ESTERASE ACTIVITY IN MOSQUITOES AND ITS POSSIBLE RELATIONSHIP TO ORGANOPHOSPHATE AND CARBAMATE RESISTANCE

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Resistance in mosquitoes to organophosphate (OP) [1] insecticides in this country is presently confined to a few species. However, in California where *Aedes nigromaculis* (Ludlow) has become resistant to parathion, methyl parathion, malathion, and fenthion (Brown *et al.*, 1963), the problem is considered serious. We have found no published reports on the nature of the mechanism responsible for this resistance.

Another species where OP resistance occurs is *Culex tarsalis* Coquillett where a specific malathion resistance is associated with an increased ability to detoxify malathion (Matsumura and Brown, 1961b, 1963b; Bigley and Plapp, 1962). In *Aedes aegypti* (L.) where several malathion-resistant strains are known, Brown and coworkers (Brown and Abedi, 1960; Matsumura and Brown, 1961a, 1963a) found no evidences of a greater detoxifying ability, but only of less efficient absorption of the insecticide.

Recently, Georghiou and Metcalf (1963) attempted to select for carbamate resistance in *Anopheles albimanus* Wiedemann. Their results indicated that vigor tolerance only was present, not resistance. The lack of evidence for resistance to OP's and carbamates in mosquitoes may be contrasted with the numerous reports of their resistance to DDT and related chlorinated hydrocarbons and to cyclodiene insecticides such as dieldrin.

On the other hand, many instances of OP resistance in the house fly, *Musca domestica* L., can be cited. In this insect, resistance is often associated with the presence of mutant forms of organophosphate-sensitive (OP-S) ali-esterases (Ali-E) which, instead of being inhibited by OP's, now degrade them (Oppenoorth and Van Asperen, 1960). These mutant Ali-E's which have an increased ability to degrade OP's, have a decreased ability to hydrolyze the substrate methyl *n*-butyrate. Thus, resistant strains are characterized as having "low Ali-E activity." Similar work from our own laboratory indicated that the same mechanism may also be responsible for carbamate resistance in the house fly (Plapp *et al.*, 1964).

With *C. tarsalis*, previous measurements indicated the presence of an organophosphate-insensitive (OP-I) Ali-E hydrolyzing tri *n*-butyrin, but not methyl *n*-butyrate in both susceptible and resistant strains (Plapp *et al.*, 1961). The present study was undertaken to determine the levels of OP-S and OP-I Ali-E and of cholinesterase in mosquito species of the genera *Anopheles, Aedes,* and *Culex,* and to assess possible relationship of these esterases to organophosphate and carbamate resistance. In addition, experiments were conducted to determine whether selection for resistance to parathion could be accomplished in *C. tarsalis*.

MATERIALS AND METHODS. Insects.—The following species and strains of mosquitoes were used: (1) A susceptible strain of *Culex tarsalis* reared in the laboratory for many years without exposure to insecticides; (2) a highly malathion-resistant strain of *C. tarsalis,* selected periodically as fourth instar larvae and about 100 times resistant to the insecticide; (3) an autogenous strain of *C. tarsalis* derived from the highly malathion-resistant strain and of moderate resistance to the insecticide;

[1] Abbreviations used in the text are as follows:
OP —organophosphate
OP-S —organophosphate-sensitive
OP-I —organophosphate-insensitive
Ali-E—ali-esterase

(4) a local strain of *Culex peus* Speiser, of unknown resistance to insecticides, supplied by L. F. Lewis of this laboratory; (5) *Culex pipiens quinquefasciatus* Say, an insecticide-susceptible strain obtained from the California Bureau of Vector Control, State Health Department, Fresno; (6) a susceptible strain of *Anopheles quadrimaculatus* Say obtained from the U. S. Dept. of Agriculture, Entomology Research Division, Gainesville, Florida; (7) a susceptible strain of *Aedes aegypti* (L) from the same source; and (8) a strain of *Ae. aegypti,* originally from Malaya and about 5 times resistant to malathion (Brown and Abedi, 1960), supplied by Dr. A. W. A. Brown.

