The Effects of DDT, Toxaphene, and Dieldrin on Succinic Dehydrogenase Activity in Insecticide-Resistant and Susceptible *Gambusia affinis*

Gladys B. Moffett and James D. Yarbrough*

Succinic dehydrogenase activity in mitochondria of insecticide-resistant and susceptible mosquitofish (*Gambusia affinis*) was assayed manometrically by the phenazine methosulfate method. Intact and disrupted mitochondria from livers and brains were used. The enzymatic activity in intact mitochondria from resistant fish was either stimulated or not affected by dieldrin or DDT. Toxaphene inhibited intact mitochondrial preparations from resistant brain tissue. Succinic dehydrogenase activity in intact susceptible mitochondria was inhibited by all three insecticides. In mitochondria with disrupted membranes, enzymatic activity was inhibited by the insecticides in both resistant and susceptible fish. Inhibition of succinic dehydrogenase activity by the insecticides only after disruption of the resistant mitochondrial membrane indicates that a membrane barrier exists in insecticide-resistant mosquitofish.

Many studies have involved insecticide effects on cellular respiratory enzymes (Sacktor, 1950; Johnson, 1950; Morrison and Brown, 1954; Sova, 1966; Colvin and Phillips, 1968). However, until recently no enzymatic study had involved vertebrate insecticide-resistant tissues or attempted to present evidence that vertebrate insecticide resistance involved a specific membrane function. Based on succinic dehydrogenase assay, Yarbrough and Wells (1971), working with mitochondrial preparations from endrin-resistant and susceptible mosquitofish, reported inhibition of enzymatic activity from 10^{-6} to 10^{-4} *M* endrin in susceptible preparations, whereas there was either no effect or stimulation in preparations from endrin-resistant mosquitofish. When mitochondria from resistant tissues were disrupted and assayed there was enzymatic inhibition at all endrin concentrations.

For the three insecticides used in this study, the levels of resistance, based on 48-hr LC_{50} values, have established dieldrin as most toxic to susceptible fish and DDT as the least toxic. In resistant fish, DDT is most toxic and toxaphene is the least toxic (Boyd and Ferguson, 1964). This study is intended to expand our original observations for endrin to include DDT, toxaphene, and dieldrin; these organochlorine compounds represent a wide toxicity range for insecticide-resistant and susceptible mosquitofish.

MATERIALS AND METHODS

Mosquitofish (*Gambusia affinis*), known to be resistant to DDT, toxaphene, and dieldrin (Vinson *et al.*, 1963; Boyd and Ferguson, 1964), were collected from drainage ditches near Belzoni, Humphreys County, Mississippi. Susceptible fish were collected from ponds near Starkville, Oktibbeha County, Mississippi. All fish were kept in the laboratory at least 1 week prior to enzyme assay under the same conditions of light, temperature, and diet. About 95% of the fish used were mature females.

Recrystallized technical grades of dieldrin and DDT were used. Toxaphene was not recrystallized. For enzymatic assay each insecticide was dissolved in a solution mixture of 5% ethanol, 5% acetone, and 0.5% Triton X-100.

Livers or brains from 10–15 fish were pooled and homogenized by hand in ice-cold, 0.3 *M* Tris-HCl buffer, pH 7.6 for 1 min. Hogeboom's method of centrifugation (1955) was used to prepare the mitochondria. All mitochondrial preparations were washed three times. Intact mitochondria were resuspended by hand homogenation in Tris-HCl buffer to give a 0.3% solution (initial tissue weight/volume). Disrupted mitochondria were prepared by suspending the final pellet in 5 ml of water. The suspension was then repeatedly frozen and thawed and diluted with buffer to make a 0.3% solution.

Succinic dehydrogenase was assayed manometrically by the phenazine methosulfate method (Bernath and Singer, 1962). Each reaction flask contained 0.1 ml of 0.1 *M* $CaCl_2$, 0.3 ml of 0.01 *M* KCN, and 2.1 ml of homogenate in the main compartment. One side arm of each flask contained 0.3 ml of 0.2 *M* sodium succinate and 0.2 ml of 10% (w/v) phenazine methosulfate. The other side arm contained either 0.2 ml of insecticide or solvent. Total flask content was 3.2 ml and air was the gas phase.

