The Relationship of Malathion and Its Metabolities to Fish Poisoning[1]

Gary H. Cook, James C. Moore, and David L. Coppage
U.S. Environmental Protection Agency
Environmental Research Laboratory
Sabine Island, Gulf Breeze, Fla. 32561

INTRODUCTION

Malathion is a widely used organophosphate insecticide with an annual production estimated to be in excess of 1 X 10^7 kg in the United States (ENVIRONMENTAL PROTECTION AGENCY 1972a). It may enter surface water through surface runoff (ENVIRONMENTAL PROTECTION AGENCY 1972b,c,d) or through direct spray for mosquito control (GUERRANT et al. 1970, COPPAGE and DUKE 1971, PINKOVSKI 1972). Concentrations ranging from 0.08 to 500 µg malathion/ℓ in some surface waters have been reported (GUERRANT et al. 1970, DUPUY and SCHULZE 1972, ENVIRONMENTAL PROTECTION AGENCY 1972b) but interpretation of effects of residues on non-target species is difficult because the toxic agent during poisoning is a "persistent" metabolite bound to an enzyme in a form not identifiable by analytical chemical analysis of animal tissue (ALDRIDGE 1971, FUKUTO 1971). Poisoning results from accumulation of a neurotransmitter substance (acetylcholine) because the active site of its hydrolyzing enzyme (acetylcholinesterase) of nerve cells is phosphorylated by dimethyl or methyl phosphate after conversion of malathion to its oxygen analog (O'BRIEN 1960, KOELLE 1963, KARCZMAR 1970, ALDRIDGE 1971, FUKUTO 1971). In animals from the natural environment, enzyme inhibition is measurable in nerve tissue and indicates poisoning even though chemical residues of the enzyme-bound pesticide metabolites are not measurable.

After inhibition of acetylcholinesterase, the enzyme-bound metabolites of malathion may cause inhibition for several weeks after exposure is discontinued (WEISS 1961, CARTER 1971, POST and LEASURE 1974) and the parent compound disappears from water. Although acetylcholinesterase inhibition in animals from the environment indicates poisoning, numerous anticholinesterase pesticides are applied to the environment and the specific agent or agents causing poisoning need to be identified. To infer what parent compound caused poisoning, it may be necessary to find metabolites that are readily measurable during poisoning. In this report, we determine the relation of short-term measurability of malathion and some of its metabolites in fish to poisoning of fish in the laboratory. Degree of poisoning is determined by brain acetylcholinesterase inhibition and deaths in exposed populations.

---

[1]Gulf Breeze Contribution Number 275

Bulletin of Environmental Contamination & Toxicology,
Vol. 16, No. 3 © 1976 by Springer-Verlag New York Inc.

MATERIALS AND METHODS

Exposure of fish in the laboratory: Three laboratory exposures of fish were made. The first exposure was to 75 µg malathion/ℓ of flowing seawater. The second exposure was to 30 µg malathion/ℓ and the third to 20 µg malathion/ℓ. Pinfish, Lagodon rhomboides, (52-101 mm total length) were obtained from wild fish populations and acclimated to laboratory conditions at least 2 weeks before testing. In each test, 8 replicates of 10 fish each were exposed to technical grade malathion (95% pure) in 8-liter acrylic plastic aquaria. The malathion was dissolved in acetone and infused into the water by means of a Lambda® pump or syringe pump. Solvent infusion never exceeded 5 mg/ℓ of water and did not affect acetylcholinesterase activity (COPPAGE et al. 1975). Temperature ranged from 23-29°C and salinity from 11-29 parts per thousand during the tests. The fish exposed to 75 µg malathion/ℓ were sacrificed after 24 h for residue analyses of whole body, brain, liver, flesh (muscle) and gut (whole gut with contents). In exposure to 30 µg malathion/ℓ, 3 replicate samples of fish were removed from the replicate aquaria at 0.5, 1, 4, 8, 24, 48 and 72 h for analyses of brain acetylcholinesterase and residues in gut. In the 20 µg malathion/ℓ exposure, fish samples were removed for analyses at 1, 6, and 24 h exposure and at 24, 48, 120 and 192 h after exposure was discontinued (depuration).

Determination of enzyme activity in laboratory exposures: The acetylcholinesterase of the pinfish brain was characterized and assayed with a recording pH stat (COPPAGE 1971). Each assay sample consisted of pooled brains taken from 3 fish. Normal enzyme activity was determined from 27 samples of unexposed fish taken throughout the testing period from the same populations as fish exposed to malathion. Inhibition was determined by assay of fish that survived a designated time, and percentage inhibition was determined by comparison with mean normal activity. Specific enzyme activities of exposed fish were statistically compared to control activity by Student's t-test ($p < 0.005$).

