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SUSCEPTIBILITY OF MALARIA VECTORS TO DDT IN GREECE

Laboratory Findings

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SYNOPSIS

The authors describe an investigation, started in Greece in the autumn of 1953 and continued during the 1954 malaria-transmission season, to determine the variations in the susceptibility to DDT of the anophelines in different localities, with a view to establishing a point of departure which would make it possible to follow any future changes in susceptibility to this insecticide.

The degree of susceptibility of the mosquitos to DDT was assessed numerically by both the technique of topical application of microdoses of the insecticide and the technique devised by Busvine & Nash. The villages of Elos and Asterion (Skála area), the Agoulinitsa area, and the Georgioupolis (Crete) area were chosen for the investigation.

The findings disclosed considerable differences in the susceptibility to DDT in the above areas, but showed that, on the whole, the tendency for anophelines to acquire resistance to DDT seemed to be continuing in Greece.

The first signs of weakening in the effectiveness of DDT against *Anopheles sacharovi* appeared in Greece in 1951, i.e., six years after the commencement of the nation-wide application of this insecticide (Livadas, 1951, unpublished report). In the following year, the development of this resistance to DDT by the adult stage of *A. sacharovi* in the area of Skála-Peloponnese was experimentally proved by Livadas & Georgopoulos.[9] Recently, Garrett-Jones & Gramiccia [2] reported that *A. sacharovi* also showed resistance to the insecticide in two villages of Lebanon.

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The development of other DDT-resistant anopheline species has been reported—namely, *A. quadrimaculatus* (aquatic stage) in the USA (Krusé, Hawkins & Ludvik [3]), and, quite recently, *A. sundaicus* in some areas of Java (Van Goor & P. C. Issaris, personal communication).

During the period from 1952 to 1954 further epidemiological investigations were carried out in many areas of Greece and the following observations were made (Livadas [4, 5, 6]).

1. It was ascertained epidemiologically that *A. sacharovi* developed resistance to DDT, not only in Skála (Peloponnese) but also in other areas of the country where the extended residual-spray applications of DDT had not resulted in the eradication of that species. In these areas, the responsible service of the Ministry of Health had tried repeating the house-spraying two or three times during the transmission season or replacing DDT by other chlorinated-hydrocarbon insecticides (chlordane, BHC, lindane, dieldrin).

2. The effect of these other insecticides, especially the first three, also soon showed signs of weakening.

3. The two other local vectors (*A. superpictus* and *A. maculipennis*) also showed, in some areas, indications of developing some degree of resistance to chlorinated-hydrocarbon insecticides.

4. In some areas the development of resistance to residual insecticides by the local vector was not followed by any marked increase in the number of malaria cases, in spite of the obvious increase in vector density within the sprayed quarters. The explanation for this is very probably to be found in the absence of important local sources of infection. In other areas it resulted in an increase in the prevalence of malaria, which in some instances during 1954 assumed epidemic proportions.[7, 8]

5. The limited local epidemic outbreaks were due exclusively to *Plasmodium vivax*, although a few sporadic cases of *P. falciparum* were detected.

In view of the situation thus created, and considering that no uniform method of recording numerically the degree of resistance had been used in the past, we planned a wider investigation aiming at an evaluation of the degree of susceptibility of Greek anopheline species to chlorinated-hydrocarbon insecticides by using the technique of the topical application of microdoses of these insecticides and the technique devised by Busvine & Nash.[1] The purpose of this investigation, therefore, was specifically to study and record the variations in the resistance shown by the anopheles in different localities, thus establishing a point of departure which would make it possible, in the coming years, to follow any changes in susceptibility to DDT.

The investigation started in the autumn of 1953 and continued during the 1954 period of transmission. It included, initially, the measuring of the

susceptibility of *A. sacharovi* and *A. maculipennis* to DDT. The areas chosen for these investigations were those of Skála, Agoulinitsa, and Georgioupolis (see map below).

