1968

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Bernard Felix Stone

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NL-101(1/66)

MECHANISMS OF FENTHION-RESISTANCE IN *CULEX FATIGANS* WIED.

by

Bernard F. Stone

Department of Zoology

Submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

Faculty of Graduate Studies

The University of Western Ontario

London, Canada

March 1968

ABSTRACT

A strain of the tropical house mosquito Culex fatigans Wied. from Rangoon, Burma, in which an 8-fold resistance to fenthion had been induced by laboratory selection, was compared with the original fenthion-susceptible Rangoon strain, and with a Rangoon strain of only 3-fold tolerance to fenthion. The possible mechanisms of resistance were investigated in vivo and in vitro by means of radiotracer, chromatographic, enzymatic and electrophoretic methods. The absorption and detoxication of fenthion was measured, the metabolites were estimated qualitatively and quantitatively, the enzymatic activity of homogenates towards fenthion and other substrates was determined, and the esterases were separated and partially quantitated on zymograms.

The resistant strain was found to absorb as little as one half and hydrolyzed as much as twice the amount of fenthion as the susceptible strain, while the tolerant strain showed significantly less absorption but not significantly more by hydrolysis than the susceptible strain. Both the resistant strain and the tolerant strain retained

significantly less of the toxic oxygen analogue of fenthion and its toxic metabolites in their larval bodies than the susceptible strain.

The oxidative and hydrolytic metabolism of fenthion was similar in both the resistant and susceptible strains, but the resistant strain produced proportionately less of the oxygen analogue and its toxic oxidation products, and proportionately more dimethyl phosphoric acid, a water-soluble metabolite derived only from the oxygen analogue or its oxidation products. However, dimethyl phosphorothioic acid derived from fenthion or its sulphoxide or sulphone was the most abundant hydrolytic metabolite in both resistant and susceptible strains, indicating that the hydrolysis mainly involved fenthion itself or its sulphoxide or sulphone.

In vitro the resistant strain degraded slightly more fenthion than the susceptible strain, but the difference lacked statistical significance. Assay of esterases hydrolyzing α-naphthyl and β-naphthyl acetate showed that in the resistant strain the supernatant fraction from centrifuged homogenates was up to twice as active as in the susceptible strain. Agar-gel zymograms showed that the major esterase, acid phosphatase or alkaline phosphatase bands,

insensitive to fenthion and its oxygen analogue, were quantitatively more active in the resistant than in the susceptible strain. Evidence was obtained in the thin-layer agar-gel zymograms that these phosphatase bands hydrolyzed the oxygen analogue of fenthion.

This research was financially assisted by a grant-in-aid from the Public Health Service, Department of Health, Education and Welfare, U.S.A., per grant number A102320-09.

ACKNOWLEDGMENTS

I would like to express my sincere thanks to Dr. A.W.A. Brown, Head of the Department of Zoology, to whom I am particularly indebted for his interest, encouragement and advice during the course of the research and for his practical help in the preparation of the manuscript.

I am also most grateful to the Commonwealth Scientific and Industrial Research Organization, Australia for the award of a C.S.I.R.O. Divisional Overseas Postgraduate Studentship in Pesticide Resistance and for granting the leave of absence necessary for this period of study. I would also like to thank Dr. D.F. Waterhouse, Chief, Division of Entomology, C.S.I.R.O., Canberra, A.C.T. and Dr. R.H. Wharton, Officer in Charge, Cattle Tick Investigation, C.S.I.R.O., Yeerongpilly, Queensland for their encouragement and for their sympathetic understanding during my absence from duty.

Special thanks are due to Mrs. E. Wambera and Mrs. G. Riekhoff for culturing the mosquito larvae required and to Mrs. H. Kyle, Mr. S. Pluzak and Mr. A. Gage, all of the Department of Zoology, for their willingness to help in

every possible way.

It is a pleasure to thank Drs. C.A. Anderson, D. MacDougall and R. von Rümker for their having kindly provided several samples of radioactive fenthion.

It is also a pleasure to acknowledge the instruction received from Dr. Z. Ogita, Department of Genetics, Osaka Medical School, Japan in the use of his thin-layer electrophoretic tenhnique, and for practical hints on this subsequently given by Mr. M. Ziv, Graduate Student in the Department of Zoology, University of Western Ontario.

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INTRODUCTION

Although criticism has been levelled at the widespread and indiscriminate use of the modern synthetic insecticides in recent years, largely as a result of Rachel Carson's "Silent Spring", it is quite clear that these chemicals have made a tremendous contribution towards the control of arthropod-borne diseases of man and his domestic animals. The discovery in 1939 of the insecticidal effectiveness of DDT by Paul Müller, for which he received the Nobel Prize in Medicine in 1948, marked the beginning of a remarkable new advance in vector control which has benefited many millions of people. Commencing with the spectacular arrest of an outbreak of louse-borne typhus by DDT in Naples in 1944 (Knipling, 1946), it is in the control of mosquito-borne diseases such as malaria, yellow fever and encephalitis that the modern insecticides have made their greatest contribution.

Malaria, for example which formerly caused half the world's deaths, has been largely eliminated from vast areas by insecticidal control of the Anopheles vectors in a world-wide eradication programme. The mosquito Aedes aegypti L., vector of

yellow fever has been successfully eradicated from the many countries of South and Central America. The various vectors of encephalitis have also been systematically and effectively attacked with insecticides.

However, these toxicants have in many instances proved equally effective as selecting agents for individuals with resistance alleles pre-existing in the population, and thus they have produced resistant strains of arthropods at an alarming rate following the first appearance of DDT-resistance in the housefly in Sweden in 1946 (Wiesmann, 1947). Since then, the number of resistant pest species has increased to a total of 225 insects and acarines by 1967 (Brown, 1968), of which 97 are of public-health or veterinary importance. As resistance to DDT developed and spread, it was replaced by HCH (BHC) and the cyclodiene derivatives such as chlordane and dieldrin, and as cyclodiene-resistance (extending to HCH) in turn developed, high hopes were held for the organophosphorus compounds which were by then appearing in great numbers. Moreover, since the OP compounds are more degradable, undesirable residues do not accumulate in wildlife, domestic animals or man. Apart from tetranychid mites, arthropods do not develope resistance to OP compounds very readily, although the number of species developing resistance to these compounds now totals 54 insects, mites and ticks (Brown, 1968).

One programme in vector control for which these chemicals

seemed particularly promising was that aimed at Bancroftian filariasis, caused by the nematode Wuchereria bancrofti (Cobbold) which is transmitted chiefly by the tropical house mosquito, Culex pipiens fatigans Wiedemann. This vector breeds in polluted water in urban areas where sanitation is poor and where the human population density is increasing rapidly. Thus it is that this insidious disease is increasing in the cities of the humid tropical zone where it has an almost world-wide distribution (Edeson and Wilson, 1964) and particularly in southeast Asia. Attention became focussed on the use of organophosphorus insecticides for filariasis control when it became apparent that C.p. fatigans was naturally somewhat tolerant to DDT and subsequently could develope strong resistance to HCH and dieldrin.

Fenthion (originally known as Bayer 29493 and marketed under the name of Baytex) is one promising new OP compound which proved highly effective against C.p. fatigans (Cerf and Lebrun, 1959). However a degree of fenthion-resistance has been produced by laboratory selection in two strains of this species (Tadano and Brown, 1966) which although insufficient to annul the larvicidal effectiveness of fenthion is nonetheless high enough to warrant an investigation into the physiological mechanisms of the resistance. Although the absorption and metabolism of fenthion has been studied in an OP-resistant

strain of houseflies (Metcalf, Fukuto and Winton, 1963) similar studies have not been made on fenthion-resistance in mosquitoes or on any type of OP-resistance in C.p. fatigans. This thesis therefore describes a study of the absorption and metabolism of fenthion by strains of C.p. fatigans specifically selected with fenthion as compared to the original unselected strain. Attempts have also been made to separate and identify the metabolites, and to characterise the esterases which may be involved in OP-resistance mechanisms in larvae of this mosquito.

LITERATURE REVIEW

This review will outline only the more noteworthy historical stages in the development of OP-resistance in arthropods, but will concentrate on OP-resistance in mosqqitoes in general and in C. fatigans in particular. Special attention will be given to mechanisms of OP-resistance, and general resistance mechanisms involving other types of insecticides will be considered where necessary and relevant,

Arthropod resistance to OP compounds appeared first in mites, when Garman (1950) reported parathion-resistance in a field population of Tetranychus urticae (bimaculatus), in U.S.A. The artificial induction of OP-resistance in insects was first reported by Chadwick (1952) and Decker and Bruce (1952), who selected laboratory strains of houseflies with diisopropyl fluorophosphate (DFP) and paraoxon respectively. Four years later Keiding (1956) reported that field populations of houseflies had become resistant to the OP compounds coumaphos and diazinon in Denmark. By 1967 there were 8 species of mosquitoes among the 54 arthropod species in which OP-resistance had developed (Brown, 1968).

OP-resistance is more prevalent among the culicines than

in the anophelines, among which Anopheles albimanus has developed malathion-tolerance in Nicaragua (WHO, 1966). The first suggestion that culicines might develop tolerance to the OP compounds was reported by Kellen and Chapman (1953), who found that the salt marsh mosquitoes Aedes sollicitans and A. taeniorhynchus in Florida, that had developed resistance to DDT and later to HCH, showed a 4-fold cross-tolerance to malathion and EPN. It is unusual for selection with DDT or HCH to produce cross-tolerance to OP compounds, although the converse is to be expected (Brown, 1958); in fact this OP-tolerance in Florida was reported (WHO, 1963) to fall short of real resistance. But by 1965 it became evident that A. taeniorhynchus had developed a 6-10 fold malathion-resistance in Lee county (Glancey, Lofgren and Miller, 1966). Subsequently a 13-30 fold malathion-resistance was found in this species in Brevard and St. Johns counties (Gahan, Smith and Glancey, 1966).

The irrigation-water mosquito A. nigromaculis in California was discovered to have developed resistance to parathion by 1957 (Lewallen and Brawley, 1958; Lewallen and Nicholson, 1959). By 1962 the level of resistance had increased to a maximum of 4,000-fold to parathion, 20-fold to methyl parathion and fenthion, and 10-fold to malathion (Brown, Lewallen and Gillies, 1963). Where fenthion had been used to replace parathion and methyl parathion, control

failures eventually appeared in 1965 (Lewallen and Peters, 1966). A. melanimon, also an irrigation-water mosquito, has shown some parathion-resistance in northern California but no control failures (Gillies, 1964). Resistance to fenthion has recently been observed in A. dorsalis in New Mexico (Harmston, 1967).

On the other hand the yellow fever mosquito Aedes aegypti has failed to develop malathion-resistance although increased tolerance to malathion has been found in several Caribbean populations. Laboratory selection with malathion applied for 8 generations to a susceptible strain from Penang, Malaya produced only 5-fold tolerance to malathion, although the cross-resistance induced to DDT was more than 30-fold (Brown and Abedi, 1960). Subsequent selection for at least 20 generations has proved unable to increase the malathion-tolerance by more than 10-12 times in any laboratory strain (Ziv and Brown, unpublished data 1968).

The western encephalitis mosquito Culex tarsalis developed a malathion-resistant population in an irrigated pasture near Fresno, California in 1956 (Gjullin and Isaak, 1957), and a laboratory colony of it, maintained under malathion pressure, attained a resistance 50 times that of a normal strain (Darrow and Plapp, 1960). The resistance did not extend to any other OP compound except malaoxon. Although this malathion-resistance has been very thoroughly studied with respect to its mechanism (see below), in the field it regressed and almost

completely disappeared by 1959 (Lewallen, 1960).

In Culex fatigans, the subject of this thesis, the larvae and adults had become resistant to DDT after 6 years of its use in India (Pal, Sharma and Krishnamurthy, 1952) and larvae in Malaya became resistant to HCH within 3 years (Reid, 1955); these two types of resistance now occur throughout the global range of this species (Brown, 1961). The OP compounds malathion and diazinon were therefore introduced as larvicides, but populations resistant to both compounds developed after the use of malathion at Doula, Cameroun (Mouchet et al.,1960) and of malathion and diazinon at Freetown, Sierra Leone (Thomas, 1963).

Experiments in Leopoldville, Congo (Cerf and LeBrun, 1959), Rangoon, Burma (Rosen, 1967) and California, U.S.A. (Mulla, Axelrod and Isaak, 1962) had shown fenthion to be a powerful insecticide for C. fatigans. The resistance hazard to this and other OP compounds was assessed in this species by Tadano and Brown (1966), who selected larvae of laboratory strains from Rangoon, Doula, Freetown and Fresno for 10-23 generations with either fenthion, diazinon, malathion or parathion, amongst other insecticides. In contrast to the strong resistance rapidly developed to DDT and dieldrin, the OP compounds induced only modest tolerances between 2 and 6 times the normal. The highest tolerance obtained was to fenthion in a DDT-resistant Rangoon strain, which developed

a 9-fold resistance to fenthion after 23 generations of selection with that chemical. In the process it lost its initial DDT-resistance, which declined to a level below the DDT-tolerance of a normal Rangoon strain. It is this fenthion-resistant strain of C. fatigans which provided the principal material for the investigation of resistance mechanisms described in this thesis.

Before considering OP-resistance mechanisms as such, it may be appropriate to point out that many OP compounds require activation to the real toxicants. This is usually, but not exclusively, brought about by replacement of the phosphoryl sulphur (P=S) of a phosphorothionate or phosphorodithioate by an oxygen atom, thus producing a much more effective inhibitor of the target enzyme which is most probably a cholinesterase; this type of activated toxicant is often referred to as the oxygen analogue of the insecticide, or an "oxon" as distinct from a "thion". This and other types of activation, full coverage of which is given by O'Brien (1960, 1967), will be discussed more fully later in this review.

Studies of OP-resistance among insects and acarines made by a number of workers have indicated that 4 main types of resistance mechanism may be implicated, namely (i) reduced uptake or increased excretion of the insecticide or its oxygen analogue, (ii) increased metabolism of these toxicants to non-toxic or less toxic metabolites, (iii) decreased activation

of the insecticide, and (iv) decreased sensitivity of the target enzyme to inhibition by the oxygen analogue.

The first type of resistance, that due to reduced uptake or increased excretion, is infrequent; the examples may be found among mosquitoes and houseflies. Reduced larval uptake and retention of malathion was found to be the only factor in the malathion-tolerance of the Penang (Matsumura and Brown, 1961b) and the Trinidad and Kongolikan (Matsumura and Brown, 1963a) strains of Aedes aegypti. In the Penang strain malathion and malaoxon were detected in the excreta, recalling the observation of Roan, Fernando and Kearns (1950) in Periplaneta americana, that TEPP or one of its metabolites was excreted into the lumen of the foregut. Reduced uptake of diazinon or fenthion was found to be a contributing factor, but not the only one, in the diazinon-resistance or fenthion-resistance respectively of adult houseflies of a diazinon-resistant New Jersey (Rutgers A) strain (Forgash, Cook and Riley, 1962), of a diazinon-resistant English (SKA) strain (Farnham, Lord and Sawicki, 1965) and of a fenthion-resistant (SC) strain (Metcalf, Fukuto and Winton, 1963). Although reduced absorption and increased excretion usually involve only low-order non-specific tolerance, it may often be additive to a more significant mechanism and thus produce a critical level of resistance.

The second type of resistance mechanism, increased

detoxication, is usually the decisive one; there are many examples of OP-resistance among arthropods which depend on this mechanism. Increased enzymic hydrolysis of the phosphatase type of the OP insecticide was one of the mechanisms involved in the fenthion-resistance (Metcalf, Fukuto and Winton, 1963) and diazinon-resistance (Farnham, Lord and Sawicki, 1965) of the houseflies described above. Plapp et al. (1961) showed that parathion-resistant houseflies degraded paraoxon in vivo at a greater rate than normal flies. The OP-resistant strains of March (1959) showed a similar effect with malaoxon in vitro, but no differentiation was made between phosphatase and carboxyesterase attack. The level of resistance to malathion and malaoxon was much less by injection than by topical application; March (1959) explained this as due rather to the differential rate of release of active inhibitor and its differential destruction than to a difference in rate of penetration or degree of storage. Jarczyk (1966) measured the in vitro hydrolysis of paraoxon, parathion and fenitrothion by partially-purified homogenates of parathion-resistant and susceptible housefly pupae and found that the resistant homogenates degraded these compounds more rapidly than the normal.

This type of resistance mechanism had first been reported by Oppenoorth (1958) who found that there was much less paraoxon present in a parathion-resistant than in a susceptible

strain of houseflies after treatment with parathion, and attributed it to a greater detoxication of paraoxon or parathion. Van Asperen and Oppenoorth (1959) noted that the OP-resistant houseflies maintained in their laboratory at Utrecht were characterized by a low level of aliesterase, and Oppenoorth (1959) established that the mutant allele a responsible for the low enzyme level was also responsible for the OP-resistance. The congruence of low aliesterase with OP-resistance was confirmed by Bigley and Plapp (1960) in their housefly strains. Since increased metabolism of parathion was responsible for the resistance (van Asperen and Oppenoorth, 1960), it was therefore postulated by Oppenoorth and van Asperen (1960) that the aliesterase has been modified in the OP-resistant strains to a degrading enzyme of the phosphatase type.

Yet another diazinon-resistant strain, the Ka strain, obtained by selection for low aliesterase activity, was studied biochemically at Utrecht by Matsumura and Hogendijk (1964b). They found that one fraction of the crude homogenate separated on DEAE cellulose degraded 7 times as much parathion to phosphatase products as that of the susceptible strain. On a further chromatographic separation of the active fraction, the enzyme activity in the resistant strain went into a different sub-fraction from that in the susceptible

strain. This association of low-aliesterase selection with increased enzymic degradation of parathion provides strong evidence favouring the "mutant aliesterase" theory of van Asperen and Oppenoorth (1960), although no *in vivo* differences in rate of parathion degradation between the strains were detected.

The malathion-resistant strains of low-aliesterase titre studied by van Asperen and Oppenoorth (1960) were biochemically investigated by Matsumura and Hogendijk (1964a), who showed that malathion was degraded more, *in vivo* as well as *in vitro*, by the resistant strains than by the susceptible strain employed. Radio-isotope techniques revealed that whereas both phosphatase and carboxyesterase activities were higher in the resistant strain *in vitro*, only the carboxyesterase activity was significantly higher *in vivo*. The preponderant water-soluble metabolite produced by the homogenates was the monocarboxylic acid derivative of malathion, indicating that carboxyesterase attack was the more important route of metabolism. Purified enzyme fractions obtained from the homogenates showed an even greater difference between resistant and susceptible strains. It is therefore evident that increased OP-degrading esterase activity parallels OP-resistance in houseflies of low aliesterase activity, thus supporting the view of Oppenoorth and van Asperen (1960) that an aliesterase is converted to an OP-degrading esterase, which

although not conclusively proven at least constitutes the most coherent theory available for OP-resistance in general (O'Brien, 1967).

However low aliesterase activity is not always associated with OP-resistance even in houseflies: for example Franco and Oppenoorth (1962) reported a susceptible strain in which 50% of the flies had low activity, and Oppenoorth (1967) described an OP-resistant strain with normal aliesterase activity; six of the OP-resistant strains of houseflies at the Riverside laboratory have a high aliesterase level (R.B. March, personal communication, 1967). A normal level of aliesterase activity was found in a parathion-resistant strain of Drosophila melanogaster (Ogita, 1961) and a malathion-resistant strain of Culex tarsalis (Matsumura and Brown, 1961a; Plapp et al., 1965). In the green rice leafhopper, the aliesterase activity was actually higher in the malathion-resistant than in the normal strain (Hayashi and Hayakawa, 1962; Kojima et al., 1963) and so was the general esterase activity (Ozaki, 1964). It is only in Leverkusen strain of the mite Tetranychus urticae that a lower aliesterase level than normal has been observed in the OP-resistant colony, when naphthyl acetate was used as a substrate (Smissaert,1965).

