Biochemical Responses to Heavy Metals in *Oreochromis mossambicus* (Peters) with special reference to Metal Detoxifying Mechanisms.

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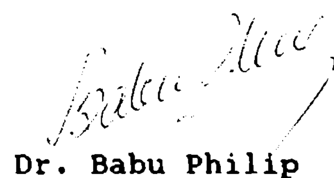
CERTIFICATE

This is to certify that the thesis entitled '**Biochemical Responses to Heavy Metals in** ***Oreochromis mossambicus*** **with special reference to Metal Detoxifying Mechanisms**' submitted herewith by Miss. Rema. L. P. is an authentic record of research work carried out by her, in the Division of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Kochi-16, under my supervision and guidance, in partial fulfillment of the requirements for the award of Ph.D.degree of Cochin University of Science and Technology and that no part thereof has been presented before for any other degree in any university.



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DECLARATION

I hereby declare that the thesis entitled `**Biochemical Responses to Heavy Metals in** *Oreochromis mossambicus* **(Peters) with special reference to Metal Detoxifying Mechanisms**', is an authentic record of research work carried out by me under the supervision and guidance of Dr. Babu Philip, in partial fulfillment of the requirements for the award of the Ph. D. degree in the Faculty of Marine Sciences, Cochin University of Science and Technology, and that no part of it has previously formed the basis for the award of any degree, diploma or associateship in any university.

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C H A P T E R 1

1. GENERAL INTRODUCTION

1.1 GENERAL SCIENTIFIC BACKGROUND

Metal ions form an integral part of living system. Lack of essential metals causes serious defects and even death. This points out that, in nature, organisms have evolved sophisticated ways of absorbing, transporting, storing and recycling them within cells. However, environmental levels of many of these essential and non-essential metals are constantly being raised by an increased influx from different sources.

Investigations on pollution due to metals are of importance, since metals are immutable and once they accumulate in biological systems, they disturb the biochemical processes leading to perturbations of the metabolic pathways and the biochemistry of different subcellular structures.

Mercury is the most extensively studied element in the field of heavy metal toxicology. The sources of this hazardous metal to the aquatic environment include effluents from chlor-alkali plants and paper and pulp industries. Anthropogenic sources of mercury arise mostly from mineral processing and fossil fuel combustion. It shows little sign of being regulated and a linear relationship between concentrations in sea water and those in flesh of teleosts has been observed in the field (Gardner, 1978). Mercury shows a great affinity for sulfhydryl

groups and appears to exert toxic effects largely by combining with such groups on protein, thus disrupting enzyme-mediated processes and / or damaging cellular structure leading to catastrophic consequences (Goldwater, 1971).

Zinc is released in effluents from newsprint mills, where, zinc hydrosulphite is used as a brightening agent for the ground wood pulp. Like mercury, zinc also gets accumulated in the tissues of animals. But, unlike mercury, it is essential for the organism in trace amounts. Once this level is exceeded, symptoms of toxicity appear. Thus, although mercury and zinc are classified as heavy metals and have similar electronic characteristics, due to differences in atomic number, electronegativity etc., their affinity for biological ligands varies greatly (Angelici, 1973).

The physico-chemical nature of the ambient environment is of crucial importance, while considering the homeostatic mechanisms of an animal which make it a harmoniously orchestrated system. Pollution of the aquatic environment with metals as well as other xenobiotics is highly detrimental when compared to other environments. In addition to the entry of pollutants through food and breathing air as in the case of terrestrial animals, the aquatic fauna gets a pollutant load from the dissolved and particulate materials in the medium also.

Compared to water or sediment analyses, biological

monitoring is more ideal and advantageous in the study of aquatic pollution (Philips, 1980). Even though quantitative structure - activity relationship (QSAR) techniques may give good approximation, toxicity cannot be estimated without testing biota. Metals are concentrated within the organisms to levels that make them easier to detect. Moreover, biological responses may be elicited by these chemicals at levels well below their analytical detection limits. Furthermore, the quantity accumulated within the organism is the one which is biologically available to the organism. Since the body loads of metals are accumulated over a prolonged period of time, they probably represent more precisely than any other system the sum of environmental insults that have occurred in the recent history of the particular ecosystem. Finally, use of food organisms as monitors, provides a direct measure of human exposure.

Heavy metals produce toxic effects and endanger the life of aquatic fauna. Among these, fishes form the most sensitive group and are obviously the most economically important ones affected. The continuous exposure of both the external and internal organs and almost complete lack of protection of the soft body through which osmotic influx can occur make them especially sensitive to the contaminant. The mucus coating of the body surface with which the metal can form complexes aggravates the situation further. Experiments to establish a relationship between a pollution gradient and the degree of

response have mostly concentrated on fresh water fisheries. This may be due to the increased bioavailability of heavy metals in fresh water and the more or less restricted niche of the fresh water system where an escape to a clean environment is impracticable.

Recently, many authors have outlined the importance of environmental impact assessment programmes including methods which measure the biological effects of pollutants on the health condition of organisms (Moore, 1985). Many studies have been carried out to develop stress indices at different levels of biological organization. These biological responses can be considered as biomarkers of toxicity to central metabolic pathways. Ideally, measurements of biological responses should be based on an understanding of the intracellular mechanisms by which a fish responds to exposure to heavy metals. It would be especially useful if it could be shown that the toxic effects of a metal are related to the perturbations of a particular key biochemical process. Measurement of the extent to which such an alteration has occurred in a given situation would provide a good indication of the significance of the toxic effects (Roch *et al.*, 1982).

The xenobiotic-induced sublethal cellular pathology reflects perturbations of function and structure at the molecular level. In many cases, the earliest detectable changes

or *primary events* are associated with a particular type of subcellular organelle such as the lysosomes, endoplasmic reticulum and mitochondria. Thus, investigations at the subcellular level can reveal alterations at an earlier stage of response before integrated cellular damage shifts to the level of organ or whole organism. The structure and / or function of organelles and cells can be disturbed by toxic contaminants in many different ways. Slater (1978) has classified these into four main categories, viz, depletion or accumulation of metabolites or co-enzymes, inhibition or stimulation of enzymes and other proteins, activation of a xenobiotic to a more toxic molecular species and disturbances of biological membranes. The cellular responses to pollutant-induced cell injury thus provide rapid and highly sensitive indicators of environmental impact. It should also be possible to observe alterations in the structural and functional organization in individual target cells or groups of cells at an early stage of reaction to cell injury before an integrated cellular response would manifest at the level of organism and long before appearance of perceptible changes at the population level (Moore, 1980).

1.2 EXPERIMENTAL ANIMAL

The natural habitat of the test fish, *Oreochromis mossambicus*, locally called tilapia (family - Cichilidae, order - Perciformes, subclass - Actinopterigii and class - Teleostomi)

is the rivers on the east coast of Africa. In India, the first consignment of the fish was brought by the CMFRI, Mandapam on August 7, 1952 from Bangkok (Panikkar and Thampi, 1954).

Tilapia can tolerate a wide range of salinity from fresh water to waters of 30 to 48 ppt salinity (Panikkar and Thampi, 1954). However, sudden changes are fatal to the fish. It has got an omnivorous feeding habit, with different growth stages exhibiting varying food habits. Artificial feeds like rice bran, oil cakes, flour, chopped leaves and kitchen refuses are readily taken by the fish.

A comparative study of tilapia and *Etroplus suratensis* has revealed that both have more or less similar nutritive value and belong to low oil, high protein category (John and Samuel, 1993). Tilapias are gaining increasing importance as food fish in India. The industry is growing rapidly as tilapia has become more accepted by consumers. Consumer demand for the fish is increasing dramatically.

Its euryhaline nature, high fecundity and growth rate etc. account for the suitability of tilapia as a culture fish. The above qualities along with its local availability throughout the year, low cost, reasonable size, its restricted niche, omnivorous feeding habit etc. make them ideal candidates for laboratory studies.

1.3 SCOPE OF THE STUDY

The present work is focused on the organelle and biochemical responses to heavy metal exposure in the fish *Oreochromis mossambicus* giving particular importance to the metal detoxifying machinery of the organism. The thesis is an outcome of the effort aimed at developing practicable monitoring techniques to deliver guidelines for biological effect monitoring and the need for specific biochemical methods to detect biological effects of heavy metals that can be interpreted in terms of the health status of the individual organism and eventually alterations in vital processes as growth and reproduction. The efficiency of the metal detoxifying metallothioneins which is an attractive tool for biological monitoring, their role as scavengers of trace metal ions and thus in relieving the biological machinery from their toxicity effects are important themes of this study. Efforts have also been made to test the reliability of the *spill over* hypothesis of the action of metallothioneins (Winge *et al.*,1973) and their use as a biological barometer of heavy metal stress.

The unit membrane of lysosomes is stable with its contained hydrolytic enzymes. This fundamental biochemical property is a direct consequence of the impermeability of the lysosomal membrane to many substrates as well as the internal membrane bound nature of many of its enzymes (Bayne *et al.*,

1985). Though the structural specialities of this unit membrane which cause this impermeability are still under a cloak of secrecy, works have been carried out to compare the relative stability of the lysosomal membranes of a mollusc, a fish and a mammal.

Lysosomes are capable of sequestering heavy metals and are the most important sites of metal compartmentation in the cell (Sternlieb and Goldfischer, 1976). But once the storage capacity is exceeded, they destabilize the integrity of the lysosomal membrane with subsequent activation and release of the degradative lysosomal enzymes. These enzymes initiate catabolism of cellular components and in severe cases cause death of cells. The lysosomal stability test - the lysosomal enzyme release assay (LERA) which has been effectively applied to mammals by Bitensky *et al.* (1973) is here extended to the fish and it has been found to be an effective and sensitive index of heavy metal stress. The applicability of LERA technique as an early warning system for detection of environmental disturbances is checked and its use as a tool for biomonitoring studies is delineated.

Heavy metals affect the activity of key metabolic enzymes of the organism either by controlling the hormonal mechanism or by directly affecting the structure or biosynthesis of the enzymes. The utility of the enzyme monitoring method based on enzyme induction, activation or inhibition, in fish as an index

of stress has been studied. The efficiency of the biochemical actions of the animal exposed to heavy metals is a function of the amount of the metal accumulated within the specific tissues. This in turn depends on the dose and duration of exposure to the heavy metals, and also on the degree of regulation of the metabolism of the metal by the animal. Hence, the quantity of the metal getting accumulated within the fish, as the period of exposure increases is worked out since it is expected to play an important role in any toxicological investigation.

C H A P T E R 2

2. METALLOTHIONEINS OR METALLOTHIONEIN LIKE PROTEINS

2.1 INTRODUCTION

Fishes are equipped with a variety of mechanisms for the detoxification of excess heavy metals entering into their system. This relieves the key metabolic and biochemical pathways from the adverse effects of heavy metals. The major ones of the above category include, binding of the metal to nonspecific high molecular weight proteins or polysaccharides and to specific low molecular weight proteins like metallothioneins or lipoprotein complexes like lipofuscin granules. Studies carried out by Thomas *et al.* (1982) have shown the conjugation and detoxification of heavy metals in cells by glutathione, the major non-protein thiol. The other means of detoxification include sequestration in intracellular organelles, immobilization into non-living tissues such as shells, scales etc.

Metallothioneins are a class of soluble low molecular weight cytosolic proteins (MW ≈ 6800-7000) characterized by their high affinity for heavy metal cations, heat stability, virtual lack of aromatic amino acids and histidine and by an unusually high content (30-35%) of cysteine (Nordberg and Kojima, 1979; Kagi and Nordberg, 1979). One molecule of metallothionein containing about 60-63 amino acids can bind up to seven metal ions. Their role in detoxifying metals has been

identified as a promising area for the development of stress index specific for metals as pollutants (Bayne *et al*., 1980; Lee *et al*., 1980). These metalloproteins are ubiquitous both in animal and plant kingdom - in the contaminated as well as uncontaminated organisms and are increasingly being demonstrated to play a central role in metal metabolism. Metallothioneins were first shown to be present in the tissues of fish exposed to cadmium and mercury (Marafante *et al*., 1972). It has been proposed that the physiological role of metallothioneins of uncontaminated organisms is in the regulation of metabolism of zinc or copper (Webb and Cain, 1982). The metallothioneins or metallothionein like proteins have an important role in the regulation of the metal dependent cellular activities of metalloenzymes, nucleic acids and membranes (Kojima and Kagi, 1978).

Exposure of animals to specific metal ions induces the synthesis of metallothionein which preferentially bind those metals.They thus have a sparing effect on the metabolic activities. This reduction in the extent to which essential metabolic activities would otherwise be inhibited, explains the development of tolerance by the animal to the transition metal ions (Winge *et al*., 1973). The value of the metallothionein as an indicator of heavy metal contamination of natural fresh water ecosystems has been demonstrated by Roch *et al*. (1982). Works of Hogstrand and Haux (1990) have substantiated the role of

metallothionein as an indicator of heavy metal exposure in two subtropical fish species.

Studies by Overnell and Coombs (1979) have shown that the physical and chemical properties of plaice metallothionein closely resemble those of mammalian metallothioneins. This finding demonstrates a remarkable conservation of primary and tertiary structure in metallothionein during many evolutionary stages. The resemblance in amino acid composition between metallothioneins isolated from different species suggests a conservation of function during the evolution of these proteins. Similarity in the position of cysteine, serine and basic amino acids provides further evidence for the functional importance of these residues (Andersen *et al.*, 1978).

That all metallothioneins that have been isolated from the living system are saturated with respect to the metal, points to the fact that there never occur free apothioneins. This specific association of a metal with a biological macromolecule is an important step towards the eventual definition of the biological function of such an element. The native metallothionein is zinc or copper bound, but their presence and / or relative importance depends on the tissue and species studied (Hidalgo *et al.*, 1985). Entry of divalent cations into the cell, causes redistribution of the native essential metals among the appropriate proteins. This buffer effect of pre-existing

thioneins represents the first step in the process of heavy metal homeostasis. When the concentration of the challenge metal taken up by the cell increases and saturates the existing physiological pool, a rise in the metal to protein ratio occurs. The metals that are not bound to the protein and are thus free to exert toxicity induce *de novo* synthesis of thioneins which may be triggered at the transcriptional or translational level of protein biosynthesis.

McCarter and Roch (1983) have shown that the concentration of metallothionein in the liver of the heavy metal exposed fish increased to a maximum at four weeks exposure and thereafter remained more or less constant. This has been explained to be due to the fact that, once the synthesis of metallothionein is *turned on*, the capacity for the fish to make metallothionein and bind metal to it exceeds its necessity to do so. After four weeks of exposure, the rates of synthesis of metallothionein and its degradation become equal and the level gets stabilized at a value determined by the concentration of the metal to which the fish is exposed. The initial low concentration of metallothionein in exposure studies may be hypothesized as due to selective accumulation of the metal at low concentrations initially in the kidney and later in the liver and then to the non-specific binding of metal to other macromolecules (Task Group on Metal Accumulation, 1973). Subsequent elevated metallothionein levels in liver may be correlated with the

accumulation of metal in liver during chronic exposure (Piotrowski *et al.*, 1974).

Winge *et al.* (1973) proposed the *spill over* hypothesis to explain the mechanism of toxicity due to heavy metals in relation to metallothionein levels. According to this hypothesis metal exerts toxicity when the two mechanisms for detoxification - displacement of less toxic native zinc and copper from metallothionein by the challenge metals and the metal-induced *de novo* synthesis of metallothionein - become insufficient to sequester the xenobiotic metal, due to its high influx. This results in the *spill over* of excess metal and results in the perturbations of enzyme activity causing cellular toxicity. However, there are different views both for and against this classical hypothesis. Reports of Brown and Parsons (1978), whose studies were the earliest to implicate directly the metal binding capacity of metallothionein to metal toxicity in fish, demonstrated pathology when metallothionein became saturated with mercury. Since this pathology coincided with the appearance of mercury in the high molecular weight fraction, *spill over* hypothesis was found to be a valid explanation to elucidate the role of metallothionein in protecting the fish against the toxicity of mercury and other heavy metals. Contradictory observations have been described by McCarter *et al.* (1982) and Roch *et al.* (1982). These investigators reason that if *spill over* hypothesis is valid, the fish showing an increase in copper

concentration in the high molecular weight protein fractions should be at the threshold of toxic effects and that slight increases in copper should be harmful. They, however, found that such animals can withstand large increases in copper. The fact that the concentration of the metal in the high molecular weight fraction increased in parallel with the metallothionein levels, clearly proves the invalidity of the *spill over* hypothesis that the concentration of the metal in the high molecular weight proteins is kept down until the capacity of the animal to synthesize metallothioneins is exceeded. To conclude, the rate of metallothionein synthesis, rather than the actual concentration of the protein is the critical factor determining the efficiency of fish to acclimate to heavy metals. The induction of metallothionein synthesis may be only a part of the protective mechanisms employed by fishes.

2.2 MATERIALS AND METHODS

Male specimens having an average length of 10 cm were collected from Rice Research Institute, ICAR, Cochin and were brought to the laboratory immediately. They were acclimated in large aquarium tanks for one month, under defined environmental and nutritive conditions. The water in the tank was changed daily after consumption of the supplied food.

The acclimated fishes were transferred to a large fibre glass tank containing dechlorinated tap water to serve as

control and to other two tanks containing 0.42 ppm zinc and 0.1 ppm mercury (1/10 of 96 hr LC50 value) respectively. The stocking density for the experiment was one fish per 5 l of the medium. The water in the tanks were replaced daily. The toxicant levels were kept constant after each replacement of water. The medium was changed in such a manner that there occurs least disturbance for the fish. Care was taken to maintain more or less constant environmental conditions during the experiment. Feeding of the fish was suspended 24 h prior to dosing and throughout the tenure of the experiment.

After 8 days of exposure to the toxicant the fishes were collected by a net producing minimum disturbance to the test specimen. The fishes were immobilized by a blow on the head, the body cavity was cut open and the liver tissue was excised. The removed tissue was immediately blotted of the adhering fluid and weighed accurately. Separation of the metal binding protein was done following the procedure of Brown (1985). The tissue was homogenized in sufficient volume of 0.9% NaCl and the suspension was centrifuged at 5,000g for 20 min. The supernatant obtained was incubated in a water bath at 70^0C for 5 min. so as to remove the heat coagulable proteins. The suspension was then centrifuged again at 10,000g for 20 min. The supernatant obtained was employed as the sample.

About 2g of Sephadex G 75 (Sigma) was suspended in excess

of 0.01 M ammonium bicarbonate buffer (pH 7.8), and kept for few hours for the complete swelling of the gel particles. The swollen gel was then packed carefully in a column (0.9 x 60 cm), and was washed with the buffer until it is equilibrated to the pH of the buffer. 1 ml of the prepared sample was applied to the top of the column with least disturbance and was allowed to percolate through the column material. Once the applied sample has completely entered into the column, elution was started with the ammonium bicarbonate buffer. The absorbance of the eluting drops was monitored at 254 nm using Uvicord (LKB) and 50 fractions of 1 ml each were collected at defined flow rate in a fraction collector (LKB, Redirac). The fractions were then monitored in a UV-Visible Spectrophotometer (Hitachi) at 254 and 280 nm. The absorbance obtained at 254 nm is converted into equivalent amounts of protein by interpolating from the standard graph of bovine serum albumin in ammonium bicarbonate buffer, read at the same wavelength.

The metal content of each fraction from the control sample was analysed for copper and zinc using Atomic Absorption Spectrophotometer (Perkin-Elmer, Model No.2380) and the relative concentration of the metal bound to the separated protein is confirmed. Eluant fractions from the dosed samples were analysed for zinc and mercury. Concentration of mercury bound to the protein was determined using Mercury Analyser (MA 5800D) after digestion using acid.

2.3 RESULTS

The concentration of Cu, Zn and Hg in different peaks of each experimental group is given in Table 1.

The relative distribution of protein and metal in the eluant fractions of proteins extracted from control fish is given in Fig.1. UV monitoring showed that the fractions constituting the second peak have got a high absorbance at 254 nm and a comparatively low absorbance at 280 nm. A screening of the eluant fractions for the metals confirmed that the native metallothionein or metallothionein like protein of *O.mossambicus* is predominantly zinc bound, copper being present in lesser amounts. Zinc is also present in the fractions corresponding to the high molecular weight proteins. But the concentration here is comparatively less.

Concentration of zinc in the fractions corresponding to metallothionein or metallothionein like proteins , of the sample from the fish exposed to zinc is significantly less, when compared to its concentration in the respective fractions of the control (Fig.2). But in the high molecular weight protein fractions there is a significant increase in the concentration of zinc when compared to those in the control.

When dosed with mercury, there occurs ~~a reduction~~ an increase in the concentration of zinc present in the fraction of metallothionein or metallothionein like proteins. ~~It is seen~~ that mercury has ~~displaced zinc from the metallothionein or metallothionein like proteins fractions (Fig.3 and 4). C~~oncentration of zinc in the

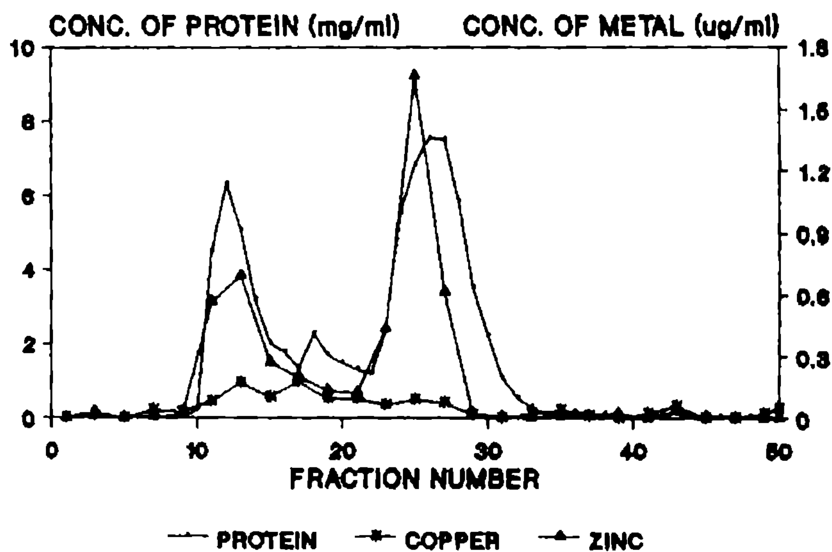

Fig.1 Distribution of metals in relation to protein content of the eluant fractions from the Sephadex G 75 column in the control fish.

