TOXICOLOGICAL STUDIES
ON
OREOCHROMIS MOSSAMBICUS (PETERS)

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BY
P. J. CYRIAC

DIVISION OF MARINE BIOLOGY, MICROBIOLOGY AND BIOCHEMISTRY
SCHOOL OF MARINE SCIENCES

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
COCHIN - 682 016

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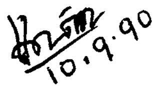
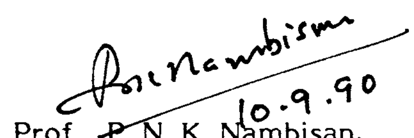


Dr. A. Antony,
Reader,
School of Marine Sciences,
Cochin University of Science
and Technology,
Cochin - 682 016.



Prof P N K Nambisan,
Director,
School of Marine Sciences,
Cochin University of Science
and Technology,
Cochin - 682 016

PREFACE

Heavy metal pollution of the aquatic environment has received considerable attention in recent years owing to the toxicity of heavy metals at very low levels, persistence in the environment and ability to get incorporated in the tissues of organisms. In organisms, including fish, usually negative consequences of pollution manifest themselves as detrimental deviations from the normal state affecting behavioural, physiological and biochemical systems.

Since some metals are required in the life processes, most organisms have a capability of concentrating heavy metals in the body. Copper, though an important micronutrient, is toxic to the animals above threshold levels. Mercury has no biological function to serve and is harmful to the animal even at low concentrations. Apart from direct absorption, both copper and mercury undergo a number of biological concentration steps through food chains.

A clear understanding of heavy metal toxicity on the aquatic biota demands detailed investigations on the sublethal effects of heavy metals at various concentrations which are more realistic with reference to environmental concentrations. Studies of sublethal effect of metals in fish aim at analysing the biological responses of an organism to metal exposure. When these responses are quantified, it forms a basis for bioassay procedures. Hence an attempt was made to study the sublethal effects of copper and mercury on the fish *Oreochromis mossambicus* (Peters).

The thesis is laid out in nine chapters. The Chapter 1 introduces the topic and outlines the aim and scope of the present investigation. The second chapter deals with the effects of copper and mercury on the glycogen and protein content of the liver and muscles. The variation of acid and alkaline phosphatase activity in the liver and kidney after exposure to copper and mercury is described in chapter 3. In the fourth chapter, aspartate and alanine aminotransferase activity in the liver and kidney of metal-treated fishes are reported. In the fifth chapter the effect of copper and mercury on the haematology of the fish is described. The effect of copper and mercury on the blood glutathione content is presented in the sixth chapter. The seventh

chapter deals with the blood catalase activity in fish, dosed with copper and mercury. The eighth chapter describes the effects of copper and mercury on the ascorbic acid content of the liver, kidney and blood. The last chapter gives a summary of the thesis followed by a list of references.

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CHAPTER 1

GENERAL INTRODUCTION

The term pollution in relation to aquatic systems can be defined as "the introduction by man, directly or indirectly, of substances or energy into the aquatic environment resulting in such deleterious effects as harm to living resources, hazards to human health, hindrance to aquatic activities including fishing, impairment of quality for use of water and reduction of amenities" (GESAMP, 1980). The aquatic environment is increasingly being polluted by anthropogenic inputs. The alteration of the ecosphere by human activities may be physical, chemical, biological or radioactive. Chemical alteration of the environment appears to be the major type which threatens the living system extensively. All organisms maintain their "internal milieu" more or or less constant by making use of a variety of regulatory mechanisms. When the level of pollutants in the environment exceeds the assimilatory capacity of these regulatory mechanisms, it leads to biochemical changes, finally resulting in death. It was the effects of 'Minamata' tragedy caused by the consumption of mercury contaminated fish and shell fish, and the effect of 'Itai itai' caused by the consumption of food, contaminated with cadmium and concerns about the effects of atmospheric lead, that forced man to take action for the control of various pollutants. The 'Minamata' incident resulted in an awareness of the problem of bio-accumulation of pollutants by aquatic organisms and spurred research in examining the levels of the metals in aquatic organisms and other food items.

Heavy metal Pollution

In recent years, concern has increased over heavy metal pollution, as all heavy metals are potentially harmful to most organisms at some level of exposure. The release of increasing quantities of heavy metals and their salts in terrestrial and aquatic environment and their accumulation in living and non-living systems endanger life. The nature of environment is of crucial importance when considering homeostatic mechanisms of an animal. Whereas terrestrial animals are generally only exposed to metals in their diets, or in the air they breathe, aquatic organisms are also exposed to dissolved and particulate metals in the medium.

Bio-availability of heavy metals in aquatic medium

Heavy metals may be considered as a threat to the environment and biota, only if these could be taken up by biota through whatever route. It is not possible to equate a given emperically defined fraction of water sample with the biological availability of a contaminant. Thus, both soluble and particulate fraction of water sample contain some components which are highly available to certain part of biota only. In the soluble fraction trace metal ions generally exhibit high biological availability. By contrast some chelates or complexes present in solution may be unavailable. Pagenkopf et al. (1974) cited an example of this effect for copper in fresh waters and postulated that the effect of chelation may be responsible for the observed variation in the toxicity of most trace metals in hard and soft natural waters.

The particulate fraction can be divided into two groups, organic part and inorganic part. The bio-availability of trace metals attached to organic particulate matter is high and that of inorganic matter is low. All these factors make the availability of trace metals and their actual composition in water samples different.

Heavy metal uptake and bio-concentration

The mechanism of metal uptake has not yet been fully elucidated. The evidence indicates that metals cross the cell membrane essentially by a passive transport process although endocytosis may also occur (Viarengo, 1985). Studies by Simkiss (1983) suggest that the metal complex goes through the biological membranes as a lipophilic compound. Moreover in fish, metals like Hg, Cd etc. are able to disrupt the ionic balance, altering the permeability characteristics of the cell membrane. Thus they effect passive ion movements as well as the active transport process either by directly inhibiting the activity of Na/K dependent ATPase or as a secondary effect by reducing the availability of ATP (Bouquegneau and Gilles, 1979). When metals cross the cell membrane the metals react with cytosolic components and are usually complexed in different ways (by sulphydrylic binding, chelation, salt formation) to cytosolic compounds such as high affinity specific ligands, substrates, products of enzymatic activity or enzymes themselves.