Increased detoxication as a resistance mechanism has also been found in other insects besides the housefly. It

was shown by Kojima (1963) that a strain of the rice stem borer with a 10-fold resistance to parathion hydrolyzed about twice as much parathion or paraoxon *in vivo* as a susceptible strain. Similarly, Mengle and Lewallen (1966) showed that larvae of a strain of *Aedes nigromaculis* with a 96-fold resistance to parathion produced water-soluble metabolites more rapidly than susceptible larvae *in vivo*. Larvae of a malathion-resistant strain of *Culex tarsalis* when exposed to malathion came to contain 1/3 as much malaoxon as susceptible larvae (Matsumura and Brown, 1961a); mainly due to higher phosphatase activity degrading malaoxon. The principal *in vitro* difference however was in the carboxyesterase level and this difference could be shown *in vivo* when the larvae were cleared of their gut contents. The difference in phosphatases on the other hand was apparent in larvae whose guts had not been cleaned, indicating that much of the phosphatase products had been produced by extracorporeal hydrolysis, probably by enzymes of the alimentary canal. Both enzymes were most abundant in the mitochondrial fraction and least abundant in the microsomal fraction.

Further evidence for the importance of carboxyesterases was obtained in hybridization experiments in which Matsumura and Brown (1961a) found that the increase in carboxyesterase content was inseparable genetically from the malathion-resistance. Subsequently Matsumura and Brown (1963b) purified the

carboxyesterase at least 100 times, and separated it from phosphatase activity, by column chromatography through DEAE cellulose. It proved to be a small protein of M. W. 16,000 in both strains, the electrophoretic charge and U-V spectra also being similar in the enzymes from both sources. This information, coupled with the similarity in K_m values obtained previously (Matsumura and Brown, 1961a), indicated that the 13-fold increase in enzyme activity observed in the resistant strain was quantitative rather than qualitative.

In the greenhouse mite T. urticae the OP-resistant Blauvelt strain was characterized by elevated phosphatase and carboxyesterase levels (Matsumura and Voss, 1964). The former enzyme hydrolysed parathion more effectively in the BLauvelt than in the Leverkusen resistant strain. Although both enzymes attacked malathion, as might be expected from its molecular structure with its phosphate and carboxyester linkages, the most vigorous attack and the greatest inter-strain difference was due to carboxyesterases. These findings obtained both in vitro and in vivo provide strong evidence that enzymatic hydrolysis is the main factor in the resistance of these strains. The two resistant strains differed in their ability to metabolize malaoxon, the Blauvelt strain being much more efficient in this regard

than the Leverkusen strain, which did not differ from the susceptible strains.

Therefore in these mites studied by Matsumura and Voss (1964), resistance in the Blauvelt strain was due to "thionase" and "oxonase" detoxication. In the Leverkusen strain, however, it was only "thionase" attack; it is known that there are specific "thionases" as contrasted with "oxonases" (O'Brien, 1967), one enzyme in liver microsomes degrading parathion but not paraoxon (Nakatsugawa and Dahm, 1965). No differences were detected between either of the resistant strains and the susceptible strain of these mites in paraoxon degradation, but the rates were very low (Matsumura and Voss, 1964). A parathion-resistant Niagara strain of T. urticae also showed a resistance mechanism of increased detoxication when assayed with C^{14}-malathion as a substrate (Herne and Brown, 1968). On the other hand a dioxathion-resistant strain of the cattle tick (Boophilus microplus), showed no increase of paraoxon (Lee and Batham (1966), but again the degradation rates were very low.

The precise type of enzymic detoxication of a particular OP compound by a particular arthropod depends largely on its molecular structure and the biochemistry of its metabolism. Since the OP insecticides are usually tri-esters of phosphoric, thiophosphoric or thiophosphonic acids with various alcohols,

they are quite readily hydrolysed by enzymes. These may be generically described as hydrolases (tri-esterases) and more specifically as phosphatases which attack the ester linkages attached to the phosphorus atom; where the OP compound contains a carboxyester in the side chain, e.g. malathion, the enzymes involved are carboxyesterases. The hydrolytic products are generally ionic and non-toxic, the fragment containing the phosphorus atom usually having an anionic group attached to or capable of approaching the phosphorus atom, making it less positive and much less active as a cholinesterase inhibitor. The most likely enzymic mechanism, and the one most often explored in OP-resistance studies, is the phosphatase attack which splits off the "leaving group", namely the third alcohol substituent which splits away when an OP toxicant phosphorylates the target enzyme cholinesterase.

Hydrolysing enzymes of this type are common in mammals, as the metabolism studies of Aldridge (1953a, 1953b) have revealed. These studies showed that the sera and tissues of various laboratory animals contain one group of enzymes which are capable of hydrolysing paraoxon and p-nitrophenyl acetate (A esterases), and another group of aromatic esterases (B esterases) which hydrolyse p-nitrophenyl acetate but are sensitive to inhibition by 10^{-8} M paraoxon. There is a number of such serum phosphatases which attack OP insecticides such

as dichlorvos, butonate and naled, but not others (O'Brien 1967).

Metcalf et al. (1956) studied A esterases in insects and found them to be abundant in the abdomen of the honey bee and in the gut of Periplaneta; these esterases could hydrolyse paraoxon about half as effectively as p-nitrophenyl acetate. In vivo studies have shown that insects such as the housefly and the American cockroach, although less active in this respect than laboratory mice, nevertheless vigorously degrade OP insecticides (Krueger, O'Brien and Dauterman, 1960). It is usually difficult to demonstrate such active phosphatase degradation in vitro; however, Krueger and Casida (1961) found that activators such as Mn^{++} ions increased the rate of hydrolysis.

As part of a general in vitro study of the influence of insect esterases degrading OP compounds of the E605 (parathion) series, Jarczyk (1966) showed that the midgut of Saturniid, Lymantriid, Bombycid and Noctuid caterpillars contained at least two enzymes highly active against OP compounds with an optimum pH in the range 7.5 to 8.5. However a typical alkaline phosphatase hydrolysing disodium-p-nitrophenyl phosphate at pH 8.5 was found to be unable to hydrolyse OP compounds. Furthermore, this enzyme was distinct from the OP hydrolases, since the alkaline phosphatase could be separated from them by fractional centrifugation followed by column chromatography on Sephadex G 100. The OP hydrolases

termed P=O and P=S enzymes, were also separated from one another by the same method and found to differ in their specific activities, velocity constants and substrate specificities, although they were able to degrade both thiophosphoric and phosphoric acid esters.

The general esterases in OP-resistant strains have been compared with those in susceptible strains by means of electrophoresis, specific inhibitors and specific substrates. Separation by electrophoresis was the method employed by van Asperen (1962), Menzel, Craig and Hoskins (1963) and Ogita and Kasai (1965), who showed that the esterase zymograms differed in different houseflies but they failed to detect any correlation of the zymogram patterns with OP-resistance. Using specific inhibitors and substrates in studies on the esterases of malathion-resistant and susceptible C. tarsalis and A. aegypti, Plapp et al. (1965) also found no relationship between esterase constitution and resistance. However, a malathion-resistant strain of the green rice leafhopper possessed esterases more active against naphthyl esters than those of a susceptible strain; by means of electrophoretic separation these were identified as alkaline phosphatases and an aliesterase or aromesterase (Kasai and Ogita, 1965).

Another type of enzymic hydrolysis involves the other two alcohol substituents of the phosphorus ester; it is

termed O-dealkylation, or specifically demethylation, deethylation etc., and involves the removal of only one of the alkyl groups, thus destroying the toxicity of the OP molecule. Plapp and Casida (1958) showed that demethylation of ronnel occurred in rats. Shishido and Fukami (1963) and Fukami and Shishido (1963) reported that demethylation of methyl parathion, methyl paraoxon and fenitrothion was an important pathway in the detoxification of these pesticides by rats, being sulphydryl-dependent and mainly due to the supernatant fraction of the liver homogenate. They concluded that hydrolysis rather than microsomal oxidation was responsible, since the exclusion of air and the presence of reduced pyridine nucleotides had little effect on the rate of degration by the supernatant fraction. Hollingworth, Metcalf and Fukuto (1967) found that O-dealkylation was an important mechanism ensuring the relative non-toxicity of fenitrothion to mice; the sensitivity of fenitrothion to O-dealkylation by the laboratory mouse was lost when the phosphorothionate was replaced by the phosphonothionate structure. Dealkylation is however considered by O'Brien (1967) to be of minor importance for the degradation of OP insecticides in general.

Enzyme preparations from insects such as the rice stem borer and the American cockroach have much less dealkylating activity than mammalian tissue preparations. Although glutathione greatly increased the demethylation of methyl

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Enzyme preparations from insects such as the rice stem borer and the American cockroach have much less dealkylating activity than mammalian tissue preparations. Although glutathione greatly increased the demethylation of methyl

parathion by mid-gut and fat-body preparations from the silkworm and horn beetle, (Fukami and Shishido, 1966) the activity was far less than in rat liver; it was this lack of demethylating activity which, coupled with the lower glutathione content in these insects, was considered to account for the higher toxicity of methyl parathion to insects than to mammals. However, Bull and Lindquist (1964, 1966) found O-demethylation (as distinct from N-demethylation) to be a major degradative route for Bidrin in bollworm larvae and for Bidrin and Azodrin in boll weevils.

N-dealkylation of substituted amino or amidic OP compounds (e.g. Bidrin, Azodrin, Famphur, Amiton) on the other hand is probably due to microsomal oxidation; the alkyl group is hydroxylated and then splits away as an aldehyde (O'Brien 1967). For example Scaife and Campbell (1959) showed that Amiton was degraded by the microsomal fraction of liver homogenates, the process requiring $NADH_2$ and oxygen; although the breakdown product was not identified, O'Brien (1967) considered that N-deethylation was involved. In insects, N-dealkylation proved to be a significant pathway in the detoxication of Azodrin by the cotton bollworm, the tobacco budworm and the American cockroach (Bull and Lindquist, 1966), of Bidrin by the housefly (Menzer and Casida, 1965) and of Famphur by the milkweed bug and the American cockroach (O'Brien, Kimmel and Sferra, 1965).

Examples of dealkylation as a resistance mechanism are rare but Kojima et al. (1963) and Saito (1964) reported that a detoxifying enzyme which converts paraoxon to desethyl paraoxon showed higher activity in parathion-resistant than susceptible strains of the rice stem borer Chilo suppressalis.

The third possible type of OP-resistance mechanism would be a reduction in the amount of active toxicant produced. In order to inhibit AChE efficiently, OP compounds (except the phosphates and certain sulphoxides and sulphones) must be converted to a structural configuration with much greater affinity for AChE. This most commonly involves desulphuration (oxidation) of the P=S of a phosphorothionate to the P=O of a phosphate. It is considered (O'Brien, 1967) that the P of a P=S group is insufficiently positive to attract the hydroxyl group of the serine in the AChE molecule, whereas the =O, being much more electrophilic than =S, increases adequately the positivity of the P. Although the inhibition of AChE by OP compounds is often described as "irreversible", frequently there is spontaneous recovery of the enzyme at a very low rate (Hobbiger, 1951; Wilson,1951).

Thus the level of activated toxicant in an OP-resistant strain is often compared with that in the susceptible strain to see whether less activation or more degradation has occurred. In houseflies Oppenoorth (1958) and Plapp et al. (1961) found less paraoxon present in resistant than in susceptible flies, and concluded that the difference was due to a higher detoxication rate of the oxygen analogue in the

resistant strain. Krueger, O'Brien and Dauterman (1960) obtained similar inter-strain differences in diazoxon and likewise concluded that it was due to greater detoxication. In Lucilia cuprina, however, Roulston and Schuntner (personal communication, 1967) found that the lower diaoxon content in 2 diazinon-resistant strains was not due to any greater metabolism of diazinon by the resistant strains, thus suggesting either that the degrading enzyme was an "oxonase" acting on diaoxon or that there was less activation of diazinon by the resistant strains.

The enzymes responsible for degrading certain insecticides may be inhibited by the pyrethrin synergists; sesamex, for example, synergizes the OP compounds phosphamidon, mevinphos and dichlorvos, among others (Sun and Johnson, 1960). This synergism is probably due to the inhibition of the microsomal system which catalyses many enzymic reactions including oxidations, reductions, hydroxylations, hydrolyses, dealkylations and sulfoxidations (Netter, 1962: Gillette, 1962). On the other hand, the action of certain other OP compounds is antagonised by sesamex, perhaps because it inhibits the enzyme which oxidizes the insecticide to the active cholinesterase inhibitor. In insects it is the microsomal fraction that is the source of activating enzymes; the fat-body is particularly rich in these enzymes in the American cockroach, although all parts of the gut and nerve

cord are also active (Metcalf and March, 1953; O'Brien and Spencer, 1953; Kok and Walop, 1954). The activation of parathion by fat-body microsomes was proved and characterized by Nakatsugawa and Dahm (1965) in this species. Subsequently they found (Nakatsugawa, Tolman and Dahm, 1967) that the *in vivo* metabolism of parathion is suppressed by sesamex, which suggests that the microsomal enzymes participate in the degradation of parathion. Recently Oppenoorth (1967) has shown that sesamex suppresses dithion-resistance in houseflies, which also suggests that this OP-resistance is due to microsomal detoxication.

Another modifier of microsomal activity in the presence of reduced nucleotide and oxygen is SKF 525A, which inhibits the activation of parathion and schradan by rat liver (Davison,1955) and of parathion by mouse liver slices (O'Brien,1961). However, the latter author also showed that this inhibitor also blocked the degradation of paraoxon more than it inhibited the oxidation of parathion, so that the net effect was a lack of protection by SKF 525A against parathion poisoning in the mouse. Thus SKF 525A may also act as a synergist, particularly if degradation rather than activation is the limiting factor. It may also increase microsomal enzyme levels by induction (O'Brien, 1967), since the hydrolysis of fenthion by liver slices from white rats was increased when they had been pretreated with

SKF 525A (Brady and Arthur, 1961). The same effect was noted in Periplaneta (Shoffeitt and Arthur, 1961).

The use of EPN as a specific carboxyesterase inhibitor having a strong synergistic effect against the resistant strain, and of sodium fluoride as a phosphatase inhibitor having little synergistic effect against either strain, showed that carboxyesterases were more important than phosphatases in the malathion-resistance of Culex tarsalis (Matsumura and Brown, 1961a). Mention should be made of TOCP, which in addition to being an inhibitor of aliesterase (Stegwee, 1960) also inhibits the carboxyesterase which hydrolyses malathion in mammals and insects (O'Brien, 1967). TOCP is synergistic after activation for malathion not only in mammals but also in resistant houseflies and C. tarsalis (Plapp et al., 1963), thus providing evidence that the resistance mechanism depends on carboxyesterase. Other pairs of OP compounds may also be synergistic towards resistant strains if one inhibits the enzyme that degrades the other. For instance, n-propyl paraoxon was reported to synergize diazinon against diazinon-resistant houseflies (Oppenoorth and van Asperen, 1961). The propyl paraoxon apparently irreversibly phosphorylated the phosphatase which degrades diazinon, while being itself non-toxic, since it has little affinity for cholinesterase. The synergistic effect was not restricted

to the resistant strain, however, and did not completely eliminate resistance.

The fourth type of resistance mechanism is a reduction in the vulnerability of the target system, which is generally conceded to be AChE (O'Brien, 1960, 1967), to attack by the activated toxicant. Smissaert (1964) was the first to detect such an AChE of reduced sensitivity in the parathion-resistant Leverkusen strain of T. urticae; it was not only less sensitive, but was present at a level which was much lower than normal. All of the AChE present appeared to be of the insensitive type in these mites, which were homozygous for the single gene for OP-resistance, while all the AChE in mites of a susceptible Leverkusen strain was of the sensitive type. Furthermore the hybrids obtained in reciprocal crosses between the two strains had both types of AChE, as shown by two distinct bimolecular rate constants each coinciding with those found in either parental strain. Smissaert (1964) pointed out that the only effect of the lower rate of inhibition would be to delay death, and that resistance would eventuate only if the inhibitor was detoxified or counteracted in some other way. In fact, Matsumura and Voss (1964) showed that this strain was able to degrade more malathion and parathion in vitro than the susceptible strain.

The relative insensitivity of the AChE in this resistant Leverkusen strain was confirmed by Voss and Matsumura (1964). Additional evidence of the interstrain difference in sensitivity was provided by Voss and Matsumura (1965), who showed that the esteratic site of the resistant AChE possessed an abnormally weak affinity for the substrate as well as for the inhibitor. This interstrain difference was greatest for inhibitors with the shortest O,O-dialkyl side chain. These authors concluded, from their studies on various cholinester substrates and OP inhibitors, that the AChE in mites differs from that of insects and mammals in having a slightly broader esteratic site and a very general anionic site.

A similar resistance mechanism was discovered by Lee and Batham (1966) in a dioxathion-resistant strain of the cattle tick, Boophilus microplus, from Ridgelands, Queensland. They detected the presence in these ticks of a type of AChE which was slower to combine with organophosphates than the AChE of the susceptible strain. The greatest interstrain difference was shown with those OP compounds for which the resistance ratios were the highest. In the resistant strain of the cattle tick, in contrast to that of the red mite, only about 60 per cent of the AChE was of the insensitive type; it was considered probable that many of the resistant individuals were heterozygotes and would therefore have both

types of AChE. In fact, it was shown by Stone (1968) that OP-resistance in this strain of cattle tick was almost completely dominant, so that the heterozygotes could not be readily distinguished from the resistant homozygotes.

The main types of OP-resistance mechanisms have been ascertained from studies with parathion, malathion or diazinon; it now remains to review what is known about the metabolism of fenthion, a more recent OP insecticide which is the subject of this thesis. Fenthion is a phosphorothionate (Fig. 1), and its possible metabolites may arise from desulphuration of the P=S to the P=O group or from oxidation of the thioether sulphur. Both types of metabolites will be referred to collectively as oxidative metabolites, although "desulphurative" is more correct for the former type (O'Brien, 1967). Additional metabolites and pathways of metabolism are hydrolytic (Fig. 2). Some or all of these possible oxidative and hydrolytic metabolites are readily produced in a number of different animals and plants, although the number and proportion vary considerably from species to species.

