In Vivo and *In Vitro* Epoxidation of Aldrin by Aquatic Food Chain Organisms

by M. A. Q. KHAN, A. KAMAL, R. J. WOLIN, and J. RUNNELS

Department of Biological Sciences
University of Illinois at Chicago Circle
Chicago, Ill. 60680

The contamination of aquatic environments with pesticides and other chemicals and their pick-up, storage and concentration through food chain has resulted, on several occasions, in ecological hazards to fish and birds (RUDD 1964, WOODWELL et al. 1967, PIMENTAL 1971). These hazards have prompted investigation on the significance of organisms at low trophic levels in the concentration and the transfer of biocides to terminal components as well as on the mechanisms of the survival of these organisms in toxic environments. The detoxication of foreign compounds is considered as one of the most important mechanisms of survival in toxic environments (BRODIE AND MAICKEL 1962, BROWN 1964, WILLIAMS 1969). Since a number of xenobiotics including pesticides, drugs, chemosterilants, carcinogens, etc., are metabolized by the microsomal mixed-function oxidase in vertebrates and insects (MASON et al. 1965, GILLETTE 1966, CASIDA 1969), studies of such a system in food chain organisms can contribute to understanding the species differences in sensitivity to biocides. Investigations are being carried out in this laboratory to characterize the mixed-function oxidase in aquatic invertebrates and to relate the activity of this system with their ability to stand toxicants. Preliminary results to demonstrate the activity of this system in some freshwater organisms are presented.

Materials and Methods

The organisms used were either collected in the field (Tinley Park, Chicago) or purchased from Carolina Biological Supply Co., Burlington, North Carolina and Coe-Palm Biological Supplies, Chicago. Some of these organisms are maintained in this laboratory.

In vivo studies involved the exposure of the algae, Chlorella and Diatoms and the protozoa, Dinoflagellates and mixed-protozoa (at least 100 cells of each) in 100 mls of water in 1-pint Mason jars containing 0.1 p.p.m. aldrin. After a 24-hour exposure, the water from each jar was extracted 3-times with 200 mls of hexane by

Bulletin of Environmental Contamination & Toxicology,
Vol. 8, No. 4, 1972

shaking vigorously for 2 hrs in a separatory funnel. The combined hexane extract was filtered over anhydrous sodium sulfate and then evaporated to dryness. The residue was redissolved in 10 mls of hexane and, after adding 0.5 gm of sodium sulfate, analyzed by gas chromatography.

The *in vivo* exposure of invertebrates was carried out similarly using 50 organisms per jar in the case of *Hydra*, *Dugensia*, Leech (*Halobdella stagnalis*), *Asellus*, *Gammarus*, *Daphnia*, *Cyclops* and *Aedes* larvae. They were exposed to 0.1 p.p.m. aldrin for 2 hrs. Crayfish, *Cambarus* (small 1-2 inch long) and Dragon fly nymphs, *Aeschna* were exposed for the same period but to 0.25 p.p.m. aldrin. The freshwater mussel, *Anodonta*, and snail, *Lymnaea*, were exposed to .25 p.p.m. aldrin in 1 liter of water for 4 hrs. After the exposure, the water was filtered off on fine mesh screen. The small animals were homogenized as whole in 20 mls of water. The larger animals, crayfish, snail and mussel, were dissected and their viscera homogenized in 50 mls of water. The homogenate from smaller organisms was extracted first with 100 mls of isopropanol-hexane (1:4) and then twice with 100 mls of hexane. The homogenate from larger animals was extracted first with 400 mls of isopropanol-hexane and then twice with 400 mls of hexane. The crayfish skeleton was refluxed in 200 ml hexane for 2 hrs and this extract combined with the tissue extract. The combined extracts were filtered on anhydrous sodium sulfate and then evaporated to dryness under vacuum. The residue was redissolved in hexane and analyzed by gas chromatography.

