA* parents. Table IV shows the location of the factors of resistance in the *SKA* strain and Fig. 1 the resistance of this strain to organophosphate insecticides. The properties of each *SKA* autosome are described in turn. All resistance factors with the exception of *Deh* were homozygous.

Parental strains

(a) *ac;ar;bwb;ocra* SRS.—The response of this strain was tested against *ocra* SRS, the most susceptible of the four single-marker SRS strains. The quadruple-marker strain was half to three-quarters as susceptible as the *ocra* SRS strain against most of

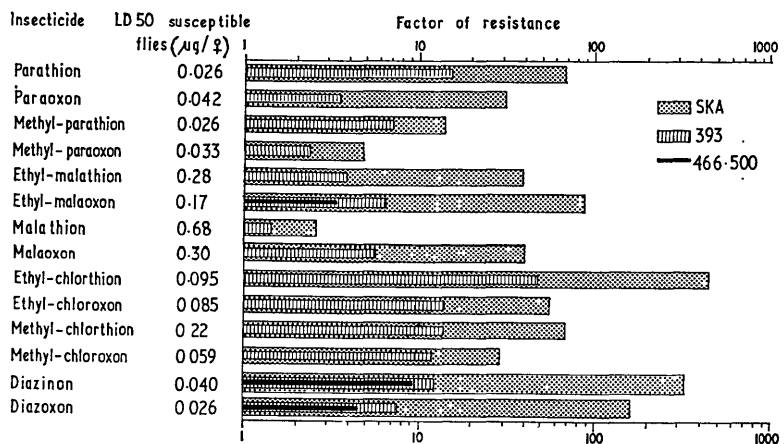


Fig. 1.—Resistance spectra against several organophosphates of the SKA strain and two strains derived from it. Strain 393 has gene *a* on the II autosome, and strain 466.500 the sesamex-inhibited factor of resistance on the V autosome.

the organophosphates tested. It was fully susceptible to DDT and dieldrin and dimethylan, but was about five times more resistant to Zectran than the Cooper strain, our most susceptible strain.

(b) SKA.—The LD50's of SKA flies resembled those reported elsewhere (El Basheir & Sawicki, 1963). The flies were more resistant to ethyl-chlorthion, with which they had never been selected, than to diazinon, with which they had been selected in this laboratory for over 12 years. They were almost susceptible to malathion (resistance factor (R.F.) 2.6); this small resistance could be interpreted wrongly as "vigour tolerance". SKA flies were also more resistant to the ethoxy than the methoxy organophosphates, and, for the malathion analogues, were more resistant to the thioates than the phosphates (Fig. 1). They were very resistant to malaoxon although only slightly resistant to malathion, and were about three times more resistant to ethyl-malaoxon than to the corresponding thioate.

TABLE IV. Location of the resistance factors in SKA

Linkage groups				
I	II gene <i>a</i>	III <i>Pen</i>	IV <i>Dld</i>	V <i>Ses</i>
chlorthion chloroxon <i>c.</i> × 2	parathion × 15 paraaxon × 3.6 methyl-parathion × 7 methyl-paraaxon × 2.4 ethyl-malathion × 4 ethyl-malaoxon × 6.5 malathion × 1.4 malaoxon × 6 ethyl-chlorthion × 48 ethyl-chloroxon × 14 chlorthion × 14 chloroxon × 12 diazinon × 13 diazoxon × 8 <i>Deh</i> DDT × 1000 >	diazinon × 2* chlorthion × 5* dieldrin × 2* DDT × 2* tri-butyl tin acetate × 12 Zectran × ?	dieldrin × 700	diazinon × 9 diazoxon × 4.6 ethyl-malaoxon × 3.3 DDT × 10 methoxychlor × 20

*Measure of delayed penetration, not resistance to kill.

Strains with individual autosomes of the SKA strain

(a) SKA flies autosome I (Strain 338 *ar;bwb;ocra*).—The flies of this strain were susceptible to most of the organophosphates tested, except against malaoxon (R.F. 1.9), chlorthion (R.F. 2.3) and chloroxon (R.F. 1.8). They were also fully susceptible to DDT and dieldrin.