The form of the heavy metal (ionic form, oxidised, reduced, complexed by organic substances, adsorbed to inorganic or organic particulate materials, acting singly or in combination with other cations) to which the organisms are exposed is extremely important in its overall toxicity to aquatic organisms and its uptake by them. Metals are taken up by aquatic organisms usually across respiratory surfaces and strongly bound by sulphydryl groups of proteins. Because of this ability, there is a tendency for them to be fixed in tissue and not to be excreted. In other words they have a long biological half life. Metal also changes the structure and enzymatic activities of proteins and causes toxic effects, evident at the whole organism level.

Requirements for a monitoring organism

Certain organisms have long been known to accumulate pollutants from the ambient water. Many bivalves have been known to accumulate a wide range of metals. Thus different heavy metals may be accumulated by aquatic biota to levels far above those found in the surrounding medium, thereby enabling to use selected organisms to monitor the levels of the metals in water bodies. They have several advantages over the classical method of water or sediment analysis. The greatest advantage is that the biological availability of the pollutant is measured directly. In addition to this, such animals would produce a time averaged index of pollution availability. The much higher concentration of pollutants in the body, compared with those in the surrounding water makes it easier to analyse the samples, and the biochemical, haematological and enzymatic changes caused by pollutants are much more evident.

Industry and regulating authorities concerned with the environmental management increasingly recognize the need for biological monitoring to detect changes, either deterioration or improvement in environmental quality. Numerous and varied biological responses have been suggested as potential techniques for monitoring biological impact of waste discharges to the aquatic environment.

The indicator organisms selected for monitoring the chemical and biological effects of pollution should possess a number of important attributes. According to Widdows (1985) such an indicator organism should have the following attributes:

1) a wide geographic distribution;
2) a dominant member of coastal and estuarine communities;
3) ability to accumulate contaminants in the body tissue; and
4) be responsive to many environmental pollutants but do not show a prolonged handling stress.

Philips (1980) regarded biomonitoring data more direct and less inferred than water or sediment analysis because information on bioavailability and biomagnification is included. Other reasons to use biomonitoring in addition to physico-chemical monitoring are:

1) toxicity cannot be estimated without testing biota, although quantitative structure activity relationship (QSAR) techniques may give good approximation;
2) biological response may be elicited by chemicals below analytical detection limits;
3) biological responses to toxicity may be different in mixture (ecosystem, species, different chemicals, etc.) than tested individually; and
4) environmental quality strongly influences toxicity.

Criteria for a standard test fish

Many authors have mentioned the desirability of using a standard fish species as a bioassay animal for reproducibility of test results (Marking, 1966; Lennon, 1967; Cairns, 1969; Sprague, 1970). In some western countries, acute toxicity tests with a fish, Daphnia and an algal species have been made mandatory for the acceptence of new substances (Smeets, 1980). Though the species of fish that should be employed in such tests is not specified, the use of a standard fish has been suggested in order to prove the results, and to compare the results of one laboratory with another (Cairns, 1980). Furthermore, the results of tests conducted at different moments in the same laboratory can also be compared by employing a standard fish (Fogels and Sprague, 1977).

The following criteria have been listed by Adelman and Smith (1976) for the choice of a standard test fish:

1) It must have a constant response and have neither high nor low sensitivity to a broad range of toxicants tested under similar conditions.
2) It must be available throughout the year.
3) A constant size group of that species should be available all through the year.
4) It should be easy to collect, transport and handle.
5) The adults should be small enough to facilitate the conducting of acute or chronic tests without imposing undue difficulties in maintaining the recommended loading densities.
6) It should be possible to breed the species in the laboratory.
7) It should complete its life cycle within one year or less.

Though in the industrialized nations various species like common carp, fathead minnow, guppy, rainbow trout etc. have been recommended and employed as standard fish, under Indian conditions, no fish species has been identified as standard test fish for diverse reasons like variation in the temperature and climatic conditions in the Indian subcontinent, differences in standard test protocols, divergent species etc.

Suitability of *Oreochromis mossambicus* for the toxicological studies

Oreochromis mossambicus (Peters) selected for the present study, fulfils most of the criteria listed for a standard test fish. They are found in abundance in the rivers, lakes and backwaters of Kerala. They have been described as a 'miracle fish' owing to their bio-economic advantage such as quick growth, fewer bones, tasty flesh, good market acceptance, ease of reproduction and adaptability to wide range of environmental features, ready acceptance to artificial feed, direct assimilation of blue green algae (Jhingran, 1984) and effectiveness in controlling insect and weed. As it is a sturdy fish it can be easily maintained in the laboratory. Being euryhaline, *O. mossambicus* can withstand a wide range of salinities and thrives well in freshwater as well as in brackish water. They breed throughout the year. Being herbivorous, they are used to control weeds and other aquatic plants in rivers and lakes. The fish is neither too big to rear in the laboratory nor too small for

experimental purposes. It is available throughout the year. Considering all these factors, the cichlid fish, Oreochromis mossambicus has been selected for the toxicological studies.

Trace metals selected for the experiment

The term 'trace metals' identifies a large group of metallic elements which are present in living organisms in limited amounts. Trace metals are usually divided into two sub-classes. The first includes Fe, Mn, Cu, Co, Mg and Zn which are essential micronutrients. Such nutrient metals are usually key elements in metalloenzymes or cofactors in enzymatic reactions. However, the micronutrient when present above threshold levels becomes toxic. Cd, Hg, Cr, Pb etc. belong to the second category which is made up of metals without any established biological function and include the more important contaminants in the aquatic environment.

Mercury

The fact that inorganic mercurial compounds are transformed into biological active methylmercury have prompted comprehensive investigations into environmental problems created by mercurials (Goldberg, 1980; Ray, 1984; Patel et al., 1985 a, b). Wobeser (1975) confirmed the ability of fish to concentrate Hg rapidly from water.