For the *in vitro* assay of the microsomal mixed-function oxidase only crayfish, snail and mussel tissues were used. The animals were dissected on ice in 0.1 M phosphate buffer, pH 7.4. The tissues were rinsed twice with cold buffer, soaked on paper towels and weighed in cold. The tissue was homogenized (0.6%, W/V) in phosphate buffer, pH 7.4, unless otherwise mentioned. The homogenate was filtered through 4 layers of cheese cloth and centrifuged as follows to obtain various subcell fractions: post-nuclear fraction, 2000g/10 min - supernatant; post-mitochondrial fraction, post-nuclear supernatant spun at 9,000g/10 min; microsomes, post-mitochondrial supernatant spun at 78,000g/1-1/2 hr-pellet. The supernatant of the last one is the post-microsomal supernatant. The microsomal pellet was resuspended in 0.1 M phosphate buffer at the required pH (KHAN et al. 1972)

The microsomal mixed-function oxidase activity was assayed as reported elsewhere (KHAN and TERRIERE 1968) by incubating the enzyme preparation with 100

nano-moles of aldrin at 26°C for 1 hr. and analyzing the reaction product, dieldrin, by gas chromatography. Enzyme activity was expressed as picomoles of dieldrin formed per hr per milligram tissue (fresh weight). The 0.1 M phosphate buffer, pH 7.4 at 26°C incubation temperature appeared optimum for most enzyme preparations. Any deviation from these optima drastically reduced the enzyme activity (KHAN et al. 1972).

To study the effect of pH on in vitro oxidase activity, the microsomal pellet was resuspended in the respective buffer using 0.1 M phosphate buffer up to pH 8.0 and 0.1 M tris-HCl above this pH.

The gas chromatography of the extracts of organisms, tissues and in vitro reaction products was carried out using a 4 ft x 1/8" column of 2.5% SE-30 coated on chromosorb-W (acid-washed and HMDS treated). The gas chromatograph used was Packard Model 7300 with a Nickel^{-63} electron capture detector (Packard Instruments, Chicago) employing the conditions reported earlier (KHAN et al. 1970).

Results

The planktonic part of the freshwater ecosystem is capable of absorbing insecticides as judged by the amount of dieldrin present in these organisms (Table 1). The amount of aldrin extracted from both organisms and the water is not shown here. However, since no dieldrin was detected in control jars, without any living organisms, the dieldrin recovered was assumed to be produced by biological epoxidation. The amount of dieldrin formed is low in the algae and high in the protozoa. These organisms thus appear to be endowed with an enzyme system which can epoxidize aldrin. The pickup of insecticides by these planktons is apparently the first step in the introduction of a pesticide in the aquatic food chain (RUDD 1964, WURSTER et al. 1967).

The amount of aldrin absorbed and its subsequent epoxidation by aquatic invertebrates is shown in Table 2. These invertebrates can absorb aldrin directly from the medium. The amount of insecticide, aldrin and its epoxide dieldrin, present in the bodies of four smaller invertebrates increases in the following order (on per individual basis): Gammarus < Dugensia < Cyclops < Asellus < Daphnia with a range of 21.5-28.8 nanogram per animal. However, if the insecticide concentration is expressed on wet wt basis then this relative rate of absorption changes in the following order: Asellus < Dugensia < Gammarus < Daphnia < Cyclops with about 10-fold difference between Asellus and Cyclops. Although the mosquito larvae are of the same size and weight as a leech or flatworm or Gammarus,

TABLE I

Epoxidation of Aldrin by Aquatic Microorganisms

Culture	Dieldrin recovered: nanogram
Control	NIL
Chlorella	65
Diatoms	82
Dinoflagellates	120
Mixed protozoa	200

they absorb about 5-times more insecticide than the other smaller invertebrates. The differences among smaller organisms may be due to differences in size, activity, cuticular structure and methods of feeding as well as their tolerance to aldrin and dieldrin.

The highest amount of insecticide is present in the mussel and this may apparently be related with its rate of filtration (JORGENSEN 1966). All the other animals are intermediate between these two extremes so far as the insecticide concentration in their bodies is concerned.

All these organisms are capable of oxidizing aldrin to dieldrin (Table 2). The rates of epoxidation show considerable variations among species. These organisms can be grouped in the increasing order of their ability to oxidize the absorbed aldrin as follows: (1) Hydra, Dugensia, Leech and Asellus; (2) Gammarus, Daphnia, Cyclops and Cambarus; (3) Anodonta, Lymnaea and Aeschna; and (4) Aedes. The dragonfly nymphs and mosquito larvae appear to be the most active organisms to epoxidize aldrin.