(b) SKA flies autosome II (Strain 393 *ac;bwb;ocra*).—Two major factors of resistance are present on the II autosome of the SKA strain, the gene *a* for low ali-esterase (Oppenoorth & van Asperen, 1961) and the gene *Deh* for high DDT-dehydrochlorinase. Gene *a* conferred resistance to all the organophosphates tested, but resistance was much less than that of SKA flies (Fig. 1). This factor gave two- to three-fold greater resistance against the ethoxy than the methoxy thioates, but this difference almost disappeared with the methoxy and ethoxy phosphates. It was more effective against the thioates than the phosphates except for the malathion analogues. The resistance of strain 29 isolated previously (Sawicki & Farnham, 1967*b*) was identical to those of strains 393 and 270, the latter strain discarded because of contamination, and there is evidence that the greater resistance of strain 52 (Sawicki & Farnham, 1967*b*) was through the interaction of gene *a* and the penetration factor *Pen*, which was heterozygous. All four strains had low ali-esterase.

DDT-dehydrochlorinase was heterozygous in strains 29, 52 and 393, and the response to DDT within each of these strains varied from full susceptibility to complete immunity. However, DDT-dehydrochlorinase did not protect strain 393 against methoxychlor. The resistance against this compound is on the V autosome. Many attempts to isolate DDT-dehydrochlorinase in a homozygous condition from strains 29, 52 and 393 failed. Strain 270 was fully susceptible to DDT. This was one of very few strains with resistance to organophosphate insecticides and full susceptibility to DDT.

(c) SKA flies autosome III (strain 348 *ac;ar;ocra*).—This autosome carries the penetration factor *Pen* which controls the mechanism delaying knockdown and the penetration of several insecticides into the flies (Table V) but has little effect on protecting the insects at death except against tri-butyl tin acetate (R.F. 12). About twice as much diazinon, DDT and dieldrin, and five times more chlorthion, were washed off strain 348 four hours after treatment than from susceptible flies.

TABLE V. Percentages of doses of insecticides recovered four hours after treatment from external washings of strain 348 and susceptible strains lacking *Pen*

Insecticide	% of dose recovered from strains	
	348	Susceptible
chlorthion (0.65 µg/fly)	59	12
diazinon (0.10 µg/fly)	74	38
DDT (0.50 µg/fly)	56	30
dieldrin (1.0 µg/fly)	41	21

Results obtained by gas/liquid chromatography (Sawicki & Farnham, 1968).

Recently Plapp & Hoyer (1968) isolated a similar factor, *organotin R*, which they introduced into the susceptible *stw;w^s* strain. The two genes are probably close alleles because both strain 348 and *tin;stw* and their *F*₁ progeny respond similarly to DDT and diazinon, and the delay in penetration of diazinon and dieldrin are alike (Table VI) and both parents are equally resistant to tri-butyl tin acetate.

Strain 348 responds to organophosphates at death in almost the same way as the susceptible parent, except with diazinon to which it is slightly resistant (R.F. c. 1.5). It is also slightly resistant to dieldrin (Sawicki & Farnham, 1968), DDT and methoxychlor.

TABLE VI. *Percentage of dose of insecticide recovered four hours after treatment from external washings of strains 348, tin;stw and F₁ (tin;stw × 348)*

Insecticide	% of dose recovered		
	348	F ₁ (tin;stw × 348)	tin;stw
dieldrin (0.5 µg/fly)	69	75	72
diazinon (0.1 µg/fly)	48	38	38

This strain also has a factor of resistance, unaffected by sesamex, to the carbamate Zectran. Its resistance has not been measured because of interference by the resistance factor(s) to Zectran inherited from the *ac;ar;bwb;ocra* SRS.

(d) SKA flies autosome IV (Strain DR *ac;ar;bwb;ocra*).—Flies with the SKA autosome IV respond to organophosphates and DDT in the same way as the susceptible parent. The major factor of dieldrin resistance DR of the SKA strain, which is on this linkage group, is described elsewhere (Sawicki & Farnham, 1968).