Eventhough toxicity and bioaccumulation of the mercury in fishes have been studied by many authors, information on the Hg-induced biochemical and haematological alterations in Oreochromis mossambicus is meagre. As most fishes are responsive to deleterious effects of mercury (Wobeser, 1975; Panigrahi and Misra, 1978; Christensen et al., 1977; Gill and Pant, 1981; Dange, 1986a) the early detection of specific physiological abnormalities provide an indication of exposure prior to manifestation of any gross damage. The measurement of biochemical changes in blood and tissues of the fish under exposure to the toxicant may be used to predict effects upon chronic exposure.

Source of mercury pollution in the aquatic medium

The major sources of water contamination by mercury are effluents from Chlor-alkali plants which use mercury cells for producing chlorine and

caustic soda. These factories discharge large quantities of mercury compounds into the aquatic medium. Agricultural application of mercury also contaminate waterways. Over seventy mercury compounds known to have been used to control seed-borne, or soil-borne fungal diseases. They get washed with rain and irrigation waters and pollute the waterways. Mercury metal and mercury compounds are also used in a variety of industrial applications like electrical equipment manufacture, antifouling paint manufacture, industrial and scientific equipment manufacture, dental uses and laboratory uses. Miscellaneous uses include use as a catalyst and fungicide and slimicide in pulp and paper industry in pharmaceutical and cosmetic preparations and in amalgamation process. Burning of fossil fuels or sewage sludge, municipal wastes, Hg-containing fertilizer are also important sources of Hg. The release of Hg compounds from industrial liquid wastes has been shown to greatly influence the immediately adjacent environment of the source.

Effects of Mercury on fish

Mercury has no known metabolic function in human beings and therefore even low concentrations in the body may be considered potentially harmful. Mercury in fish and seafood occurs mainly as methylmercury and partly as inorganic mercury bound to organic molecules.

Mercury compounds also exert their action by altering the membrane structure, thereby seriously affecting the permeability character of these cell types. Lock et al. (1981) found a decrease of the Na^+ and Cl^- and osmolality of the blood in rainbow trout following exposure of the fish to mercury in the water. The inability of Hg-exposed fish to maintain its ionic balance could be attributed to either a decreased uptake of ions via gills or to an increased loss of ions via gills or kidneys. As the gills function as the main route of mercury intake (Olson et al., 1973), the osmoregulatory function of this organ is likely to be affected.

When fishes are exposed to Hg in their environment, the gill tissue rapidly accumulates these compounds in such a manner that their concentration reaches values exceedingly high by far those in other organs and tissues (Olson et al., 1973, 1978; Lock, 1979). This conspicuous accumulation is often accompanied by structural changes in the gill epithelium (Lindahl and Hell, 1970; Wobeser, 1975; Lock, 1979). Wobeser (1975) found that in rainbow trout exposed to

mercuric chloride severe epithelial necrosis occurs.

Methylation of mercury drastically alters the properties of Hg. It loses polarity and ceases to behave as a typical metallic ion. It becomes much less water soluble and much more fat soluble (Craig, 1986 a). This affects the environmental fate of Hg. Methylmercury becomes mobile entering any substrate containing fat and is accumulated by aquatic organism directly from water. In fish methylmercury may comprise 80% or more of total mercury and it occurs primarily in muscle as a cysteine complex (Craig, 1986 b). While energy is lost with each trophic transfer, methylmercury is conserved and biomagnified in aquatic food chains.

Toxicologically increased fat solubility means not only rapid uptake and retention of mercury, but more rapid penetration of sensitive tissues, particularly the lipid membranes of neurons (Craig, 1986 a). The high affinity of methylmercury for sulphydryl group causes significant neurotoxic effects by combining with cysteine containing protein.

The different Hg species have different pathways and routes in the environment. Many of the routes can be taken both by biologically mediated processes and by a biological process. Olson and Fromm (1973) found Hg in the gills of rainbow trout which had been exposed to inorganic Hg and suggested that Hg enters the gill across the general lamellar surface.

Copper

Copper and its compounds have been used by man since prehistoric times. Like other metals it is potentially toxic and is a widespread pollutant of water (Slowey and Hood, 1970; Rehwoldt et al. 1971; Abdullah et al., 1972). Copper has been added to marine culture system to control pathogens such as parasitic protozoan (Dempster, 1970). Numerous studies (Ozaki et al., 1971; McKim and Benoit, 1971; O'Hara, 1971; Spear and Pierce, 1979) have shown that addition of copper salts to natural water can seriously threaten aquatic life.

Source of copper pollution in the aquatic medium

The largest amount of copper enters the oceans as pollutant in river

runoffs from industrial and agricultural sources and appears to concentrate in nearshore areas (Jernelov, 1975) posing serious water pollution problem (Pickering and Henderson, 1966; Rehwoldt et al., 1971). Copper is widely used as an algicide and in the treatment of diseases and parasitism in fishes. The latter application sometimes poses the problem of toxicity to the fish treated. Chronic copper poisoning can be a serious problem in domestic animals, since copper is stored in fish and other organisms, but the greatest hazard to fish is from acute poisoning. (Cardeilhac, 1972; Takeshita, 1975).

Effects of copper on fish

Copper is an essential element for animals for the synthesis of haemoglobin, formation of bone, maintenance of myelin within nervous system and an essential component of key metalloenzymes. It is intimately associated and involved in normal haematopoiesis since it is essential for the absorption of iron available for haemoglobin (Hb) synthesis (Goodman and Gilman, 1956).

Both excess and deficiency of copper cause pathological changes. The behaviour of fish, flounder exposed to medium and high levels of copper, was uncoordinated with spontaneous movement which was similar to Wilson's disease (Peisach et al., 1967). It seems possible that the symptoms shown by the flounder could be the result of copper acting on their central nervous system. Excessive copper intake causes hepatic necrosis, haemolytic anaemia, necrosis of kidney tubules and death of brain neurones. Copper also attacks various renal enzymes that mediate the resorption of several compounds. Copper has been shown to cause haemolytic anaemia by inhibiting erythrocytic glycolysis, denaturing Hb and oxidising glutathione (Fairbanks, 1967).

