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Carbamate Resistance in Mosquitos

The Metabolism of Propoxur by Susceptible and Resistant Larvae of *Culex pipiens fatigans* *

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Toxicity tests on Culex pipiens fatigans *with propoxur (*o*-isopropoxyphenyl methylcarbamate) and carbofuran (2,2-dimethyl-2,3-dihydrobenzofuranyl-7-methylcarbamate) indicated that both compounds are fast-acting insecticides. Transfer of treated larvae to fresh water results in their partial recovery from knockdown.*

Propoxur is metabolized by resistant and susceptible larvae by their homogenate-reduced nicotinamide–adenine dinucleotide phosphate ($NADPH_2$) enzyme system and by the microsome-plus-soluble fraction of mouse-liver extracts to at least 10 organosoluble metabolites with the isopropoxy group intact. The major metabolites, which are primarily hydroxylation products or the result of degradation of these products, have tentatively been identified as: acetone plus o*-hydroxyphenyl methylcarbamate, 2-isopropoxy-5-hydroxyphenyl methylcarbamate, 2-isopropoxyphenyl carbamate, and 2-isopropoxyphenyl* N*-hydroxymethylcarbamate. Upon incubation of water-soluble products from treated larvae with β-glucosidase, β-glucuronidase, aryl sulfatase and acid phosphatase, the conjugates are hydrolysed, liberating mainly hydroxylated carbamates.*

The results indicate that slower absorption as well as faster detoxification by hydroxylation mechanisms, together with conjugation with polar molecules and elimination, are major factors in resistance of mosquito larvae to substituted-aryl methylcarbamate insecticides.

Knowledge of the pathways and rates of metabolism of toxicants in insects is important for an understanding of the mechanisms of selective toxicity, resistance and synergism. The information available at the present time concerning these mechanisms in mosquitos, especially with respect to carbamate insecticides, is extremely limited. The present investigation concerns the fate of propoxur (*o*-isopropoxyphenyl methylcarbamate) in the mosquito *Culex pipiens fatigans* Wiedemann. Propoxur (Baygon) was selected for intensive study because (1) it plays an important role in the control of insect vectors, especially in malaria eradication; (2) it is highly toxic to mosquitos and its structure contains a variety of functional groupings which might be susceptible to metabolic attack; (3) it is moderately synergized by piperonyl butoxide in resistant strains of *C. p. fatigans* (Georghiou et al., 1966); and (4) extensive information is available on its metabolism *in vivo* and *in vitro* in the housefly (*Musca domestica* L.) (Tsukamoto & Casida, 1967a, 1967b; Shrivastava, 1967; Shrivastava et al., 1969) and on the biochemical genetics of resistance to propoxur (Tsukamoto et al., 1968). More limited tests were performed with mouse-liver enzyme systems and on the metabolism of carbofuran (Furadan) (2,2-dimethyl-2,3-dihydrobenzofuranyl-7-methylcarbamate) by enzyme preparations. The present study is a continuation of earlier published work on the development of

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resistance to propoxur in *C. p. fatigans* (Georghiou et al., 1966).

MATERIALS AND METHODS

Chemicals

The sources and specifications of isotope-labelled and non-labelled chemicals and enzyme preparations used during this study were as follows: propoxur (Baygon), Chemagro Corporation, Kansas City, Mo., USA; ^{14}C-propoxur (*o*-isoprop-1,3-^{14}C-oxyphenyl methylcarbamate), specific activity 10 μCi per mmol, WHO, Geneva, Switzerland; *N*-demethylpropoxur (*o*-isopropoxyphenyl carbamate) and *o*-isopropoxy phenol synthesized in this laboratory; *N*-hydroxymethyl propoxur (*o*-isopropoxyphenyl *N*-hydroxymethylcarbamate), Dr S. Abd El-Aziz; carbofuran (Furadan), ^{14}C-carbofuran (2,2-methyl-2,3-dihydrobenzofuranyl-7-methyl-^{14}C-carbamate) specific activity 0.104 μCi per mmol, 3-hydroxy carbofuran (2,2-dimethyl-3-hydroxy-2,3-dihydrobenzofuranyl-7-methylcarbamate), and 3-keto-carbofuran (2,2-dimethyl-3-keto-2,3-dihydrobenzofuranyl-7-methylcarbamate) were synthesized or obtained as reported (Metcalf et al., 1968); acid phosphatase (potato) lyophilized, B grade, 2 enzyme units/mg, β-glucosidase (almond emulsion), 500 enzyme units/mg, and β-glucuronidase (bovine liver), 60–70 enzyme units/mg, were each obtained from Calbiochem, Los Angeles, Calif., USA; and aryl sulfatase, type III (limpets), 5 enzyme units/mg was supplied by the Sigma Chemical Company, St. Louis, Mo., USA. Both ^{14}C-propoxur and ^{14}C-carbofuran had a radiochemical purity of more than 99%.

Mosquitos

The mosquito strains used were as follows: insecticide-susceptible laboratory strain (S-Lab.) and propoxur-resistant (Baygon L) strain selected by propoxur in the larval stage (Georghiou et al., 1966). The resistant strain (R) has been maintained since June 1963 under continuous selection by propoxur; its resistance level was approximately 25 times greater than that of the susceptible (S) strain, the LC_{50} values being 5.8 ppm and 0.23 ppm, respectively.

Toxicity tests

In order to gain more information on the mode of action of carbamate insecticides against mosquitos, larvae of the S and R strains were tested with propoxur and carbofuran at various concentrations, and mortality was recorded at frequent intervals. Exposure of larvae to a treated medium was either continuous or short-term. In continuous-exposure tests, the larvae were exposed to an insecticide solution continuously for 24 hours, and the mortality was recorded after various intervals of time. In the short-term-exposure experiments, the larvae were first exposed to the insecticide solution for the indicated period, i.e., 15, 30, 45, or 60 minutes, and were subsequently washed thoroughly and transferred to fresh water in standard bioassay paper cups in which mortalities were determined. Bioassay tests were made by the standard procedure reported by Georghiou et al. (1966), except for indicated variations.

