BIOCHEMISTRY OF INSECTICIDE RESISTANCE IN MOSQUITOES

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Among the 160 insect species that have developed resistance to insecticides in the past two decades, mosquitoes occupy a prominent position. Thirty or more anopheline, aedine, and culicine species are known to be resistant to one or more of the commonly used insecticides, and there is reason to believe that this list will keep growing.

Many theories have been advanced as to the nature of this resistance, but no one theory alone can account for the change in tolerance in terms of one specific mechanism common to all species. Thus, each species or strain possesses a combination of attributes for resistance which may be different from that found in other strains.

To understand the mechanisms involved in insecticide resistance it is necessary to delineate the factors which account for the toxic action of the particular insecticide. In general, the toxicity of a compound depends on (a) penetration of the chemical through the insect's integument (b) penetration at the site of action (c) enzymic conversion of the chemical to more toxic compounds (d) enzymic conversion to less toxic metabolites (e) excretion of the insecticide and/or toxic metabolites, and (f) storage of the insecticide or toxic metabolites in nonsensitive tissues.

Increased tolerance to an insecticide may be due, therefore, to genetic selection or enhancement of those processes favoring survival of the individual organism.

RESISTANCE TO DDT-TYPE COMPOUNDS. Recent reviews on this subject have been published by Brown (1964), Perry (1964) and Oppenoorth (1965). Therefore, only the highlights will be considered here, and these will cover mainly the type of enzymic mechanisms associated with resistance.

It has been established (see reviews given above) that most DDT-resistant strains of *Aedes aegypti* convert substantially more DDT to the nontoxic derivative DDE than their susceptible counterparts. Some strains, however, notably some Asiatic resistant strains (Chattoraj and Brown, 1960) show little increase in DDE production. Analyses by gas chromatography (unpublished results from this laboratory) showed that susceptible *Aedes aegypti* larvae produce substantial quantities of DDE. Although more DDE was produced by the resistant Trinidad strain, the differential in detoxication was not as marked as that found between susceptible and resistant houseflies. Typical examples are shown in Table 1.

The weight of evidence indicates that, in *Aedes aegypti,* detoxication of DDT *in vivo* is causally associated with DDT resistance, but that enzymic studies *in vitro* have not yet reached the degree of refinement necessary for quantitative enzyme assessment.

For example, an increase of 100-fold in the LC_{50} of the Trinidad strains resulted in an increase in DDE production of only 8.5 percent. In the Asiatic strains the increase in DDE was almost nil (Kimura and Brown, 1964).

Agreement as to the correlation between resistance and DDT dehydrochlorination in culicine species is also lacking. Thus, *Culex pipiens quinquefasciatus* converted 67 percent of a topical dose of DDT to DDE whereas its susceptible counterpart converted only 36 percent of a similar dose (Bami *et al.,* 1957). This difference in detoxication is even more pronounced in a similar subspecies from California (Hoskins *et al.,* 1958).

A 10-fold increase in DDT dehydrochlorinase was recently reported by Brown *et al.* (1965) to be responsible for DDT

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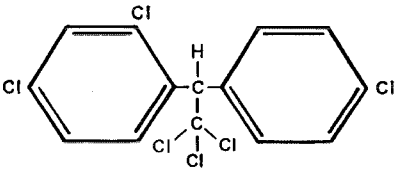
TABLE 1.—Toxicity and metabolism of DDT derivatives in susceptible and resistant *Culex fatigans* and *Aedes aegypti* larvae exposed for 24 hours to 0.5 ppm of the insecticide