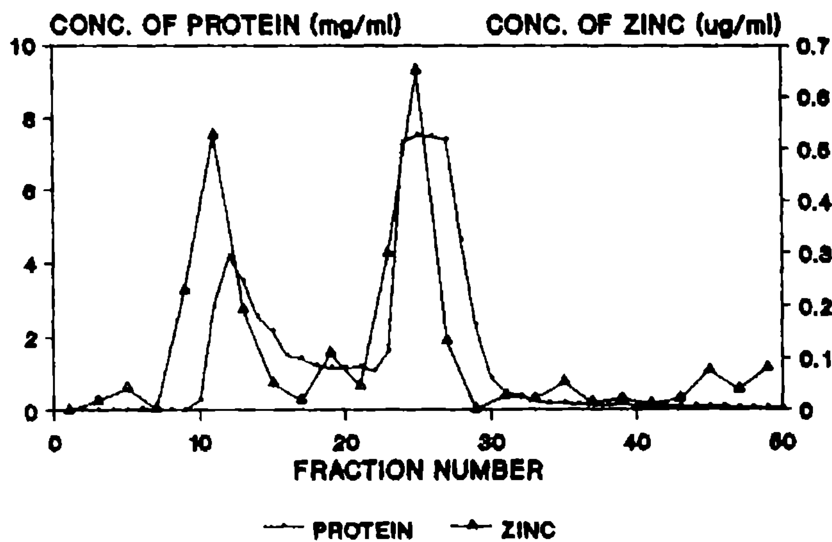

Fig.2 Distribution of zinc in relation to protein content of the eluant fractions from the Sephadex G 75 column in the fish exposed to zinc.

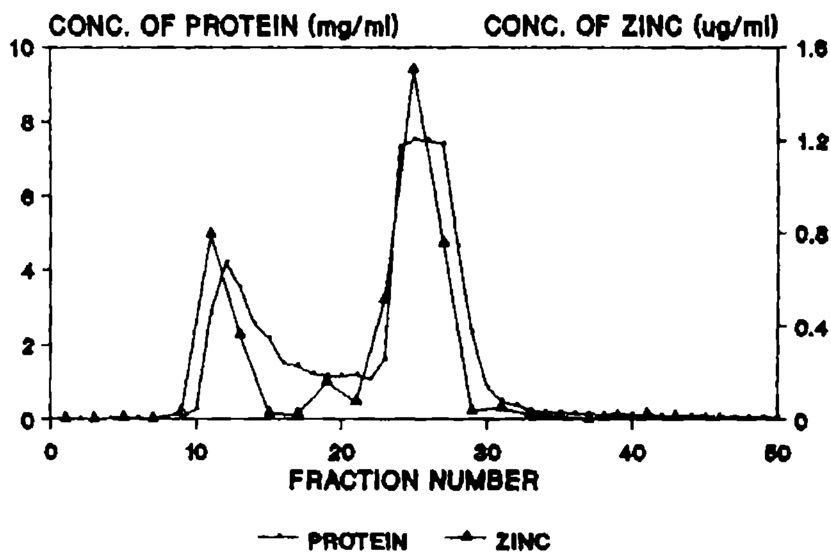

Fig.3 Distribution of zinc in relation to protein content of the eluant fractions from the Sephadex G 75 column in the fish exposed to mercury.

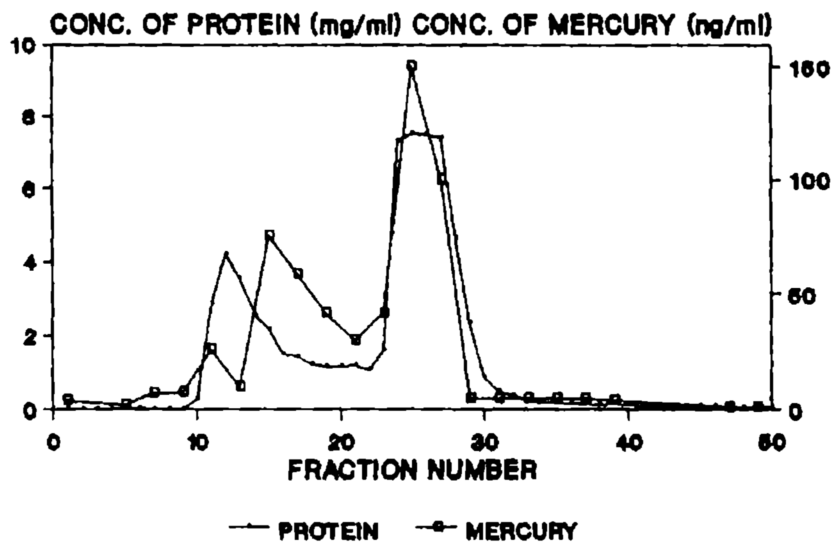

Fig.4 Distribution of mercury in relation to protein content of the eluant fractions from the Sephadex G 75 column in the fish exposed to mercury.

high molecular weight protein fractions is greater in this case when compared to the control.

Conspicuous quantitative variation in the amount of metallothionein or metallothionein like proteins is not observed between the control and test groups. It is also noticed that mercury appears in detectable amounts in the high molecular weight protein fractions, along with its increase in metallothionein or metallothionein like proteins.

2.4 DISCUSSION

The development of tolerance to transition metals by the induction of synthesis of metallothioneins, which by preferentially binding the metal ions, reduce the extent to which essential metabolic activities would otherwise be inhibited, has been proposed by Winge *et al.* (1973). However, metallothioneins have also been detected in uncontaminated organisms. An apparent metallothionein or metallothionein like proteins peak with a high absorbance at 254 nm and high levels of zinc are seen in the control fish, representing high levels of metal binding protein. Eel liver has been shown to contain, in the absence of any experimental intoxication, appreciable amounts of metallothioneins (Noel-Lambot *et al.*, 1978; Hidalgo *et al.*, 1985). Metals bound to the native metallothioneins are usually zinc and copper whose presence and / or relative

importance depends on the tissue and species studied. Zinc is found to be the principal constituent in the metallothionein from liver. The physiological role of the metallothionein from unpolluted organisms could be related to the control of zinc and copper metabolism, either through binding the excess quantity of the divalent cations that penetrate into the cell or by permitting a redistribution of these essential trace metals among the appropriate apoenzymes.

Present studies clearly demonstrate that the native metallothionein or metallothionein like protein from the liver of *O. mossambicus* is predominantly bound with zinc, though copper also is present to a minor extent. However, amount of copper did not show considerable variation among different groups. Zinc bound nature of piscine liver metallothionein and its role in the metabolism of the metal has been pointed out by Hidalgo *et al.* (1985).

When exposed to metal toxicants, variation in the relative content of metals bound to the native metallothionein or metallothionein like proteins is noticed due to their replacement by the exposed ones. It appears that increased tolerance to trace metals may be effected by the production of metallothionein or metallothionein like proteins with a portion of binding sites occupied by relatively non-toxic and readily displaceable metals. When excess toxic metals penetrate into the cells, they displace the loosely bound essential metal from the thioneins normally present in the cytosol. This buffering effect

of pre-existing thioneins represents the first step in the process of heavy metal homeostasis (Viarengo, 1985). Zinc thus appears to play a particularly important role in the metabolism of the toxic metals.

Entry of excess of zinc to the system causes a redistribution of this essential trace metal among the proteins. This is indicated by the presence of zinc in the high molecular weight fractions in amounts much greater than that present in control animals though the concentration of zinc is reduced in the metallothionein peak. Zinc being an essential metal no metallothionein is synthesized to keep the zinc in reactive form...

The amount of metallothionein or metallothionein like proteins cannot be taken as a direct measure of the degree of detoxification by that protein, because it is unlikely that there always occur a detectable increase in the relevant protein. An increased tolerance to toxic metal could also be due to the displacement of the bound non-toxic metal. However, a distinct induction of metallothionein or metallothionein like proteins is seen in mercury treated fish as evidenced by the rise in the amount of total metal bound to the protein.

Several factors could be related to the high control values of metallothionein or metallothionein like proteins and low levels of induction in mercury treated fish. The duration and / or dose of mercury treatment could be one important criterion in determining the level of induction of the metal

binding protein. High protein levels in control animals could result from acclimation, stress and / or starvation which has been shown, at least in mammals to induce metallothionein (Bremner and Davies, 1975). A reduction in the level of zinc present in the fractions corresponding to metal binding proteins would occur, simultaneous with the increased binding of mercury to the fractions. However, the decrease in the concentration of zinc ~~is~~ not equal to the increase in the mercury level, since detoxication involves both *de novo* synthesis and subsequent binding with mercury and also, replacement of zinc from the native metal binding protein by mercury. The present study indicates a stimulated *de novo* synthesis of the metallothionein or metallothionein like proteins evidenced by the increased amount of ~~zinc and hence total amount of~~ metal per gram protein in the mercury dosed animals than those present in the control animals. It thus appears that exposure to any toxic metal results in the synthesis of metallothionein or metallothionein like proteins containing approximately equimolar amounts of zinc and the exposure metal (Winge *et al.*, 1975) which would result in increased tolerance to any other toxic metal upon subsequent exposure.

The classical approach towards the mechanism of action of metal binding proteins in confronting metal toxicity is as follows:- the metal binding protein binds the metal ions, preventing them from exerting toxic effects through binding to

enzymes or other sensitive sites. However, if the rate of influx of metals into the cells exceeds the rate at which metallothionein or metallothionein like proteins can be synthesized, there may occur a *spill over* of metals from the metallothionein or metallothionein like proteins into the enzyme pool (Winge *et al.*, 1973; Brown and Parsons, 1978). Toxic effects can then be due to the displacement of essential metals from metalloenzymes by non-essential metal (Friedberg, 1974). This displacement can change the conformation of enzymes so that the substrate molecules can no longer fit the binding sites, resulting in the loss of enzyme activity.

The toxic effects of trace metals thus possibly occur only when the binding capacity of the metallothionein or metallothionein like proteins has been exceeded and there is a resultant interaction of the toxic metals with the enzyme pool. But the present studies show that the binding of mercury in the metallothionein or metallothionein like proteins containing fraction of cytosol precedes elevation of mercury levels in the high molecular weight protein fractions in fish continuously exposed to the metal in the medium. This rise in mercury level occurs, even when there is enough metallothionein or metallothionein like protein molecules, the zinc bound to which can be displaced and the incoming toxic mercury can be kept in a bound non-toxic form. These results seem to be inconsistent with the *spill over* hypothesis that the binding of mercury by

metallothionein or metallothionein like proteins should protect other proteins from the inhibitory effects of mercury which would have otherwise bound to them. According to this hypothesis an increase in the levels of mercury in the high molecular weight fractions can be an indication of the termination of the ability of the animal to sequester the metal and maintain homeostasis. This may not be true, since the concentration tested is too small (1/10 of 96 hr LC 50) and that it can survive well in much higher concentration without any external manifestation of the toxicity. This hypothesis therefore does not account for the experimental facts we have observed.

One probable explanation for the occurrence of high levels of toxic metal in the enzyme pool before metallothionein or metallothionein like proteins become saturated may be an the result of an established deficiency of an essential trace metal in the enzyme pool. The toxic metal has got an increased ability to compete for the binding sites of high molecular weight proteins. But if the enzymes are replete with the essential metal, the toxic metal would be outcompeted for the binding sites on the enzymes.

It may be true that the induction of metallothionein or metallothionein like proteins synthesis is a part of the protective mechanism employed by *O. mossambicus* exposed to mercury and it seems probable that the measurement of the

crease in the amount of total metal bound to the protein might a sensitive indicator of the response of the fish to environmental exposure to the challenge metal. It may be substantiated that the *spill over* hypothesis as a whole appears to be inadequate to account for the data obtained in the present study and also those of McCarter *et al.* (1982) and Buckley *et al.* (1982).

Metal binding proteins, however fulfill the criterion of an index of sublethal stress, namely that the changes in the profile of metal binding are responses to variation in the metal composition of the ambient environment. It is thus a primary biochemical response that is likely to be specific to the environmental stressor. Changes in the metal composition of this metallothionein or metallothionein like protein fractions presumably will identify the environmental metal that elicits the response (Leber, 1974). Total levels of the metals measured in tissues do not make any distinction between the quantity which causes a biological response in fish and that which is non-specifically bound. But the metal analyses of the eluant fractions give an explicit idea of the amount of metal which is bound to the high molecular weight proteins that is responsible for the toxicity of metals and the quantity which is bound to the low molecular weight metallothionein or metallothionein like protein fractions which may provide a detoxifying effect.

C H A P T E R 3

3. RESPONSE OF LYSOSOMES TO HEAVY METAL STRESS

3.1 INTRODUCTION

Lysosomes are a heterogenous group of cytoplasmic organelles that mediate digestive and other lytic processes of cells. Lysosomes with their diverse content of hydrolytic enzymes have the capacity to degrade virtually every molecule of biological origin. But this digestion forms only the central theme in a broad spectrum of biological functions which include regulation of secretory processes, cellular defense mechanisms, cell death, protein and organelle turn over, accumulation and sequestration of xenobiotics and mediation of target tissue specific hormone action (Moore, 1982).

Lysosomes exhibit considerable polymorphism. The primary lysosomes or storage granules are dense particles surrounded by a unit membrane. Their enzymatic content is synthesized by ribosomes in the endoplasmic reticulum and appears in the golgi region where it is finally packed into the so called lysosomes. The secondary lysosomes or digestive vacuoles result from the association of primary lysosomes with vacuoles containing phagocytized material. The phagosome fuses with the lysosome and is digested by hydrolytic enzymes. Residual bodies contain undigested materials and are eliminated or stored. The autophagic vacuole or cytolysosome is a special instance where parts of the cells are digested.

One important property of lysosomes is their stability in the living cell. The unit membrane of lysosome is stable with its contained hydrolytic enzymes. Some, yet not clear, structural peculiarities of this single lipoprotein layer enclosing the enzymes, make the lysosomal membrane impermeable to many substrates. The membrane is resistant to the enzymes that it encloses and the entire process of digestion is carried out within the lysosomes. This impermeability of the lysosomal lamina as well as the internal membrane bound nature of the lysosomal enzymes are the reasons for the fundamental biochemical property of structure linked latency of lysosomal enzymes (Bayne *et al*., 1985). In this way, it protects the rest of the cell from the destructive effects of the enzymes and this stability is essential for the normal functioning of the cell. Any interference which makes the membrane labile, permits the egress of the enzymes, resulting in catastrophic situations.

Pharmacologically, lysosomes have got outstanding significance, since they are involved in many pathological conditions. Alterations produced in the membrane cause release of lysosomal enzymes followed by an acute inflammation of the tissues. It is well established that the lysosomal enzymes possess the capacity to degrade the various components of the connective tissue protein such as collagen (Woessner Jr., 1971; Kalindi and Nimni, 1973), proteoglycans (Kocchar and Larsson, 1977) and glycoproteins (Mahadevan and Tappel, 1968). The injury

caused by enzymes of lysosomal origin and the enzyme mediated modification of tissue constituents to new antigen are of great interest in the inflammatory disturbances (Tseudo *et al.*, 1976). The lysosomal enzymes are also implicated in the pathogenesis of articular tissue degradation in several rheumatic diseases.

Lysosomes are one of the most fascinating targets of heavy metals. Although the acidic pH prevailing in the internal milieu of these organelles would not seem ideal for metal accumulation, investigations of Sternlieb and Goldfischer (1976) have demonstrated that lysosomes are the most important sites of metal compartmentation in the cell. Their ability to store and sequester a wide range of metal ions has been acknowledged by Allison (1969). Research on flounder liver by Myers *et al.* (1987) has shown an activation of lysosomal system in response to xenobiotic exposure. This activation involves an increase in number and size of lysosomes which accumulate foreign compounds and lipids in the attacked liver. Ultrastructural studies by Studnicka (1983), Daoust *et al.* (1984), Weis *et al.* (1986) and Sauer and Watabe (1989) have also shown similar results. This step clearly represents an adaptive and protective response to injury.

Heavy metals which accumulate in lysosomes, stimulate the lipid peroxidation process and at the same time, inhibit the native defense mechanisms involved in the prevention of lipid

peroxidation. This results in the formation of lipofuscin granules within the lysosomes and is a cardinal mechanism in heavy metal homeostasis (Viarengo, 1985). Lipofuscin can complex metals, due to the increase in acidity of the lipidic component and to the contribution of the associated protein fraction (George, 1982). This peroxidation product is later transformed into an insoluble polymer that includes part of the bound metals which then become unavailable to the cell. Thus, though the metals within the lysosomes enhance lysosomal lipid peroxidation and alter the normal physiology of these organelles, they augment the amount of lipofuscin granules that can trap toxic metals in relatively stable form (Viarengo, 1985). An apparent accumulation of heavy metals within lysosomes has also been shown by the histochemical studies of Weis *et al.* (1986), Baatrup *et al.* (1986), Baatrup and Danscher (1987), Andersen and Baatrup (1988) and Sauer and Watabe (1989).

A particularly efficient double way of elimination of heavy metals via lysosomes is proved by the presence of an insoluble protein polymer of amino acid composition similar to metallothionein within the lysosomes (Porter, 1974). Studies concerning metal detoxication in the digestive gland of molluscs have shown that like other cytosolic proteins, thioneins are also taken up into the lysosomes (George, 1983; Viarengo *et al.*, 1981). Once inside these organelles, they follow different metabolic pathways, probably related to the particular metal

associated with the apoprotein. It appears that, part of the non-toxic, essential metal which is only loosely bound to the thionein, is lost due to the acidic environment of the lysosomes, but not the toxic one. This makes the metalloprotein rich in free sulphydrylic groups and they polymerise by disulphide bridges. They subsequently get accumulated in the residual bodies. However, investigations on vertebrates have disclosed the fact that elimination of the residual bodies containing the metals both in the lipofuscin bound form and in the form of insoluble polymer of thionein is not properly utilised in the hepatocytes (Sternlieb and Goldfischer, 1976). The foregoing descriptions thus reveal that the lysosomal actions provide the necessary environment for the cell to adapt itself to the elevated levels of metals in the ambience.

Though lysosomes are noted for their sequestration and accumulation of metals and various other chemicals, many of these substances are capable of destabilizing the lysosomal membrane, if the storage capacity is surpassed. A subsequent activation and leakage of the previously latent degradative lysosomal enzymes which have got a high potential for the catabolic disruption of cellular systems, ensue (Bayne *et al.*, 1978) and is thus of considerable environmental consequence. This alteration in the lysosomal stability is due to the functional modifications in the lysosomes and in certain instances, the structural changes which are all induced by the

stressor and are indicative of cytotoxicity (Moore *et al*., 1978; Bayne *et al*., 1978). Many xenobiotics evoke alterations directly in the bounding membrane of the lysosomes (Moore and Lowe, 1985). A gain in the lysosomal volume resulting from the increased accumulation of contaminants involves the formation of pathologically enlarged or *giant* lysosomes. This variation leads to enhanced permeability to or in other words reduced latency of lysosomal enzymes (Moore and Clarke, 1982; Moore *et al*., 1985). Destabilization may also entail increased lysosomal fusion with other intracellular vacuoles leading to pathologically enlarged lysosomes. Supporting evidence for this clear-cut, two step response namely an initial adaptive and protective response followed by a damage of the lysosomal digestive and detoxifying system due to overloading, has been furnished by Myers *et al*. (1987) in the English sole *Parophrys vetulus*. A significant negative correlation between the lysosomal stability and extension of liver lesion denoting the overcharge and break down of the detoxifying capacity of liver has been observed by Kohler (1989b). A distinct decline in the stability of lysosomal membrane in relation to contaminant burden has been reported by Kohler *et al*. (1986), Kohler (1989a, 1990) and Ward (1990).

Evidence of the value of the lysosomal stability as a measure of cellular condition and catabolic potential is provided by significant positive correlation between this index and the physiological scope for growth (Bayne *et al*., 1976,

1979). Also, the level of destabilization bears a quantitative relationship to the degree of stress (Bayne *et al.*, 1976; Moore and Stebbing, 1976; Moore *et al.*, 1978). Investigations at the subcellular level can reveal alterations at an early stage of response, before integrated cellular damage shifts to the level of organ or whole organism. In many instances, the earliest detectable alterations are associated with the lysosomes (Moore, 1985). This sensitivity of lysosomes to environmental pollutants including heavy metals ranks lysosomal responses as early warning systems for detection of the disturbances in the surroundings. The lysosomal stability measured in terms of the lysosomal enzyme release assay (LERA), thus can clearly reflect any break down in the adaptive capacity of the organism to toxic injury. Thus, a test battery measuring lysosomal perturbations should be recommended as a tool for biological effect monitoring (Kohler, 1991). The utility of lysosomal membrane lability assay as a health monitoring tool has also been suggested by Chvapil *et al.* (1972).

The lysosomal enzyme release assay has been substantiated as a sensitive indicator of numerous environmental stresses in molluscs (Bayne *et al.*, 1976; Moore and Clarke, 1982; Widdows *et al.*, 1982). However a few studies have been conducted on aquatic vertebrates (James, 1986; Kohler *et al.*, 1986; Kohler, 1989a, 1990). The measurement of lysosomal perturbations in fish liver as an integrative biological warning system for biological

effect monitoring was suggested by Kohler (1990, 1991).

In this chapter, effort has been made to extend the lysosomal enzyme release assay technique to *Oreochromis mossambicus* and to assess its effectiveness as a sensitive index of heavy metal stress and its applicability as a biochemical warning of environmental alterations. Attempts have also been made to compare the lysosomal membrane stability of different animals, both aquatic and terrestrial. and hence to evaluate the evolutionary status and adaptive strategy of our experimental animal.

3.2 MATERIALS AND METHODS

Collection and acclimation of fish, method of dosing and the collection of tissue samples were the same as that described in the previous chapter except that the sampling was done at 2 days intervals.

The samples were homogenized in ice-cold isotonic sucrose solution (0.35 M) containing 2 mM mercaptoethanol (Philip, personal communication). Stability of the lysosomal membrane was determined following the procedure of Philip and Kurup (1977, 1978) and Rao and Sisodia (1986) with slight modifications. All the steps were done at temperatures below $4^{\circ}C$. The homogenate was centrifuged at 6,00g for 20 min. at $0^{\circ}C$ in a refrigerated centrifuge. The pellet was resuspended in isotonic sucrose and was centrifuged again at the same conditions as above. The

supernatants were pooled and was centrifuged at 8,000g to remove mitochondria. The resulting supernatant was then centrifuged at 23,000g for 30 min. at $0^{o}C$ in a refrigerated centrifuge. The pellet resulted was rich in lysosomes and was washed once in the sucrose solution to remove the lighter less dense particles. The lysosome rich fraction was resuspended in the isotonic sucrose solution containing 2 mM mercaptoethanol. To a definite volume of this sample equal volume of citrate buffer (pH 4.8) containing 0.2% Brij-35 was added and was kept for the determination of total lysosomal acid phosphatase activity. The lysosomal suspension obtained was incubated at $37^{o}C$ and samples were removed after 0, 10, 20 and 30 min. preserving the withdrawn samples at $0^{o}C$. The incubated samples were centrifuged at 23,000g for 30 min. at $0^{o}C$ in a refrigerated centrifuge. Acid phosphatase - the marker enzyme of lysosomes released from that organelle was assayed following the procedure of Anon (1963) with slight modification. 0.5 ml of citrate buffer (100 mM, pH 4.8) and 0.5 ml of paranitrophenyl phosphate (400 mg in 100 ml distilled water) were incubated for 3 min. at $37^{o}C$. 0.3 ml of the enzyme extract and 0.3 ml of 0.01 mM EDTA (Verity and Reith, 1967) were added to the above mixture and incubated for 30 min. at $37^{o}C$. The reaction was stopped by the addition of 4 ml of 0.1 N NaOH. The paranitrophenol formed was measured at 410 nm. The amount of paranitrophenol was calibrated from a standard graph. Specific activity of acid phosphatase was expressed as millimoles of paranitrophenol formed per hour per gram protein.

