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Investigations on the Mechanism of DDT Resistance in Certain Anopheline Mosquitos*

ALBERT S. PERRY[1]

*DDT resistance, first observed some thirteen years ago in houseflies, is rapidly spreading in an alarming number of other insects of public health importance, particularly mosquitos. But while much has been learned of the mechanism of this resistance in houseflies, little is known as yet of the corresponding mechanism in mosquitos. A WHO-sponsored study was designed to find out primarily whether, as in houseflies, the breakdown of DDT to DDE—a non-toxic metabolite—*in vivo *is the predominant factor responsible for the development of resistance in anopheline mosquitos.*

No definite correlation could be established between the detoxication of DDT and resistance in the anophelines tested, but the interesting fact emerged that the detoxication potential was a measure of the ratio of DDE to absorbed DDT rather than of the ratio of DDT recovered. A multiplicity of factors governs the development of resistance in mosquitos, each species possessing a " combination of attributes for resistance " that may differ from that of other species ; some of these are discussed.

Since the appearance of DDT resistance in houseflies in 1946 much has been learned concerning the physiology, ecology, and genetics of this phenomenon. More recently, the occurrence of resistance to many of the halogenated-hydrocarbon insecticides in other insects of public health importance, especially in mosquitos, has given impetus to an expanded programme of research covering diverse fields of endeavour.

The present research, which was sponsored by the World Health Organization, was designed to find out if the mechanism of DDT resistance in certain anopheline mosquitos is similar to or different from that found in houseflies, i.e., to investigate, among other things, the detoxification of DDT through its conversion *in vivo* to a non-toxic metabolite such as DDE.

Investigations were carried out at the Malaria Field Station in Skala, Greece; at the Institute of Malariology in Adana, Turkey; at the Institute of Malariology in Rome, Italy; and at the Parasitology Laboratory, Istituto Superiore di Sanità, Rome.

The history of malaria vector control and of the development of resistance to the newer insecticides in the anopheline species of the areas mentioned above has been masterfully reviewed by Brown (1958). In addition, de Zulueta (1959), who preceded the present writer with investigations on anopheline resistance to various insecticides in Greece and Turkey, has given a detailed account of control measures and the past history of the resistance problem in those areas. It would appear superfluous here to review an already well-reviewed subject; hence, the reader is referred to the above publications for details and original contributions to this problem.

METHODS AND PROCEDURES

The analytical procedures involved in the chemical assay of DDT by the method of Schechter et al. (1945) require certain equipment and apparatus which, under ordinary circumstances, is not available in malaria field stations or in small entomological laboratories. Consequently, most of the equipment needed for this work was transported by the author to the various stations where investigations were carried out. The equipment included a Bausch and Lomb " Spectronic 20 " spectrophotometer, Soxhlet extractors, chromatographic columns, glass-stoppered Erlenmeyer flasks and graduated cylinders,

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[1] Technical Development Laboratories, Communicable Disease Center, Bureau of State Services, Public Health Service, United States Department of Health, Education, and Welfare, Savannah, Ga., USA.

a mechanical evaporator for the preparation of exposure chambers, shell vials, a mortar and pestle, and various other pieces of glassware as well as the special reagents needed for the analysis. Ordinary chemicals were obtained locally.

Preparation of exposure chambers

The exposure chambers (Hoskins & Messenger, 1951) are made of glass shell vials, 2.5 cm in diameter and 5 cm high. The open end of the vial is drawn inwards to an extent of 2-3 mm, so as to form an inverted lip. This is accomplished by placing the vial in an adapter which is mounted on the chuck of a mechanical stirrer and applying heat to the upper end of the vial while the stirrer is rotating at a speed of 500-1000 r.p.m. A slight pressure on the heated portion of the vial forms the inward lip. The complete operation takes 1-2 minutes.

Preparation of residual deposits

Into each vial is placed 0.5 ml of a solution of DDT in acetone, the desired concentration being adjusted by proper dilution from a stock solution.

The vial is placed in a horizontal position on a mechanical evaporator [1] designed to keep the vial in a rotating motion at a slow speed while a heating element at the bottom of the evaporator, which is controlled by a rheostat, supplies the necessary heat to evaporate the acetone at a uniform temperature. The evaporator accommodates 10 vials and uniform deposits of the desired dosage of insecticide are obtained in 3-5 minutes.

The insecticide dosage is always given in micrograms per vial. Each vial has an area of 44.27 cm^2. To convert micrograms per vial into grams per m^2 the following formula may be applied:

$$\text{conversion factor} : \frac{m^2}{\text{area of vial}} = \frac{10\,000\ cm^2}{44.27} = 226$$

$$1\ \text{gram} = 1\,000\,000\ \text{micrograms}$$

$$\text{grams/m}^2 = \frac{\text{micrograms/vial} \times 225}{1\,000\,000}$$

For example, a dosage of 100 micrograms per vial is equivalent to 0.023 grams per m^2.

The vials may be used immediately after their preparation or at any suitable time thereafter.

[1] The mechanical evaporator was designed and manufactured at the Technical Development Laboratories, Communicable Disease Center, Savannah, Ga., USA.

Exposure of test insects

As in the previous experiments with houseflies, the first few experiments in Greece were carried out by introducing 15-20 adult *Anopheles sacharovi* into each vial and exposing them to various DDT dosages for 30 minutes. However, it soon became apparent that the number of mosquitos was excessive, and in subsequent tests only 10 adults per vial were used. This proved to be satisfactory both in Greece and in Turkey. In Italy, 10 mosquitos per vial proved to be somewhat crowded for *A. atroparvus* and *A. maculipennis* and, consequently, the number was reduced to 5 mosquitos per vial. However, little difference in mortality was noted in comparative tests with 5 and 10 mosquitos per vial.

The mosquitos were aspirated from a stock cage and were gently transferred to the exposure vial by tapping lightly on the aspirating tube. Blowing the mosquitos into the chamber was avoided. The open end of the tube was covered with gauze and secured with a rubber band. The tubes were placed on a level surface in a horizontal position in the order of their preparation and at intervals of 5-10 minutes they were rotated through 90°, the insects being agitated by a slight tap on the open end of the tube. Trivial as they may seem, these details are nevertheless important in obtaining uniform results.

After a standard 30-minute exposure, or in some cases exposure for one or two hours, the mosquitos were transferred to suitable containers where they were held for 24 hours. Wads of cotton soaked in 10% glucose solution were provided as food.