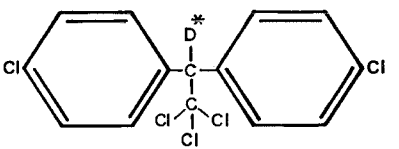
	Culex pipiens fatigans						*Aedes aegypti*			
	Susceptible		Savannah		Delhi (R)		Susceptible		Resistant (TR)	
Insecticide	LC_{50}	% Metabol.	LC_{50}	% Metabol.	LC_{50}	% Metabol.	LC_{50}	% Metabol.	LC_{50}	% Metabol.
p,p'-DDT	0.042	60.5 27.6*	0.18	52.2*	>2.5	60.2 51.3*	0.006	20.4	>4.0	55.6
deutero-DDT	0.045	33.9	0.13	..	1.4	42.4	0.009	6.5	0.5	39.5
ortho-chloro-DDT	0.090	6.3	0.07	..	0.29	7.0	0.04	4.0	6.0	15.9
o,p-DDT	0.450	0.4	0.23	..	>2.5	0.9	>2.5	1.4	>2.5	1.6
TDE	0.014	11.0	0.027	..	1.2	36.4	..	..	..	..

* Exposed for short duration.



ORTHO-CHLORO-DDT

	HOUSEFLY LD_{50} μG/FLY		MOSQUITO (*A. AEGYPTI* LARVA) LC_{50} P.P.M.	
	P, P' -DDT	O-CL-DDT	P, P'-DDT	O-CL-DDT
SUSCEPTIBLE	0.2	0.3	0.006	0.04
RESISTANT	> 50	1.0	4.0	6.0



DEUTERO-DDT

	HOUSEFLY LD_{50} μG/FLY		MOSQUITO (*A. AEGYPTI* LARVA) LC_{50} P.P.M.	
	P, P'-DDT	DEUTERO-DDT	P, P'-DDT	DEUTERO-DDT
SUSCEPTIBLE	0.2	0.15	0.006	0.009
RESISTANT	> 50	5.0	4.0	0.5

FIG. 1.—Comparative toxicity of p,p'-DDT, ortho-chloro-DDT, ortho-chloro-DDT, and deuterated DDT to houseflies and mosquitoes.

resistance in *Culex fatigans*. The enzyme is thought to be slightly different from that found in *Aedes aegypti* or in *Musca domestica*. In contrast, a highly resistant strain of *Culex pipiens molestus* produced little DDE (Perry, 1960), and both resistant and susceptible *Culex pipiens fatigans* from India, dehydrochlorinated DDT at an equal rate (Kalra *et al.*, 1966).

Among anopheline species, correlation of DDT resistance with dehydrochlorination is even less satisfactory (Perry, 1960; Lipke and Chalkley, 1964). However, there are notable exceptions such as DDT-resistant *Anopheles sundaicus* (Kearns, 1957) and *Anopheles sacharovi* (Perry, 1960) which produce large amounts of DDE.

Lack of correlation between resistance and dehydrochlorination extends to DDT derivatives such as deuterated DDT and ortho-chloro-DDT. Theoretically, the ortho-chloro compound should be refractory to dehydrochlorination because of steric hindrance due to the ortho chlorine atom (Hennessy *et al.*, 1961) and, therefore, its toxicity toward the DDT-resistant strain should be greater. This hypothesis has been proven to be correct with many DDT-resistant strains of house flies, but DDT-resistant *Aedes aegypti* larvae tolerate even larger quantities of the ortho-chloro derivative. On the other hand, deuterated DDT which is metabolized quite readily by DDT-resistant *Aedes aegypti* larvae is considerably more toxic to the resistant strains than p,p'-DDT (Figure 1). In contrast with the results shown above, Kimura and Brown (1964) obtained exidence that *in vitro* metabolism of ortho-chloro-DDT proceeds at a faster rate than that of deuterated DDT. This finding conforms with the toxicity data but negates the steric hindrance hypothesis.

Judging from all available information it is impossible at present to reconcile the hypothesis of enhanced detoxication with resistance to several DDT derivatives.

METABOLIC FATE OF OTHER CHLORINATED HYDROCARBON INSECTICIDES. Little is known about the fate of chlorohydrocarbon insecticides other than DDT, in mosquitoes. The γ-isomer of benzene hexachloride undergoes little degradation (10 percent in 24 hours) following its administration to BHC-resistant and susceptible *Anopheles gambiae* adults (Bradbury and Standen, 1956). This contrasts immensely with the BHC-resistant house fly which is able to metabolize 80 percent or more of the absorbed dose during the same interval.

