The *in vitro* Effects of Mirex on Succinic Dehydrogenase Activity in *Gambusia affinis* and *Lepomis cyanellus*

by

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Mirex, an organochlorine compound (1, 3, 4-metheno-2H-cyclobuta-cd-pentalene), is characterized by high chemical stability and low solubility in aqueous solutions. To date no breakdown product of this compound has been reported as the result of biological activity. Further, no studies have been reported which deal specifically with mirex and its effects on cellular enzymes.

The in vitro organochlorine compounds effects on cellular respiration have been reported in both invertebrate and vertebrate tissues. In houseflies resistant to DDT, cytochrome oxidase activity increased when compared to houseflies susceptible to DDT (SACKTOR, 1950). Aldrin and dieldrin at 10^{-3} M completely inhibited cytochrome oxidase activity in in vitro muscle studies of the American cockroach (MORRISON and BROWN, 1954). JOHNSON (1950) found no inhibition of succinic dehydrogenase activity in rat heart homogenates with DDT, DDA, and BHC at treatment concentrations of 10^{-4} and 10^{-5} M. Endrin has been shown to inhibit both succinic dehydrogenase and cytochrome oxidase activity in liver homogenates of catfish (CALVIN and PHILLIPS, 1950).

The first in vitro study on the effects of an organochlorine compound of succinic dehydrogenase activity in insecticide-resistant vertebrate tissues indicated a membrane barrier to endrin as a factor in resistance (YARBROUGH and WELLS, 1971). It was later shown that DDT, toxaphene and dieldrin give essentially the same response as that reported for endrin (MOFFETT and YARBROUGH, 1972). This study was undertaken to expand earlier observations to include the effect of mirex on succinic dehydrogenase activity in tissue preparations from Gambusia affinis (mosquitofish) and Lepomis cyanellus (green sunfish).

MATERIALS AND METHODS

Insecticide-resistant mosquitofish (BOYD and FERGUSON, 1964) and green sunfish (MINCHEW and FERGUSON, 1969) from

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drainage ditches at Belzoni, Humphreys County, Mississippi and susceptible fish from ponds in Oktibbeha County, Mississippi, were used in this study. All fish were held in the laboratory for at least one week prior to use.

Succinic dehydrogenase was assayed manometrically by the phenazine methosulfate method (BERNATH and SINGER, 1964). All mitochondria were prepared from brains or livers homogenized in 0.3 M Tris-HCl buffer, pH 7.6. The homogenate was centrifuged at 600 g for 10 min at 0° C. The supernatant was centrifuged at 8,000 g for 10 minutes at 0° C. The pellet was resuspended in fresh Tris-HCl buffer and centrifuged at 32,000 g for 10 minutes at 0° C. The final pellet was suspended in fresh Tris-HCl buffer to make a 0.3% mitochondrial suspension. Protein determinations were by the method of LOWRY et al. (1951), with a Tris-HCl buffer standard prepared to correct for Tris-HCl interference.

All manometric assays were performed on a Gilson Differential Respirometer at 37° C with a final volume of 3.2 ml per flask. The following were placed in the main compartment of each reaction flask: 2.0 ml of the homogenate; 0.3 ml of 0.01 M KCN; and 0.2 ml of 0.1 M $CaCl_2$. One side arm contained 0.2 ml of mirex in a solvent mixture of 5% ethyl alcohol, 5% acetone and 0.5% Triton X-100, and the other side arm had 0.3 ml of 0.2 M sodium succinate and 0.2 ml of fresh 10% phenazine methosulfate.

After an 8 min temperature equilibration period, the sodium succinate and phenazine methosulfate solutions were introduced from the side arm into the main compartment of the reaction vessel. The enzyme assay consisted of two-30 min periods, in which the first period was used as a control and the second period was the treatment. Oxygen uptake was recorded for 10 min intervals during the first 30 min period. After this period either the mirex or the solvent mixture was introduced into the main compartment and oxygen uptake was recorded for a second 30 min period at 10 min intervals. The values from vessels containing the solvent were used to correct for any effect caused by the solvent during the experimental periods. The solvent effect averaged about 10% of the total enzyme activity.

