Bull. Environ. Contam. Toxicol. (1994) 52:893–897

Environmental Contamination and Toxicology

Effect of Cadmium, Mercury, and Zinc on the Hepatic Microsomal Enzymes of *Channa punctatus*

R. Dalal,* S. Bhattacharya

Environmental Toxicology Laboratory, Department of Zoology, School of Life Sciences, Visva-Bharati, Santiniketan 731235, India

Received: 15 July 1991/Accepted: 30 September 1993

The increased use of heavy metals like cadmium and mercury in industry and agriculture, and their subsequent intrusion in indeterminate amounts into the environment has caused ecological and biological changes. In vivid contrast, zinc, one of the essential elements, and used in the cosmetic industry, is known to play a pivotal roles in various cellular processes (Chvapil et al. 1972). The seriousness and longevity of these metals in the environment are compounded by the fact that they are non-degradable with significant oxidizing capacity and substantial affinity for electronegative nucleophilic species in proteins and enzymes. Exposure of aquatic animals, especially fish, to these toxic metals for a prolonged period produces an intrinsic toxicity in relation to susceptible organs and / or tissues, although no serious morphological or anatomical changes in the animal or even their feeding behavior may occur.

The p-hydroxylation of aniline by aniline hydroxylase (AH) and the N-demethylation of amines to generate formaldehyde (HCHO) by aminopyrine demethylase (APD) are the two oxygen-dependent reactions of microsomal mixed-function oxidase (MFOs) which control the pharmacological and toxicological activities of xenobiotics in mammalian and other species (Kato 1979). While both these classical enzymes in fish are reported to demonstrate relatively low specific activity (Buhler and Rasmusson 1968), they are used as criteria for delineating polluted areas (Payne et al. 1987). Unlike mammalian species, however, intoxication and interference of MFO enzymes by metal toxicants, especially during prolonged exposure, has not been investigated.

The present report describes the results of studies from the concurrent exposure for 28 d to cadmium ($CdCl_2$), mercury ($HgCl_2$) or zinc ($ZnCl_2$) individually, on the AH and APD activities and microsomal protein content in liver of freshwater teleost Channa punctatus (Bloch).

MATERIALS AND METHODS

Male Channa punctatus (30 ± 5 g) were collected during April-May and acclimated to laboratory conditions in all- glass aquaria of 48 L capacity with well water (pH 7.6 ± 0.1, dissolved O_2 8.0 ± 0.2 ppm, alkalinity as $CaCO_3$ 150 ppm, temperature 25.0 ± 3.0 ^{o}C). Fish were fed commercial fish food (Shalimar Fish Food, India), and water in each aquarium was renewed daily to remove metabolic detritus.

Present address: Department of Biochemistry and Molecular Biology, Medical Science Building, University of Medicine and Dentistry of New Jersey, Newark, NJ 07103
Correspondence to: R. Dalal

Preliminary range-finding tests were done to establish the mortality range of 0-100%, suitable for calculating LC-50 using Probit- analysis. Feeding was stopped during the test period. Mortality was recorded after 96 hr.

For chronic study, groups of 36 fish were exposed for 28 d to sublethal concentrations of either $CdCl_2$ (30.0 ppm), $HgCl_2$ (16.7 ppb) or $ZnCl_2$ (6.0 ppm). A concurrent control was maintained at identical conditions without toxicants. Water in aquaria was renewed daily to maintain the initial concentration of the respective toxicants.

Six fish from each treatment group and concurrent control were sacrificed on 1, 2, 7, 14, 21, and 28 d. Livers were excised and blanched in ice cold 150 mM KCl. Subsequent procedures were carried out at 4 °C unless otherwise mentioned. Liver homogenate (20 %) was prepared in 10 mM tris-HCl, pH 7.4, containing 250 mM sucrose, using Potter-Elvejham homogenizer (operated at 600 x g with 10 up and down strokes to prevent microsomal membrane damage). Microsomes were isolated by $CaCl_2$ precipitation (8.0 mM), followed by differential centrifugation twice at 25,000 x g for 15 min, and resuspended in 100 mM potassium phosphate buffer, pH 7.4, containing 150 mM KCl, 1 mM K_2EDTA, 1 mM dl-dithiothreitol and 20 % glycerol (w/v), according to the procedure of Schenkman and Cinti (1978). The suspension was held at 20 °C and further processed immediately.

AH activity was measured according to the method of O'Brien and Rahimtula (1978). The assay mixture (1.5 mL) consisted of 3.0 mg microsomal protein in 100 mM tris-HCl, pH 7.4 at room temperature, and 0.2 mL of 100 mM aniline-HCl. Reaction was initiated by adding 0.5 mL of 30 mM cumene hydroperoxide. After 2 min of incubation, 0.3 mL of 70 % TCA was added to terminate the reaction. The mixture was centrifuged at 3,000 x g for 10 min. To 1 mL of the resultant supernatant, 1 mL each of 1 M Na_2CO_3 and 2 % phenol in 0.5 M NaOH were added. The reaction mixture was allowed to stand at room temperature for 30 min and absorbance of p-aminophenol formed measured at 630 nm.