McKim and Benoit (1971) found that sublethal exposure of copper to brook trout decreased survival and growth in adult fish and reduced both number of viable eggs produced and hatchability. Baker (1968) found that effects of copper exposure resulted in fatty metamorphosis in the liver, necrosis in the kidney, destruction of haemopoietic tissue and gross changes in gill architecture of winter flounder. The respiratory system of fish seems to be the prime target at exposure to acute lethal levels of copper. The toxic effects of copper on fish are considered to be mainly attributed to copper ions (Pagenkopf et al., 1974).

Gardner and LaRoche (1973) observed cellular changes attributable to copper in the mechanoreceptors of the lateral line canals in mummichihog, *Fundulus heteroclitus* and Atlantic silverside, *Menidia menidia*. Renal lesions were observed in *F. heteroclitus*. Hepatic and renal disorders have been reported for the winter flounder (Baker, 1968) while the metal was described as being both nephrotoxic and neurotoxic to the goldfish *Carassius auratus* (Vogel, 1959). Liver disorders have been reported in the mummichihog (Lett et al. 1976; Skadhauge, 1977; Drummond et al., 1973). Cardeilhac et al. (1979) found that in the fish Sheephead, copper sulphate exposure resulted in the appearance of swollen and congested kidney, and gill lamellae of copper poisoned fish were blunt and thickened. They also observed the failure of osmoregulation in such fishes.

From these accounts it is clear that copper, though a micronutrient, is also a pollutant at high threshold levels. Hence an attempt was made to study the effects of both copper and mercury on some biochemical activities of the fish.

Tissues selected for the study

Liver

Xenobiotics undergo a wide variety of biochemical transformation to allow for their more rapid elimination in water soluble forms. These biotransformations include conjugation, aromatic hydroxylation, deamination, epoxidation, dealkylation, nitro and azo reduction and ester and other cleavages (Chambers and Yarbrough, 1976). Although there are oxidations and reductions catalysed by enzymes that are non-hepatic and non-microsomal, the major metabolic biotransformation occur in the liver (Backstrom, 1967, Hogan and Knowles, 1968) and as specifically associated with endoplasmic reticulum. It was generally accepted that fishes process lipid soluble organic substances by the rapid elimination of these substances into the external aqueous environment through the activity of gills. However it was later demonstrated that fish could detoxify xenobiotics and the toxicity of several xenobiotics could be related to hepatic metabolic activity (Backstrom, 1967; Lech, 1974). Hogan and Knowles (1968) observed that the livers of fishes are major sites of detoxification. Enzyme estimations are used to study the liver function and differential diagnosis of

liver diseases. Hence the study of hepatic response to chemical injury is of prime importance to toxicologists.

Since the liver of teleosts is important in the maintenance of internal homeostasis and the metabolism of xenobiotics (Chambers and Yarbrough, 1976) and has also been shown to accumulate foreign compounds (Statham et al., 1978) and to be susceptible to damage by hepato-toxic agents (Racicot et al., 1975; Gingerich et al. 1978) the functional integrity of the liver in fish can be affected by xenobiotics (Gingerich, 1982).

The liver is specifically affected by a large number of chemical agents. The liver of mammals act as a major organ for copper storage as also many other metals. Backstrom (1967) observed that liver is one of the most important Hg accumulating organs in animals treated with phenyl mercurials. According to Buck (1978) liver is the first line of defence against copper poisoning. Copper becomes toxic only when the high binding capacity of the liver is exceeded and copper is released into blood stream. In fish also, liver is the major storage organ for copper (Buckley et al., 1982; Shearer, 1984).

Kidney

El-Domiaty (1987) found that highest concentration of copper was in the liver and kidney of Clarias lazera after exposure to copper and suggested that the liver and kidney are vital organs in the regulation of metal as they are detoxification centres. Kidney is second only to the gills as an affector organ in ionic regulation and played an important role in the removal of the heavy metals from the body. According to Adamson (1967) the gill of fish is a poor excretory unit whereas kidney is capable of active excretion of many biotransformed derivatives of toxicants. Hence, examination of the changes in the biochemical composition and enzymatic activity in the liver and kidney are essential to understand the detoxification mechanisms in these organs.

Blood

Blood chemistry has been used for the detection of various diseases. Further knowledge of blood chemistry offers possibilities for a physiological evaluation

of environmental effects on fish under different conditions. In addition, the measurement of specific physiological and biochemical changes in the blood of fish exposed for short periods to sublethal environmental stress may provide a sensitive method for predicting the effects of chronic exposure on survival and growth. Hence blood analysis may be a good tool for quantitatively assessing sublethal effects without having to resort to sophisticated techniques of cell culture and electron microscopy.

Objectives of the present study

Heavy metals are one of the most active polluting substances as they cause serious impairment in the metabolic, physiological and structural systems of the body, when high concentrations are present in the milieu.

A continuing study of specific physiological, biochemical, metabolic and enzymatic changes of aquatic organism exposed for short periods to environmental stressors is essential to provide a rational basis for anticipating and understanding the ecological effects in the aquatic environment. Such studies may provide a sensitive method for predicting the effects of chronic exposure as survival, reproduction and growth. This would allow a relatively rapid evaluation of the chronic toxicity of a compound.

In order to understand the sublethal effects of two metals-copper, a micronutrient, and mercury, a xenobiotic on *O. mossambicus,* changes in the glycogen and protein content in liver and muscle, phosphatase activities in liver and kidney, transaminase activity in liver and kidney, changes in the blood glutathione and blood catalase, changes in the haemoglobin, haematocrit and Mean Cell Haemoglobin concentration values and changes in the ascorbic acid content of liver, kidney and blood were studied after exposing the fish to two sublethal concentrations of copper (100 µg/l and 200 µg/l) and mercury (100 µg/l and 150 µg/l) for 24, 72, 120 and 168 hours.

Effect of heavy metals on the glycogen and protein content

As the respiratory potential of fish is related to energy expenditure and metabolism in general, any change in the oxygen supply to the tissue may interfere with the glycogen stores and many pollutants are known to influence

the respiratory potential of fishes. A change in the glycogen content indicates a change in the energy demand and expenditure since anoxia or hypoxia increases carbohydrate consumption. When the glycogen reserves decrease, tissue protein supply ketoacids by the deamination of amino acids. So a study of the glycogen and protein content of the liver and muscle is necessary to understand the changing demands and expenditure of energy during metal stress.