All assays were run in triplicate on both fresh mitochondrial preparations (intact) and preparations that were freeze-thawed three times (disrupted). A 99% confidence interval analysis, and a standard error analysis were performed.

RESULTS

The mean values of succinic dehydrogenase activity for intact and disrupted mitochondrial preparations of mosquitofish and green sunfish are shown in Table 1. The enzyme activity

was essentially unchanged in both preparations of resistant mosquitofish. Brain preparations from susceptible fish decreased in activity, while liver preparations increased in activity. Except in liver preparations from insecticide-susceptible green sunfish, enzymatic activity decreased in all preparations after freeze-thawing.

TABLE 1

Succinic dehydrogenase activity of intact and disrupted mitochondrial preparations from mosquitofish and green sunfish. Sample size is represented by N. Mean values are expressed as ul O_2/5 min/mg Protein.

	Mosquitofish ul O_2/5 min/mg Protein			Green Sunfish ul O_2/5 min/mg Protein		
	N	Intact	Disrupted	N	Intact	Disrupted
r-brain	27	18.4±0.87	22.0±0.32	27	40.8±0.74	28.9±0.95
s-brain	27	18.0±0.84	13.3±0.39	27	38.0±0.47	26.3±1.29
r-liver	27	19.5±0.29	21.8±0.58	27	35.4±1.33	28.4±0.53
s-liver	27	13.8±0.32	27.1±0.91	27	28.6±1.51	43.9±2.37

At 10^{-4} M mirex there was stimulation in all intact and disrupted mitochondrial preparations with the exception of disrupted brain mitochondrial preparations from resistant mosquitofish (Tables 2 and 3). There was stimulation in brain and liver preparations from susceptible fish. In disrupted brain and liver preparations from resistant and susceptible fish, there was inhibition of enzymatic activity. With the exception of the brain preparations from susceptible fish, inhibition occurred at 10^{-6} M mirex in both intact and disrupted mitochondrial preparations. There was no difference in the overall mirex effect on succinic dehydrogenase activity in either insecticide-resistant or -susceptible mosquitofish.

Stimulation of succinic dehydrogenase activity occurred at 10^{-4} M mirex in both intact and disrupted mitochondrial preparations from green sunfish (Tables 4 and 5). Except for the preparations from the livers of susceptible fish, there was inhibition in all intact mitochondrial preparations at 10^{-6} M mirex. There was enzymic inhibition of preparations from liver and brain of resistant fish, but stimulation in liver and brain preparations from susceptible fish. In general there was no difference in the mirex effect on enzyme preparations from the two sunfish populations.

DISCUSSION

A comparison of the effects of mirex on succinic dehydrogenase in mitochondrial preparations from insecticide-susceptible and -resistant populations show no great

TABLE 2

Succinic dehydrogenase activity of intact mitochondrial preparations from mosquitofish. For each mirex concentration tested, column 1 is a mean value of six 5-min periods (control) and column 2 is a mean value of six 5-min periods (treatment). All values are corrected for the effect of the solvent. Sample size is three replicates each in triplicate.[1]

Mitochondrial preparation	Mirex concentration (M)	ul O_2/5 min/mg Protein 1	2	Mirex effect[2] (%)
Resistant brain	10^{-4}	19.8±0.19	22.2±0.10	112.0
	10^{-5}	16.8±0.15	17.7±0.10	105.6
	10^{-6}	18.5±0.10	13.9±0.14	75.5
Susceptible brain	10^{-4}	17.1±0.17	19.7±0.11	115.0
	10^{-5}	17.3±0.12	15.6±0.19	90.4
	10^{-6}	19.7±0.20	17.4±0.19	88.1
Resistant liver	10^{-4}	20.0±0.19	21.4±0.15	107.0
	10^{-5}	19.0±0.11	20.7±0.30	108.9
	10^{-6}	19.5±0.14	15.3±0.18	78.4
Susceptible liver	10^{-4}	14.2±0.14	16.5±0.11	115.9
	10^{-5}	14.1±0.18	11.6±0.11	82.2
	10^{-6}	13.2±0.12	12.2±0.13	92.1

[1] All values are significant at the 99% level based on confidence interval analysis.