HCHO formation during N-demethylation of amines by microsomal APD was estimated according to the procedure of Werringloer (1978), introduced by Nash (1953). To 2.4 mL of microsomal suspension, 1.5 mL of 12.5 % TCA was added, followed by thorough mixing at room temperature and subsequently centrifuged for 15 min. Volumes of 2 mL of the clear supernatant were transferred to test tubes and 1 mL of Nash reagent containing 6 M ammonium acetate, 60 mM acetylacetone, and 150 mM glacial acetic acid (pH 6.7) was added and heated at 58 °C for 10 min. Upon cooling to room temperature, the samples were analyzed for HCHO generated at 412 nm.

Microsomal protein was determined according to the method of Bradford (1976) using BSA as standard.

Enzyme activities were expressed in terms of umoles of product formed / min / mg microsomal protein, and microsomal protein as mg protein / g liver wt. Data were analyzed by Student's t Test at the 5 % significance level. Each point represents the mean $\pm$ S.D. of triplicates of each assay.

RESULTS AND DISCUSSION

The 96 hr LC_{50} value for Channa punctatus (Bloch) were 405.0 ppm ($CdCl_2$),

1.15 ppm ($HgCl_2$), and 80.0 ppm ($ZnCl_2$). Higher concentrations of the toxicants produced increased hypersensitivity, loss of equilibrium, descaling and inhibition of schooling behavior. Toxicity signs showed edema in the moribund fish exposed to $CdCl_2$, while shrunken viscera and hemorrhagic spots in the $HgCl_2$- and $ZnCl_2$-exposed fish.

Biochemical analyses of the liver microsomal enzyme activities are shown in the Table 1. Among the toxicants, $CdCl_2$ elicited decreased AH activity throughout the exposure period, while inhibition of APD activity upto day 7 and increased microsomal protein content were observed till day 14. One of the most important cytotoxic effects of cadmium relates to its affinity for sulfhydryl moieties of proteins (Rabenstein et al. 1979). The significant enzymicidal effect evidenced during the early stages of exposure can be attributed to the specific accumulation of Cd^{++} in liver and spilling over of Cd^{++} from Cd-sequestering, low molecular weight proteins to other macromolecules, eliciting a toxic response.The steady depletion of AH activity in contrast to reversal of APD inhibition during the subsequent days of exposure indicates that these two MFO enzymes are differentially localized in the liver of fish. This result further suggests that the persistence of toxic response despite the reversal of APD inhibition and normalization of microsomal protein level on day 14 is due to the induction of hepatic metallothionein in the fish by Cd^{++} (Dalal 1990), which probably could not completely detoxify intracellular Cd^{++} by the 28 th day of exposure.

Table 1. Microsomal aniline hydroxylase (AH) and aminopyrine demethylase (APD) activities[a], and protein content[b] in liver of Channa punctatus after chronic exposure to sublethal doses of metals.

Metal		1	2	7	14	21	28
Control	AH	3.45±08	3.6±0.1	3.5±0.7	3.82±0.7	3.2±1.0	3.9±0.2
	APD	5.23±0.3	5.31±0.2	5.21±0.7	5.26±0.3	5.45±0.7	5.9±0.7
	Protein	6.61±1.0	6.59±0.9	6.7±0.8	6.9±0.3	6.73±0.6	6.5±0.8
$CdCl_2$	AH	2.1±0.2*	2.21±0.3*	3.22±0.1*	3.11±0.1*	2.66±0.4*	2.1±0.3*
	APD	4.1±0.5*	3.96±0.7*	4.21±0.3	5.07±0.7	5.39±0.2	5.5±0.3
	Protein	13.41±0.2*	13.91±0.9*	10.2±0.5*	7.64±0.3*	6.67±0.3	6.4±0.9
$HgCl_2$	AH	3.36±0.9	3.47±0.5	3.7±1.6	3.91±0.3	7.45±0.3*	6.6±0.7*
	APD	5.1±0.3	5.19±0.7	5.37±0.9	3.16±0.5*	3.0±0.1*	3.8±0.7*
	Protein	6.56±0.2	6.73±0.3	6.38±0.9	6.85±0.2	10.87±0.8*	6.99±0.5*
$ZnCl_2$	AH	3.26±0.3	3.49±0.5	3.5±0.9	4.17±0.5	5.38±0.3*	3.39±0.8
	APD	5.42±0.9	4.52±0.3*	4.97±0.3	5.13±1.0	4.99±0.7	5.61±0.3
	Protein	6.99±0.7	7.2±0.5	6.46±0.5	2.91±0.6*	6.92±0.5	6.37±0.9

Values depict means ± SD of triplicates of assay; [a] represents enzyme activities in umoles/min/mg microsomal protein; [b] mg protein/g liver wt; and * values significantly different from means of control ($P<0.05$).