(e) SKA flies autosome V (Strain 466.500 *ac;ar;bwb*).—The response of the flies with the sesamex-inhibited factor *Ses* on the SKA autosome V varies with different organophosphate insecticides (Fig. 1). *Ses* gives moderate resistance to diazinon (R.F. 9), slight resistance to diazoxon (R.F. 4.5) and ethyl-malaoxon (R.F. 3), and possibly very slight resistance to parathion (R.F. c. 1.5), but none to most of the other organophosphates, and it enhances the susceptibility by 1.5–2 to methyl- and ethyl-paraoxon. This factor also gives slight to moderate resistance to DDT (LD₅₀ 1.9 µg/♀, R.F. 10) and methoxychlor (LD₅₀ c. 12 µg/♀, R.F. 20) which is completely reversed by pretreatment with sesamex (2 µg/fly) but is unaffected by FDMC (bis-(*p*-chlorophenyl)-trifluoromethyl carbinol). This factor, which has been described as giving extremely great resistance against DDT (Oppenoorth, 1965*a*, *b*; Oppenoorth & Houx, 1968) in the Fc strain, is called *DDTmd*. However, as discussed later, the resistance to DDT in Fc is almost certainly caused by the interaction of two or more factors. Gene *DDTmd* increases the microsomal breakdown of DDT c. 15 times (Oppenoorth & Houx, 1968) which is in reasonable agreement with the 10-fold resistance of strain 466.500 to DDT.

Effect of sesamex and TBTP on the response of the different strains to organophosphate insecticides

The determination of the effect of the two additives was less accurate than the estimate of the response to the insecticides alone. In spite of this, the variations between tests done at different times were small.

Only strains 393 (gene *a*), 466.500 (*Ses*) and SKA differed significantly from the others in their response to organophosphates after pretreatment with the additives. The other strains differed little from the susceptible parent and the pooled results are discussed under 'susceptible strains'.

Response after pretreatment with TBTP (Fig. 2–3).—TBTP synergised the thioates only marginally both against susceptible strains and strain 466.500. The phosphates were slightly better synergised (synergistic factor (S.F.) 1.5–3.2). SKA flies also showed little or no synergism with TBTP, except with chlorthion (S.F. 3) and paraoxon (S.F. 2.8), and retained very great resistance. The only consistent and large synergism was with flies of strain 393 (gene *a*) especially with ethyl-chlorthion (S.F. 16) and methyl-parathion (S.F. 8). In this strain TBTP almost eliminated resistance to methyl-parathion, its phosphate and malathion, decreased resistance to seven other compounds to a half or one-quarter, and only slightly decreased resistance to parathion (× 6), chlorthion, diazoxon (× 8) and diazinon (× 10) (Fig. 3).

Response after pretreatment with sesamex (Fig. 2–3).—Pretreatment with sesamex elicited two types of response:—antagonism, when kill after treatment was less than kill by organophosphates alone, and synergism when pretreatment increased kill. Antagonism occurred only with thioates, whereas most phosphates were well synergised.

The general pattern of response to organophosphates after pretreatment with sesamex was similar in all strains (Fig. 2-3). Synergism decreased, or antagonism increased, with the following thioates: malathion, parathion, chlorthion, and the decreasing order of synergism with the phosphates was: malaoxon, paraoxon, chloroxon. The ethyl phosphates were slightly better synergised than the methyl phosphates. Diazinon and diazoxon obeyed the general pattern, but diazinon was considerably more synergised than other thioates in strain 466.500. The strains differed most in the extent to which the individual compounds were synergised or antagonised by sesamex. SKA flies responded to sesamex least, and synergism or antagonism were either small or none. Synergism was greatest in strain 466.500 especially with ethyl-malaoxon (S.F. 52), malaoxon, diazinon and diazoxon; on the other hand, antagonism was greatest in strain 393 where almost all the thioates were antagonised by sesamex, especially methyl-parathion and ethyl-chlorthion which even failed to kill 10% at 16 $\mu\text{g}/\text{fly}$. It is interesting to note that the thioates most synergised by sesamex in strain 466.500 were least antagonised in strain 393, and that the three thioates antagonised by sesamex in strain 466.500 were those most antagonised in strain 393. Unexpectedly large synergism was obtained with the phosphates in the susceptible strains. Also, 466.500 flies pretreated with sesamex were more susceptible to the analogues of parathion and malathion than flies of the 'susceptible strains' but were fully susceptible to diazinon and diazoxon.