Enzyme Assays.—Assays were carried out with crude homogenates of fourth instar larvae of the various mosquito species. Homogenates were prepared in pH 7.2 phosphate buffer with a Potter-Elvehjem homogenizer and filtered through glass wool. The final concentration of tissue was 20 mg. of larvae (wet weight) per ml. of buffer. All measurements of enzyme activity were made at least in quadruplicate.

Substrates used for measuring esterase activity were acetylcholine bromide at a concentration of 2.5×10^{-4} M, methyl *n*-butyrate and phenyl acetate at concentrations of 4×10^{-3} M, and tri *n*-butyrin at a concentration of 1×10^{-3} M. The addition of a small amount of Triton X-100 ® was necessary to obtain dispersion with phenyl acetate and tri *n*-butyrin. Analyses were performed by incubating 1 ml. of the various substrates with 1 ml. of homogenates of the different mosquito strains. Samples were incubated for 15 minutes at 37° C., at which time the reaction was stopped by the addition of hydroxylamine and the remaining substrate was determined colorimetrically by the method of Hestrin (1949). Details of the procedure as used have been previously described (Bigley and Plapp, 1960).

Inhibitors.—Esterase activity, as measured in gross homogenates, was differentiated into three types, each based on whether or not inhibition by eserine and paraoxon takes place. Cholinesterase is defined as esterase activity inhibited by both eserine and paraoxon; OP-S Ali-E is inhibited by paraoxon and other OP's, but not by eserine; and OP-I Ali-E is esterase activity not inhibited by 10^{-5} M concentrations of either inhibitor. For determining enzyme inhibition, mosquito homogenates were incubated with 1-ml. amounts of 10^{-5} M concentrations of the appropriate inhibitor for 15 minutes, after which substrate was added, the samples were incubated for an additional 15 minutes, and the remaining substrate was determined as previously described.

Development of Resistance to Parathion.—For 3 years we have been selecting for resistance to parathion in our susceptible colony of *Culex tarsalis*. Early selection techniques consisted of exposing larvae of different instars to a given concentration of parathion and removing the survivors after 75–90 percent mortality was obtained. More recently, the procedure has been to expose populations of several thousand fourth instar larvae to a solution of parathion in 5 liters of distilled water. After 24 hours the survivors are removed and placed in their original rearing water. Concentrations of parathion used have been from 1 to 5 parts per billion. Mortalities have ranged from 65 to 100 percent. When necessary, progenies of survivors or unpressured siblings are allowed to rebuild their numbers for several generations before selection pressure is again applied.

RESULTS. Esterase Activity.—Measurements pertaining to hydrolysis of the four substrates by homogenates of the several mosquito populations are summarized in Table 1. All substrates used were degraded at measurable rates by some of the mosquito populations.

Cholinesterase activity, as measured with acetylcholine, was highest in *C. tarsalis,* lower in other *Culex* species and in *Anopheles,* and lowest in *Aedes*. As reported in a previous study on *C. tarsalis* (Plapp *et al.,* 1961), we found no differences in rate of cholinesterase hydrolysis that could be related to organophosphate resistance.

Phenyl acetate was readily hydrolyzed

TABLE 1.—Esterase activity in larvae of several species and strains of mosquitoes

Species	Strain	Substrate and micromoles hydrolyzed per 15 minutes per 20 mg. of whole larval homogenate			
		Acetylcholine 2.5 μM	Phenyl acetate 4 μM	Methyl *n*-butyrate 4 μM	Tri *n*-butyrin 1 μM
Culex					
tarsalis	Insecticide susceptible	0.67	2.01	0	0.52
"	Malathion resistant	.69	2.74	0.04	.58
"	Autogenous	.93	2.20	0	.46
peus	Wild collection	.36	0.96	0	.42
pipiens quinquefasciatus	Insecticide susceptible	.43	1.64	.13	.54
Anopheles					
quadrimaculatus	Insecticide susceptible	.28	2.44	.01	.55
Aedes					
aegypti	Insecticide susceptible	.06[1]	2.20	.84	.52
"	Malathion resistant	.16[1]	2.52	.94	.56

[1] Determined with 40 mg./ml. mosquito homogenate.

by all homogenates tested with the single exception of *C. peus* in which activity was less than half that found in any other strain. Tri *n*-butyrin was also readily hydrolyzed by all mosquito species studied with no large differences in rate being noted.