Limited tests were performed with various synergists in order to extend the data obtained earlier by Georghiou et al. (1966) and to analyse further the mechanism of synergism. Propoxur alone at a concentration of 1 ppm and in combination with 5 ppm of the given synergist was tested on R-strain larvae. Studies also involved the simultaneous exposure of S- and R-strain larvae to combined insecticide–synergist solutions or 2-hour pretreatment of larvae with the synergist alone, followed by 24-hour exposure to synergist–insecticide solutions. In the latter tests, a 5-ppm dose of piperonyl butoxide was followed by varying dosages of propoxur or carbofuran.

General procedures for in vivo *metabolism studies*

Altogether, 200 fourth-instar mosquito larvae were placed in 1.9 ml of distilled water in a 5-ml beaker and 100 μl of a ^{14}C-propoxur saline solution (Shrivastava et al., 1969) was added to give a final concentration of insecticide solution of 0.2 ppm–1 ppm. The larvae and insecticide solution were further treated in one of two ways, either for continuous-exposure or for short-term-exposure studies.

In the continuous-exposure studies, the larvae were exposed to the insecticide solution in the beakers for periods ranging from 0 to 24 hours and at the end of the exposure the medium and larvae were transferred to a conical glass homogenizer tube and homogenized. Each homogenate was then transferred to a conical centrifuge tube. The beaker, homogenizer tube and pestle were washed twice with 1.5-ml portions of distilled water and the rinses were added to the homogenate. The organosoluble metabolites were extracted with 5-ml portions of diethyl ether, and the procedure of extraction was repeated 3 times. The combined ether extracts were evaporated to dryness and spotted on thin-layer chromatographic plates for further analyses.

In order to understand the sequence of events taking place in propoxur-treated larvae and in the medium, an attempt was made to simplify the larval medium. This was achieved partly by allowing the larvae to pick up a certain quantity of propoxur during a short-term exposure and then allowing them to recover and metabolize the picked-up dose in an untreated medium. At the end of the experiment, an analysis of radioactivity in each medium and group of larvae was made. In such studies, the larvae were exposed to the insecticide medium for only 1 hour, after which they were filtered off in a 1.5-inch (3.75 cm) porcelain funnel and further washed with a 3-ml portion of distilled water. The combined medium and wash thus obtained is referred to as "exposure medium". The larvae were transferred from the filter-funnel to a fresh 2-ml volume of distilled water and allowed to recover for various periods. At the end of the recovery period, the larvae were again filtered off and washed and the 5-ml volume of water thus obtained is referred to as "recovery medium". The exposure and recovery media were extracted with ether and the larvae were extracted 3 times with acetone (1.5 ml, 1.5 ml, 2 ml). The acetone extract was also used for thin-layer chromatographic radioautography and radioactivity determinations. The radioactivity of the water layer was determined by counting two 0.1-ml portions of this layer. The times given in Table 1 indicate the length of the recovery period after a given exposure; a zero recovery time indicates that the radioactivity of various fractions was determined immediately after the treatment of the larvae.

In other experiments, water-soluble metabolites of propoxur were analysed by treating the water layer with hydrolases. R-strain larvae (6 g) were added to 30 ml of a 1-ppm solution of ^{14}C-propoxur. The larvae were exposed for 24 hours and then homogenized, 5-ml portions of the homogenate being extracted with 20 ml of ether as described above. After evaporation of the residual ether, the pH of the water layer was adjusted to 4.8 by the addition of citric acid and disodium phosphate. β-Glucosidase, β-glucuronidase, aryl sulfatase or acid phosphatase were added in 10-mg quantities to 5 ml of the water layer and the mixture was incubated at 37°C for 4 hours. At the end of the incubation period, the reaction was stopped by the addition of 5 ml of ether. Organosoluble metabolites were extracted again with 20 ml of ether. In sequential hydrolysis studies, the second hydrolase was added to the water layer after evaporation of the residual ether, and other processes were repeated accordingly. Acid hydrolysis of the conjugate with 1 *N* hydrochloric acid at 37°C for 4 hours was also studied. No attempt was made to determine the radioactivity in the solid residue.

For determinations of the production of acetone during the metabolism of ^{14}C-propoxur, 2 g of larvae of the S or R strains were held in 10 ml of distilled water in a 100-ml conical flask. The medium was fortified with 1 μg or 10 μg of ^{14}C-propoxur for the S and R strains, respectively. The oxygen in the container was replenished every 6 hours. At the end of 24 hours, the larval medium was transferred to a test-tube and fortified with 2 mmol of cold acetone, 2 mmol of semicarbazide hydrochloride and 4 mmol of sodium acetate. The reaction was completed by shaking the mixture at room temperature (24.5°C). The procedure for crystallization and recrystallization of acetone semicarbazone described by Casida et al. (1968) was used.