Ten-ounce (280-ml) paper cups, similar to those provided by the World Health Organization, were found to be the most suitable holding containers. However, 600-ml beakers, as well as cages of various sizes, were used in some cases, depending on local conditions. Irrespective of the type of container used, filter-paper was always placed at the bottom of the container.

The exposure method described above allows of a safe and rapid transfer of the test insects, and thus obviates the use of anaesthetics.

Extraction and analysis

At the end of the holding period the mortality was recorded. The test insects, whether dead or alive, were subjected to treatment with ether or chloroform and were ground in a mortar with anhydrous sodium sulfate to a fine dry powder. The powder was extracted in a Soxhlet apparatus with carbon tetrachloride for 3-4 hours, after which

the solvent was evaporated to approximately 5 ml. The concentrate was chromatographed through a glass column (15 mm in diameter and 250 mm long, fitted with a No. 1½ stopcock) containing 15 g of Florisil,[1] 60-100 mesh, using carbon tetrachloride as eluent until 75 ml of eluate had been collected. The eluate was evaporated to dryness and the residue was nitrated and processed for colorimetric analysis by the Schechter-Haller method. After addition of 1.5 ml of benzene and 3 ml of sodium methylate reagent the coloured complex was read in a Bausch and Lomb spectrophotometer at wave-lengths of 597 and 530 mμ for DDT and DDE, respectively. Resolution of DDT and DDE in a mixture was accomplished by analysis of a two-component colour system (Perry & Hoskins, 1951). In many instances a complete spectrum in the visible range was taken to ascertain the nature of the coloured end-product. The method of analysis is outlined below:

Nitration of Sample

Add 5 ml of 1:1 mixture of fuming HNO_3 and concentrated H_2SO_4. Heat in steam-bath at 100°C for one hour.

Cool in cold water, add 25 ml of cold distilled water and 50 ml of ether or benzene. Shake for two minutes and allow to separate.

Discard lower layer and wash solvent with 10 ml of 2% aqueous NaOH. Repeat until washings are alkaline.

Discard lower layer and wash solvent with two 10-ml portions of saturated NaCl.

Discard lower layer and evaporate the solvent completely.

Development of Colour

Add 1.5 ml of benzene and 3 ml of sodium methylate.

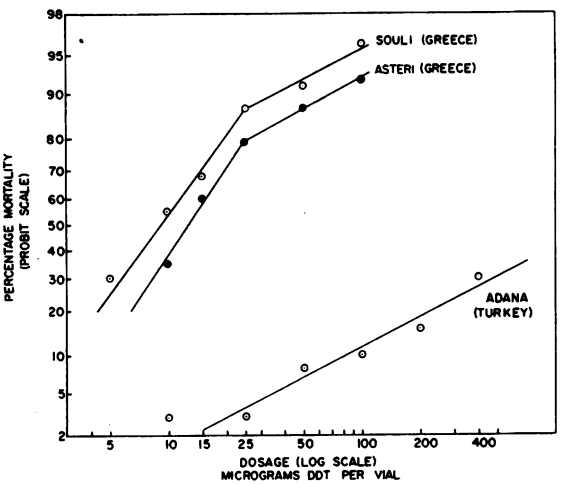
After 15 minutes read absorbance at wave-lengths of 597 and 530 mμ for DDT and DDE, respectively.

It is worth noting that in some localities, such as Skala, Greece, where electricity was not available most of the day, the spectrophotometer as well as the evaporator were operated from a 6-volt automobile battery. A 6-110-volt portable convertor was connected to the battery and the instrument was plugged into the convertor. The performance of the apparatus under these conditions was very satisfactory and this system is highly recommended for field stations where electricity is not available or where voltage fluctuation is too great to permit accurate measurements.

[1] Florisil is a synthetic adsorbent produced by the Floridin Co., Warren, Pa., USA. It consists of 15.5% MgO, 84% SiO_2, and 0.5% Na_2SO_4.

FIG. 1

LOG-DOSAGE/PROBIT-MORTALITY CURVES FOR ADULT FEMALE *A. SACHAROVI* COLLECTED NEAR SKALA, GREECE, AND ADANA, TURKEY



RESULTS

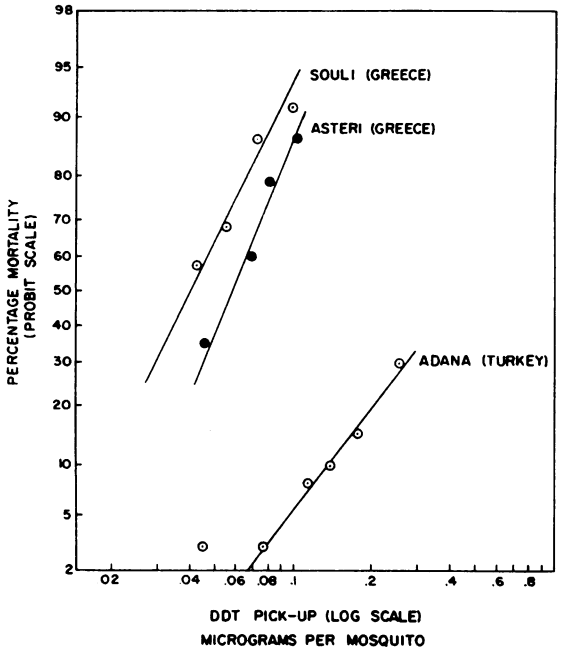
Work at Malaria Field Station, Skala: A. sacharovi

Approximately 5000 mosquitos were exposed to various DDT dosages. About 1500 mosquitos were collected in Asteri village, south-east of Skala, and about 3500 in Souli village, west of Skala. The results are shown graphically in Fig. 1. It will be seen that DDT dosages of 5-25 μg per vial produced average mortalities ranging from 35% to 84%. By the standards of the method employed, these values indicate that the major part of the field populations of *A. sacharovi* in the Skala area is only slightly resistant to DDT.

Another approach in interpreting the dosage-mortality curve shown in Fig. 1 is illustrated in a plot of DDT pick-up against mortality. This type of plot takes into consideration the response of the organism only to the amount of insecticide actually collected, disregarding the dosage of the residue. In a sense, it is similar to plotting a dosage-mortality curve by topical application. It may be seen (Fig. 2) that the response of *A. sacharovi* follows a normal distribution regression line and that the break in the curve disappears. The latter plot implies that there

FIG. 2

LOG-PROBIT CURVES RELATING DDT PICK-UP TO PERCENTAGE MORTALITY OF ADULT *A. SACHAROVI* 24 HOURS AFTER EXPOSURE TO RESIDUAL DDT *



* Values derived from Tables 1 and 2.

are no two distinct types of physiologically resistant individuals in the population and that the break in the curve is due to other phenomena not clearly understood.