AREAS CHOSEN FOR INVESTIGATION INTO SUSCEPTIBILITY OF MALARIA VECTORS TO DDT IN GREECE

(*a*) *Skála area (villages of Elos and Asterion).* The development of resistance to DDT by *A. sacharovi* was reported for the first time in this area.[9] The village of Elos was sprayed with DDT [a] continuously from 1946 to 1951; in 1952 it was sprayed with chlordane, in 1953 it remained unsprayed, and in 1954 it was again sprayed with DDT. The village of Asterion

[a] This DDT house-spraying was done once in a year with 1.80-2.0 g of DDT per m² of surface.

was sprayed without interruption from 1946 to 1954 with DDT. It should be noted that, besides house-spraying, air-spraying of breeding-places with DDT was carried out in the Skála area from 1946 to 1951. In the years 1952 and 1954, chlordane was used for the air-spraying in addition to DDT.

(*b*) *Agoulinitsa (Elia) area.* In this area, as indicated by the epidemiological investigations made recently, *A. sacharovi* has developed resistance not only to DDT but also to chlordane and BHC. Agoulinitsa was sprayed with DDT for three successive years (1946-48), was left unsprayed in 1949, and was sprayed again during the following three years (1950-52). In 1953, Agoulinitsa was again left unsprayed and in 1954 it was sprayed with chlordane and BHC. In the breeding-places of the Agoulinitsa area air-spraying was also carried out, from 1946 to 1953 with DDT and in 1954 with DDT and chlordane.

(*c*) *Georgioupolis (Crete) area.* The prevailing species in this area is *A. maculipennis.* DDT house-spraying was carried out there from 1947 for four consecutive years. From 1951 up to the time of writing the area has not been sprayed. It should be noted, however, that in this area, just as in other areas of Greece where house-spraying has been discontinued, the farmers, in order to get rid of the nuisance of domestic insects, are using, to a varying extent, the chlorinated insecticides furnished by the Agricultural Bank of Greece for the control of agriculturally significant insects.

Techniques Used in the Investigation

Topical application of different solutions of DDT under field conditions

Female adult anopheline mosquitos were collected in their natural shelters by means of a standard sucking-tube. Immediately after their capture, they were carried in clean wire-mesh cages to the field laboratory, all necessary precautions, such as covering the cage with a wet cloth, being taken to avoid mishandling. In the laboratory, the mosquitos were taken out of the cages with the aid of a sucking-tube and, after being immobilized with chloroform, were placed by means of a pair of forceps in a suitable position to receive the solution of technical DDT prepared for the test.

Ethyl alcohol was used as solvent. The preparation of the DDT solutions was made locally from a 1% stock solution.

The DDT solution was applied to the back of the thorax of each mosquito by means of a calibrated microloop of 0.24-mm^3 capacity.[a] After each application, the microloop was cleaned by dipping it in acetone to remove any residue of insecticide that might have remained on it.

[a] The estimate of the capacity of the microloop used was made by Dr Perry of the Biochemistry Section, Oak Island Laboratories, Savannah, Ga., USA.

The mosquitos treated by each solution were then transferred with forceps to clean wire-mesh cylindrical cages, 20 cm high and 8 cm in diameter, fitted at the base with white paper, properly fastened with rubber bands, and sealed on their upper surface with pads of damp cotton-wool dipped in a sugar solution. The mosquitos used as controls were transferred to similar cages after the application of only ethyl alcohol to their thoraxes with the microloop. Fifty mosquitos were placed in each cage.

After 24 hours the mortality rates for the treated and control mosquitos were determined. The above tests, carried out at a temperature of 25°-28°C and a relative humidity of 60%, were continued until the dose given resulted in a mortality of approximately 90%, the aim being to get more accurate values of LD_{90} for comparison. Later it was pointed out to the authors that LD_{50} was a more satisfactory basis for comparison and that the sequential procedure was not desirable since it would vitiate application of the usual statistical methods for such estimations.

Application of the Busvine & Nash technique under field conditions

Anopheline mosquitos caught in their natural shelters were introduced immediately after their capture into vials (5-6 per vial) lined with filter-paper uniformly impregnated with DDT solution of a determined strength, in accordance with the technique described by Busvine & Nash.[1] Shell " Risella " oil was used as solvent, and the solution prepared was diluted before use with two parts of ether. The paper-lined vials were corked and placed vertically in the field-laboratory darkroom at a temperature of 25°-27°C and a relative humidity of 60%. After the mosquitos had been exposed to the insecticide for one hour they were transferred from the vials to clean wire-mesh cages supplied with pads of damp cotton-wool dipped in a sugar solution and with white-paper bases as in the previous test. About 50 mosquitos were placed in each cage. After 24 hours, the mortality among the caged mosquitos was determined.