Bovine serum albumin was used as the standard for protein estimation.

Similar procedure was followed for the in vitro studies of the lysosomal enzyme latency, where the lysosomal rich fraction obtained from liver of acclimated fish was suspended in isotonic sucrose solution containing mercaptoethanol and was incubated with various concentrations (0.002, 0.02, 0.2, 2, 20 and 200 ppm) of zinc and mercury solutions. A comparative study of the lysosomal latency of a mollusc, a fish and a mammal was also conducted following the same procedure. The isotonic sucrose solutions used were of 0.45 M for mollusc, 0.35 M for fish and 0.25 M for mammal.

3.3 RESULTS

The data of lysosomal lability index obtained as a function of time, in different groups were analysed using ANOVA technique. Wherever the effects were found to be significant, least significant difference (LSD) at 5% level was calculated.

The stability of the lysosomes of different species versus time of incubation is shown in Table 1 and Fig.5. Statistical analyses have shown a significant difference between the lysosomal stability of *R. norwegicus, O. mossambicus* and *S. scripta* ($P < 0.001$). A significant difference is noticed between

the lysosomal lability index due to different time of incubation also ($P<0.01$). The least significant difference for various species is 1.006. The lysosomal stability is maximum for *R.norwegicus* whereas, *S. scripta* is having the minimum. The LSD for time of incubation is 0.616.

Studies on the effect of in vivo exposure of *O. mossambicus* to zinc and mercury for a period of 2 days (Table 2A and Fig.6) show that the lysosomal stability varies significantly between the control and the test ($P< 0.01$). LSD at 5% level is 0.87. The labilization of the lysosomal membrane brought about by zinc and mercury is significantly higher when compared to control. There is also significant difference between the lysosomal stability due to different times of incubation ($P< 0.01$). LSD for the different times of incubation at 5% level is 0.754. The labilization resulting after 10, 20 and 30 min. of incubation is significantly higher when compared to that of 0 time, though there is no significant difference between the extent of destabilization due to 10, 20 and 30 min. of exposure among themselves.

Labilization of lysosomal membrane caused by the exposure of the organism to zinc and mercury for 4 days is shown in Table 2B and Fig.7. Here also a significant variation between groups ($P< 0.001$) is noticed. LSD at 5% level is 1.038. But here the labilization due to mercury is significantly higher when

compared to that of control and zinc. The lability index for zinc is less than that for control indicating a stabilizing effect. There is significant difference between the labilization brought about by different periods of incubation ($P < 0.05$). The LSD at 5% level is 0.899. Though there is no significant difference between the lability indices of 0 and 10 min., there occurs a significant elevation in the lability index from the values at 0 time, after 20 and 30 min.

Table 2C and Fig.8 illustrate the effect of exposure to zinc and mercury for 6 days. Here also, there is significant difference between the lability of lysosomes of fish dosed with various xenobiotics ($P < 0.01$). The LSD at 5% level is 9.18. The difference between the lability of lysosomes in control and zinc dosed animals is not significant. But the labilization caused by mercury is significant when compared to those by control and zinc. The labilization brought about by different periods of incubation is not significant at 5% level.

The labilization brought about by in vivo exposure to zinc and mercury for 8 days is summarized in Table 2D and Fig.9. A significant difference is seen between the lability index of different groups ($P < 0.001$). LSD at 5% level is 2.62. The lability index of lysosomes from the mercury dosed animals is significantly higher when compared to those from control and zinc dosed ones. The variation between lability index of

lysosomes from zinc dosed and control organisms is not significant. Different times of incubation has, however, produced a significant difference in labilization of lysosomes ($P < 0.05$). LSD at 5% level is 2.27. The labilization occurring after 10, 20 and 30 min. of incubation is significantly higher when compared to that of 0 time, even though there is no significant difference between themselves.

Effect of in vitro exposure to zinc and mercury (2 and 20 ppm) on the cell free preparations of lysosomes from *O. mossambicus* is presented in Table 3A and 3B and Fig.10 and 11 respectively. Variation in the effect of 2ppm solutions of zinc and mercury on lysosomal stability is not significant between themselves and between control. The increase in lability index is significant after 20 and 30 min. of incubation. 20 ppm solutions have a significantly different effect between groups ($P < 0.001$). LSD at 5% level is 0.69. The lability index of control and mercury exposed lysosomes is significantly higher relative to those of zinc exposed ones. The labilization arising due to mercury is not significant when compared to control. The effect of different times of incubation is also significantly different ($P < 0.05$). LSD at 5% level is 0.599. Though the labilization at 10 min. of incubation is not significant, it has become conspicuous at 20 and 30 min. of incubation.

TABLE : 1

In vitro studies on the stability of lysosomal membrane of different animals as a function of time at 37°C.

Stability of lysosomes is assessed by following the activity of acid phosphatase (millimoles of p-nitrophenol formed/hour/gm protein) released.

TIME min.	*R. NORWEGICUS*		*O. MOSSAMBICUS*		*S. SCRIPTA*	
	ACTIVITY	LLI	ACTIVITY	LLI	ACTIVITY	LLI
00	01.76 ± 0.14	11.01	01.67 ± 0.05	18.72	21.14 ± 0.32	35.76
10	01.92 ± 0.27	12.00	01.85 ± 0.16	20.72	21.84 ± 0.19	36.95
20	02.20 ± 0.04	13.74	02.03 ± 0.15	22.70	22.97 ± 0.43	38.86
30	03.01 ± 0.08	18.80	02.13 ± 0.18	23.81	23.01 ± 0.92	38.93
40	03.61 ± 0.19	22.56	02.09 ± 0.11	23.42	23.33 ± 1.39	39.47
60	03.92 ± 0.38	24.51	02.13 ± 0.18	23.87	23.31 ± 0.42	39.44
90	04.61 ± 0.58	28.78	02.16 ± 0.21	24.26	23.26 ± 0.13	39.36
120	04.60 ± 0.39	28.70	02.11 ± 0.09	23.64	23.38 ± 0.21	39.56

Lysosomal lability index (LLI) is the activity of acid phosphatase released expressed as the percentage of the total activity of lysosomal acid phosphatase. Values are the mean of six different experiments ± SD.

TABLE : 2

In vivo effect of Zn and Hg on the stability of lysosomal membrane of *O. mossambicus* as a function of period of exposure. Stability of lysosomes is assessed by following the activity of acid phosphatase (millimoles of p-nitrophenol formed/hour/gm protein) released as a function of time at 37°C.

A. TWO DAYS

TIME	CONTROL		MERCURY		ZINC	
min	ACTIVITY	LLI	ACTIVITY	LLI	ACTIVITY	LLI
00	02.96 ± 0.36	16.99	04.23 ± 0.26	15.68	03.52 ± 0.32	16.36
10	03.84 ± 0.18	22.07	05.27 ± 1.95	19.53	05.32 ± 0.20	24.76
20	03.87 ± 0.26	22.24	05.50 ± 0.23	20.38	06.04 ± 0.17	28.11
30	04.22 ± 0.11	24.26	05.73 ± 0.17	21.25	06.59 ± 0.21	30.67

B. FOUR DAYS

TIME	CONTROL		MERCURY		ZINC	
min	ACTIVITY	LLI	ACTIVITY	LLI	ACTIVITY	LLI
00	08.13 ± 0.91	34.80	11.00 ± 2.17	35.60	03.89 ± 1.11	19.13
10	08.40 ± 0.33	35.95	10.93 ± 0.88	35.12	05.39 ± 0.38	26.45
20	08.67 ± 0.37	37.12	12.80 ± 0.44	41.13	05.44 ± 0.17	26.72
30	09.00 ± 0.29	38.53	12.82 ± 0.34	41.22	06.21 ± 0.15	30.48

(contd.)

C. SIX DAYS

TIME min.	CONTROL		MERCURY		ZINC	
	ACTIVITY	LLI	ACTIVITY	LLI	ACTIVITY	LLI
00	09.28 ± 1.05	22.60	15.71 ± 0.84	21.18	04.42 ± 0.26	17.87
10	09.55 ± 0.29	23.25	19.37 ± 0.85	26.12	05.58 ± 0.30	22.58
20	10.47 ± 0.45	25.50	24.51 ± 1.75	33.04	05.53 ± 0.46	22.37
30	09.84 ± 0.74	23.97	34.67 ± 1.20	46.74	05.52 ± 0.07	22.38

D. EIGHT DAYS

TIME min.	CONTROL		MERCURY		ZINC	
	ACTIVITY	LLI	ACTIVITY	LLI	ACTIVITY	LLI
00	02.53 ± 0.20	13.45	02.94 ± 0.57	09.77	02.29 ± 0.57	10.31
10	04.06 ± 0.39	21.56	09.71 ± 0.23	32.29	04.17 ± 0.31	18.83
20	04.70 ± 0.38	24.95	09.57 ± 0.29	31.85	04.81 ± 0.17	21.68
30	05.63 ± 0.49	29.92	09.61 ± 0.50	31.96	04.98 ± 0.45	22.47

Lysosomal lability index (LLI) is the activity of acid phosphatase released expressed as percentage of the total activity of lysosomal acid phosphatase.

Values are the mean of six different experiments ± SD.

TABLE : 3

In vitro effect of different concentrations of Zn and Hg on the stability of lysosomal membrane of *O. mossambicus*.
Stability of lysosomes is assessed by following the activity of acid phosphatase (millimoles of p-nitrophenol formed/hour/gm protein) released as a function of time at 37°C.

A. 2 ppm

TIME	CONTROL		MERCURY		ZINC	
min.	ACTIVITY	LLI	ACTIVITY	LLI	ACTIVITY	LLI
00	01.33 ± 0.13	12.54	01.58 ± 0.11	14.89	00.61 ± 0.06	05.80
10	01.99 ± 0.10	18.74	02.11 ± 0.12	19.94	03.03 ± 0.56	28.59
20	02.34 ± 0.10	22.04	02.45 ± 0.07	23.12	03.74 ± 0.57	35.29
30	02.96 ± 0.15	27.92	02.53 ± 0.07	23.87	04.83 ± 0.25	45.62

B. 20 ppm

TIME	CONTROL		MERCURY		ZINC	
min.	ACTIVITY	LLI	ACTIVITY	LLI	ACTIVITY	LLI
00	01.64 ± 0.11	06.70	01.20 ± 0.19	04.91	00.23 ± 0.04	00.93
10	02.15 ± 0.10	08.77	01.80 ± 0.13	07.34	00.61 ± 0.17	02.48
20	02.75 ± 0.14	11.23	02.63 ± 0.13	10.71	00.61 ± 0.06	02.50
30	03.30 ± 0.15	13.48	02.53 ± 0.35	10.34	00.61 ± 0.07	02.48

Lysosomal lability index (LLI) is the activity of acid phosphatase released expressed as percentage of the total activity of lysosomal acid phosphatase.
Values are the mean of six different experiments ± SD.

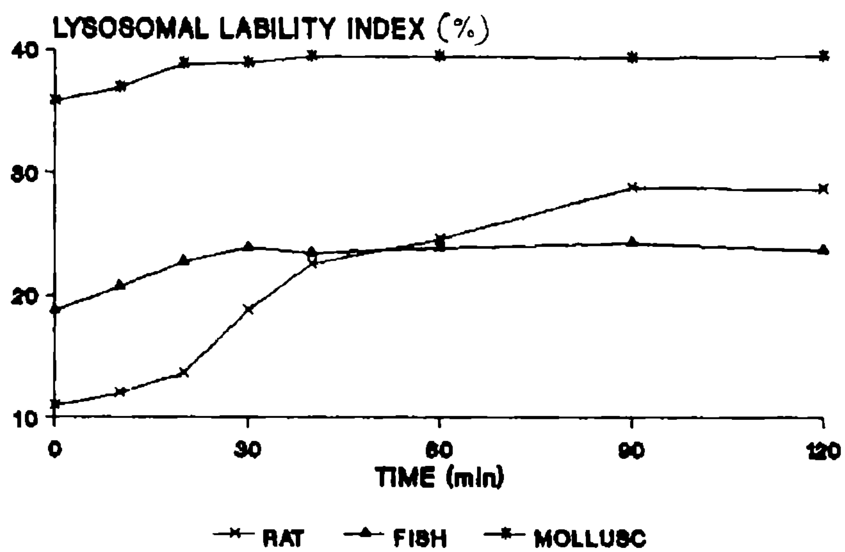

Fig.5 Stability of lysosomal membrane of rat, fish and mollusc.

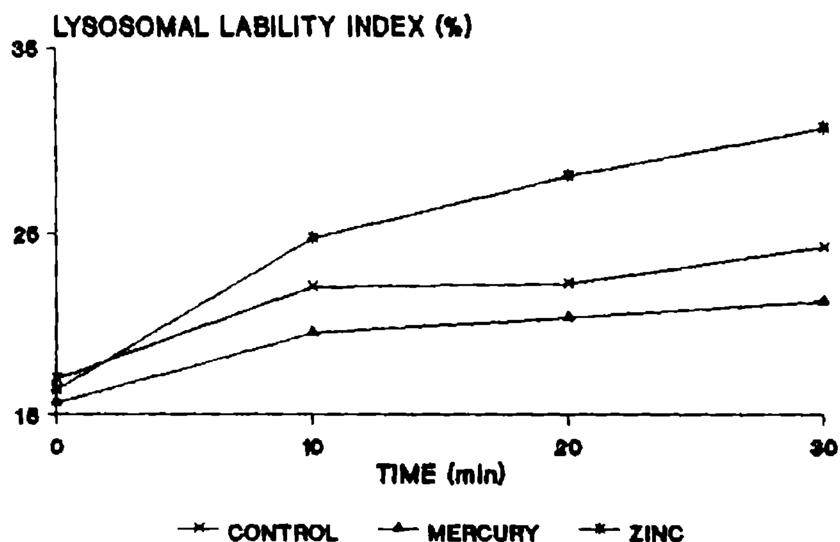

Fig.6 Stability of lysosomal membrane in *Oreochromis mossambicus* exposed to zinc and mercury for 2 days in vivo.

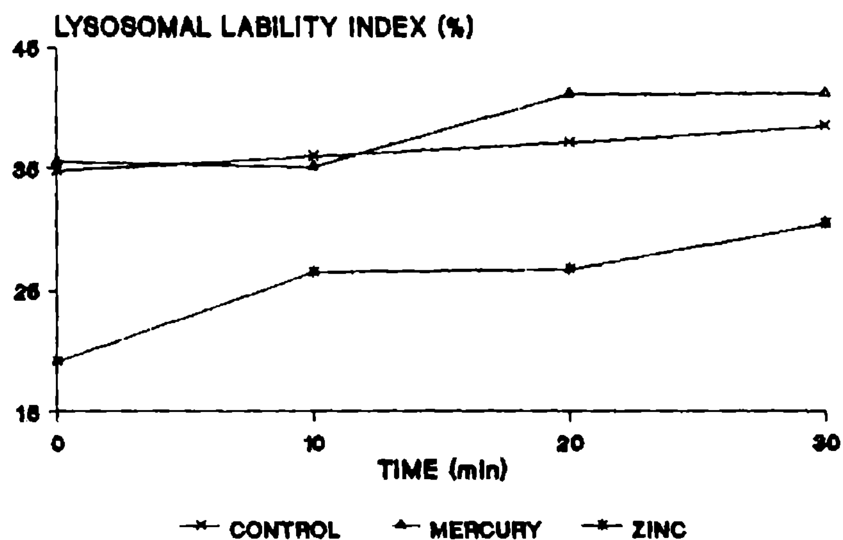

Fig.7 Stability of lysosomal membrane in *Oreochromis mossambicus* exposed to zinc and mercury for 4 days in vivo.

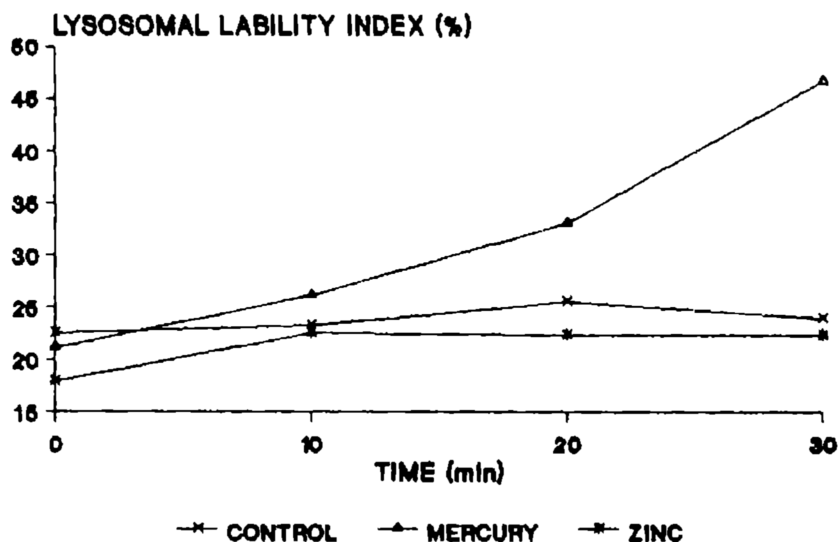

Fig.8 Stability of lysosomal membrane in *Oreochromis mossambicus* exposed to zinc and mercury for 6 days in vivo.

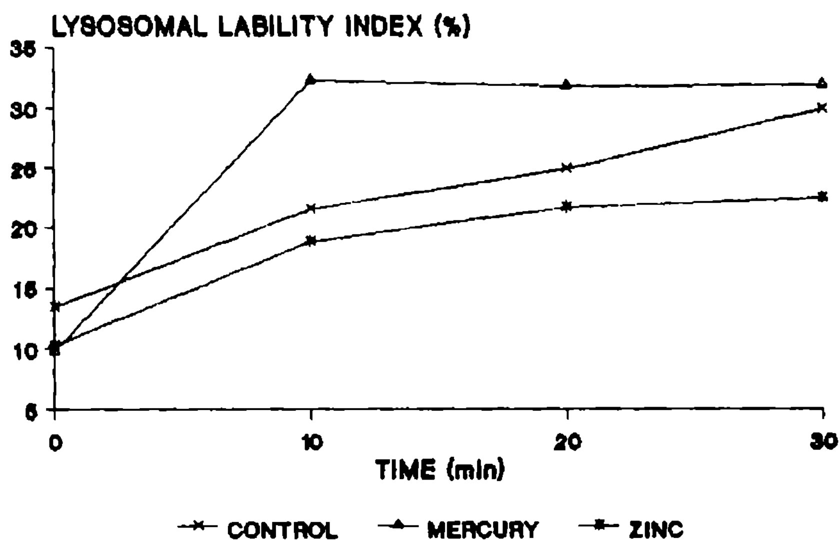

Fig.9 Stability of lysosomal membrane in *Oreochromis mossambicus* exposed to zinc and mercury for 8 days in vivo.

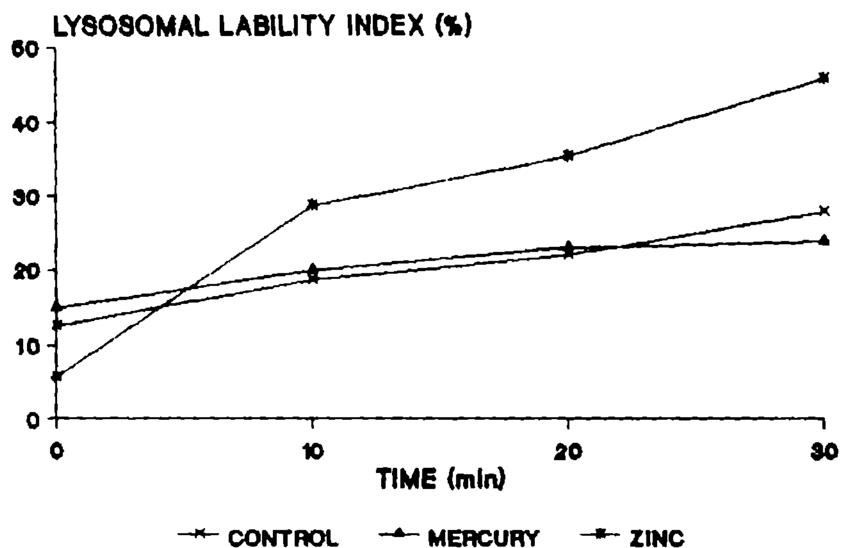

Fig.10 Stability of lysosomal membrane in *Oreochromis mossambicus* exposed to 2 ppm solutions of zinc and mercury in vitro.

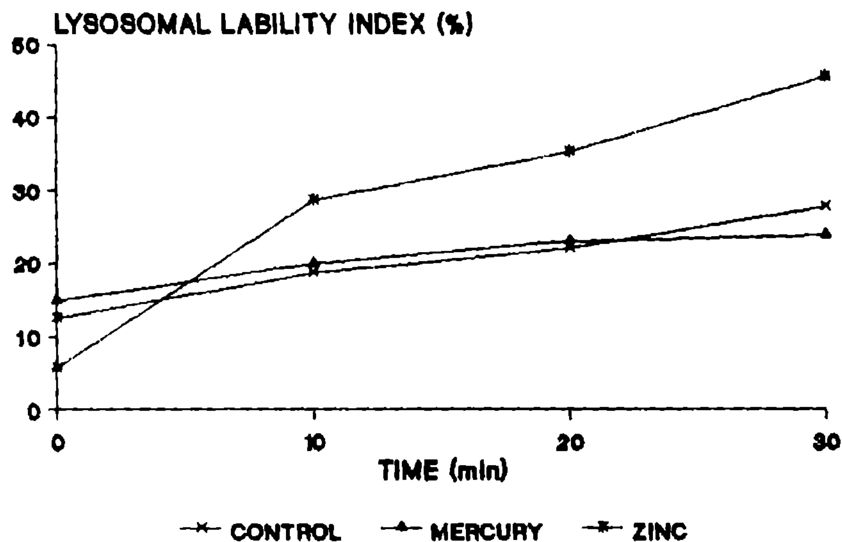

Fig.11 Stability of lysosomal membrane in *Oreochromis mossambicus* exposed to 20 ppm solutions of zinc and mercury in vitro.