Procedure for in vitro *metabolism studies*

Mosquito-larvae enzyme preparations. Tests were performed to determine the differences in the ability of freshly prepared enzyme preparations of S- and R-strain larvae to degrade propoxur. Larvae were homogenized in buffer solution to produce a 20% homogenate concentration (wet weight/volume). The buffer consisted of 1 M sodium-potassium phosphate in a 0.25 M sucrose solution at pH 7.5. A typical incubation mixture contained 10 μg of ^{14}C-propoxur, 200-mg equivalents of fresh larval homogenate, 2 μmol of reduced nicotinamide-adenine dinucleotide phosphate ($NADPH_2$) cofactor, with 0.5 mmol of sucrose and 2 mmol of phosphate buffer at pH 7.5 in a total volume of 2 ml. The mixture was incubated in air at 30°C for 2 hours. All the enzyme-preparation operations were carried out at 5°C. At the end of the incubation period, the reaction was stopped by the addition of 5 ml of ether followed by extraction of organosoluble materials with four 5-ml portions of ether, resolution of radioactive metabolites on thin-layer chromatographic plates and quantitation of radioactivity by the procedure described by Shrivastava et al. (1969). A detailed investigation of the appropriate conditions for enzymatic studies in mosquito larvae will be reported elsewhere (Shrivastava et al., unpublished data).

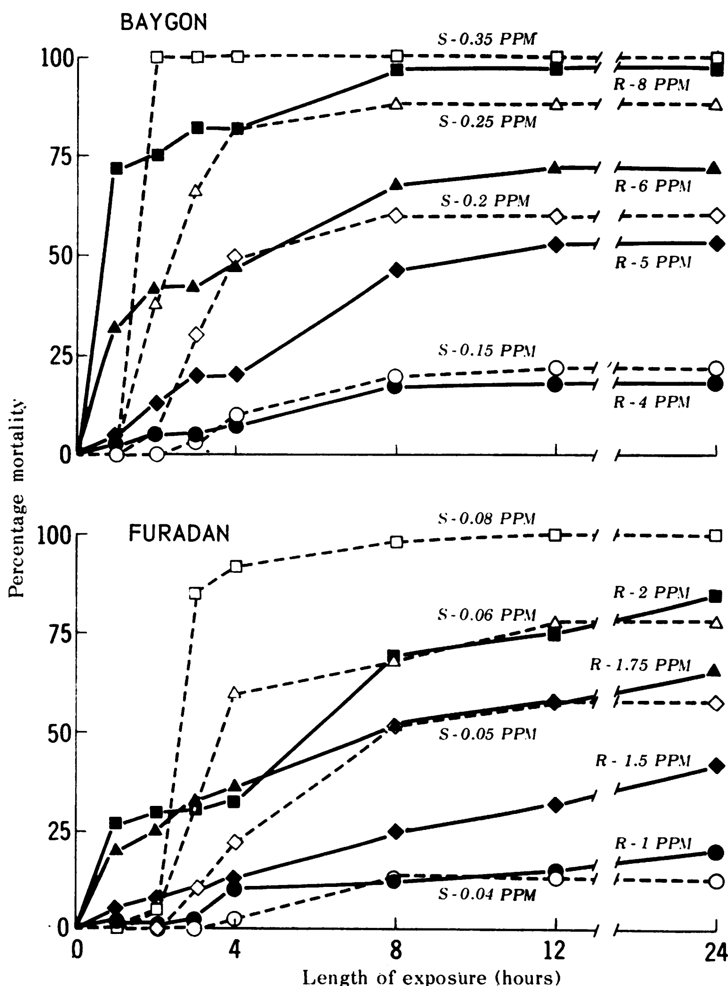
Mouse-liver enzyme preparations. A microsome-plus-soluble fraction was prepared from mouse liver as described by Oonnithan & Casida (1968). The

typical incubation mixture contained a 200-mg equivalent of liver enzyme preparation, 0.2–5.0 μg of ^{14}C-propoxur or 10 μg of ^{14}C-carbofuran, 2 μmol of co-factor or chemical or both, 500 μmol of sucrose and 50 μmol of sodium-potassium phosphate buffer at pH 7.5 in a total volume of 2 ml. The mixture was incubated at 37°C for 4 hours. Further procedures for the extraction of organosoluble metabolites, thin-layer chromatographic radioautography and quantitation of radioactivity have previously been described for houseflies by Shrivastava et al. (1969).

Thin-layer chromatography and identification of metabolites

The thin-layer chromatographic procedures for the resolution of organosoluble metabolites of propoxur were the same as those described by Shrivastava et al. (1969) in which silica-gel G, thin-layer chromatographic plates, 0.25 mm thick, were used. The quantitative analysis of resolved radioactive metabolites, cochromatography and tentative identifications of metabolites were made by procedures described by the following authors: Krishna et al. (1962), Oonnithan & Casida (1968) and Shrivas-

FIG. 1
CUMULATIVE MORTALITY OF SUSCEPTIBLE (S) AND RESISTANT (R) STRAINS OF *C. P. FATIGANS* LARVAE AT VARIOUS TIMES DURING CONTINUOUS EXPOSURE TO DIFFERENT DOSAGES OF PROPOXUR (BAYGON) AND CARBOFURAN (FURADAN)



tava et al. (1969). The major organosoluble metabolites of propoxur formed in living mosquito larvae and in enzyme preparations from mosquito larvae and mouse liver, were tentatively identified by co-chromatography and by comparison with propoxur metabolites in houseflies. The solvent systems used were benzene–ethanol–ether (90 : 10 : 3), and chloroform–ether–*n*-butanol (50 : 1 : 1). The manner in which they were applied has previously been described by Shrivastava et al. (1969). Production of acetone as one of the ^{14}C-propoxur metabolites was identified by making a ^{14}C-acetone-semicarbazone derivative and purifying it to a constant specific activity (Casida et al., 1968; Shrivastava et al., 1969). Gibbs' reagent for detection by colour and authentic samples of propoxur analogues were used for co-chromatographic identification.

