Endrin Uptake in Insecticide-Resistant and Susceptible Mosquitofish (*Gambusia affinis*)

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Insecticide-resistant and susceptible mosquitofish were exposed to 10 and 1500 ppb of endrin. Brain, liver, and muscle tissue concentrations of [^{14}C]endrin were compared based on time of endrin exposure and whether fish were exhibiting symptoms of insecticide poisoning (symptomatic) or not exhibiting symptoms of insecticide poisoning (asymptomatic) at the time of sampling. Within each population there was a direct relationship between symptoms of poisoning and tissue concentrations of endrin. Insecticide-resistant fish generally showed lower endrin concentrations than susceptible fish except when both populations were exhibiting symptoms of poisoning. This lower concentration of endrin suggests a membrane barrier that is more effective in the resistant than in the susceptible fish. The higher concentrations of endrin in asymptomatic than in symptomatic susceptible fish are suggestive of a less sensitive target site in the more insecticide tolerant susceptible fish. This same pattern was seen when analogous comparisons for resistant fish were made. The data indicate a significant difference in endrin uptake between resistant and susceptible fish and suggest a target site that is less sensitive to endrin as a factor in insecticide resistance in mosquitofish.

Although insecticide resistance has been demonstrated and studied in many invertebrate groups, only a limited number of vertebrates have been reported to be resistant (Vinson et al., 1963; Ferguson and Bingham, 1966; Ludke et al., 1968; Dziuk and Plapp, 1973). Of these, organochlorine insecticide resistance in the mosquitofish (*Gambusia affinis*) has been the best documented. This population is found in drainage ditches in the Mississippi Delta and has been shown to be resistant to many organochlorine insecticides (Culley and Ferguson, 1969) and resistant and/or tolerant to organophosphorus insecticides (Chambers and Yarbrough, 1974).

Ferguson et al. (1966) reported uptake in susceptible (S) and resistant (R) mosquitofish exposed to lethal endrin concentrations to be the same. They proposed that the major mechanism of endrin resistance was a physiological tolerance of the active toxicant within the body. Fabacher and Chambers (1971) reported that at high endrin concentrations, endrin entered the nervous tissue at a slower rate in R fish than in S fish and suggested that binding to nonessential proteins in R fish might be a factor in insecticide resistance.

Wells and Yarbrough (1972) reported that cell membranes of brain tissue from R fish bound more endrin than comparable preparations from S fish, and suggested that a brain barrier played a part in resistance. Yarbrough and Wells (1971) had earlier shown a membrane barrier to endrin in studies of succinic dehydrogenase activity in intact and disrupted mitochondria. In a subsequent study, a similar response was reported in R fish to DDT and dieldrin (Moffett and Yarbrough, 1972).

Most uptake studies are based solely on the time of exposure to a given insecticide and do not consider insecticide tolerance within a population as a factor. This study is an attempt to provide information on endrin uptake in which resistant and susceptible mosquitofish are selected and compared on the basis of tolerance variations within both populations. To accomplish comparisons within and between populations, R and S mosquitofish exhibiting symptoms of insecticide poisoning (symptomatic) and those not exhibiting symptoms of insecticide poisoning (asymptomatic) were grouped within a selected time period.

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METHODS

The insecticide-resistant mosquitofish (*Gambusia affinis*) used in this study were collected from drainage ditches in Humphreys County, Mississippi. A single population of susceptible fish from one pond in Oktibbeha County, Mississippi, was selected for this study. All fish used were adults (about 95% female) ranging from 2.5 to 5 cm in length and were held in the laboratory for at least 2 days prior to use. The 48-hr LC_{50} value of endrin for susceptible fish is 0.6 ppb and for resistant fish is 314.1 ppb. This represents a 499-fold difference in endrin toxicity between the S and R fish populations (Culley and Ferguson, 1969).

