DDT/PYRETHROID RESISTANCE INTER-RELATIONSHIPS IN *ANOPHELES STEPHENSI*

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ABSTRACT. Cross-resistance to (1*R*)-*trans*-permethrin (12-fold at LC_{50}) and to (1*R*)-*cis*-permethrin (18-fold) was induced in *Anopheles stephensi* larvae by selection in the laboratory with DDT for 6 generations to a level of 144-fold DDT-resistance. Selection by (1*R*)-*trans*-permethrin of a sub-strain in F_4 and F_5 increased resistance to (1*R*)-*trans*-permethrin and (1*R*)-*cis*-permethrin to 15-fold and 20-fold the initial levels, respectively. The highest cross-resistance to (1*R*)-*trans*-permethrin (19-fold) and to (1*R*)-*cis*-permethrin (23-fold) were detected in a sub-strain that had been selected in F_4 and F_5 by DDT in combination with the synergists DMC and piperonyl butoxide. In none of the selected strains were DDT or pyrethroids synergized significantly by these metabolic inhibitors thus suggesting reduced sensitivity of active site as the major factor responsible for the observed resistance.

Intracellular recordings from larval muscles of susceptible and resistant strains revealed that the concentrations of (1*R*)-*cis*-permethrin needed to cause an increase in miniature excitatory postsynaptic potentials were $5 \times 10^{-11}M$ and $1 \times 10^{-9}M$, respectively. Miniature excitatory postsynaptic potential frequency is a measure of the poisoning of motor nerve terminals, these being particularly susceptible to pyrethroid and DDT poisoning. Thus, the observed resistance appears to be due largely to change(s) in the nervous system of resistant strains, producing a lower sensitivity to the toxic action of permethrin.

Several DDT-resistant strains of insects have been shown to exhibit cross-resistance to pyrethrins and synthetic pyrethroid insecticides. Busvine (1953) reported that a strain of the house fly, which had been selected for knockdown-resistance by DDT, proved to be cross-tolerant to pyrethrin. Similarly, DDT cross-resistance was found by Farnham and Sawicki (1976) in a pyrethroid-selected strain of the house fly. Other species, such as *Cimex* spp. (Busvine 1958) and *Boophilus* spp. (Whitehead 1959, Nolan *et al.* 1977), were also reported to show DDT/pyrethroid resistance.

Among mosquitoes, *Culex tarsalis* Coquillett possibly possesses a gene that confers resistance to both DDT and pyrethrins (Plapp and Hoyer 1968). DDT-resistant females of *Aedes aegypti* L. also showed cross-tolerance to several pyrethroids, the extent of which seemed to vary with the regional background of the strains (Chadwick et al. 1977, Prasittisuk and Busvine 1977). *Anopheles gambiae* Giles and *An. quadrimaculatus* Say (Prasittisuk and Busvine 1977), but not *An. stephensi* Liston (Chadwick *et al.* 1977), apparently displayed the same correlation.

The reverse is also true: Strains of *Cx. quinquefasciatus* Say that had developed high levels of resistance to pyrethroids by laboratory selection with (1*R*)-*cis*- and (1*R*)-*trans*-permethrin (Priester and Georghiou 1978) were found to show high levels of cross-resistance to DDT (Priester *et al.*, 1979). A *kdr*-like mechanism expressed as "site insensitivity" was postulated as the major component in the pyrethroid/DDT resistance.

Pyrethroids are currently receiving considerable attention as candidate chemicals for indoor spraying in malaria control programs. In view of the widespread occurrence of DDT resistance (WHO 1976), further work on the inter-

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relationships of DDT and pyrethroid resistance in *Anopheles* species is necessary. This study was performed on *An. stephensi*, a species that is widely resistant to DDT and dieldrin (WHO 1976) and is also beginning to show resistance to malathion (Manouchehri et al. 1976).

MATERIALS AND METHODS

STRAINS. The following strains of *An. stephensi* were used:

Susceptible (S): This was obtained from Walter Reed Army Institute of Research, Washington, D.C. It was originally brought from India in 1964.