Since in vivo epoxidation of aldrin is catalyzed by the microsomal mixed-function oxidase in insects (CASIDA 1969, KHAN et al. 1969), crayfish (KHAN et al. 1972) and vertebrates (CASIDA 1969), the distribution of this system was carried out in various organs of the crayfish, the snail and the clam. Mixed-function oxidase activity was generally present in the gut, liver, kidneys and nervous tissue (Table 3). In snail and clam, the liver possessed the highest oxidase activity while in the case of the crayfish, the highest activity was present in the green gland (Kidney).

TABLE 2

Pick-up of Aldrin and its Subsequent Epoxidation by Freshwater Invertebrates

ıimal	Insecticide absorbed: ng/animal			Epoxidation of aldrin
	aldrin	dieldrin	Total	% dieldrin/animal
. Hydra littoralis (Coelentrate)	45.02	1.20	46.25	2.59
. Dugensia (plannarian)	23.01	0.24	23.25	1.03
. Leech (Annelid)	34.14	0.54	35.65	1.51
. Asellus (Crustacea, isopod)	25.02	0.59	25.61	2.30
. Gammarus (Crustacea, amphipod)	19.98	1.52	21.50	7.07
. Daphnia pulex (Crustacea, Cladocera)	27.16	1.64	28.80	5.69
. Cyclops (Crustacea, copepoda)	26.23	1.13	27.36	4.13
. Cambarus (Crustacea, Decapoda)	86.43	8.01	94.44	8.48
. Aeschna (Insecta, Odonata)	48.59	16.11	64.70	24.90
. Aedes aegypti (Insecta, Diptera)	95.06	69.82	164.88	42.35
. Anodonta (Mollusk, pelecypoda)	553.89	130.81	684.70	19.10
. Lymnaea palustis (Mollusk, Gastropoda)	69.15	13.95	83.10	16.79