The flasks were suspended in a 37°C water bath. After a temperature equilibration of 8 min, the sodium succinate and phenazine methosulfate were introduced into the main compartment. The assay consisted of two 40-min periods, the first a control and the second period the treatment. Oxygen uptake was recorded at 10-min intervals during the first period of 40 min. At the end of this period either the insecticide or solvent was introduced from the side arm into the main compartment and oxygen uptake was recorded at 10-min intervals for the second 40-min period. The values obtained from vessels containing only solvent were used to correct for any change in oxygen uptake caused by the solvent during the experimental period. In no case reported did the solvent effect exceed 15% of the total activity and there was no difference in mean solvent effect on either susceptible or resistant preparations.

Protein was estimated by the method of Lowry *et al.* (1951), in which a standard calibration curve using Tris-HCl buffer was prepared to correct for buffer interference.

RESULTS AND DISCUSSION

Insecticide effects on intact and disrupted mitochondria preparations from resistant and susceptible mosquitofish are shown in Table I. Succinic dehydrogenase activity in susceptible intact mitochondrial preparations was inhibited by all insecticide concentrations (Table I). However, enzymatic activity in resistant intact mitochondria was stimulated by DDT and dieldrin in all cases at the two lower concentra-

Department of Zoology, Mississippi State University, State College, Mississippi 39762.

Table I. DDT, Toxaphene, and Dieldrin Effects on Succinic Dehydrogenase Activity. For Each Concentration Assayed, Column 1 is the Mean Value of Six 5-min Periods (Control) and Column 2 is the Mean Value of Six 5-min Periods (Treatment). All Values Are Corrected for Solvent Effect. Sample Size is Three Replicates Each in Triplicate