The results of the colorimetric analyses for DDT and metabolites are somewhat confusing. Fairly large amounts of DDE were found in all cases analysed, but as shown in Table 1 there was little difference in DDE content between the mosquitos that died and those which survived dosages of 10 and 15 μg of DDT per vial. Even at a dosage of 25 μg of DDT per vial, which produced up to 85% mortality, there was more DDE than DDT in the extracts. All possibilities of contamination or artifacts were exhaustively investigated and eliminated. Complete spectra taken of many of the extracts showed distinct absorption peaks characteristic of DDE. A typical spectrum is shown in Fig. 3.

An important finding in the analysis of mosquitos used as controls and not exposed to DDT in the laboratory was that they, too, contained significant amounts of DDE (see Table 1). Spectrophotometric data showed the same characteristic absorption as that obtained from treated mosquitos (Fig. 3).

Although, by its characteristic absorption spectrum the metabolite appeared to be DDE, at least one of its properties was found to be different. Ordinarily, the colour complexes of the nitrated products of DDT and of its known metabolites are stable for 2-3 hours at room temperature after addition of benzene and sodium methylate reagent. The colour, either blue or pink for DDT and metabolites, respectively, fades out rapidly after standing

TABLE 1

RECOVERY OF DDT AND DDE FROM *A. SACHAROVI* 24 HOURS AFTER 30-MINUTE EXPOSURE OF ADULT FEMALES TO VARIOUS DOSAGES OF RESIDUAL DDT [a]

DDT dosage (μg/vial)	No. of adults extracted	Amount recovered (μg/mosquito)			Mortality range (%)
		DDT	DDE	Total	
Source: Asteri					
10	206	0.016	0.027	0.043	32-37
10	211	0.019	0.031	0.050	
15	272 [b]	0.031	0.037	0.068	55-66
15	181 [c]	0.029	0.042	0.071	
25	310	0.037	0.048	0.085	68-88
25	354	0.035	0.045	0.080	
50	110	0.048	0.057	0.105	83-92
Control	380	0.012	0.030	0.042	3-10
Source: Souli					
10	520 [b]	0.012	0.027	0.039	55-56
10	340 [c]	0.013	0.034	0.047	
10	266 [b]	0.010	0.030	0.040	
10	248 [c]	0.016	0.032	0.048	
15	317	0.020	0.036	0.056	63-74
15	336	0.018	0.034	0.052	
15	230	0.022	0.037	0.059	
25	346	0.028	0.048	0.076	80-89
25	202	0.030	0.042	0.072	
50	113	0.055	0.045	0.100	87-94
Control	215	0.015	0.022	0.037	2-12
Control	480	0.014	0.025	0.039	

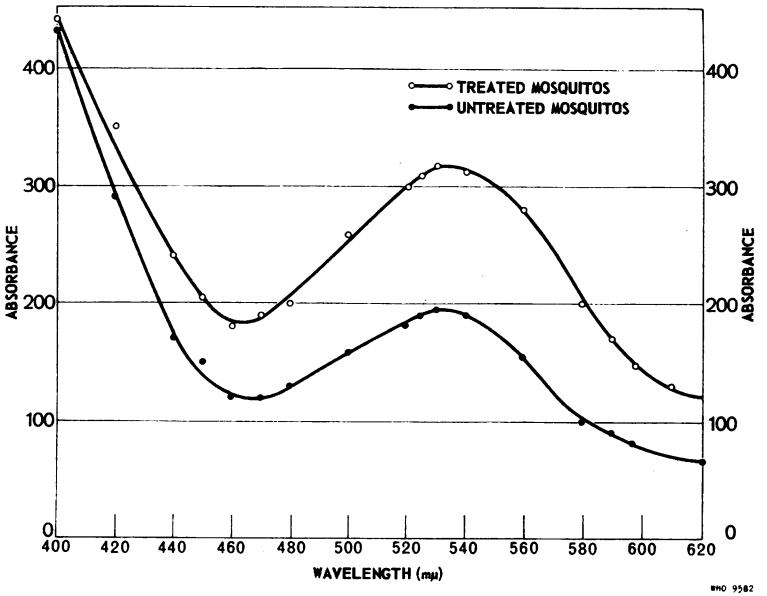
[a] The mosquitos were collected from Asteri and Souli in the Skala area of southern Greece. The values shown are corrected for controls.

[b] Dead only.

[c] Survivors only.

FIG. 3

TYPICAL ABSORPTION SPECTRA OF THE COLOURED REACTION PRODUCTS OBTAINED FROM EXTRACTS OF UNTREATED AND DDT-TREATED *A. SACHAROVI* FROM THE SKALA AREA OF GREECE



for more than 6 hours and turns yellow or light-brown. The pink-coloured complex resulting from the analysis of extracts of treated and untreated *A. sacharovi* was stable at room temperature for 16 hours and possibly longer. This abnormal characteristic casts a shadow of doubt on the identity of the metabolite as DDE. This point needs further clarification.

In order to arrive at a logical conclusion and at a satisfactory interpretation of the biochemistry of DDT resistance in *A. sacharovi* in the Skala area it is necessary to repeat some of the tests reported above and to perform other experiments not envisaged in this work.

Work at Institute of Malariology, Adana: A. sacharovi

The findings of de Zulueta (1959) and of Dr C. D. Gökberk (unpublished report to WHO, 1958) just four months prior to the commencement of the investigations reported below clearly demonstrated the widespread occurrence of DDT resistance in *A. sacharovi* in Adana and its surroundings. However, owing to the introduction of dieldrin in those areas in the summer of 1958 some difficulty was encountered in obtaining sufficient numbers of mosquitos in the immediate vicinity of Adana.

For this reason all of the mosquitos used in the experimental work were collected in the village of Kilbas, approximately 40 km east of Adana, where dieldrin had not yet been introduced.

Approximately 2000 adult mosquitos were exposed to various dosages of DDT. The procedure was essentially the same as that used in Greece except that 10-ounce paper cups were used for holding the mosquitos after exposure. Ten mosquitos were introduced into each vial and the exposure period varied from 30 minutes to as long as 3 hours. The results of these tests are shown in Fig. 1. There is no doubt that a resistance of high magnitude was demonstrated here. On all tests performed and even at the highest dosage used, i.e., 400 μg per vial, the mortality never exceeded 35%. In many instances, an exposure period of 2-3 hours was long enough to cause some knock-down but, after being transferred to the holding containers at the end of the exposure period, many mosquitos presumed to be dead eventually recovered completely. There was no mortality in the controls, even though the mosquitos were brought to the laboratory from quite a distance.