Effects of heavy metals on the phosphatase and aminotransferase activity

In the field of environmental toxicology, serum and tissue analyses are becoming increasingly important for the detection of toxic effects of chemical pollutants (Eisler, 1972; Jackim, 1974). A number of enzymes have been shown as possible indicators of pollutant contamination (Magurie and Watkin, 1975). It appears that structural and other properties as well as activities of enzymes can be affected by exposure to pollutants, possibly leading to loss of cellular metabolic flexibility (Gould et al., 1976). Many metals have been reported to alter the activity of various enzymes in marine organisms (Dixon and Webb, 1964; Jackim et al., 1970; Moore and Stebbing, 1976). Some of these responses are likely to be of a more general nature ie., indicating the organism's response to a situation of stress brought about by a general deterioration of water quality (Oikari and Soivio, 1977).

Cell membranes which surrounds the cell and the continuous endoplasmic reticulum (ER) are the first to confront pollutants. They are susceptible to the effects of pollutants as they bind the lipo-protein layer of the membrane and induce variation in the permeability which upset the whole cellular systems. So a study of the membrane bound enzymes like acid phosphatase and alkaline phosphatase become a useful index of the extent of pollution imposed.

Transamination represents one of the principal metabolic pathways for the synthesis and deamination of acids. Ammonia can be incorporated into a variety of amino acids by the action of glutamate dehydrogenase on alpha ketoglutaric acid to form glutamate. Transamination, therefore, allows an interplay between carbohydrate, fat and protein metabolism an activity which can serve the changing demands of the organism (Cohen and Sallach, 1961). The activities of the enzymes are likely to reflect the metabolic state of the fish in the same way as they do in mammals (Bouck et al., 1975). Hence

study of two important aminotransferases, aspartate aminotransferase and alanine aminotransferase would provide much information on the metabolic state of the fish under metal stress.

Effects of heavy metals on the haematology

Fishes are in direct contact with the environment and are susceptible to any change that may occur. It is expected that such changes would be reflected in the physiology of fish and particularly in the values of haematological parameters (Blaxhall, 1972). Blood takes part directly or indirectly in almost all the activities of fish and acts as a good indicator of stress conditions. When values are obtained under abnormal conditions, it should be possible to monitor changes in the physical and chemical properties of water (Mawdesley-Thomas, 1971). The use of haematological parameters as indicators of sublethal effects of stress can provide information on the physiological responses of the fish in a changing external environment. This is a result of close association of the circulatory system with the external environment and with every tissue. Changes in haematology of fishes in response to stressing agents enable to initiate prophylactic measures in fish culture.

Effects of heavy metals on the catalase activity

It is becoming apparent that many environmental toxicants function through free radical or related oxidant-mediated reactions (Bus and Gibson, 1979; Mason, 1982; Mason et al., 1982). Superoxide (O_2^-) and hydroxyl radicals (OH^-) are the two primary oxygen-based radicals. H_2O_2 though not a free radical is an important related oxidant. Superoxide, hydrogen peroxide and hydroxyl radicals are able to cause much damage to organisms through effects such as oxidation of cellular reductants, formation of methaemoglobin, the peroxidation of polyunsaturated fatty acids and the degeneration of nuclear materials. The enzyme superoxide dismutase (SOD) present in the body converts superoxide radicals into hydrogen peroxide which is converted into water and oxygen by the action of catalase. Exposure to metals increases the production of superoxide and hydrogen peroxide. If these toxic oxygen based radicals are not removed, they damage different cell organelles and important cell constitutents. Biochemical antioxidant defence mechanisms protect the body

from these radicals by scavenging them. Catalase is one of the important enzymes involved in the enzymic antioxidant defence mechanism. Catalase, alongwith SOD, removes many of these oxidant mediated radicals.

There is another nonenzymatic antioxidant defence system which becomes active as a result of the introduction of toxicants to the living system and that is the glutathione cycle.

Effects of heavy metals on the glutathione content

The glutathione cycle operates using endogenous glutathione through the involvement of glutathione peroxidase (GPX), glutathione-S-transferase (GST) and glutathione reductase (GR) and they can eliminate hydroperoxides and other lipid radicals thereby eliminating the toxic, free oxygen radicals. Many xenobiotics are eliminated from the body by oxidative metabolism and subsequent conjugation.

Glutathione (GSH) is a major non-protein thiol representing more than 90% of the thiol content in animals (Meister, 1981 a, b). The biological importance of tripeptide lies in the fact that it represents together with NADH and NADPH, the reducing system of the cell involved in the maintenance of redox potential (Reed and Beatty, 1980).

Glutathione is involved in a number of important physiological and detoxification process (Kosower and Kosower, 1978; Moron et al., 1979; Hazelton and Lang, 1980; Reed and Beatty, 1980). Nonprotein thiol interact with metals in several ways. The cysteine and GSH content of mammalian tissues influences the uptake, distribution and toxicity of mercury (Congui et al., 1978; Alexander and Aaseth, 1982; Johnson, 1982). Methylmercury glutathione is a major methylmercury derivative excreted in rat bile and its rate of excretion is related to the ability of the liver to excrete glutathione (Hirata and Takahashi, 1981; Ballatori and Clarkson, 1982).

Glutathione is a substrate for a variety of enzymes involved in conjugation, and reduction/oxidation reaction important for the detoxification and excretion of foreign compounds and their metabolites (Orrenius and Jones, 1978; Reed and Beatty, 1980).

Effects of heavy metals on the ascorbic acid content

Amongst the various morphological anomalies in fish that may be caused by chemicals in the aquatic environment, spinal column damage and craniodidymus have been reported by Dawson (1971) and Imada and Yoshizumi (1973). Reports of experiments on the development of vertebral anomalies due to agrochemicals have been made by Meyer (1966), McCann and Jasper (1972) and Mehrle and Mayer (1975 a, b). Malformation caused by heavy metals have been described by Muramoto et al. (1972) and Muramoto (1979) in relation to vertebral anomalies in carp. In addition, Pickering and Gast (1972) reported a case of malformation in fathead minnow due to cadmium whilst, Eaton (1974) described a similar effect in bluegill, Nakamura (1975) in dace and Fujimagari et al. (1974) in guppies.