Kasur-P: This strain was collected by Dr. W. K. Reisen from Kasur, Pakistan, during February and March, 1978, and was made available through the courtesy of Pakistan Medical Research Center, Lahore. At the time of initiation of this study, it was calculated that the strain contained DDT resistance genes at a frequency of 0.038 as evident by 92.5% mortalities caused by a 2-hr exposure of adults to 4% DDT-impregnated papers. These calculations assumed monofactorial inheritance of DDT resistance, as shown for the species by Davidson and Jackson (1961). This strain served as the parental stock in the selection of the following strains:

DDT-R: This strain was derived by applying DDT selection pressure to adults of the Kasur-P strain and to larvae of subsequent generations.

Permethrin-R: This strain was derived by selection with (1*R*)-*trans*-permethrin of a subcolony of DDT-R F_4 for 2 successive generations.

DDT/Syn.-R: This strain was derived by selection of another subcolony of DDT-R F_4 with a mixture of DDT + DMC + p.b.

CHEMICALS. The following chemicals were used for tests on larvae: *p,p'*-DDT, >96% pure.

(1*R*)-*cis*-permethrin, [3-phenoxybenzyl - (1*R*) - *cis* - 3 - (2,2 - dichlorovinyl) - 2,2 - dimethylcyclopropane carboxylate]; NRDC 167, 97% pure.

(1*R*)-*trans*-permethrin, [3 - phenoxybenzyl-(1*R*)-*trans*-3-(2,2-dichlorovinyl) - 2,2 - dimethylcyclopropane carboxylate]; NRDC 147, 99% pure.

DMC, [1,1-di-(*p*-chlorophenyl)-ethan-1-ol], 82%a.i. + 18% related compounds.

Piperonyl butoxide (p.b.), *d* - (2 - (2 - *n* - butoxyethoxy) - ethoxy - 4,5 - methylenedioxy - 2 - propyltoluene, >80% pure.

For adults the following chemicals were used:

DDT-impregnated papers, 4%, obtained from the World Health Organization.

(1*R*)-*cis,trans*-permethrin, [3-phenoxybenzyl - (1*R*) - *cis,trans* - 3 - (2,2 - dichlorovinyl) - 2,2 - dimethylcyclopropane carboxylate], NRDC 143, 1.7%: 96% *cis:trans*.

SELECTION. Selection of adults was performed in standard WHO adult-susceptibility kits (WHO 1970). Groups of 20 virgin mosquitoes were exposed for 2 hr, followed by a 24-hr holding period, and survivors were reared to obtain the F_1.

Larvae were selected in enamel pans containing water that had been treated with the appropriate concentration of insecticide or insecticide plus synergists in acetone solution, producing ca. 95% kill. Ten ml of the insecticide solution were applied per 1 liter of water, in which about 250 larvae were exposed for 24 hr. For selections and bioassay, synergists were used at constant sub-lethal concentrations of 2.5 ppm DMC and 5 ppm p.b.

BIOASSAY. Groups of 20 early 4th-instar larvae were exposed to the insecticide, or insecticide plus synergist(s), in 100 ml of water contained in waxed cups as described by Georghiou et al. (1966). Controls treated with acetone, or synergist(s) in acetone, generally yielded no mortalities.

The standard WHO test was used for adults (WHO, 1970). For tests with (1*R*)-*cis,trans*-permethrin, papers were prepared in the laboratory 2–3 hr before use. The insecticide solution (0.05%) was made in a 4:1 mixture of petroleum

ether:Risella Oil #921 (Shell Chem. Co., Modesto, Calif.). Of this solution, 5 ml were applied per 15 cm diam. glass fiber filter paper yielding a deposit of 14 $\mu g/cm^2$. Treated papers were held in darkness until use. Batches of 20 mosquitoes were exposed for varied durations and then held for 24 hr at which time mortalities were recorded. Sexes were tested separately, but because of the absence of significant differences the results were analyzed jointly.

Data were subjected to probit analysis. Resistance ratios (RR) were calculated by comparing resistant strains with S strains at the LC_{50} level. Synergism ratios (SR = LC_{50} insecticide ÷ LC_{50} insecticide + synergists) were discussed with reference to known resistance mechanisms.