3.4 DISCUSSION

Various chemicals are capable of initiating cellular disturbances which may rapidly spread into a complex net work of associated secondary and higher order perturbations which become progressively more difficult for the cell to reverse or modify. However, during the course of evolution, many of the mechanisms of detoxification have developed the capability of increasing their ability to meet a particular environmental challenge. Thus living organisms have a profound ability to survive within an environment containing toxic chemicals, in spite of the tendency of these chemicals to overload the normal physiological mechanisms of biotransformation or detoxification present in the cell.

Lysosomes play a crucial role in the isolation and sequestration of metals, and thus relieve biological machinery from their toxic effects. But once the storage capacity is exceeded, there ensues a leakage of hydrolytic lysosomal enzymes into the cytoplasm and nucleoplasm bringing about a derangement of cell functions. Thus, despite the role of lysosomes in heavy metal sequestration, the lysosomal membrane is often the target of injury by the xenobiotics. Concrete evidence for contaminant related pathological modification in cell structure can thus be obtained from investigations on lysosomal structure. One of the first alterations due to metal exposure that can be detected at

lysosomal level is the variation in the membrane stability (Viarengo *et al.*, 1981; Moore *et al.*, 1984) and these changes indicate severe dysfunction of lysosomal system. These interfere with the intracellular digestion of food, the normal turn over of proteins and organelles and the regulation of fusion processes associated with the lysosomal vacuolar system. Injury resulting in destabilization of the lysosomal lamina bears a quantitative relationship to the magnitude of stress response (Bayne *et al.*, 1979, 1982) ie., to the intensity of catabolic or degradative effects as well as the level of pathological changes. Lysosomal membrane stability is thus a highly sensitive measure of the functional state of the cell and provides an ideal starting point for probing into the generalized cellular deterioration. Assessment of this type of injury has been confirmed as an extremely sensitive index of cellular condition by Pickwell and Steinert (1984).

An inquiry of the effect of heavy metal ions on membrane function may furnish us with a means for the exploration of membrane properties as well as for seeking the types of the toxic effects of the metal at an ultrastructural level. The variation in the stability of the lysosomal particles is considered to be a reflection of the characterestic of the lipoprotein membrane of the particles. This is further substantiated by the fact that a large number of a variety of chemical agents which cause stabilization or labilization of the

membrane are lipophilic. The possible significance of the lipid fraction of the lysosomal lamina in determining the stability of lysosomes has been discussed by Wynn and Iqbal (1985) but is not yet fully understood.

An exceptional affinity of mercury for thiol groups of proteins has been demonstrated by Satchell (1984). In view of the ligand binding qualities of mercury in various membrane systems, the investigations of the effect of mercury on the integrity of lysosomal membrane and the structure linked latency of lysosomal enzymes, especially acid phosphatase - the marker enzyme of lysosomes, seem to be appropriate. Studies conducted with a purified preparation of lysosomes demonstrate the loss of enzyme latency in the presence of low concentrations of mercurials which may be attributed to the irreversible damage of the membrane after the formation of mercaptide ligands. The same conclusion has also been accomplished by Verity and Reith (1967). The increased free enzyme activity caused by the addition of mercury ions to a particulate fraction of lysosomes appears paradoxical when considering the known inhibition of acid phosphatase by heavy metal ions (Verity and Brown, 1964). However, this effect of mercury ions has been got rid off in the present experiment by removing any mercury remaining free to influence the system by chelating to EDTA. Formation of stable mercaptide with thiol groups, in preference to other ligand groups that may be present, has been reported by Gurd and

Willcox (1956). The presence of the thiol and disulphide groups in membrane systems is well established (Riley and Lehninger, 1964; Passow *et al.*, 1961; Jacob and Jandl, 1962). Such functional groups may play a role in the integrity and permeability properties of the membrane and the phenomenon of structure linked latency. Mercuric compounds irreversibly damage the limiting membrane with the formation of mercaptide bridges.

In addition to the above processes, mercury entering the cell and thence to the interior of lysosomes may attack the unsaturated fatty acids of the membrane as an absolute step towards the process of lipid peroxidation. The lipofuscin granules formed are transformed into an insoluble polymer that include part of the bound metal and become unavailable to the metabolic machinery. Though this peroxidation and the consequent formation of lipofuscin granules turn out to be of fundamental importance in heavy metal homeostasis, they leave an unfavourable alteration in the physiology of lysosomal lamina.

In the cell several defense mechanisms implicated in the prevention of lipid peroxidation occur naturally. Mercury is able to reduce the activity of these protective machinery, thus enhancing the lipid peroxidation (Bus and Gibson, 1979) resulting in an augmented attack on the unsaturated fatty acids of the membrane. Thus, mercury intensifies the injury of the lysosomal membrane directly as well as indirectly causing

release of degradative enzymes.

Studies have demonstrated that correct ratios between zinc in the external medium, that bound to the membrane and to the internal environment are essential in the maintenance of the structure and function of the cell membrane (Bettger and O'dell, 1981). Zinc ions stabilize the plasma and internal membranes either by binding to the structural components or by inhibiting metal catalyzed lipid peroxidation (Chvapil, 1973). Ability of zinc to stabilize the lysosomal membrane has been demonstrated by Sternlieb and Goldfischer (1976). This is in agreement with the results obtained in the present study also. Earlier works have also indicated that the activity of metal ions in lipid peroxidation is probably dependent upon the balance between the amount of zinc and other metals. This helps to explain the role of zinc in membrane stability and latency of enzyme activity.

Considering the *all or none* effect of heavy metal ions on individual lysosomal particles (Verity and Reith, 1967), the maximal values of acid phosphatase released, represent the proportion of particles in which the threshold has been surpassed and the sigmoid curve demonstrates the distribution of thresholds in the lysosomal population. Enzyme activation is a function of the proportion of lysosomes whose threshold has been exceeded. Studies by Shibko *et al.* (1965) on the release of lysosomal enzymes from kidney have also shown that at no stage

uring a graded release, did the particles lose only part of their electron opaque matrix. The interaction of mercury with thiol groups of membrane suggests that no single ligand plays a significant functional role in membrane integrity, but that a critical number of ligands if cross linked will disrupt the membrane contiguity with resultant permeability. The differences in individual lysosomal responses can be attributed to morphological and biochemical parameters that may decide the relative availability of thiol groups assuming a constant stoichiometric ratio and mass law distribution of mercury ions in the pre-incubation system (Clarkson and Magos, 1966).

The information obtained in the current study indicates that as with other detoxication systems, the accumulation and sequestration of these metals also is effective only until the storage capacity of the lysosomes is overloaded, or the lysosomes are damaged directly by the accumulated contaminant as shown by Moore *et al.* (1985). An initial stabilization of the lysosomal membrane followed by a progressive labilization is seen, specifying that sequestration of mercury by lysosomes which relieves the biochemical machinery from their toxic effects is efficient only up to a limit. Thereafter, this detoxifying machinery becomes ineffective resulting in a decline in the structure linked latency of lysosomal enzymes. An initial labilization in the case of zinc may be due to the fact that the onset of operation of detoxifying mechanisms for this essential

Results of the in vitro studies with 2 and 20 ppm solutions of zinc substantiate this hypothesis. The optimum internal level of zinc is achieved in the animals exposed to 20 ppm solution

element is not as fast as that for the toxic mercury. A direct effect of metals on the membrane rather than on the lysosomal enzyme is possible since all the metals remaining, which can cause any difference in activity of acid phosphatase, whose rate of release is taken as the index of the lability of lysosomes, are chelated and made unavailable to the system. Such a direct interaction, suggesting that metals that form redox systems catalyze lipid peroxidation and cause membrane damage has also been reported by Chvapil (1973).

A comparison of the lysosomal lability index of ~~the~~ control fish for various days ~~samples which are incubated for 0 time~~ shows that there may occur an increased production of lysosomes as an adaptive response to stress. This is evidenced by the increased lysosomal lability index after four days of exposure. A further decrease observed may be due to the fact that a large proportion of lysosomes have already got affected without contributing to our lysosomal preparation. These statements are especially applicable to the control and mercury dosed animals whereas in zinc dosed animals an almost uniform initial lability index is seen at all periods of exposure which may indicate that no significant *de novo* synthesis of lysosomes occur in this case. The difference between the control values of 2 and 20 ppm test group is not significant and can be considered as biological variation (t value 1.053 for degrees of freedom 8)

An index of lability or latency - the lysosomal property of requiring membrane alterations for enzyme expression - permits the comparison of the relative amount of enzyme leaking

out of the lysosomal preparation. This lysosomal enzyme release assay (LERA) is a sensitive signal of the functional state of the lysosomes. It is seen that the molluscan lysosomes are having a higher lysosomal lability index. It may be because of the fact that molluscs have comparatively more number of lysosomes and are the most sensitive to environmental alterations. This along with other qualities account for the validity of molluscs as *sentinel* organisms. Fishes have relatively low numbers of lysosomes which are physiologically more tolerant, whereas mammalian lysosomes are very few in number and are the most resistant of the three.

To sum up, the lysosomal test clearly reflects the break down of the adaptive capacity of fish liver to toxic injury. However, it may be generalized that the instability of lysosomal membrane is not caused by special chemicals but by many factors such as binding ability to protein which may be involved in the disruption of lysosomal membrane, or attack to the unsaturated fatty acids of membranes to form lipid peroxides. The assay using lysosomes makes possible to monitor the effects of environmental pollutants on biomembranes. But lysosomal membrane stabilizing or destabilizing effects are not specific to certain chemicals (Tabata *et al.*, 1990). Thus responses of lysosomes to heavy metals may be primary or secondary and can be considered only as a general index of stress.

C H A P T E R   4

4. ENZYMES AS INDICATORS OF HEAVY METAL STRESS

4.1 INTRODUCTION

Enzymes are a major class of proteins in the living system which function as catalysts that direct and accelerate biochemical reactions. They offer a potential area for the monitoring of biological effects and have attracted a great deal of attention of scientists of various fields. As each step in any metabolic process is dependent on a specific enzyme, inhibition or acceleration of one enzyme is bound to cause a series of serious metabolic disorders. In clinical enzymology, levels of activity of enzymes such as aspartate aminotransferase, lactate dehydrogenase etc. in serum are determined for diagnostic purposes. Some enzymes including lactate dehydrogenase and aspartate aminotransferase have already been used and others are potentially useful as toxicological indices. The effects of toxicants on the key enzymes of metabolism have become a topic of common interest to toxicologists and biochemists.

Studies by Gould *et al.* (1976), Gould (1977) and Thurberg *et al.* (1977) have demonstrated that structural and other properties of enzymes as well as specific activities can be affected by exposure of animals to pollutants possibly leading

to loss of metabolic flexibility. Response of enzymes also varies with the rate and magnitude of absorption of toxicant. Thus, the impact of xenobiotics at cellular and subcellular levels can be perceived by enzymological studies. Many heavy metal ions have been reported to alter significantly the activity of various enzymes in plants, microbes and aquatic as well as terrestrial organisms (Dixon and Webb, 1964; Webb, 1966; Moore and Stebbing, 1976). Metals influence the rate of action of certain enzymes by activation, inactivation, uncoupling reactions or mechanisms yet to be defined. Hence, a study of metabolic and enzymatic activities of aquatic organisms is essential to provide a rational basis for anticipating and understanding the ecological effects of an accelerated input of heavy metals into the fresh water or marine environment.

The prime objective of the present study is to determine the feasibility of the measurements of enzyme activity for diagnosing sublethal metal poisoning in fish. The study also provides an insight into the mechanism of action of heavy metals on enzymes. Experiments were conducted by adding the metal salts directly to enzyme reaction mixtures and to tanks containing fish. Thus, a comparison could be made between direct (in vitro) effects of the metal on enzymes and the indirect (in vivo) effects as measured in liver preparation from fish exposed to metal ions. The key enzymes selected for the study are acid phosphatase, aspartate aminotransferase, β-hydroxy, β-methyl

glutaryl coenzyme A reductase (HMG Co A reductase), lactate dehydrogenase and serine hydrolyase.

Acid phosphatase from lysosomes is a typical marker enzyme of that organelle and is concerned with the biosynthesis of mucopolysaccharides (Kroon, 1952), nucleic acids and proteins (Cox and Griffin, 1965; Tewari and Sood, 1969). This enzyme is located within the lysosomes in a latent form and can be activated by labilization of the particle membrane by a number of stressors. Lysosomal hydrolases are involved in the degradation and dissolution of damaged cells and thereby facilitate their replacement by normal tissues (De Duve *et al.*, 1955). The stimulation or inhibition of these enzymes can result in the disturbance of metabolism and hence are good indicators of stress in the biological system (Gupta *et al.*, 1975; Verma *et al.*, 1980). Enhanced activity of acid phosphatase, in particular due to any stress, seems to be characteristic of tissue damage and have become a useful diagnostic and experimental tool (Teitz, 1970). Activity of acid phosphatase in the liver of fish exposed to heavy metals provides a measurement of the hydrolase latency and lysosomal membrane stability. It furnishes information on mechanisms involving molecular alterations in the lysosomal membrane, which undoubtedly contributes to disturbances of integrated cellular function (Slater, 1978).

Aminotransferases are a group of enzymes that catalyze the

process of biological transamination. Aspartate aminotransferase forms a major one in this group of enzymes. They represent a link between carbohydrate, fat and protein metabolism (Cohen and Sallach, 1961) providing a source of keto acids for Kreb's cycle and gluconeogenesis. Their importance in amino acid metabolism and gluconeogenesis has also been reported by Wilson (1973). These mechanisms provide additional energy to meet the increased demand under stress (Weber, 1963). The role of aspartate aminotransferase in the nitrogen metabolism and the production of animal energy has been demonstrated by Gould (1977). These enzymes are widely distributed in all organisms and serve as an indicator of cell destruction and altered physiological or stress condition (Knox and Greengard, 1965). Weiser and Hinterleitnar (1980) have reported the utility of the estimations of aspartate aminotransferase as a tool in diagnosis of water quality where they noticed high enzyme activity as pollutant load increases. The use of transaminases in diagnosis of tissue damage in fishes was investigated by Mollander *et al.* (1955), Wroblewski and La Due (1956), Wroblewski *et al.* (1956) and Bell (1968). Bell (1968) has discussed the practical value of aspartate aminotransferases estimation in serum of salmon for the detection of apparently healthy fish from those treated with hepatic poison, bromobenzene or those affected by bacterial kidney disease. Apart from the above works, serum aspartate aminotransferase and alanine aminotransferase were frequently used by fish pathologists to diagnose sublethal insult or injury

to liver by pollution (Mehrle and Bloomfield, 1974; Racicot *et al.*, 1975).

Glutamate dehydrogenase is a mitochondrial enzyme containing zinc. It is one of the primary enzymes concerned with amino acid metabolism and gluconeogenesis (Wilson, 1973). The reversible conversion of L-glutamic acid to α ketoglutarate - a member of citric acid cycle - is mediated by glutamate dehydrogenase. The enzyme serves as a link between the metabolisms of amino acids and carbohydrates. The reaction between L-glutamic acid and the pyridine nucleotides is readily reversible and is probably of prime importance in the nitrogen metabolism of all living systems. Role of glutamate dehydrogenase has been proposed to explain the sparing effects exerted by carbohydrates and/or fats in higher animals (Munro, 1951). Although the exact mechanism by which carbohydrates spare proteins is not understood, it has been proposed that the oxidation of excess carbohydrate and/or fat during stress results in an increase of available NADH (Miller *et al.*, 1955). An enhanced glutamate dehydrogenase activity results in the formation of glutamic acid from ammonia that would otherwise be excreted. The glutamic acid-nitrogen is then used in transamination reactions and the resultant amino acids are *stored* as protein.

HMG Co A reductase catalyzes the conversion of β-hydroxy β

methyl glutaryl Co A to mevalonate and is found attached to the endoplasmic reticulum. HMG Co A and mevalonate are the important intermediates in the biosynthesis of both cholesterol and ketone bodies. The primary regulation of cholesterol biosynthesis is centered on the HMG Co A reductase reaction (Zubay, 1988). Hepatic lipogenesis has been reported as a defense mechanism in fish by Basaglia *et al.* (1992). Hence the enhanced synthesis of cholesterol mediated by this enzyme may be important in counteracting the increased influx of heavy metals into the body of the fish.

Like glutamate dehydrogenase, lactate dehydrogenase also is a zinc containing enzyme. These are generally associated with cellular metabolic activities and play a key role in energy metabolism. Lactate dehydrogenase acts as a pivotal enzyme between the glycolytic pathway and the tricarboxylic acid cycle. They catalyze the conversion of pyruvate into lactate under anaerobic conditions. A fish under stress preferentially meets its energy requirements through anaerobic oxidation. Lactate dehydrogenase can thus be used as an indicator in monitoring metal-induced toxicity in fish (Balavenkatasubbaiah *et al.*, 1984).

Serine hydrolyase or serine dehydratase plays a very significant role in gluconeogenesis and metabolism of amino acids. The glycogenic action of serine is explained by the

production of pyruvic acid. Serine in vivo can give rise to pyruvic acid. The α and β carbon atoms of the amino acid can also be considered as a source of C-2 units and thus to fatty acid and cholesterol (Zubay, 1988). Thus like HMG Co A reductase, serine hydrolyase also plays a significant role in lipogenesis and hence they form an important defense mechanism against heavy metal toxicity (Basaglia *et al.*, 1992).

4.2 MATERIALS AND METHODS

Collection and acclimation of fish, mode of dosing and the collection of liver sample were same as that described in the previous experiments.

Acid phosphatase (EC 3.1.3.2) : The tissue was homogenized in citrate buffer (pH 4.8) and was centrifuged at 20,000g for 30 min. at $0^{o}C$ in a refrigerated centrifuge. The supernatant was employed as the enzyme sample. The acid phosphatase activity was assayed following the procedure of Anon (1963) with slight modification. 0.5 ml of citrate buffer (pH 4.8) and 0.5 ml of p-nitrophenyl phosphate (400 mg in 100 ml distilled water) were incubated for 3 min. at $37^{o}C$. 0.3 ml of the enzyme sample and 0.3 ml of 2 mM mercaptoethanol (Philip, personal communication) were added to the above mixture and was incubated for 30 min. at $37^{o}C$. Reaction was stopped by the addition of 4 ml 0.1 N NaOH. The p-nitrophenol formed was measured at 410 nm. Amount of

p-nitrophenol released was calibrated from a standard graph. Specific acid phosphatase activity was expressed as millimoles of p-nitrophenol formed per hour per gram protein, using bovine serum albumin as standard for protein estimation.

Aspartate aminotransferase (EC 2.6.1.1) : The tissue was homogenized in 50 mM phosphate buffer (pH 7.5). The homogenate was centrifuged at 20,000g for 30 min. in a refrigerated centrifuge at $0^{o}C$. The supernatant was saved and was employed as the enzyme sample. The activity was assayed following the method of Reitman and Frankel (1957) and Wootton (1964). 0.5 ml of the buffered substrate was warmed for 3 min. at $37^{o}C$. 0.3 ml of enzyme sample was added to all the tubes except the blank and incubated for 1 hour at $37^{o}C$. 1 ml of 1 mM 2,4-Dinitrophenylhydrazine was added to stop the enzyme activity. The mixture was shaken and allowed to stand for 20 min. at room temperature. The color reaction was stopped by the addition of 5 ml of 0.4 N NaOH. The hydrazone formed was measured spectrophotometrically at 510 nm, after 15 min. The pyruvate released was determined from a standard graph prepared using sodium pyruvate. The activity of the enzyme was expressed as micromoles of pyruvate formed per hour per gram protein. Protein content of the extract was determined following the procedure of Lowry *et al.* (1951) using bovine serum albumin as standard.

Glutamate dehydrogenase (EC 1.4.1.2): The tissue was homogenized in 5 mM Tris-HCl buffer (pH 7.8) containing 0.1% (v/v) Triton X-100. The homogenate was centrifuged at 20,000g for 30 min. in a refrigerated centrifuge at $0^{o}C$. The supernatant was saved. The enzyme assay was carried out using this supernatant (Hayashi, 1987). The enzyme activity was assayed following the method of Reiss *et al.* (1977) and Hayashi (1987). The reaction mixture (2.6 ml) consisted of 100 mM Tris-HCl buffer (pH 7.8), 10 mM α-ketoglutarate, 100 mM ammonium acetate, 0.2 mM NADH, 1 mM ADP and enzyme preparation. The reaction was initiated by the addition of enzyme and was performed at $30^{o}C$. Change in absorbance at 340 nm corresponding to the conversion of NADH to NAD^{+} was measured using a spectrophotometer. The activity of glutamate dehydrogenase was found out from the standard graph of NADH prepared and was expressed as micromoles of NADH oxidized per hour per gm protein. Protein content of the extract was estimated by the procedure of Lowry *et al.* (1951) with bovine serum albumin as standard.

HMG Co A reductase (EC 1.1.1.34): HMG Co A reductase activity of the liver was estimated following the method described by Rao and Ramakrishnan (1975). The tissue was homogenized in 10% saline arsenate. Equal volumes of the above solution and dilute perchloric acid were mixed, kept for 5 min. and was centrifuged at 6,000g for 10 min. at $0^{o}C$ in a refrigerated centrifuge. To 1 ml of the filtrate, 0.5 ml of

freshly prepared hydroxyl amine reagent was added and mixed. This was meant for the assay of mevalonate. For the assay of HMG Co A, alkaline hydroxyl amine reagent was used. After 5 min. at room temperature, 1.5 ml of ferric chloride was added. The mixture was kept at room temperature for 10 min. and the absorbance was measured at 540 nm after shaking the mixture well, against a similarly treated saline arsenate blank. The ratio between the HMG Co A and the mevalonate was taken as an index of the activity of the enzyme, which was expressed as activity of enzyme per minute per gm protein. Protein content of the extract was estimated by the procedure of Lowry *et al.* (1951) with bovine serum albumin as standard.

Lactate dehydrogenase (EC 1.1.1.27): Preparation of the enzyme sample was done as given for aspartate aminotransferase. Lactate dehydrogenase activity was measured following the method of Bergmeyer and Bernt (1974). The reaction mixture consisted of 50 mM phosphate buffer (pH 7.5), 0.6 mM pyruvate, 0.18 mM NADH and the enzyme preparation. The reaction was initiated by the addition of enzyme and the assay was carried out at 30°C. The activity was determined from the rate of oxidation of NADH. The change in extinction at 340 nm was measured in a spectrophotometer. A standard graph of NADH was prepared and the activity of the enzyme was expressed as mg of NADH oxidized per hour per gm protein in the sample. Protein content of the sample was estimated by following the procedure of Lowry *et al.* (1951)

using bovine serum albumin as standard.