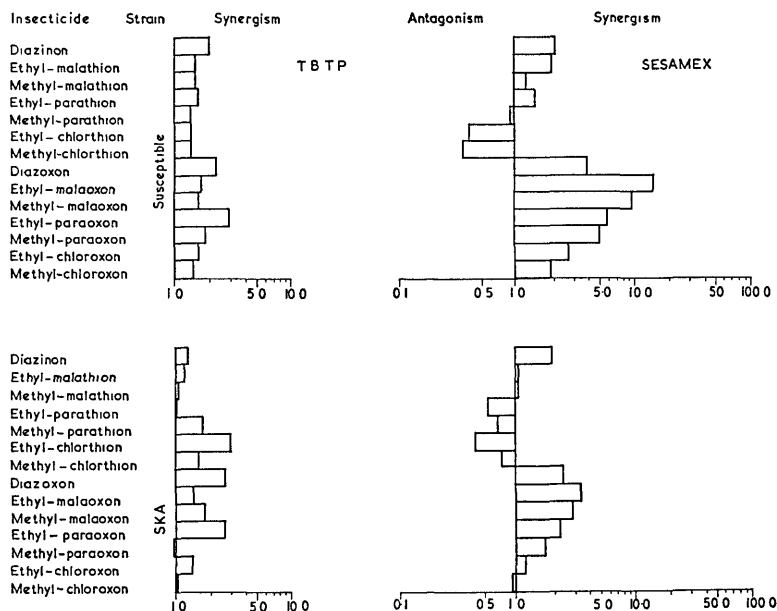


Fig. 2.—Synergism or antagonism of strains SKA and *ac;ar;bwb;ocra* SRS (susceptible) to several organophosphates after pretreatment with TBTP and sesamex. Synergism and antagonism were calculated from ratios of the LD50's of the insects treated with the insecticide alone and with the insecticide plus additive.

Discussion

The SKA strain is the first multi-resistant strain from which each autosome has been isolated and studied in detail. Five factors of resistance were located of which four were isolated in a homozygous condition.

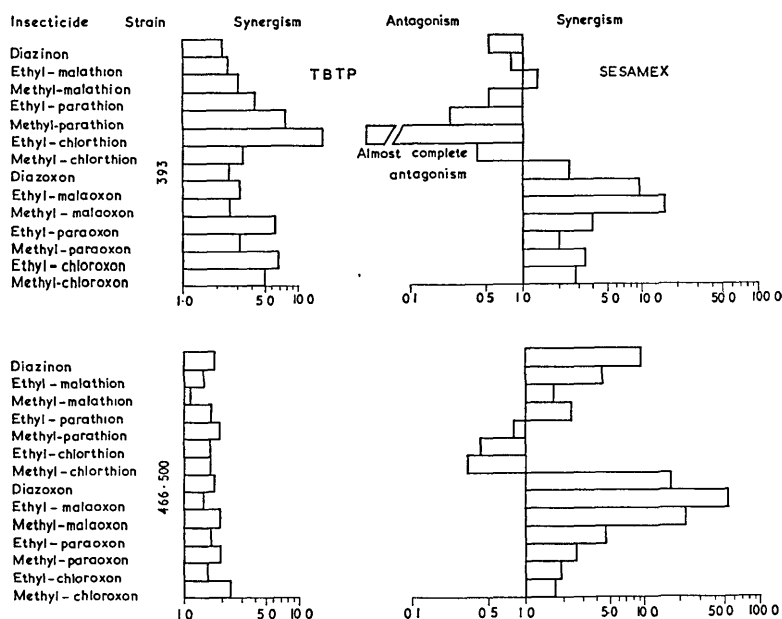


Fig. 3.—Synergism or antagonism of strains 393 and 466.500 to several organophosphates after pretreatment with TBTP and sesamex. For calculation of synergism and antagonism, see Fig. 2.