KNOCKDOWN. Following preliminary tests, groups of 20 4th-instar larvae were exposed to 10 ppm (S) or 100 ppm (DDT-R F_6) DDT for 6 hr, followed by 18 hr in clean water. Records of knockdown were made at various intervals up to 24 hr. Larvae that were unable to swim to the surface and remain there were considered knocked down.

All tests and selections were carried out under temperatures of 27°C and >60% relative humidity.

INTRACELLULAR RECORDING. Larvae were dissected by dorsal mid-line incision, the gut removed and intracellular recordings from ventral longitudinal muscle $(VLM)_2$ (Samtleben 1929) made and monitored using standard electrophysiological techniques. Preparations were bathed in saline (Hayes 1953) containing (1*R*)-*cis*-permethrin at the concentrations indicated. After 30 min, VLM_2 muscles were monitored for miniature excitatory postsynaptic potentials. Forty muscles (4 larvae, 10 muscles per larva) were used for each assay.

RESULTS AND DISCUSSION

EFFECT OF DDT SELECTION ON DDT RESISTANCE AND PERMETHRIN-SUSCEPTIBILITY. The Kasur-P strain contained a moderate larval resistance of 8-fold (LC_{50} = 4.2 ppm) by comparison with the S strain (LC_{50} = 0.52 ppm) (Table 1). Selection of adults of the former strain with 4% DDT-papers increased larval resistance to 15-fold in the F_1. This selection probably removed the susceptible individuals, as was shown by Davidson and Jackson (1961). The intense selection pressure exerted on larvae of subsequent generations (over 95% kill) resulted in a rapid increase of DDT resistance; viz. from RR = 15 in the F_1 to RR = 98 in the F_4, where heterozygous individuals had probably been eliminated. Larvae of F_1, F_2 and F_3 were selected with 40, 70 and 100 ppm DDT, respectively. The slopes of regression lines (Fig. 1; Table 1) steepened from 2.2 in the Kasur-P strain to 5.5 in the DDT-R F_4 strain. The appearance of a plateau in the 1d-p lines of F_4-F_6 beyond 100 ppm is probably due to saturation of the test water with DDT.

Results of susceptibility tests of larvae

Table 1. Development of resistance to DDT and cross-resistance to permethrin in DDT-selected larvae of *Anopheles stephensi*.

STRAIN	DDT			(1*R*)-*trans*-permethrin			(1*R*)-*cis*-permethrin		
	LC_{50}	RR[a]	slope	LC_{50}	RR[a]	slope	LC_{50}	RR[a]	slope
S	0.52	1	2.7	0.013	1	2.5	0.0035	1	3
Kasur-P	4.2	8.1	2.2	0.014	1.1	1.8	0.0031	0.9	2.6
DDT-R									
F_1	7.8	15	2.3	0.028	2.2	2.2	0.013	3.7	2.5
F_2	24	46	2.9	0.044	3.4	2.7	0.018	5.1	2.6
F_3	38	73	4	0.055	4.2	2.7	0.029	8.3	2.5
F_4	51	98	5.5	0.072	5.5	2.9	0.041	12	2.5

[a] Resistance ratio=LC_{50} R strain ÷ LC_{50} S strain.

of the DDT-R strain to (1*R*)-*trans*-permethrin and its *cis*-isomer are illustrated in Fig. 2 and summarized in Table 1. The responses of the Kasur-P strain to (1*R*)-*trans*-permethrin (RR = 1.1) and 1*R*)-*cis*-permethrin (RR = 0.9) were not different from those of the S strain. However, development of DDT resistance induced an appreciable increase in cross-tolerance to both isomers (at F_4 RR = 5.5 for (1*R*)-*trans*-permethrin, and 12 for (1*R*)-*cis*-permethrin). There was little increase in the slopes of regression lines from one generation to the next.

It should be noted (Table 1) that although the *cis*-isomer (LC_{50} = 0.0031 ppm) was initially about 4 times as toxic to the Kasur-P strain as was the *trans*-isomer (LC_{50} = 0.014 ppm), larvae of subsequent generations exhibited a relatively higher RR to the former than to the latter chemical.

