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Studies on the Mechanism of DDT-Resistance in *Culex pipiens fatigans*

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Since the appearance of DDT-resistance in houseflies in 1947, over 30 mosquito species have developed resistance to this and other commonly used insecticides. Much knowledge has been gained concerning the mechanism of resistance in insects but, in general, this phenomenon cannot be explained in terms of a single factor common to all resistant species.

Enzymic detoxication of DDT to DDE has been found in the study reported to be a major factor in the resistance of several mosquito species, particularly Aedes aegypti. *However, such correlation could not be established in a highly DDT-resistant strain of* Culex pipiens fatigans *originating in Delhi, India. Furthermore, resistance of the latter strain to* o-*chloro-DDT and* o,p-*DDT cannot be attributed to a detoxication process since these compounds are refractory to dehydrochlorination both* in vivo *and* in vitro. *Quantitative data on metabolism of DDT derivatives are given and other possible mechanisms of resistance are discussed.*

The metabolism of DDT to DDE was found to be a significant defence mechanism against the lethal action of DDT on houseflies and certain other insects (see review by Perry, 1964). This relationship holds true for several mosquito species although it is by no means universal.

Bami et al. (1957) observed that DDT-resistant *Culex pipiens fatigans* adults produced more DDE than their normal counterparts. Kalra & Joshi (1962) found that DDT-resistance in this species extended to both *p,p'*-DDT and *o,p*-DDT. Further work by Kalra [1] suggested that a single defence mechanism could not possibly account for the resistance to both isomers, since *o,p*-DDT is more stable to alkali dehydrochlorination than *p,p'*-DDT is. On the other hand, Kimura et al. (1965) observed that *o*-chloro-DDT which also is refractory to alkali dehydrochlorination, was detoxified *in vitro* by the enzyme DDT-dehydrochlorinase isolated from a DDT-resistant strain of *C. p. fatigans* originating in Rangoon.

Substitution of deuterium for the aliphatic hydrogen in the DDT molecule could produce an isotopic effect on dehydrochlorination (Barker, 1960; Dachauer et al., 1963), thereby reducing the rate of DDT breakdown and increasing its toxicity. The results of Pillai et al. (1963) proved that deutero-DDT was highly toxic to DDT-resistant *Aedes aegypti* larvae but only slightly more toxic than *p,p'*-DDT to their susceptible counterparts.

Recently, Hooper (1967) detected TDE and two other unidentified metabolites as products of DDT metabolism by *C. p. fatigans*. Our findings confirm the presence of small amounts of TDE and the dehydrochlorinated product TDEE in several *C. p. fatigans* strains but the source of these metabolites has not been ascertained.

The present investigation was undertaken to obtain quantitative data on the comparative toxicity and metabolism of *p,p'*-DDT, deutero-DDT, and *o*-chloro-DDT, and to correlate these findings with DDT resistance in *C. p. fatigans*.

MATERIALS AND METHODS

Insects

Susceptible and DDT-resistant strains of *C. p. fatigans* were used during this investigation. The

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[1] Kalra, R. L.—unpublished data, 1965.

susceptible strain was obtained from Dr P. Georghiou, University of California, Riverside, and was reared at the National Communicable Disease Center insectary. The Savannah strain is being maintained at the Savannah laboratory under continuous exposure to DDT by treating a plywood panel comprising 15% of the surface of the colony cage with 25 mg of *p,p'*-DDT per square foot (0.25 mg/m²). Adults of a strain designated as Delhi were originally collected from sprayed areas around Delhi, India. Larvae of this strain were reared in the laboratory under selection pressure with *o,p*-DDT for 10 generations. This strain was transported from Delhi to the National Communicable Disease Center, Savannah, and reared without further selection before use.