Among the oxidative metabolites produced from fenthion in plants, the sulphoxide is the most important in the bean (Niessen, Tietz and Frehse, 1962) in cabbage and tea (Tomizawa, et al., 1962) and in rice (Fukuda, Masuda and Miyahara, 1962). On the other hand sulphoxide and sulphone are the

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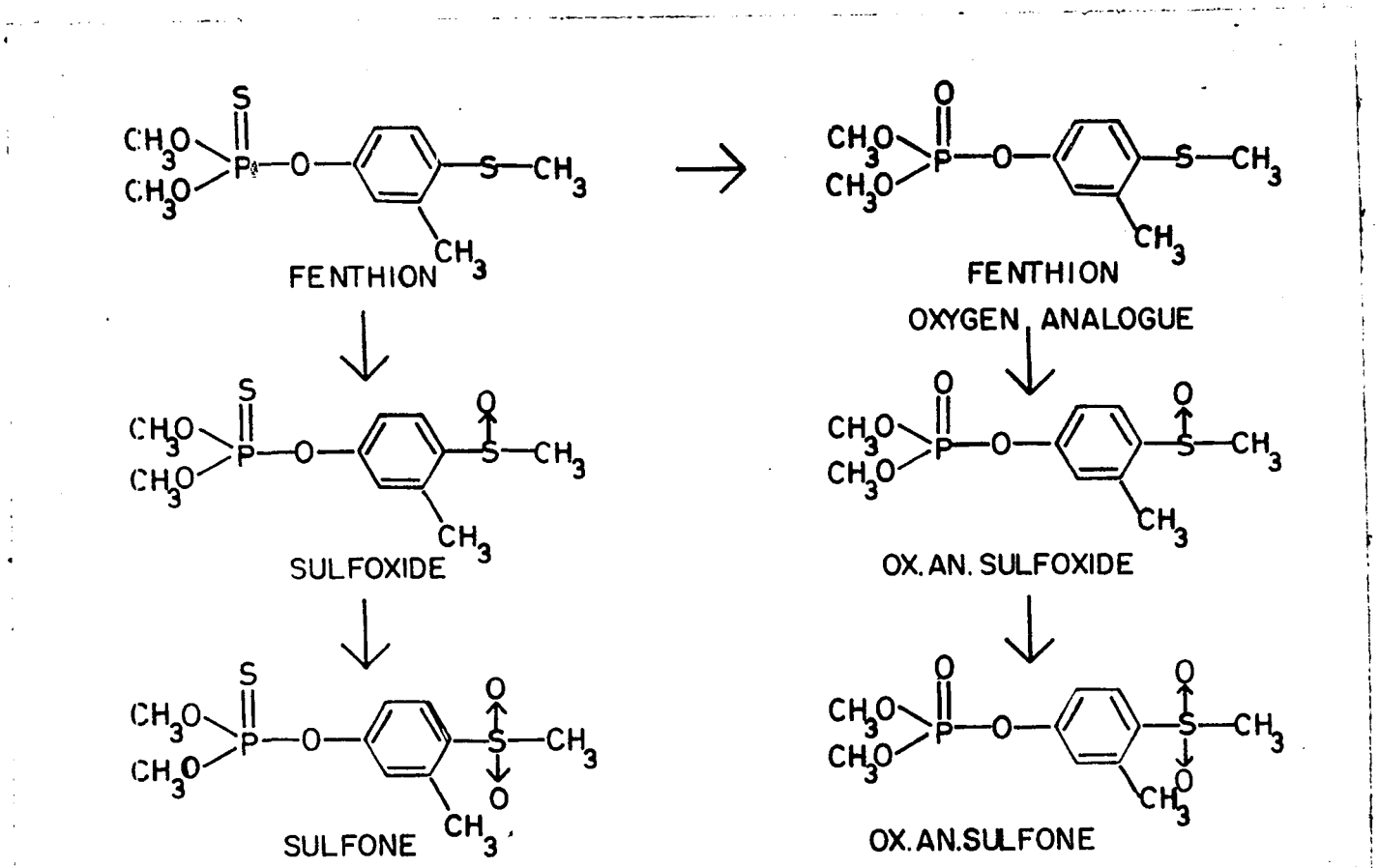

Fig. 1. Some desulphurative and oxidative metabolites of fenthion and possible metabolic routes.

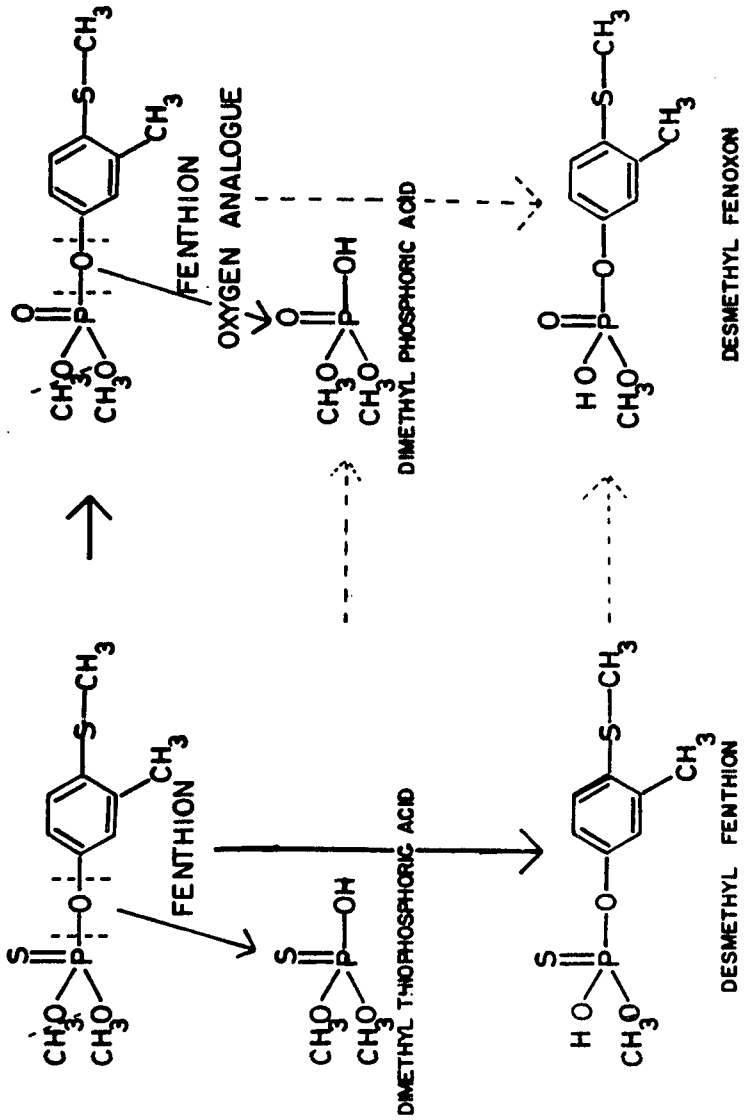

Fig. 2. Some hydrolytic metabolites of fenthion and possible metabolic routes.

main products in cotton (Metcalf, Fukuto and Winton, 1963), the sulphone being the most important (Brady and Arthur,1961). In white rats the major metabolite in the faeces was the oxygen analogue of fenthion, while in urine it was the sulphoxide and/or sulphone of the oxygen analogue (Brady and Arthur, 1961). In dairy cows, fenthion itself was the most common single component in faeces and in milk, whereas in urine the most common material was an inseparable complex of the sulphone of fenthion and/or the sulphoxide and/or the sulphone of the oxygen analogue (Knowles and Arthur, 1966).

In houseflies treated with fenthion 4 hours previously the most common metabolites were the sulphoxide and sulphone of the oxygen analogue (Brady and Arthur, 1961) and the sulphoxide and sulphone of fenthion itself (Metcalf, Fukuto and Winton, 1963). The boll weevil produced almost equal quantities of the sulphoxide of fenthion and the oxygen analogue, while the German cockroach produced the sulphone of fenthion (Brady and Arthur, 1961). In insects in particular, fenthion itself was often present in substantial amounts, frequently making up more than half of the chloroform-soluble fraction. Most of the animal and plant species under investigation produce a great variety of the minor metabolites and often retain some unchanged fenthion.

The hydrolytic metabolites produced in quantity by all species consist mainly of dimethyl phosphoric and dimethyl

phosphorothioic acids. Cotton plants yielded the latter as the principal hydrolytic metabolite whereas white rats produced predominantly dimethyl phosphorothioic acid in urine and dimethyl phosphoric acid in the faeces (Brady and Arthur, 1961). Dairy cows gave almost equal quantities of both of these components in urine but mainly dimethyl phosphoric acid in the faeces (Knowles and Arthur, 1966). In insects the hydrolytic products have not been separated, although 4 hours after treatment the housefly had hydrolysed 56 per cent, the boll weevil 76 per cent and the German cockroach 39 per cent of the fenthion absorbed (Brady and Arthur, 1961).

Demethylation appeared at best to be only a minor degradative mechanism, although some des-methyl fenthion was detected by Fukuda, Masuda and Miyahara (1962) in rice plants. It was suggested by Brady and Arthur (1961) and by Knowles and Arthur (1966) that unknown water-soluble fractions from urine of rats and faeces of dairy cows may include des-methyl fenthion or one of its oxidation products. The S-methyl isomer of fenthion was a rare metabolite, being recorded by Fukuda, Masuda and Miyahara (1962) only in rice plants; it was present to the extent of 5-6 per cent as an impurity in the original fenthion (Niessen, Tietz and Frehse, 1962; Metcalf, Fukuto and Winton, 1963).

The only group to compare the uptake and metabolism of fenthion in resistant and susceptible insects was that of Metcalf, Fukuto and Winton (1963) working with houseflies, They found that fenthion penetrated more slowly into the OP-resistant SC strain than the susceptible NAIDM strain. Although there was no interstrain difference in oxidative metabolism the hydrolytic breakdown of fenthion was faster in the resistant strain. These writers concluded that the combination of these two factors, decreased absorption and increased hydrolysis, could account for the 15-fold fenthion resistance in the SC strain.

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MATERIALS AND METHODS

Biological Material

Five strains of Culex fatigans were reared in the laboratory. The two fenthion-resistant strains (R and I), following 3-4 generations of relaxed fenthion pressure, were maintained under a regime of reapplied fenthion selection for another 17 generations. The description of the strains is as follows:

Rangoon N: derived from 4 females surviving from a field collection sent in May by Dr. B. de Meillon, WHO Filariasis Unit, Harcourt Butler Institute, Rangoon, Burma. This strain was originally dieldrin-resistant and DDT-tolerant.

S: a strain with a large gene pool derived from about 100 females received from the above source in December1963, being the Rangoon LP strain of Tadano and Brown (1966). It was already DDT-resistant and slightly dieldrin-tolerant, but entirely susceptible to fenthion; it was used as the susceptible reference strain throughout most of this investigation.

DDT-R: derived from the Rangoon-N strain by selection with DDT for 3 generations and with not more than 100-fold

resistance to DDT.

R: derived from the DDT-R strain by selection with fenthion for 23 generations, being the DDT:fenthion-R strain of Tadano (1966). The LC_{50} to fenthion was 10 times the normal, and the cross-resistance to malathion and diazinon was 2-4 times, and to parathion 1.5 times the normal; it constituted the resistant reference strain in this investigation.

I: obtained by selecting Rangoon-N larvae with fenthion for 18 generations, being the Fenthion-R strain of Tadano (1966). The LC_{50} to fenthion was 4 times the normal and the cross-tolerance to malathion and diazinon was 2-4 times, and to parathion, methyl parathion, Bromophos and Abate it was less than twice the normal.

Rearing Methods: The adult mosquitoes were kept in screened $3\frac{1}{2}$ cu. ft. cages, in two insectaries maintained at approximately 24°C and 75% relative humidity. The females were given blood meals usually every second night by exposing mice overnight in the cages. Illumination was provided during the day by daylight bulbs; by means of a system of time switches this was progressively reduced to darkness every evening between 7 and 9 p.m. and increased again every morning between 7 and 9 a.m. (Brennan and Harwood, 1953). A 10% sucrose solution was provided as food for the males, being absorbed on cotton dental swabs

in small bottles. The egg rafts produced by the females were collected three times a week from water-filled finger-bowls and exposed in the cages.

A larval culture was established by placing 3 or 4 egg-rafts in an enamel basin 12 inches in diameter, containing about 3,000 ml of distilled water, and covered with wire gauze. Larvae were fed a mixture of 5 parts brewers yeast, 2 parts blood albumin and 1 part RNA three times a week, the scum which formed on the water surface being removed by means of strips of paper towelling.

Toxicological Methods: Larval selection was performed on each successive generation of the R and I strains by exposing 100-200 larvae in the 3rd-4th instar for 24 hr in 450-ml glass jars to a concentration of fenthion that was expected to cause 90% mortality. The survivors were carefully rinsed with distilled water and transferred to clean water for pupation and emergence. One generation occupied about 1 month.

The susceptibility of the strain to fenthion in a given generation was determined by the WHO standard method for mosquito larvae (WHO, 1963), batches of 25 larvae in the late 3rd or early 4th instar being exposed in 450-ml glass jars containing 1-ml aliquots of ethanol solutions of the chemical and 249 ml distilled water. Tests were made in duplicate at each of a number of concentrations, which

for the susceptibility tests formed a geometric series.

The toxicity of candidate metabolites to R and S larvae and the effect of synergists on the toxicity of fenthion was measured by similarly exposing larvae in suspensions prepared as above from ethanol solutions or in aqueous solutions. The percentage mortalities based on the dead and moribund larvae counted together after 24-hr exposure were plotted on logarithmic probability paper; the dosage-mortality lines fitted by eye yielded the LC_{50} values.

Chemical Material

Two P^{32}-labelled samples of fenthion, O,O-dimethyl O(4-(methylthio)-m-tolyl) phosphorothioate, with specific activities of 3.42 and 2.23 mc./g, were kindly provided by the Chemagro Corporation, Kansas City, Missouri. The radiochemical purity of both batches was found to be at least 98% by thin-layer chomatography; there may have been traces of the S-methyl isomer present, but they went undetected since a reference sample was not available at the time. Non-radioactive fenthion (98.8% pure) was also provided by Chemagro along with the following candidate oxidative metabolites:

O,O-dimethyl O-(4-(methylsulphinyl)-m-tolyl) phosphorothioate, the sulphoxide of fenthion

0,0-dimethyl O-(4-(methylsulphonyl)-m-tolyl) phosphorothioate, the sulphone of fenthion

0,0-dimethyl O-(4-(methylthio)-m-tolyl) phosphate, the oxygen analogue of fenthion

0,0-dimethyl O-(4-(methylsulphinyl)-m-tolyl) phosphate, the sulphoxide of the oxygen analogue

0,0-dimethyl O-(4-(methylsulphonyl)-m-tolyl) phosphate, the sulphone of the oxygen analogue

O-methyl S-methyl O-(4-(methylthio)-m-tolyl) phosphate, the S-methyl isomer of fenthion

Sodium 0,0-dimethyl phosphate and potassium 0,0-dimethyl phosphorothioate, kindly supplied by American Cyanamid Company, Princeton, New Jersey, when dissolved in 0.01N HCl provided the hydrolytic reference metabolites, dimethyl phosphoric and dimethyl phosphorothioic acids.

The toluene scintillation fluid used in radioactivity measurements was the Solution I of Hayes (1963), consisting of 0.5% PPO (2,5-diphenyloxazole) and 0.03% POPOP (2,2-p-phenylene bis (5-phenyloxazole)) in scintillation-grade toluene (Matheson, Coleman and Bell). The dioxane scintillation fluid was described by O'Brien (1964) and consisted of 1% PPO, 0.05% POPOP or dimethyl POPOP, and 5% naphthalene (Packard) in a 5:1 mixture of spectro-quality dioxane (Matheson, Coleman and Bell) and ethylene glycol (Eastman). Packard scintillation quality PPO, POPOP and dimethyl POPOP were used throughout.

Chemical Procedure

Absorption and Metabolism: Larvae of susceptible and resistant strains were exposed in vivo at 23° C to aqueous suspensions of P^{32}-fenthion containing up to 0.4% ethanol, at dosages ranging from 0.025 to 1.25 p.p.m. The exposure was short (1-12 hr) and the larval densities were high (600-6,000 larvae/ 250 ml, equivalent to 1-10g/250 ml). Thus inter-strain differences in mortality were minimized, but at the same time sufficiently high levels of radioactivity could be obtained in the larval extracts despite the high larvicidal activity of fenthion. The unabsorbed fenthion was removed from the treated larvae by washing them several times with distilled water until no further radioactivity could be detected; these washings were usually then combined with the exposure water for assay.

Larvae were homogenized in ice-cold 0.2% trichloracetic acid(5ml/g tissue) by means of a Sorvall Omni-Mixer Homogenizer with or without micro-attachment. The denaturation of the water-soluble proteins by the TCA (Matsumura, 1959) facilitated the extraction of fenthion and its metabolites; the precipitation of protein was made more complete by storing the homogenates at 4°C for several hours following the recommendation of Kinnory et al. (1958). The homogenates were then adjusted to pH 7.0 and extracted twice with an equal volume of chloroform in a separating funnel; the two

phases were allowed to separate and then were centrifuged at 3,000 r.p.m. for 15 min to separate debris and break any emulsions formed.

The chloroform fraction was filtered through glass wool to remove debris, and then evaporated to dryness on a rotary vacuum evaporator; this residue was taken up in hexane and the solution was extracted twice with an equal volume of acetonitrile, which took up most of the radioactive material while leaving the lipids in the hexane phase. The acetonitrile fraction was taken to dryness in a rotary vacuum evaporator, and this residue was redissolved in hexane or chloroform in sufficient volume that a 1-ml aliquot added to 15 ml toluene scintillation fluid gave a count significantly higher than background (15 - 30 c.p.m.).

The aqueous fraction was similarly adjusted in volume, concentrating if necessary, and 1 ml of this aqueous solution was added to 15 ml dioxane scintillation fluid. The exposure water left after removal of the larvae was also separated into chloroform-soluble and water-soluble fractions by simple extraction with chloroform, and the level of radioactivity similarly measured.

The liquid scintillation counting followed standard procedure, the samples being placed in 22-mm polyethylene counting vials (Packard) and counted for 10 min at +2°C, to avoid freezing of aqueous solutions. With the toluene

scintillation fluid, low K^{40} glass counting vials were substituted. The Packard Model 314X Liquid Scintillation Spectrometer (Fig. 3) was employed, the high-voltage control being set at position 1 to give the minimum voltage of 630 volts and thus the highest c.p.m. readings for P^{32}. Although attenuation of A and B channels at position 2 gave a higher counting efficiency, position 1 gave a better balance between channels and was adopted as the standard setting. A-A1, B and C discriminators were set for 10, 50 and 100 volts respectively.

Under these conditions it is known that P^{32} counting efficiencies are normally very high even for chromatogram strip counting (Davidson, 1962). Although P^{32} standards were unavailable for precise determination of counting efficiency, nevertheless calculations made from sample counts, the original specific activity of the P^{32} fenthion and a standard decay curve showed it to be approximately 75% at attenuation position 1 and 94% at position 2. Correction factors for quenching were taken from an approximation to a standard curve as described for the "channels ratio" method of Bruno and Christian (1961); they were seldom needed since P^{32} is such a strong emitter. Total recovery of radioactivity was usually satisfactory and was over 90% in the most critical experiments.

Metabolites: The oxidative metabolites, which were found exclusively in the chloroform fraction, were separated by means of thin-layer chromatography in silica-gel coatings on polyester film with or without a fluorescent indicator (Eastman Chromogram K301R Sheet), in a "sandwich" type chamber (Eastman Chromagram Developing Apparatus). Clean-up of extracts was unnecessary when the specific activity of the P^{32}-fenthion was high, but in later experiments as the P^{32} (half-life 14.1 days) decayed the clean-up method of Storherr et al. (1964) was employed before chromatography. The extracts were spotted on the chromogram sheet in 2-10µl samples, and chromatographed to a height of 10-15 cm. Of the different solvent systems tested, heptane plus acetone (7:3) and benzene plus ethyl acetate (3:1) were the most useful, the latter giving the best separation of metabolites in general. Samples of the pure metabolites and of fenthion itself were co-chromatographed on the same sheet as the radioactive extract.

The radioactive strip was cut off and scanned (Fig. 4) by a thin end-window gas-flow Geiger tube in a shielded continuous scanner (Tracerlab SC-59S), connected to a ratemeter (Tracerlab SC-79) and a recorder (Texas Instrument Inc. Recti/riter) under standard conditions as specified by

Fig. 3. Packard 314X Liquid Scintillation Spectrometer.