The metabolism of cyclodiene insecticides by mosquitoes is largely in a state of confusion. Whereas most investigators agree that aldrin is converted to dieldrin by several mosquito species, there is no agreement as to the production or identity of other metabolites. At this laboratory, the writer (unpublished results, 1962, 1965) found only dieldrin-C^{14} as a product of aldrin-C^{14} metabolism by dieldrin-resistant *Aedes aegypti* and *Anopheles quadrimaculatus*. Similarly, Gerolt (1965) found less than 5 percent conversion of dieldrin to hydrophilic material by resistant or susceptible *Aedes aegypti* larvae. Korte *et al.* (1962), on the other hand, found that aldrin-C^{14} was converted by *Aedes aegypti* larvae not only to dieldrin but to a large extent (72–92 percent) into a more polar compound which was largely excreted. The same unidentified metabolite was obtained following exposure of larvae to dieldrin.

Dieldrin-resistant *Culex pipiens quinquefasciatus* were found by Oonnithan and Miskus (1964) to convert dieldrin-C^{14} to a polar metabolite having an R_F value on paper chromatography similar to that of aldrin glycol (hexachloro-octahydro-6,7-dihydroxy-dimethano-naphthalene). The metabolite was largely excreted, but sufficient unchanged dieldrin remained in the tissues to cause mortality of many susceptible individuals. A dieldrin metabolite with similar properties, identified as 6,7-dihydroxy-dihydro-aldrin was isolated and purified by Korte and Arent (1965).

Again we are faced here with the perpetual question, whether or not the re-

maining unchanged insecticide in the tissues is in a physiologically active state and at the site of action. Until these questions can be answered and the mode of action of dieldrin is better understood, resistance to the cyclodiene compounds will remain a highly speculative issue.

RESISTANCE TO ORGANOPHOSPHORUS AND CARBAMATE INSECTICIDES. Resistance to organophosphorus (OP) compounds such as parathion, malathion, diazinon, etc., is biochemically limited to levels peculiar to each compound. The resistance is rather specific, relatively unstable and the pattern varies from strain to strain. It is possible that more than one mechanism may be responsible for this type of resistance. Selection with organophosphorus compounds usually results in high levels of cross-resistance to chlorinated insecticides, especially of the DDT type.

Malathion is hydrolyzed extensively by many insect species, and the degradation involves two major pathways: one due to attack by phosphatase enzymes at the phosphorus-sulfur or carbon-sulfur linkages yielding dialkyl phosphates and thiophosphates; another owing to hydrolysis of the diethyl succinate moiety by carboxyesterase enzymes yielding the nontoxic mono- and dicarboxylic acid derivatives of malathion. In malathion-resistant *Culex tarsalis,* resistance is associated with enhanced malathion detoxication, and carboxyesterase action predominates (Figure 2, Table 2). (Matsumura and Brown, 1961a, 1963a; Bigley and Plapp, 1962). In *Aedes aegypti,* Brown and Abedi (1960) and Matsumura and Brown (1961b, 1963b) found no evidence of a greater malathion-detoxifying ability in the resistant strain but only a less efficient absorption of the insecticide.

Evidence was also obtained for the presence of OP-sensitive and OP-insensitive aliesterase enzymes in larvae of several *Culex* species as well as in *Aedes aegypti* and *Anopheles quadrimaculatus,* but not all of these species react in the same manner to different substrates such as phenyl acetate, methyl butyrate and tributyrin (Plapp *et al.,* 1965). Also, no gross changes in methyl butyrate-hydrolyzing enzymes could be discerned between resistant and susceptible *Aedes aegypti* larvae. Hence, the OP-resistance mechanism(s) in mosquitoes might differ in some respects from that found in the house fly.

TABLE 2.—Distribution of malathion degradation products resulting from phosphatase and carboxyesterase action in various insects.