Comparable published data for anopheline larvae are lacking. DDT-selection of larvae of *Cx. tarsalis* similarly developed cross-tolerance to pyrethrin (ca. 10-fold) (Plapp and Hoyer 1968), while larvae of *Cx. quinquefasciatus* selected with (1*R*)-*cis*- and (1*R*)-*trans*-permethrin (Priester and Georghiou 1978) each displayed high levels of cross-resistance to DDT (Priester et al. 1979).

TESTS OF DDT-R F_4 WITH SYNERGIZED CHEMICALS. The results of tests with synergized insecticides are given in Table 2. DDT-susceptibility of the S strain was little affected by DMC (SR = 1.3), p.b. (SR = 1.6) or a mixture of both synergists (SR = 2.1). Likewise, susceptibility of the same strain to (1*R*)-*trans*-permethrin or (1*R*)-*cis*-permethrin was minimally depressed by the presence of p.b. (SR = 1.8). When DDT-R F_4 larvae were tested, DMC (SR = 1.2), p.b. (SR = 1.9) and DMC + p.b. (SR = 2.2) were found not to enhance the toxicity of DDT substantially. These results indicate that neither DDT-dehydrochlorination nor oxidative degradation are important defense mechanisms in DDT resistance of *An. stephensi*. They are comparable to the findings of Perry (1960) and Lipke and Chalkey (1964), who found no correlation between DDT resistance and the production of DDE in this species.

Since the results of this study (Table 2)

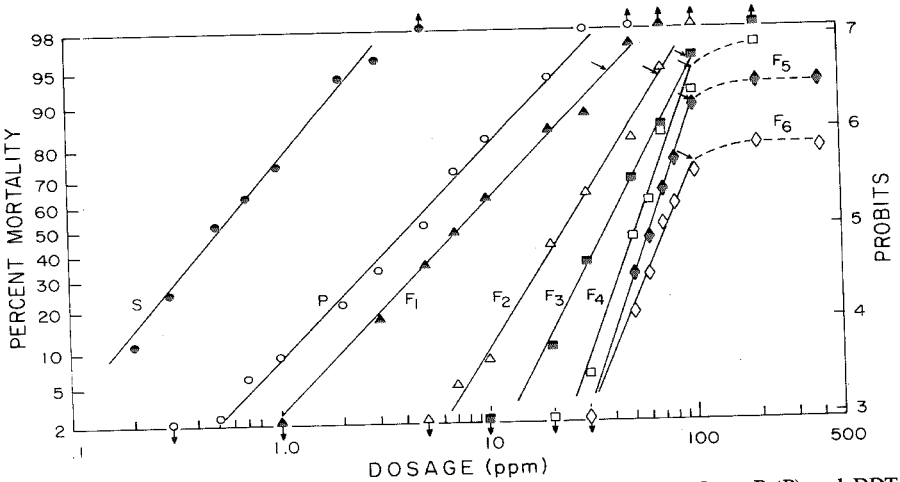

Figure 1. Dose-mortality relationships of DDT against susceptible (S), Kasur-P (P) and DDT-selected (F_1-F_6) larvae of *Anopheles stephensi*.