		Intact			Disrupted		
		μl O_2/5 min/mg protein			μl O_2/5 min/mg protein		
Mitochondrial preparation	Concentration, *M*	1	2	DDT effect, %	1	2	DDT effect, %
Resistant brain	9×10^{-9}	12.74 ± 1.04	15.78 ± 0.42	+23.9	20.32 ± 0.43	18.91 ± 0.79	−6.9
	9×10^{-8}	12.69 ± 0.83	14.25 ± 0.73	+12.3	20.92 ± 0.86	19.14 ± 1.29	−8.5
	9×10^{-7}	13.59 ± 1.11	10.58 ± 0.80	−22.2	19.12 ± 1.19	19.61 ± 0.92	+2.6
Susceptible brain	9×10^{-9}	13.31 ± 1.20	13.12 ± 0.66	−1.4	18.26 ± 0.91	12.69 ± 0.55	−30.5
	9×10^{-8}	14.54 ± 1.16	12.87 ± 0.75	−11.5	19.51 ± 1.05	14.90 ± 0.95	−23.6
	9×10^{-7}	14.24 ± 1.12	11.42 ± 1.05	−19.8	19.15 ± 0.96	18.42 ± 0.72	−3.8
Resistant liver	9×10^{-9}	20.66 ± 0.42	23.93 ± 0.55	+15.8	20.64 ± 1.09	18.97 ± 1.05	−8.1
	9×10^{-8}	20.02 ± 0.82	21.03 ± 0.93	+5.0	22.56 ± 1.12	18.46 ± 1.29	−18.7
	9×10^{-7}	20.64 ± 0.86	21.41 ± 0.60	+3.7	21.72 ± 0.89	14.82 ± 0.79	−31.8
Susceptible liver	9×10^{-9}	21.36 ± 0.80	19.15 ± 0.86	−10.4	20.63 ± 0.70	18.79 ± 0.49	−8.9
	9×10^{-8}	22.53 ± 0.73	18.70 ± 0.83	−17.0	19.77 ± 0.82	15.44 ± 0.88	−21.9
	9×10^{-7}	22.08 ± 0.63	16.13 ± 0.48	−26.9	20.72 ± 0.83	13.63 ± 0.71	−34.2
				Toxaphene effect, %			Toxaphene effect, %
Resistant brain	6×10^{-8}	12.76 ± 0.46	12.03 ± 0.83	−5.7	19.84 ± 1.06	17.70 ± 0.91	−10.8
	6×10^{-7}	12.54 ± 0.93	11.69 ± 0.85	−6.8	19.80 ± 0.83	16.76 ± 0.93	−15.4
	6×10^{-6}	12.80 ± 0.86	11.34 ± 0.85	−11.4	19.91 ± 0.91	14.24 ± 0.80	−28.5
Susceptible brain	6×10^{-8}	14.78 ± 0.84	13.97 ± 0.90	−5.5	18.74 ± 0.69	17.76 ± 1.08	−5.2
	6×10^{-7}	13.78 ± 0.60	12.19 ± 0.84	−11.5	18.85 ± 0.69	17.35 ± 0.75	−8.0
	6×10^{-6}	14.70 ± 0.54	12.41 ± 0.72	−15.6	16.95 ± 0.87	13.80 ± 0.96	−18.6
Resistant liver	6×10^{-8}	22.32 ± 1.20	24.96 ± 0.78	+11.8	22.71 ± 1.48	21.10 ± 0.84	−7.1
	6×10^{-7}	20.34 ± 0.79	20.82 ± 0.50	+2.4	22.10 ± 0.74	18.54 ± 1.20	−16.1
	6×10^{-6}	21.15 ± 0.96	21.81 ± 1.21	+3.1	22.67 ± 0.45	15.96 ± 0.24	−29.6
Susceptible liver	6×10^{-8}	21.31 ± 0.96	19.92 ± 1.08	−6.5	22.29 ± 0.67	18.07 ± 0.92	−18.9
	6×10^{-7}	21.91 ± 0.77	18.35 ± 0.70	−16.3	22.38 ± 0.86	18.48 ± 0.82	−17.4
	6×10^{-6}	22.78 ± 0.73	17.61 ± 0.47	−22.7	21.18 ± 0.84	16.18 ± 0.66	−23.6
				Dieldrin effect, %			Dieldrin effect, %
Resistant brain	6×10^{-5}	18.60 ± 0.71	20.09 ± 0.90	+8.0	20.53 ± 0.68	18.11 ± 0.54	−11.8
	6×10^{-4}	17.88 ± 0.74	19.06 ± 0.74	+6.6	19.74 ± 0.81	16.26 ± 0.72	−17.6
	6×10^{-3}	17.83 ± 0.62	18.50 ± 0.82	+3.8	17.68 ± 0.96	12.20 ± 0.73	−31.0
Susceptible brain	6×10^{-5}	16.81 ± 0.79	13.66 ± 0.71	−18.7	20.31 ± 0.72	15.12 ± 0.72	−25.6
	6×10^{-4}	19.19 ± 0.42	14.10 ± 0.81	−26.5	19.44 ± 0.90	16.14 ± 0.59	−17.0
	6×10^{-3}	19.55 ± 1.07	11.35 ± 0.58	−41.9	18.78 ± 1.10	18.70 ± 0.61	−00.4
Resistant liver	6×10^{-5}	17.32 ± 0.42	20.42 ± 0.45	+17.9	21.27 ± 1.22	17.39 ± 0.67	−18.2
	6×10^{-4}	17.17 ± 0.96	18.65 ± 1.00	+8.6	21.02 ± 1.17	15.52 ± 0.34	−26.2
	6×10^{-3}	19.58 ± 1.09	18.27 ± 0.33	−6.7	20.96 ± 0.77	13.25 ± 0.81	−36.8
Susceptible liver	6×10^{-5}	20.31 ± 0.96	17.56 ± 0.73	−13.5	21.84 ± 0.71	20.39 ± 1.10	−6.6
	6×10^{-4}	21.33 ± 0.45	15.85 ± 0.79	−25.7	20.92 ± 1.10	18.41 ± 1.17	−12.20
	6×10^{-3}	22.60 ± 0.91	12.40 ± 0.16	−45.1	19.58 ± 1.10	14.05 ± 0.65	−28.2

tions used. When the mitochondria were disrupted the insecticides inhibited enzymatic activity in both susceptible and resistant preparations.

Only once in the dieldrin treatments (Table I) did inhibition occur in resistant liver preparations. Comparing the effects of the two lower insecticide concentrations, one would expect inhibition at 6×10^{-3} *M* dieldrin in resistant intact liver preparations.

