METABOLISM OF MALATHION BY A RESISTANT AND A SUSCEPTIBLE STRAIN OF *CULEX TARSALIS*: I. DEGRADATION *IN VIVO* AND IDENTIFICATION OF ORGANIC SOLUBLE METABOLITES[1]

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Organophosphate insecticides have been used successfully to combat species of arthropod pests which have developed resistance to chlorinated hydrocarbons; however, the development of resistance to certain organophosphates may limit their use as well. Resistance to organophosphate insecticides has been reported in 17 species of insects and 12 species of plant-feeding mites (Brown 1961). Organophosphate resistance in mosquitoes was first reported by Gjullin and Isaak in 1957; they observed populations of *Culex tarsalis* Coq. from Fresno, California, which had developed resistance to malathion after the insecticide had been used in the field for less than three years. Laboratory bioassays indicated LC_{50} and LC_{90} values which were 21 and 33 times greater, respectively, than values for larvae from untreated areas located near Fresno.

The mechanism of organophosphate resistance has been most frequently investigated in house flies (van Asperen and Oppenoorth 1959, 1960, March 1959, Mengle and Casida 1960, Plapp and Bigley 1961). Recently attention has also been directed toward the study of phosphate resistance in mosquitoes (Bigley and Plapp 1962, Dauterman and Matsumura 1962, Matsumura and Brown 1961). Preliminary studies of the metabolism of malathion in resistant and susceptible *C. tarsalis* were reported by Lewallen and Nicholson (1959). Using chromatographic methods in a purely qualitative study, they did not detect unchanged malathion in the larval water extracts of either strain; however, a compound corresponding to the R_f of malaoxon was present in the chromatograms from both strains. This method favored detection of the more water soluble malaoxon over malathion.

To obtain more quantitative data on the metabolism of malathion in both resistant and susceptible mosquito larvae, the tracer studies reported below were undertaken.

MATERIALS AND METHODS. Since 1957 a resistant strain of *C. tarsalis* has been reared in this laboratory under malathion pressure. When the tests reported in this paper were performed, the LC_{50} and LC_{90} values for the strain were 7 and 16 times greater, respectively, than values for larvae from untreated areas. The normal or susceptible strain used in these tests was a subcolony obtained in 1956 from the U. S. Public Health Service Encephalitis Laboratory at Bakersfield, California.

Malathion incorporating the phosphorus-32 atom was synthesized following the technique of Krueger and O'Brien (1959), using an isotope exchange technique as developed by Casida (1958). Fifty millicuries of radioactive phosphoric acid, obtained from the Oak Ridge National Laboratory, were used in the exchange with phosphorus pentasulfide. Solvent extraction according to O'Brien (1960) and column partition chromatography (Bowman and Casida, 1957) were used for purifying the final product. The

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fraction used was identical in infrared spectrum analysis with non-labeled malathion, obtained from the manufacturer (Beckman IR 5 Spectrophotometer, 10 percent solution in chloroform).

The LC_{50} values for *C. tarsalis* after 24 hours treatment in malathion solution were identical for the radioactive and the authentic non-labeled compound. The product was formed in 75 percent yield with an activity of about 10,000 counts per gamma per minute. All counting was done with a Nuclear-Chicago gas flow counter and a decade scaler. Bioassays of both the susceptible and resistant strains with radioactive malathion were conducted according to the procedure of Lewallen and Nicholson (1959) for non-labeled malathion. The mortality endpoint of the tests was the point at which larvae failed to respond when probed lightly with a sharp instrument.

The *in vivo* fate of radiolabeled malathion was determined as follows: Fourth instar larvae were exposed to the insecticide at 10 p.p.m. in distilled water; at this concentration to obtain 50 percent mortality the susceptible larvae required an exposure of 30 minutes, while resistant larvae required 4 to 5 hours. Solvent extraction-partitioning studies were conducted with groups of 100 larvae treated ¼, ½, 1, 2, 4, 8 and 24 hours. Larvae were then removed from the solutions, washed with distilled water, and homogenized in acetone. The same procedure was followed for paper chromatography studies, except that analysis was made after ½, 4 and 8 hours exposure.

Extracts were partitioned between chloroform and water, and the solvent evaporated. The amount of radioactivity was determined in each portion. Organic solubles were identified for P^{32}=S and P^{32}=O content (Krueger, O'Brien, and Dauterman, 1960). The solvent was evaporated from the organic solubles and the residue was dissolved in hexane and chromatographed on an alumina column. One hundred ml. of benzene were used to elute malathion and 100 ml. of chloroform to elute malaoxon. Finally, 100 ml. of methanol were passed through the column to remove any remaining radioactivity. More than 80 percent of the radioactivity recovered from the alumina column was malathion or malaoxon.