Chemical analyses of extracts (Table 2) showed rapid degradation of DDT to a Schechter-Haller-positive metabolite presumed to be DDE. A

TABLE 2
RECOVERY OF DDT AND DDE FROM *A. SACHAROVI* 24 HOURS AFTER EXPOSURE OF ADULT FEMALES TO VARIOUS DOSAGES OF RESIDUAL DDT [a]

DDT dosage (μg/vial)	Length of exposure (hours)	No. of adults extracted	Amount recovered (μg/mosquito)			Mortality range (%)
			DDT	DDE	Total	
10	½	176	0.012	0.033	0.045	3-34 [b]
25	½	190	0.031	0.046	0.077	3-16
50	½	192	0.041	0.073	0.114	3-13
100	1	200 [c]	0.115	0.015	0.130	—
100	1	200	0.060	0.080	0.140	6-16
100	3	98	0.073	0.115	0.188	17-27
200	1	200 [c]	0.161	0.022	0.183	—
200	1	200	0.078	0.100	0.178	4-17
200	2	95	0.096	0.122	0.218	23-35
400	1	96	0.115	0.141	0.256	25-32
400	1½	100	0.127	0.150	0.277	28-40
Control	½-1	300	0.022	0.036	0.058	0
Control	2-3	197	0.018	0.034	0.052	0

[a] The mosquitos were collected from Kilbas village near Adana, Turkey. The values shown are corrected for controls.
[b] 100 mosquitos tested three days after collection showed 34% mortality.
[c] These mosquitos were ground and extracted immediately after the exposure period (0-hour test).

complete spectrum of the metabolite in the visible range (Fig. 4) showed absorption characteristics similar to those of DDE, and unlike the metabolite found in *A. sacharovi* in Greece, the colour complex evinced stability characteristics similar to those of the degradation products of DDT. In addition, mosquitos which were ground and extracted immediately after the exposure period (so-called "zero-hour" test) contained predominantly DDT and only a small amount of DDE (see Table 2). A typical spectrophotometric curve showing the recovery of DDT from such extracts is shown in Fig. 4.

Mosquitos used as controls and not exposed to DDT residues in the laboratory contained measurable amounts of DDT and DDE (Table 2). This is to be expected since the DDT residual deposit must have been very high from previous sprayings of the area in which the mosquitos were collected.

It will also be noted that fairly large amounts of unchanged DDT were found in the extracts of treated mosquitos. Time did not permit the analysis of samples in terms of external and internal DDT. Hence, it is not known what fraction of the DDT had remained unabsorbed at the end of the observation period. However, judging from the amounts of DDE recovered, it is evident that, on the average, at least 60% of the amount of DDT picked up was absorbed during the 24-hour period of observation. If most of the recovered DDT remained unabsorbed it would then indicate that practically all the absorbed DDT was converted to DDE and would imply the presence of a very efficient dehydrochlorinating system, which might contribute to a large extent to the physiological resistance of this species to DDT.

Attempts to demonstrate the breakdown of DDT to DDE *in vitro* by *A. sacharovi* adults were unsuccessful. In three separate experiments, one of which was performed by Dr Gökberk after the writer had left Turkey, only unchanged DDT was found in extracts of homogenates incubated with DDT for several hours. It must be noted, however, that the preparations were rather crude, having been prepared in physiological saline and incubated at room temperature without the benefit of cofactors which might be essential for the normal functioning of the enzyme.

Work at Institute of Malariology, Rome: various mosquito species

Owing to the lateness of the season (20 October) it was found difficult to obtain large numbers of mosquitos from the field, but the expert and untiring efforts of Dr A. Coluzzi and his staff provided the writer with small numbers of adults of several field-collected species. The following anopheline species were tested:

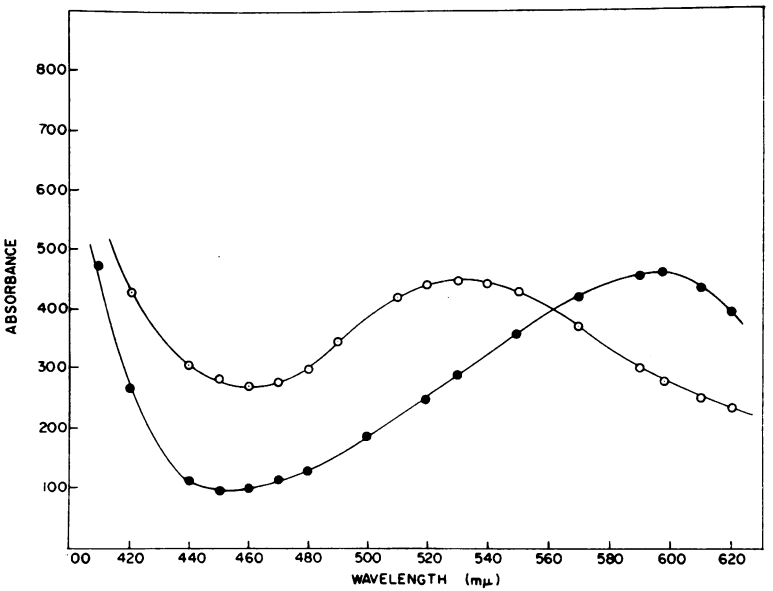
A. atroparvus: from Follonica (Grossito)
A. atroparvus: from Macchia d'Isernia (Campobasso)
A. maculipennis typicus: from Vicalvi (Frosinone)
A. labranchiae: from Vieste (Foggia)
A. sacharovi: from Vieste (Foggia)
A. claviger: from Macchia d'Isernia (Campobasso)
A. superpictus: from Macchia d'Isernia (Campobasso)

In addition, laboratory strains of *A. atroparvus* and *A. stephensi* were also tested.

Because of their high activity inside the exposure chamber and in order to reduce crowdedness and

FIG. 4

TYPICAL SPECTROPHOTOMETRIC ABSORPTION CURVES OF EXTRACTS OF DDT-TREATED *A. SACHAROVI* FROM KILBAS, TURKEY*



* The solid circles represent an analysis of 200 adults extracted immediately after exposure to deposits of 200μg of DDT pervial. The open circles represent a similar analysis of 200 mosquitos extracted 24 hours after exposure to 200 μg of DDT per vial.

traumatism, only five adults were used per vial and the exposure period was held at 30 minutes. Ten-ounce paper cups were used as holding containers and 20-25 treated mosquitos were placed in each cup. The results of tests with various DDT dosages are summarized in Table 3. It is evident that all the species tested were susceptible to DDT by comparison with the normal, laboratory-reared strain of *A. atroparvus*.