The major factors of resistance to organophosphates and DDT are on autosomes II and V. One of those, *Ses* (V) and the minor factor *Pen* (III), which controls penetration and acts as a modifier by enhancing the action of the major factors (Sawicki & Farnham, 1969), are common to both groups of insecticides (Sawicki & Farnham, 1968). Dehydrochlorinase controlled by *Deh* on the II autosome is heterozygous in SKA (Sawicki & Farnham, 1967a) and was not isolated by us in a homozygous condition. Because gene *a* is also on the II autosome, flies with the SKA genotype maintain resistance to organophosphates and DDT whether selected with diazinon or DDT. There may also be a minor factor of resistance to chlorthion on the I linkage group, but its function or contribution towards resistance to chlorthion is not known. *Dld*, the factor responsible for dieldrin resistance on the IV autosome, was probably inherited from the parents of SKA, which were treated with chlordane in the field, and has persisted for over 12 years, although the percentage of SKA flies with this gene is now very small (Sawicki & Farnham, 1968) and plays no part in resistance to DDT or the organophosphates.

The factors of resistance found in SKA flies seem to occur in widely separate fly populations. Gene *a* or a similar gene on the II linkage group has been reported in many strains resistant to organophosphates. *Ses* (V) has only been identified so far in Fc, where it is called *DDTmd*, and in SKA; both strains are derived from strain 203a. A factor of resistance to diazinon similar to *Ses* may occur in a Japanese resistant strain (Tsukamoto & Suzuki, 1966). *Pen* (III) occurs both in a European strain (203a) and American strains (Plapp & Hoyer, 1968) and perhaps in Japanese strains (Tsukamoto & Suzuki, 1966). The dieldrin-resistance factor *Dld* is located on the same linkage group as the dieldrin-resistant factor in U2, a Uruguayan strain (Oppenoorth & Nasrat, 1966).

The only factor that conferred resistance to all the organophosphates tested was gene *a*, which is probably the gene controlling low ali-esterase (Oppenoorth, 1959). In

our experience heterozygosity of ali-esterase was always linked with heterozygosity for resistance in all flies having the factor of resistance on the II autosome. The degree of resistance of strain 393 resembles that of the Italian strain (Latina) (Bell, 1968a), which was one of the parents of SKA. Latina differs from 393 in its greater resistance to parathion, but selection with parathion (Busvine, 1959) may be responsible for the two-fold difference in resistance between the strains. Resistance conferred by gene *a* to other strains is also small (Oppenoorth, 1959; Hoyer *et al.*, 1965; Bell, 1968a), and its contribution to the total resistance of SKA flies is variable, viz. it contributes half the resistance to methyl-parathion but only one-twenty-sixth against diazinon (Fig. 1).

The second major factor of resistance to diazinon is *Ses*, the sesamex-inhibited factor on the V linkage group. It is most probably the same as *DDTmd* of strain Fc (Oppenoorth & Houx, 1968) and has been inherited by strains Fc and SKA from the Danish strain 203a. However, the Fc strain differs from strain 466.500 in having factors of resistance which modify its response to DDT and organophosphates. These factors are: *Pen* on the III linkage group (Plapp & Hoyer, 1968), an esterase controlled by a factor on the II linkage group (Bell, 1968b) and intermediate *Deh* (Oppenoorth, 1965b). The last two factors may have been inherited from the susceptible strain, with which strain F (strain 203a) was crossed to remove gene *a*. Strains Fc and 466.500 differ considerably in their resistance spectra both to organophosphates (Bell, 1968a) and DDT (Oppenoorth, 1965a; Oppenoorth & Houx, 1968). The most interesting feature of factor *Ses*, in addition to the ease with which it is inhibited by sesamex, is its specificity. *Ses* by itself gives moderate resistance to DDT, methoxychlor, diazinon, diazoxon, very little to ethyl-malaoxon and none to any other organophosphate tested. It even increases susceptibility to the analogues of paraoxon. In the presence of gene *a*, however, it interacts to give high resistance, even with compounds against which it is inactive by itself, such as ethyl-chlorthion (R.F. 150) (Sawicki & Farnham, 1969).

Although the penetration factor *Pen* does not protect the flies against most insecticides when tested in the laboratory, it may protect insects in the field when they come into contact with small amounts of insecticides. It is primarily an intensifier of the expression of other factors of resistance. For example, when present in homozygous condition with the sesamex-inhibited factor *Ses*, which alone gives $\times 10$ resistance, it makes the flies immune to DDT (Sawicki & Farnham, 1969). Interaction between *Ses* and *Pen* may be responsible for the great resistance of Fc to DDT. *Pen* is non-specific and delays the penetration of most groups of insecticides, but the delay in penetration differs in different insecticides (Table V).