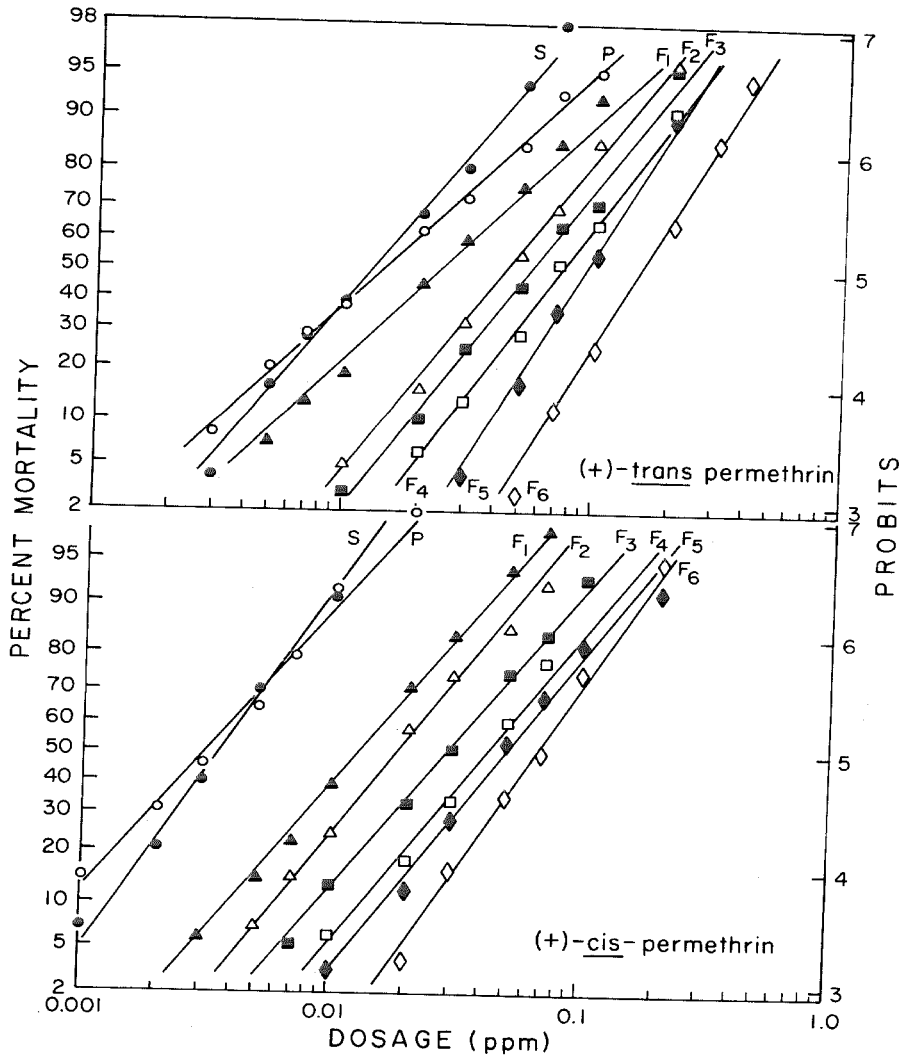

Figure 2. Dose-mortality relationships of (1*R*)-*cis*-permethrin and (1*R*)-*trans*-permethrin against susceptible (S), Kasur-P (P) and DDT-selected (F_1-F_6) larvae of *Anopheles stephensi*.

and those of published work (Chadwick et al. 1977, Prasittisuk and Busvine 1977, Priester et al. 1979, Priester and Georghiou 1979) indicated that cross-resistance between DDT and pyrethroids may be dependent primarily on non-metabolic factors (possibly on *kdr*-like factors) it was reasoned that further selection by DDT in the presence of synergists that block detoxication mechanisms would more effectively concentrate the non-metabolic factor(s). It was also assumed that selection by pyrethroids would contribute to pyrethroid resistance as well as further enhance DDT resistance, provided that pyrethroid selection pressure would also select for modifier genes.

DDT-R F_4 was thus subdivided into 3 lines, each of which was selected in 2 consecutive generations as follows: 1 line (strain DDT-R) was continued with DDT selection at 100 ppm, the 2nd (strain Permethrin-R) was selected with (1*R*)-*trans*-permethrin at 0.2 and 0.3 ppm, and the 3rd (strain DDT/Syn.-R) was selected with a mixture of DDT + DMC + p.b. at 50 and 70 ppm DDT.

FURTHER COMPARATIVE SELECTIONS. The results of these selections are also summarized in Table 2. Continued DDT selection increased DDT resistance to about 144-fold in the F_6 (Fig. 1), whereas selection by (1*R*)-*trans*-permethrin and a mixture of DDT + synergists resulted in even higher levels of DDT resistance (RR = 168 and 187, respectively).