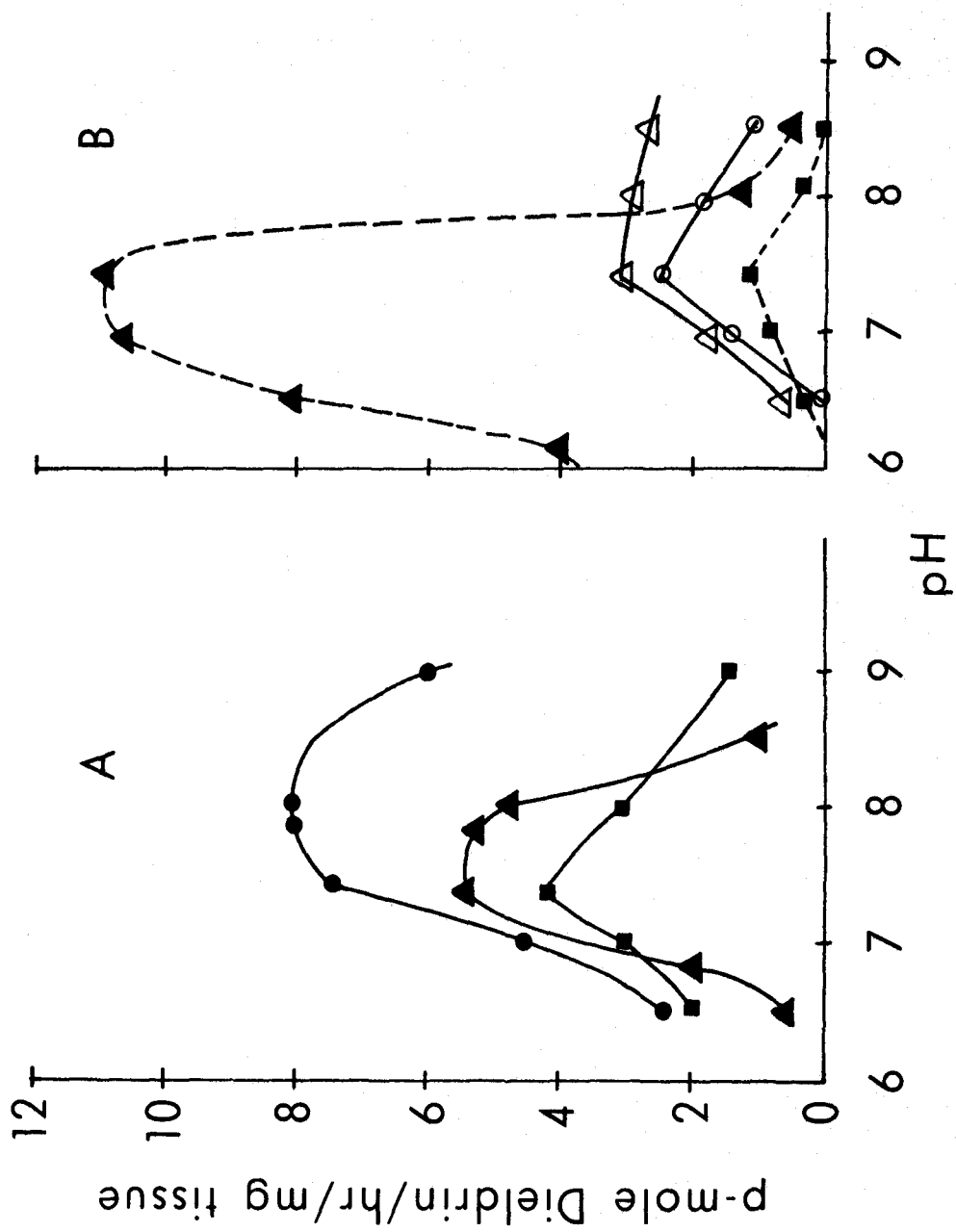

Fig. 1. Effect of pH on microsomal aldrin epoxidase from various organs. A, crayfish: ●-● green gland, ▲-▲ liver, ■-■ gut; B. clam: ▲-▲ liver, o-o gut; snail ▲---▲ liver, ■--■ gut.

TABLE 3

Aldrin Epoxidation by the Post-Nuclear Supernatant from Various Organs of the Crayfish, the Snail and the Clam.

Organ/System	Dieldrin produced: p-mole/hr/mg tissue		
	Crayfish	Snail	Clam
alimentary canal	4.04	1.28	2.61
gills	NIL	NIL	NIL
muscle*	NIL	NIL	NIL
mantle	-	NIL	NIL
nervous system	0.62	not detected	0.36
heart and reproductive system	NIL	NIL	NIL
kidneys	8.86	not detected	not detected
liver (hepatopancreas)	5.48	10.13	3.07

*abdominal muscle in crayfish and adductor muscle in clam.

An assay of various subcellular fractions from the livers of these animals showed that 80% of the total oxidase activity resided in the microsomal fraction (Table 4).

The effect of the pH on the oxidase activity in microsomes from the gut and liver of these animals and also from the green gland of the crayfish is presented in Fig. 1. The gut and liver microsomes from these animals show a pH optimum around 7.4. Only the green gland of the crayfish showed a broad peak with maximum around 7.8. The shapes of the pH-activity curves show differences among these species. In the crayfish there is a sharp decline on both sides of the optimum pH in the gut microsomes. The peak is considerably broadened in the case of the liver microsomes. The pH-activity curve for the green gland is even broader and covers an optumum pH range of 7.4 - 8.5. In the case of the snail, both liver and gut microsomes show sharp curves. In the clam, the gut microsomes show a somewhat sharp peak but the liver microsomes seem to be active on the

TABLE 4

Aldrin Epoxidation by Subcellular Fractions from Livers of the Crayfish, the Snail and the Clam.

Subcell fraction	Dieldrin produced: p-mole/hr/mg tissue		
	Crayfish*	Snail	Clam
whole homogenate	4.88	10.04	3.02
nuclear supernatant	5.07	10.16	3.10
mitochondrial supernatant	4.78	9.84	2.96
microsomes	3.87	9.75	2.92
microsomal supernatant	0.84	0.46	0.28

*hepatopancreas

alkaline side of the pH. Thus there seems to be more similarity in the case of gut and liver microsomes in these animals, with the exception of the clam. Probably these conditions were not optimum for clam liver oxidase and therefore the observed activity for clam liver may not be maximum. Investigations are in progress to characterize the mixed-function oxidase in each of these animals.

Discussion

These results provide a laboratory examination of the absorption of aldrin from aqueous suspensions by food chain organisms. There seem to be obvious differences in the rate of absorption of aldrin which may be due to various physiological mechanisms in these animal species. The rate of absorption seems to be very high in freshwater clam and may be related to its higher filtration rate (JORGENSEN 1966). Significance of similar observations in relation to ecological effects of insecticides has been reported (RUDD 1964, WESTLAKE and GUNTHER 1966).