Methyl *n*-butyrate hydrolysis was either nonexistent or very nearly so in the several *C. tarsalis* strains studied. In addition, hydrolytic activity toward this substrate was absent or low in the other *Culex* species tested and in *An. quadrimaculatus* as well. In *Ae. aegypti* activity was present in both strains tested.

Inhibition and Types of Esterase Activity.—Inhibition data are summarized in Table 2. Based on this data, the hydrolysis of acetylcholine in the strains studied appeared to be due entirely to enzymes with the properties of cholinesterases. In all strains complete inhibition was obtained with both eserine and paraoxon. The largest portion of the hydrolysis of phenyl acetate in *Culex* and *Anopheles* was also due to cholinesterase. In *Ae. aegypti,* in which hydrolytic activity toward acetylcholine was very low, only a small portion of the phenyl acetate hydrolysis was due to cholinesterases.

There was significant OP-S Ali-E activity toward phenyl acetate in all the strains studied, as indicated by that portion of the hydrolysis susceptible to inhibition by paraoxon, but not by eserine. Methyl *n*-butyrate hydrolysis, absent in *Culex* and *Anopheles,* was entirely due to an OP-S Ali-E in *Aedes.* Based on inhibition data, tri *n*-butyrin hydrolysis in *Culex* and *Anopheles* was not primarily due to OP-I Ali-E's. In *Aedes,* however, a measurable portion of the hydrolysis of this substrate was due to OP-S Ali-E's. In neither *C. tarsalis* nor *Ae. aegypti* were there any large differences in OP-S Ali-E's between susceptible and malathion-resistant strains. Similar results for *Ae. aegypti* were reported previously (Matsumura and Brown, 1961a).

Based on the hydrolysis of tri *n*-butyrin, evidence was obtained for the presence of OP-I Ali-E in all strains studied. In *Culex* and *Anopheles,* hydrolysis of tri *n*-butyrin was mostly due to OP-I esterases whereas in *Aedes* only a portion of the total was due to these esterases. The results are different from those reported with house flies (Van Asperen and Oppenoorth, 1959) in which most of the hydrolysis of tri *n*-butyrin was due to OP-S Ali-E's.

Development of Resistance to Parathion.—The attempt to select a strain of parathion-resistant *Culex tarsalis* has so far been unsuccessful. Based on bioassay data

TABLE 2.—Inhibition of esterase activity in larvae of several species and strains of mosquitoes by 1×10^{-5} M esterine and paraoxon.

Species	Strain	Substrate and % inhibition of hydrolytic activity of homogenates toward each substrate with eserine (Es) and paraoxon (PO)							
		Acetylcholine		Phenyl acetate		Methyl *n*-butyrate		Tri *n*-butyrin	
		Es	PO	Es	PO	Es	PO	Es	PO
Culex									
tarsalis	Insecticide susceptible	100	100	56	98	..[1]	..	10	20
"	Malathion resistant	100	100	62	99	..	..	14	24
"	Autogenous	100	100	64	100	..	..	6	16
peus	Wild collection	100	100	75	100	..	..	2	10
pipiens quinquefasciatus	Insecticide susceptible	100	100	61	100	..	..	9	23
Anopheles									
quadrimaculatus	Insecticide susceptible	100	100	51	98	..	..	10	20
Aedes									
aegypti	Insecticide susceptible	100	100	30	100	0	100	9	46
"	Malathion resistant	100	100	22	95	0	90	17	77

[1] Insufficient activity to determine % inhibition.

obtained during the 3 years the study was being conducted, only slight differences could be detected between the selected and the laboratory susceptible colony. These differences, as shown in a recent bioassay of progeny of selected and susceptible larvae, have never been greater than 2-fold at either the LC-50 or LC-90 levels (Table 3). The results are smilar to those

TABLE 3.—Toxicity of parathion to larvae of susceptible and parathion-selected strains *of Culex tarsalis.*

Dosage (p.p.b.)	Mortality after 24 hours (%)	
	Susceptible	Parathion-selected
1	0	0
2	25	12
3	83	60
4	100	65
5	100	100
0 (Control)	0	0

reported by Georghiou and Metcalf (1963) for *Anopheles albimanus* in which selection with a carbamate also failed to produce resistance.