Insecticides

Purified samples of *p,p'*-DDT, *o,p*-DDT, *o*-chloro-DDT (2-(2,4-dichlorophenyl)-2-(4-chlorophenyl)-1,1,1-trichloroethane), deutero-DDT, and TDE and their metabolites were used. Technical-grade DMC (bis(*p*-chlorophenyl)methylcarbinol) was used to study its synergistic effect with *p,p'*-DDT.

Bioassay

The susceptibility of the strains of *C. p. fatigans* was assessed by exposing groups of 20 third-instar larvae, each group in 100 ml of water containing the desired concentration of insecticide in 1 ml of acetone. Mortality counts were made after 24 hours of continuous exposure. The results were plotted on log-probit paper and the LC_{50} and LC_{90} of various insecticides were determined.

Exposure and extraction

For the studies on metabolism of *p,p'*-DDT and related compounds, 250 larvae were exposed to 0.5 ppm of the insecticide in 500 ml of water for 24 hours. After exposure, the larvae were thoroughly rinsed with distilled water and then washed with 3 successive 10-ml portions of chloroform to remove the unabsorbed insecticide. The larvae were then ground with sodium sulfate and extracted with ether in a Soxhlet apparatus for 6 hours. After evaporation of the solvent, the external and internal extracts were dissolved in *n*-hexane and chromatographed through a column containing 10 g of Florisil [1] using 100 ml of ether in *n*-hexane (1 : 20) as the eluting solvent. The solvent was evaporated and the residue was taken up in nanograde petroleum ether and analysed by gas–liquid chromatography. For each insecticide parallel tests were made with susceptible and resistant strains.

Larvae of the susceptible and resistant strains were also exposed to insecticides for short periods (approximately 3 hours) to study the comparative rate of metabolism before the manifestation of toxic symptoms. The synergistic effect of DMC on the toxicity of DDT was investigated by exposing susceptible and resistant larvae for 24 hours to various combinations of DDT and DMC. Following exposure, the larvae were removed from the treated water and placed in fresh water for an additional period of 24 hours. Mortality counts were taken at the end of this period and the results plotted on probit paper.

Gas–liquid chromatography

The residues were redissolved in nanograde petroleum ether and 5-μl aliquots were injected into a MicroTek gas chromatograph (Model GC-2500-R) equipped with an electron-capture detector using either tritium or ^{63}Ni as a source of electrons. The columns were constructed of 6 ft by ¼-in (1.8×6 mm) external diameter aluminium tube packed with 5% OV-17 on 110/120-mesh Anakrom SD or with 5% QF-1 on 60/80-mesh chromosorb GAW and were maintained at 195°C. The carrier gas was nitrogen at a flow-rate of 100 ml/min. The electron-capture detectors were held at 230°C for tritium and 260°C for ^{63}Ni. The chromatograms obtained were compared with standards of known concentrations injected on the same day under identical operating conditions. The areas under the peaks were measured and computed to obtain the quantitative data.

RESULTS

The LC_{50} and LC_{90} values of *p,p'*-DDT and related compounds for the susceptible Savannah and Delhi strains of *C. p. fatigans* are given in Table 1. The results showed that the Delhi strain was highly resistant to *p,p'*-DDT, *o,p*-DDT, and TDE. The degree of tolerance to *o*-chloro-DDT and deutero-DDT was much lower than that to *p,p'*-DDT but, nevertheless, was significantly higher than the tolerance of the susceptible strain to the same insecticides. The Savannah strain showed a

[1] A product of Floridin Co., Warren, Pennsylvania. The use of trade names is for purposes of identification only and does not constitute endorsement by the Public Health Service, US Department of Health, Education, and Welfare.