Residue analyses: Residues for malathion, malaoxon, malathion monoacid (MCA), and malathion di-acid (DCA) in fish were determined (COOK and MOORE). Pooled tissue from selected organs was placed in a 25 x 150 mm culture tube, spiked with 20 µg phenthoate and its acid degradation product PHA, and extracted twice with 10 ml acetonitrile which had been acidified with 2% 2 N HCl. The extraction was carried out on a Willems polytron for 30 sec at 20,000 rpm. After extraction, the culture tubes were centrifuged and the acidified acetonitrile decanted into 100 ml of 2% aqueous sodium sulfate. The pH was adjusted to 8 and

---

® Harvard Apparatus Co., Millis, Mass. Mention of commercial products does not constitute endorsement by the Environmental Protection Agency.

malathion and phenthoate separated by extraction twice with 20 ml petroleum ether. Malaoxon was removed by extracting the aqueous solution twice with 20 ml methylene chloride. MCA, DCA, and PHA were removed by adjusting the pH of the aqueous solution to 2, adding 5% (wt/vol) solid sodium chloride, and extracting twice with 50 ml ethyl acetate. The petroleum ether and methylene chloride extracts were dried by eluting through a plug of anhydrous sodium sulfate and collected in a crystallizing dish. The ethyl acetate extracts were dried by eluting through a plug of acidified sodium sulfate, collected in a crystallizing dish, and methylated with diazomethane as described by SCHINK and GELLERMAN (1960).

The extracts were concentrated by placing the crystallizing dish in a hood on a slide warmer at 50°C and passing a gentle stream of air over them. Recovery of the internal standard, phenthoate, was determined as a measure of integrity of the malathion analysis and recovery of the acid internal standard, PHA, was determined as a measure of the integrity of the MCA and DCA analyses.

All residue analyses were performed without cleanup on a Tracor MT-220 gas chromatograph equipped with a flame photometric detector operating in the phosphorus mode. The column was a 182 cm x 3 mm ID glass column packed with 2% OV-101 on Gas Chrom Q 100/120 mesh. Operating conditions were: column 175°C, inlet 225°C, detector 165°C; nitrogen 65 ml/min, hydrogen 200 ml/min, oxygen 15 ml/min, and air 50 ml/min.

Water samples were analysed as previously described (COPPAGE et al. 1975).

RESULTS AND DISCUSSION

Residues found in whole body and organs after exposure of pinfish to 75 µg malathion/ℓ are shown in Table I.

Residues of malathion or malaoxon were not found in any body tissue in concentrations greater than 0.10 µg/g after 24 hr exposure to 75 µg malathion/ℓ. However, malathion monoacid and di-acid were found in all tissues with the greatest residues in gut and liver. This indicates rapid conversion of malathion to other compounds with the major portion of the malathion being hydrolyzed by carboxyesterase enzyme to monoacid and di-acid (O'BRIEN 1960). Assuming malaoxon is the active enzyme inhibitor, the fraction of malathion converted to malaoxon must be small (below 0.10 µg/g) or malaoxon reacts very rapidly to form other compounds and to phosphorylate proteins such as acetylcholinesterase.

TABLE I

Malathion, malaoxon, malathion monoacid (MCA), and malathion di-acid (DCA) residues in pinfish after 24 h exposure to 75 µg malathion/ℓ

	Residue (µg/g)			
Organ	Malathion	Malaoxon	MCA	DCA
Whole body	ND[a]	ND	4.2	0.30
Brain	ND	ND	1.7	0.22
Liver	ND	ND	6.0	1.25
Gills	ND	ND	2.5	0.36
Flesh	ND	ND	3.9	0.34
Gut	ND	ND	31.4	3.70

[a]ND indicates less than 0.10 µg/g

The degree of brain acetylcholinesterase inhibition resulting from lethal poisoning of pinfish by exposure to 30 µg malathion/ℓ and accumulation of malathion monoacid and di-acid in the gut are shown in Table II.

These data show accumulation of monoacid in the gut that coinsides with phosphorylation of acetylcholinesterase in brain and poisoning in short-term continuous exposure to relatively constant levels of malathion in seawater. The brain acetylcholinesterase was inhibited 79% after 60% mortality and monoacid in gut had accumulated to 19 µg/g which indicates measurement of monoacid in gut is useful for determining short-term poisoning by malathion.