RESULTS

Toxicity tests

Toxicity data on continuous and short-term exposures of mosquito larvae to various propoxur and carbofuran dosages are presented in abbreviated form in Fig. 1–3. In the continuous-exposure tests, mortality increased gradually, reaching a peak at

FIG. 2
RECOVERY OF SUSCEPTIBLE (LAB.) AND RESISTANT (BAYGON-R) STRAINS OF *C. P. FATIGANS* LARVAE AT VARIOUS TIMES FOLLOWING SHORT-TERM EXPOSURE TO DIFFERENT DOSAGES OF PROPOXUR (BAYGON)

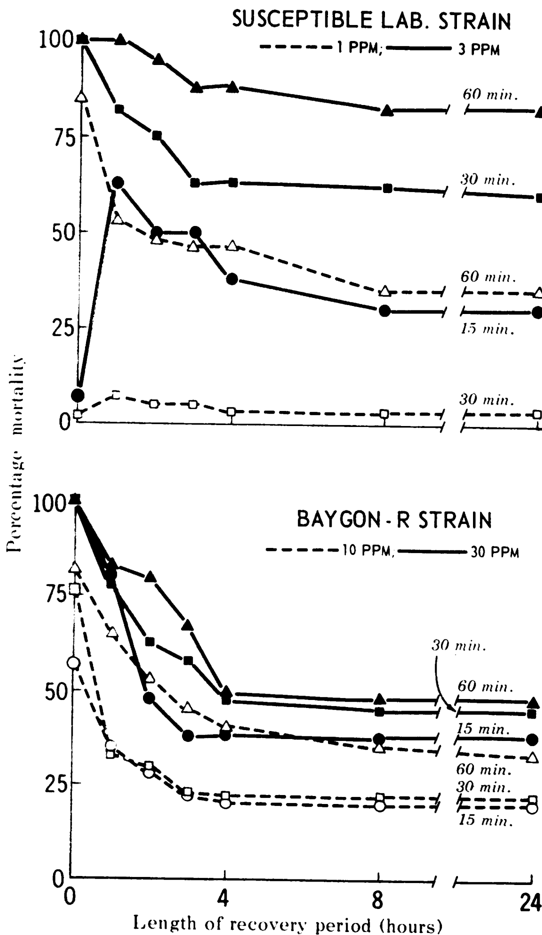
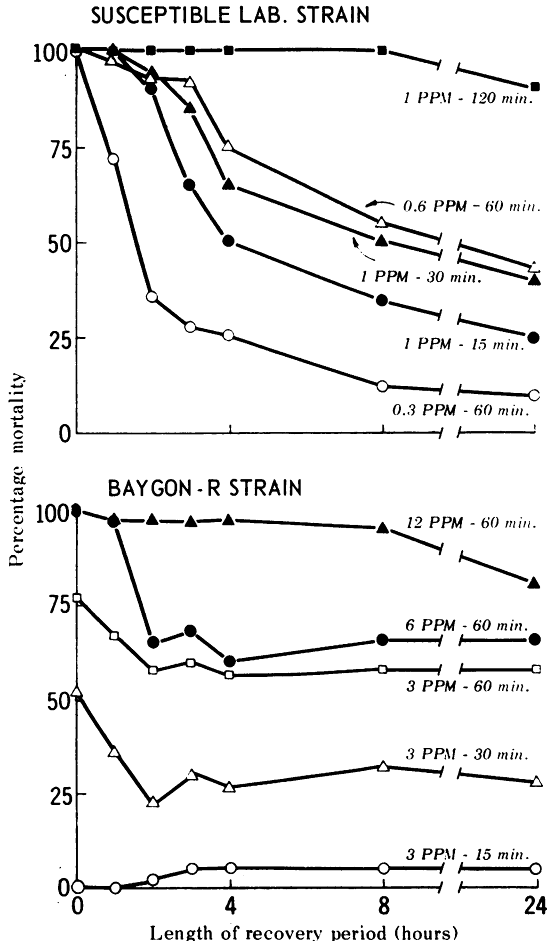


FIG. 3
RECOVERY OF SUSCEPTIBLE (LAB.) AND RESISTANT (BAYGON-R) STRAINS OF *C. P. FATIGANS* LARVAE AT VARIOUS TIMES FOLLOWING SHORT-TERM EXPOSURE TO DIFFERENT DOSAGES OF CARBOFURAN (FURADAN)



about 12 hours. Little or no further increase was noted during the next 12 hours (Fig. 1). From the short-term-exposure tests the following observations were made. (1) Larvae are able to tolerate considerably higher dosages of the carbamates during short periods of exposure; for example, 15 times more propoxur was required in the 30-minute exposure of S-strain larvae to produce the same mortality as that obtained in 24-hour exposure tests. With R-strain larvae, an approximately 6-times greater dosage was required for mortality in the 30-minute exposure to equal that in the 24-hour exposure. (2) Both compounds tested showed an extremely quick action against the larvae, especially when used at relatively high concentrations (Fig. 2, 3). Exposures as brief as 15 minutes produced considerable knockdown in larvae of both the R and S strains. (3) A large proportion of the affected larvae recovered completely when transferred to fresh water; maximum recovery was attained in about 8 hours (Fig. 2, 3). This recovery was due probably to detoxification of the carbamate by the larvae, as discussed below.

Synergism

Synergism of propoxur was obtained in decreasing order with the following compounds: 2-(2,4,5-trichlorophenyl) propynyl ether; 2,3-methylenedioxynaphthalene; α-[2-(2-butoxyethoxy)ethoxy]-4,5-methylenedioxy-2-propyltoluene (piperonyl butoxide); 2,4-dichloro-6-phenylphenoxyethyldiethylamine (Lilly, compound 18947); isobornyl thiocyanoacetate (Thanite); tri-*o*-cresyl phosphate; 2-(3,4-methylenedioxyphenoxy)-3,6,9-trioxaundecane (sesamex) and *N*-octyl bicycloheptenedicarboximide (MGK-264). Whereas no mortality occurred when propoxur was used alone, more than 60% mortality was obtained when it was combined with any of the first six compounds; sesamex and MGK-264 increased mortality only slightly. In general, the degree of synergism was considerably lower than that observed in houseflies (Georghiou, 1962). Pretreatment of S- or R-strain larvae with piperonyl butoxide prior to the application of propoxur or carbofuran did not increase mortality above that obtained by the simultaneous application of both compounds. This suggests that the synergist affects mosquito larvae relatively rapidly.