The mosquitos used as controls were subjected to the same procedure throughout, except that they were introduced into vials lined with filter-paper impregnated only with Shell " Risella " oil and ether in the same proportions as those used in the test solution.

Results

The observations made after the topical application of different DDT solutions are summarized in Tables I to VI, and those after application of the Busvine & Nash technique in Tables VII and VIII. From these observations, the LD_{50} value of DDT for the mosquitos in each village was computed. The estimation was made by using the maximum-likelihood method,

TABLE I. ELOS, 1953: MORTALITY OF "A. SACHAROVI" (FEMALE ADULTS) 24 HOURS AFTER TOPICAL APPLICATION OF DIFFERENT SOLUTIONS OF DDT

Observation date	Number of observations	Treated					Controls		
		concentration of DDT solution (%)	number of mosquitos treated	number of dead mosquitos	mortality rate (%)	micrograms of DDT per mosquito	number of mosquitos treated with ethyl alcohol	number of dead mosquitos	mortality rate (%)
14 August	1	0.01	80	16	20.0	0.024	112	17	15.2
14 August	1	0.05	50	18	36.0	0.120	112	17	15.2
15-20 August	6	0.10	807	696	86.2	0.240	615	52	8.4
21-23 August	3	0.15	319	295	92.5	0.360	260	12	4.6
23 August	1	0.20	88	88	100.0	0.480	90	2	2.2

TABLE II. ELOS, 1954: MORTALITY OF "A. SACHAROVI" (FEMALE ADULTS) 24 HOURS AFTER TOPICAL APPLICATION OF DIFFERENT SOLUTIONS OF DDT

Observation date	Number of observations	Treated					Controls		
		concentration of DDT solution (%)	number of mosquitos treated	number of dead mosquitos	mortality rate (%)	micrograms of DDT per mosquito	number of mosquitos treated with ethyl alcohol	number of dead mosquitos	mortality rate (%)
23 August	1	0.10	50	17	34.0	0.240	60	5	8.3
23 August	2	0.15	210	130	62.8	0.360	120	10	8.3
30 August	9	0.20	1 321	1 203	91.0	0.480	510	36	7.6
30 August	1	0.25	115	115	100.0	0.600	50	4	8.0

TABLE III. ASTERION, JUNE 1954: MORTALITY OF "A. SACHAROVI" (FEMALE ADULTS) 24 HOURS AFTER TOPICAL APPLICATION OF DIFFERENT SOLUTIONS OF DDT

Observation date	Number of observations	Treated					Controls		
		concentration of DDT solution (%)	number of mosquitos treated	number of dead mosquitos	mortality rate (%)	micrograms of DDT per mosquito	number of mosquitos treated with ethyl alcohol	number of dead mosquitos	mortality rate (%)
17 June	1	0.15	60	43	71.7	0.360	70	10	14.2
17-25 June	9	0.20	1 170	1 061	90.7	0.480	651	64	9.8

TABLE IV. ASTERION, AUGUST 1954: MORTALITY OF "A. SACHAROVI" (FEMALE ADULTS) 24 HOURS AFTER TOPICAL APPLICATION OF DIFFERENT SOLUTIONS OF DDT

Observation date	Number of observations	Treated					Controls		
		concentration of DDT solution (%)	number of mosquitos treated	number of dead mosquitos	mortality rate (%)	micrograms of DDT per mosquito	number of mosquitos treated with ethyl alcohol	number of dead mosquitos	mortality rate (%)
8 August	1	0.10	106	44	41.5	0.240	60	4	6.7
8 August	1	0.15	125	85	68.0	0.360	60	4	6.7
8-20 August	12	0.20	1 016	932	91.7	0.480	687	42	6.1
20 August	1	0.25	60	60	100.0	0.600	71	6	8.4

TABLE V. AGOULINITSA, 1954: MORTALITY OF "A. SACHAROVI" (FEMALE ADULTS) 24 HOURS AFTER TOPICAL APPLICATION OF DIFFERENT SOLUTIONS OF DDT