DISCUSSION. The experiments reported here provided evidence that all three esterases studied—cholinesterase, OP-S Ali-E, and OP-I Ali-E—are present in the three genera of mosquitoes tested. There are, however, differences between genera, and differences between mosquitoes and the house fly, the species where perhaps most is known about OP resistance. The most striking difference is with regard to OP-S Ali-E activity which is present in all genera when phenyl acetate was used as a substrate, but present only in *Aedes* when methyl *n*-butyrate or tri *n*-butyrin were used.

In the house fly, mutant forms of an OP-S Ali-E, more efficient in OP detoxication (and less efficient in hydrolyzing methyl *n*-butyrate), have frequently been shown to be characteristic of resistant strains. In *Culex* and *Anopheles,* we found no butyrate-hydrolyzing OP-S Ali-E. In *Aedes aegypti* where such an esterase was present, no gross changes in level of the enzyme occurred between susceptible and resistant strains.

It follows then, that the mechanism of resistance to organophosphates in mosquitoes differs from that known to be present in a number of house fly strains. The lack of a butyrate-hydrolyzing OP-S Ali-E in *Culex* and *Anopheles* may explain why very little OP-resistance (and this only a specific resistance to malathion) has occurred in mosquitoes of these genera. *Aedes nigromaculis* is apparently the only mosquito species where a broad-spectrum type of OP-resistance has occurred and, based on the present work with *A. aegypti,* it is quite possible that an OP-S Ali-E is involved in this species, although the possibility has not yet been investigated.

Similarly, the results suggest an explanation for the lack of carbamate resistance in mosquitoes. If, as suggested earlier (Plapp *et al.,* 1964), carbamate and OP-resistance are due to allelic forms of the same gene, the lack of a butyrate-hydrolyzing Ali-E would also explain the absence of carbamate resistance in mosquitoes.

SUMMARY. Evidence was obtained for the presence of cholinesterase, organophosphate-sensitive ali-esterase, and organophosphate-insensitive ali-esterase in larvae of several species of *Culex* and in those of one species each of *Aedes* and *Anopheles.* Cholinesterase activity was greatest in larvae of *Culex,* less in *An. quadrimaculatus,* and lowest in *Ae. aegypti.* Organophosphate-sensitive ali-esterase activity was present in all strains when phenyl acetate was used as the substrate. When methyl *n*-butyrate was used as the substrate, activity was present only in *Ae. aegypti.* Organophosphate-insensitive ali-esterase activity was present at similar levels in all strains tested.

In contrast to previously reported results with house flies, lower levels of organophosphate-sensitive ali-esterase activity were not found in organophosphate-resistant strains of either *C. tarsalis* or *Ae. aegypti.* The results indicated that the mechanism responsible for organophosphate-

resistance in house flies, altered forms of a butyrate-hydrolyzing ali-esterase, is not present in *Culex* or *Anopheles* and may be the reason there is almost no resistance to organophosphates in mosquitoes of these genera.

A 3-year selection of a susceptible strain of *C. tarsalis* with parathion resulted in a strain with not more than 2-fold resistance to the insecticide.

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AN AQUATIC TRAP FOR SAMPLING MOSQUITO PREDATORS

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In studying the natural control of mosquitoes near Belleville, Ontario, it was necessary to investigate the role of predacious arthropods, especially aquatic Coleoptera. A new type of aquatic trap was devised that was particularly effective in capturing water beetles. The trap requires no source of attraction or bait and captures beetles at various depths with litttle disturbance of the habitat. A series of traps may be used to obtain information on beetle activity and numbers and also provide specimens for dissection.

The trap (Fig. 1, top) consists of a holding cage (A), a trap-jar (B), and an anchor rod (C). The holding cage is constructed of 16-mesh brass screening on a frame of ¼ inch x 1/32 inch thick copper angle. Dimensions are 5 inches long and 4 inches wide and deep. The entrance at the front is of screening shaped to form a four-sided funnel with a terminal opening (D)