Identification of organosoluble metabolites

More than 10 organosoluble metabolites of ^{14}C-propoxur were detected *in vivo* and *in vitro* in mosquito larvae and *in vitro* in mouse-liver preparations. The metabolites tentatively identified were: acetone, *N*-hydroxymethyl propoxur and *N*-demethyl propoxur. In addition, one of the metabolites of propoxur in larvae shows chromatographic behaviour similar to that of 2-isopropoxy-5-hydroxyphenyl methylcarbamate. Since acetone has been identified as one of the metabolites, it is evident that *o*-hydroxyphenyl methylcarbamate (*o*-hydroxy propoxur) is also a metabolite of propoxur in mosquito larvae. Upon incubation of the water layer with hydrolases, many of the metabolites were cleaved; thus, *N*-hydroxymethyl propoxur, 2-isopropoxy-5-hydroxyphenyl methylcarbamate and possibly other metabolites with the isopropyl group intact, are released. Among various hydrolases tested, β-glucosidase appeared to be the most active, producing about 50% cleavage of the conjugates, followed by acid phosphatase, β-glucuronidase and sulfatase; 1 N hydrochloric acid produces about 50% cleavage. Sequential treatment with hydrolases produces more complete hydrolysis of the conjugates which had remained uncleaved in the previous treatment.

Metabolism in living mosquito larvae as related to the resistance level

The fate of propoxur when applied to the larval medium is indicated by the results obtained from short-term-exposure studies (Table 1). Comparison of the exposure medium, with and without larvae, treated with the same amount of ^{14}C-propoxur, indicates that only 2.5% of the applied dose is degraded into various other compounds in 24 hours as a result of non-enzymatic reactions, whereas 73% of propoxur is metabolized by larvae in 1 hour. Thus, degradation of propoxur is truly larval.

The metabolites are eliminated into the medium as organosoluble and water-soluble products. In the recovery medium, in which each group of larvae has the same initial amount of radioactivity, the organosoluble metabolites decrease and the water-soluble products increase with the length of the recovery period. This suggests that propoxur and the metabolites, present in larvae at the end of exposure period (zero recovery period), are eliminated into the recovery medium, reabsorbed by the larvae, and further metabolized and re-eliminated into the medium. This cycle of absorption, metabolism and elimination appears to continue until all organosoluble metabolites are changed into water-soluble products. Finally, in the larval extract, where again each group of larvae begins with the same amount of radioactivity at zero recovery

TABLE 1

ANALYSIS OF METABOLITES OF ^{14}C-PROPOXUR IN PROPOXUR-RESISTANT MOSQUITO LARVAE AND IN THEIR "EXPOSURE" AND "RECOVERY" MEDIA AT VARIOUS TIMES AFTER EXPOSURE FOR 1 HOUR [a]

Compound or fraction	Radioactivity (%) in relation to recovery at zero period [b]														
	Exposure medium		Recovery medium (hours)						Larval extract (hours)						
	No larvae, 24 hours' exposure	With larvae, 1 hour's exposure [c]	4	8	12	16	20	24	0	4	8	12	16	20	24
Organosoluble [d]															
Unknowns A–I [e]	1.72	2.21	0.33	0.33	0.33	0.29	0.31	0.23	0.15	0.06	0.02	0.03	0.01	0.01	0.02
5-hydroxy propoxur	0.24	1.02	0.11	0.10	0.08	0.06	0.06	0.05	0.28	0.12	0.04	0.02	0.01	0.01	0.00
N-hydroxymethyl propoxur	0.12	0.15	0.03	0.03	0.03	0.03	0.03	0.02	0.08	0.01	0.00	0.00	0.00	0.00	0.00
N-demethyl propoxur	0.19	0.22	0.02	0.03	0.03	0.02	0.03	0.02	0.22	0.02	0.01	0.01	0.00	0.00	0.00
Propoxur	97.43	72.99	0.11	0.11	0.10	0.05	0.03	0.03	0.81	0.07	0.03	0.02	0.02	0.01	0.01
Water-soluble	0.32	13.87	4.30	5.53	5.83	6.37	6.58	6.89	8.01	3.03	1.78	1.91	1.67	1.78	1.67
^{14}C-recovery	100.02	90.46	4.90	6.13	6.40	6.82	7.04	7.24	9.55	3.31	1.88	1.99	1.71	1.81	1.70
Insoluble residues [f]	—	—	—	—	—	—	—	—	0.00	1.33	1.53	1.15	1.01	0.69	0.60

[a] Dose: 2 μg (1 ppm). Radioactivity recovered from a medium containing no larvae was almost equal to that recovered in the exposure medium and the larval extract at zero time, i.e., at the end of the exposure period.

[b] Radioactivity at zero recovery period consisted of total extractable organosoluble radioactivity in larvae and exposure medium, immediately after exposure for 1 hour.

[c] The values are the averages of values obtained after 7 time intervals, each replicated 3 times.

[d] The R_f values for organosoluble products with silica-gel G thin-layer chromatogram, and benzene–ethanol–ether (90 : 10 : 3) solvent system were; A, 0.0; B, 0.03; C, 0.1; D, 0.3; E, 0.35; F, 0.4; 5-hydroxy propoxur, 0.42; G, 0.48; *N*-hydroxymethyl propoxur, 0.55; H, 0.6; *N*-demethyl propoxur, 0.64; propoxur, 0.7; I, 0.85.