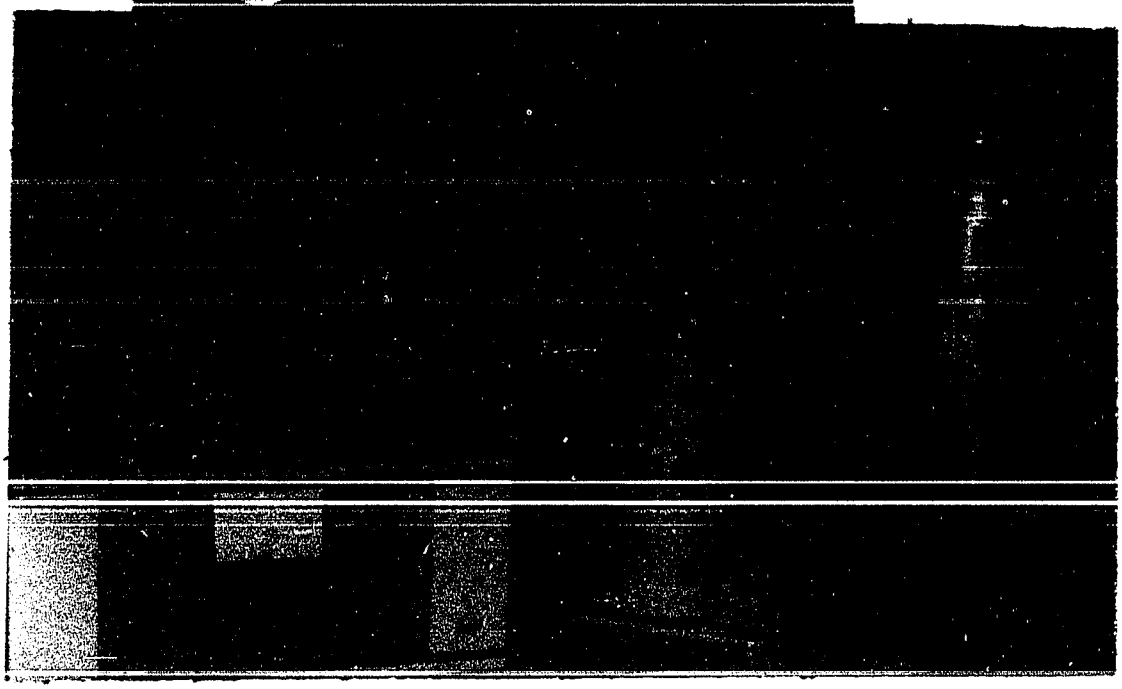
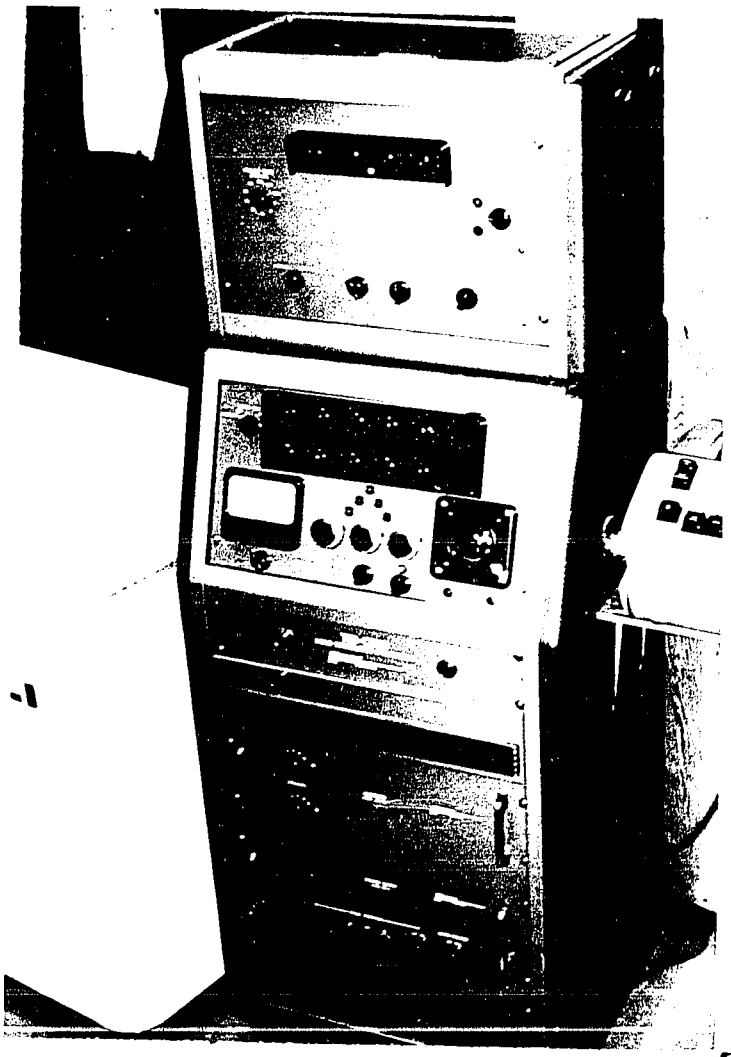


Fig. 4. Ratemeter with chromatogram strip scanner and recorder.



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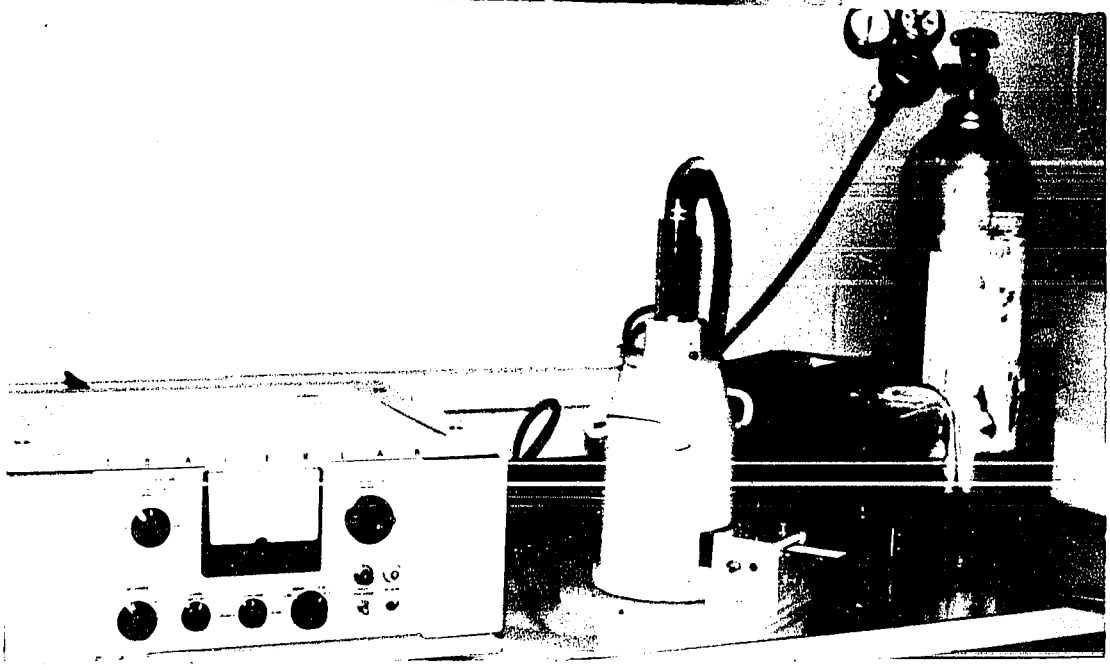


Fig. 4. Rate meter with chromatogram strip scanner and recorder.

the instrument manufacturers. Peaks of radioactivity due to the metabolites were measured with a polar planimeter, or were quantitated by snipping out the active spots and counting their radioactivity in the toluene scintillation fluid in the spectrometer (Davidson, 1962). In certain instances the entire chromatogram was examined under a germicidal ultraviolet lamp (G.E. G15T8) and the spots where quenching of the fluorescent indicator occurred were marked; these sections were sprayed with 1% DCQ (2,6-dibromo-N-chloro-p-benzoquinoneimine) in acetone, heated at 110°C, for 30 min, resprayed with DCQ, exposed to bromine vapour, resprayed with DCQ and re-exposed to bromine vapour (Menn, Erwin and Gordon, 1957; Chemagro Corp. Analytical Method TM C-8.44, 1965).

The hydrolytic metabolites, present only in the aqueous fraction, were usually separated by anion-exchange column chromatography (Fig.5) on Dowex 1X8, following essentially the method of Plapp and Casida (1958) with the modifications of Hollingworth, Metcalf and Fukuto (1967). In this method the Dowex 1X8 resin, after washing with 1N HCl was packed into a 2.5-cm column to a depth of 29 cm; the aqueous fraction, concentrated to a volume of 15 ml, was added to the column and followed by 20 ml distilled water; for identification by co-chromatography a mixture of candidate

metabolites was included. Gradient elution was performed with the following series of mixtures:

0.01 to 0.1N HCl, 450 ml each

0.1N HCl:CH_3OH (1:3) to 1N HCl:CH_3OH (1:3), 300 ml each

1N HCl:CH_3OH (1:3) to concentrated HCl:H_2O:CH_3OH (1:1:6), 200 ml each

Concentrated HCl:H_2O:CH_3OH (1:1:6) to concentrated HCl:H_2O:acetone (1:1:6), 200 ml each

Concentrated HCl:H_2O:acetone (1:1:6), 200 ml.

Using the system of separating funnels exactly as described by Plapp and Casida (1958), successive fractions of approximately 20 ml were collected by means of a fraction collector, and their total phosphorus content was determined by the method of Allen (1940).

Degradation by homogenates: The metabolism of radioactive fenthion *in vitro* by larval tissues of R and S strains was assessed by a modification of the method of Matsumura and Brown (1961a). A 1-ml aliquot of the supernatant fraction, obtained by centrifuging a crude homogenate (40 mg larval tissue per ml) at 15,000 r.p.m. for 20 min at -4°C, was incubated with 0.5 ml of an aqueous suspension containing 15 µg P^{32}-fenthion and 2% ethanol in M/15 phosphate buffer at pH 7.7 for 3 hr in a shaker water-bath at 23°C; at the end of the incubation 23.5 ml of 0.2% trichloracetic acid was added and the flask was stored

at +3°C for 1 hr. Then the pH was adjusted to 7.0 and the homogenate was extracted with chloroform according to the method described above, and the radioactivity was measured in the aqueous and chloroform fractions.

Esterase Assay: Esterase activity in larvae was measured colorimetrically by the method of van Asperen (1962). Batches of 140 mg larval tissue were homogenized in 0.5 ml ice-cold phosphate buffer at pH 6.8 and diluted to 100 ml with buffer solution; some homogenates were centrifuged at 15,000 r.p.m. and -4°C for 20 min before being diluted. A 1-ml aliquot of the diluted homogenate was incubated with 5 ml of α-naphthyl or β-naphthyl acetate (3 x 10-4M) in phosphate buffer, containing 1% acetone, at pH 7.0 for 30 min at 27°C. Then 1 ml of diazo-blue lauryl sulphate solution was added (van Asperen, 1962) and the developed colour was measured in a Bausch and Lamb Spectronic 20 spectrophotometer at wave-lengths of 600 mμ for the a-naphthyl and 550 mμ for the β-naphthyl acetate.

The esterases were separated and detected by thin-layer electrophoresis (Fig. 6) in agar-gel coatings on glass plates (15 x 10 cm or 15 x 20 cm) following the method of Ogita (1964). Samples of 1-10 μl of the undiluted homogenate, containing 0.5-1 g larval tissue per ml phosphate buffer (pH 6.8 and ionic strength 0.025) or 1 x 10^{-4}M phenylthiourea in buffer to inhibit melanisation, were

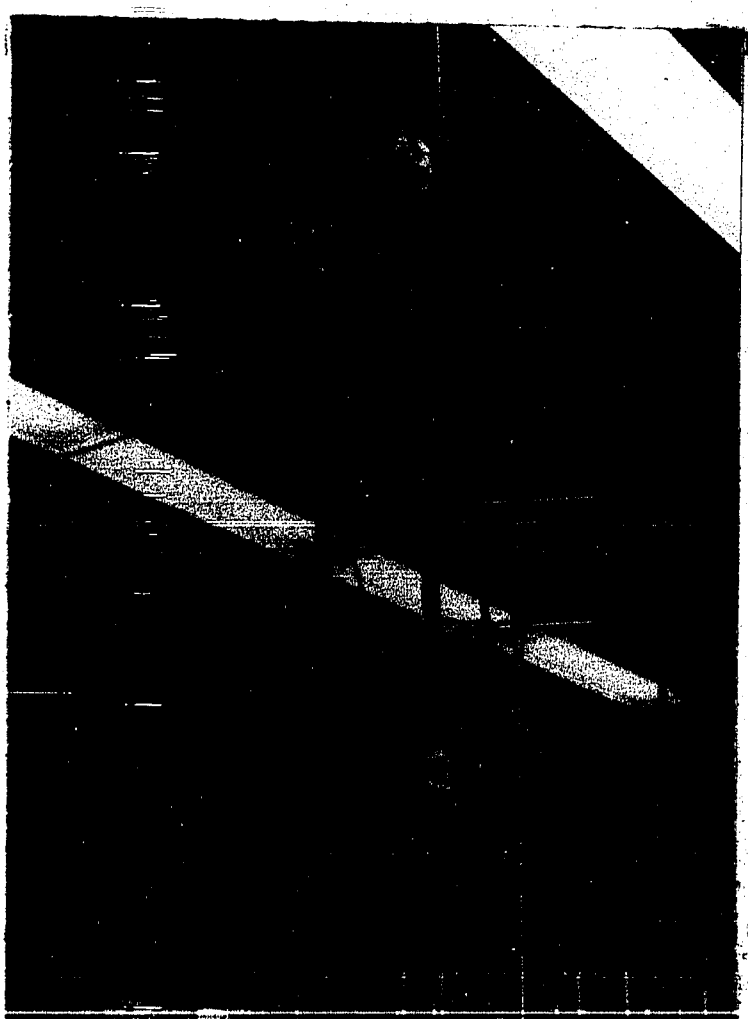
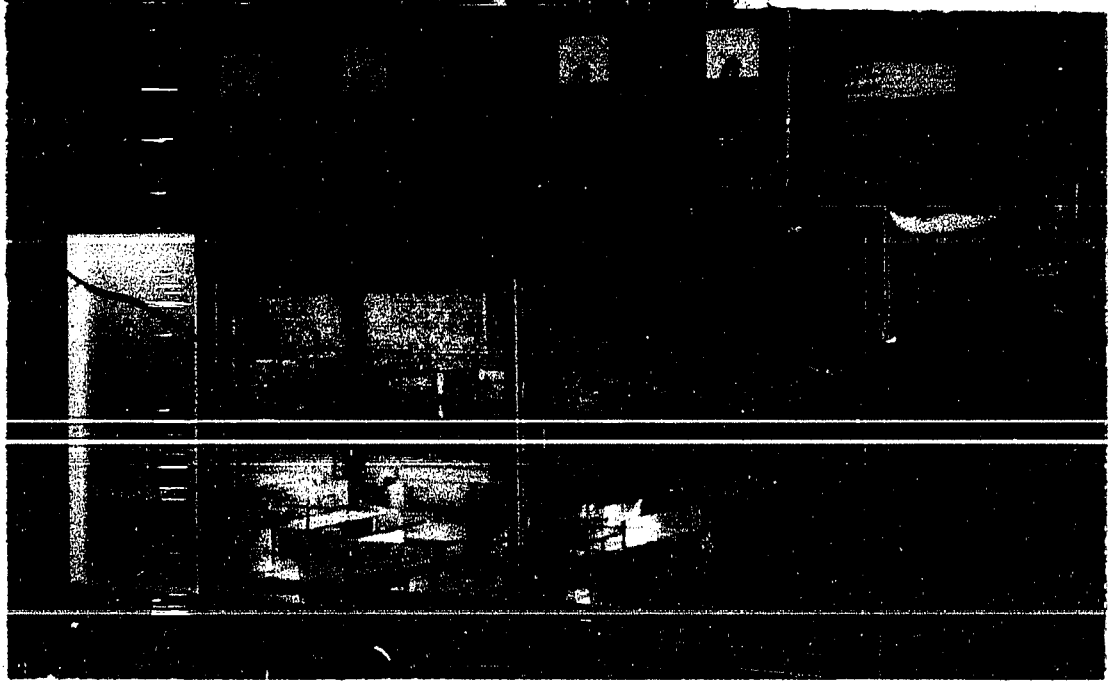


Fig. 5. Gradient elution system, ion-exchange chromatographic column and fraction collector.

Fig. 6. (below) Voltage regulators and refrigerated cabinet containing thin-layer electrophoresis units (Ogita, 1964).



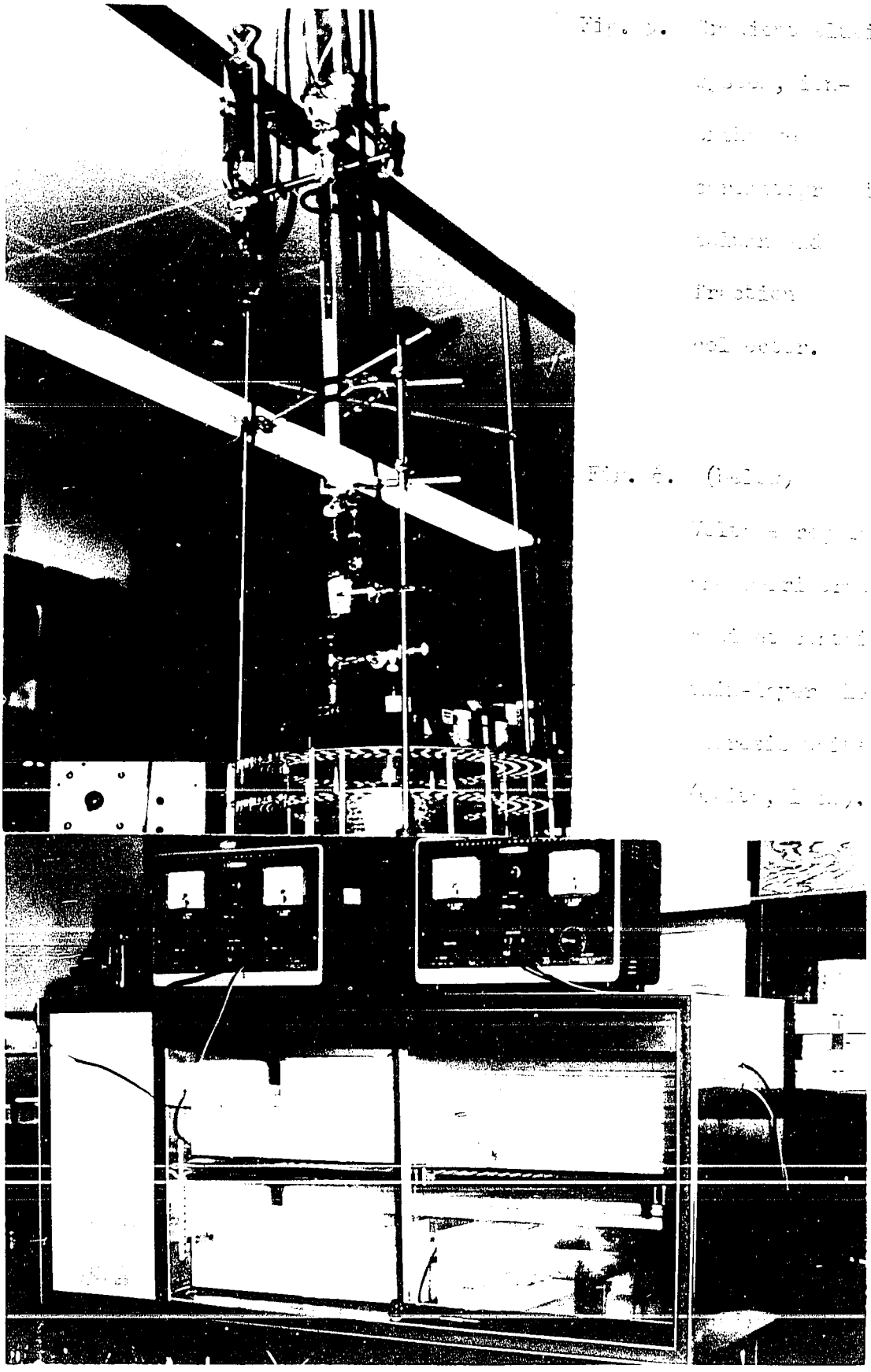


Fig. 5. [illegible]

Fig. 6. (Left,

applied to depressions made in the agar surface by leaving 2 × 15 or 20 mm filter-paper strips for 5 min before removal (for semi-quantitative tests these strips were dipped in the homogenate and applied to the plate for 20 min). The electrophoresis was performed at 300 volts and 1.0-2.0 mA per cm width for 1.5 to 2 hr at 5-10°C, the plates were sprayed with deionized water and then with 1% β-naphthyl acetate solution in acetone, and the enzyme action was assessed by incubating the plate for 20 min at 37°C. The esterase bands were visually developed by spraying the plates with a 2% aqueous solution of diazo-blue B (fast blue B, Sigma Chemical Co.). The developed plates were fixed by immersion in 5% acetic acid for 10 min, washed in running water for 2-4 hr, and dried for storage.