Serine hydrolyase (EC 4.2.1.1.3): The liver removed from the fish was homogenized in 0.1 M phosphate buffer (pH 8.0). The homogenate was centrifuged at 20,000g for 30 min. in a refrigerated centrifuge at 0°C. The supernatant was saved and employed as the enzyme sample. The activity of serine hydrolyase was assayed following the method of Suda and Nakagawa (1971). The mixture containing 0.1 ml of 0.5 mM pyridoxal phosphate, 0.5 ml of 0.1 M phosphate buffer (pH 8.0) containing 2 mM EDTA and 0.2 ml enzyme extract was incubated at 37°C for 5 min. To this mixture was added 0.2 ml of serine, previously warmed to 37°C. The reaction mixture was incubated at 37°C for 5 min. To all the tubes were added 0.5 ml of 10% trichloro acetic acid. The tubes were then placed in ice bath for 10 min. and precipitate formed was removed by centrifugation. 0.5 ml of the supernatant was mixed with 0.5 ml of 0.033% 2,4-Dinitrophenylhydrazine (in 2N HCl). After keeping the mixture at room temperature for 5 min., 2 ml of 2 N NaOH was added to all the tubes. The extinction was read at 520 nm, within 5 min. Amount of pyruvate formed was calculated from a standard graph and the specific activity was expressed as millimoles of pyruvate formed per hour per gm protein. Protein content was assayed following the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

The effect of in vitro exposure to zinc and mercury on all

the above enzymes was studied following the same procedures as for in vivo study. The concentrations of metals employed were 0.002, 0.02, 0.2, 2.0, 20 and 200 ppm.

4.3 RESULTS

The data were analyzed statistically using ANOVA technique. Wherever the effects were found to be significant, least significant difference at 5% level was calculated.

The effects of in vivo and in vitro exposure to zinc and mercury on the activity of acid phosphatase are summarized in Table 4A (Fig.12) and Table 4B (Fig.13) respectively. It is seen that in vivo exposure has produced no significant difference between various groups studied. In vivo exposure to mercury has got an activating effect on the enzyme. Maximum activity is observed on 6 th day of exposure and this activity varies significantly from the enzyme activity at 2 nd day. After 6 th day of exposure the activity remains without any marked variation. Zinc has got an evident stimulatory effect on acid phosphatase. But this stimulatory effect goes on decreasing as the period of exposure increases. The stimulatory or inhibitory effect produced by in vitro exposure to different concentrations of zinc and mercury, is not significant at 5% level except that between the effect of 0.002 ppm and 200 ppm concentrations of both the metals.

Activity of aspartate aminotransferase when exposed to zinc and mercury in vivo and in vitro is shown in Table 5A (Fig.14) and Table 5B (Fig.15) respectively. In vivo exposure to both the xenobiotics has no significant effect on the activity of this enzyme at 5% level. Presence of mercury is eliciting an inhibitory response at all periods of exposure. Maximum activity due to mercury exposure which is still inhibitory is occurring after 6 days of exposure. Zinc also is inhibitory except on 2 nd day. There is, however, significant difference between the activity of enzyme along different periods of exposure ($P<0.05$). Least significant difference at 5% level is 27.23. The effects of 4 days of exposure to the toxicants are found to be significantly different from that due to 2 days of exposure.

In vitro studies reveal a similar pattern, which also is not significant at 5% level. Mercury is inhibitory at all concentrations tested. The intensity of inhibitory effect increases up to 0.2 ppm, above which a relieving tendency from the inhibitory action is noticed. The enzyme activity thus shows a rise and attains maximum value at 200 ppm, which is still less than the control. In the case of zinc the inhibitory effect at 0.002 ppm is followed by an increase in activity, which attains control values at 0.2 ppm. However, after 0.2 ppm the activity shows a regular decline.

Table 6A (Fig.16) and Table 6B (Fig.17) depict the effect

of in vivo and in vitro exposure to zinc and mercury on the activity of glutamate dehydrogenase. The difference in activity produced by the two xenobiotics is not significant at 5% level. But the difference in activity produced by various periods of exposure is significant at 5% level ($P<0.001$). Least significant difference at 5% level is 140.31. The enzyme activity due to in vivo exposure to mercury for different periods has got a sigmoidal activity curve with maximum activity attained at 6 th day. Mercury on 4 th and 6 th day of exposure is stimulatory whereas that on 2 nd and 8 th day is inhibitory. Zinc has got a significant stimulatory effect on 2 nd day of exposure after which the stimulatory effect goes on diminishing.

In vitro studies clearly indicate that the effect of zinc and mercury is significantly different ($P<0.01$) from the control. Both zinc and mercury have a stimulatory effect. The activation due to mercury is maximum at 0.002 ppm. This stimulatory effect is found to be inversely proportional to the concentration. But the activation due to zinc is directly proportional to the concentration of the metal.

Modifications in the activity of HMG Co A reductase produced by exposure to zinc and mercury in vivo and in vitro are summarized in Table 7A (Fig.18) and Table 7B (Fig.19) respectively. The difference between the effect of both the xenobiotics is not significant at 5% level. But significant

difference is noticed between the effect of different periods of exposure to both zinc and mercury ($P<0.001$). Least significant difference at 5% level is 7.58. In vivo studies of the effect of different periods of exposure show a sigmoid pattern with maximum activity attained on 4 th day of exposure to zinc and mercury. At 2 nd day of exposure both have an inhibitory effect, whereas at all other periods the enzyme is showing higher levels of activity.

In vitro studies reveal an inhibitory effect for zinc at all concentrations tested. But mercury activates the enzyme at 0.02, 2 and 20 ppm levels, where the enhancement due to 20 ppm mercury is about seven times that of control.

The effect of exposure to zinc and mercury on the activity of lactate dehydrogenase in vivo and in vitro is represented in Table 8A (Fig.20) and Table 8B (Fig.21) respectively. The difference in activity of lactate dehydrogenase produced due to in vivo exposure to zinc and mercury is not significant at 5% level. But significant difference is noticed between the activity of enzyme due to different periods of exposure ($P<0.05$). The least significant difference at 5% level is 271.98. Highest activity of the enzyme as a result of zinc exposure is seen at 2 nd day, after which the activity goes on decreasing. The enzyme activity in presence of mercury over different periods of exposure shows a sigmoidal

curve. The effect of 2 days of exposure is inhibitory, whereas an accelerating effect is noticed at all other periods of exposure.

There is no significant variation between the effects produced by various concentrations of zinc and mercury on the enzyme in vitro. However between the effect of different concentrations of individual xenobiotic there is significant difference. The enzyme exhibits a sigmoidal activity curve along different concentrations of both zinc and mercury. The maximum enzyme activity occurs in presence of 0.2 ppm zinc and 2 ppm mercury. 0.002 ppm and 200 ppm of mercury have an inhibitory effect whereas all other concentrations of both the metal are having an enhancing effect.

Serine hydrolyase activity in presence of zinc and mercury both in vivo and in vitro is given in Table 9A (Fig.22) and Table 9B (Fig.23)respectively. The variation between the effects of zinc and mercury on the enzyme is not significant at 5% level. But the effects due to different periods of exposure to each metal varies significantly ($P<0.001$). Least significant difference at 5% level is 9.96. Both zinc and mercury exert a stimulatory effect at all periods of exposure. The maximum activity is observed at the 2 nd day of exposure and thereafter the stimulatory effect goes on decreasing in both cases.

TABLE : 4

Effect of Zn and Hg on the activity of acid phosphatase of *O. mossambicus*.

The activity of the enzyme is expressed as millimoles of p-nitrophenol formed/hour/gm protein.

A. IN VIVO

TIME	CONTROL		MERCURY		ZINC	
Days	ACTIVITY	%	ACTIVITY	%	ACTIVITY	%
2	09.58 ± 0.06	100	10.18 ± 0.05	106.26	16.24 ± 0.05	169.52
4	11.42 ± 0.21	100	12.88 ± 0.10	112.78	14.30 ± 0.09	125.22
6	16.10 ± 1.16	100	18.49 ± 0.59	114.84	14.95 ± 0.08	92.86
8	12.32 ± 0.09	100	14.11 ± 0.03	114.50	12.87 ± 0.03	104.45

B. IN VITRO

CONC. OF Zn/Hg (ppm)	MERCURY		ZINC	
	ACTIVITY	%	ACTIVITY	%
00.002	145.13 ± 05.66	110.68	133.01 ± 00.00	101.44
00.02	113.62 ± 18.85	086.65	133.28 ± 03.50	101.65
00.20	139.20 ± 01.88	106.16	126.28 ± 01.88	096.30
02.0	136.24 ± 00.00	103.90	126.01 ± 01.61	096.10
20.0	131.40 ± 05.93	100.21	133.82 ± 07.27	102.06
200.0	88.58 ± 01.34	067.56	135.16 ± 00.54	103.08

Values are the mean of eight different experiments ± SD.

TABLE : 5

Effect of Zn and Hg on the activity of aspartate aminotransferase of *O. mossambicus*.

The activity of the enzyme is expressed as micromoles of pyruvate formed/hour/gm protein.

A. IN VIVO

TIME	CONTROL		MERCURY		ZINC	
Days	ACTIVITY	%	ACTIVITY	%	ACTIVITY	%
2	71.56 ± 5.37	100	59.08 ± 3.64	82.56	111.46 ± 2.98	155.76
4	47.59 ± 0.00	100	40.98 ± 5.01	86.11	28.28 ± 10.4	59.42
6	75.56 ± 0.00	100	72.89 ± 8.50	96.47	73.98 ± 2.18	97.91
8	91.92 ± 5.16	100	76.64 ± 6.02	83.37	84.68 ± 0.00	92.12

B. IN VITRO

CONC. OF Zn/Hg (ppm)	MERCURY		ZINC	
	ACTIVITY	%	ACTIVITY	%
00.002	120.47 ± 08.00	39.48	240.90 ± 16.12	78.94
00.02	080.30 ± 16.06	26.31	176.64 ± 16.06	57.88
00.20	080.01 ± 15.63	26.22	305.17 ± 16.06	100.00
02.0	120.47 ± 08.00	39.48	224.78 ± 00.00	73.66
20.0	144.53 ± 16.06	47.36	176.64 ± 16.06	57.88
200.0	176.64 ± 16.06	57.88	120.47 ± 08.00	39.48

Values are the mean of eight different experiments ± SD.

TABLE : 6

Effect of Zn and Hg on the activity of glutamate dehydrogenase of *O. mossambicus*.

The activity of the enzyme is expressed as micromoles of NADH oxidized/hour/gm protein.

A. IN VIVO

TIME	CONTROL		MERCURY		ZINC	
Days	ACTIVITY	%	ACTIVITY	%	ACTIVITY	%
2	299.55 ± 22.6	100	291.88 ± 36.8	97.44	440.47 ± 49.2	147.04
4	888.48 ± 30.2	100	950.97 ± 14.7	107.03	963.83 ± 21.9	108.48
6	616.22 ± 62.7	100	714.33 ± 14.9	115.92	519.80 ± 36.3	84.35
8	634.89 ± 35.2	100	553.50 ± 29.8	87.18	654.03 ± 59.6	103.01

B. IN VITRO

CONC. OF Zn/Hg (ppm)	MERCURY		ZINC	
	ACTIVITY	%	ACTIVITY	%
00.002	547.46 ± 37.14	164.79	626.99 ± 16.24	188.73
00.02	458.53 ± 42.86	138.02	645.71 ± 28.08	194.36
00.20	495.94 ± 16.18	149.28	683.15 ± 42.88	205.63
02.0	500.64 ± 21.45	150.70	655.08 ± 16.18	197.18
20.0	458.56 ± 16.18	138.03	767.35 ± 42.87	230.98
200.0	402.36 ± 08.15	121.11	898.33 ± 00.00	270.43

Values are the mean of eight different experiments ± SD.

TABLE : 7

Effect of Zn and Hg on the activity of HMG Co A reductase of *O. mossambicus*.

The activity of the enzyme is expressed as the ratio of mevalonate to HMG Co A/min./gm protein.

A. IN VIVO

TIME	CONTROL		MERCURY		ZINC	
Days	ACTIVITY	%	ACTIVITY	%	ACTIVITY	%
2	23.08 ± 0.05	100	18.82 ± 0.05	81.55	18.97 ± 0.00	82.20
4	15.58 ± 0.00	100	22.09 ± 0.05	141.78	23.94 ± 0.00	153.66
6	30.83 ± 0.07	100	43.56 ± 0.09	141.29	31.49 ± 0.08	102.14
8	41.44 ± 0.00	100	47.32 ± 0.66	114.19	44.76 ± 0.56	108.01

B. IN VITRO

CONC. OF Zn/Hg (ppm)	MERCURY		ZINC	
	ACTIVITY	%	ACTIVITY	%
00.002	297.35 ± 01.53	68.66	160.11 ± 00.00	36.97
00.02	652.64 ± 00.00	150.70	193.66 ± 00.00	44.72
00.20	378.16 ± 01.53	87.32	152.49 ± 07.63	35.21
02.0	491.00 ± 01.53	113.38	198.23 ± 15.25	45.77
20.0	2023.48 ± 00.00	467.25	228.73 ± 15.25	52.82
200.0	294.30 ± 01.53	67.96	378.16 ± 01.53	87.32

Values are the mean of eight different experiments ± SD

TABLE : 8

Effect of Zn and Hg on the activity of lactate dehydrogenase of *O. mossambicus*.

The activity of the enzyme is expressed as micromoles of NADH oxidized/hour/gm protein.

A. IN VIVO

TIME	CONTROL		MERCURY		ZINC	
Days	ACTIVITY	%	ACTIVITY	%	ACTIVITY	%
2	45.72 ± 6.09	100	30.78 ± 5.20	67.32	103.69 ± 10.6	226.79
4	61.15 ± 8.15	100	83.81 ± 12.1	137.06	73.34 ± 2.04	119.93
6	70.40 ± 11.1	100	104.58 ± 20.9	148.55	69.17 ± 3.87	98.25
8	154.04 ± 3.65	100	174.78 ± 6.43	113.46	149.48 ± 51.2	97.04

B. IN VITRO

CONC. OF Zn/Hg (ppm)	MERCURY		ZINC	
	ACTIVITY	%	ACTIVITY	%
00.002	3.36 ± 00.24	97.67	3.59 ± 00.36	104.54
00.02	3.98 ± 00.24	115.92	3.94 ± 00.07	114.78
00.20	3.67 ± 00.14	106.81	4.22 ± 00.23	122.73
02.0	4.09 ± 00.21	119.00	3.87 ± 00.12	112.51
20.0	3.51 ± 00.00	102.27	4.18 ± 00.07	121.59
200.0	3.05 ± 00.20	88.62	3.44 ± 00.49	100.03

Values are the mean of eight different experiments ± SD

TABLE : 9

Effect of Zn and Hg on the activity of serine hydrolyase of *O. mossambicus*.

The activity of the enzyme is expressed as millimoles of pyruvate formed/hour/gm protein.

A. IN VIVO

TIME	CONTROL		MERCURY		ZINC	
Days	ACTIVITY	%	ACTIVITY	%	ACTIVITY	%
2	22.94 ± 0.82	100	27.13 ± 0.00	118.27	41.30 ± 1.47	180.04
4	42.94 ± 1.90	100	43.18 ± 5.35	100.55	59.43 ± 1.43	138.39
6	70.28 ± 1.55	100	78.19 ± 6.40	111.25	73.06 ± 0.00	103.95
8	51.76 ± 1.16	100	55.02 ± 2.45	106.29	54.08 ± 7.21	104.47

B. IN VITRO

CONC. OF Zn/Hg (ppm)	MERCURY		ZINC	
	ACTIVITY	%	ACTIVITY	%
00.002	529.19 ± 54.73	082.85	511.00 ± 72.95	080.00
00.02	492.76 ± 36.43	077.14	474.52 ± 36.52	074.29
00.20	365.00 ± 00.00	057.14	383.24 ± 18.22	060.00
02.0	438.03 ± 73.03	068.57	401.48 ± 72.95	062.85
20.0	401.48 ± 18.22	062.85	438.03 ± 00.00	068.57
200.0	428.85 ± 18.22	067.14	438.03 ± 73.03	068.57

Values are the mean of eight different experiments ± SD.

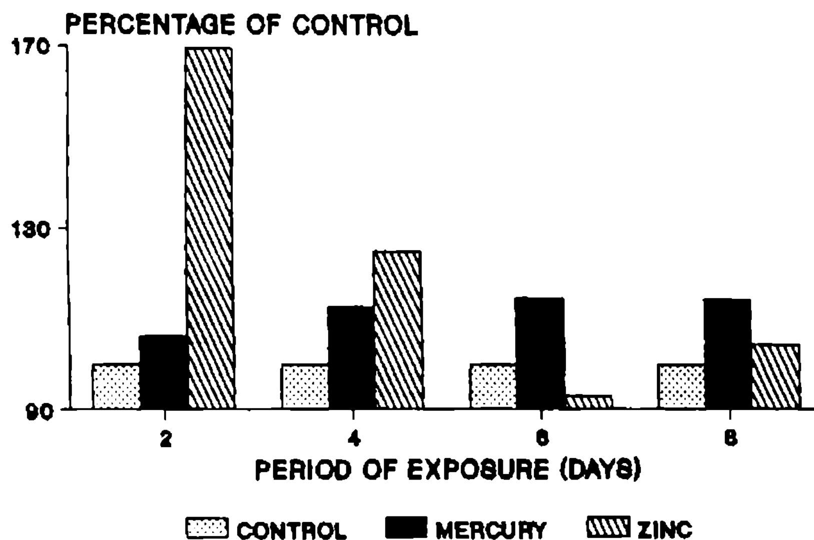

Fig.12 Activity of acid phosphatase in *Oreochromis mossambicus* exposed to zinc and mercury for different periods of time in vivo.

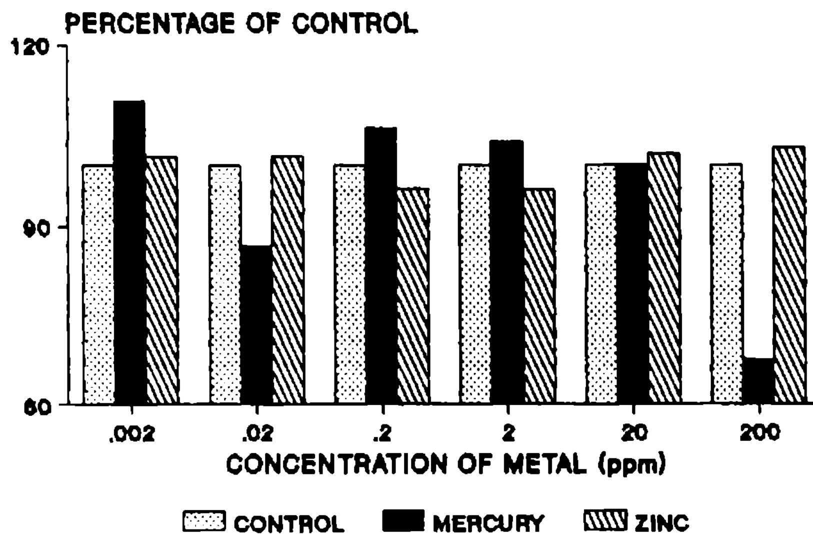

Fig.13 Activity of acid phosphatase in *Oreochromis mossambicus* exposed to different concentrations of zinc and mercury in vitro.

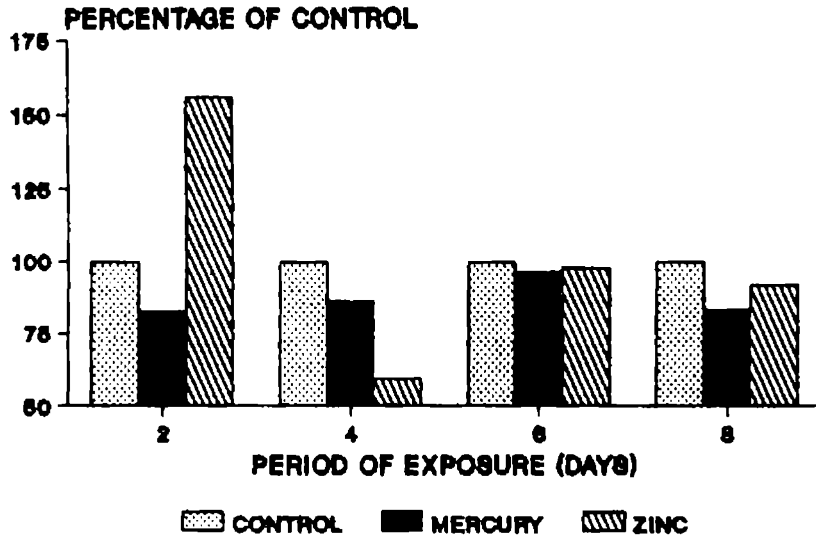

Fig.14 Activity of aspartate aminotransferase in *Oreochromis mossambicus* exposed to zinc and mercury for different periods of time in vivo.

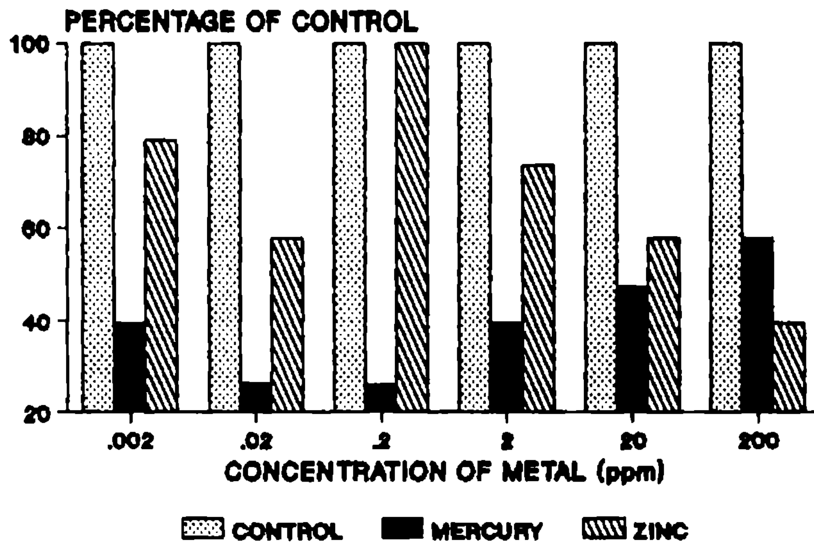

Fig.15 Activity of aspartate aminotransferase in *Oreochromis mossambicus* exposed to different concentrations of zinc and mercury in vitro.