The results of tests with organophosphates after pretreatment with TBTP and sesamex indicate that at least two different mechanisms are involved in the breakdown of organophosphates in all the strains we investigated, and that the differences between the susceptible and resistant strains are mainly qualitative. The two major mechanisms of breakdown are: (i) on the II linkage group a factor which is inhibited or the activity of which is decreased by TBTP, and (ii) detoxication by microsomes on the V linkage group which is inhibited by sesamex (Wilkinson, 1968). Antagonism, almost complete only with ethyl-chlorthion, most probably results from inhibition of the activation of phosphorothioate into phosphate (Sun & Johnson, 1960) and the simultaneous breakdown of thioates, which is most pronounced in strains with low ali-esterase (Sawicki & Farnham, 1967b). In strain 393, which breaks down diazinon into O,O-diethyl phosphorothioate and O,O-diethyl phosphate more than strains with normal ali-esterase (Lewis, 1969), synergism by TBTP, an ali-esterase inhibitor (Plapp *et al.*, 1963), is greatest as is antagonism by sesamex (Fig. 3). However, there is little or no antagonism of malathion because strain 393 has only a parathion-type ali-esterase (Nguy & Busvine, 1960). In strains with normal ali-esterase, significant antagonism occurs only with the chlorthion analogues and slight antagonism with methyl-parathion, whereas the other thioates are synergised by sesamex (Table II). This is probably because those compounds are only slightly affected by the factor on the II linkage group and are mainly broken down by microsomes. Therefore the inhibition by sesamex of microsomal

detoxication, coupled with the incomplete inhibition of activation, results in the synergism of the thioates. Microsomal detoxication of the phosphates must be fast even in the susceptible strain, because these compounds are well synergised by sesamex (Fig. 2). They are even better synergised in strain 466.500, which has the factor that breaks down DDT by microsomes (Oppenoorth & Houx, 1968; Gil *et al.*, 1968), and the synergistic factor of 52 with ethyl-malaoxon is the largest yet reported. However, it is not known why the strain 466.500 resists DDT, diazinon, diazoxon and ethyl-malaoxon, but not the other 11 organophosphorus insecticides tested. It is possible that DDT and the organophosphates are broken down by two closely linked factors on the V autosome in strain 466.500. It is even more puzzling that the two major factors of resistance to organophosphates when together, as in SKA flies, decrease the response to TBTP or sesamex. A similar result was obtained with a strain bred from 466.500 and 393, indicating that the penetration factor is apparently not involved in the lack of response of SKA flies to the additives.

Summary

The factors of resistance to many insecticides were located and isolated in a homozygous condition from the diazinon-selected SKA strain of house-flies (*Musca domestica* L.) by inbreeding each of the five autosomes of the SKA strain into a susceptible multi-marker strain *ac;ar;bwb;ocra* SRS. The insecticides were: DDT, methoxychlor, tri-butyl tin, the methoxy and ethoxy analogues of parathion, chlorthion and malathion and their corresponding phosphates, diazinon and diazoxon.

The SKA flies' autosomes had the following factors of resistance:—autosome I—very slight resistance to ethyl-chlorthion; autosome II—gene *a* for low ali-esterase activity which conferred weak to moderate resistance against all organophosphates tested, and *Deh* (DDT-dehydrochlorinase) which gave great resistance to DDT, but none against methoxychlor; autosome III—*Pen*, which delayed knock-down by slowing down the entry of insecticides into the flies and had a negligible effect at death except against tri-butyl tin and methoxychlor, and a factor of resistance to Zectran unaffected by sesamex; autosome IV—*Dld*, the major factor of resistance to dieldrin; and autosome V—*Ses*, a sesamex-suppressed factor, which gave weak to moderate resistance against ethyl-malaoxon, diazoxon, diazinon, DDT and methoxychlor, but was ineffective against the other organophosphates tested.

TBTP (S,S,S tri-butyl phosphorotrithioate), an ali-esterase inhibitor, greatly synergised organophosphates only against insects with gene *a*. Pretreatment with sesamex, an inhibitor of microsomal activity, elicited two types of response: antagonism with most of the thioates, most pronounced in flies with gene *a*, and synergism with the phosphates, especially evident with flies with factor *Ses*. The possible reasons for differences in the response to organophosphates after pretreatment with TBTP or sesamex, and the nature of the resistance factors are discussed. High resistance in SKA flies against organophosphates arises through the interaction of resistance factors which singly give only weak to moderate resistance.