Because of the very small number of individuals available for testing, the results, particularly with *A. sacharovi* and *A. superpictus*, cannot be considered significant. It is surprising, however, that *A. sacharovi* showed the lowest mortalities of all species tested. One might argue that this was due only to the higher fat content, because the species was already in hibernation, but for the fact that most of the other species tested were also in hibernation, especially *A. labranchiae*, which was collected in the same area. This point needs further clarification.

Chemical analyses of the extracts are shown in Table 4. Since the samples were small it was found impossible to analyse each extract separately. Therefore, in most instances, several samples were pooled and were analysed collectively. Values for DDT and DDE recovery which are below the limit of accuracy of the colorimetric procedure have been pointed out in Table 4. It will be seen that small amounts of DDE were found in all samples, but the content of DDT was always higher than that of DDE. The presence of DDE in what are considered to be susceptible mosquitos suggests that a dehydrochlorinating mechanism is present in both susceptible and DDT-resistant populations.

In the course of this work Dr Coluzzi suggested that a comparison be made between the Busvine & Nash method (Busvine & Nash, 1953; WHO Expert Committee on Malaria, 1954) and the newer WHO method (WHO Expert Committee on Insecticides, 1958) employing the test kit provided by WHO. Five mosquitos per tube were used in the former technique and 25 mosquitos per tube were used in the latter. Two hundred *A. atroparvus* adults were exposed for one hour to papers impregnated with 4% DDT in Risella oil. The results showed 45% mortality with the Busvine & Nash method and 85% mortality with the WHO method. Similar differences have been found on previous occasions (de Zulueta, 1959). Analysis for DDT

TABLE 3

DOSAGE-MORTALITY RELATIONSHIPS FOR SEVERAL ANOPHELINE SPECIES AND STRAINS COLLECTED FROM VARIOUS LOCALITIES IN ITALY [a]

Species or strain	Source	DDT dosage (μg/vial)	Number tested	24-hour mortality (%)
A. atroparvus	Laboratory	5	44	57
"	"	10	100	72
"	"	25	100	89
"	"	50	100	99
"	Follonica	5	50	68
"	"	10	50	77
"	"	25	50	90
"	Macchia d'Isernia	5	50	42
"	"	10	50	62
"	"	25	50	88
A. maculipennis	Vicalvi	5	50	48
"	"	10	70	70
"	"	25	25	84
"	"	50	25	96
A. stephensi	Laboratory	2	50	70
"	"	5	50	88
"	"	10	50	96
A. labranchiae	Vieste	5	60	28
"	"	10	65	36
"	"	25	50	74
A. sacharovi	Vieste	5	25	20
"	"	10	25	28
"	"	25	20	35
A. superpictus	Macchia d'Isernia	5	20	35
"	"	10	20	50
"	"	25	20	90
A. claviger	Macchia d'Isernia	2	20	35
"	"	5	25	72
"	"	10	25	84
"	"	25	25	96

[a] There was no mortality in the controls.

and DDE gave the following values in micrograms per mosquito:

	DDT	*DDE*	*Total*	*24-hour mortality (%)*
Busvine & Nash method	0.024	0.015	0.039	45
WHO method	0.038	0.022	0.060	85

It is clear that in the WHO method each mosquito, on the average, picked up 0.021 μg, or 54%, more DDT than in the Busvine & Nash method. This difference in pick-up is perhaps a function of the greater activity of the insect in the WHO method.

If one were to compare the values given above with those obtained by the vial method using the laboratory colony of *A. atroparvus* (see Table 4), a remarkable agreement in results will be found. Thus, by the vial method of exposure a pick-up of 0.048 μg and 0.062 μg of DDT per mosquito produced 72% and 89% mortality, respectively. By the Busvine & Nash method 0.039 μg of DDT caused 45% mortality, and by the WHO method 0.060 μg yielded 85% mortality. If these values are plotted on log-probit paper a straight line is obtained, indicating that, in the final analysis, mortality is a function of the amount of insecticide removed from the residues by the insect, irrespective of the method of testing used. The latter should be evaluated in terms of practicability, convenience and sensitivity.

Work at Istituto Superiore di Sanità, Rome: various mosquito species

The mosquitos used in these tests were obtained from the various colonized strains of *A. atroparvus* maintained by Dr E. Mosna, Chief of the Parasitology Laboratory at the Istituto Superiore di Sanità. Several of these strains had been under DDT-selection pressure for 39 generations at the time of testing.

The following strains of *A. atroparvus* were tested:

SR: Rome susceptible strain;

RAFM: adult males and females under DDT-selection pressure for 39 generations;

RL: selected for DDT resistance in the larval stage only (F-39);

RLAF: larvae as well as adult females under DDT-selection pressure for 39 generations.

In addition, the following species were tested:

A. stephensi: adult males and females under DDT-selection pressure for 21 generations;

TABLE 4

RECOVERY OF DDT AND DDE FROM VARIOUS ANOPHELINE SPECIES 24 HOURS AFTER EXPOSURE OF ADULT FEMALES TO RESIDUAL DDT [a]

Species	Source	DDT dosage (μg/vial)	No. of adults extracted	Amount recovered (μg/mosquito)		
				DDT	DDE	Total
A. atroparvus	Laboratory	10	100	0.028	0.020	0.048 [b]
,,	,,	25	100	0.033	0.029	0.062
,,	,,	50	100	0.045	0.025	0.070
,,	Macchia d'Isernia	10 & 25	100	0.048	0.028	0.076
,,	Follonica	10 & 25	100	0.034	0.021	0.055
A. maculipennis	Vicalvi	5	50	0.039	0.026	0.065 [b]
,,	,,	10	70	0.054	0.036	0.090
,,	,,	25 & 50	50	0.115	0.072	0.187
A. labranchiae	Vieste	10 & 25	100	0.053	0.022	0.075
A. stephensi	Laboratory	5 & 10	100	0.017	0.011	0.028 [b]
A. claviger	Macchia d'Isernia	10 & 25	50	0.079	0.026	0.105

[a] The values shown are corrected for controls.

[b] These values are considered to be below the limit of accuracy of the analytical method and, therefore, are not reliable.