[^{14}C]Endrin (Mallinckrodt Nuclear, St. Louis, Mo.), specific activity 2.37 mCi/mmol, was diluted to a concentration of 1 ppm in acetone. This was combined with nonlabeled endrin in acetone in proportions that would yield either 10 or 1500 ppb of endrin when added to a 5-l. test aquarium. The concentrations were chosen to yield symptoms of poisoning within a 3–9 hr exposure period in sufficient numbers of fish for adequate sampling.

Fish were described as symptomatic when they began to show signs of disorientation, increased opercular activity, and hypersensitivity; asymptomatic fish showed none of these signs. All comparisons of symptomatic fish to asymptomatic fish were for essentially the same time periods of exposure. Fish which did not exhibit signs of insecticide poisoning were sampled at 3, 6, 9, and 12 hr of exposure. Sampling consisted of removing the fish from the test aquarium, rinsing the fish in water, and dissection of brain, liver, and muscle tissues. Coronal cuts were made immediately anterior and posterior to the optic lobes to divide the brain into forebrain, midbrain, and hindbrain. After scaling and the removal of the epithelium, muscle samples were obtained from an area just anterior to the caudal peduncle. All samples consisted of pooled tissue from three fish. All tissues were kept on ice during sample preparation.

Samples were homogenized in cold, glass-distilled, deionized water in TenBroeck glass homogenizers. A 1-ml portion was taken from each homogenized sample and placed in a scintillation mixture for counting. Aliquot samples were taken for protein determinations (Lowry et al., 1951). The values reported are means of three 10-min counting periods. For conversion of counts (cpm) to nanograms of endrin, standards were prepared from each test aquarium solution and counted. Counting efficiency and quench curves were determined using a [^{14}C]endrin standard in a series of tissue homogenates. The scintillation mixture con-

Table I. Tissue Concentrations of Endrin in Mosquitofish Exposed to 10 ppb of [^{14}C]Endrin and Not Exhibiting Symptoms of Poisoning[a]

Exposure time, hr	Population	Forebrain[b]	Midbrain[b]	Hindbrain[b]	Liver[b]	Muscle[b]
3	S	38.37 ● 7.27**	31.24 ± 5.56**	38.00 ± 8.40*	38.80 ± 5.76***	8.49 ± 0.92***
	R	7.43 ± 0.72	6.90 ± 0.44	8.00 ± 0.48	9.83 ± 1.88	2.73 ± 0.25
6	S	39.43 ± 7.43**	34.64 ± 4.66***	39.20 ± 4.20***	36.97 ± 4.22***	9.84 ± 1.22***
	R	7.75 ± 0.54	7.81 ± 0.55	7.93 ± 0.76	7.94 ± 0.59	3.22 ± 0.59
9	S	46.54 ± 9.06**	39.00 ± 5.53***	47.30 ± 6.11***	46.47 ± 7.68***	12.77 ± 2.76*
	R	8.54 ● 0.47	8.10 ± 0.25	9.04 ± 0.33	7.29 ± 0.43	2.69 ± 0.13
12	S	51.05 ± 4.77***	41.53 ± 4.57**	48.43 ± 4.80***	58.14 ± 3.47****	12.64 ± 3.43
	R	19.04 ± 1.97	19.43 ± 1.52	23.66 ± 1.17	17.39 ± 1.85	6.58 ± 1.09

[a] Values are expressed as a mean of five determinations in nanograms of endrin/milligram of protein ± SE. [b] Tissue concentration in S fish is significantly greater than in R fish at $P < 0.05$ (*), $P < 0.02$ (**), $P < 0.01$ (***), or $P < 0.001$ (****) as determined by the student's t test.