TABLE II

Brain acetylcholinesterase (AChE) inhibition and malathion monoacid (MCA) and di-acid (DCA) residues in gut of pinfish exposed to 30 µg malathion/ℓ for 72 h[a]

Hours exposed	Percent killed	AChE[b] activity	Inhibition (%)	Residues (µg/g) MCA	DCA
Control	-	2.01 ± 0.19(27)[c]	-	0	0
0.5	0	1.91 ± 0.23(3)	5	0.95	0.29
1	0	1.85 ± 0.12(3)	8	1.9	0.19
4	0	1.83 ± 0.13(3)	9	2.1	0.48
8	0	1.49 ± 0.14(3)	26[d]	4.8	0.50
24	0	0.90 ± 0.30(3)	55[d]	11.1	0.70
48	0-10	1.05 ± 0.16(3)	48[d]	10.9	1.00
72	60	0.43 ± 0.10(3)	79[d]	19.0	0.92

[a]The measured concentration of malathion in water was 26.9 ± 0.57 µg/ℓ; no residues of malathion or malaoxon (0.10 µg/g) were detected in gut during the test.

[b]AChE activity = µmoles acetylcholine hydrolyzed/h/mg brain tissue.

[c]Numbers in parentheses indicate replicate samples assayed.

[d]Significantly inhibited at $p < 0.005$ (Student's t-test).

Data from the exposure of pinfish to 20 µg malathion/ℓ for 24 h followed by 192 h depuration are shown in Table III.

Exposure of pinfish to the lower concentration of malathion (20 µg/ℓ) produced correspondingly less brain acetylcholinesterase inhibition and monoacid residues in gut than the exposure to greater concentration. Only 29% brain acetylcholinesterase inhibition was produced in 24 h and a maximum residue of 7.2 µg monoacid/g of gut was found after 24 h - the greatest residue found. Monoacid concentration decreased sharply to 2.2 µg/g of gut 24 h after exposure was discontinued but brain acetylcholinesterase remained inhibited. Residues and enzyme inhibition were lower 48 h

TABLE III

Brain acetylcholinesterase (AChE) inhibition and malathion monoacid (MCA) and di-acid (DCA) residues in gut of pinfish exposed to 20 µg malathion/ℓ for 24 h and through 192 h after exposure[a]

Hours		AChE[b]	Inhibition	Residues (µg/g)	
Exposure	Post-exposure	activity	(%)	MCA	DCA
Control	-	2.01 ± 0.19(27)[c]	-	0	0
1	-	1.94 ± 0.15(3)	3	1.2	0.13
6	-	1.88 ± 0.03(3)	6	3.0	0.17
24	-	1.42 ± 0.12(3)	29[d]	7.2	0.37
-	24	1.35 ± 0.15(3)	33[d]	2.2	0.28
-	48	1.65 ± 0.05(3)	18[d]	0.94	0.29
-	120	1.84 ± 0.15(3)	8	0.14	0.09
-	192	-	-	ND	ND

[a]The measured concentration of malathion was 19.8 ± 0.77 µg/ℓ; no residues of malathion or malaoxon (>0.10 µg/g) were detectable in gut during the test.

[b]AChE activity = µmoles of acetylcholine hydrolyzed/h/mg brain tissue.

[c]Numbers in parentheses indicate replicate samples assayed.

[d]Significantly inhibited at $p < 0.005$ (Student's t-test)

after exposure was discontinued. However, measurable concentrations of monoacid and di-acid remained in gut 120 h after exposure but brain acetylcholinesterase inhibition had returned to normal range. No residues were found in the gut 192 h after exposure was discontinued.

These findings suggest that, although monoacid and di-acid residues in gut are probably not causally related to poisoning, they are the most readily measurable metabolites produced during short-term acute poisoning by malathion. Since malathion and malaoxon are converted to other compounds during poisoning and are not readily measurable, monoacid residues in gut concomitant with brain acetylcholinesterase inhibition should confirm malathion as a cause of poisoning in fish.

It has been shown that some members of the Order Cypriniformes can accumulate malathion in body tissues. KANAZAWA (1975) exposed "Motsugo", Pseudorasbora parva, to 1.2 mg malathion/ℓ freshwater in static tests and reported about 2.5 µg/g in whole-body on day 1 with rapid decrease to 0.001 mg/ℓ water and about 0.1 µg/g in fish by day 7. BENDER (1969) exposed carp, Cyprinus carpio, to 5 mg malathion/ℓ freshwater (a concentration not likely to occur in natural water) for 96 h and reported concentrations of 2.58 µg/g brain, 4.97 µg/g blood, 3.23 µg/g gills, 66.59 µg/g liver and 28.43 µg/g flesh.