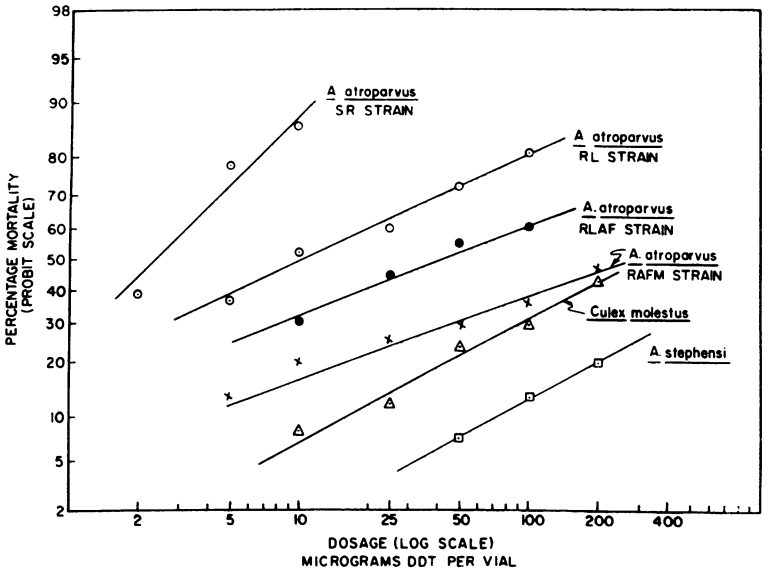
Culex pipiens autogenicus (C. molestus) : collected from the Latina district near Rome. Originally resistant to most of the halogenated-hydrocarbon insecticides. Maintained in the laboratory for 12 years without selection.

In all, 30 separate tests were performed, 15 of which were made by Dr Mosna and Mr Carta while the writer was on leave. Approximately 5100 adult female mosquitos were exposed to various dosages of DDT. The summarized results are shown graphically in Fig. 5. It is obvious that selection pressure with DDT for 39 generations in the adult stage (RAFM strain) produced a strain that was highly resistant in comparison with the susceptible SR strain. Although the highest dosage used was only 200 μg per vial it is doubtful whether higher dosages would have produced much higher mortalities in view of the flatness of the curve (Fig. 5).

The results of analyses for DDT and DDE in the various strains are given in Table 5. It is immediately apparent that the values for DDE are low in relation to the magnitude of resistance encountered. Although in some cases the selected strains of *A. atroparvus* converted more DDT to DDE than the susceptible strain it is difficult to correlate DDT breakdown with the level of resistance, since the amount of DDT absorbed was not measured. A plot of the DDT picked up against dosages of residual deposit (Fig. 6) shows wide differences in DDT pick-up among the various strains, especially at the higher dosages. These differences are due perhaps to the insect's activity inside the exposure chamber (the so-called "irritability" characteristic), since it is reasonable to assume that the more active insects will collect more of the insecticide. This, in fact, was the case with some species. For example, *A. stephensi* adults were very quiescent inside the vial throughout the exposure period whereas *Culex molestus* adults were very active. Fig. 6 shows that *C. molestus* picked up 3-4 times as much DDT as *A. stephensi* during the same exposure period and under the same conditions. Of the three DDT-selected *A. atroparvus* strains it was noted that the RL and RLAF strains suffered 100% knock-down when exposed to a residue of 100 μg per vial, whereas the RAFM strain

FIG. 5

LOG-DOSAGE/PROBIT-MORTALITY CURVES FOR ADULT FEMALES OF SEVERAL RESISTANT *A. ATROPARVUS* STRAINS, OF *A. STEPHENSI* AND OF *CULEX MOLESTUS* FROM THE LABORATORY COLONIES AT THE ISTITUTO SUPERIORE DI SANITÀ, ROME



remained active throughout the exposure period. The DDT pick-up is partly reflected in this differing activity, being 0.130, 0.160 and 0.187 μg for the RLAF, RL and RAFM strains, respectively. In this connexion it is also of interest to note that adult mosquitos of the RAFM strain which were given a blood meal prior to exposure to DDT picked up only half as much DDT as adults which were fed glucose solution. This peculiarity is again reflected in the more sluggish activity of the blood-fed adults as compared with the glucose-fed adults. It is not understood, however, why the blood-fed mosquitos suffered greater mortality, since published reports in the literature point in the opposite direction.

It is also evident from Fig. 6 that removal of DDT is less efficient from heavy deposits than from lighter ones. This is perhaps one of the reasons why many so-called plateaux are obtained when plotting dosage-mortality curves with resistant insects. One is apt to measure the limitations of a technique rather than the insect's response to the chemical.

Another approach in interpreting the dosage-mortality data of Fig. 5 is illustrated in a plot of DDT pick-up against mortality (Fig. 7). Barring differences in absorption (which is, however, a very important variable), it may be seen that these strains were truly physiologically resistant strains since they could tolerate larger amounts of insecticide as compared with the susceptible strain. Even *A. stephensi*, which removed much less DDT from residual deposits, was shown to tolerate larger amounts of the insecticide than the SR *A. atroparvus* strain or the extremely susceptible *A. stephensi* from the Institute of Malariology (see Fig. 6 and Table 4).

It must also be remembered that unless a fairly straight line is obtained for dosages yielding from 30% to 90% mortalities, the LD_{50} is not a true criterion of the response of the population as a whole. This is especially true of resistant species where, owing to some factor inherent in the population or, in many cases, to limitations in the sensitivity of the method used, an LD_{90} cannot be obtained. In the experiments presented above no attempt was made to reach an LD_{90}, but from the data plotted in Fig. 7 one must be cognizant of the limitations inherent in the method. Hence, a criterion other than the LD_{50} should be of greater value when dealing with heterogeneous populations of resistant insects.