Observation date	Number of observations	Treated					Controls		
		concentration of DDT solution (%)	number of mosquitos treated	number of dead mosquitos	mortality rate (%)	micrograms of DDT per mosquito	number of mosquitos treated with ethyl alcohol	number of dead mosquitos	mortality rate (%)
26 July	2	0.05	184	112	60.9	0.120	120	10	8.3
17-26 July	11	0.08	2 056	1 910	92.9	0.192	700	58	8.3
17 July	2	0.10	203	203	100.0	0.240	120	16	13.3
16-17 July	2	0.15	235	235	100.0	0.360	120	16	13.3
16 July	2	0.20	215	215	100.0	0.480	120	16	13.3

TABLE VI. GEORGIOUPOLIS, 1954: MORTALITY OF "A. MACULIPENNIS" (FEMALE ADULTS) 24 HOURS AFTER TOPICAL APPLICATION OF DIFFERENT SOLUTIONS OF DDT

Observation date	Number of observations	Treated					Controls		
		concentration of DDT solution (%)	number of mosquitos treated	number of dead mosquitos	mortality rate (%)	micrograms of DDT per mosquito	number of mosquitos treated with ethyl alcohol	number of dead mosquitos	mortality rate (%)
15 September	1	0.03	50	23	46.0	0.072	50	2	4.0
15 September	1	0.05	50	40	80.0	0.120	50	2	4.0
17-21 September	6	0.06	648	586	90.4	0.144	300	8	2.7
15-21 September	3	0.08	150	150	100.0	0.192	150	4	2.7

with the usual assumption that the probit values corresponding to the 24-hour mortality of mosquitos were linearly related to the logarithmic dosage of DDT. The possible chance deviations in the natural mortality of mosquitos were also taken into account in the computation. Although, as is pointed out in a previous paragraph, the sequential experimental procedure utilized in this study was not satisfactory from the statistical point of view, the mortality data were analysed as if they were the results of independent observations. The results of the probit analysis are summarized in Table IX.

As shown in Table IX, the LD_{50} of DDT for *A. sacharovi* in the village of Elos (Skála) in 1953 was 0.080 μg per mosquito, whereas in 1954 it rose to 0.322 μg per mosquito. In the village of Asterion (Skála), situated two kilometres from Elos, the LD_{50} of DDT in two series of tests made in June and August 1954 were 0.302 μg and 0.258 μg per mosquito, respectively. The LD_{50} of DDT in Agoulinitsa (Elia) in 1954 for *A. sacharovi* was much smaller than in the other two villages, i.e., 0.115 μg per mosquito, and the value found in Georgioupolis (Crete) for *A. maculipennis* was still smaller, i.e., 0.081 μg per mosquito. Moreover, in Agoulinitsa in 1954, the average mortality rate for *A. sacharovi* exposed for one hour to a 2% DDT solution and kept for 24 hours, in accordance with the Busvine & Nash technique, was 71.2% (Table VII). On the other hand, in Asterion in 1954, the average mortality rate after 24 hours for *A. sacharovi* exposed for one hour to the same solution was 22.2%, but the rate increased progressively when the mosquitos were exposed to stronger solutions, reaching as high a value as 96.9% after exposure to a 4% DDT solution (Table VIII). The probit analysis has revealed that the values of LD_{50}, measured in percentage concentrations of DDT solution, were 1.39 in Agoulinitsa and 2.71 in Asterion.

Trapido,[10] using the Busvine & Nash technique on *A. albimanus* in Panama, observed a mortality of approximately 90% 24 hours after a 12-minute exposure to a 0.5% DDT solution. The difference in the technique used, especially in regard to the period of exposure, does not permit a comparison between our figures and those of Trapido. However, the strong resistance to the DDT solutions shown by *A. sacharovi* and the difference in the susceptibility of this species in Asterion and in Agoulinitsa, ascertained by both the techniques used, are quite indicative of a general trend towards decreased susceptibility to DDT in Greece.

Discussion

The lack of data on the degree of susceptibility to DDT shown by the malaria vectors in Greece in the early stages of the application of this insecticide does not permit an accurate evaluation of the results obtained.