The *in vivo* epoxidation of aldrin indicates that the microsomal mixed-function oxidase type enzymes may be present in algae, protozoa, coelentrates, worms, arthropods and molluscs. A similar *in vivo* epoxidation of aldrin by an ostracod (KAWATSKI and SCHMULBACK 1971) and of oxidation of several drugs

by a crayfish (BRODIE and MAICKEL 1962) have been reported. Brodie and Maickel (1962) have mentioned the in vitro presence of the microsomal mixed-function oxidase in the crayfish hepatopancreas but have not included any data in their paper. Recently, Khan et al. (1972) have characterized this system in the crayfish green gland. Other than this no information is available on the in vitro demonstration of the microsomal mixed-function oxidase type enzymes in aquatic invertebrates.

As compared with insecticide-resistant strains of their terrestrial relatives, insects (KHAN and TERRIERE 1968, CASIDA 1969, KHAN 1970), these fresh-water invertebrates show low levels of microsomal oxidase activity. Either the experimental conditions are not yet optimum or, like the susceptible insects with low oxidase levels (KHAN and TERRIERE 1968, CASIDA 1969, KHAN 1970), these aquatic invertebrates have not yet been selected by chemicals in their environment (BROWN 1964).

The in vitro oxidase activity levels differ in tissues of the crayfish, the snail and the clam. The highest activity is present in the livers of the two molluscs and in the green gland of the crayfish. In fish and other vertebrates, liver microsomes contain the highest oxidase activity (BRODIE and MAICKEL 1962, GILLETTE 1969, CASIDA 1969). These molluscs may thus be showing a phylogenetic trend in the evolution of these enzymes similar to aquatic vertebrates. In some orthopteroid insects, malpighian tubules (excretory organs) have been reported to possess higher oxidase levels than other tissues (BENKE and WILKINSON 1971). The high levels of oxidase in the green gland of the crayfish therefore are not unusual for an arthropod.

The dependance of the oxidase activity on a narrow pH range, buffer molarity and temperature may be related with the environmental stress on homeo-stasis. The work is in progress to understand these phenomena and to characterize further this system in aquatic invertebrates to realize its adaptive value in their survival in toxic environment.

Acknowledgements

We express gratitude to Professor E. B. Hadley, Head of the Department of Biological Sciences, for the use of facilities in his laboratory and for support from his grant NSF-GY-7234. This research was supported mainly by grants from National Institutes of Environmental Health Sciences (ES-FD-00808-01) and from the University of Illinois at Chicago Circle Research Board.

References

1. BENKE, G. M. and C. F. WILKINSON: J. Econ. Entomol. 64, 1032 (1971).

2. BRODIE, B. B. and R. P. MAICKEL: Proceed. 1st Internat. Pharmacol. Meeting, Vol. 6, Pergammon Press, 209 pp. (1962).

3. BROWN, A. W. A.: in "Handbook of Physiology" Section 4, Waverly Publishers, Baltimore, U.S.A. (1964).

4. CASIDA, J. E.: in "Microsomes and Drug Oxidations" (J. R. Gillette, A. H. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts and G. J. Mannering, editors), Acad. Press, 547 pp. (1968).

5. JORGENSEN, C. B.: Biology of Suspension Feeding, Pergammon Press, 210 pp. (1966).

6. WILLIAMS, R. T.: in "Biochemical Aspects of Anti-metabolites and Drug Hydroxylation" (D. Schugar, editor), Vol. 16, Acad. Press, 295 pp. (1969).

7. KAWATSKI, J. A. and J. C. SCHMULBACK: J. Econ. Entomol. 64, 1082 (1971).

8. KHAN, M. A. Q. and L. C. TERRIERE: J. Econ. Entomol. 61, 732 (1968).

9. KHAN, M. A. Q., J. D. ROSEN and D. J. SUTHERLAND: Science 164, 318 (1969).

10. KHAN, M. A. Q.: Biochem. Pharmacol. 19, 903 (1970).

11. KHAN, M. A. Q., J. L. CHANG, D. J. SUTHERLAND, J. D. ROSEN and A. KAMAL, J. Econ. Entomol. 63, 1807 (1970).

12. KHAN, M. A. Q., W. COELLO, A. A. KHAN and H. PINTO: Life Science 11(8), 405 (1972).

13. MASON, H. S., J. C. NORTH and M. VANNESTE: Feder. Proceed. 24, 1172 (1965).