TABLE 5

RECOVERY OF DDT AND DDE 24 HOURS AFTER EXPOSURE OF THE INDICATED SPECIES AND STRAINS TO VARIOUS DOSAGES OF RESIDUAL DDT [a]

Species and strain	DDT dosage (μg/vial)	No. of adults extracted	Amount recovered (μg/mosquito)			Mortality range (%)
			DDT	DDE	Total	
A. atroparvus						
SR strain	5 & 10	100	0.026	0.015	0.041	77-86
RAFM strain	5 & 10	139	0.022	0.018	0.040	8-28
	25	148	0.028	0.028	0.056	24-40
	50	309	0.068	0.032	0.100	20-46
	100	221	0.141	0.045	0.186	28-42
	200	197	0.185	0.041	0.226	40-52
	200	200 [b]	0.092	0.026	0.118	90
RL strain	5 & 10	196	0.023	0.010	0.033	29-58
	25	140	0.044	0.023	0.067	45-71
	50	199	0.082	0.030	0.112	62-80
	100	239	0.125	0.036	0.161	76-88
RLAF strain	25	196	0.031	0.022	0.053	24-56
	50	197	0.057	0.021	0.078	42-58
	100	198	0.106	0.024	0.130	50-68
A. stephensi	50	200	0.022	0.011	0.033	4-12
	100	145	0.036	0.024	0.060	8-16
	200	192	0.067	0.035	0.102	12-24
Culex molestus	25 & 50	51	0.056	0.048	0.104	12-24
	100	168	0.164	0.064	0.228	28-36
	200	169	0.300	0.067	0.367	38-56

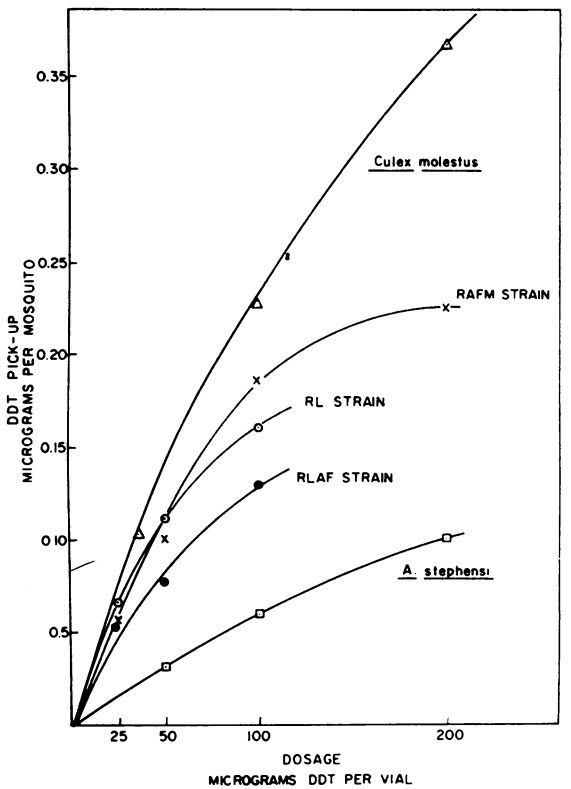
[a] The values shown are corrected for controls.
[b] These mosquitos were fed on blood prior to exposure to DDT.

DISCUSSION

The reported findings on the resistance problem in Greece over the past several years (for reviews, see Brown, 1958; de Zulueta, 1959) clearly demonstrated the presence of a high degree of physiological resistance to DDT in *A. sacharovi* in the Skala area of southern Peloponnese. The present study, however, indicates only slight DDT resistance in the majority of *A. sacharovi* adults collected in the Skala area.

The technique for assessing the degree of resistance employed in this study differed in many respects from methods used on previous occasions and, in general, tended to yield higher mortalities. On the other hand, using the highly standardized WHO method, de Zulueta (1959), who carried out earlier investigations in the same area as the writer, arrived at essentially the same conclusion. It appears likely that the high degree of DDT resistance previously encountered might have declined over the past few years owing to the discontinuance of DDT spraying. Since no record is available of the susceptibility of this species before DDT resistance was detected in 1951, the only evaluation that can be made at present is to compare its resistance with that of the same species in a different locality. Although one must be cognizant of the limitations involved in

FIG. 6
DDT PICK-UP IN RELATION TO CONCENTRATION OF RESIDUAL DEPOSIT *

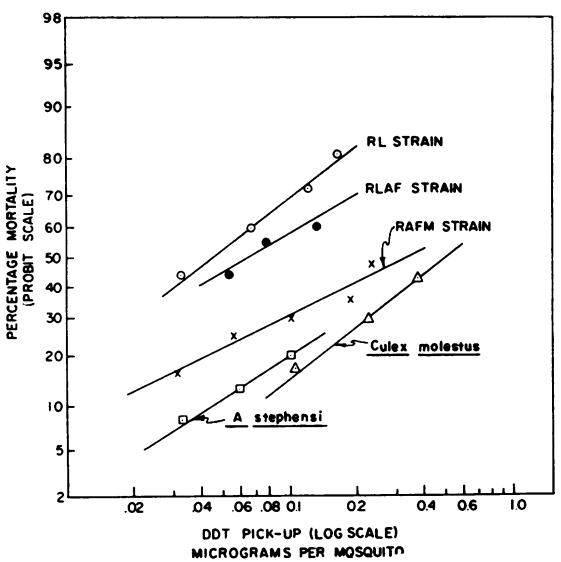


* Values derived from Table 5.

making such comparisons, it is none the less evident that *A. sacharovi* in the Skala area in Greece is a good deal more susceptible than *A. sacharovi* in Adana, Turkey. This is shown not only in terms of dosage-mortality curves (Fig. 1), but also in relation to the response of the respective strains to the actual amount of insecticide picked up from the residual deposits to which they were exposed (Fig. 2). Whereas the LD_{50} of the Souli strain from Skala was 0.040 μg of DDT per mosquito, that of *A. sacharovi* from Adana was approximately 0.42 μg of DDT per mosquito. The difference in the LD_{90}'s might have been even greater, but this could not be measured. It is also apparent that the Asteri strain requires approximately twice as much DDT as the Souli strain for equivalent mortalities.

The presence of measurable amounts of what was presumed to be DDE in the Greek strains complicates the picture even further. If these strains have only slight physiological resistance, as has been suggested in the text, one might attribute control failure in the field to behaviouristic resistance patterns. This might be the case; however, even slight physiological resistance can produce control failure because of the extremely small amount of insecticide that certain insects are able to pick-up from residues under natural conditions. Field investigations conducted in 1951 at the Technical Development Laboratories, United States Public Health Service, Communicable Disease Center, Savannah, Ga., showed that resistant houseflies which were collected at weekly intervals from two dairies sprayed with 200 mg of DDT per square foot (2 g per m^2) had picked up, on the average, less than 0.5 μg of DDT per fly, most of which had already been degraded to DDE (US Public Health Service, 1951). Hence, the level of resistance need not be particularly high to account for control failure in the field. However, the fact that more DDE than DDT was found in most extracts even though the mortality was high suggests, perhaps, that conversion of DDT to DDE may not be an important protective mechanism in the Greek

FIG. 7
LOG-PROBIT CURVES RELATING DDT PICK-UP TO PERCENTAGE MORTALITY OF SEVERAL SPECIES AND STRAINS OF MOSQUITOS 24 HOURS AFTER EXPOSURE TO RESIDUAL DDT *



* Values derived from Table 5.

strains of *A. sacharovi*. Additional tests are necessary to ascertain the nature of this phenomenon.