In fishes ascorbic acid is known to play an important role in the synthesis of collagen (Halver et al., 1975; Lim and Lovell, 1978; Mauck et al., 1978). As most of the fishes lack the ability to synthesise ascorbic acid, a deficiency of ascorbic acid in the diet, results in various skeletal deformities (such as lordosis and scoliosis) and haemorrhage.

When fishes are exposed to metals, the body uses the stored ascorbic acid in the body to detoxify the metals and in the process consumes the ascorbic reserves. This creates a deficiency of ascorbic acid in the body and consequently ascorbic acid deficiency symptoms appear.

CHAPTER 2

EFFECT OF COPPER AND MERCURY ON THE GLYCOGEN AND PROTEIN CONTENT OF THE LIVER AND MUSCLE

Eventhough effects of environmental pollutants on the mortality of aquatic animals have been studied by many authors, very little is known about the disturbed physiological and biochemical processes within the organism following exposure to environmental pollutants. This is all the more important as contamination of natural water resources by heavy metals threaten fish culture and population (McKim and Benoit, 1971; Christensen, 1975).

When heavy metal ions exceed a threshold concentration in the aquatic ecosystem, they act as pollutants and create stress in fish. Environmental pollution is reported as one of the major factors causing hypoxemia in animals (Black et al., 1962). The respiratory potential of an animal is an important physiological parameter to assess the toxic stress because it is a valuable indicator of energy expenditure in particular and metabolism in general. This also helps for making valid inferences on its environmental requirements. Basha et al. (1984) found that the activity levels of succinate dehydrogenase and malate dehydrogenase decreased in toxicant-exposed fish suggesting the prevalance of hypoxia. Pesticides, heavy metals and other xenobiotics are known to affect the oxygen consumption and metabolic pathways.

The respiratory system of fish seems to be the prime target of many pollutants. When tissues of the animal do not receive sufficient oxygen they must either reduce the overall energy demand or respire anaerobically. Since glycogen is the ready source of energy even in anaerobic condition, the depletion of glycogen from the tissue is expected to be an immediate manifestation of hypoxemia. During severe hypoxia, flounder reduces its oxygen consumption and partially compensates by increasing anaerobic energy metabolism based on fermentation of glycogen or glucose with lactic acid as the major anaerobic end product (Jorgensen and Mustafa, 1980). This strategy is also employed by other fishes such as carp (Johnston, 1975), goldfish (Van den Thillart, 1977) and trout (Burton and Spehar, 1971). A decrease in the glycogen content confirms the prevalance of hypoxic condition at the tissue level since anoxia or hypoxia increases carbohydrate consumption (DeZwaan and Zandee, 1972)

thereby creating a sort of stress in the fish even at the sublethal level, resulting in extra expenditure of energy.

The biochemical and physiological adaptations made by fish in response to change in environmental oxygen levels can be correlated to the ecology of the species. Species such as the European carp, Cyprinus carpio, which has been shown to live for several months in water of very low oxygen content (Blazka, 1958) are able to survive by reducing their oxygen uptake and changing to anaerobic metabolism (Johnston, 1975). Other species such as salmonids, adapted to environments of high oxygen tension, are less able to survive hypoxia (Itazawa, 1971). Since different fishes react differently to hypoxic situation, the procedure of using oxygen consumption as a yard-stick to measure the metabolic activities of the body may not produce satisfactory results. Although a number of methods are now available to measure sublethal effects of pollutants (Sprague, 1971) most of them are long term and are not suitable for routine monitoring programmes.

Extensive investigations have revealed that different tissues of fish can sustain varying levels of anaerobic metabolism. In most teleosts fermentation of glucose to lactate provided the main source of energy under hypoxic condition (Heath and Pritchard, 1965; Burton and Spehar, 1971). Black et al. (1961) found that the endurance of fast swimming fish is limited by the anaerobic energy released when stored glycogen is transformed by the Emden-Meyerhof cycle to form lactic acid within muscle cells. Johnston (1977) postulated that while skeletal muscle of fish, in common with most vertebrate tissues, responds to periods of anoxia by an increase in anaerobic glycogenolysis.

In almost all these circumstances the major share of stored energy comes from the carbohydrate or glycogen reserves. Thus carbohydrates form the central point in energy production because of its great mobility in the living systems, together with its capacity to get compartmentalised within cells and tissues. The mobility is provided by glucose and compartmentalization by glycogen and glucose-6-phosphate.

It is widely accepted that carbohydrate deposits in the form of glycogen in tissues like liver and muscle provide the immediate energy requirements

in teleost fishes under a variety of stressors including exercise (Black et al., 1960, 1961, 1962), physical disturbance (Nakano and Tomlinson, 1967), starvation (Black et al., 1966), environmental hypoxia (Heath and Pritchard, 1965; Narasimhan and Sundararaj, 1971), salinity changes (Bashamohideen and Parvatheswara Rao, 1972).

Effects of environmental stress due to chemical pollution on tissue glycogen levels of fish have also been reported (McLeay and Brown, 1975; Mazmanidi and Kovaleva, 1975; Gill and Pant, 1981; Dange and Masurekar, 1982). These studies, involving exposure of fishes to different pollutants have indicated that the pollution stress stimulates glycogenolysis in fish tissue.

From a biochemical point of view, life is uniquely characterised by its association with protein. Tissue proteins as energy sources for fishes during thermal stress, spawning, and muscular exercise have been demonstrated by several investigators (Fontaine and Hatley, 1953; Idler and Clemens, 1959). Though considerable information is available dealing with the determination of acute toxic levels of several pollutants and their influence on oxidative metabolism, studies on the tissue energy sources are relatively few.

Adrenocortical hormones are known to influence mammalian intermediary metabolism by stimulating protein metabolism (Long et al., 1940; Storer, 1967; Freeman and Idler, 1973). It is now known that these hormones are produced during stress. Hence the stress created by the exposure of metals interferes with the intermediary metabolism and affect the protein content of the body.