Acid and alkaline phosphatases also were similarly detected and separated, but using a variety of buffers and conditions of incubation (Ogita, 1964; Kasai and Ogita, 1965; Wieme, 1965). After electrophoresis the substrate was applied to the plates either by spraying it on at 0.5-hr intervals or by uniformly covering the plate, and the incubation was maintained for 1.5 to 18 hr at either 23°C or 37°C. The substrate solutions consisted of 0.05% disodium β-naphthyl phosphate (Borden Chemical Co.) or 0.5% disodium α-naphthyl phosphate (Borden Chemical Co.) in pH 4.0 or 5.0 acetate

buffer, pH 7.7 tris buffer, or pH 8.6 veronal buffer; all solutions contained 0.3% magnesium chloride. The enzyme bands were visualised as before by spraying with 2% aqueous diazo-blue B.

In an attempt to detect and separate the enzymes which may hydrolyze the oxygen analogue of fenthion, agar plates were prepared containing various concentrations of this inhibitor and Mn^{++}ions added as an activator in $5x10^{-4}$ M concentration obtained from $MnCl_2 \cdot 4H_2O$ (Krueger and Casida, 1961). Samples of the homogenate were applied, and the electrophoresis was performed. After an incubation for 4 hr at 37°C and high humidity to allow hydrolysis of the inhibitor to proceed, the plates were liberally sprayed with a solution of bovine-erythrocyte acetylcholinesterase (Nutritional Biochemicals Corp.) containing approximately 0.27 mg AChE per ml. After allowing 20 min for penetration of the enzyme, the presence of uninhibited AChE at the sites of hydrolyzing enzyme bands was detected by the INT method of Uriel (1961) as copied by Wieme(1965). By this method the plates are covered with a substrate solution consisting of 0.1% acetylthiocholine iodide (Nutritional Biochemicals Corp.) in barbital buffer at pH 8.2, which also contains 0.025% INT which is p-iodonitrotetrazolium violet C.P., (Mann Research Labs.). Upon incubation for 2.5 hr at 23°C., red areas appear where the homogenate has hydrolyzed the oxygen-analogue inhibitor.

RESULTS

Preliminary Toxicological Tests

Resistance Levels in the Strains: Periodical checks on the larval resistance of the R and I strains showed that the LC_{50} levels were maintained within the following limits:

R 0.012 - 0.015 p.p.m.

I 0.0035 - 0.0090 p.p.m.

S 0.0014 - 0.0020 p.p.m.

Thus the resistance level of strain R was fairly stable at about 8-fold, while strain I which was a reverted strain, was recovering its tolerance on the reapplication of pressure from an initial 2-fold to a final 4-fold tolerance level to fenthion.

Toxicity of the Oxidative Metabolites: Larvae of the R and S strains were exposed for 24 hr to a range of concentrations of each metabolite and the LC_{50} values were determined (Table 1). All the metabolites and the S-methyl isomer showed some toxicity but none are as toxic as fenthion itself when applied in this way. The least toxic compounds to strain S were the sulphoxide of the oxygen analogue and the sulphoxide of fenthion, which were both only about 1/40 as toxic as fenthion; while the most toxic metabolite was

Table 1. Toxicity of fenthion, its S-methyl isomer and its oxidative metabolites to larvae of the R and S strains of *C. fatigans*

	LC_{50} p.p.m.		Resistance Ratio*	Toxicity relative to fenthion**
	R	S		
Fenthion	0.015	0.0020	7.5	1.0
Oxygen analogue of fenthion	0.062	0.0080	7.8	0.25
Sulphone of fenthion	0.16	0.017	9.4	0.12
S-methyl isomer of fenthion	0.72	0.052	13.8	0.039
Sulphone of oxygen analogue	0.56	0.070	8.0	0.029
Sulphoxide of fenthion	0.45	0.076	5.9	0.026
Sulphoxide of oxygen analogue	0.40	0.078	5.1	0.026

* Ratio between LC_{50} to R strain and LC_{50} to S strain

** Based on LC_{50} for S strain

the oxygen analogue, which was about 1/4 as toxic as fenthion. Strain R showed increased tolerance to all the metabolites, the resistance ratio being highest with the S-methyl isomer, which was the least toxic compound to this strain.

Studies on Larvae in vivo

Uptake and Retention When lots of 1.25 g of R, I and S larvae were exposed to 0.25 p.p.m., P^{32}-fenthion for 1.5 hr, there was little mortality, although in the last 15 min toxic symptoms commenced to set in rapidly resulting in a knock-down of about 30-40% in Strain R, 60-70% in strain I and 80-90% in strain S. When similar lots of these larvae were exposed to 0.025 p.p.m. for 12 hr, the main effect was a rapid knockdown towards the end of the exposure period which amounted to about 50% in strain R and 90% in strain S, with some mortality probably occurring just before homogenization.

The total amount of radioactivity found in these larvae (Table 2) indicates that both R and I strains took up and retained significantly less fenthion or metabolites than strain S; the difference between strains R and S was greatest for the 1.5-hr exposure period, where the R strain had come to contain only about 1/2 as much radioactivity as strain S. These results suggest that a difference in

Table 2. Total radioactivity taken up and retained by larvae exposed to aqueous suspensions of P^{32}-fenthion. Expressed as micrograms fenthion per gram larvae tissue.

Strain	0.25 p.p.m. for 1.5 hr.	0.025 p.p.m. for 12 hr.
R	$1.75 \pm .02$[a]	$0.90 \pm .04$[c]
I	$3.07 \pm .09$[b]	–
S	$3.53 \pm .06$	$1.38 \pm .01$

[a] t_{10} 27.97; [b] t_{10} 4.22; [c] t_4 20.15; as compared to S strain

Table 3. Radioactivity present as water-soluble products in larvae exposed to aqueous suspensions of P^{32}-fenthion. Expressed as micrograms per gram and as the percentage of the total radioactivity

Strain	0.25 p.p.m. for 1.5 hr.		0.025 p.p.m. for 12 hr.	
	µg/g	%	µg/g	%
R	$0.95 \pm .02$	54.6[a]	$0.81 \pm .04$	90.4[c]
I	$1.44 \pm .06$	46.9[b]	—	—
S	$1.23 \pm .04$	34.7	$0.74 \pm .02$	53.6

[a] Significant at 5%; [b] not significant; [c] significant at 1%; as compared to S strain

the amount of fenthion absorbed or excreted may be a factor in the fenthion-resistance of strain R and the fenthion-tolerance of strain I.

When the water-soluble fraction was separated from the chloroform-soluble fraction (Table 3), it was found that the R strain had converted a significantly higher proportion of the total radioactivity to water-soluble metabolites than the S strain. In terms of absolute amounts, the water-soluble metabolites in the R strain were only slightly more abundant (long exposure) or even less abundant (short exposure) than the S, because the R strain had absorbed less total radioactivity. In the I strain also a greater proportion of the activity was present as water-soluble products than the S strain, although the inter-strain difference was not statistically significant. When larvae were exposed for 1.5 hr to concentrations of P^{32}-fenthion from 0.3 up to 1.0 p.p.m. (Table 4), the proportion of radioactivity in the bodies which had become water-soluble was above 49 per cent in the R strain and below 38 per cent in the S strain.

If the results reported in Table 3 are retabulated according to the proportion of the radioactivity in the chloroform-soluble fraction, then it is seen (Table 5) that R-strain larvae retained significantly smaller amounts

Table 4. Percentage of radioactivity converted to the water-soluble fraction in larvae of the R and S strains exposed to P^{32}-fenthion for 1-5 hr.

p.p.m.	R	S	R/S
0.3	49	28	1.8
0.5	59	29	2.0
0.7	53	37	1.4
1.0	53	38	1.4

Table 5. Radioactivity remaining as chloroform-soluble fraction in larvae exposed to aqueous suspensions of P^{32}-fenthion; expressed as micrograms and as the percentage of the total radioactivity

Strain	0.25 p.p.m. for 1.5 hr		0.025 p.p.m. for 12 hr	
	µg/g	%	µg/	%
R	$0.79 \pm .02$[a]	45.4	$0.086 \pm .004$[c]	9.6
I	$1.57 \pm .03$[b]	53.1	—	—
S	$2.31 \pm .01$	65.3	$0.63 \pm .01$	46.4

[a] t_{10} 67.8; [b] t_{10} 19.6; [c] t_4 30.5; as compared to S strain

of unchanged or oxidized toxicant than the S strain, being only about 1/3 of that retained by S larvae after exposure to 0.25 p.p.m. for 1.5 hr and only 1/7 when the exposure was to 0.025 p.p.m. for 12 hr. I-strain larvae had about 7/10 of the chloroform-soluble radioactivity of strain S, and this difference though small was statistically significant.

Oxidative Metabolism: When the candidate metabolites of fenthion were separated by thin-layer chromatography on silica-gel, they gave R_f values (Table 6) which were on the whole in inverse proportion to the degree of oxidation; good separation of metabolites was obtained with 3-1 benzene-ethyl acetate (Fig. 7).

When the chloroform fraction from R and S larvae exposed to 0.25 p.p.m. P^{32}-fenthion for 1.5 hr was chromatographed in heptane-acetone, the main peaks of radioactivity (Fig. 8A) were tentatively identified as fenthion, the sulphone of the oxygen analogue, and the sulphoxide of the oxygen analogue. Both strains showed similar peaks. When the peaks were quantitatively estimated it became clear (Table 7) that the two strains did not differ greatly in the proportions of chloroform-soluble oxidative metabolites remaining. However, there was more unchanged fenthion left in the R strain and more of the oxidation products of the oxygen

Table 6. Approximate R_f values for fenthion and its oxidative metabolites* by thin-layer chromatography on silica-gel** using 2 solvent systems

Compound	Colour with DCQ + Bromine	Heptane + Acetone (7:3)	Benzene + Ethyl Acetate (3:1)
Fenthion	Red	0.67-0.73	0.92-0.97
Sulphone of fenthion	Red	0.47-0.54	0.82-0.87
Oxygen analogue of fenthion	Faint yellow brown	0.50-0.56	0.57-0.65
Sulphoxide of fenthion	Red	0.31-0.37	0.34-0.40
Sulphone of oxygen analogue	No colour	0.21-0.27	0.25
Sulphoxide of oxygen analogue	No colour	0.11	0.05

*All compounds quenched strongly except the sulphone of fenthion

**Eastman Chromagram K301R sheet with or without fluorescent indicator

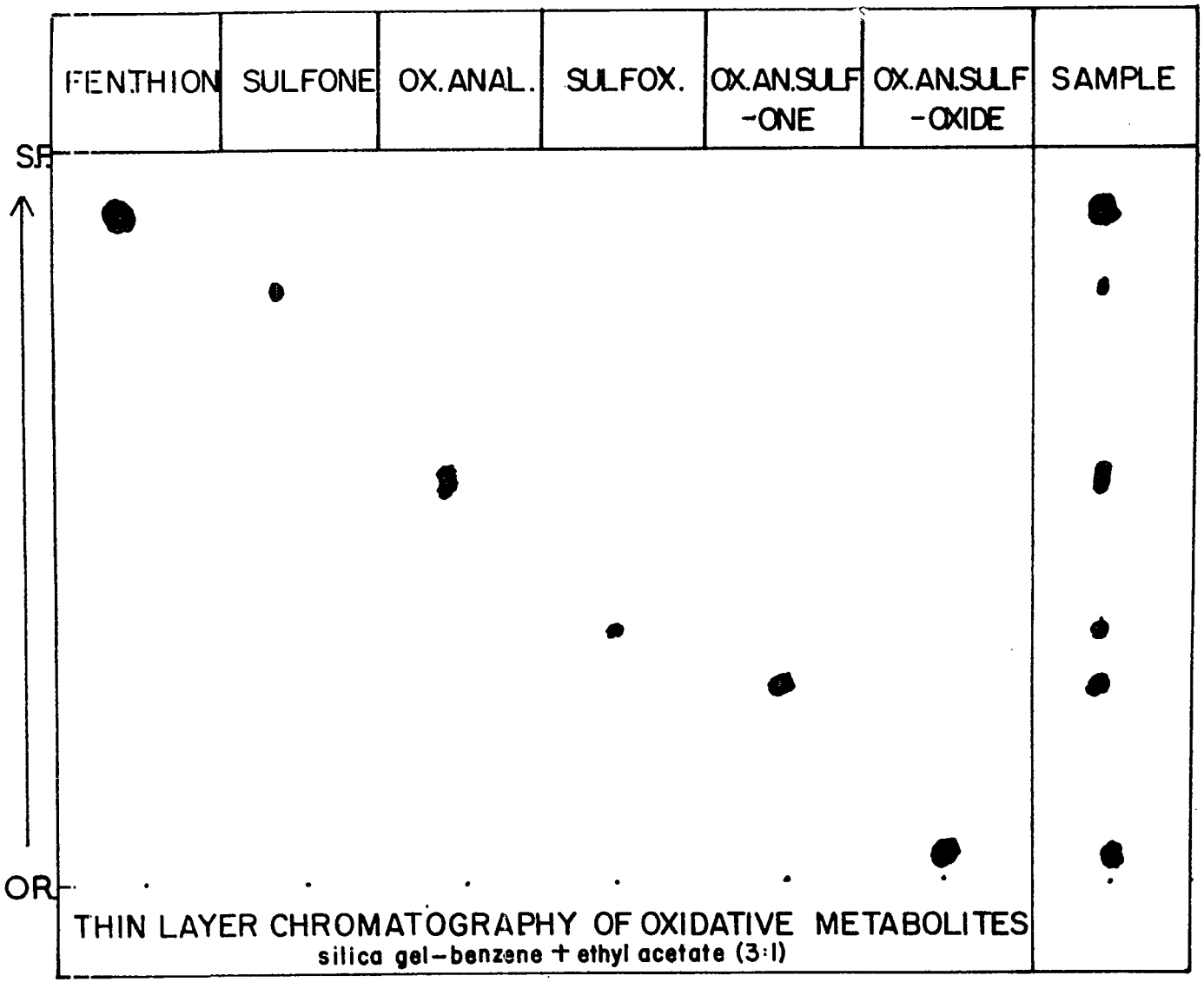


Fig. 7. Schematic separation of oxidative metabolites by thin-layer chromatography.

analogue remaining in the S strain. Fenthion, although the most active compound when applied externally (see Table 1), is a much less efficient cholinesterase inhibitor internally than the sulphoxide and sulphone of the oxygen analogue (Metcalf, Fukuto and Winton, 1963). Therefore, the overall toxicity of the chloroform fraction from R larvae, containing a smaller proportion of the oxygen-analogue sulphoxide and sulphone and a smaller absolute amount of fenthion toxicants (see Table 5), would be less than that from S larvae.

The chloroform extract from the exposure water left after removal of larvae of strains R and S following exposure to 0.25 p.p.m. P^{32}-fenthion for 1.5 hr was also subjected to thin-layer chromatography. The radiochromatograms obtained for both strains showed 3 main peaks (Fig. 8B); here the sulphone of the oxygen analogue is absent, but the sulphoxide of fenthion itself is present. There was also a small amount of the sulphoxide of the oxygen analogue in the exposure water. It may be seen (Table 8) that here again fenthion is in higher proportion to its metabolites in the R strain rather than the S strain.

Enlarged samples of 10 g larvae were exposed in 250 ml water to the increased dosage of 1.25 p.p.m. for the increased

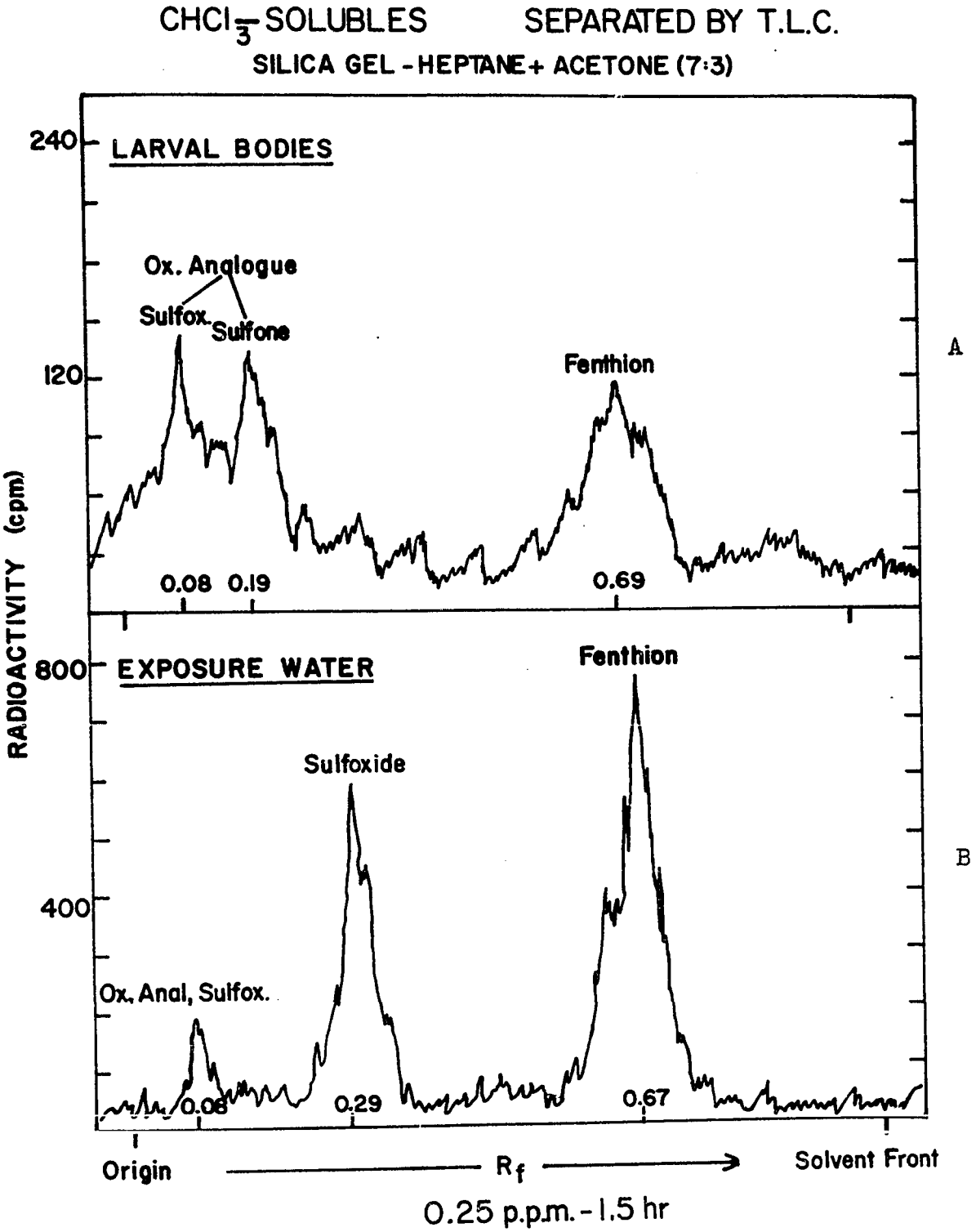


Fig. 8. Radiochromatograms of oxidative metabolites separated by thin-layer chromatography.

Table 7. Proportions of fenthion and metabolites in the chloroform-soluble fraction from R and S larvae exposed to 0.25 p.p.m. P^{32}-fenthion for 1.5 hr. Identified by co-chromatography with reference metabolites; heptane-acetone 7-3

	R strain		S strain	
	R_f	%	R_f	%
Fenthion	0.69	35.3	0.72	22.7
Sulphoxide of oxygen analogue	0.08	34.6	0.07	37.1
Sulphone of oxygen analogue	0.19	30.1	0.19	40.2

Table 8. Proportions of fenthion and metabolites in the chloroform extract of the exposure water from R and S larvae exposed to 0.25 p.p.m. P^{32}-fenthion for 1.5 hr. Identified by co-chromatography with reference metabolites; heptane-acetone 7-3

	R strain		S strain	
	R_f	%	R_f	%
Fenthion	0.67	72.1	0.67	57.5
Sulphoxide of fenthion	0.29	23.8	0.29	35.0
Sulphoxide of oxygen analogue	0.08	4.1	0.09	7.5

time of 2 hr, the R and I strains being compared (Table 9). The thin-layer chromatograms of the chloroform extract, made with benzene-acetone (3-1), showed no fenthion remaining in larvae of either strain. The main metabolite in the comparatively-susceptible I strain was the oxygen analogue of fenthion, i.e. the active cholinesterase inhibitor. Only small amounts of this toxicant were present in the extract of the R strain, which consisted mostly of the sulphoxide and sulphone of the oxygen analogue. The complete degradation of fenthion in both strains may be due either to hydrolysis or oxidation, or to both. The low level of the oxygen analogue in the R strain may be due to its rapid hydrolysis or to fenthion being oxidized at the thioether rather than the phosphoryl sulphur.