TABLE VII. AGOULINITSA, 1954: MORTALITY OF "A. SACHAROVI" (FEMALE ADULTS) 24 HOURS AFTER EXPOSURE FOR 1 HOUR TO DIFFERENT SOLUTIONS OF DDT IN PAPER-LINED VIALS (BUSVINE & NASH TECHNIQUE)

Observation date	Number of observations	Exposed				Controls
		concentration of DDT solution (%)	number of mosquitos exposed	number of dead mosquitos	mortality rate (%)	average mortality rate (%)
16-17 July	2	0.2	101	18	17.8	12.0
16-17 July	2	0.5	100	21	21.0	
16-17 July	2	1.0	100	40	40.0	
16-17 July	11	2.0	1 142	813	71.2	

TABLE VIII. ASTERION, 1954: MORTALITY OF "A. SACHAROVI" (FEMALE ADULTS) 24 HOURS AFTER EXPOSURE FOR 1 HOUR TO DIFFERENT SOLUTIONS OF DDT IN PAPER-LINED VIALS (BUSVINE & NASH TECHNIQUE)

Observation date	Number of observations	Exposed				Controls
		concentration of DDT solution (%)	number of mosquitos exposed	number of dead mosquitos	mortality rate (%)	average mortality rate (%)
8-9 August	2	1.5	60	10	16.6	7.5
8-25 August	17	2.0	1 031	229	22.2	
8-25 August	17	2.5	1 008	414	41.1	
8-25 August	17	3.0	1 014	675	66.5	
8-25 August	17	3.5	1 053	882	83.7	
8-25 August	17	4.0	1 334	1 293	96.9	

TABLE IX. SUMMARY RESULTS OF PROBIT ANALYSIS OF DATA RELATING TO THE SUSCEPTIBILITY OF MALARIA VECTORS TO DDT IN GREECE DURING 1953 AND 1954

Place	Period	Malaria vector	Type of DDT application	LD_{50}*	95% confidence interval of LD_{50}
Elos	August 1953	*A. sacharovi*	topical application	0.080	(0.063, 0.101)
Elos	August 1954	,,		0.322	(0.303, 0.342)
Asterion	June 1954	,,	,,	0.302	(0.285, 0.320)
Asterion	August 1954	,,	,,	0.258	(0.238, 0.281)
Agoulinitsa	July 1954	,,	,,	0.115	(0.107, 0.124)
Georgioupolis	September 1954	*A. maculipennis*	,,	0.081	(0.071, 0.092)
Agoulinitsa	July 1954	*A. sacharovi*	Busvine & Nash technique	1.39	(1.26, 1.54)
Asterion	August 1954	,,	,,	2.71	(2.67, 2.75)

* Unit—for topical application: micrograms per mosquito; for Busvine & Nash technique: percentage concentration of DDT solution.

It is, however, possible on the basis of the observations made to draw certain relevant conclusions.

The increase in the LD_{50} value of DDT from 0.080 μg in 1953 to 0.322 μg in 1954 in the village of Elos is statistically significant, showing a lowering in the level of susceptibility of *A. sacharovi* to DDT. It is noteworthy that, in 1954, *A. sacharovi* presented a similar degree of susceptibility in the village of Asterion, where malarial conditions are practically the same as in the former village and where house-spraying as well as air-spraying of breeding-places has been carried out over a period of many years. However, there is insufficient evidence to ascertain whether this was caused solely by a development of resistance to DDT by *A. sacharovi* during the one-year period. Investigations carried out on similar lines in the future will no doubt serve to indicate whether the same tendency continues.

The findings in the Agoulinitsa area are also important, for although the epidemiological observations in 1954 showed a clear development of resistance by *A. sacharovi* to insecticides—not only to DDT but also to BHC and chlordane—the susceptibility of this species to DDT was found to be considerably higher (LD_{50}, 0.115 μg per mosquito) than that observed in the villages of Elos and Asterion (LD_{50}, 0.322 μg and 0.302 μg per mosquito, respectively). The higher susceptibility of *A. sacharovi* to DDT in Agoulinitsa was also confirmed by the Busvine & Nash technique.