TABLE 1

TOXICITY [a] OF *p,p'*-DDT AND DDT-DERIVATIVES AGAINST SUSCEPTIBLE AND DDT-RESISTANT *C. P. FATIGANS* LARVAE

Insecticide	Susceptible		DDT-resistant			
			Savannah		Delhi	
	LC_{50}	LC_{90}	LC_{50}	LC_{90}	LC_{50}	LC_{90}
p,p'-DDT	0.04	0.09	0.18	0.35	5.0	20.0
o,p-DDT	0.17	0.34	0.41	1.0	>2.5	—
o-chloro-DDT	0.07	0.13	0.11	0.19	0.5	1.5
Deutero-DDT	0.02	0.04	0.05	0.09	0.6	1.5
TDE	0.01	0.02	0.03	0.05	1.2	2.1

[a] Values given in parts per million of insecticide.

slight increase in tolerance to *p,p'*-DDT and *o,p*-DDT, but was almost as susceptible to deutero-DDT, *o*-chloro-DDT, and TDE as the normal strain.

DDE was the principal metabolite of *p,p'*-DDT obtained by gas chromatography. However, small amounts of *p,p'*-TDE and *p,p'*-TDEE were identified in several samples (Fig. 1). Using the solvent systems described by Abedi et al. (1963) and Perry et al. (1963), paper chromatography revealed only DDE as the product of *p,p'*-DDT metabolism since the small amounts of TDE which might have been present could not be resolved by this method. The source of TDE in these samples has not been ascertained. Judging from the fact that production of this metabolite was not consistent in all experiments and from the minute amounts obtained in relation to DDE, it is conceivable that TDE was a product of DDT metabolism by micro-organisms. Extracts of the DDT-treated water plus acetone rinses of the glass bowls after removal of the water showed mostly DDT and small amounts of DDE. Hence, metabolism of DDT to TDE by micro-organisms in the aqueous medium can be excluded from consideration. Other possible factors are currently being investigated.

When exposed to 0.5 ppm of *p,p'*-DDT for 24 hours, larvae of the two resistant strains contained appreciable amounts of DDE; although they differed considerably in their degree of tolerance to DDT, the percentage of DDT metabolized was practically the same in both strains (Table 2). The percentage of DDT metabolized by the susceptible strain was lower in all cases, but it must be taken into account that these larvae were exposed to a DDT concentration 5 times their LD_{90}.

Deutero-DDT was metabolized at an approximately equal rate by the susceptible and the resistant strains even though their respective tolerances for this insecticide differed markedly. Similarly, no difference in the metabolism of *o*-chloro-DDT and *o,p*-DDT was observed among the strains, and both compounds were found to be refractory to dehydrochlorination *in vivo* (Table 2).

The results obtained with the 3-hour exposure periods showed a similar trend in that the Savannah

FIG. 1

GAS–LIQUID CHROMATOGRAMS OF INTERNAL EXTRACTS OF SUSCEPTIBLE AND DDT-RESISTANT *C. P. FATIGANS* AFTER EXPOSURE OF LARVAE TO 0.5 PPM *p,p'*-DDT FOR 24 HOURS