Table II. Tissue Concentrations[a] of [^{14}C]Endrin in Insecticide-Resistant (R) and Susceptible (S) Mosquitofish Exhibiting Symptoms (s) and Not Exhibiting Symptoms (a) of Endrin Poisoning (Fish Exposed to 10 ppb of Endrin for 6 hr)

Tissue	Susceptible asymptomatic	Susceptible symptomatic	Resistant asymptomatic	Sa/Ss[b]	Ss/Ra[b]	Sa/Ra[b]
Forebrain	39.43 ● 7.43	23.78 ± 2.71	7.75 ± 0.54	1.67*	3.07**	5.09*
Midbrain	34.64 ± 4.66	22.43 ± 1.67	7.81 ± 0.55	1.54	2.87**	4.44**
Hindbrain	39.20 ± 4.20	24.17 ± 1.52	7.93 ± 0.76	1.61*	3.05**	4.94**
Liver	36.97 ± 4.22	24.69 ± 3.29	7.94 ± 0.59	1.49	3.11*	4.66**
Muscle	9.84 ● 1.22	10.15 ± 0.67	3.22 ● 0.59	0.97	3.15**	3.06**

[a] Values are expressed as a mean of five determinations in nanograms of endrin/milligram of protein ± SE. [b] Endrin tissue concentrations differ significantly at $P < 0.05$ (*) or $P < 0.01$ (**) as determined by the t test.

Table III. Tissue Concentrations[a] of [^{14}C]Endrin in Insecticide-Resistant (R) and Susceptible (S) Mosquitofish Exhibiting Symptoms (s) and Not Exhibiting Symptoms (a) of Endrin Poisoning (Fish Exposed to 1500 ppb of Endrin for 6 hr)

	Susceptible symptomatic	Resistant symptomatic	Resistant asymptomatic	Rs/Ss[b]	Rs/Ra[b]	Ss/Ra[b]
Forebrain	1.99 ± 0.09	4.25 ± 0.36	1.15 ● 0.14	2.13**	3.70**	1.73*
Midbrain	2.03 ± 0.37	4.47 ± 0.21	0.94 ± 0.08	2.22**	4.76***	2.16*
Hindbrain	2.15 ± 0.41	4.87 ± 0.24	1.10 ± 0.13	2.27**	4.43***	1.95
Liver	1.67 ± 0.24	5.59 ± 0.48	0.86 ± 0.05	3.33**	6.50***	1.94*
Muscle	0.31 ± 0.05	0.63 ± 0.11	0.31 ± 0.03	2.04	2.03*	1.00

[a] Values are expressed as a mean of five determinations in micrograms of endrin/milligram of protein ± SE. [b] Endrin tissue concentrations differ significantly at $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***) as determined by the t test.

sisted of 50 mg of 2,2′-p-phenylenebis(5-phenyloxazole), 4.0 g of 2,5-diphenyloxazole, 40 g of Cab-o-sil, 500 ml of scintillation grade toluene, and 500 ml of Triton X-100. All samples were counted by a Packard Model 3320 Tri-Carb liquid scintillation spectrometer.

RESULTS

In S and R fish treated with 10 ppb of endrin, and sampled at 3, 6, 9, and 12 hr, the endrin concentrations of tissues from S fish were higher than those from R fish (Table I). These differences were statistically significant in all cases except the muscle samples at 12-hr endrin exposures. The endrin concentrations in brain segments of S fish showed a marked increase in uptake between the 6- and 9-hr sampling periods. In R fish, a marked increase in endrin concentrations occurred between 9- and 12-hr sampling periods. In general, endrin concentrations in liver tissue showed increases after 9 hr of exposure. There was a noticeable change in the endrin concentrations of most tissues between 6 and 9 hr in S fish and between 9 and 12 hr in the R fish. The concentration of endrin in the test aquaria remained essentially constant throughout the test period.

Tables II and III present data in which comparisons of endrin concentrations in selected tissues are between fish exposed for 6 hr and grouped by symptomology. At the 10-ppb endrin exposure level in comparisons of tissue endrin levels in S fish, the forebrain and hindbrain showed higher levels in asymptomatic than in symptomatic fish (Table II). When tissues from asymptomatic fish were compared, the S fish had significantly higher tissue concentrations than the R fish. No comparisons to symptomatic R fish were possible at the 10-ppb endrin treatment because R fish did not exhibit symptoms of poisoning at this endrin concentration within the 6-hr exposure period.