[2] Per cent of uninhibited controls.

TABLE 3

Succinic dehydrogenase activity of disrupted mitochondrial preparations from mosquitofish. For each mirex concentration tested, column 1 is a mean value of six 5-min periods (control) and column 2 is a mean value of six 5-min periods (treatment). All values are corrected for the effect of the solvent. Sample size is three replicates each in triplicate.[1]

Mitochondrial preparation	Mirex concentration (M)	ul O_2/5 min/mg Protein 1	2	Mirex effect[2] (%)
Resistant brain	10^{-4}	21.6±0.33	19.9±0.21	92.3
	10^{-5}	22.6±0.33	17.5±0.29	77.4
	10^{-6}	21.7±0.33	16.3±0.21	75.8
Susceptible brain	10^{-4}	14.1±0.14	16.3±0.10	115.7
	10^{-5}	12.8±0.12	12.0±0.13	93.6
	10^{-6}	13.1±0.18	15.2±0.13	115.7
Resistant liver	10^{-4}	21.7±0.14	27.2±0.10	125.2
	10^{-5}	20.8±0.15	19.2±0.23	92.4
	10^{-6}	22.8±0.25	19.7±0.31	86.4
Susceptible liver	10^{-4}	26.6±0.46	26.8±0.36	100.6*
	10^{-5}	25.9±0.40	22.0±0.32	84.8
	10^{-6}	28.9±0.50	24.5±0.37	84.2

[1] All values are significant at the 99% level based on confidence interval analysis except (*).

[2] Per cent of uninhibited controls.

* Not significant at the 99% level based on confidence interval analysis.

TABLE 4

Succinic dehydrogenase activity of intact mitochondrial preparations from green sunfish. For each mirex concentration tested, column 1 is a mean value of six 5-min periods (control) and column 2 is a mean value of six 5-min periods (treatment). All values are corrected for the effect of the solvent. Sample size is three replicates each in triplicate.[1]

Mitochondrial preparation	Mirex concentration (M)	ul O_2/5 min/mg Protein 1	2	Mirex effect[2] (%)
Resistant brain	10^{-4}	40.4±0.21	48.8±0.33	120.7
	10^{-5}	39.7±0.10	40.8±0.33	102.7
	10^{-6}	42.2±0.13	36.9±0.30	87.4
Susceptible brain	10^{-4}	37.1±0.17	42.4±0.24	114.3
	10^{-5}	38.5±0.17	34.5±0.17	89.7
	10^{-6}	38.5±0.23	34.4±0.17	89.4
Resistant liver	10^{-4}	35.3±0.17	39.2±0.33	111.0
	10^{-5}	37.7±0.20	34.2±0.33	90.8
	10^{-6}	33.1±0.33	28.7±0.22	86.8
Susceptible liver	10^{-4}	31.4±0.32	34.2±0.17	108.9
	10^{-5}	26.2±0.28	28.5±0.17	108.6
	10^{-6}	28.2±0.10	32.4±0.11	114.8

[1] All values are significant at the 99% level based confidence interval analysis.

[2] Per cent of uninhibited controls.

TABLE 5

Succinic dehydrogenase activity of disrupted mitochondrial preparations from green sunfish. For each concentration tested, column 1 is a mean value of six 5-min periods (control) and column 2 is a mean value of six 5-min periods (treatment). All values are corrected for the effect of the solvent. Sample size is three replicates each in triplicate.[1]

Mitochondrial preparation	Mirex concentration (M)	ul O_2/5 min/mg Protein 1	2	Mirex effect[2] (%)
Resistant brain	10^{-4}	28.9±0.25	36.7±0.31	127.0
	10^{-5}	30.6±0.27	32.1±0.15	105.0
	10^{-6}	27.3±0.13	25.7±0.10	94.0
Susceptible brain	10^{-4}	26.7±0.18	32.0±0.33	112.0
	10^{-5}	28.3±0.33	25.6±0.32	90.6
	10^{-6}	23.9±0.33	26.5±0.29	101.1
Resistant liver	10^{-4}	29.2±0.21	33.9±0.28	116.1
	10^{-5}	27.4±0.30	24.7±0.17	90.1
	10^{-6}	28.6±0.11	22.0±0.19	77.0
Susceptible liver	10^{-4}	45.5±0.33	56.4±0.20	123.9
	10^{-5}	46.9±0.18	44.4±0.15	94.6
	10^{-6}	39.2±0.23	45.6±0.23	116.3

[1] All values are significant at the 99% level based confidence interval analysis.