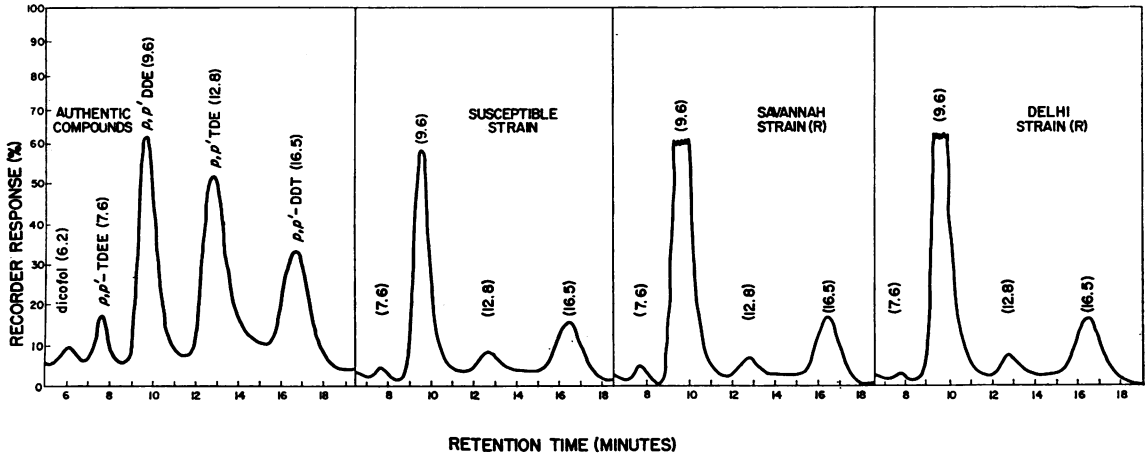


TABLE 2
PICK-UP, ABSORPTION, AND METABOLISM [a] OF *p,p'*-DDT AND DDT DERIVATIVES BY SUSCEPTIBLE AND DDT-RESISTANT *C. P. FATIGANS* LARVAE AFTER EXPOSURE TO INSECTICIDE [b] FOR 24 HOURS

Insecticide	Susceptible				DDT-resistant							
					Savannah				Delhi			
	External	Internal		Metabolized (%)	External	Internal		Metabolized (%)	External	Internal		Metabolized (%)
	DDT	DDT	DDE		DDT	DDT	DDE		DDT	DDT	DDE	
p,p'-DDT	1.5	5.5	4.7	46.1	1.2	2.8	10.2	78.4	2.6	5.0	12.3	71.1
o,p-DDT	7.2	14.3	0.2	1.4	4.6	11.9	0.8	6.3	4.0	17.4	0.7	3.9
o-chloro-DDT	2.3	2.7	0.3	10.0	0.7	4.2	0.3	6.6	2.0	4.0	0.4	9.1
Deutero-DDT	4.2	6.6	4.5	40.5	1.7	4.4	4.3	49.4	2.5	9.5	8.5	47.2

[a] Values given in μg per 250 larvae. [b] 0.5 ppm.

and Delhi strains, while differing considerably in their level of resistance to DDT, metabolized DDT to DDE to the same extent (Table 3). As previously, the susceptible strain metabolized less DDT than the resistant strains.

Synergistic effect of DMC

When combined with *p,p'*-DDT, DMC showed a slight synergistic activity against the susceptible and Savannah strains, and moderate activity against the resistant Delhi strain, provided the concentration of DMC was kept below 10 ppm. At a concentration of 10 ppm and higher, DMC was very toxic to *C. p. fatigans* larvae. The effective range of dosages over which the synergist can be used successfully is limited by its own toxicity which, at 15 ppm, exceeds the toxicity of DDT. A plot of these results with the Delhi strain (Fig. 2) shows the limit of synergistic activity at the lower dosages, the additive effect at moderate dosages, and the somewhat antagonistic effect at higher dosages. These combinations are therefore impracticable for effective control operations.