Finally, in Georgioupolis (Crete) in 1954 the LD_{50} of DDT (0.081 μg) for *A. maculipennis* showed a high susceptibility of the species to this insecticide. In this area the house-spraying programme was discontinued in 1951, although some use was reported to have been made of chlorinated insecticides by the population during the ensuing period. It is not possible to estimate how this LD_{50} compares with the original LD_{50} levels of DDT for *A. maculipennis* in Greece.

Conclusions

From the results of the investigation made to assess numerically the degree of susceptibility of *A. sacharovi* and *A. maculipennis* to DDT, and in which 21 723 of the former species and 1448 of the latter species were used, the following conclusions can be drawn.

1. The susceptibility of *A. sacharovi* to DDT in the village of Elos was significantly lower in August 1954 than in August 1953.

2. The susceptibility of *A. sacharovi* to DDT in 1954 showed a considerable difference from place to place under investigation.

3. *A. maculipennis* in Georgioupolis registered a higher susceptibility to DDT than did *A. sacharovi* in other places in 1954.

4. The continuation of similar investigations in Greece and elsewhere will provide the further information necessary for determining the degree of change in the susceptibility of various anopheline species to insecticides.

RÉSUMÉ

C'est en 1951, soit six ans après le début des pulvérisations, qu'apparurent en Grèce les premiers signes d'une baisse d'efficacité du DDT à l'égard d'*Anopheles sacharovi*, vecteur du paludisme. Les recherches poursuivies de 1952 à 1954 révélèrent que *A. sacharovi* avait acquis une résistance en diverses régions de la Grèce; les pulvérisations répétées de DDT n'avaient pu y supprimer cette espèce. Cette résistance s'étendait au chlordane, au HCH et au lindane, et il apparut bientôt que deux autres vecteurs locaux (*A. superpictus* et *A. maculipennis*) avaient acquis eux aussi une résistance aux hydrocarbures chlorés. Dans certaines régions cependant, le nombre de cas de paludisme n'avait pas augmenté à la suite de cette résistance, malgré une forte densité anophélienne, ce qui peut s'expliquer par l'absence d'importants foyers locaux d'infection. Dans d'autres, au contraire, la fréquence était plus élevée, et avait pris, en 1954, des proportions épidémiques.

Afin d'étudier la situation d'un point de vue plus général et sur des bases plus précises, on procéda à l'évaluation de la résistance aux hydrocarbures chlorés des espèces d'anophèles actives en Grèce. Il s'agissait d'étudier les variations de la sensibilité dans diverses régions et d'établir des données de référence d'après lesquelles on pourrait estimer à l'avenir d'éventuels changements de sensibilité. La méthode par dépôt de microdoses d'insecticide sur le corps des moustiques et celle de Busvine & Nash ont été appliquées dans trois régions: celle de Skála (villages d'Elos et d'Asterion), celle d'Argoulinitsa, toutes deux dans le Péloponnèse, et celle de Georgioupolis en Crète.

Faute de connaître la sensibilité des anophèles au début des campagnes de lutte antipaludique, on ne peut évaluer numériquement la diminution de la sensibilité observée en 1954. On peut cependant tirer quelques conclusions des résultats obtenus par les deux méthodes mentionnées.

Dans le village d'Elos, la DL_{50} de DDT, qui a passé de 0,08 μg par moustique en 1953 à 0,332 μg en 1954, indique une baisse significative de la sensibilité d'*A. sacharovi*. A Agoulinitsa, les observations épidémiologiques indiquaient en 1954 une résistance d'*A. sacharovi* au DDT, au HCH et au chlordane; mais en fait, la sensibilité de l'espèce était beaucoup moins élevée qu'à Elos (DL_{50} 0,115 μg par moustique). A Georgioupolis, où les pulvérisations générales ont été suspendues en 1951, *A. maculipennis* présentait en 1954 une sensibilité au DDT beaucoup plus forte que celle d'*A. sacharovi* dans les localités du Péloponnèse (DL_{50} 0,081 μg par moustique). La sensibilité des anophèles avant le début des pulvérisations étant inconnue, on ne peut savoir si elle a varié au cours des années de traitement.

Les recherches seront poursuivies au cours des prochaines années, sur la base de ces premiers résultats.

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