[2] Per cent of uninhibited controls.

differences in the two species studied. After disruption of the mitochondrial membrane, a mixed effect of inhibition and stimulation occurred in both species. This response is not the same pattern that has been reported for other organochlorine compounds using insecticide-resistant and -susceptible mosquitofish populations. YARBROUGH and WELLS (1971) found that endrin at 1.25 X 10^{-6} M caused stimulation of succinic dehydrogenase activity in intact brain and liver preparations from insecticide-resistant mosquitofish, and inhibition of intact brain and liver preparations from susceptible mosquitofish. Upon disruption of the mitochondrial membrane, there was inhibition of all preparations from resistant and susceptible fish. Essentially the same pattern has been reported for DDT, toxaphene, and dieldrin (MOFFETT and YARBROUGH, 1972). Because mirex appears to be less toxic than DDT, the membrane barrier in resistant mosquitofish could only be demonstrated for mirex at much lower concentrations. The barrier, if present at all, is slight and the concentrations used in this study probably far exceed the capacity of the membrane barrier. Mosquitofish show only marginal resistance to DDT, and the concentration required to produce the same effects on enzyme activity as that reported for endrin was much less (YARBROUGH and WELLS, 1971; MOFFETT and YARBROUGH, 1972). In comparing the effects of mirex to those of DDT on enzyme activity it should be noted that the mirex concentrations used were from 10 to 100 times more concentrated than the levels used for DDT (MOFFETT and YARBROUGH, 1972). The toxicity of mirex in the two species studied has not been established, but this study would support a mirex toxicity that would be much less than DDT.

In comparing the mean values of succinic dehydrogenase activity to intact and disrupted preparations from susceptible and resistant mosquitofish, there is an increase in activity after disruption in all preparations with the exception of mitochondrial brain preparations from susceptible fish. This is essentially what has been previously reported (YARBROUGH and WELLS, 1971). In green sunfish there is a decrease in enzyme activity after disruption of the mitochondrial membrane in all preparations except in those from the livers of susceptible fish. The loss in activity after disruption by freeze-thawing is unexplained.

Although mirex does not fit the pattern of effect reported for other organochlorine compounds on enzyme activity in resistant species, it is consistent with what has been reported for non-resistant species. In mosquitofish, succinic dehydrogenase activity was inhibited at 10^{-6} M mirex in intact brain and liver and disrupted brain and liver preparations. In green sunfish, 10^{-6} M mirex inhibited enzyme activity in most all mitochondrial preparations. This is similar to what has been reported for heavy beef heart mitochondrial succinoxidase, NADH-oxidase and succinoxidase systems using arochlor (1232) (PARDINI et al., 1971).

There was stimulation at 10^{-4} M mirex in all intact mitochondrial preparations. Stimulation also occurred at 10^{-5} M mirex in intact brain and liver preparations of resistant mosquitofish and intact brain preparations of resistant and liver preparations of susceptible green sunfish. This is similar to what has been previously reported for other organochlorine compounds. Cytochrome oxidase activity in the American cockroach was stimulated before inhibition by 10^{-3} and 10^{-5} M DDT and dieldrin (MORRISON and BROWN, 1954). PARDINI et al. (1971) reported stimulation of succinoxidase and NADH-oxidase with 10^{-6} M dieldrin in heavy beef heart mitochondrial preparations. The stimulation by certain organochlorine compounds, including mirex, is difficult to explain, but might be related to increase in substrate concentration. The insecticide may affect the membranes' permeability to the substrate allowing more substrate to penetrate the outer mitochondrial membrane. Therefore, in this system, the insecticide does not directly affect the enzyme, but affects substrate concentration.

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