The responses of each of the 3 strains to synergized DDT closely followed the pattern exhibited by DDT-R F_4 (Table 2). Generally, DDT resistance experienced little change due to DMC (SR = 1.2–1.4), p.b. (SR = 1.4–2.2) or the combined synergists (SR = 1.7–2.4). The toxicity of

Table 2. Effects of synergists on the responses of *Anopheles stephensi* larvae to DDT and permethrin.

STRAIN	DDT	DDT + DMC	DDT + p.b.	DDT + DMC + p.b.	(1*R*)-*t*-permeth.	(1*R*)-*t*-permeth. + p.b.	(1*R*)-*c*-permeth.	(1*R*)-*c*-permeth. + p.b.
S								
LC_{50}[a]	0.52	0.4	0.33	0.25	0.013	0.0072	0.0035	0.0019
RR[b]	1	0.76	0.63	0.48	1	0.55	1	0.54
SR[c]	—	1.3	1.6	2.1	—	1.8	—	1.8
DDT-R F_4								
LC_{50}	51	41	27	23	0.072	0.033	0.041	0.014
RR	98	102	158	176	5.5	4.6	12	7.4
SR	—	1.2	1.9	2.2	—	2.2	—	2.9
DDT-R F_6								
LC_{50}	75	61	46	38	0.16	0.050	0.063	0.022
RR	144	153	139	152	12	6.9	18	12
SR	—	1.2	1.6	2	—	3.2	—	2.9
Permethrin-R F_2								
LC_{50}	87	64	40	36	0.19	0.065	0.070	0.026
RR	168	160	121	144	15	9	20	14
SR	—	1.4	2.2	2.4	—	2.9	—	2.7
DDT/Syn.-R F_2								
LC_{50}	97	82	69	57	0.25	0.11	0.82	0.033
RR	187	205	209	228	19	15	23	17
SR	—	1.2	1.4	1.7	—	2.3	—	2.5

[a] LC_{50} values in ppm.
[b] Resistance ratio.
[c] Synergism ratio=LC_{50} insecticide ÷ LC_{50} insecticide + synergist.

DDT was least enhanced by the presence of synergists in the DDT/Syn.-R strain, thus affirming early indications of the concentration of non-metabolic factor(s).

The same pattern of responses was also true for permethrin (Table 2, Fig. 2). Continued DDT selection of the F_6 further enhanced the level of cross-tolerance to (1*R*)-*trans*-permethrin (RR = 12) as well as to (1*R*)-*cis*-permethrin (RR = 18). Selection by (1*R*)-*trans*-permethrin induced relatively higher levels of resistance to both the selecting chemical (RR = 15) and its *cis*-isomer (RR = 20) than those due to DDT selection. The highest levels of resistance to (1*R*)-*trans*- and (1*R*)-*cis*-permethrin were shown by larvae of DDT/Syn.-R F_2 (RR = 19 and 23, respectively).

Like DDT resistance, pyrethroid resistance in larvae of the 3 strains was only slightly affected by the presence of p.b., least so in DDT/Syn.-R F_6 (Table 2). The fact that a large part of the pyrethroid resistance (or cross-resistance) remains after synergism suggests that a major component of DDT/pyrethroid resistance in *An. stephensi* is due to non-metabolic factor(s). A *kdr*-like "site insensitivity" mechanism was also postulated as being largely responsible for DDT/pyrethroid resistance in *Cx. tarsalis* (Plapp and Hoyer 1968) and *Cx. quinquefasciatus* (Priester et al. 1979, Priester and Georghiou 1979).

INTRACELLULAR RECORDING. To further investigate this "site insensitivity," intracellular recordings were made from larval muscles. It has recently been shown that the terminals of motor axons are very susceptible to pyrethroid and DDT poisoning, producing abnormalities in neuromuscular transmission (Adams and Miller 1979, V. Salgado et al. in preparation). A convenient assay of motor nerve terminal function is to monitor miniature excitatory postsynaptic potentials (mepps). These potentials represent the spontaneous and continuous release of transmitter from the nerve terminals.

During poisoning, mepp frequency increases dramatically, and this increase corresponds to the onset of poisoning symptoms (S. N. Irving et al., in preparation).