For paper chromatographic studies groups of 200 larvae were homogenized in acetone and centrifuged at 1000 x gravity for 5 minutes. The solution applied to paper strips was obtained by evaporating the supernatant liquid to dryness and then reconstituting with 0.2 ml. of acetone. A 0.25 cc. syringe with a blunt 27 gauge needle was used to triple-spot one-lambda drops on the paper strips.

One-dimensional reversed phase paper chromatograms of malathion and malaoxon gave R_f values similar to the pure non-labeled compounds spotted in acetone. Strips of Whatman number 1 filter paper impregnated with 1 percent Dow-Corning Silicone 550 in petroleum ether were employed (March *et al.*, 1954); the mobile phase was the lower portion of a 10:10:1 mixture of N-butanol, water, and propionic acid. Radioactivity in the paper chromatographic strips was counted by adding a 5 mm. slit window to the gas flow detector; a modified planchet holder was employed to allow hand feeding of the strips in 5 mm. increments.

Treatment solutions were partitioned with chloroform and the amount of radioactivity in both fractions was determined immediately after removal of larvae by solvent extraction and paper chromatography techniques.

RESULTS. Data on solvent extraction, partitioning and column separation of of metabolites in larval homogenates are summarized in Figures 1–4. The number of micrograms of water soluble P^{32} compounds increased progressively in both strains (Fig. 1). A two-fold or greater difference between the susceptible and resistant strains occurred by two hours and continued through 24 hours, indicating more rapid hydrolysis in the resistant larvae. The amounts of chloroform solubles were similar at early treatment times, indicating similar rates of penetration (Fig. 2). The amounts of water and

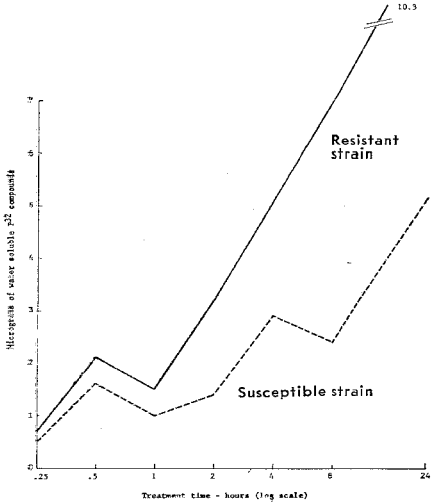

FIG. 1.—Water soluble P^{32} compounds recovered from homogenates of 100 larvae of resistant and susceptible strains of *Culex tarsalis* treated with radiolabeled malathion for various time periods.

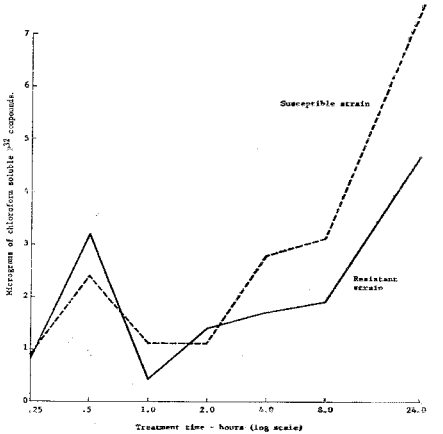

FIG. 2.—Chloroform soluble P^{32} compounds recovered from homogenates of 100 larvae of resistant and susceptible strains of *Culex tarsalis* treated with radiolabeled malathion for various time periods.

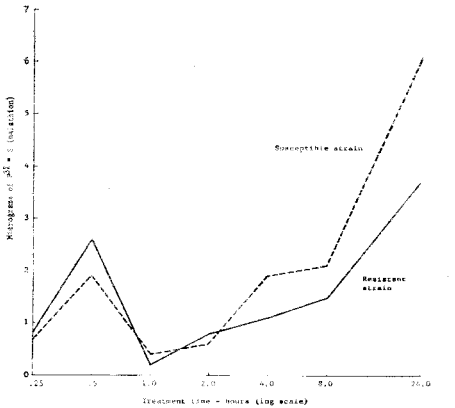

FIG. 3.—P^{32}=S (malathion) content of chloroform soluble compounds recovered from homogenates of 100 larvae of resistant and susceptible strains of *Culex tarsalis* larvae treated with radiolabeled malathion for various time periods.