Aspartate aminotransferase (GOT) and alanine aminotransferase (GPT) are known to play a strategic role in mobilising L-amino acid for gluconeogenesis and also function as links between carbohydrate and protein metabolism under altered physiological, pathological and induced environmental stress conditions (Nichol and Rosen, 1963; Knox and Greengard, 1965; Harper et al., 1977).

Glycogen and protein present in liver and muscles provide energy to the body. Animal under stress deplete the energy sources at different rates. Hence a study was conducted to examine the effect of copper and mercury on the glycogen and protein content of liver and muscles.

MATERIAL AND METHODS

Specimens of Oreochromis mossambicus were collected from Cochin by cast net and were brought to the laboratory immediately. Fishes of length 9 to 11 cm and weighing 30-40 gms were selected for the experiment. They were examined for diseases, external parasites and injuries and totally healthy fishes were acclimatized in large aquarium tanks containing dechlorinated tap water (pH 6.8 - 7.1, dissolved oxygen 4.8 ml/l, hardness 22 mg/l as $CaCO_3$) for one month. They were fed daily with boiled eggs, clam meat and hydrophytes maintained in the laboratory. After feeding, the water was changed daily.

For the experiments the acclimatized fishes were transferred to five large experimental tanks, each containing 200 l of dechlorinated tap water. To two tanks, 1000 ppm $CuSO_4$ (BDH) solution was added to give a concentration of 100 μg/l and 200 μg/l and to the other two tanks, mercury was added as mercuric chloride (Merck) to give a concentration of 100 μg/l and 150 μg/l respectively. Fishes transferred to the fifth tank identically kept but without any of the toxicants, served as controls. Water in the experimental and control tanks were removed daily to remove the debris and to maintain the required dissolved oxygen content in the tanks, taking care to give minimum disturbance to the fish and replacing the various concentrations of mercury and copper in the experimental tanks. The temperature of the tanks were maintained at 28 $\pm$ 1°C and particular care was taken that O_2 is not a limiting factor. The fishes were not fed 24 h prior to the experiment and during the entire period of the experiment.

The fishes from each experimental tank were caught by a hand net at intervals of 24, 72, 120 and 168 h. They were immobilised with a hard blow on the head. Immediately the body was cut open and the liver, and a piece of white epaxial muscle from a definite area below the dorsal fin were removed and weighed accurately. The samples were homogenized in 5 ml of 10% Trichloro acetic acid (TCA) and centrifuged at 3000 rpm for 10 minutes.

Estimation of glycogen

The supernatant of the tissue extract in TCA was used for the estimation

of glycogen. The glycogen content of the extract was determined following the phenol sulfuric acid method of Montgomery (1957); 0.75ml of the supernatant of the muscle tissue extract and 0.3 ml of the supernatant from the liver tissue extract was used for the glycogen estimation. To each sample 1.5 ml of 95% ethyl alcohol was added, mixed and kept overnight in a refrigerator. It was centrifuged at 3000 rpm for 15 minutes. The supernatant was very carefully decanted. The precipitate was dissolved in 2 ml of distilled water and to this 0.1 ml 80% phenol was added. The mixture was shaken well. 5 ml of conc. H_2SO_4 was added forcefully to this mixture. It was then kept at room temperature for 30 minutes. After cooling to room temperature, the colour developed was read at 490 nm in a spectrophotometer (Hitachi, light path 1 cm). The glycogen concentration of the samples were determined from the calibration curve prepared by employing oyster glycogen (Sigma) as the standard. The significant difference between controls and experimental fishes was determined using student's 't' test (Zar, 1974).

Estimation of Protein

The residue of the tissue extract in TCA was used for the estimation of protein. The protein of the residue was estimated by the method of Lowry et al. (1951). The precipitated protein in the residue was dissolved in 5 ml in NaOH. 0.3 ml of the liver sample and 0.2 ml of the muscle sample were used for the experiment. The samples were made up to 1 ml with distilled water. To these samples 5 ml of alkaline copper reagent was added and shaken well. After 10 minutes 0.5 ml of Folin's Phenol reagent was added and mixed well. After 45 minutes the blue colour developed was read at 500 nm. The concentration of the protein in the samples were calculated from the standard graph prepared by using bovine serum albumin as the standard. The results were analysed statistically.

RESULTS

Results of the experiments are presented in Tables 1, 2, 3 and 4 and Figs. 1, 2, 3 and 4.

Glycogen content of the liver

In the liver of controls, the glycogen concentration showed a non-

significant decline between days. In the liver of fishes exposed to lower concentration (100 µg/l) of copper there was no significant variation in the glycogen content at 24 h. However, there was a very significant decrease ($P < 0.01$) in these fishes from 72 h onwards, when compared to controls. In the fishes exposed to higher concentration (200 µg/l) of copper and to mercury (100 µg/l and 150 µg/l) the significant ($P < 0.01$) decrease in the glycogen level started at 24 h itself and continued till the end of the experiment.

Glycogen content of the muscles

Here also the controls showed a nonsignificant decrease in the glycogen of the tissue. The lower concentration of copper did not cause any change in the glycogen content in the muscles. But there was a significant decrease ($P < 0.05$) in the glycogen level of muscles of *O. mossambicus* exposed to the higher concentration of copper at 120 h and 168 h. However, in the mercury treated fishes the picture was different. A significant decline of the glycogen was observed at 24, 72, 120 and 168 h.

Protein content of the liver

The lower concentration of copper did not affect the protein content. In those fishes exposed to lower concentration of copper, the protein content of the liver did not vary significantly throughout the experiment from that of controls and in the liver of fishes exposed to higher concentration of copper, the significant decrease in protein was observed at 120 and 168 h. A similar change was observed in the fishes exposed to lower concentration of Hg when a significant decrease in the protein content occurred at 120 and 168 h. But in the liver of fishes exposed to mercury (200 µg/l), the protein level declined at 72 h ($P < 0.05$). This significant decrease continued at 120 and 168 h ($P < 0.01$).