Hydrolytic Metabolism: The aqueous fraction from the extract of the larvae exposed in 10-g lots to 1.25 p.p.m. P^{32}-fenthion for 2 hr was chromatographed on an anion-exchange column of Dowex 1x8 resin by means of gradient elution with HCl solutions of increasing concentration. It was found (Fig. 9) that 3 principal peaks of radioactivity were present in the R-strain extract; two of these were identified by co-chromatography as dimethyl phosphoric acid (peak A) and dimethyl phosphorothioic acid (peak C). Peak B remained

Table 9. Proportions of fenthion metabolites* in the chloroform-soluble fraction from R and S larvae** exposed to 1.25 p.p.m. P^{32}-fenthion for 2 hr. Identified by co-chromatography with reference metabolites; benzene-acetone 3-1

	R strain		I strain	
	R_f	%	R_f	%
Sulphoxide of fenthion	—	—	0.38	7.6
Sulphone of fenthion	—	—	0.84	2.9
Oxygen analogue of fenthion	0.56	6.1	0.58	77.4
Sulphoxide of oxygen analogue	0.11	29.7	0.07	8.0
Sulphone of oxygen analogue	0.24	64.2	0.23	4.1

*Calculated from radioactivity of strips containing peaks cut from chromagram sheet and dissolved in toluene scintillation fluid

**10 gm larvae per 250 ml water

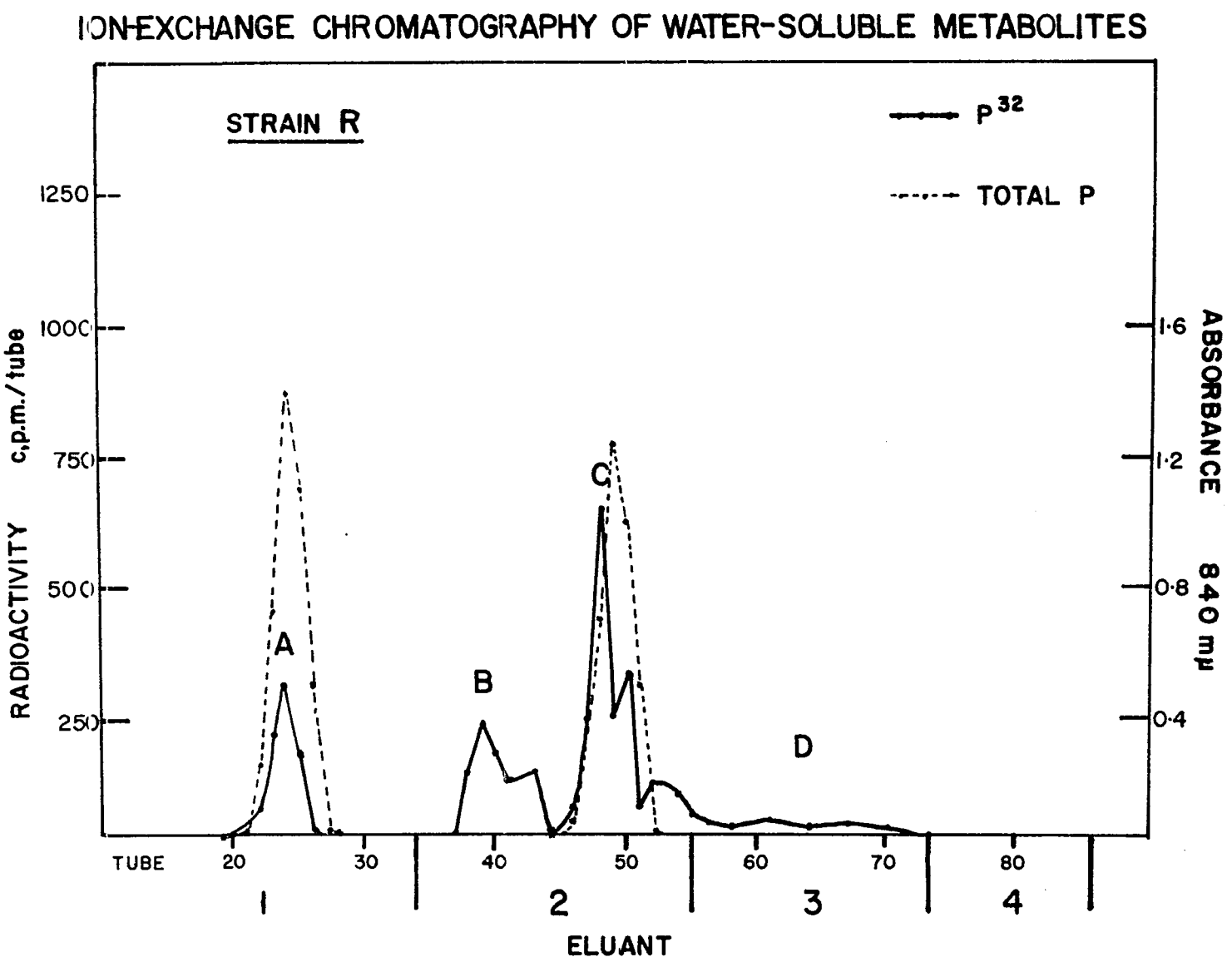


Fig. 9. Radiochromatogram of hydrolytic metabolites of strain R separated by ion-exchange chromatography.

unknown, and there was some unresolved residual radioactivity (plateau D). The aqueous fraction of strain S chromatographed similarly (except that 480 ml of eluant 2 was used instead of 600 ml, and the fractions collected were slightly larger) and showed the same 3 peaks (Fig. 10). The total recovery of radioactivity from the column was approximately 85% with either strain. It was confirmed by thin-layer chromatography on silica-gel using a mixture of 2-propanol and concentrated NH_4OH (75:25) that 3 main peaks were separable in strain S, the R_f values for two of these approximating to those previously established for dimethyl phosphoric acid (0.67) and dimethyl phosphorothioic acid (0.81).

Peak B remained unidentified, there being only two reference metabolites available at the time of the co-chromatography. However, it seems likely by analogy with published work (Plapp and Casida, 1958; Fukuda et al. 1962; Hollingworth, Metcalf and Fukuto, 1967) that this peak was neither phosphoric acid nor monomethyl phosphoric acid, for these have quite different elution characteristics. It is equally unlikely that it could have been any of the more polar oxidative metabolites, since it is now clear (Table 8) that they would have partitioned into the chloroform phase; although Hollingworth, Metcalf and Fukuto (1967) had found

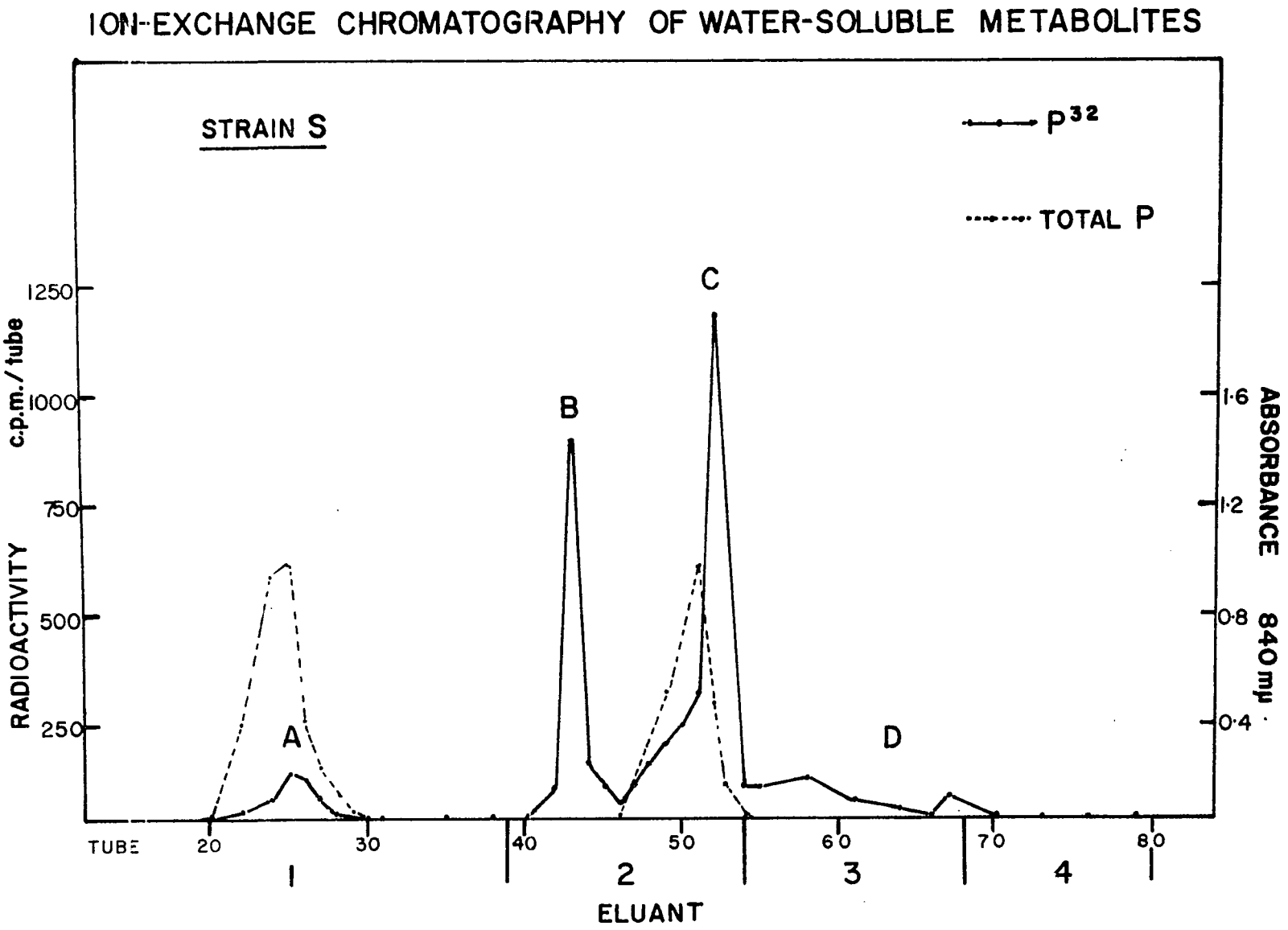


Fig. 10. Radiochromatogram of hydrolytic metabolites of strain S separated by ion-exchange chromatography.

methyl paraoxon and sumioxon in rat urine chromatographed from an aqueous fraction, this was before chloroform extraction. Peak B might have been one of the various desmethyl derivatives of fenthion or of its oxygen analogue, which are water-soluble; however, desmethyl fenthion requires stronger HCl solutions for it to be eluted from the column (Fukuda et al, 1962) than those used in eluant 2. In fact, the unresolved residual radioactivity D eluting after peak C is much more likely to have contained desmethyl derivatives, there being a suggestion of the presence there of one or possibly two very minor peaks in strain S.

On the radioactive assessment of the column eluate, it became clear (Table 10) that dimethyl phosphorothioic acid constituted about 50 per cent of the water-soluble fraction in both strains. Dimethyl phosphoric acid was more than twice as abundant in the R-strain than the S-strain, but was not as abundant as the unknown peak B metabolite. When expressed as the percentage of the total radioactivity absorbed and retained by larvae, these figures show that the R larvae converted 13 times as much fenthion into dimethyl phosphoric acid as the S larvae, and 53 times as much into dimethyl phosphorothioic acid.

Studies on Homogenates in vitro: When larval homogenates were incubated with 15 μg P^{32}-fenthion for 3 hr at 23°C and

Table 10. Proportions of hydrolytic metabolites* in the water-soluble fraction from R and S larvae** exposed to 1.25 p.p.m. P^{32}-fenthion for 2 hr.

Peak	Metabolite	% of water-soluble radioactivity		% of total*** Radioactivity	
		R	S	R	S
A	Dimethyl phosphoric acid	18.9	7.6	9.1	0.7
B	Unknown	23.2	26.9	11.2	2.4
C	Dimethyl phosphorothioic acid	50.3	51.8	24.3	4.7
D	Unresolved radioactivity	7.6	13.7	3.7	1.2

* Calculated from radioactivity collected from a column of Dowex 1x8 resin

** 10 gm larvae per 250 ml water

*** Calculated from previous column (water-soluble fraction) and from Table 9 (chloroform-soluble fraction)

pH 7.7, it was found that only 9.5% and 6.7% was hydrolysed by strains R and S respectively. The hydrolysis rates were very low, being of the order of 10 µg/g/hr; thus although the R-strain homogenate degraded fenthion slightly faster than the S-strain, the difference was not statistically significant.

Esterase Assays: When homogenates from R and S larvae were incubated with α-napthyl acetate and β-naphthyl acetate (Table 11), no interstrain difference was found with the crude homogenates. But with the supernatant fractions after centrifugation there was a statistically significant difference, the R-strain homogenates being almost twice as active as the S-strain in hydrolysing these substrates. With the centrifuged enzyme preparation, I-strain material was also significantly more active than the S-strain on α-naphthyl acetate but not on β-naphthyl acetate.

The DDT-R strain from which the fenthion-resistant R strain was derived showed no greater esterase activity than the S strain; it had a 36-fold DDT-resistance at the time of the enzyme assay. It will be noted that the homogenates of both R and S strains were slightly more active in hydrolysing the α-naphthyl than the β-naphthyl acetate, and that towards both substrates the crude homogenates were much more active than the supernatant fractions.

pH 7.7, it was found that only 9.5% and 6.7% was hydrolysed by strains R and S respectively. The hydrolysis rates were very low, being of the order of 10 µg/g/hr; thus although the R-strain homogenate degraded fenthion slightly faster than the S-strain, the difference was not statistically significant.

Esterase Assays: When homogenates from R and S larvae were incubated with α-napthyl acetate and β-naphthyl acetate (Table 11), no interstrain difference was found with the crude homogenates. But with the supernatant fractions after centrifugation there was a statistically significant difference, the R-strain homogenates being almost twice as active as the S-strain in hydrolysing these substrates. With the centrifuged enzyme preparation, I-strain material was also significantly more active than the S-strain on α-naphthyl acetate but not on β-naphthyl acetate.

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Table 11. Esterase activity* of crude and centrifuged** homogenates of R and S larvae on α-naphthyl and β-naphthyl acetate
Activities expressed as optical density x 100

Strain	α-naphthyl acetate			β-napthyl acetate		
	Crude	Centrifuged		Crude	Centrifuged	
		Exp. 1	Exp. 2		Exp. 1	Exp. 2
R	65.0±0.5	52.0±5.0[a]	—	56.0±0.5	48.0±1.4[c]	
I	—	—	42.0±2.9[b]	—	—	37.0±2.5
DDT-R	—	—	35.0±1.1	—	—	31.0±0.7
S	66.0±2.2	28.0±1.4	32.0±1.6	59.0±1.4	25.0±1.4	31.0±0.7

*1 ml. homogenate incubated with 5 ml 3×10^{-4} M naphthyl acetate at pH 7.0 for 30 min. at 27°C.

**15,000 r.p.m. for 20 min. at -4°C.

[a] t_2 4.62; [b] t_4 3.02; [c] t_2 11.62; as compared with S strain

Esterase Zymograms: When the centrifuged homogenates from the R and S strains were subjected to agar-gel electrophoresis and the activity of the esterase bands towards β-naphthyl acetate was visualized by Fast Blue B (Fig. 11), it became evident that the major band was conspicuously more prominent in Strain R than in strain S (Fig. 11). These main esterase bands in the R and S strains are not appreciably inhibited by treatment with 1 x 10^{-6}M fenthion included in the agar (Fig. 12), although the various esterase bands in the OP-resistant and susceptible strains of Aedes aegypti, included for comparison, are almost completely inhibited by the fenthion. In a separate test with 1 x 10^{-4}M oxygen analogue of fenthion (Table 12), the inhibition was estimated to be less than 50 per cent, but with 1 x 10^{-7}M paraoxon, the inhibition of the major esterase band was quite marked, reducing that in the R strain to roughly the same intensity as in the S strain. It is noteworthy that this fenthion-resistant R strain had no appreciable cross-resistance to parathion.

Agar-gel electrophoresis was carried out for acid and alkaline phosphatases with the disodium salts of α-naphthyl and β-naphthyl phosphate as substrates. In the acid phosphatase zymograms at pH 4.0 and 5.0, only one band was prominent, (Fig. 13). This was more active in the R than the S strain, and was insensitive to 1 x 10^{-4}M fenthion

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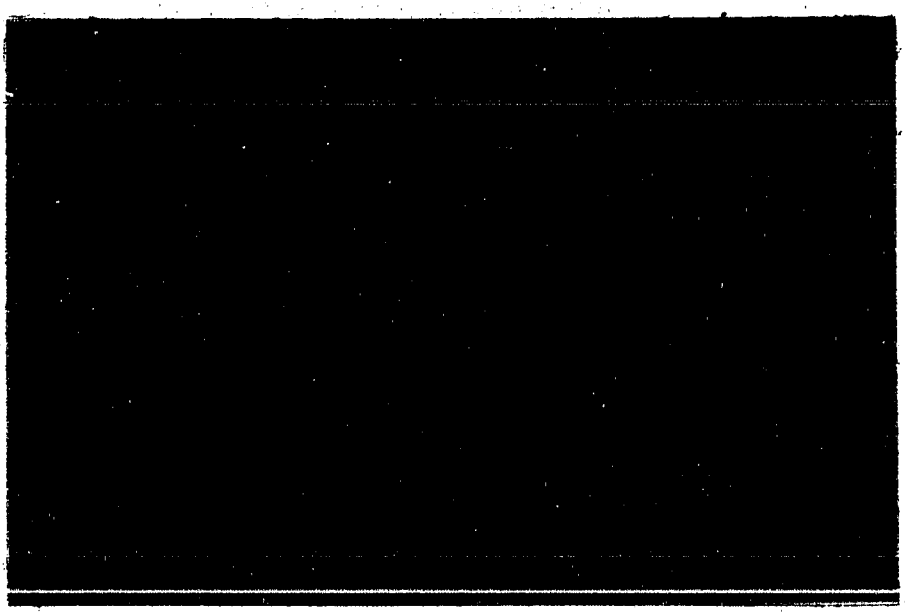
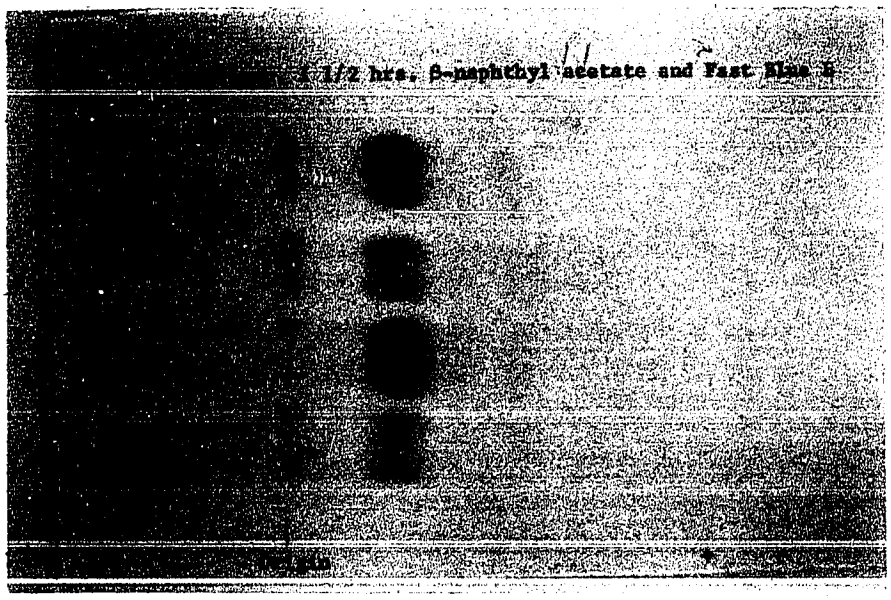


Fig. 11. Zymogram showing separation of major esterase bands of the R and S strains.