At 1500-ppb endrin exposure, all S fish were exhibiting symptoms of insecticide poisoning by 6 hr of exposure. Therefore, the only comparisons that could be made were between symptomatic S fish and symptomatic or asymptomatic R fish (Table III). In fish exhibiting symptoms of

poisoning, all tissue concentrations in R fish were statistically higher than those in S fish with the exception of muscle. Although all tissue samples were taken at 6 hr of endrin exposure, S fish generally demonstrated symptoms of poisoning in a shorter period of time than R fish. When comparisons were made within the R population, there were higher endrin concentrations in all tissues from asymptomatic than from symptomatic fish. All the tissue concentrations of symptomatic S fish were significantly higher than those of asymptomatic R fish, except for hindbrain and muscle.

DISCUSSION

Within a fish population, increases in tissue endrin concentrations occur concurrent with the appearance of symptoms and death. However, the time of appearance of symptoms within a treatment was variable, that is, within the two populations some fish are more susceptible to endrin than others. Therefore, random sampling based solely on time of exposure to a given organochlorine insecticide does not consider insecticide tolerance within individuals of a population. Furthermore, most uptake studies are really selecting the more tolerant individuals within a population for study.

Although not statistically significant, in S fish exposed to 10 ppb of endrin all tissues examined from asymptomatic fish had higher endrin concentrations than comparable tissues from symptomatic fish with the exception of muscle tissue. Within R fish exposed to 1500 ppb of endrin, all tissues from symptomatic fish exhibited higher concentrations than those from asymptomatic fish. All tissue concentrations of asymptomatic R fish did increase with time, although the increases were not constant. There was a definite change in the pattern of uptake between 9 and 12 hr of endrin exposure. This may indicate a decrease with time in the effectiveness of the membrane barrier and suggests that there is a relationship between rate of uptake and insecticide resistance. However, there was a definite difference in the uptake of endrin between S and R populations. In all tissues of S fish, there were higher endrin concentrations than in comparable tissues of R fish. This was true regardless of whether symptoms were visible or not, and indicated a significant difference in the rate of endrin uptake.

Except in muscle tissues, when both S and R fish were exhibiting symptoms, the R fish showed a significantly greater tissue level of endrin. This does not agree with the work of Wells (1971), who found that tissues of S fish always had higher concentrations of endrin than tissues of R fish. However, the levels of endrin used in that study were not sufficiently high to induce toxic symptoms in R fish.

Although not effectively demonstrated, it is possible that the overall lower endrin tissue levels in R fish as compared to S fish are due in part to detoxication and elimination of endrin by R fish. Preliminary thin-layer chromatography studies suggested that some metabolism was taking place, although methods of quantification and identification were not achieved. However, endrin is not readily metabolized by most animals and therefore only a small fraction of the measured radioactivity would be expected to be related to biotransformed material.

Within the two populations, comparison of endrin levels in muscle tissue between symptomatic and asymptomatic fish could provide an indication of actual uptake not related to retention or compartmentation in lipid as in brain and liver tissues. The endrin levels in muscle from S symptomatic as compared to asymptomatic fish were the same indicating uptake to be equal between symptomatic and asymptomatic fish even though all other tissues examined showed higher endrin levels in the asymptomatic than in the symptomatic S fish. Therefore, in S fish, the barrier to endrin is apparently lacking, but there is an insensitivity at the target site. Making the same comparison in R fish, there is twice as much endrin in muscle tissue of the symptomatic as in the asymptomatic fish. This indicates that there is a barrier to endrin penetration in R fish. However, this is at a level of endrin exposure (1500 ppb) that may well be beyond the most effective functional range of the membrane barrier in R fish. At this level, if the comparison between symptomatic S and symptomatic R fish is made, there is more endrin in every tissue examined from R fish than S fish. This could be interpreted as an insensitivity at the target site in R fish that is only demonstrable where the membrane barrier system is negated by extremely high levels of endrin. This lack of sensitivity could be expressed as a threshold level which a toxicant must exceed before disruption of nerve function is possible. Thus, in vertebrate resistance there is both a membrane barrier, which is protective at the site of action, and a decrease in the sensitivity of the target tissue to the toxicant.