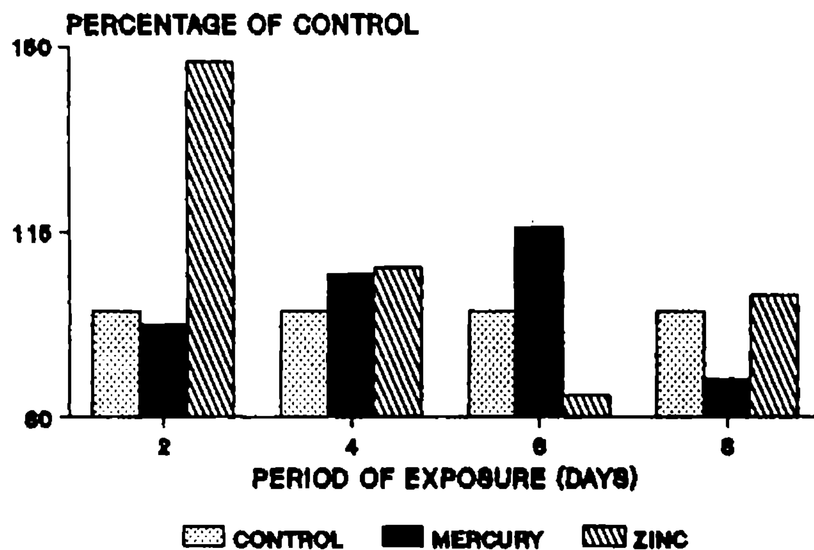

Fig.16 Activity of glutamate dehydrogenase in *Oreochromis mossambicus* exposed to zinc and mercury for different periods of time in vivo.

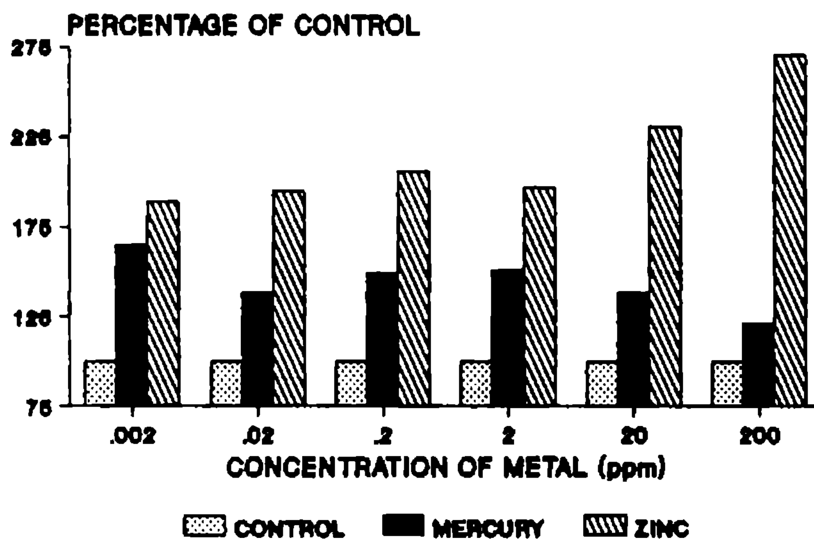

Fig.17 Activity of glutamate dehydrogenase in *Oreochromis mossambicus* exposed to different concentrations of zinc and mercury in vitro.

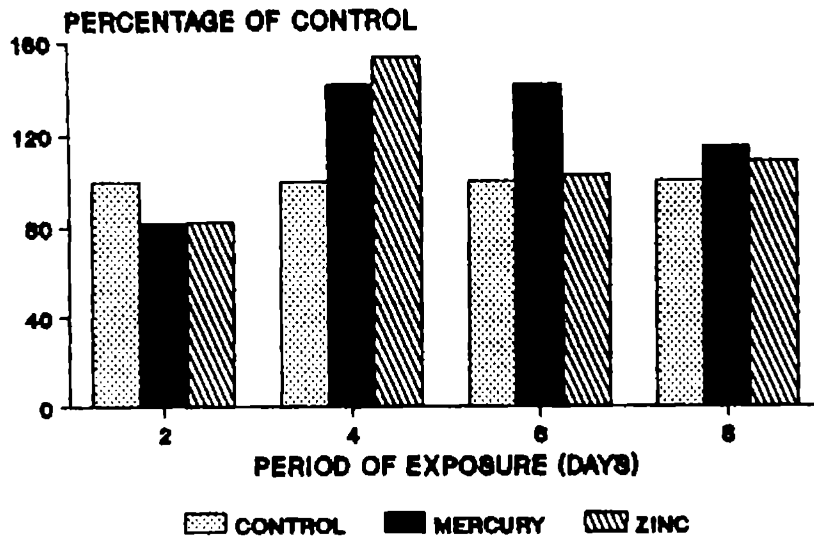

Fig.18 Activity of HMG Co A reductase in *Oreochromis mossambicus* exposed to zinc and mercury for different periods of time in vivo.

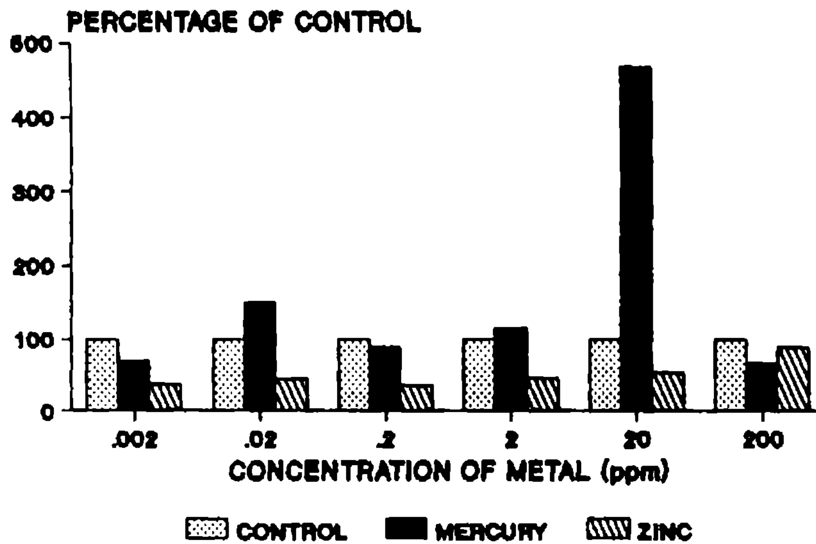

Fig.19 Activity of HMG Co A reductase in *Oreochromis mossambicus* exposed to different concentrations of zinc and mercury in vitro.

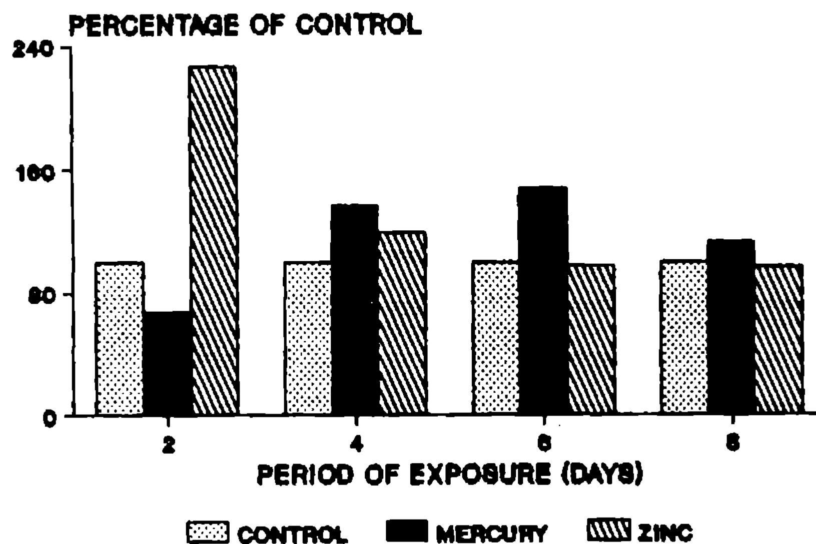

Fig.20 Activity of lactate dehydrogenase in *Oreochromis mossambicus* exposed to zinc and mercury for different periods of time in vivo.

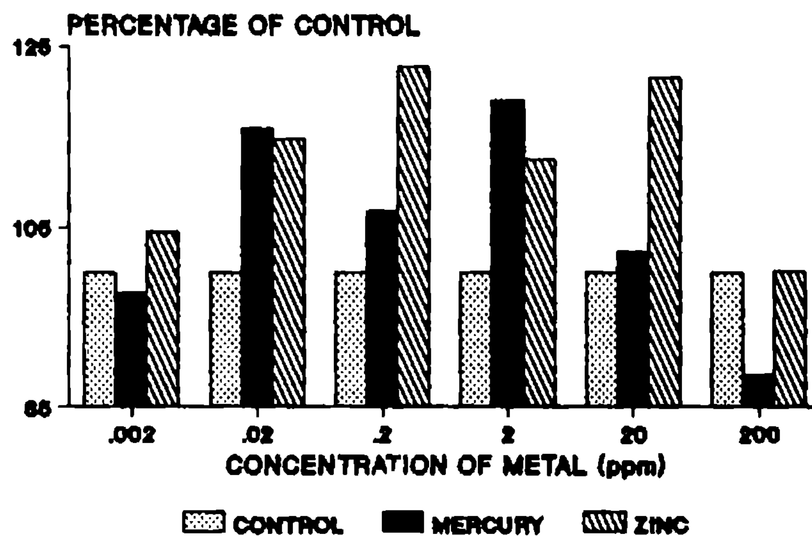

Fig.21 Activity of lactate dehydrogenase in *Oreochromis mossambicus* exposed to different concentrations of zinc and mercury in vitro.

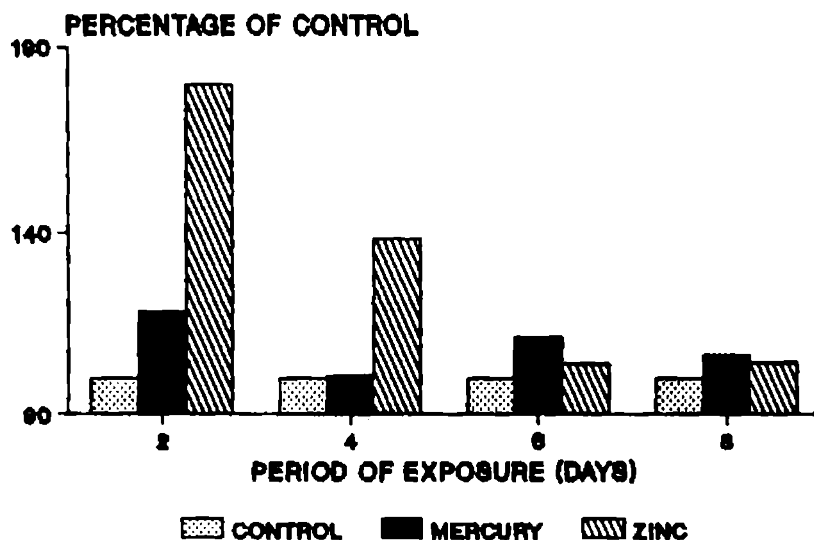

Fig.22 Activity of serine hydrolyase in *Oreochromis mossambicus* exposed to zinc and mercury for different periods of time in vivo.

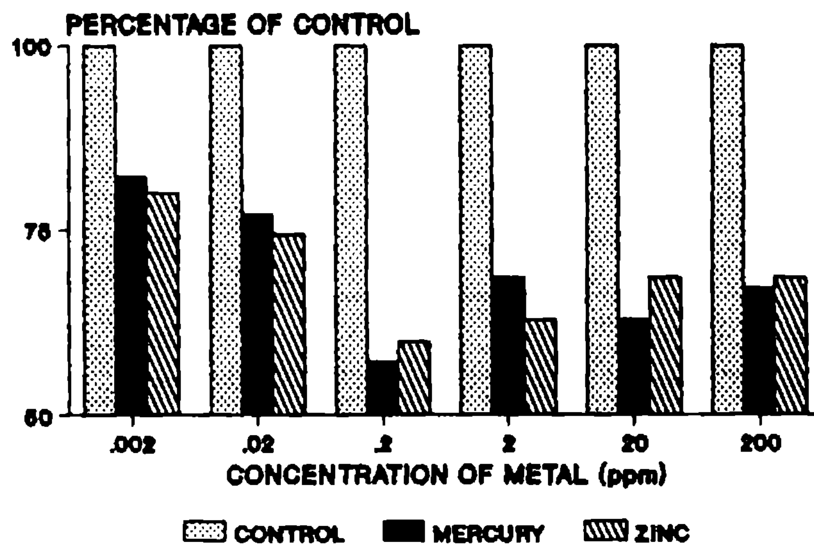

Fig.23 Activity of serine hydrolyase in *Oreochromis mossambicus* exposed to different concentrations of zinc and mercury in vitro.

In vitro exposure to zinc and mercury also does not produce any marked variation in the enzyme activity. However the variation in enzyme activity produced by different concentrations of each metal is significant ($P<0.001$). Least significant difference at 5% level is 28.7. All concentrations of both the metal are inhibitory. The inhibitory effect is intense when subjected to 0.2 ppm concentration of both the metals.

4.4 DISCUSSION

Studies on the effect of exposure of *O. mossambicus* to sublethal concentrations of zinc and mercury revealed that the activity of almost all enzymes monitored, varied depending on the dose and duration of exposure to the toxicant. Results of the in vitro studies show that there was an optimum level of the metal for the enzyme to be active, before and above which the metal inhibits the activity of the enzyme.

In vivo exposure to sublethal concentrations of both zinc and mercury for different periods produced an enhanced acid phosphatase activity. The elevation in acid phosphatase activity in the present study could be attributed to the heavy metal-induced changes in lysosomal latency. The induction of acid phosphatase seems to be needed for the conversion of the toxic material into less toxic products (Hinton *et al.*, 1973; Armstrong, 1979; Doull *et al.*, 1980; Passino, 1981). Several mechanisms have been suggested for the release of hydrolase from lysosomes.

Increase in acid phosphatase activity may be due to alteration in osteoblasts resulting in more production and liberation of acid phosphatase (Cantarow and Schepartz, 1967), proliferation of smooth endoplasmic reticulum in the parenchymatous cells that leads to enhanced production and release of microsomal enzymes (Hart and Fouts, 1965), peroxidation of lysosomal membrane resulting in membrane break down or increase in permeability or both, leading to an enhanced activity of acid phosphatase (Novikoff, 1961), or degeneration and necrosis of tissues (Nagarathnamma, 1982) which results in the release of acid phosphatase. Tissue necrosis and elevated acid phosphatase activity has also been observed by Onikieno (1963). Increased acid phosphatase activity may also be attributed to hyperglycemia to meet energy requirements during stress. In the present study a direct positive correlation is noticed between the duration of exposure to mercury and the activity of acid phosphatase. This indicates that increased internal content of the toxicant on prolonged exposure causes a greater tissue damage and increase in the activity of acid phosphatase. Nagarathnamma (1982) has made a similar observation in *Cyprinus carpio* exposed to organophosphate pesticide.

Stimulation of acid phosphatase activity in presence of sublethal levels of mercury has been reported by Hossain and Dutta (1986). A mercury induced enhancement of acid phosphatase activity has also been demonstrated by Jackim *et al.* (1970) and

Hinton and Koenig (1975). It may be thus generalized that chronic toxicity of mercury causes phosphatase activation.

A gradual reduction in acid phosphatase activity from the 2 nd day onwards is noticed during exposure to zinc. A similar decline in acid phosphatase activity in crabs exposed to sublethal concentrations of methyl parathion for a period of 30 days has been reported by Reddy *et al.* (1986). It has been explained that on prolonged exposure, the organism might counteract the deleterious effects of zinc at cellular level and the activity of acid phosphatase becomes more or less similar to that of control. Studies by Nagarathnamma (1982) have shown that on prolonged exposure to sublethal concentrations of methyl parathion, signs of histological recovery occur on 10 th day. With the beginning of recovery the activity of acid phosphatase started declining and it gradually approached control values, indicating the major role of this enzyme during damage rather than in recovery. A gradual decline in acid phosphatase activity with increasing concentration or extended exposure to the toxicant has also been observed by Saxena *et al.* (1982) and Sharma (1990). This reduction in the activity of acid phosphatase may be due to the direct effect of zinc on the enzyme, stabilization of lysosomal membrane by zinc, impairment of lysosomal metabolism in liver or the decreased synthesis of the enzyme. Variation in activity of acid phosphatase with concentration and duration of exposure to the toxicant metal as

seen in the present study has also been reported by Hinton and Koenig (1975) and Sastry and Gupta (1978).

An increased activity of acid phosphatase than that of the control is seen on 2 nd day in fish exposed to zinc. This is in agreement with the labilization of lysosomal membrane observed after 2 days of exposure and hence the high acid phosphatase activity due to zinc established by our earlier works (chapter 3) and also of others. This may be due to the fact that the onset of the regulatory response for this essential metal is not as fast as those for toxic mercury. Thus the increased acid phosphatase activity may represent increase in number, function or damage of lysosomes. The decline in the acid phosphatase activity following may reflect the progressive stabilization.

The general trend followed by acid phosphatase activity on exposure to zinc and mercury is in consistence with the results of the study of their effect on lysosomal membrane stability. However, from 4 th day onwards there occurs a significant stabilization of the lysosomal membrane. Though the activity of acid phosphatase from 4 th day onwards is less than that at 2 nd day, it is greater than the control. On 2 nd day, mercury induces a stabilizing effect on lysosomal membrane which should have resulted in a lesser amount of acid phosphatase than the control. But here, the acid phosphatase activity is greater than the control. From this observation it may be inferred that

though lysosomes are the major source of acid phosphatase, there exists some other sources such as mitochondria etc., which contributes to the total acid phosphatase activity in the liver of stressed fish.

Data indicate that during the whole period of exposure mercury has an inhibitory effect on aspartate aminotransferase. It seems probable that the inhibitory effect of the toxicant on aspartate aminotransferase is at the point of pyridoxal phosphate synthesis. The sole source of pyridoxal phosphate which is an absolute requirement for aminotransferase activity was the tissue extract. Hence variation in intracellular pyridoxal phosphate can affect the activity of aspartate aminotransferase. Biosynthesis of this essential cofactor requires a divalent metal cation (Meister, 1955). Mercury may affect the availability of the divalent metal cations, thereby influencing the synthesis of pyridoxal phosphate. Other possible reasons for the decreased aspartate aminotransferase activity are the damage caused to mitochondrial membranes, loss of matrix and swelling of mitochondria which contains aspartate aminotransferase (Chow and Pond,1972). The decrease may also be attributed to the decreased availability of oxaloacetate or the direct inhibition of the enzyme by the metals as observed in our study. A significantly lower activity of aspartate aminotransferase in the liver of the fish exposed to toxicants has also been observed by Gould and Karolus (1974), MacInnes *et*

al. (1977), Christensen (1975), Dawson *et al.* (1977), Chetty *et al.* (1980), Koyama *et al.* (1985), Parashari and Saxena (1986) and Hilmy *et al.* (1985).

A biphasic response of aspartate aminotransferase, indicated by an initial induction followed by an ~~progressive~~ inhibition is produced by exposure to zinc. The initial stimulation of aspartate aminotransferase observed at 2 nd day possibly reflects the increased conversion of the amino acids to keto acids and vice versa, thus resulting in the disturbance of protein metabolism. An enhanced activity of aspartate aminotransferase has been reported by Verma *et al.* (1981) and Gupta *et al.* (1983). A dose and duration dependent stimulation or inhibition of aspartate aminotransferase suggests a potential for the toxicant to interfere with processes related to amino acid metabolism inside the body of the fish (Verma *et al.*, 1982). The decline in the activity resulted from 4 th day onwards may be attributed to the direct inhibition of the enzyme by the metal as noticed in the present study. Such a response of aspartate aminotransferase to the toxicant has also been demonstrated by Jackim *et al.* (1970) and Christensen *et al.* (1972).

An enhanced activity of glutamate dehydrogenase at almost all periods of exposure suggests an increased amino acid metabolism and gluconeogenesis. The excess carbohydrates serve

as a source of energy for metabolic requirements during stress. At the same time, they exert a sparing effect on proteins. Thus, during toxicant induced stress, increased break down of carbohydrates results in elevated levels of NADH. This in turn affects the activity of liver glutamate dehydrogenase to form glutamic acid from ammonia which would have been otherwise excreted. This glutamic acid-nitrogen is then stored as protein reserves for use at emergencies (Miller *et al.*, 1955). The increase in glutamate dehydrogenase activity may also be due to the rupture of mitochondria which contains the major amount of glutamate dehydrogenase in the cell. An elevated glutamate dehydrogenase activity has been reported by Sastry and Rao (1982) in *Channa punctatus* in response to mercuric chloride. Prolonged exposure to both zinc and mercury ~~however~~ causes a decrease in the activity of glutamate dehydrogenase. This may be assigned to the reduction in the deamination process in the exposed fish. An inhibition of glutamate dehydrogenase activity in gill, muscle, brain, liver and kidney of fishes exposed to toxicants has also been revealed by Ghosh (1985).

Present studies reveal an initial inhibition of the HMG Co A reductase in presence of both the metals. This may be due to the damage produced to the endoplasmic reticulum to which the enzyme is bound (Zubay, 1988). Another possibility is the direct inhibition of the enzyme by both the metals as observed in the present study. However, a progressive stimulation of the enzyme

is seen from 4 th to 6 th day of exposure. This enhanced activity, which can lead to increased production of cholesterol and ketone bodies works as a defense mechanism against metal toxicity (Basaglia *et al.*, 1992). The in vivo stimulation of HMG Co A reductase may be achieved by regulation at the level of gene expression, by controlling the degradation of the enzyme or by activating the enzyme by phosphorylation (Zubay, 1988). A further decrease after 6 th day indicates the inefficiency of lipogenesis by this enzyme to trap the metal, the influx of which again exerts a deleterious effect on the enzyme. An increased HMG Co A reductase protein and mRNA levels in the small intestine has been reported in hyperglycemic condition (Feingold *et al.*, 1994).

In general, an enhanced activity of lactate dehydrogenase is noticed in presence of sublethal concentrations of zinc and mercury. This suggests that the stressed fish is meeting the lion-share of its energy requirements through anaerobic oxidation. The stimulated lactate dehydrogenase activity points to the fact that the pyruvate is preferentially transformed to lactate, thus favouring the anaerobic metabolism. It has been explained that the structural damage - the separation of epithelia from the underlying pillar cells of gill lamella - produced in the gill membranes increases the effective distance that oxygen must diffuse to reach the blood. This reduced efficiency of oxygen uptake leads to hypoxia and pyruvate is

preferentially converted to lactic acid (Skidmore, 1970). Enhanced lactate dehydrogenase activity thus means a stepped up glycolytic rate. An enhanced lactate dehydrogenase activity in *Tilapia mossambica* in response to toxicant stress has been reported by Koundinya and Ramamurthi (1978) and Balavenkatasubbaiah *et al.* (1984). An elevation in serum lactate dehydrogenase has been reported by Hilmy *et al.* (1985) and Christensen *et al.* (1972, 1977).