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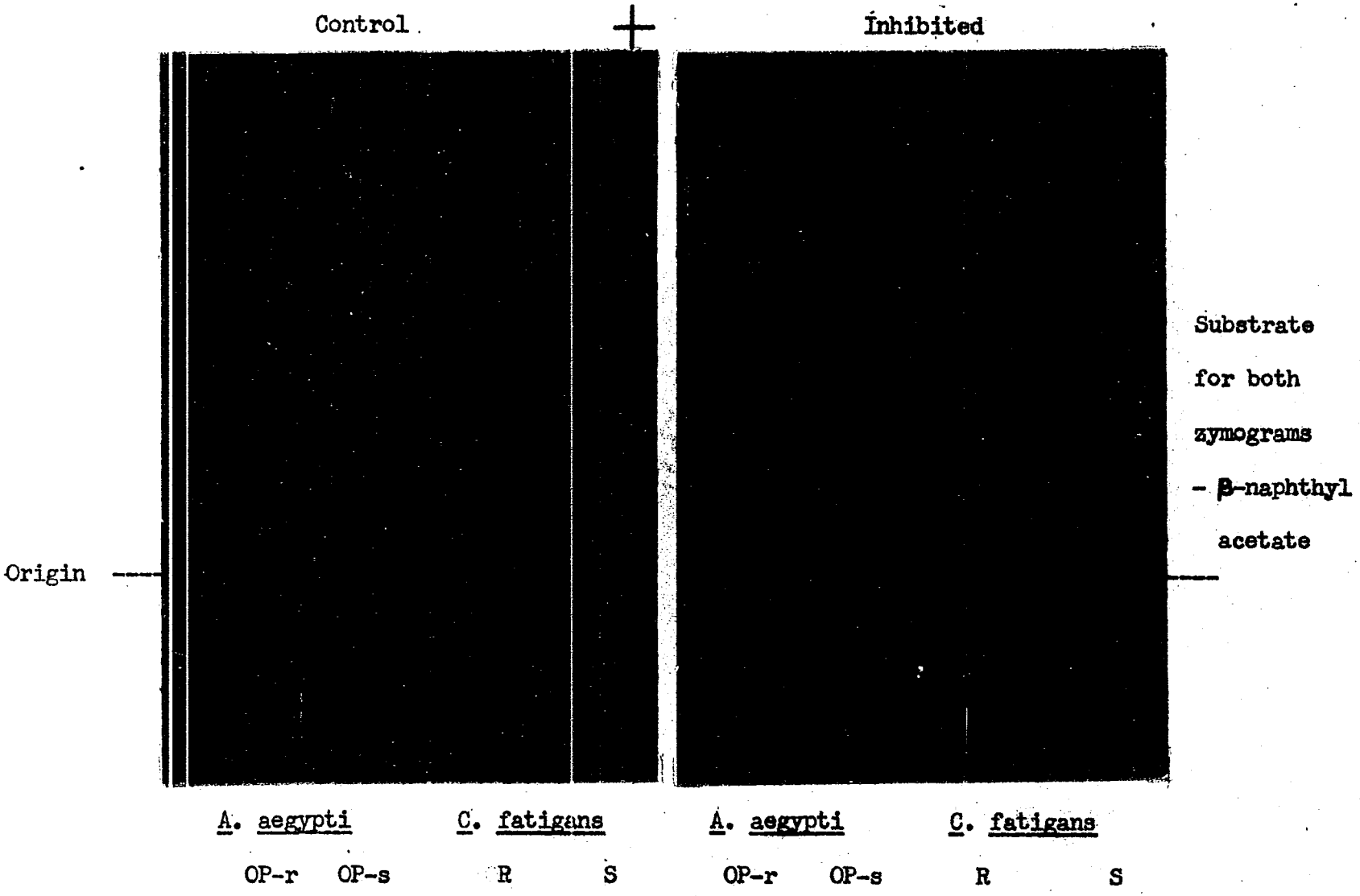


Fig. 12. Zymograms showing effect of 1 x 10^{-6}M fenthion on esterase bands of the R and S strains and of Aedes aegypti included for comparison.

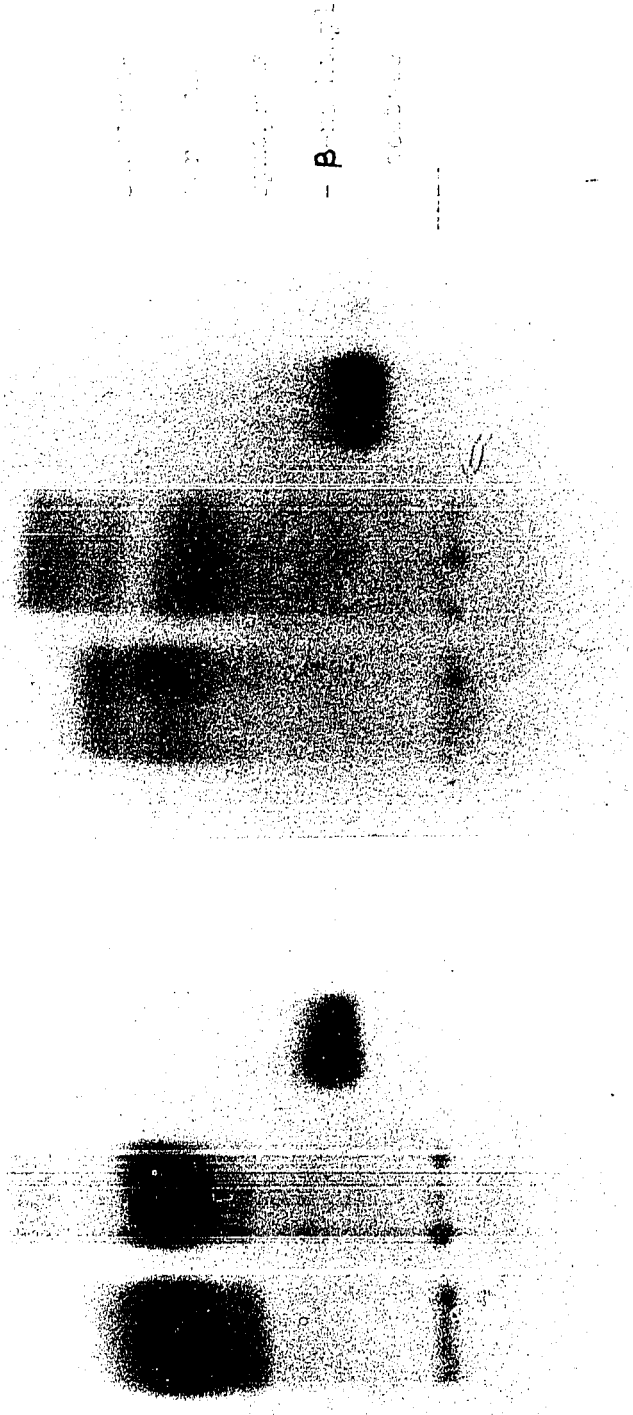


Table 12. Effect of inhibitors in the agar gel on relative intensity* of esterase bands in zymograms after incubation with various substrates. The homogenates were centrifuged in experiments with fenthion and paraoxon

Inhibitor	Substrate	R strain Control	R strain Inhibitor	S strain Control	S strain Inhibitor
1×10^{-4}M oxygen analogue of fenthion	β-naphthyl acetate (pH 6.8)	+ + + + + + + + + +	+ + + + + + +	+ + +	+ +
	α-naphthyl phosphate (pH 5.0)	+ + + + + + + +	+ + + + + + + +	+ +	+ +
	β-naphthyl phosphate (pH 7.7)	+ + + + + + +	+ + + + +	+	+
1×10^{-6}M fenthion	β-naphthyl acetate (pH 6.8)	+ + + + + +	+ + + + + +	+ +	+ +
1×10^{-7}M paraoxon	β-naphthyl acetate (pH 6.8)	+ + + + + + +	+ + +	+ +	+

*Number of +'s indicates intensity of colour (i.e. of hydrolysis) on a 10-point scale.

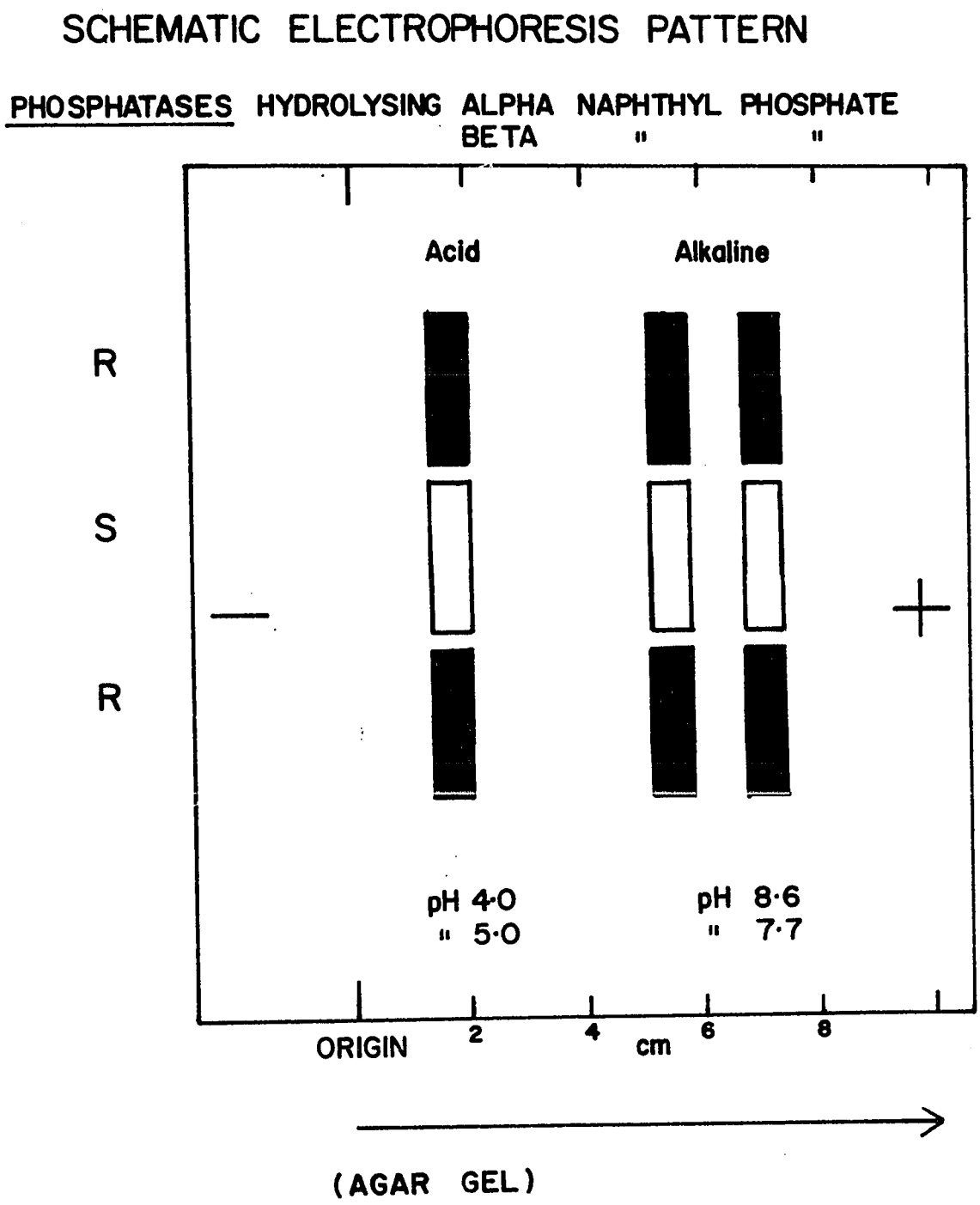


Fig. 13. Schematic zymogram pattern for acid and alkaline phosphatases.

oxygen analogue at pH 5.0. It was essentially congruent with the esterase band previously discovered (see Figs. 11 and 12) which had been found to be insensitive to fenthion and its oxygen analogue. This acid phosphatase, therefore, is similar in electrophoretic, substrate-preference and inhibition characteristics to the principal esterase band hydrolysing the naphthyl acetates. In the alkaline phosphatase zymograms at pH 7.7 and 8.6, two closely-adjacent faint bands were detected with both substrates (Fig. 13) and they were more prominent in the R than the S strain. These bands were only slightly inhibited by 1×10^{-4} M oxygen analogue of fenthion (see Table 12).

An attempt was made to ascertain whether the esterase bands which were not inhibited by the oxygen analogue of fenthion were able to degrade it at 1×10^{-4} M concentration. Bovine erythrocyte AChE was applied to the plate after a period had been allowed for degradation of the OP compound, and then a mixture of acetylthiocholine iodide and the tetrazolium salt INT was added; the presence of red bands due to the 'formazan' obtained by reduction of the INT by the thiocholine liberated from acetylthiocholine would indicate that the oxygen analogue had been degraded there since the AChE remained active. Some persistence of the ability to produce the red colour was indeed found in the zymogram so treated,

and it was located in the general region where the major esterase band, the acid phosphatase band and the alkaline phosphatase bands occur (Fig. 14). There was also some indication that strain R had been more active in degrading the anticholinesterase, but the results were not clear-cut due probably to diffusion of the enzymes.

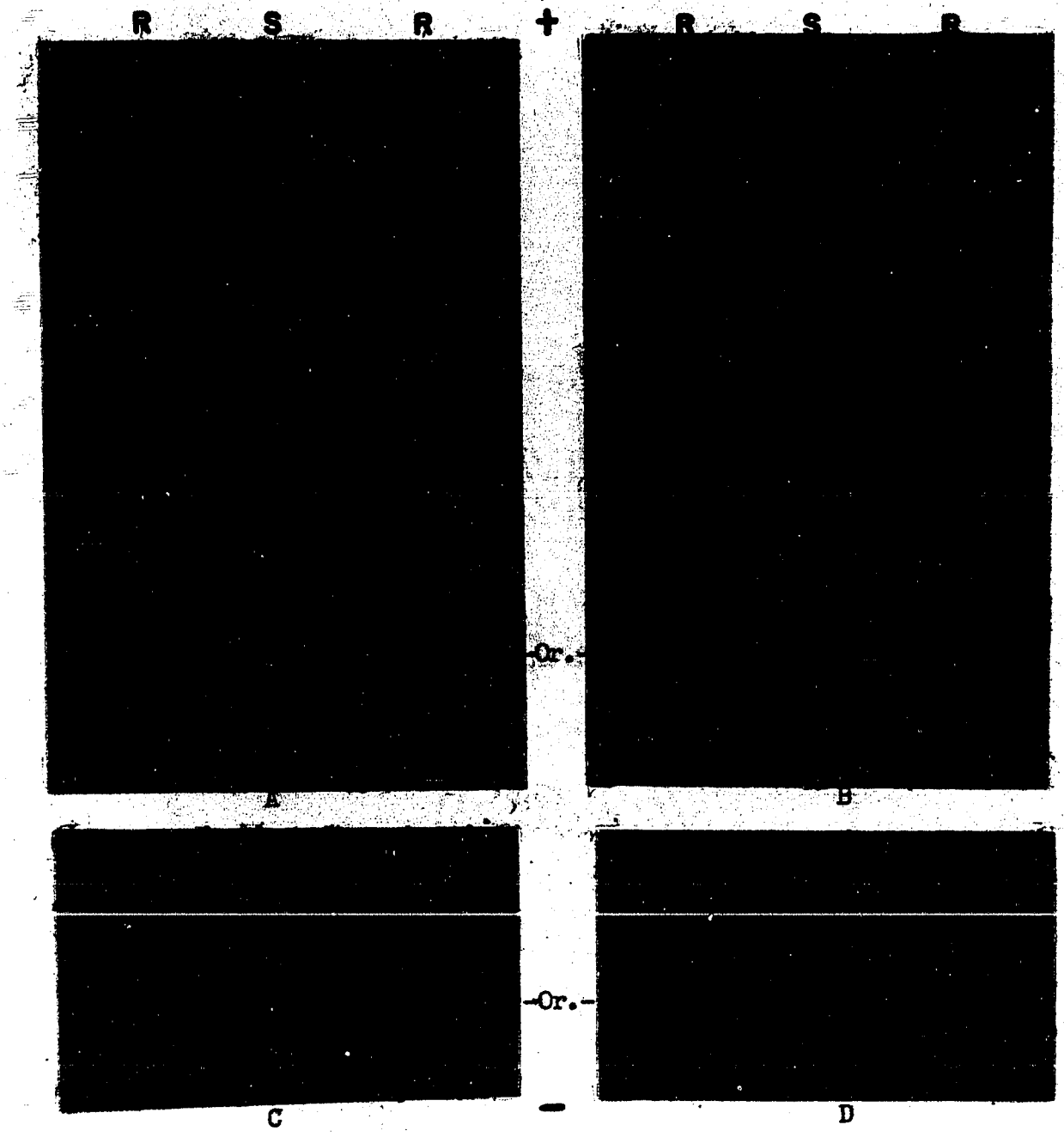


Fig. 14. Zymograms illustrating the possible degradation of 1 x 10^{-4}M oxygen analogue of fenthion by phosphatases. All plates contained inhibitor.

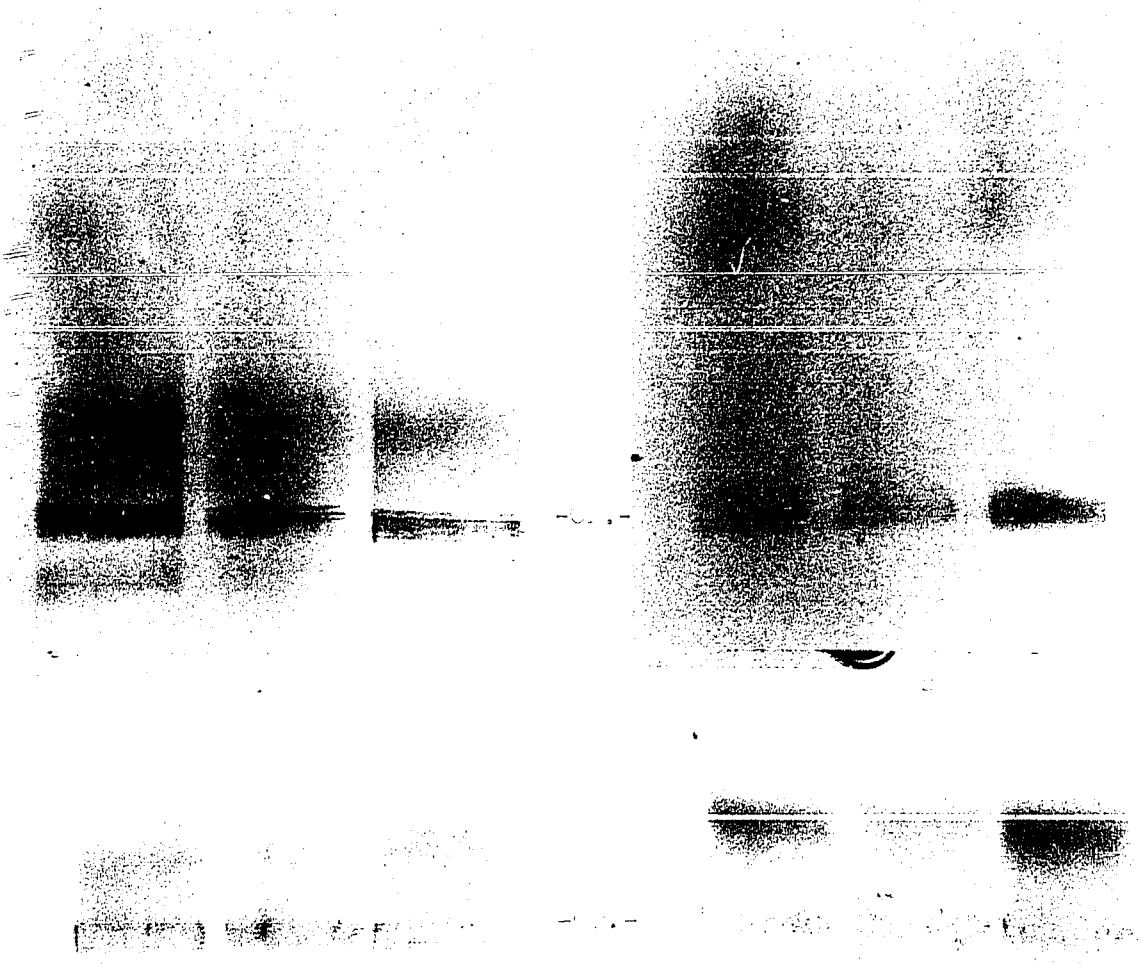
A. Areas of uninhibited AChE (INT method) and of hydrolysis of inhibitor.