	Total Phosphatase Products (%)	Total Carboxyesterase Products (%)	Total Unknown (%)	Reference
Culex tarsalis				
in vivo Susc.	48.9	37.7	11.9	Matsumura & Brown (1961a)
in vivo Res.	54.8	35.0	7.7	Matsumura & Brown (1961a)
in vitro Susc.	6.3	9.6	....	Matsumura & Brown (1963a)
in vitro Res.	14.4	24.3	....	Matsumura & Brown (1963a)
Musca domestica				
in vivo Susc.	67.0	12.0	21.0	Krueger & O'Brien (1959)
in vitro Susc.	8.6	10.8	....	Matsumura & Hogendijk (1964)
in vitro Res.	10.8	59.6	....	Matsumura & Hogendijk (1964)
Blattella germanica	47.0	36.0	17.0	
Periplaneta americana	50.0	44.0	6.0	Krueger & O'Brien (1959)
White mouse	20.5	68.0	11.5	

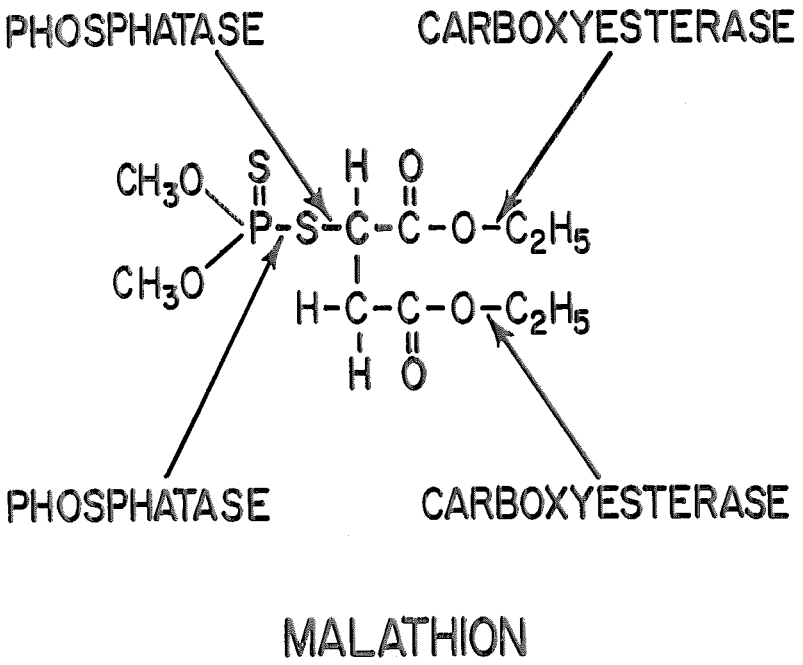


FIG. 2.—Pathways of malathion metabolism in *Culex tarsalis* and other insects.

Carbamate insecticides, too, are subject to destructive hydrolysis by insect enzymes. Thus, the carbamate insecticide carbaryl is metabolized to the nontoxic derivative 1-naphthol. In mosquitoes, resistance to carbaryl is attributed to accelerated metabolism, slower penetration, and enhanced excretion of the chemical.

The paucity of data on the biochemistry of organaphosphorus and carbamate insecticide resistance in mosquitoes is perhaps due to the slow acquisition of resistance to these compounds by mosquitoes in general. For example, Georghiou and Metcalf (1963) attempted to select *Culex pipiens quinquefasciatus* and *Anopheles albimanus* with a carbamate, but only vigor tolerance was obtained after 20 generations of selection.

Aedes nigromaculis seems to be the only mosquito species in which a broad spectrum type of OP-resistance has occurred (Brown *et al.*, 1963).

RESISTANCE COUNTERACTION. From a biochemical viewpoint, resistance counteraction is based on interference with the resistance mechanism. Thus, piperonyl butoxide and other methylenedioxyphenyl compounds will effectively synergize several organophosphorus and carbamate insecticides against resistant insects. Similarly, EPN, TOCP, and several nontoxic trisubstituted phosphoric acid derivatives will enhance the toxicity of malathion by blocking carboxyesterase activity; and n-propyl paraoxon will enhance paraoxon effectiveness by inhibiting corresponding enzyme activity (Table 3).