However, prolonged exposure registers a gradual decrease in lactate dehydrogenase activity which eventually approaches control. This drop in lactate dehydrogenase activity may represent a decline in the efficiency of anaerobic metabolic pathways. This may be related to the low rate of metabolism in the liver following extended exposure to sublethal concentrations of these toxicants (Verma *et al.*, 1979; Saxena *et al.*, 1982). A temporary metabolic adaptation with increased dependence on glycolysis followed by a decline in their metabolism as evidenced by our study, has also been reported by John (1974). A low lactate dehydrogenase activity due to impaired oxidative activities has been demonstrated by Sastry and Gupta (1980), Sastry and Sharma (1980), Sastry and Sunita (1983), Basaglia *et al.* (1992) and Ramesh and Manavalaramanujam (1993). The initial lactate dehydrogenase activity in the mercury dosed animals is very low and is significantly less than the control. This may refer to a situation where the regulatory

mechanisms are set in fast and mercury is maintained at a level which is not enough to impair the aerobic oxidative pathways. The organism is preferentially resorting to aerobic metabolic pathways and maintains a decreased lactate dehydrogenase activity.

Maximum serine hydrolyase activity at the 2 nd day of exposure followed by a gradual decrease on continued exposure is observed in the case of both the metals. This may indicate an augmented production of pyruvic acid and hence gluconeogenesis. An elevated synthesis of fatty acids and cholesterol whose carbon residues are derived from the amino acids may also result. This lipogenesis serves as a defense mechanism against heavy metal toxicity (Basaglia *et al.*, 1992). However, extended exposure to the metals makes this defense mechanism inefficient and the metals exert direct inhibitory effect on the enzyme as observed in the present study. This may explain the gradual decrease in the enzyme activity noticed after 2 nd day of exposure.

In this perspective backdrop, it may be concluded that considerable changes occur in the activities of various enzymes representing a disturbance in the metabolic processes of the organism. The difference in the response of the enzymes associated with the in vivo and in vitro exposure to zinc and mercury suggest that different molecular processes are involved

in the two types of exposure. Lack of correlation between in vivo and in vitro studies seen in some cases could be explained as due to the cellular or tissue barrier that excludes specific cations from active sites. In vivo activation could be indirect, through endocrine mechanisms as demonstrated in mammals (Knox *et al.*, 1956) or by interaction with regulators or cofactors. In short, the measurement of enzyme activity could indicate the effects of stress at an earlier stage than when the effects become evident at the level of organ or organism and represents a valuable tool in biological monitoring.

CHAPTER 5

5. BIOACCUMULATION OF HEAVY METALS

5.1 INTRODUCTION

The processes of accumulation and retention of a number of environmentally available materials by organisms provide an excellent means of screening the environment. Among these materials, heavy metals are of particular significance. This approach of understanding the nature of the environment has a number of obvious advantages. It is possible to detect pollutants in organisms even when the concentrations in the water are too low or too variable to be determined by routine analytical tools. Even if the detection of metals in the water is possible, the interpretation of the results may be difficult since some form of metal may be unavailable to the living system. Moreover, there occur analytical limitations in separating the metals from sediments, nutrients and a variety of other organic molecules. Furthermore, the relative importance of heavy metals varies from place to place in the aquatic system and their occurrence is not continuous. The intermittent events such as tides, influx of pollutants from rivers or industries may not be concomittent with the time of sampling. The process of bioaccumulation has attracted the environmentalists because ofthe following additional advantages it offers. It gives direct indication of pollution and may also have important influences on food chains. Moreover, it provides some easily measurable

indication of the ecological consequences of pollution.

From a physiologist's point of view, bioaccumulation is the phenomenon that is relatively easy to detect, but rather difficult to explain at the cellular level. In a number of cases, it provides a way to identify cellular sites of activity either by electron probe micro analysis (Walker *et al.*, 1975; Brown, 1977) or by cell fractionation (Coombs and George, 1978). It has been implied that physiological processes exist specifically for regulating and removing such interfering cations so that the deposits represent the end product of a detoxification system.

Fishes are good bioaccumulators and they concentrate the metals to a level that makes them easier to detect - an increase of thousands of times greater than that in the ambient medium (Onwumere and Oladimeji, 1990). Also, the accumulated levels reflect the concentration present in the environment. The process thus have two components, the uptake and retention, both showing a direct proportionality with the environmental concentration.

Studies of Ray *et al.* (1990) with *Clarias batrachus* have pointed out that monitoring bioaccumulation in specific tissues provides a better basis than whole body analysis. In the present study, liver, gill, caudal muscle, scale and skin of the heavy

metal exposed *Oreochromis mossambicus* were monitored for the extent of bioaccumulation.

Liver is the central metabolic machinery and the site of detoxification reactions in an animal. Hence, the metal accumulated within the liver is of biochemical significance. The content of fat in the liver has been found to be an important factor in the accumulation of metals (Grimaas *et al.*, 1985). Gills are the main site of entry of the xenobiotics. Gill provides the largest vascular and permeable area exposed to the medium. A temporal accumulation of mercury within gills of brown shrimp has been shown by Andersen and Baatrup (1988). It has been reported that the total mercury is uniformly distributed in the edible muscle of fin fish. Hence, a small sample of muscle tissue taken from any region will be representative of the whole muscle tissue (Freeman and Horne, 1973). Validity of muscle as a tool for biomonitoring has also been substantiated by Cossa *et al.* (1982), by working on the flounder *Platichthys flesus*. Skin with its extensive fatty tissues underneath represents another site for the storage of the metals. A high accumulation of heavy metals by the skin of plaice has been reported by Pentreath (1973a,b).

However, the bulk analyses of these soft tissues to monitor the bioaccumulation of heavy metals result only in a time-averaged metal content of the tissues. This masks any short

term changes in environmental metal exposure. But the elemental composition of scales of teleost fishes has been shown to reflect that of the aqueous environment. The incorporation of metals into the circuli - the concentric ridges on the scales formed during exposure period has been reported in *Fundulus heteroclitus* by Sauer and Watabe (1989). Fish scales as possible records of short term variability in environmental heavy metal exposure has also been reviewed by Sauer and Watabe (1985). Though partial remobilisation of metals from scales can occur, the residence time of metals in skeletal tissues is much longer than that in soft tissues (Pentreath, 1973a,b) and *metal signatures* may remain for long periods.

The rate at which accumulation occurs in an organism depends not only on the availability of the pollutant, but also on a whole range of biological, chemical and environmental factors. The ultimate level reached is governed by the ability of the organism to excrete the pollutant or alternately store it. Thus, even though in a number of cases there is evidence that the accumulation of the xenobiotic metal by the organism is proportional to the concentration in the external medium, this is true only in the case of non-essential metals. Mercury is a typical non-essential metal that has no known physiological function and has a deleterious effect on living system. It has a high affinity for molecules containing sulfhydryl groups. This strong affinity would tend to cause a considerable decrease in

mobility of mercury within the animal. Thus, tissues such as liver and gill which have more of sulfhydryl group containing molecules like metallothioneins, enzymes etc., would tend to accumulate greater amounts of mercury (Boudou and Ribeyre, 1985).

The biological level of essential metals like zinc has been reported to be independent of its ambient concentration. The bioaccumulation of this essential trace element is actively controlled by the animal (Bryan, 1979; Devineau and Triquet, 1985). It may be assumed that the internal zinc load could be under metabolic control. This ability of fish to regulate zinc has been reported by Saward *et al.* (1975) and Pentreath (1976a). Many studies have shown that the rate of ^{65}Zn accumulation decreases for several days following commencement of exposure and the internal ^{65}Zn concentration may only stabilize after many weeks as in carp (Lebedeva and Kuznetsova, 1969) and plaice (Pentreath, 1973a).

Considering the effect of heavy metals on the organism, what is important is the nature in which it is present in the biological system. Metals found in many fishes would be lethal if they existed as free ions. However, it has already been established that metals occur as deposits which represent sites of detoxification where they are compartmentalised or precipitated in a form which renders them harmless to the

organism. Both mercury and zinc are type B metals or soft acids with preference for ligands in the order S>N>O. Liver, gill etc. which are rich in sulfhydryl containing molecules bind these metals as tight complexes. Though comparatively less preferred, these metals are also bound by pyrophosphate ions in the liver. It is therefore not surprising that these metal ions are removed from the blood and are immobilized as complexes in liver, gills etc. (Simkiss, 1981). Studies by Sauer and Watabe (1989) indicate that osteoblast lysosomes may be involved in the enzyme mediated release of the metal bound to metallothioneins or other metal binding proteins and thus may play a significant role in the accumulation of heavy metals by fish scales. The accumulation of heavy metals in osteoblasts thus represents an important detoxifying mechanism in fishes.

In short, a xenobiotic metal entering into an organism may be retained in the body for one of two reasons. It may either parasitise the metabolic pathway of an essential element and if it disrupts that produces toxic effects or it may be trapped by some detoxifying sink. The extent to which an organism modifies its metabolism to compensate for a pollutant metal will, of course influence the final tissue load. Thus the bioaccumulation and detoxification are associated and the former can be explained on the basis that it is the net result of detoxification and subsequent retention in the system. In other words, there exists a causal relationship between the general

problem of metal toxicity and the accumulation of free metal ions that occurs in the various tissues.

5.2 MATERIALS AND METHODS

Collection and acclimation of fish and the dose and duration of exposure to the xenobiotic were same as that described in the previous experiments. After the specified period of exposure, liver, gill, caudal muscle, scale and skin were removed. An accurately weighed quantity was digested with acid in a digester (Tecator,Digestion System 12, 1009 Digester) according to the method given in AOAC (1975). The digested samples were made up to a definite volume and this was used as the sample for metal analysis.

Zinc present in the sample was determined using Atomic Absorption Spectrophotometer (Perkin Elmer, Model No.2380). Mercury content of the sample was measured using a Mercury Analyzer (MA 5800D). The quantity of metal accumulated in each tissue was expressed as micrograms of metal per gram wet weight of the tissue.

5.3 RESULTS

The accumulation of mercury and zinc by the different tissues studied are summarized in Table 10A to 10E and Fig.24 to

TABLE: 10

Accumulation of zinc and mercury in different tissues of *O. mossambicus* as a function of period of exposure.

Concentration of the metal is expressed as ug\g wet weight.

A. LIVER

PERIOD OF EXPOSURE (DAYS)	ZINC		MERCURY	
	CONTROL	TEST	CONTROL	TEST
2	63.3 ±1.8	38.6 ±1.7	0	34.3 ±1.02
4	32.4 ±1.6	49.8 ±1.4	0	62.9 ±2.5
6	25.5 ±0.82	41.5 ±1.41	0	92.5 ±2.96
8	26.6 ±0.96	23.1 ±0.76	0	215.0 ±5.17

B. GILL

PERIOD OF EXPOSURE (DAYS)	ZINC		MERCURY	
	CONTROL	TEST	CONTROL	TEST
2	34.2 ±1.74	36.0 ±1.26	0	18.9 ±0.6
4	38.7 ±1.19	37.6 ±1.88	0	19.6 ±0.05
6	24.4 ±0.87	32.1 ±1.15	0	20.6 ±0.97
8	24.7 ±1.03	25.1 ±1.13	0	28.8 ±1.03

(contd.)

C. CAUDAL MUSCLE

PERIOD OF EXPOSURE (DAYS)	ZINC		MERCURY	
	CONTROL	TEST	CONTROL	TEST
2	126.2±4.4	61.6±2.5	0	2.5±0.07
4	73.2±2.7	57.6±2.0	0	4.0±0.12
6	44.3±1.7	62.5±2.4	0	5.0±0.12
8	60.6±2.1	48.1±1.4	0	10.6±0.36

D. SCALE

PERIOD OF EXPOSURE (DAYS)	ZINC		MERCURY	
	CONTROL	TEST	CONTROL	TEST
2	49.4±1.55	56.6±[illegible]	0	2.4±0.07
4	44.2±2.21	57.7±2.01	0	3.1±0.1
6	39.7±1.42	42.4±1.52	0	3.5±0.13
8	38.4±1.15	47.3±2.56	0	6.9±0.2

E. SKIN

PERIOD OF EXPOSURE (DAYS)	ZINC		MERCURY	
	CONTROL	TEST	CONTROL	TEST
2	99.8±3.55	54.6±1.69	0	2.1±0.06
4	44.7±1.37	48.6±1.56	0	2.3±[illegible]
6	35.6±1.23	36.8±1.58	0	5.0±[illegible]
8	33.8±1.38	36.9±1.66	0	5.0±0.15

Values are the mean of six different experiments ± SD

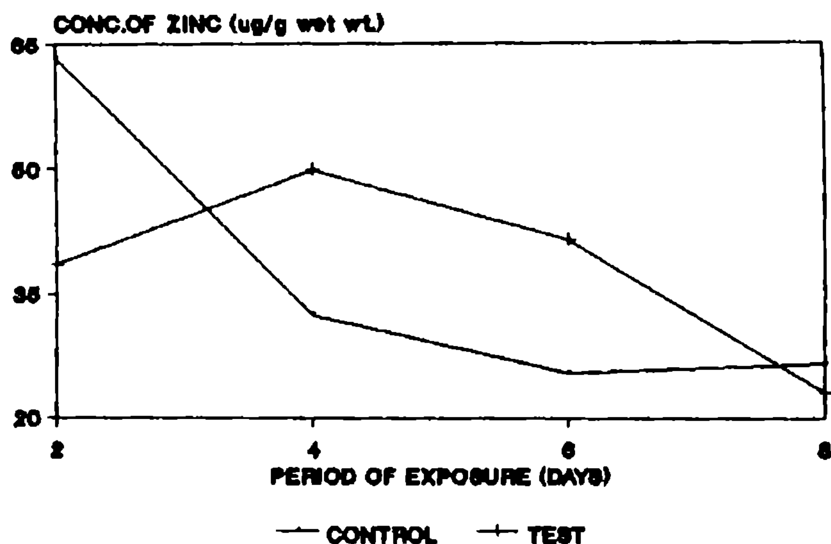

Fig.24 Concentration of zinc in the liver of control and zinc dosed *Oreochromis mossambicus*.

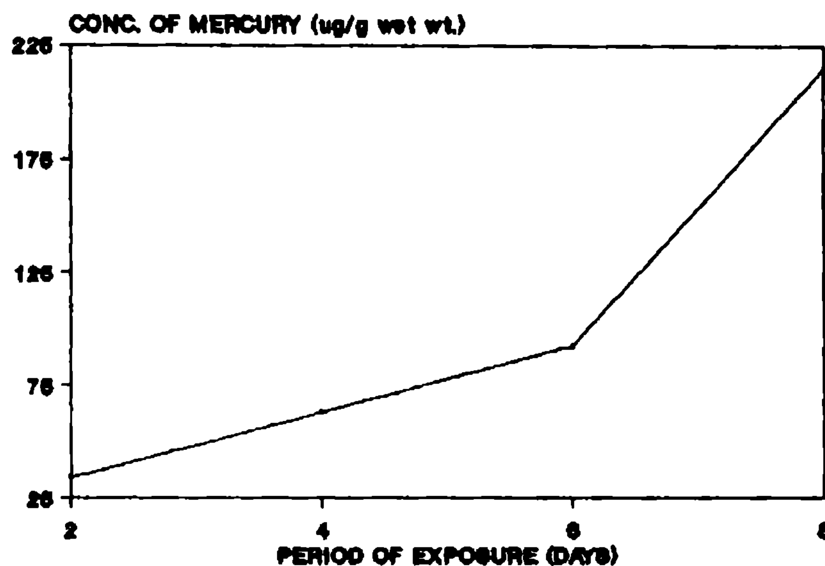

Fig.25 Concentration of mercury in the liver of mercury dosed *Oreochromis mossambicus*.

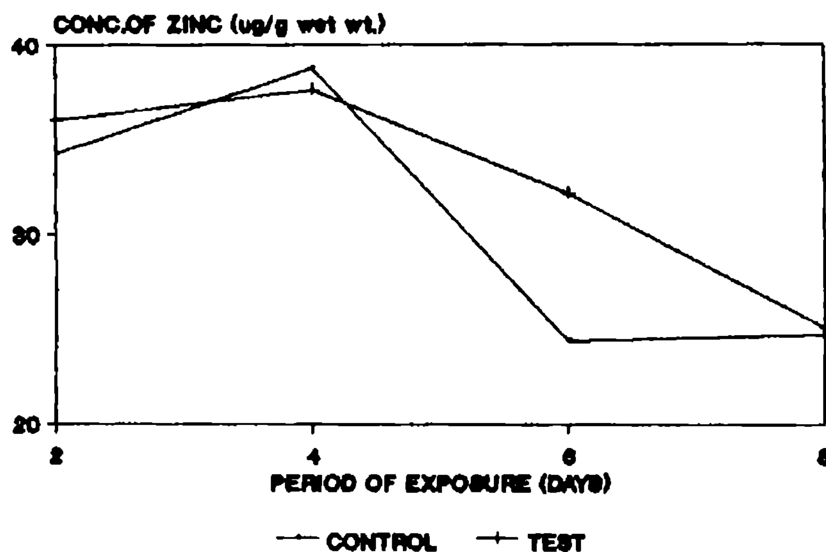

Fig.26 Concentration of zinc in the gill of control and zinc dosed *Oreochromis mossambicus*.

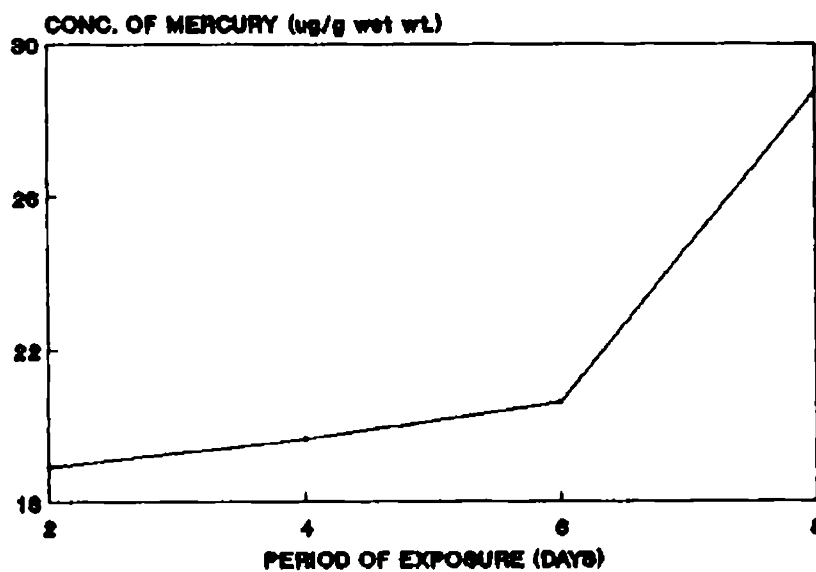

Fig.27 Concentration of mercury in the gill of mercury dosed *Oreochromis mossambicus*.

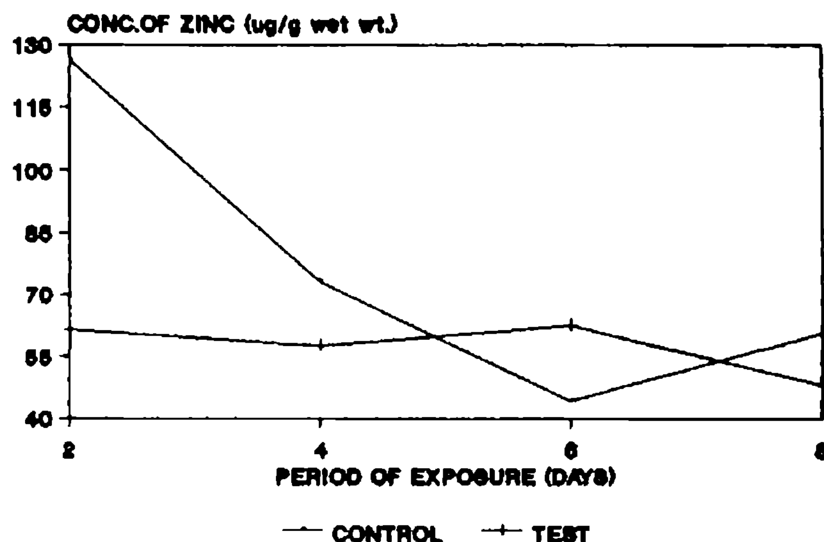

Fig.28 Concentration of zinc in the caudal muscle of control and zinc dosed *Oreochromis mossambicus*.

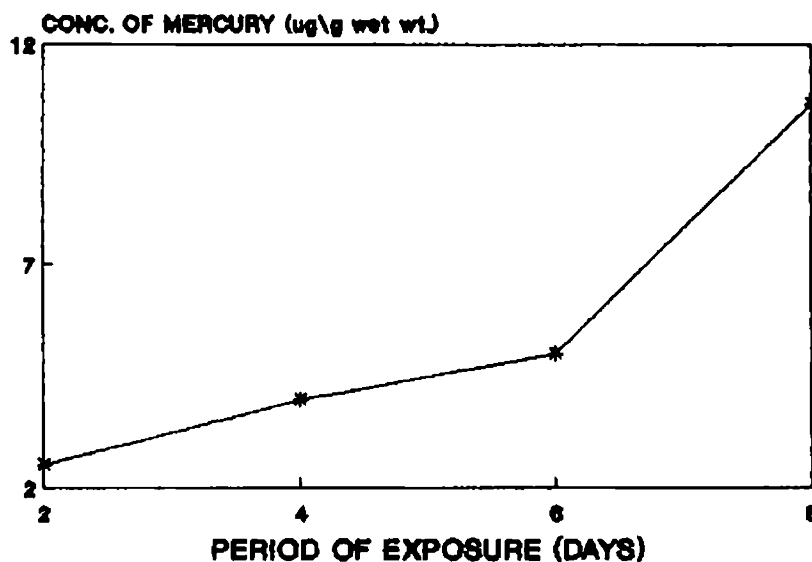

Fig.29 Concentration of mercury in the caudal muscle of mercury dosed *Oreochromis mossambicus*.