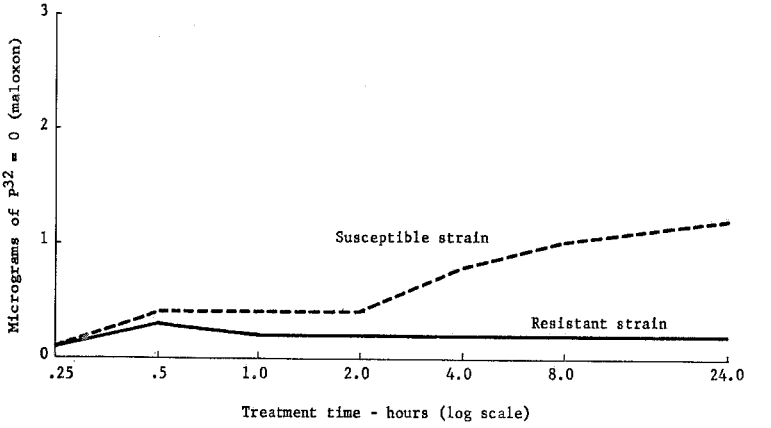

FIG. 4.—P^{32}=O (maloxon) content of chloroform soluble compounds recovered from homogenates of 100 larvae of resistant and susceptible strains of *Culex tarsalis* larvae treated with radiolabeled malathion for various time preiods.

chloroform solubles at 30 minutes were high in both resistant and susceptible strains.

Data from longer periods of treatment indicate that resistant larvae contained smaller amounts of chloroform solubles than were found in susceptible larvae. Chloroform solubles were further analyzed for P=S (malathion) and P=O (malaoxon) content. The P=S levels increased in both strains at nearly the same rate (Fig. 3); the P=O content remained consistently lower in the resistant larvae, especially after four or more hours of treatment (Fig. 4).

Data summarizing the number of micrograms of P^{32} recovered by extracting and partitioning the treatment solution are given in Figure 5. These results as well as those in Figures 1–4 are averages of 3 determinations, each replicated twice and each replicate counted 3 times. Non-mosquito related hydrolysis is also indicated. Analysis of chloroform solubles from the treatment solution for P=S and P=O content was not made. Analysis of water solubles in the treatment solutions showed little variation between strains when larvae were exposed for less than 8 hours; however, more hydrolytic products and less chloroform soluble material were produced than when no mosquitoes were present. Chloroform solubles in the treatment solution were slightly greater for the resistant than susceptible strains when exposed up to 8 hours. Twenty-four-hour data indicated that resistant larvae produced more hydrolytic products and removed more chloroform solubles than did the susceptible larvae. The amount of chloroform and

TABLE 1.—Paper chromatographic analysis of homogenates of groups of 200 susceptible and malathion resistant *C. tarsalis* larvae for metabolites of radiolabeled malathion.[1]

Treatment time	Mg. P^{32} recovered from larval homogenates at indicated R_f's	
	0.07 (malathion)	0.90 (malaoxon)
30 minutes		
Susceptible	0.18	0.10
Resistant	0.08	0.13
4 hours		
Susceptible	0.06	0.04
Resistant	0.02	0.10
8 hours		
Susceptible	0.08	0.16
Resistant	0.01	0.05

[1] These results, and those in Table 2 are the average of two separate determinations, each with three replicates, and each replicate counted three times.