[e] Amounts of radioactivity for the individual unknown products in the exposure medium were as follows; A, B, 0.79 %; C, 0.3 %; D, 0.1 %; E, 0.11 %; F, 0.15 %; G, 0.0 %; H, 0.25 %; I, 0.51 %.

[f] Based on the difference between the total recovery at zero recovery time and total recovery at the indicated time.

period, the amounts of both organosoluble and water-soluble metabolites decrease with the length of recovery period. This indicates that each of the metabolites present in the larvae at the end of a 1-hour exposure period is further metabolized and eliminated into the recovery medium; this again supports the findings described above.

In other tests S- and R-strain larvae were exposed to a 0.1 ppm concentration of ^{14}C-propoxur solution for various times and the radioactivities in the exposure medium and larval extract were separately analysed (Table 2). The following observations are noteworthy. (1) The amount of propoxur after each exposure time in S-strain larvae is twice as high as in the respective R-strain larvae. This indicates that S-strain larvae pick up propoxur faster than the R-strain larvae. (2) The same major organosoluble metabolites are formed both in S and R strains. (3) The major quantitative interstrain differences in metabolites are the small production of 5-hydroxy, *N*-hydroxymethyl and *N*-demethyl propoxur derivatives by S-strain larvae into the exposure medium. (4) The amount of organosoluble metabolites in the exposure medium does not appear to increase with time, probably because they are further metabolized to water-soluble products (Table 1).

Metabolism by the larval and mouse-liver enzyme preparations

The comparative study on the metabolism of propoxur by the homogenate–$NADPH_2$ enzyme system of S- and R-strain mosquito larvae indicates that, in agreement with the *in vivo* tests, metabolism of propoxur is faster in R-strain than in S-strain larvae (Fig. 4). Thus, the amounts of propoxur and its major metabolites, 5-hydroxy propoxur, *N*-hydroxymethyl propoxur and *N*-demethyl propoxur in S- and R-strain larvae were 98.6%–93.1%, 0.04%–1.1%, 0.1%–2.0% and 0.1%–1.7% of the recovered radioactivity, respectively.

TABLE 2

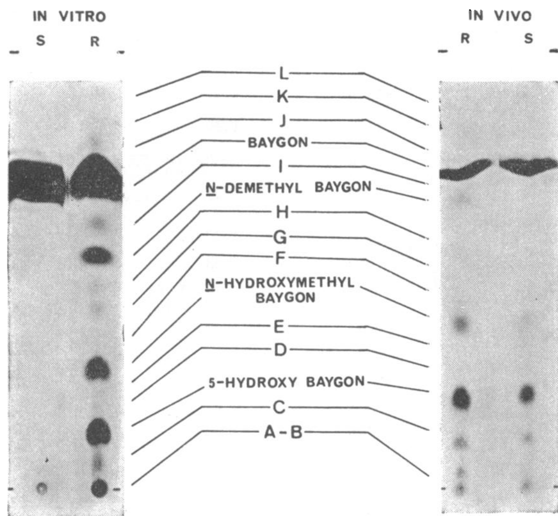
METABOLISM OF ^{14}C-PROPOXUR [a] BY SUSCEPTIBLE AND RESISTANT MOSQUITO LARVAE [b] AFTER VARIOUS TIME INTERVALS

Compound or fraction	Radioactivity (%) recovered at hours indicated							
	Susceptible strain				Resistant strain			
	0	8	16	24	0	8	16	24
Exposure medium								
Organosoluble [c]								
Unknowns A–C	1.14	1.15	1.31	2.01	1.88	1.71	1.75	1.54
5-hydroxy propoxur	0.50	0.68	0.55	0.80	0.74	0.45	1.33	1.58
N-hydroxymethyl propoxur	0.24	0.26	0.32	0.71	0.38	0.32	0.20	0.61
Unknowns F–H	0.70	0.97	1.42	1.28	1.98	1.29	1.94	2.14
N-demethyl propoxur	0.49	0.46	0.65	0.50	1.14	3.24	0.70	0.62
Propoxur	84.57	65.09	52.00	47.87	81.68	68.37	53.45	45.91
Unknowns J–L	1.44	0.57	1.57	0.68	0.96	0.95	0.71	0.82
Water-soluble	5.17	14.25	22.03	32.89	6.42	13.43	27.65	32.55
Larval extract								
Organosoluble [c]								
Unknowns A–C	1.10	3.75	5.78	4.58	1.08	2.64	3.75	5.01
5-hydroxy propoxur	0.23	0.50	0.36	0.31	0.33	0.30	0.39	0.31
N-hydroxymethyl propoxur	0.23	0.23	0.22	0.10	0.27	0.23	0.22	0.26
N-demethyl propoxur	0.19	0.16	0.17	0.18	0.25	0.62	0.27	0.43
Propoxur	2.33	4.40	4.81	5.36	1.23	1.56	3.32	1.94
Unknowns J–L	0.29	0.38	0.19	0.30	0.43	0.19	0.23	0.5
Water-soluble	1.39	7.13	8.60	2.40	1.21	4.68	4.07	5.77

[a] Dose; 0.2 μg (1 ppm).
[b] Altogether, 500 mg (wet weight) of larvae were used in each test.
[c] The chromatographic position of organosoluble compounds and their designations are given in Fig. 4; other unknowns constituted less than 0.1 %.