DISCUSSION AND CONCLUSIONS

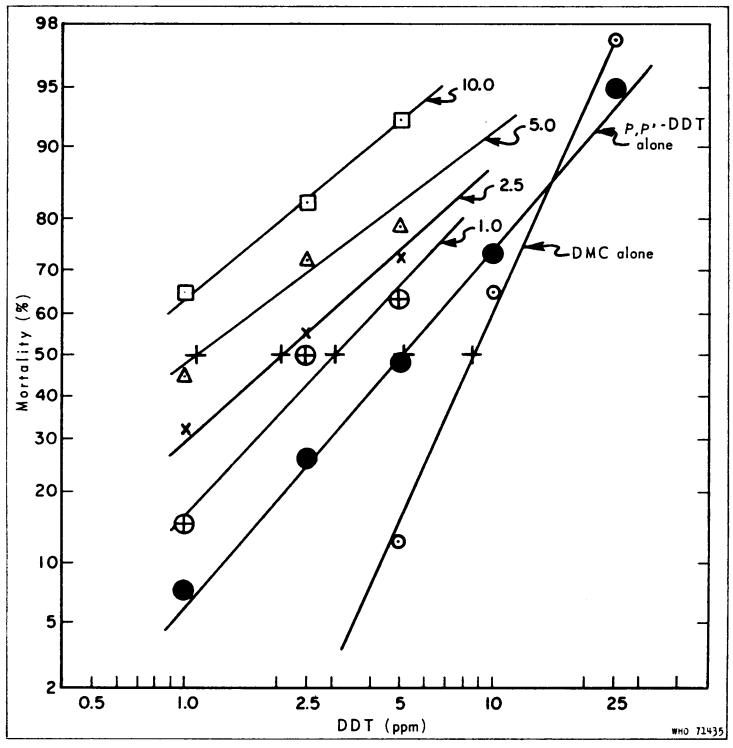
The results of susceptibility and insecticide metabolism tests indicated that the Savannah strain possesses a defence mechanism that is specific for *p,p'*-DDT but does not extend to *o*-chloro-DDT and *o,p*-DDT. The resistant Delhi strain, however, is more tolerant to *p,p'*-DDT but the resistance extends also to *o*-chloro-DDT, *o,p*-DDT deutero-DDT, and TDE. Since the *ortho*-substituted compounds are refractory to dehydrohalogenation, the resistance of the Delhi strain to these compounds must involve more than one defence mechanism. Furthermore,

TABLE 3
PICK-UP, ABSORPTION, AND METABOLISM [a] OF *p,p'*-DDT AND DDT DERIVATIVES BY SUSCEPTIBLE AND DDT-RESISTANT *C. P. FATIGANS* LARVAE AFTER EXPOSURE TO INSECTICIDE [b] FOR 3 HOURS

Insecticide	Susceptible				DDT-resistant							
					Savannah				Delhi			
	External	Internal		Metabolized (%)	External	Internal		Metabolized (%)	External	Internal		Metabolized (%)
	DDT	DDT	DDE		DDT	DDT	DDE		DDT	DDT	DDE	
p,p'-DDT	1.3	1.8	1.2	39.3	1.2	0.5	1.8	78.3	1.1	0.8	2.0	71.5
o,p-DDT	4.1	7.0	0.6	7.9	3.2	7.1	0.7	8.9	5.4	9.1	0.9	9.0
o-chloro-DDT	2.7	3.3	0.2	5.7	4.6	4.5	0.2	4.3	3.4	4.7	0.3	6.0
Deutero-DDT	1.4	2.4	0.2	7.7	1.7	3.0	0.5	14.3	1.5	2.0	0.6	23.0

[a] Values given in μg per 250 larvae. [b] 0.5 ppm.

FIG. 2
LOG DOSAGE-PROBIT MORTALITY LINES FOR DDT-RESISTANT *C. P. FATIGANS* LARVAE (DELHI STRAIN) EXPOSED TO VARIOUS DDT-DMC COMBINATIONS [a]



[a] Values adjacent to curves indicate parts per million of DMC.

the fact that DMC (present report) and WARF anti-resistant compound [1] do not synergize DDT to a great extent against this strain indicates that dehydrochlorination might be supplemented by other resistance mechanisms. Another point of interest is the lack of correlation between quantitative metabolism of DDT and the LD_{50} values of the Savannah and Delhi strains, the Delhi strain being 28 times more resistant to DDT but producing no more DDE than the Savannah strain. Similar results were obtained by Perry (1960) and Lipke & Chalkley (1964) with certain anopheline species.

Pick-up and absorption of the insecticide were found to play no significant role in the resistance of the strains reported in this paper.