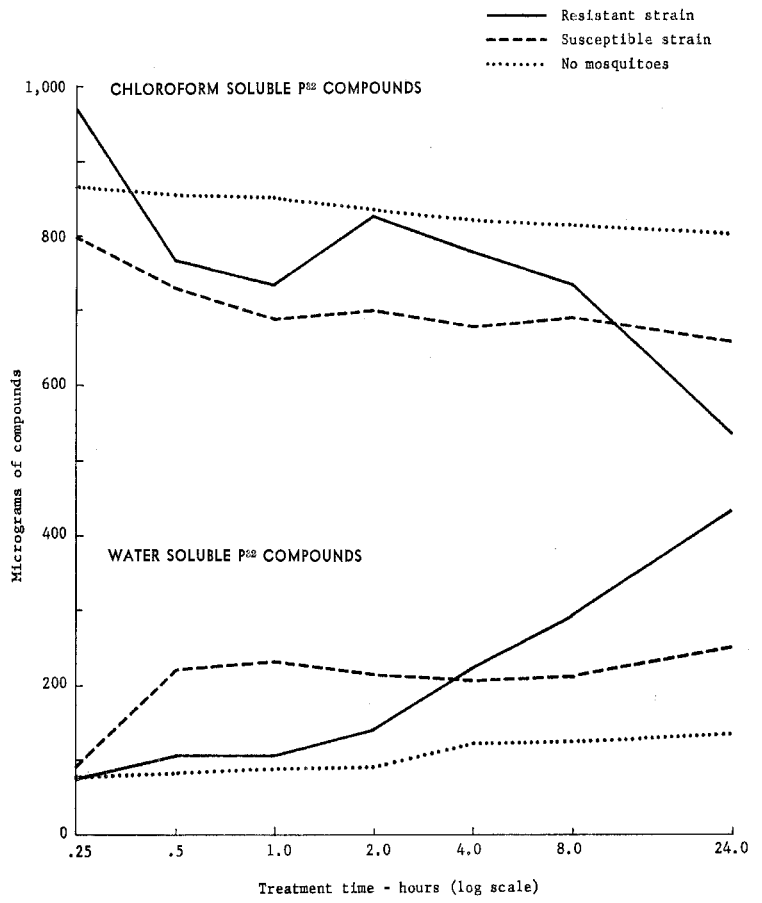

FIG. 5.—Compounds recovered from radiolabeled malathion treatment solutions following removal of resistant and susceptible *Culex tarsalis* larvae treated for various time periods.

water solubles recovered from treatment solutions of both susceptible and resistant strains are similar at the time larvae have greater than 50 percent but less than 100 percent mortality.

Analysis of homogenates of susceptible and resistant larvae showed no similarity in amounts of water solubles when there was greater than 50 percent but less than 100 percent mortality (at 30 minutes in the susceptible and 4 hours in the resistant strain). There was, however, some similarity between strains in the amount of malathion recovered at these times. Malaoxon levels, on the other hand, were twice as high in the susceptible larvae as in the resistant at these times when the symptoms were the same.

Table 1 summarizes the data from paper chromatographic studies of larval homogenates of both strains following treatment with radiolabeled malathion. The concentrations of malathion and malaoxon at their R_f's are indicated. In general, the malathion levels decreased in both susceptible and resistant larvae as the treatment time increased.

Malaoxon levels were nearly the same

in the larvae of both strains of mosquitoes after 30 minutes of treatment; the levels then decreased in both strains as treatment time increased to 4 hours. After 8 hours' treatment the susceptible larvae, which by then were all dead, contained higher levels of malaoxon than at 30 minutes. Meantime, malaoxon content in the resistant larvae which were partially affected by 8 hours continued to decrease. nearly the same rate. The P^{32} labeled chloroform solubles consisted entirely of malathion; malaoxon was not detected at any time in the treatment solution. The malathion levels are nearly alike in the treatment solutions of both strains.

DISCUSSION. Analysis of larval homogenates indicated similar penetration of both strains, similar concentrations of malaoxon formed from malathion, and similar de-

TABLE 2.—Paper chromatographic analysis of radiolabeled malathion treatment solution following removal of susceptible and malathion resistant *C. tarsalis* larvae.

	Mg. P^{32} recovered from treatment solutions at indicated R_f's		
	0.07 (malathion)	0.90 (malaoxon)	1.0 (unknown)
Chloroform solubles			
Treatment time			
30 minutes			
Susceptible	1.9	None	None
Resistant	1.5	None	None
4 hours			
Susceptible	2.1	None	None
Resistant	1.0	None	None
8 hours			
Susceptible	1.1	None	None
Resistant	0.9	None	None
Water solubles			
Treatment time			
30 minutes			
Susceptible	0.3	None	0.20
Resistant	0.3	None	0.17
4 hours			
Susceptible	None	None	0.48
Resistant	None	None	0.65
8 hours			
Susceptible	None	None	1.05
Resistant	None	None	0.90

Results of the paper chromatographic examinations of both water and chloroform solubles in the treatment solution are shown in Table 2. The concentrations are indicated for malathion, malaoxon, and unidentified material with an R_f of 1.0, a highly polar material or materials which follow the mobile phase to the front (similar material was observed on chromatograms of the larval homogenates). After the first 30 minutes the water soluble P^{32} portion apparently consisted entirely of this unknown material. The amount of this material increased in the treatment solutions of both strains at gradation of malathion; however, hydrolysis of malaoxon occurred more rapidly in the resistant strain. These results agree with the findings of Matsumura and Brown (1961) who concluded that modest malathion resistance of about 9 fold in *C. tarsalis* can be accounted for by destruction of malaoxon alone.