On the contrary, $HgCl_2$ elicited no significant effects on the hepatic microsomal enzyme activities and protein profile in the fish up to 7 d (Table 1), indicating organspecificity for Hg^{++} accumulation at low doses in organ /s other than the liver. In mammals, high levels of mercury are reported to bind avidly to heme moieties of cytochrome P-450 (Maines and Kappas 1977) and decrease APD and heme oxygenase activities (Eaton et al. 1980) and AH activity (Congiu and Pani 1978). The significant modulations of both AH and APD activities on days 21 and 28 in the present study indicate the binding of Hg^{++}, non-specifically, to nucleophilic species of the MFO enzyme system. However, the increased microsomal AH activity in contrast to depleted APD activity in the liver is hypothesized to be due to binding of Hg^{++} to components that enhance AH activity in the fish. The result further validates the hypothesis that both these enzymes are differentially localized in the liver of Channa punctatus. The increased microsomal protein content on day 21 indicates a toxic response in the fish which is probably due to the inability of metallothionein, a Hg^{++}-sequestering protein in fish (Bouquegneau 1979), to protect the system from $HgCl_2$, and may be correlated with the depleted hepatic metallothionein content in the fish on day 21 (Dalal 1990).

$ZnCl_2$ caused no significant effects on microsomal AH and APD activities almost throughout the exposure period except it produced decreased APD activity on day 2, an elevated AH activity on day 21 and a significant depletion in microsomal protein on day 14. The enzymicidal activity of zinc has been extensively cited (Rabenstein et al. 1979; Eaton et al. 1980). The subsequent reversal of binding of Zn^{++} to MFO enzyme systems is probably due to the induction of metallothionein synthesis byy the increasing levels of intracellular Zn^{++}, a potent inducer, which effectively sequesters the excess Zn^{++} intracellularly in liver, thereby reducing the bioavailability of the metal ion to other cellular macromolecules during prolonged exposure of fish to $ZnCl_2$.

The results of the present study indicate that sublethal concentrations of $CdCl_2$, $HgCl_2$ and $ZnCl_2$ have direct effects on the MFO enzymes in Channa punctatus. However, further study on the interference of heavy metal at asymptomatic doses, their bioaccumulation and detoxification in fish is warranted.These parameters may serve as bioindices to pollution-prone areas.

Acknowledgement. The authors are thankful to the University Grants Commission, New Delhi, India, for financial assistance in this study.

REFERENCES

Bouquegneau JM (1979) Evidence for the protective effect of metallothionein against inorganic mercury injuries in fish.Bull Environ Contam Toxicol 23 : 218-219

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72 : 248-54

Buhler DR, Rasmusson ME (1968) The oxidation of drugs by fishes. Comp Biochem Physiol 25 : 223-239

Chvapil M, Elias SL, Ryan JN (1972) Pathophysiology of zinc. In : J.R.Smythies and R.J.Bradley (eds.) International Review of Neurobiology, Academic

Press, New York, pp 105
Congiu L, Pani P (1978) Heavy metals induced in liver injury. In : T.F. Slater (ed.) Biochemical Mechanisms of Liver Injury. Academic Press, New York, pp 591
Dalal R (1990) Involvement of hepatic metallothionein and aniline hydroxylase in the detoxication of industrial pollutants in an air breathing freshwater teleost, Channa punctatus (Bloch) : Evaluation of Toxic Response Syndrome, Ph.D Thesis, Visva-Bharati, Santiniketan, India.
Eaton DL, Stacey NH, Wong K-L, Klaassen CD (1980) Dose response effects of various metal ions on rat liver metallothionein, glutathione, heme oxygenase and cytochrome P-450. Toxicol Appl Pharmacol 55 : 393-402
Kato R (1979) Characteristics and differences in the hepatic mixed function oxidases of different species. Pharmacol Therapeut 6 : 48-98
Maines MD, Kappas AJ (1977) Metals as regulators of heme metabolism. Science 198 : 1215-1221
Nash T (1953) The calorimetric estimation of formaldehyde by means of the Hantzsch reaction. Biochem J 55 : 416-421
O'Brien PJ, Rahimtula AD (1978) A peroxide assay for cytochrome P-450. In : S. Fleisher and L. Packer (eds.) Methods in Enzymology, Vol LII C, Academic Press, New York,pp 407
Payne JF, Fancey LL, Rahimtula AD Porter EL (1987) Review and perspective on the use of mixed function oxygenase enzymes in biological monitoring. Comp Biochem Physiol 86C: 233-245
Rabenstein DL, Guevremont R, Evans CA (1979) Glutathione and its metal complexes. In : H.Sigel (ed.) Metal ions in biological systems,Vol 9, Marcel Dekker Inc, New York and Basel, pp 103
Schenkman JB, Cinti DL (1978) Preparation of microsomes with calcium. In : S. Fleisher and L. Packer (eds.)Methods in Enzymology, Vol LII C, Academic Press, New York, pp 83
Werringloer J (1978) Assay of formaldehyde generated during microsomal oxidation reactions. In : S. Fleisher and L. Packer (eds.) Methods in Enzymology, Vol LII C,Academic Press, New York, pp 297