Concentrations in flesh of carp exposed to 1.0, 2.5, 5.0, and 7.5 mg/ℓ water for 96 h were progressively greater with greater concentration (low = 1 µg/g, high = 42 µg/g). Carp exposed for 8 days to 5 mg malathion/ℓ attained relatively constant concentrations in flesh at day 4 (3 µg/g in 1 day, 39 µg/g in 4 days and 32 µg/g in 8 days). Carp weighing about 1 kg were fed capsules containing 200 mg malathion and their flesh was analyzed for malathion residues 24 h later. Flesh contained only 0.82 µg/g and 0.87 µg/g in duplicate tests indicating low uptake of malathion through gut, rapid conversion in gut, or rapid conversion elsewhere in the body after absorption through gut. Carp and other fishes of the Order Cypriniformes are many-fold more tolerant to malathion than other fishes (MACEK and McALLISTER 1970) which allows them to survive in concentrations that would kill more sensitive species such as pinfish before malathion reached detectable levels in tissues.

We conclude that periodic environmental monitoring by chemical analyses of sensitive fishes or water for malathion or malaoxon would not show poisoning caused by enzyme-bound metabolites because malathion and malaoxon are rapidly absorbed and metabolically altered in fish. Parent pesticide remaining in water is probably dispersed or altered (GUERRANT et al. 1970). Our data indicate analysis for malathion monoacid in gut and measurement of brain acetylcholinesterase activity in fish from the natural environmental are a practical measure of poisoning caused by malathion in sensitive fish. Measurable concentrations of parent compound need not be present, but acetylcholinesterase inhibition in brain must occur for fish to be poisoned.

ACKNOWLEDGMENT

We thank Mr. Edward Matthews for assistance in assays.

REFERENCES

ALDRIDGE, W. N.: Bull. W. H. O. 44, 25 (1971).

BENDER, M. E.: Prog. Fish Cult. 31, 155 (1969).

CARTER, F. L.: Ph. D. dissertation, Louisiana State Univ. Baton Rouge, LA. (1971).

COOK, G. H. and J. C. MOORE: To be published.

COPPAGE, D. L.: Bull. Environ. Contam. Toxicol. 6, 304 (1971).

COPPAGE, D. L. and T. W. DUKE: Proc. 2nd Gulf Coast Conf. Mosq. Suppr. Wildl. Manage. New Orleans, LA, pp. 24-31 (1971).

COPPAGE, D. L., E. MATTHEWS, G. COOK and J. KNIGHT: Pestic. Biochem. Physiol. 6, (1975).

DUPUY, A. J. and J. A. SCHULZE: Texas Water Development Board, Report 149, Austin, TX. (1972).

FUKUTO, R.: Bull. W. H. O. 44, 31 (1971).

GUERRANT, G. O., L. E. FETZER, Jr., and J. W. MILES: Pestic. Monit. J. 4, 14 (1970).

KANAZAWA, J.: Bull. Environ. Contam. Toxicol. 14, 346 (1975).

KARCZMAR, A. G., Ed.: Anticholinesterase Agents. New York: Pergamon Press 1970.

KOELLE, G. B., Ed.: Cholinesterases and Anticholinesterase Agents. Berlin: Springer-Verlag 1963.

MACEK, K. J. and W. A. McALLISTER: Trans. Amer. Fish. Soc. 99, 20 (1970).

O'BRIEN, R. D.: Toxic Phosphorus Esters. New York: Academic Press 1960.

PINKOVSKI, D. D.: Mosq. News. 32, 332 (1972).

POST, G. and R. A. LEASURE: Bull. Environ. Contam. Toxicol. 12, 312 (1974).

SCHINK, H., and J. L. GELLERMAN: Anal. Chem. 32, 1412 (1960).

ENVIRONMENTAL PROTECTON AGENCY: The Pollution Potential in Pesticide Manufacturing, Pesticide Study Series No. 5. U.S. EPA. Washington, D. C. 1972a.

ENVIRONMENTAL PROTECTION AGENCY: The Use of Pesticides in Suburban Homes and Gardens and their Impact on the Aquatic Environment, Pesticides Study Series No. 2. U. S. EPA. Washington, D. C. 1972b.

ENVIRONMENTAL PROTECTION AGENCY: Pesticide Usage and its Impact on the Aquatic Environment in the Southeast, Pesticide Study Series No. 8. U. S. EPA. Washington, D. C. 1972c.

ENVIRONMENTAL PROTECTION AGENCY: Patterns of Pesticide Usage and Reduction in Use as Related to Social and Economic Factors, Pesticide Study Series No. 10. U. S. EPA. Washington, D. C. 1972d.

WEISS, C. M.: Trans. Amer. Fish. Soc. 90, 143 (1961).