Acknowledgements

We thank Miss C. White for rearing the insects and Mrs. J. Forrest for the gas/liquid chromatographic determinations.

References

- VAN ASPEREN, K. (1962). A study of housefly esterases by means of a sensitive colorimetric method.—*J. Insect Physiol.* **8**, 401–416.
- BELL, J. D. (1968*a*). Patterns of cross resistance to organophosphates and carbamates in *Musca domestica*.—*Bull. ent. Res.* **58**, 137–151.
- BELL, J. D. (1968*b*). Genetical investigations on a strain of houseflies resistant to organophosphates and carbamates.—*Bull. ent. Res.* **58**, 191–200.

- BUSVINE, J. R. (1959). Patterns of insecticide resistance to organophosphorus compounds in strains of houseflies from various sources.—*Entomologia exp. appl.* **2**, 58–67.
- EL BASHEIR, S. & SAWICKI, R. M. (1964). Cross-resistance of the SKA strain of housefly.—*Rep. Rothamsted exp. Stn* **1963**, 129–130.
- FRANCO, M. G. & OPPENOORTH, F. J. (1962). Genetical experiments on the gene for low ali-esterase activity and organophosphate resistance in *Musca domestica* L.—*Entomologia exp. appl.* **5**, 119–123.
- GEORGHIOU, G. P. (1965). Genetic studies on insecticide resistance.—*Adv. Pest Control Res.* **6**, 171–230.
- GIL, L., FINE, B. C., DINAMARCA, M. L., BALAZS, I., BUSVINE, J. R. & AGOSIN, M. (1968). Biochemical studies on insecticide resistance in *Musca domestica*.—*Entomologia exp. appl.* **11**, 15–29.
- HOYER, R. F. & PLAPP jr., F. W. (1966). A gross genetic analysis of two DDT-resistant housefly strains.—*J. econ. Ent.* **59**, 495–501.
- HOYER, R. F. & PLAPP jr., F. W. (1968). Insecticide resistance in the house fly: identification of a gene that confers resistance to organotin insecticides and acts as an intensifier of parathion resistance.—*J. econ. Ent.* **61**, 1269–1276.
- HOYER, R. F., PLAPP jr., F. W. & ORCHARD, R. D. (1965). Linkage relationships of several insecticide factors in the house fly (*Musca domestica* L.).—*Entomologia exp. appl.* **8**, 65–73.
- LEWIS, J. (1969). Metabolism of organophosphorus insecticides by strains of housefly (*Musca domestica*).—*Rep. Rothamsted exp. Stn* **1968** pt. 1, 171–172.
- LICHTWARDT, E. T. (1964). A mutant linked to the DDT-resistance of an Illinois strain of house flies.—*Entomologia exp. appl.* **7**, 296–309.
- MILANI, R. & FRANCO, M. G. (1959). Compartimento ereditario della resistenza al DDT in incroci tra il ceppo *Orlando-R* e ceppi *Kdr* e *Kdr+* di *Musca domestica* L.—*Symp. genet.* **6**, 269–303.
- NAKATSUGAWA, T. & DAHM, P. A. (1967). Microsomal metabolism of parathion.—*Biochem. Pharmac.* **16**, 25.
- NGUY, V. D. & BUSVINE, J. R. (1960). Studies of the genetics of resistance to parathion and malathion in the housefly.—*Bull. Wld Hlth Org.* **22**, 531–542.
- OGITA, Z. & KASAI, T. (1965). A genetic analysis of synergistic action of sulfonamide derivatives with DDT against houseflies.—*Boryu-Kagaku* **30**, 119–128.
- OPPENORTH, F. J. (1959). Genetics of resistance to organophosphorus compounds and low ali-esterase activity in the housefly.—*Entomologia exp. appl.* **2**, 304–319.
- OPPENORTH, F. J. (1965a). DDT-resistance in the housefly dependent on different mechanisms and the action of synergists.—*Meded. LandbHoogesch. OpzoekStns Gent* **30**, 1390–1396.
- OPPENORTH, F. J. (1965b). Some cases of resistance caused by the alteration of enzymes.—*Proc. XII int. Congr. Ent.* **1964**, 240–242.
- OPPENORTH, F. J. & VAN ASPEREN, K. (1961). The detoxication enzymes causing organophosphate resistance in the housefly; properties, inhibition, and the action of inhibitors as synergists.—*Entomologia exp. appl.* **4**, 311–333.
- OPPENORTH, F. J. & HOUX, N. W. H. (1968). DDT resistance in the housefly caused by microsomal degradation.—*Entomologia exp. appl.* **11**, 81–93.
- OPPENORTH, F. J. & NASRAT, G. E. (1966). Genetics of dieldrin and γ -BHC resistance in the housefly.—*Entomologia exp. appl.* **9**, 223–231.
- PLAPP jr., F. W., BIGLEY, W. S., CHAPMAN, G. A. & EDDY, G. W. (1963). Synergism of malathion against resistant house flies and mosquitoes.—*J. econ. Ent.* **56**, 643–649.
- PLAPP jr., F. W. & HOYER, R. F. (1967). Insecticide resistance in the house fly: resistance spectra and preliminary genetics of resistance in eight strains.—*J. econ. Ent.* **60**, 768–774.
- PLAPP jr., F. W. & HOYER, R. F. (1968). Insecticide resistance in the house fly: decreased rate of absorption as the mechanism of action of a gene that acts as an intensifier of resistance.—*J. econ. Ent.* **61**, 1298–1303.
- SAWICKI, R. M. & FARNHAM, A. W. (1964). A dipping technique for selecting house-flies, *Musca domestica* L., for resistance to insecticides.—*Bull. ent. Res.* **55**, 541–546.
- SAWICKI, R. M. & FARNHAM, A. W. (1967a). Genetics of resistance to insecticides of the SKA strain of *Musca domestica* L. Location of the main factors responsible for the maintenance of high DDT-resistance in diazinon-selected SKA flies.—*Entomologia exp. appl.* **10**, 253–262.