Assuming the site of action of organochlorine insecticides to be the central nervous system, these data are indicative of varying degrees of sensitivity to the toxicant within the S population. Within the S population there are higher endrin levels in brain tissue from asymptomatic than from symptomatic fish. If penetration of the toxicant were the only consideration in tolerance of the insecticide, the reverse of this situation would be expected. Thus, it would seem that the more tolerant individuals within the S population are capable of withstanding higher internal concentrations of endrin without demonstrating a physiological response.

On the other hand, the less tolerant, i.e., symptomatic, individuals within the R population show greater brain endrin concentrations than the more tolerant individuals. This would be expected if all individuals within the population showed approximately the same sensitivity to the toxicant and insecticide penetration to the active site was the major factor in tolerance. It is reasonable to assume that the R fish are a more homogeneous population with respect to organochlorine insecticide sensitivity since they have undergone severe selective pressures by chronic insecticide exposure. Only individuals with a low sensitivity to the toxicant have survived and propagated. Differences in tolerance are due mainly to varying rates of insecticide uptake. However, it is still possible that an insensitivity factor exists within this population but that it may be masked by the membrane barrier and it may be flooded by the extremely high level of endrin used.

The insensitivity of the target tissue to organochlorine insecticides might explain why uptake data and membrane retention studies do not seem to directly relate to toxicity levels (LC_{50} values). The more tolerant individuals within the R population would probably possess a high insensitivity to organochlorine insecticides at the target site and an effective membrane barrier complex. The less tolerant might possess only one of these factors, or varying degrees of functional effectiveness of one or both factors. It is doubtful that individuals in the S population would contain both factors, or that the factors would be as functionally effective as in the individuals in the R population since no natural selection for a highly tolerant population has occurred.

In summary, this study indicates that vertebrate insecticide resistance is due in part to a membrane barrier which reduces the uptake of the insecticide in both the whole body and organs of R fish and a decrease in sensitivity of the target site to the insecticide. It also demonstrates that comparisons of insecticide uptake based solely on time of exposure do not consider the variations in individual tolerances to an insecticide within a given population. This variation may mask the more subtle differences within and between R and S populations which could give a clearer indication of the mechanism of insecticide resistance.

LITERATURE CITED

Chambers, J. E., Yarbrough, J. D., *Bull. Environ. Contam. Toxicol.* **11,** 315 (1974).
Culley, D. D., Ferguson, D. E., *J. Fish Res. Board Can.* **26,** 2395–2401 (1969).
Dziuk, L. J., Plapp, F. W., *Bull. Environ. Contam. Toxicol.* **9,** 15–19 (1973).
Fabacher, D. L., Chambers, H., *Bull. Environ. Contam. Toxicol.* **6,** 372–376 (1971).
Ferguson, D. E., Bingham, C. R., *Trans. Am. Fish. Soc.* **95,** 325–326 (1966).
Ferguson, D. E., Ludke, J. L., Murphy, G. G., *Trans. Am. Fish. Soc.* **95,** 335–344 (1966).
Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem.* **193,** 265–275 (1951).
Ludke, J. L., Ferguson, D. E., Burke, W. D., *Trans. Am. Fish. Soc.* **97,** 260–263 (1968).
Moffett, G. B., Yarbrough, J. D., *J. Agric. Food Chem.* **20,** 558–560 (1972).
Vinson, S. B., Boyd, C. E., Ferguson, D. E., *Science* **139,** 217–218 (1963).
Wells, M. R., Ph.D. Thesis, Mississippi State University, 42 pp, 1971.
Wells, M. R., Yarbrough, J. D., *J. Agric. Food Chem.* **20,** 14–16 (1972).
Yarbrough, J. D., Wells, M. R., *Bull. Environ. Contam. Toxicol.* **6,** 171–176 (1971).