Accordingly, the resting frequency of mepps was recorded (Fig. 3A). The concentration of (1*R*)-*cis*-permethrin needed to increase mepp frequency in larvae of the susceptible strain of *An. stephensi* was $5 \times 10^{-11}M$ (Fig. 3B). However, at this concentration, the mepp frequency of larvae of the resistant DDT/Syn.-R F_2 strain were unaffected. A concentration of $10^{-9}M$ was needed to increase the mepp frequency of this resistant strain.

This confirms that a highly susceptible region of the nervous system (the motor terminal) has changed in the resistant strain. The ratio of concentrations needed to produce mepp frequency in-

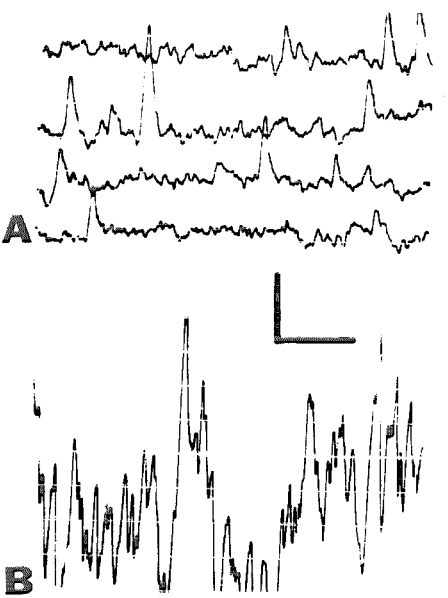

Figure 3. Miniature excitatory postsynaptic potentials recorded from ventral longitudinal muscle 2 of susceptible *Anopheles stephensi* before (A) and after (B) treatment with $5 \times 10^{-11}M$ (1*R*)-*cis*-permethrin. Calibration = 200μV: 40ms.

creases in S and DDT/Syn.-R F_2 strains is 20. Considering the crude assay, this figure agrees well with the toxicity RR of 23, confirming that the source of resistance is due to *kdr*-like factor(s) (reduced sensitivity of the active site).

CROSS-TOLERANCE TO (1*R*)-CIS, TRANS-PERMETHRIN IN DDT-RESISTANT ADULTS. At the LC_{50} level adults of the Kasur-P strain were 1.7-fold resistant to DDT and nearly as susceptible as the S strain to (1*R*)-*cis,trans*-permethrin (Table 3). DDT selection during 6 generations, mainly on larvae, yielded some 23-fold increase in DDT resistance in adults of the DDT-R F_6. It can be seen from Table 3 that, unlike in the case of larvae (Table 2), there were only insignificant differences in DDT resistance levels among the 3 strains. Such differences might have been masked by the long exposure periods used in DDT resistance tests, as it is recognized that such long exposure tests always pose problems of mortalities due to factors other than insecticide toxicity. Nevertheless, the fact remains that DDT-resistant adult *An. stephensi* displayed cross-tolerance to (1*R*)-*cis,trans*-permethrin. This was relatively higher in permethrin-R F_2 (RR = 10) and DDT/Syn.-R F_2 (RR = 11) than in DDT-R F_6 (RR = 9.4).

The work of Chadwick et al. (1977) and Prasittisuk and Busvine (1977) has shown the presence of cross-tolerance to pyrethrins, allethrin, bioresmethrin, permethrin, phenothrin and tetramethrin in DDT-resistant adult *Ae. aegypti*. Using the WHO technique, Prasittisuk and Busvine (1977) also reported that females of the Toga strain of *An. gambiae* with only 2.2-fold DDT resistance showed a 1.4-fold resistance to *cis,trans*-permethrin; corresponding data for the QTDA strain of *An. quadrimaculatus* were 2-fold and 1.8-fold, respectively. However, Chadwick *et al.* (1977) did not detect cross-tolerance to bioresmethrin + p.b. in DDT-resistant females of the ST-KAR strain of *An. stephensi*. Since they used a different testing technique, direct comparison of results cannot be made.