In comparing the substrate specificity of DDT-dehydrochlorinase from houseflies and mosquitos, it is apparent that metabolism of DDT in *C. p. fatigans* resembles that of the housefly (see Lipke & Kearns, 1960) in that *para-para-* substituents are attacked with greater ease by the enzyme, whereas the *ortho-para* substituents (see Hennessy et al., 1961) are attacked with difficulty. In *Aedes aegypti*, Abedi et al. (1963) and Kimura & Brown (1964) found a good correlation between dehydrochlorination and resistance to *p,p'*-DDT and *o*-chloro-DDT. However, there is no such clear-cut correlation in *C. p. fatigans*, as demonstrated in the experiments reported here.

Whereas deutero-DDT has been reported to be 50–100 times as toxic as *p,p'*-DDT to resistant *A. aegypti* larvae (Pillai et al., 1963) the present data indicate that deutero-DDT is only 4–12 times more toxic than *p,p'*-DDT to resistant *C. p. fatigans* larvae. The difference in dehydrochlorinating capacity between susceptible and resistant larvae is more pronounced in *A. aegypti* than in *C. p. fatigans* and the reduction in the LC_{50} is not of sufficient magnitude to make this compound of practical value against DDT-resistant *C. p. fatigans* larvae.

It is noteworthy that *o*-chloro-DDT showed the reverse trend. While DDT-resistant *A. aegypti* larvae were also found to be highly resistant to *o*-chloro-DDT (Pillai & Brown, 1965; Perry, 1966) larvae of the highly resistant strain of *C. p. fatigans* showed only moderate resistance to this compound (Table 1). These results clearly indicate a difference between the two species in toxic response to and metabolic pattern of DDT derivatives.

[1] Kalra, R. L. and Krishnamurthy, B. S.—unpublished results.

Correlation between *o,p*-DDT resistance and dehydrochlorination also leaves much to be desired since the Delhi strain of *C. p. fatigans* is quite resistant to *o,p*-DDT but metabolism of the compound is practically nil.

Mechanisms other than DDT detoxication have been demonstrated to play a major role in housefly resistance to DDT (Tsukamoto & Suzuki, 1964; Tsukamoto et al., (1965), and Pillai & Brown (1965) suggested that enhanced resistance to DDT–synergist combinations in *A. aegypti* might not be due to an increase in dehydrochlorinase activity but to an unknown defence mechanism.

The diversity of results and interpretations among workers in this field point to the need for further work on the isolation and purification of DDT-detoxifying enzymes in mosquitos and characterization of other factors involved in mosquito resistance.

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RÉSUMÉ

On a étudié chez des souches sensibles et résistantes de *Culex pipiens fatigans* le mécanisme de la résistance au *p,p'*-DDT et aux produits apparentés, en recourant à la chromatographie pour évaluer le rôle des processus métaboliques de détoxication.

Les larves des souches résistantes et sensibles ont métabolisé le *p,p'*-DDT et l'ont transformé en *p,p'*-DDE. De faibles quantités de TDE et de TDEE ont également été décelées. Bien que l'activité métabolique ait été plus élevée chez les souches résistantes, on n'a pu découvrir une corrélation satisfaisante entre ce facteur et l'intensité de la résistance. Le deutéro-DDT a fait preuve d'une toxicité 4 à 12 fois plus élevée que le *p,p'*-DDT pour les souches résistantes, mais cette réduction de la CL_{50} n'est pas suffisante pour que l'on puisse envisager de le substituer au DDT. Bien que peu sensible à l'action de la DDT-déshydrochlorinase, le *o*-chloro-DDT s'est montré peu toxique pour les souches résistantes. Chez les souches sensibles comme chez les souches résistantes, seules de très petites quantités d'*o,p*-DDT et de *o*-chloro-DDT ont été métabolisées, ce qui démontre que l'on ne peut attribuer uniquement à un phénomène de détoxication la résistance à ces composés.

Il semble que chez *C. p. fatigans* la résistance aux composés du DDT et aux agents synergiques qui leur sont éventuellement associés fasse intervenir des mécanismes auxiliaires de nature indéterminée.

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