B. Alkaline phosphatases (pH 8.6) - β naphthyl phosphate, 2 hr inc., 23°C.

C. Acid phosphatases (pH 5.0) - β naphthyl phosphate, 18 hr inc., 23°C.

D. Acid phosphatases (pH 5.0) - α naphthyl phosphate, 2 hr inc., 23°C.

R S R + R S R



β

β

α

DISCUSSION

The studies *in vivo* of the uptake and retention of P^{32}-labelled fenthion, and of its oxidative and hydrolytic metabolism, yielded the most clear-cut information on the probable mechanisms of resistance. It was quite clear that fenthion-resistant larvae of the R strain took up less fenthion than the S strain when exposed to suspensions of this OP compound and that here is undoubtedly a mechanism contributing to their resistance; this mechanism was also involved in the fenthion-tolerance of the I strain larvae. The inter-strain difference was most pronounced under the conditions of shorter exposure and higher dosage, a combination which resulted in strain R absorbing and retaining only about half as much radioactivity as strain S.

Although the two factors, decreased absorption and increased excretion, have been experimentally separated in larvae of *A. aegypti* (Matsumura and Brown, 1961b, 1963a), this was not done in this investigation since those authors' conclusions were the same as if they had not separated them. That reduced penetration as distinct from increased excretion may indeed be a factor in fenthion-resistance was shown

by Metcalf, Fukuto and Winton (1963) in the SC strain of houseflies resistant to Chlorthion and malathion. Whichever factor it is - decreased penetration or increased excretion -, in these resistant C. fatigans larvae, either or both would be quite consistent with the low-level non-specific tolerance expected in a laboratory-selected strain or one that is just starting to develope resistance.

In larvae of the moderately-tolerant I strain, reduced uptake and retention constituted in fact the only resistance factor, for this strain did not convert significantly more fenthion into water-soluble products than strain S. But the resistant R strain achieved a significantly greater hydrolysis of fenthion than strain S; this greater hydrolytic activity must be another resistance mechanism, since the water-soluble metabolites found are ionic, and thus may be expected to be non-toxic (O'Brien, 1967). The detoxication of fenthion and its oxygen analogue, as indicated by assays of the chloroform-soluble fractions of larvae exposed to high concentrations for a short time, is such that the R strain has at the most only 1/3 as much of these toxicants as the S strain. An even greater interstrain difference of 1/7 was found with the longer exposure of 12 hr (Fig. 15), correlating fortuitously with the resistance ratio of 7-9-fold characteristic of the R strain. It may

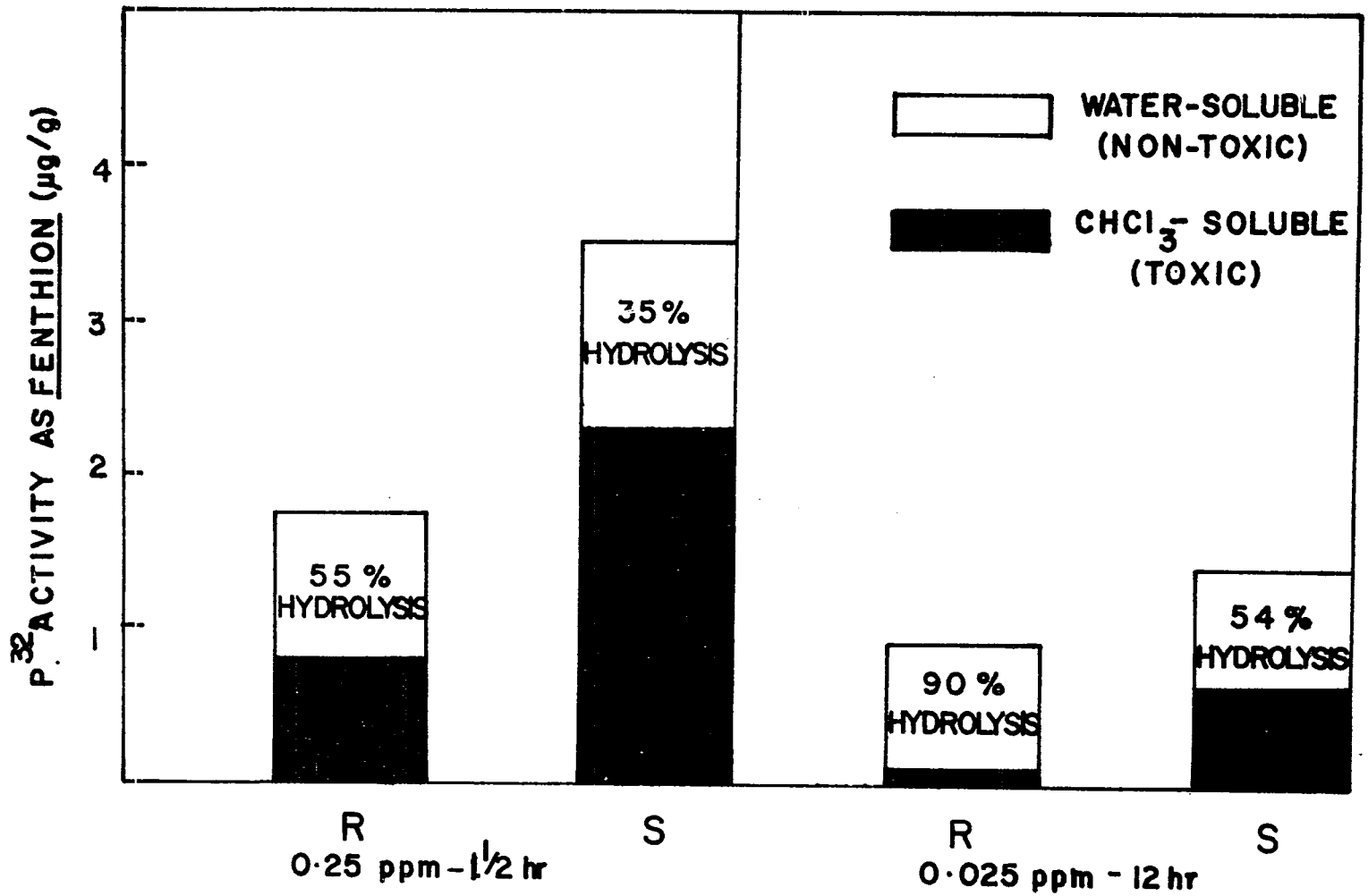


Fig. 15. Schematic diagram showing fenthion absorption and hydrolysis.

be that this additional difference was due to the longer-term toxic effects preferentially inhibiting the hydrolysis more in the S strain (Winteringham, 1962).

The larval toxicities of aqueous suspensions of the oxidative metabolites relative to fenthion were roughly in inverse proportion to the degree of oxidation, which may therefore reflect their relative stability to hydrolysis. These results were similar to those obtained with susceptible Culex pipiens quinquefasciatus larvae by Metcalf, Fukuto and Winton (1963), although for susceptible NAIDM houseflies they found that all oxidation products except the sulphone were of approximately the same degree of toxicity. The amount of resistance shown by the resistant SC strain of houseflies to these metabolites was roughly proportional to their degree of oxidation (Metcalf, Fukuto and Winton, 1963), due to the increasingly greater ease of hydrolysis of the more oxidised compounds by the resistant strain. However, such a correlation was not clearly demonstrated in the present study, except that there was almost twice as much resistance to the unstable S-methyl isomer as to fenthion; the correlation may have been obscured by the general instability of all the oxidised metabolites of fenthion in aqueous suspensions.

Thus detoxication is additive to reduced absorption in strain R, which therefore possesses two resistance mechanisms. Metcalf, Fukuto and Winton (1963) found two similar mechanisms in fenthion-resistant houseflies. However, the I strain possessed only the single mechanism of decreased uptake and retention. The difference between the resistant strains is consistent with the higher, more stable resistance of strain R and the lower, less stable tolerance of strain I.

Oxidative metabolism of fenthion obviously proceeds as readily in C. fatigans as in other insects, mammals and plants (Brady and Arthur, 1961; Fukuda and Miyahara, 1962; Niessen, Tietz and Frehse, 1962; Tomizawa, et al., 1962; Metcalf, Fukuto and Winton, 1963; Knowles and Arthur, 1966), and all of the possible oxidative metabolites are produced although not under all conditions. Fenthion or its oxygen analogue appeared to be less readily oxidized at the thio-ether sulphur by strain R than by strain S; but it is more likely that the oxygen analogue or its sulphoxide and sulphone were more easily hydrolysed in the R strain, since chemical oxidation of the methyl-thio group of derivatives of the closely related fensulphothion (Benjamini, Metcalf and Fukuto, 1959) and of fenthion itself is known to proceed very readily

(Metcalf, Fukuto and Winton, 1963), as does biological oxidation of the same group of fenthion in houseflies and the German cockroach (Brady and Arthur, 1961). Further supporting evidence of this possibility is the comparative lack of the oxygen analogue in R larvae on short-term exposure to fenthion, and the abundance of the sulphoxide and sulphone of the oxygen analogue in the R strain.

Hydrolysis of fenthion resulted in dimethyl phosphorothoic acid appearing in the water-soluble fraction; this appeared to be the main pathway of degradation and was of equal importance in both strains in terms of the percentage fenthion hydrolysed. Hydrolysis of the oxygen analogue resulted in dimethyl phosphoric acid; the R strain was relatively more active in producing this than the S strain, perhaps suggesting that it possessed a more active "oxonase" as did the fenitrothion-resistant SC strain of houseflies (Hollingworth, Metcalf and Fukuto, 1967).

The in vitro comparisons of esterases and their possible degradation of fenthion or its oxygen analogue provided confirmatory evidence of the hydrolytic mechanism, although in certain respects it was not clear-cut. Larval homogenates of the R strain did hydrolyse more fenthion than those of the S strain but the difference was not statistically significant; it may be that the difference was in the

"oxonase" rather than the "thionase", and that the oxygen analogue should have been used as the test substrate since in the organism the phosphoryl sulphur would have had to be oxidized first before an oxonase could attack. However, it is equally likely that co-factors readily available *in vivo* were lost during the homogenization, and that in the homogenate assays they should have been replaced or activators used such as Mn^{++} ions (Krueger and Casida, 1961).

The detection on zymograms of an esterase band insensitive to fenthion and its oxygen analogue which are quantitatively more active in the R than the S homogenates provides some evidence of a resistance mechanism based on hydrolysis by esterases, because OP-insensitive aromatic esterases are known to hydrolyse OP compounds (Aldridge, 1953b). This band found in *C. fatigans* may not be the true A esterase found in mammalian sera (Aldridge, 1953b) or the aromatic esterase found in the honey bee and American cockroach by Metcalf et al. (1956), since it was not completely insensitive to 10^{-7}M paraoxon. The paraoxon-insensitive esterase band in *Aedes aegypti* is also different, because the 20-min. incubation period employed for *C. fatigans* is not long enough to ensure its appearance (M. Ziv, personal communication, 1968). Therefore, the fenthion-insensitive band demonstrated may be a specific degrading enzyme which is

present in increased amount in strain R as a consequence of the fenthion selection. That a phosphatase-type hydrolysis is involved is supported by the finding in strain R of quantitatively more active acid and alkaline phosphatase insensitive to the oxygen analogue of fenthion; more active phosphatases were also detected electrophoretically in malathion-resistant houseflies (Menzel, Craig and Hoskins, 1963) and in a malathion-resistant strain of the green rice leafhopper (Kasai and Ogita, 1965).

Final supporting evidence for detoxication by phosphatases was provided by the finding that the oxygen analogue of fenthion was hydrolysed in the specific zymogram zones occupied by the phosphatase bands. This technique, suggested in part by the esterase inhibition methods developed by McKinley and Read (1961, 1962) and by Menn and McBain (1966) for detecting OP pesticides on thin-layer chromatograms, appears to be of some promise for resistance studies but requires further experimentation. Among possible artifacts, diffusion of both AChE and the degrading enzymes may have occurred in the zymograms, an effect on esterase bands previously noted when Mn^{++} ions were included in the agar. There may also have been some increased inhibition of the R esterases in the acid phosphatase-esterase zone by the Mn^{++} ions; although they were added to activate

the esterases they slightly inhibit the hydrolysis of DFP by rabbit serum (Mounter, 1954). The negatively-migrating bands seen in this zymogram but not in any of the others may be due to electroendosmotic effects. Despite the shortcomings of this technique, still in the developmental stage, it did provide confirmatory evidence for the degradation of the oxygen analogue of fenthion in the general zones of the two phosphatase enzymes. Thus hydrolytic detoxication by esterases can join decreased uptake as the important mechanisms for fenthion-resistance in C. fatigans.

SUMMARY AND CONCLUSIONS

Larvae of a strain of Culex fatigans from Rangoon, Burma, with an 8-fold resistance to fenthion, were compared with the original susceptible Rangoon strain for their uptake of P^{32}-fenthion in vivo, and for their content of specific esterases. When compared by exposure to 0.25 p.p.m. for 1.5 hr or to 0.025 p.p.m. for 12 hr, larvae of the fenthion-resistant R strain came to contain only 50% to 65% of the total radioactivity taken up and retained by the S strain. A slightly tolerant I strain with 3-fold fenthion tolerance came to contain 87% as much as the S strain.

Larvae of strain R also degraded 50% to 90% of the fenthion absorbed to non-toxic water-soluble metabolites as compared to 35% to 54% in strain S, the difference being statistically significant. Larvae of strain I degraded 47% of the fenthion absorbed to water-soluble metabolites, not significantly different from the S larvae. The combination of the factors of uptake and degradation resulted in larvae of strain R coming to contain only 1/3 to 1/7 as much fenthion and derived toxicants as those contained by strain S. The I strain contained 7/10 as much toxicants

as the S strain. It is therefore concluded that reduced uptake and retention of fenthion is a resistance mechanism in both strains, and that a major increase in hydrolytic detoxication was a strong additional mechanism in strain R.

Among oxidative metabolites, the sulphoxide and sulphone of the oxygen analogue were the most common in R and S larvae tested at 0.25 p.p.m. for 1.5 hr. However in the I strain exposed to 1.25 p.p.m. for 2 hr the most abundant metabolite was the oxygen analogue of fenthion. Among hydrolytic metabolites, dimethyl phosphorothioic acid was the most abundant, followed by an unidentified metabolite and then dimethyl phosphoric acid. Strain R came to contain a smaller proportion of the oxygen analogue and its oxidation products than the other strains. This interstrain difference was probably due to increased oxonase hydrolysis rather than thionase, since unchanged fenthion persisted in these R larvae, and they produced a higher proportion of dimethyl phosphoric acid than the S larvae.

Homogenates of the R strain were found to degrade more P^{32}-fenthion than those of the S strain, but the difference was not significant. However, agar-gel electrophoresis did reveal phosphatase bands, insensitive to fenthion and its oxygen analogue and therefore probably detoxifying enzymes , that were considerably more active in the R than

the S strain. Evidence was obtained that these bands did degrade the oxygen analogue of fenthion. Moreover, the esterases which were mainly of the same electrophoretic mobility, when tested on α-naphthyl and β-naphthyl acetate were found to be twice as active in the R strain as in the S strain.

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APPENDIX I

Effect of sodium fluoride and sesamex on the toxicity of fenthion to fenthion-resistant and susceptible larvae of *Culex fatigans*.

The finding that the phosphatase inhibitor, sodium fluoride, only slightly synergized malathion against malathion-resistant *Culex tarsalis* larvae (Matsumura and Brown, 1961a) was corroborating evidence of the relative unimportance of phosphatases as compared with carboxyesterases in the hydrolysis of malathion. The finding that sesamex suppressed dithion-resistance in the housefly (Oppenoorth, 1967) suggested the participation of microsomal detoxication (Nakatsugawa, Tolman and Dahm, 1967) in this resistance.

As phosphatases clearly were implicated in the detoxication of fenthion by larvae of the R strain of *C. fatigans* and as microsomal hydrolases may also be involved, it became desirable to test both sodium fluoride and sesamex as synergists. Accordingly sodium fluoride was added at 2 p.p.m. and sesamex at 100 p.p.m. to the suspensions of fenthion used for larval susceptibility tests, and the

following LC_{50} values were obtained:

	R	S
Fenthion	0.012 p.p.m.	0.0018 p.p.m.
Fenthion + sodium fluoride	0.010 p.p.m.	0.0016 p.p.m.
Fenthion + sesamex	0.008 p.p.m.	0.0015 p.p.m.

It is seen that sodium fluoride had little synergistic effect for either strain and sesamex was only slightly synergistic to strain R.

Although this lack of synergism may have been due to the absence of phosphatase or microsomal detoxication, it may equally have been due to the doses of synergist being sub-threshold, or unstable in water, or to the failure of the larvae to take up the synergist.

APPENDIX II

Preliminary evaluation of solvent systems for the thin layer chromatographic separation of oxidative metabolites of fenthion.

Thin-layer chromatographic separation of the oxidative metabolites of fenthion was chosen in preference to paper chromatography because of its speed and sensitivity. The remaining major advantage of paper chromatography is that radioactive spots may be readily cut out for accurate counting in scintillation fluid, without recourse to scraping as with glass plates coated with thin layers. This was offset by the use of silica-gel-coated polyester sheets which also allowed the strip on which the radioactive sample had been chromatographed to be removed (for scanning) from the rest of the sheet on which the reference metabolites have been separated.

However, difficulty was experienced in finding a suitable solvent system to separate the 6 compounds which may have been present. Although partial separation was obtained with a number of these (Appendix Table 1) none were really satisfactory. Although 7:3 heptane-acetone was adopted as the standard solvent, it was later replaced by the far superior 3:1 benzene-ethyl acetate mixture (Table6).

Appendix Table 1. Approximate R_f values for fenthion and its oxidative metabolites* in various solvents used for thin layer chromatography in silica gel**

	Colour with DCQ + Bromine	Chloroform + Methanol (40:1)***	Heptane + Acetone (7:1)	Benzene + Methanol (7:3)	Benzene + Acetone (7:2)
Fenthion	Red	1.0	0.50-0.58	0.94	0.93
Sulphone of fenthion	Red	1.0	0.12-0.18	0.83	0.92
Oxygen analogue of fenthion	Faint yellow-brown	0.70	0.12-0.16	0.69	0.80
Sulphoxide of fenthion	Red	0.48	0.04	0.54-0.65	0.72
Sulphone of oxygen analogue	No colour	-	0.0-0.03	-	-
Sulphoxide of oxygen analogue	No colour	-	0.0	0.59	0.31-0.41

*All compounds quenched strongly except the sulphone of fenthion (which was a weak quencher).

**Eastman chromagram K301R sheet with or without fluorescent indicator.

***Phase separation on sheet.