Intact mitochondria from liver and brain homogenates were affected similarly by dieldrin and DDT (Table I). However, toxaphene also inhibited resistant intact brain mitochondria (Table I). Susceptible intact brain mitochondria were inhibited by toxaphene.

With one exception, disrupted mitochondria from livers and brains of resistant and susceptible mosquitofish were inhibited at all insecticide concentrations. This exception occurred at the highest DDT concentration (Table I). Enzymatic inhibition of disrupted resistant mitochondria was neither consistently greater nor lesser than inhibition of susceptible disrupted mitochondria.

In intact mitochondria from resistant fish, stimulation was inversely proportional to increasing insecticide concentration. However, in intact mitochondria from susceptible fish, inhibition of succinic dehydrogenase was directly proportional to increasing insecticide concentration.

In disrupted mitochondria from livers of resistant and susceptible fish, inhibition of enzymatic activity was directly proportional to increasing insecticide concentration. However, in disrupted brain mitochondria, there was no pattern to the effects of insecticide concentration.

Inhibition of enzymatic activity in resistant mitochondrial preparations after membrane disruption indicates that there is a membrane barrier to DDT and dieldrin. This barrier is not clearly demonstrated for toxaphene, as inhibition occurred in resistant brain mitochondrial preparations. The membrane barrier is either absent or less effective in susceptible mitochondria, since both intact and disrupted mitochondria were inhibited by insecticide exposure. Enzymatic activity was more inhibited by the insecticides in disrupted resistant liver mitochondria than in disrupted resistant brain

mitochondrial preparations. This suggests that the barrier against DDT is most effective in resistant liver mitochondria. Intact susceptible mitochondria were inhibited by all insecticides. In DDT treatments from 10^{-9} to 10^{-8} *M* the inhibition increased in disrupted brain mitochondria but decreased at 10^{-7} *M*. The increased inhibition in susceptible preparations may indicate a slight insecticide barrier in susceptible preparations. Enzymatic inhibition by toxaphene in intact resistant brain mitochondria, at least at the concentrations reported, indicate that the membrane barrier is much less effective for toxaphene than for dieldrin or DDT.

Stimulation of enzymatic activity by organochlorine compounds in intact mitochondria is presently unexplained but has been previously recorded. Morrison and Brown (1954) reported that 10^{-5} and 10^{-3} *M* DDT and dieldrin stimulated cytochrome oxidase activity before causing inhibition. Yarbrough and Wells (1971) reported stimulation of succinic dehydrogenase activity in preparations from endrin-resistant mosquitofish livers and brains at concentrations of 10^{-7} to 10^{-4} *M* endrin.

Toxicity values for dieldrin, DDT, and toxaphene have been previously established (Boyd and Ferguson, 1964; Culley, 1968). In susceptible fish the LC_{50} values are lowest for dieldrin (8.02) and highest for DDT (18.96). In resistant fish the LC_{50} values are lowest for DDT (96.16) and highest for toxaphene (4,518.66). There is seemingly no relationship between the established LC_{50} values and the effects of the insecticides on succinic dehydrogenase activity. Several factors must be considered before one assumes that the LC_{50} values should correspond directly with enzymatic inhibition. Dieldrin, DDT, and toxaphene represent three groups of organochlorine compounds: DDT, 1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; dieldrin, a cyclodiene derivative; and toxaphene, a chlorinated camphene. Structural differences might indicate different modes of action. Furthermore, DDT is metabolized to DDE and other analogs. Prather and Ferguson (1966) suggested that the metabolism of DDT may account for low levels or resistance. In comparison to DDT which is metabolized, dieldrin has been reported to be stored unchanged in body fat (Bann *et al.*, 1956). Other factors such as molecular size, polarity, and lipid solubility in relation to membrane penetration must be considered. Odum and Sumerford (1946) compared the toxicity of DDT and four analogs in *Gambusia* and noted that the speed of action was inversely correlated with molecular size. Since DDT is lipid-soluble, apolar, and has a smaller molecular weight than dieldrin or toxaphene, it should have the least difficulty in penetrating a membrane. Other mechanisms may also be present and interact with any one species to obscure the results in a study of the comparative effects of several insecticides on one enzyme system. Ferguson and Bingham (1966) showed that a combination of two insecticides produced higher mortality than did either insecticide alone. They suggested that higher mortality from mixtures probably indicated differences in modes of action of the toxicants involved.