KNOCKDOWN. Exposure of the S strain to 10 ppm DDT caused little knockdown (ca. 10%) during the first 60 min, but subsequently, rapid knockdown did occur (Fig. 4). Fifty percent knockdown of S was achieved in 81 min., while 95% knockdown took place in 205 min. It was observed that larvae of the S strain consistently became hyperactive during the first 60 min or so of exposure to DDT before they quickly became quiescent and knocked down. On the other hand, larvae of DDT-R F_6 were virtually not affected during the first 120 min of exposure to 100 ppm DDT. An average of 30% were knocked down during the observation period. Resistant larvae appeared to be normally active until some suddenly sank to the bottom of the water where they eventually died. Once knockdown had

Table 3. Resistance to DDT and cross-resistance to (1*R*)-*cis,trans*-permethrin in adult *Anopheles stephensi*.

STRAIN	DDT (4% WHO papers)			(1*R*)-*cis,trans*-permethrin (0.05%)[b]		
	LT_{50} (min)	RR[a]	slope	LT_{50} (min)	RR[a]	slope
S	28	1	3.4	44	1	3.5
Kasur-P	50	1.7	2.4	48	1.1	3.5
DDT-R F_6	1150	40	2.6	416	9.4	3.5
Permethrin-R F_2	1170	41	2.7	461	10	3.6
DDT/Syn.-R F_2	1130	40	2.3	477	11	3.8

[a] Resistance ratio.

[b] Insecticide dissolved in 4:1 (v/v) petroleum ether:Risella Oil.

occurred in either strain, there was negligible recovery, even after transfer to clean water. Though preliminary, the present work indicates that: (a) high levels of DDT resistance in *An. stephensi* are accompanied by moderate levels of cross resistance to (1*R*)-*trans*- and to (1*R*)-*cis*-permethrin in larvae (Table 1; Figs. 1,2) and adults (Table 3); (b) selection of larvae with (1*R*)-*trans*-permethrin and DDT + DMC + p.b. each increased the degree of DDT and permethrin resistance (Table 2); (c) non-metabolic factors are involved in such resistance since neither DDT resistance nor pyrethroid resistance could be suppressed by synergists (Table 2); and (d) the observed resistance is due at least in part to changes in the nervous system of resistant strains which result in lower sensitivity to the toxic actions of the chemicals (Fig. 3).

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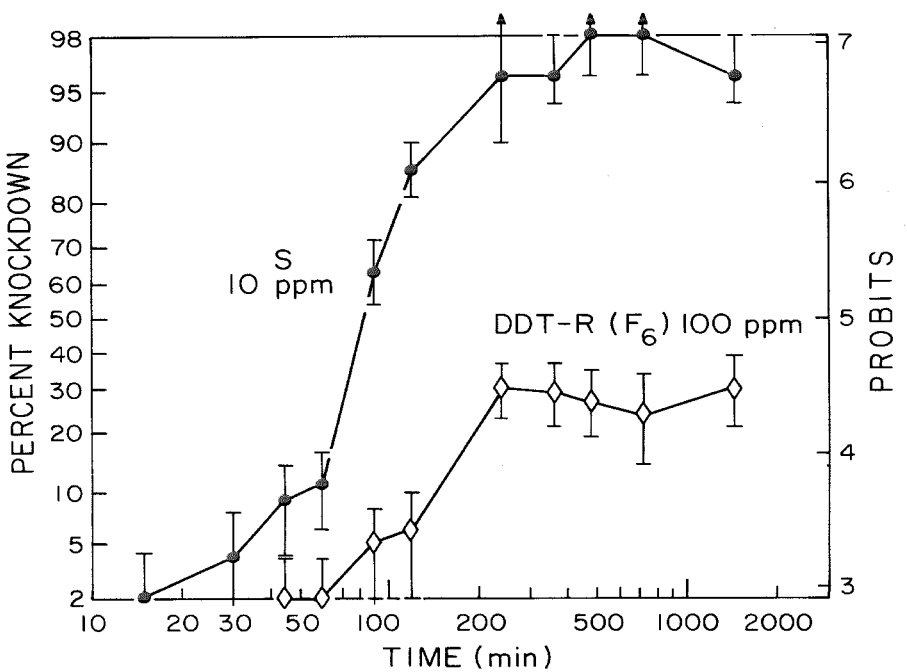

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