The effects of morbidity and death on the ability of larvae to metabolize malathion are certainly not clear-cut. While water and chloroform solubles increased throughout the length of exposure of both strains regardless of the condition of the larvae, the level of malaoxon remained

constant in the resistant strain during periods of increasing mortality; this suggests that resistant larvae have a constant conversion of malathion to malaoxon, or the excretion or degradation of all malaoxon above a certain level. These explanations are doubtful in view of increasing malathion levels in both strains (Fig. 3) and the analysis of the treatment solution for malaoxon (Table 2), indicating that none was present. A nearly constant level of malaoxon in resistant larvae was recorded by Matsumura and Brown (1961) when larvae were exposed to malaoxon in solution continuously up to 5 hours; however, removal of larvae from the treatment solution after 1.5 hours resulted in a decline in malaoxon in the larval homogenate determined 20 hours after exposure had begun.

The peak level of metabolites after 30 minutes' treatment apparently was not associated with the resistance phenomenon, since it occurred equally in both strains. Furthermore, these high levels were not necessarily associated with morbidity or mortality, since malathion and malaoxon levels in the larvae were high and effects upon the larvae were not fully manifested. It is perhaps significant, however, that large quantities of water solubles occurred with the high values of chloroform solubles after 30 minutes of exposure, indicating rapid degradation of malathion in both strains.

Results from the analysis of treatment solutions indicated that morbidity and mortality of larvae had little effect upon degradation and excretion through 8 hours of treatment. Even though larvae were moribund or dead, they apparently continued some degradation and excretion. By 24 hours, however, excretion had apparently ceased. The results showed a substantial increase in malathion and water solubles in larval homogenates at the 24-hour period. This was probably due to less excretion of metabolites or unchanged malathion while the malathion was still being absorbed. Malaoxon levels did not increase sharply at this time, indicating that a high level of degradation was still taking place. Considering that each 100 ml. of treatment solution contained 1 mg. of radioactive malathion, the total water and chloroform solubles present in the larvae of either strain at any one time was very small. The greatest observed concentration, 15 ug., (1.5 percent of the applied concentration) was present in the resistant larvae after 24 hours treatment. The rapid rate of degradation and excretion is apparent, since the solubles in the larvae represented only 3 percent of the total water soluble metabolites present in the treatment solution (Fig. 5).

The water soluble metabolites excreted into the treatment water were highly polar. Paper chromatography indicated an R_f of 1.0. Investigations are underway to determine the exact nature of these water soluble metabolites.

Data from the application of radioactive malathion in solution to resistant and susceptible larvae of *C. tarsalis* indicated that resistant larvae were more efficient in degrading the insecticide *in vivo* than were susceptible larvae. A difficulty in the interpretation of these data lies in the fact that a period existed during which the strains had different rates of toxic response. The period of greatest difference exists between 30 minutes and 8 hours in these experiments; prior to 30 minutes exposure all larvae in both strains appeared normal. By 24 hours, however, both strains were dead. It is in the period between these two extremes that correlation of insecticide metabolism is extremely difficult. However, since a single discriminating dosage had been used to treat both strains, one may examine the concentrations of metabolites in larval homogenates at the time of approximately 50 percent mortality. Results (Figs. 1–4) indicated that water soluble metabolites were 4 times more concentrated in resistant larvae at the LT_{50} (4 hrs.) than in susceptible larvae at the LT_{50} (½ hr.); unchanged malathion was about equal, and malaoxon was present in twice the concentration in susceptible larvae as in the resistant strain.

One would expect rather that similar symptoms would be caused by similar concentrations of the toxicant.

Homogenizing whole mosquitoes may mask the concentration of toxicant at some particular site where small concentrations may affect the vital system. The time required for penetration to this site may be important and the concentration of the material throughout the animal perhaps is not significant. We are now examining this aspect of the problem in our laboratory.

SUMMARY. Larvae of a resistant and a susceptible strain of *Culex tarsalis* were treated with a discriminating dose of radiolabeled malathion. At various times up to 24 hours the larvae were homogenized, extracted and the insecticide metabolites identified qualitatively and quantitatively using column and paper chromatography. While at this time the water soluble metabolites were considered as a single group, the chloroform soluble constituents were further identified as the original malathion and its toxic oxidation product, malaoxon.

This study indicates a similar rate of penetration of malathion into both the resistant and susceptible strains, similar rates of conversion of malathion to malaoxon and similar degradation of malathion; however, hydrolysis of malaoxon occurred at twice the rate in the resistant strain as in the susceptible strain. It was observed that malaoxon concentrations were not similar in the two strains at the time of similar symptoms (LT_{50}). This led to the supposition that homogenizing whole larvae had masked the concentration at a vital locus.

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