Even though no direct relationship between toxicity (LC_{50} values) and our study was found, the present study shows that enzyme activity in intact resistant fish mitochondrial preparations is not affected by the same concentrations of insecticides that inhibit activity in susceptible preparations. This fact suggests a membrane barrier that is more effective in the resistant than in susceptible mitochondrial preparations. It is evident that the "membrane barrier" is not a total barrier nor can it withstand infinite concentrations. It is reasonable to suggest that between the 10^{-4} and 10^{-3} *M* dieldrin treatments in R-liver mitochondria, we may have passed beyond the membrane's ability to function as an effective barrier. This could be true for DDT at from 10^{-8} to 10^{-7} *M* and probably is true for toxaphene at 10^{-8} *M*.

Stimulation of our enzyme system in intact resistant preparations might be used as a basis for questioning our interpretation of the results as a membrane barrier. However, the insecticide does not have to penetrate the mitochondria to cause an effect of our enzyme assay. One very simple and highly plausible explanation would involve substrate concentration. In resistant mitochondrial preparations the insecticide is prevented from penetrating the membrane; however, the membrane's permeability to the substrate is affected, allowing more substrate to enter the mitochondria, thereby increasing enzymatic activity.

Several investigators have attempted to explain how membranes act as barriers to insecticides. Mullins (1955) suggested that organochlorine compound toxicity is related to a precise fit into the intermolecular lattices of membranes. Colvin and Phillips (1968) and Yarbrough and Wells (1971) suggested that the insecticide binds to a lipid component within the membrane. O'Brien and Matsumura (1964) proposed that a charge–transfer complex occurs between the molecules of the membrane and the insecticide. Regardless of the specific mechanism responsible for a membrane barrier in mitochondria, disruption of resistant mitochondria causes a breakdown in the barrier which is present in intact mitochondria.

LITERATURE CITED

Bann, J. M., DeCino, T. J., Earle, N. W., Sun, Yun-Pei, J. AGR. FOOD CHEM. **4** (11), 937 (1956).

Bernath, P., Singer, T. P., "Methods in Enzymology," Colowick, S. P., Kaplan, N. O., Eds., vol V, Academic Press, New York, N.Y., 1962, p 597.

Boyd, C. E., Ferguson, D. E., *J. Econ. Entomol.* **57**(4), 430 (1964).

Colvin, J. H., Phillips, A. T., *Bull. Environ. Contam. Toxicol.* **3,** 106 (1968).

Culley, D. D., Ph.D. Thesis, Mississippi State University, 46 pp, 1968.

Ferguson, D. E., Bingham, C. R., *Bull. Environ. Contam. Toxicol.* **1**(3), 97 (1966).

Hogeboom, G. H., "Methods in Enzymology," Kaplan, N. O., Colowick, S. P., Eds., vol I, Academic Press, New York, N.Y., 1955, p 16.

Johnson, C. D., *Arch. Biochem. Biophys.* **31,** 375 (1950).

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem.* **193,** 265 (1951).

Morrison, P. E., Brown, A. W. A., *J. Econ. Entomol.* **47,** 723 (1954).

Mullins, L. J., *Scientist* **122,** 118 (1955).

O'Brien, R. D., Matsumura, F., *Scientist* **146,** 657 (1964).

Odum, E. P., Sumerford, W. T., *Scientist* **104,** 480 (1946).

Prather, J. W., Ferguson, D. E., *J. Miss. Acad. Sci.* **12,** 317 (1966).

Sacktor, B., *J. Econ. Entomol.* **43,** 832 (1950).

Sova, C. R., *Scientist* **154,** 1661 (1966).

Vinson, S. B., Boyd, C. E., Ferguson, D. E., *J. Econ. Entomol. Sci.* **139,** 217 (1963).

Yarbrough, J. D., Wells, M. R., *Bull. Environ. Contam. Toxicol.* **6**(2), 171 (1971).

Received for review August 18, 1971. Accepted January 17, 1972. This work was supported by NIH grant 5-ROI-ES-00412-02.