Experiments on the effects of co-factors on the metabolism of 10 μg of ^{14}C-propoxur by the microsome-plus-soluble fraction of mouse-liver preparations have shown that while no metabolism occurs with reduced glutathione (GSH), L-cysteine, L-cysteic acid, L-methionine and ethionine, a high degree of metabolism is found with co-factors $NADPH_2$ and $NADPH_2$+GSH. The combination of $NADPH_2$ with GSH helps particularly in increasing the amount of organosoluble metabolites. Thus, while 74.3 % of organosoluble metabolites were recovered with $NADPH_2$ alone, this amount increased to 90 % with $NADPH_2$+GSH. Additional tests with co-factors and their combinations with chemicals containing a free sulfhydryl group were carried out. In these studies, propoxur was used in dosages of 2 μg per test. The results (Table 3) indicate that while oxidized nicotinamide–adenine dinucleotide phosphate (NADP) and $NADPH_2$ are the most effective co-factors for degradation of propoxur, oxidized and reduced nicotinamide–adenine dinucleotide (NAD and $NADH_2$) are also effective, the amount of organosoluble metabolites being greater in the latter cases. The combination of co-factors does not improve the extent of metabolism; this may be due to the lower level of substrate used in this experiment.

FIG. 4
AUTORADIOGRAMS OF ^{14}C-PROPOXUR PRODUCED BY PROPOXUR-SUSCEPTIBLE (S) AND PROPOXUR RESISTANT (R) STRAINS OF *C. P. FATIGANS* LARVAE AND THEIR WHOLE-BODY HOMOGENATE–NADPH$_2$ ENZYME SYSTEMS [a]



[a] *In vivo* tests were carried out with doses of 0.2 μg (0.1 ppm) and 24 hours' exposure. *In vitro* studies were made with 10-μg doses of propoxur per test. In both series of tests, the extraction solvent was diethyl ether; silica-gel G thin-layer chromatograms were developed twice in one dimension in chloroform–*n*-butanol–ether (50 : 1 : 1) after development in benzene.

Metabolism of carbofuran

Ancillary studies on the metabolism of carbofuran by mouse-liver enzyme preparations indicated the production of seven labelled organosoluble metabolites from 10-μg dosages of ^{14}C-carbofuran. The metabolites were separated on thin-layer chromatography plates with the use of silica-gel G adsorbent and an ether–hexane (3 : 1) solvent system. By using co-chromatography with authentic unlabelled samples, two of the major radioactive metabolites were identified as 3-hydroxy carbofuran and 3-keto carbofuran. Another major metabolite, for which an authentic sample was not available and which was compared only on the basis of the reported R_f value, appears to be 2,2-dimethyl-2,3-dihydrobenzofuranyl-7, *N*-hydroxymethylcarbamate (*N*-hydroxymethyl carbofuran).

DISCUSSION

Studies of insecticide metabolism in mosquito larvae involve the treatment of the water medium with an insecticide solution; this is unlike the studies with houseflies in which an individual insect can be treated topically or a precise dose of the insecticide can be injected. In an aquatic system the organism picks up the insecticide, possibly with other compounds, equilibrates itself with the immediate environment, metabolizes and eliminates the compounds and these, in turn, continue through the cycle of absorption, metabolism and elimination. Thus, the medium becomes progressively more complex with time. To simplify the medium to a certain extent, a method involving short-term exposure and recovery periods was developed. As a result of this simplification, the present series of experiments has revealed the various phases through which an insecticide passes. It is evident from the short-term-exposure tests for toxicity and metabolism that an insecticide is picked up selectively or the insecticide is absorbed by the larvae, the internal environment of the larvae is equilibrated with the external medium, is metabolized to non-toxic or to less toxic compounds, and conjugated with sugars and other polar molecules to be excreted into the medium, freeing the larvae from adverse effects of the toxicant. The conduct of these experiments, however, proved to be lengthy and time-consuming. Therefore, continuous-exposure experiments were also performed which showed a similar pattern of metabolites on thin-layer chromatography plates.

The major pathways of propoxur metabolism in mosquito larvae and mouse-liver preparations are very similar to those reported in houseflies, rat-liver enzyme preparations and plants (Kuhr & Casida, 1967; Oonnithan & Casida, 1966, 1968; Shrivastava et al., 1969; Tsukamoto & Casida, 1967a, 1967b). This suggests the presence of similar mechanisms in various groups of insects, mammals and plants. The reactions involve initial hydroxylation on the ring in the 5-position, *N*-hydroxylation and *O*-dealkylation by hydroxylation of the tertiary carbon. Ester hydrolysis appears to be of minor importance in propoxur metabolism since only small amounts of isopropoxy phenol are detected in the free or conjugated form. Similar amounts are also detected in the controls. ^{14}C-acetone and *N*-demethyl propoxur are also produced; *o*-hydroxy propoxur is probably produced but could not be detected with ^{14}C-propoxur. Apart from these substances several unidentified metabolites having the isopropyl group intact were also recovered in minor quantities. The major propoxur metabolites are true detoxification products since they have lower toxicities to