- SAWICKI, R. M. & FARNHAM, A. W. (1967*b*). *Idem*. II. Isolation of the dominant factors of resistance to diazinon.—*Entomologia exp. appl.* **10**, 363–376.
- SAWICKI, R. M. & FARNHAM, A. W. (1968). *Idem*. III. Location and isolation of the factors of resistance to dieldrin.—*Entomologia exp. appl.* **11**, 133–142.
- SAWICKI, R. M. & FARNHAM, A. W. (1969). Interaction between the factors of resistance.—*Rep. Rothamsted exp. Sta* 1968 pt. 1, 173–174.
- SAWICKI, R. M., FRANCO, M. G. & MILANI, R. (1966). Genetic analysis of non-recessive factors of resistance to diazinon in the SKA train of the housefly (*Musca domestica* L.).—*Bull. Wld Hlth Org.* **35**, 893–903.
- SUN, Y. P. & JOHNSON, E. R. (1960). Synergistic and antagonistic actions of insecticide-synergist combinations and their mode of action.—*J. agric. Fd Chem.* **8**, 261–266.
- TSUKAMOTO, M. (1964). Methods for the linkage-group determination of insecticide-resistance factors in the housefly.—*Botyu-Kagaku* **29**, 51–59.
- TSUKAMOTO, M. & SUZUKI, R. (1964). Genetic analyses of DDT-resistance in two strains of the housefly, *Musca domestica* L.—*Botyu-Kagaku* **29**, 76–89.
- TSUKAMOTO, M. & SUZUKI, R. (1966). Genetic analyses of diazinon-resistance in the housefly.—*Botyu-Kagaku* **31**, 1–14.
- WAGONER, D. E. (1967). Linkage group—karyotype correlation in the house fly determined by cytological analysis of X-ray induced translocations.—*Genetics, Princeton* **57**, 729–739.
- WILKINSON, C. F. (1968). Detoxication of pesticides and the mechanism of synergism.—*In* Hodgson E. *Ed. Enzymetic oxidation of toxicants*.—229 pp. North Carolina St. Univ.

(Received 6 March 1969)

© Commonwealth Agricultural Bureaux, 1969