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A Method for the Estimation of Methylmercuric Compounds in Fish

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The methylmercuric ion ($MeHg^+$) liberated by alkaline hydrolysis from methylmercuric compounds in fish is partitioned into benzene as methylmercuric chloride (MeHgCl). This reacts with cysteine to form a MeHg sulfur complex; it is reextracted into benzene as MeHgCl and assayed using gas–liquid chromatography (GLC) and a ^{63}Ni electron capture detector (ECD). Mean recoveries for MeHgCl added to three species of fish at 0.2- to 0.6-ppm levels ranged from 73.3 to 87.2%, with percent coefficients of variation (% CV) of 8.1 to 14.1. The % CV for assay of naturally occurring MeHg compounds was 7.8. The lower limit of detectability is 0.02 ppm. Comparative assays using other methods are given. Statistical estimates include tests to examine the normal distribution of peak heights, instrumental stability, confidence limits of individual assays, and the number of assays required to provide a result with a known confidence limit.

The presence of mercury in food fish is widespread and levels exceeding 0.1 ppm are quite common in certain species of fish (Simpson et al., 1974; Uthe et al., 1972). Part of the mercury in the fish flesh has been isolated as the methylmercuric ion, $MeHg^+$, and identified as MeHgCl (Westoo, 1966; Johansson et al., 1970). Alkylmercuric compounds, including MeHg salts, cause irreversible neurological disturbances in the human (Hunter, 1969) and are considerably more toxic than other chemical forms of mercury such as the metallic, inorganic, or arylmercuric compounds.

In other methods used for the assay of MeHg compounds in fish the $MeHg^+$ is extracted as a halide into an organic solvent from the sample after the addition of a mineral acid and/or inorganic salts (Newsome, 1971; Rudling, 1971; Sumino, 1968; Uthe et al., 1972; Westoo, 1966, 1967). For any given fish sample, assays for the total mercury content usually indicate higher concentrations than that extractable as $MeHg^+$ by existing methods (Bache et al., 1971; Elkins, 1972; Uthe et al., 1972). The purpose for the development of this method, where the $MeHg^+$ is extracted as MeHgCl from fish samples after alkaline hydrolysis, includes the following: (1) to seek information on the chemical form of the mercury in the fish not extracted as a MeHg halide by other methods; and (2) to ascertain that the MeHg halide in the final step of the extraction procedures used in other methods is in fact in the fish flesh as MeHg compounds and was not synthesized in the course of the chemical reactions used to isolate and purify the MeHg halide for assay. Synthesis of the C–Hg bond of $MeHg^+$ can occur under fairly mild aqueous conditions if Hg^{2+} is present along with a protonable carbanion or an olefin (Makarova and Nesmeyanov, 1967). The rates for cleavage of the C–Hg bond of organomercuric compounds by mineral acids vary from one class of compounds to another (Makarova and Nesmeyanov, 1967). As an example, aromatic derivatives, such as the phenylmercuric ion, are decomposed at acidities exceeding 3 *M* HCl (Polley and Miller, 1952). For this reason, in the method described here, care was taken to maintain acidities of 1 *M* HCl or less in all the chemical reactions required for preparation of a benzene-soluble MeHgCl suitable for GLC assays. As a rule, alkalis do not cleave the C–Hg bond of either alkyl- or arylmercuric ions (Makarova and Nesmeyanov, 1967). Therefore, it can be assumed that alkaline hydrolysis does not alter the chemical form of the mercury as it existed in the fish.

Details of the instrumental conditions for the assay of nanogram (ng) to picogram (pg) concentrations of organomercuric halides by GLC with electron capture detection (ECD) need to be very explicit. For example, Nishi and Horimoto (1968) observed thermal degradation of the alkylmercuric compounds if stainless steel columns were used. Tatton and Wagstaffe (1969) described conditions to prevent "poisoning" of tritiated foil detectors by the mercury compounds, and Uthe et al. (1972) modified the detector design to make it adaptable to disassembly for cleaning. With the extraction method described here, sufficient instrumental stability was achieved so that data accumulated over several months could be combined for the statistical estimates. Detector disassembly for cleaning was not required.

The method consists of an eight-part extraction procedure involving four chemical reactions and a determinative step (see Scheme I).

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