TABLE 3

EFFECT OF CO-FACTORS ON METABOLISM OF ^{14}C-PROPOXUR [a] BY THE MOUSE-LIVER MICROSOME-PLUS-SOLUBLE ENZYME PREPARATION

Co-factor [b]		Radioactivity (%) in relation to total recovery in controls [c]					
I	II	Propoxur	5-hydroxy propoxur	*N*-hydroxy-methyl propoxur	*N*-demethyl propoxur	Unknowns A–L	Recovery
None (control)	—	95.68	0.15	0.40	0.28	3.49	100.00
FAD	—	98.45	0.33	0.70	0.56	6.20	106.24
NAD	—	47.55	1.96	9.03	1.60	4.50	64.64
NADP	—	0.59	1.14	2.80	0.87	7.91	13.31
$NADH_2$	—	43.92	2.51	11.24	1.98	5.54	65.19
$NADPH_2$	—	0.80	0.65	1.17	0.59	6.53	9.74
GSH	—	38.23	0.24	0.47	0.23	2.23	41.40
$NADPH_2$	GSH	0.70	1.00	1.83	0.94	8.27	12.74
$NADPH_2$	L-cysteine	0.65	1.16	1.90	0.71	9.88	14.30
$NADPH_2$	Cysteic acid	0.57	0.65	1.59	0.51	6.47	9.79
$NADPH_2$	Methionine	0.61	0.65	0.97	0.45	5.81	8.49
$NADPH_2$	Ethionine	0.41	0.52	0.93	0.48	4.66	7.00
$NADPH_2$	NADP	0.29	1.04	0.77	0.50	6.40	9.00
$NADH_2$	GSH	3.99	2.10	7.88	2.80	6.20	22.97
$NADH_2$	NAD	19.04	2.74	9.29	2.71	6.15	39.93
NADP	GSH	0.42	2.49	3.43	0.98	9.31	16.63
GSH	L-cysteine	67.03	0.10	3.55	0.20	1.23	72.11

[a] Dose; 2 µg (1 ppm).

[b] NAD = oxidized nicotinamide–ademine dinucleotide; $NADH_2$ = reduced nicotinamide–adenine dinudeotide; NADP = oxidized nicotinamide–adenine dinudeotide phosphate; $NADPH_2$ = reduced nicotinamide–adenine dinucleotide phosphate; GSH = reduced glutathione, FAD = flavin adenine dinucleotide.

[c] Chromatographic position of the organosoluble compounds and their designations are similar to those given for mosquito larvae in Fig. 4.

C. p. fatigans (S-Lab) larvae. The LC_{50} values are: propoxur, 0.3 ppm; *N*-hydroxymethyl propoxur, >10 ppm; *N*-demethyl propoxur, >10 ppm; *o*-hydroxy propoxur, >10 ppm (S. Aziz and others, unpublished data).

The major pathways of metabolism of carbofuran by mouse-liver enzyme systems involve hydroxylation of *N*-methyl and hydroxylation followed by oxidation of the 3-position of the furan ring to give *N*-hydroxymethyl, 3-hydroxy and 3-keto carbofuran analogues, respectively. It has also been reported that these compounds are produced by mouse and insect systems *in vivo* (Metcalf et al., 1968).

Initial propoxur hydroxylation products are conjugated and excreted by the larvae into the water medium. No evidence for conjugates of propoxur or of *N*-demethyl propoxur was obtained, but *O*-conjugates of *N*-hydroxymethyl propoxur, 5-hydroxy propoxur and other unidentified metabolites are probably formed since these compounds are released on incubation of water-soluble metabolites with β-glucosidase, acid phosphatase, β-glucuronidase and aryl sulfatase. Each type of conjugate has been reported for metabolites of propoxur and naphthol in houseflies (Darby et al., 1966; Shrivastava et al., 1969; Terriere et al., 1961).

Comparing the metabolism of propoxur in susceptible and resistant larvae *in vivo* and *in vitro*, and mouse-liver *in vitro* systems, it is evident that although the major metabolites in each case are the same there are quantitative, and probably qualitative, differences among the minor metabolites. Thus,

there appears to be a greater number of minor unidentified products formed in resistant larval enzyme system than in the other system studied. While qualitative differences in two species may be due to the presence or absence of certain enzymes, the differences between the S and R strains in the *in vivo* and *in vitro* results appear to be quantitative. Probably, the compounds present in trace quantities are not detected by radioautography. The larval mosquito *in vitro* system is more active because substrate, co-factor and enzyme concentrations are artificially altered and the substrate has better access to enzymes than in the *in vivo* system. The R strain shows greater enzyme activity, probably because it has a larger amount of enzyme, a more active enzyme, or both. The present study indicates that resistance to propoxur in *C. p. fatigans* is associated with increased detoxification of propoxur by enzymatic mechanisms involving hydroxylation and with reduced absorption of toxicant by the insect. More information would be needed to resolve the relative contribution of these mechanisms in resistance.

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RÉSUMÉ

RÉSISTANCE DES MOUSTIQUES AUX CARBAMATES: MÉTABOLISME DU PROPOXUR CHEZ DES LARVES DE *CULEX PIPIENS FATIGANS* SENSIBLES ET RÉSISTANTES

On a soumis des larves de *Culex pipiens fatigans* résistantes et sensibles à des épreuves de toxicité mettant en œuvre deux carbamates, le propoxur et le carbofuran, à diverses concentrations. Les larves tolèrent de très fortes doses de ces composés lorsque l'exposition est de courte durée. Les deux insecticides agissent très rapidement surtout aux concentrations élevées. Une proportion notable des larves récupèrent complètement, avec disparition des signes de knockdown, lorsqu'elles sont transférées en eau fraîche.

Des études *in vivo*, de même que des recherches *in vitro* utilisant des préparations enzymatiques obtenues à partir d'homogénats de larves, ont permis d'identifier au moins dix métabolites organosolubles, porteurs d'un radical isopropoxy intact, résultant de la dégradation du propoxur. Par incubation d'extraits hydrosolubles de larves traitées en présence de diverses hydrolases, comme la β-glucosidase, la β-glucuronidase, l'aryl-sulfatase et la phosphatase acide, on obtient un nouveau clivage des métabolites avec, principalement, libération de carbamates hydroxylés. Les larves résistantes et leurs préparations enzymatiques métabolisent le propoxur plus rapidement, mais la pénétration de l'insecticide est accélérée chez les larves sensibles.

Ces résultats indiquent qu'une absorption ralentie de l'insecticide et une détoxication plus rapide par hydroxylation, associées à la conjugaison à des sucres ou d'autres molécules polaires suivie d'élimination dans le milieu extérieur, sont les principaux facteurs qui conditionnent la résistance des larves de moustiques aux méthylcarbamates portant un groupement aryle.

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