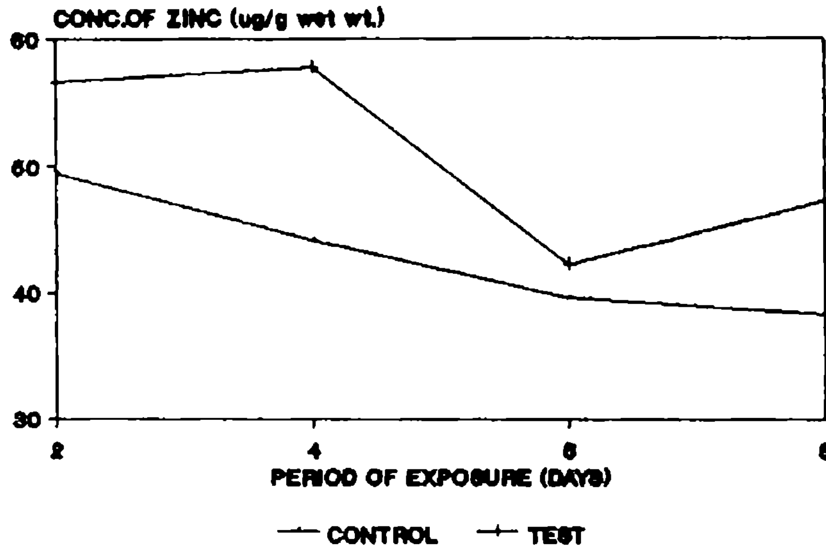

Fig.30 Concentration of zinc in the scale of control and zinc dosed *Oreochromis mossambicus*.

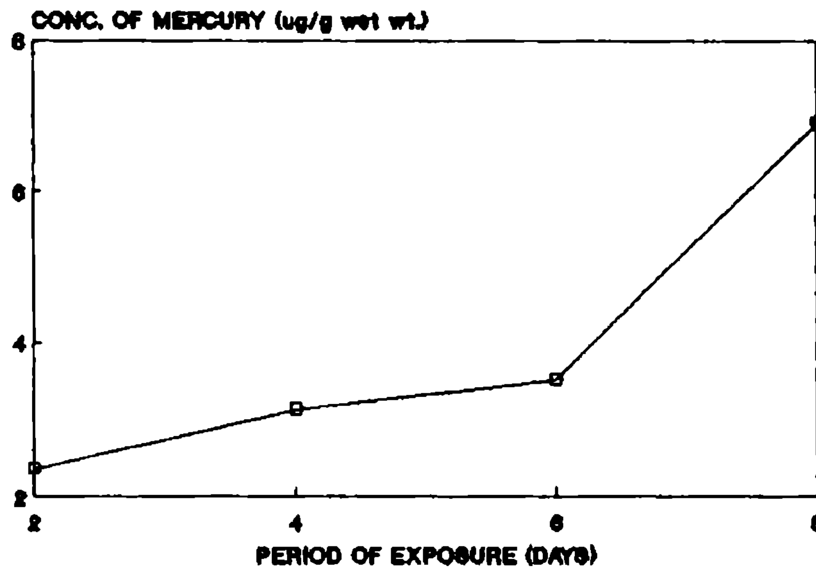

Fig.31 Concentration of mercury in the scale of mercury dosed *Oreochromis mossambicus*.

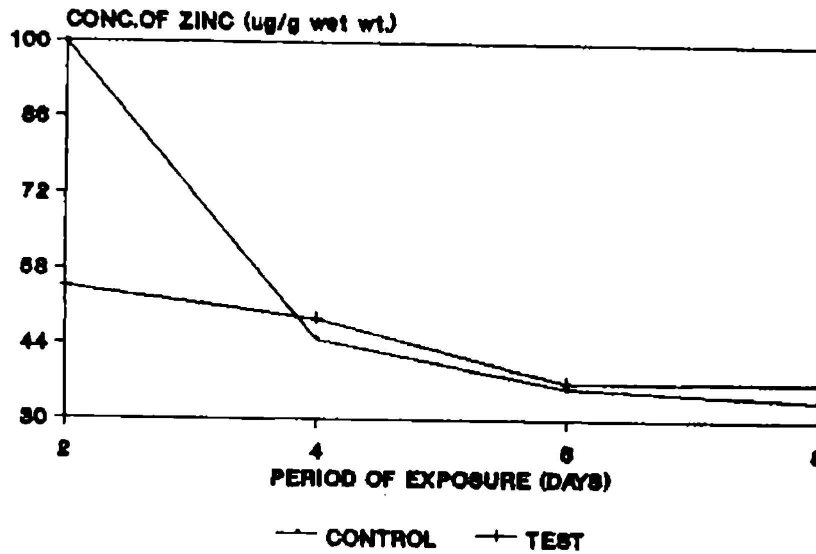

Fig.32 Concentration of zinc in the skin of control and zinc dosed *Oreochromis mossambicus*.

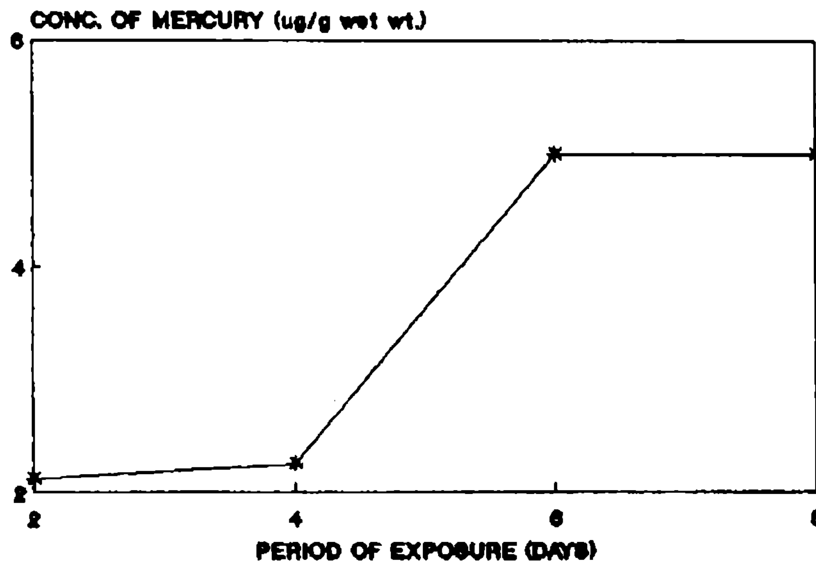

Fig.33 Concentration of mercury in the skin of mercury dosed *Oreochromis mossambicus*.

33. The data show a maximum accumulation of mercury in liver followed by gill, caudal muscle, scale and skin. The concentration of mercury in the liver is significantly higher when compared to all the other tissues. A gradual increase in the mercury level noticed up to the 6 th day of exposure in all the tissues except skin is followed by a steep elevation in the metal content. In the skin, the gradual enhancement occurring until the 4 th day is followed by a steep increase in the metal level up to 6 th day of exposure. However, from 6 th day onwards a steady state in the mercury content is maintained in the skin.

The accumulation of zinc by the various tissues of the fish exposed to the toxicant exhibits no stable pattern in any tissue except scale and skin. A slight increase in the level of zinc in the test organism is observed in scale and skin.

5.4 DISCUSSION

The patterns of bioaccumulation of mercury and zinc is distinctly different. The present studies also point to the fact that levels of essential trace metals in organisms can be regulated to some degree, whereas that of non-essential metals depend on their ambient concentration. These points are well in accordance with the reports of Bryan (1979) and Devineau and Triquet (1985).

The retention time of mercury in teleosts depends on several factors. The fish has got a rather limited ability to excrete this toxic metal (Pentreath, 1976b). The tissue load of mercury increases with the dose and duration of exposure to the metal. Due to the cumulative effect, equilibrium with the environment is not attained. Inorganic mercury has a high affinity for sulfhydryl containing molecules. The strong bonds formed would tend to greatly reduce the mobility of mercury within the animal. Mercury thus has a preferential site of accumulation, substantially limiting its transfer to the other organs of fish (Boudou and Ribeyre, 1985). The liver is endowed with a variety of kinetic traps, and the accumulation proceeds by a cascade of binding to ligands of ever increasing strength. Binding of the metal occurs to the imidazole groups in proteins, followed by thiol groups and metallothioneins, providing a final `kinetic sink' (Carpene and George, 1981). Liver is thus capable of acting as a diverse site for mercury accumulation. This explains the high content of mercury in the liver as noticed in the present study. A highest content of trace metal has been reported in the liver of trout (Baatrup and Danscher, 1987) and hepatopancreas of the cray fish (Madigasky *et al.*,1991).

Gills, however, did not show any special affinity for mercury. They may simply act as the site of influx. Weisbart (1973) has found a rapid loss of mercury from the gills at rates faster than that from the whole organism. This justifies the

very low levels of mercury in the gills, even though, it is the prime site of the metal intake. A dose response effect was also noticed in the caudal muscle, scale and skin even though the metal content in these tissues was very low when compared to that of liver and gill. The importance of scales (Sauer and Watabe, 1985, 1989) and muscle (Freeman and Horne, 1973; Cossa *et al.*, 1982) as tools of biomonitoring of heavy metals has already been demonstrated.

With increase in the period of exposure an increased residual levels of mercury was observed in all the tissues studied. A gradual increase in the metal content noticed in almost all the tissues up to the 6 th day of exposure indicates the net result of the accumulation and regulation. The same trend was seen in the case of skin up to 4 th day. This gradual elevation in all the tissues was followed by a steep enhancement in metal level denoting the insufficiency of any existing regulatory mechanisms. However, this steep increase in skin noticed up to 6 th day was followed by a plateau, indicating a steady state. This may point to the exhaustion of the capacity of the fish to incorporate any more metals to the scales.

Studies conducted on the accumulation of zinc by *Oreochromis mossambicus* showed that the fish has got an excellent capacity to regulate the tissue load of zinc. A zinc content greater than that in the control occurred only in

certain cases, where the rise was only very moderate. This indicates that the internal metal content could be under metabolic control. Similar observations has been made by Bryan (1979), Amiard and Triquet (1978-79) and Devineau and Triquet (1985). Studies by Lebedeva and Kuznetsova (1969) and Pentreath (1973a) have shown that the rate of ^{65}Zn accumulation decreases for several days following commencement of exposure. The internal ^{65}Zn concentration may only stabilize after many weeks as they have demonstrated in carp and plaice. A reduction in the internal zinc content to near normal levels, after an initial influx, has been reported in stickleback by Matthiessen and Brafield (1976). Ability of various other animal species to control the body zinc levels has been noticed by many workers.

The low levels of zinc observed in gills may be due to the fact that the initial adsorption of zinc to external gill mucus is followed by inward diffusion and eventual adsorption to unspecified internal binding sites. A similar mechanism has been reported in gills by Joyner (1961). Gills, thus have no special affinity for zinc, but works only as the site of absorption.

Though a great deal of work has been carried out on the potential usefulness of fish scales as records of environmental heavy metal exposure, short term studies have not proved any utility. An evident but slight increase seen in scales would represent the amount incorporated to the exoskeleton. The

increase in zinc content in skin would also indicate a passive accumulation on to the mucus layer of the skin.

The regulation of zinc in the fish may be achieved by the combined effects of a relatively low rate of uptake and an active excretion. Fishes probably excrete zinc through gut and gills. Excretion of ingested ^{65}Zn by way of gills has been shown by Nakatani (1966). The excretory mechanism appears to be unaffected by the external concentration of zinc, though this does not rule out the possibility that the excretion occurs partly by a passive process. The ambient calcium levels may also have some role on this protective action during zinc poisoning. The fact that *O. mossambicus* is euryhaline and thus are adapted to eliminate sudden influxes of several kinds of ions may explain the regulation of zinc in this study.

To sum up, the relatively abundant essential metals such as zinc are usually better controlled than non-essential trace metals like mercury. Clearly, the test fish can regulate zinc and study of its accumulation is unsuitable for monitoring the variations of the metal in the environment. Since mercury is least regulated by the fish they can be employed as a *sentinel* organism for this metal. The relative accumulation of the metal in various tissues studied gives insight into the sites and means of detoxification of this toxic metal.

C H A P T E R 6

6. SUMMARY AND CONCLUSIONS

Pollution due to heavy metals is a menace we are constantly confronting. Biota of the fresh water ecosystems are particularly affected by these pollutants. This may be due to the increased bioavailability of heavy metals in fresh water and also the comparatively restricted niche of these organisms. Of the different fresh water fauna, fishes are the most sensitive and obviously the most economically important species affected. Any threat to the tilapine population is worth considering, since they are getting increasing importance as food fish. The consumer demands have shot up rapidly as more and more people are convinced of their nutritive value.

The object of the present work is to develop practicable monitoring techniques to deliver guidelines for biological effect monitoring. The necessity for developing specific biochemical methods to detect biological effects of heavy metals that can be interpreted in terms of the health status of individual organism is also taken into consideration. Investigations at the subcellular level can reveal alterations at an early stage of response before integrated cellular damage shifts to the level of organ or whole organism. Measurements of biological responses, thus, are based on an understanding of the intracellular mechanisms by which the fish responds to exposure

to heavy metals.

During the course of evolution, many organisms have developed effective mechanisms of detoxification. These mechanisms have enabled the organisms to encounter the particular environmental challenge very efficiently. Living organisms, thus have a profound ability to survive in an atmosphere contaminated with toxic chemicals, in spite of their tendency to overload the normal physiological mechanisms of biotransformation or detoxification present in the cell. The pursuit on the organelle and biochemical responses to heavy metal exposure in *Oreochromis mossambicus* , therefore, is carried out giving special significance to the metal detoxifying machinery of the organism.

Various aspects subjected to study, to assess the impact of stress due to essential (zinc) as well as non-essential (mercury) heavy metals are the following:- Metallothioneins or metallothionein like proteins and their role in heavy metal detoxification, stability of lysosomal membrane as an index of heavy metal toxicity, role of some key metabolic enzymes as indicators of stress and the rate at which different tissues accumulate the metals from their ambience.

Induction of metallothionein or metallothionein like proteins was monitored in the liver samples from fishes exposed

to 1/10 th of 96 hr LC50 value of the xenobiotics for 8 days. Eluant fractions from the sephadex column was analysed for the protein as well as the metals bound to them. Present studies clearly demonstrate that the native metallothionein or metallothionein like protein from the liver of *Oreochromis mossambicus* is predominantly zinc bound. The production of metallothionein or metallothionein like proteins with the binding sites occupied by the relatively nontoxic and readily displaceable metal may very well explain their removal and the subsequent binding of a toxic metal and hence its homeostasis.

It is observed that entry of excess zinc to the system causes a redistribution of this essential metal among the apoenzymes. This is indicated by the presence of zinc in the high molecular weight fraction in amounts greater than in the control animals.

A clear-cut induction of metallothionein or metallothionein like proteins is seen in mercury treated fish. However, the rate of induction was very low. Several factors could be related to the high control value of metallothionein or metallothionein like proteins and low rates of induction in mercury treated fish. The dose and/or duration of mercury treatment could be one important criterion in determining the level of induction of the metal binding protein. High metal binding protein levels in control animals could result from

acclimation , stress and/or starvation. Studies reveal that exposure to any toxic metal results in the synthesis of metallothionein or metallothionein like proteins containing approximately equimolar levels of zinc and the metal exposed which would make the organism tolerant to any other toxic metal upon subsequent exposure.

Investigations carried out in our laboratory show that the binding of mercury in the metallothionein or metallothionein like protein containing fraction of cytosol is accompanied by elevations of mercury levels in the high molecular weight protein fractions in fish continuously exposed to the xenobiotic. This rise in mercury level occurs even when there is enough zinc-bound metallothionein or metallothionein like protein molecules, the zinc bound to which can be displaced and the incoming toxic mercury can be kept in a bound nontoxic form. The results thus seem inconsistent with the *spill over* hypothesis of action of metallothionein put forward by Winges *et al.* High levels of toxic metals in the enzyme pool before the metal binding protein becomes saturated with it may be the result of an established deficiency of an essential trace metal in the enzyme pool.

Induction of synthesis of metallothioneins or metallothionein like proteins thus proves to be a part of the protective mechanism employed by the fish. Since, the changes in

profile of metal binding reflects the variations in the metal composition in the ambient environment, these metal binding proteins fulfill the criteria of an index of sublethal stress also.

Ability of lysosomes to sequester heavy metals and to relieve the biological machinery from their toxic effects are studied using lysosomal preparations from the liver of metal exposed fishes. An initial stabilization followed by progressive labilization was noticed in the lysosomes of mercury exposed animals. This labilization may be attributed to the irreversible damage to the lysosomal membrane after the formation of mercaptide ligands. It may also be due to the fact that mercury enhances lipid peroxidation resulting in augmented attack on the unsaturated fatty acids of the membrane. Though lipid peroxidation and consequent formation of lipofuscin granules are of fundamental importance in heavy metal homeostasis, they leave an unfavourable alteration in the physiology of lysosomal lamina. The initial stabilization and the following labilization points to the fact that sequestration of mercury by lysosomes is effective only to a limited extent.

Except the initial labilization, zinc is found to have a stabilizing effect at all other periods. Stabilization by zinc may be either by the binding of the metal to the structural components of the membrane or by inhibiting the metal-catalyzed

lipid peroxidation. However, the onset of operation of detoxification mechanisms for this essential element is not as fast as those for toxic mercury as evidenced by an initial labilization of the membrane brought about by zinc.

Works carried out to compare the sensitivity of lysosomal membrane of different species showed that molluscs have the most sensitive lysosomal membrane followed by fishes and mammals. This sensitivity of molluscs to environmental alterations along with other qualities accounts for the validity of molluscs as *sentinel* organisms.

Though lysosomal enzyme release assay makes possible to monitor the effects of environmental pollutants on biomembranes, the labilizing or stabilizing effect is not specific to certain chemicals. Thus, responses of lysosomes to heavy metals may be primary or secondary and can be considered only as a general index of stress.

Activity of all enzymes monitored is found to vary depending on the dose and duration of exposure to the metal. An initial enhanced acid phosphatase activity is seen on exposure to both zinc and mercury. This increased acid phosphatase activity in the case of zinc exposed animals could be attributed to the metal-induced changes in lysosomal latency. The high enzyme activity in mercury treated fish which is inconsistent

with the initial stabilization of lysosomal membrane by mercury as seen in our study may lead to the following inference - though lysosomes are the major source of acid phosphatase, there exists some other sources such as mitochondria etc., which contributes to the total acid phosphatase activity in the liver of stressed fish. The increased internal content of mercury resulted on prolonged exposure causes a greater tissue damage and hence elevated enzyme activity. A continued exposure to zinc causes a decrease in acid phosphatase and the level approached control values. This may be due to the direct effect of zinc on enzyme, stabilization of lysosomal membrane, impairment of lysosomal metabolism in liver or decreased synthesis of enzymes. The initial high acid phosphatase activity in presence of zinc may be explained as due to the fact that the onset of regulatory responses for this essential metal is not as fast as those for toxic mercury.

Activity of aspartate aminotransferase is found to be inhibited throughout the tenure of exposure. The inhibition may occur at the point of pyridoxal phosphate synthesis. The damage caused to the mitochondrial membrane, loss of matrix, swelling of mitochondria, decreased availability of oxaloacetate, direct inhibition of enzyme etc. are the other possible reasons for the reduced levels of aspartate aminotransferase. An initial induction followed by a progressive inhibition of aspartate aminotransferase activity is noticed in presence of zinc. The

initial high level may reflect an increased conversion of amino acids to keto acids and vice versa, resulting in the disturbance of protein metabolism. The decline following may be due to the direct inhibition of the enzyme by the metal as noticed in our study.

An enhanced activity of glutamate dehydrogenase suggesting an increased amino acid metabolism and gluconeogenesis is observed at all periods of exposure to both the metals. The excess carbohydrates serve as a source of energy for metabolic requirements during stress and also exert a sparing effect on protein. The damage produced to the mitochondria may also cause an increased glutamate dehydrogenase activity. However, prolonged exposure to both zinc and mercury causes a decrease in glutamate dehydrogenase activity which may be ascribed to the reduction in the deamination process in the exposed fish.

Researches carried out reveal an initial inhibition of HMG Co A reductase in presence of both the metals. The damage produced to the endoplasmic reticulum to which the enzyme is bound or a direct inhibition of the enzyme by the metals may explain the reduced activity of the enzyme. The stimulated activity following may lead to increased production of cholesterol and ketone bodies. This lipogenesis acts as a defense mechanism against metal toxicity. A further decrease following indicates the insufficiency of lipogenesis by the

enzyme to trap the metals, the influx of which again produces destructive effect on the enzyme.

An elevated lactate dehydrogenase activity indicating a stepped up glycolytic rate is seen in presence of both the metals. This points to the fact that the stressed fish is preferentially meeting its energy requirements through anaerobic oxidation. Prolonged exposure however, causes a decrease in enzyme activity which eventually approaches control values. This is due to a decline in the efficiency of anaerobic metabolic pathways. An initial very low lactate dehydrogenase activity in presene of mercury which is less than the control activity indicates a situation where the regulatory mechanisms are set in fast and mercury is maintained at a level which is not sufficient to affect aerobic oxidative pathways. The organism does not resort to glycolysis and maintains a low lactate dehydrogenase activity.

The maximum serine hydrolyase activity observed at initial stages of exposure may indicate an augmented production of pyruvic acid and hence gluconeogenesis. An enhanced serine hydrolyase activity may also result in an elevated synthesis of fatty acids and cholesterol. This lipogenesis serves as a defense mechanism against heavy metal toxicity. Extended exposure however, makes this mechanism inefficient and a gradual reduction in enzyme activity ensues. This may be due to the

direct inhibition of the enzyme by the metals as seen in the in vitro studies.

Considerable changes occur in enzyme activities representing disturbances of metabolism of organism, either to encounter the increased energy demand during stress or as possible steps towards metal detoxifying process.

Investigations on the pattern of accumulation of zinc and mercury by different tissues, lead to the conclusion that levels of essential metals in organisms can be regulated to some degree, whereas that of non-essential metals depend on their ambient concentration. Liver acts as a diverse site of mercury accumulation resulting in high content of mercury. Even though gill is the prime site of metal intake, rapid loss of mercury from the gills at rates faster than that from the whole organism causes low levels of mercury in the gills. A dose dependent increase of mercury was also noted in caudal muscle, scale and skin even though the level was very low.

A steep increase in mercury level was noted after the initial gradual increase in liver, gill, caudal muscle and skin. The gradual increase denotes the net result of accumulation and regulation whereas steep increase indicates the insufficiency of any existing regulatory mechanisms. The plateau after the steep increase in skin represents the exhaustion of the capacity of

fishes to incorporate any more metal on to the skin.

Studies on accumulation of zinc showed that the fish has got an excellent capacity to regulate the tissue load of this metal. This metabolic regulation may be achieved by the combined effects of relatively low rate of uptake and active excretion.

In a nut shell, the effects of zinc and mercury at different levels of biological organisation give an insight into the sites and means of detoxication of the xenobiotic metal. All the four aspects studied are found to contribute a significant share to the detoxifying capacity of the organism. These different mechanisms may be interconnected also , as for example, the mobilisation of the toxic metal bound to the metal binding protein to the lysosomes of liver, scale osteoblasts etc. ; within the lysosomes the metal may be separated from the protein and sequestered and the component amino acids of the protein recycled. The effects on organisms also entail monitoring the variations of the metal concentration in the ambience.

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