Intensified efforts should be made to elucidate the ressitance mechanisms and explore the possibility of applying counter measures by structural modifications of these compounds at reactive sites and by the use of detoxication-blocking agents.

Finally, a comment on the origin of resistance.

TABLE 3.—Mechanism of action of synergists against various insecticide-resistant insects.

Synergist	Will synergize	Against
DMC,[1] BST,[2] WARF-Anti-resistant,[3] etc.	DDT	DDE-producing insects
piperonyl butoxide, sulfoxide, sesamex, propyl isome, etc.	pyrethrins: Co-Ral, Diazinon, Dipterex, Potasan; Isolan, Sevin Isopropyl-, Isopropoxy-N-methyl carbamates, etc.	Insects capable of hydrolyzing or oxidizing these compounds
EPN,[4] TOCP[5]	malathion	Carboxyesterase action, e.g. *Culex tarsalis, Musca domestica*
n-propyl para-oxon	para-oxon Diaz-oxon	Aliesterases of para-oxon-resistant house flies.

[1] bis-(*p*-chlorophenyl)methyl carbinol
[2] 4-bromobenzene sulfonotoluidide
[3] N,N-dibutyl-*p*-chlorobenzene sulfonamide
[4] O-ethyl O-*p*-nitrophenyl phenylphosphonothioate
[5] Tri-ortho-cresyl phosphate

It has generally been accepted that resistance arises by intensive selection with an insecticide followed by inbreeding of survivors; that is to say, selection of a mutant present in low frequency in an insect population. The present trend in biochemical genetics and the impetus provided by molecular biology emphasize the nature of genes and proteins as dynamic processes undergoing constant evolutionary changes. Many factors are involved in bringing about these changes including external pressures from foreign compounds and drugs. Certainly, insecticides too, fall in the same category.

From an evolutionary standpoint the greatest biochemical changes took place when marine organisms ventured onto land. According to Brodie (1962) disposal of foreign compounds, especially lipid material, became essential during the transition from aquatic to terrestrial life when waterproofing of the skin replaced a less rigid, semipermeable membrane. Thus, the land dweller developed biochemical mechanisms to oxidize non-polar foreign compounds to more polar derivatives which could be excreted.

Prominent among the drug-disposal mechanisms are the microsomal enzymes —a class of enzymes requiring $NADPH_2$ and oxygen but having no specific substrates of their own. Such enzymes have been found in several insect species to hydroxylate DDT (Tsukamoto, 1959; Agosin *et al.*, 1961; Dinamarca *et al.*, 1962); to act on parathion (Nakatsugawa and Dahm, 1965); on BHC (Ishida and Dahm, 1965); to oxidize carbaryl, pyrethrins, rotenone, etc., (Tsukamoto and Casida, 1965) and to epoxidize aldrin (Schonbrod *et al.*, 1965; Nakatsugawa *et al.*, 1965).

It is obvious that these enzymes could not have arisen as mutations for the sole purpose of detoxifying insecticides, most of which appeared on the market less than two decades ago. An alternative explanation as originally suggested by Brodie (1956, 1962), and by many other workers since, is that foreign compounds induce these detoxifying enzymes to act and destroy the compounds before the latter can produce a harmful effect on the organism. This induction process need not be a *de novo* synthesis of the enzyme such as occurs in microorganisms, but it could stimulate the activity of preadaptive microsomal enzymes. These enzymes show little discrimination and have no specific substrates of their own. This hypothesis may not hold true for the DDT-type

compounds since the DDT dehydrochlorinases show a great deal of substrate specificity and are not of microsomal origin. The induction process by DDT might be of the type proposed by Agosin *et al.*, (1963) and Ilevicky *et al.*, (1964), i.e., the stimulation of *in vivo* protein synthesis, including enzymes.

Whatever the answer may be, it is clear that elucidation of the resistance phenomenon must stem from new probes into the field of molecular biology, i.e., from studies on DNA and RNA—makers of the genetic code and primers of enzyme and protein synthesis.

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