The results in Turkey are more clear-cut. In this case DDE recovery was high and mortality was low. Most of the unchanged DDT recovered may be external but this has yet to be proven. The over-all picture indicates a protective mechanism similar to that found in houseflies—namely, the conversion of DDT to DDE.

It is of interest to note that *A. maculipennis melanoon* was also found to be highly resistant to DDT in the Adana region. One of the mosquito populations brought from the field and allowed to lay eggs in the laboratory proved to contain 30-40% of *A. maculipennis melanoon*. During the summer months of 1958, Dr Gökberk, Director of the Malaria Institute in Adana, analysed the eggs of several mosquito populations in the vicinity of Adana and found them to contain 20-50% of *A. maculipennis melanoon*. On other occasions pure populations of *A. sacharovi* were found. It is therefore difficult to estimate the exact proportion of the two species used in each test, but there is no doubt that both species were highly resistant to DDT.

The results at the Institute of Malariology in Rome indicate that there are no field populations of resistant mosquitos in Italy. With the exception of *A. sacharovi*, all the species tested showed normal susceptibility by the standards of the technique. The tests with *A. sacharovi* are not considered significant because of the small size of the test samples.

The investigations carried out at the Istituto Superiore di Sanità demonstrated the ability of *A. atroparvus* to develop DDT resistance by serial selection with this insecticide. The highest degree of resistance was found in the RAFM strain, which was selected in the adult stage. It is noteworthy that selection of larvae over the same period of time produced adults (RL strain) much less resistant than the former strain, but there was little difference either in DDT pick-up or in DDE production between the two strains (Table 5). This leaves a possibility that differences in DDT absorption might account for the difference in resistance. If the rate of absorption of DDT by the RL strain were greater than that of the RAFM strain while the rate of conversion to DDE remained about the same, the greater accumulation of unchanged DDT at a critical site might well produce the observed effect. This factor of absorption may also play a role in the resistance of the RLAF strain. Studies on the rate of absorption will be of value in elucidating the mechanism of this resistance.

The results with *A. stephensi* show definitely a diminished activity in the exposure chamber and probably, as a result, a lesser pick-up of DDT. It is evident, however, that this species is highly resistant to DDT since a pick-up of 0.102 μg of DDT per mosquito killed only 20% of the test insects, whereas approximately the same pick-up of DDT killed 30% of the RAFM strain and 72% of the RL strain. Since the amount of DDE produced by *A. stephensi* was very low, it appears that the rate of DDT absorption might also play an important role in the resistance of this species. The over-all level of resistance might then be a function of low DDT pick-up, slow rate of absorption and detoxication of the insecticide. To what extent each of these factors contributes to the survival of the insect is a matter for further investigation.

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RÉSUMÉ

Des recherches sur le mécanisme de la résistance au DDT chez certains anophélinés de l'Europe méridionale ont conduit aux constatations suivantes:

Chez toutes les espèces étudiées, sensibles ou résistantes au DDT, il y a eu transformation de DDT en DDE, mais à divers taux. Toutefois, pour plusieurs espèces, la corrélation entre le degré de résistance et la décomposition du DDT n'a pas paru concluante.

A Skala, en Grèce, *A. sacharovi* n'est apparu que faiblement résistant au DDT, bien que l'on ait extrait un métabolite supposé être du DDE, en quantités plus grandes que celles généralement prévues après l'emploi de doses de DDT provoquant de fortes mortalités. Ce métabolite a été également trouvé chez des moustiques récoltés sur le terrain et utilisés comme témoins.

A Adana, en Turquie, *A. sacharovi* est apparu extrêmement résistant au DDT. A en juger par les faibles mortalités obtenues avec de fortes doses de DDT et par les quantités relativement importantes de DDE trouvées, il semble qu'un mécanisme efficace de décomposition des chlorhydrates protège cette espèce contre l'action létale du DDT. Par ailleurs, de substantielles quantités de DDT non modifié, mais dont l'origine, externe ou interne, n'a pas été déterminée, ont été récupérées.

La sensibilité d'adultes de *A. atroparvus*, *A. maculipennis typicus*, *A. labranchiae*, *A. superpictus* et *A. claviger* récoltés sur le terrain dans diverses régions d'Italie, s'est révélée normale par rapport à celle d'une souche de laboratoire de *A. atroparvus*. Des spécimens de *A. stephensi* élevés en laboratoire ont témoigné d'une extrême sensibilité au DDT. La détermination colorimétrique a révélé la présence de petites quantités de DDT et de DDE.

Trois souches de *A. atroparvus* sélectionnées par le DDT ont manifesté divers degrés de résistance à cet insecticide. La plus forte résistance a été observée chez des mâles et des femelles adultes sélectionnés, et la plus faible chez des adultes provenant uniquement de larves sélectionnées. L'existence d'une très forte résistance a été constatée chez des adultes de *A. stephensi* sélectionnés au DDT. Des adultes de *Culex pipiens autogenicus* provenant d'une souche originaire de la région de Latina, près de Rome, mais maintenue en laboratoire pendant 12 ans sans sélection, ont manifesté une très forte résistance. Le taux de transformation du DDT en DDE par toutes ces espèces et souches a été très faible par rapport à leur degré de résistance. La forte teneur en DDT peut être d'origine externe ou interne, mais les proportions respectives de DDT interne et externe n'ont pas été déterminées.

Les données recueillies à l'occasion de ces travaux ne permettent pas de préciser le degré de corrélation éventuelle entre la détoxication du DDT et la résistance. Il serait indispensable de déterminer le taux d'absorption du DDT, car le potentiel de détoxication est donné par le rapport de la quantité de DDE formé à celle de DDT *absorbé* et non par celui de la quantité de DDE formé à celle de DDT récupéré. De même, l'hypothèse de la détoxication ne pourra être confirmée tant que l'enzyme catalysant la décomposition du DDT chez les diverses espèces de moustiques n'aura pas été isolé.

Etant donné la multiplicité des facteurs en jeu, l'ensemble du mécanisme de la résistance ne peut être évalué en fonction d'un seul processus biochimique. Les recherches dont il s'agit ont montré que chaque espèce possède une pluralité de caractéristiques favorables à la résistance, qui peuvent varier d'une espèce à l'autre.

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