Protein content of the muscles

Here, the two metals reacted differently. The protein content of the muscles of the fishes exposed to lower concentration of copper did not show any significant variation from that of controls. But there was a significant decrease ($P < 0.05$) in the protein level of the muscle of the fishes treated

Table 1. Glycogen content of the liver of *O. mossambicus* exposed to copper and mercury

Concentration of metals µg/l		Glycogen mg/gm wet weight $\bar{x} \pm$ S.D, (N = 10) Exposure time			
		24 h	72 h	120 h	168 h
Cu	100	14.71 ± 2.31	10.26** ± 2.23	9.06** ± 1.85	8.21** ± 2.09
	200	10.34** ± 1.97	9.10** ± 2.21	7.14** ± 2.12	6.13** ± 2.14
Hg	100	9.74** ± 2.08	7.43** ± 2.15	5.24** ± 2.17	5.07** ± 1.98
	150	8.14** ± 2.24	6.14** ± 2.36	4.78** ± 2.40	4.32** ± 2.28
Control		15.86 ± 2.18	14.76 ± 2.24	13.79 ± 2.32	13.03 ± 2.29

* $P < 0.05$ ** $P < 0.01$

Table 2. Glycogen content of the muscle of *O. mossambicus* exposed to copper and mercury

Concentration of metals µg/l		Glycogen content mg/gm wetweight $\bar{x}$ + S.D, (N = 10) Exposure time			
		24 h	72 h	120 h	168 h
Cu	100	1.65 ± 0.35	1.39 ± 0.48	1.06 ± 0.39	0.96 ± 0.52
	200	1.55 ± 0.31	1.22 ± 0.53	0.95* ± 0.52	0.82* ± 0.47
Hg	100	1.25* ± 0.48	1.02* ± 0.44	0.91* ± 0.43	0.78* ± 0.41
	150	1.27* ± 0.36	0.85** ± 0.38	0.80** ± 0.42	0.55** ± 0.49
Control		1.76 ± 0.41	1.54 ± 0.52	1.42 ± 0.39	1.33 ± 0.44

* $P < 0.05$ ** $P < 0.01$

FIGURE 1. GLYCOGEN CONTENT OF THE LIVER OF O. MOSSAMBICUS EXPOSED TO COPPER AND MERCURY

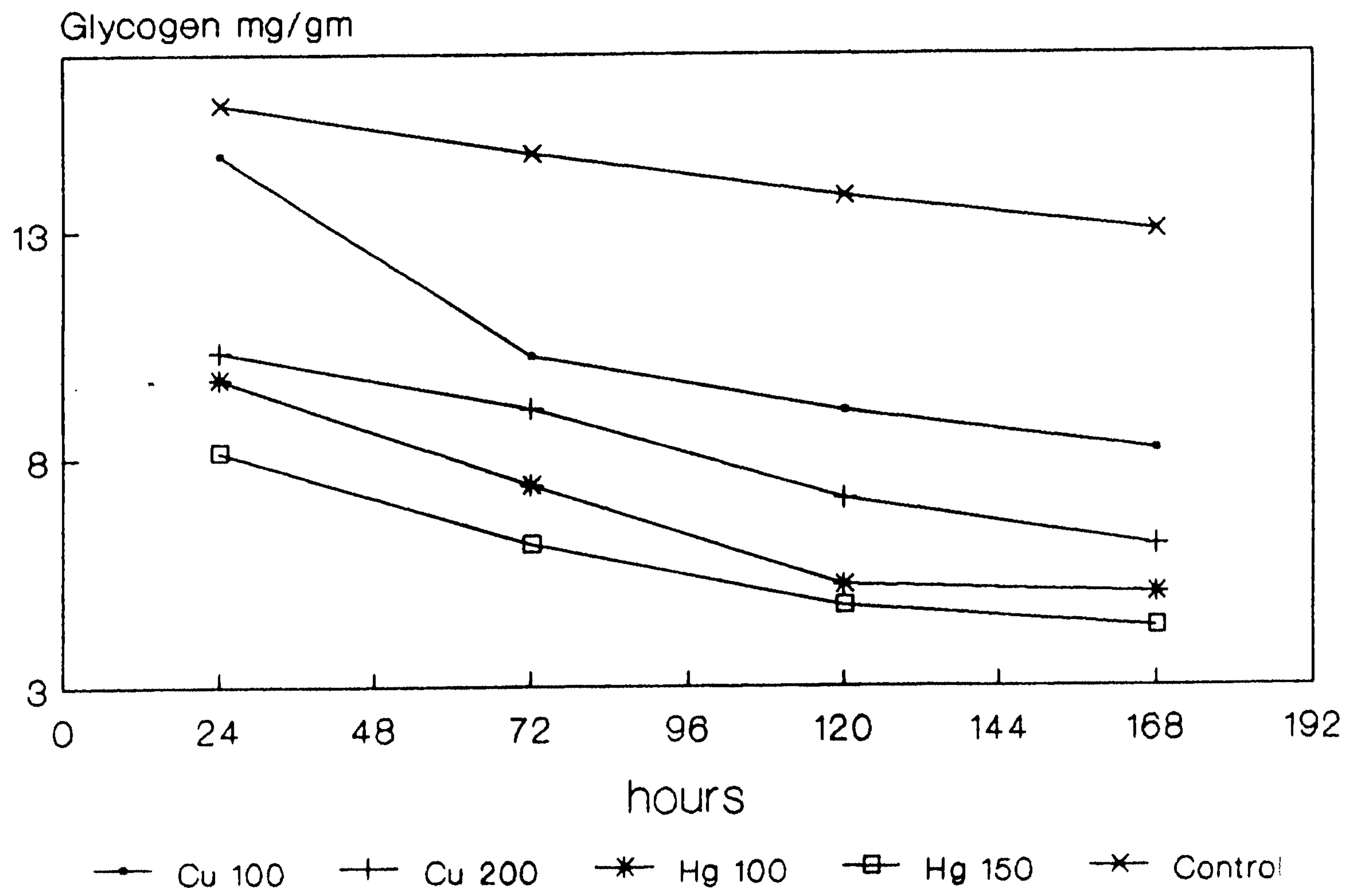
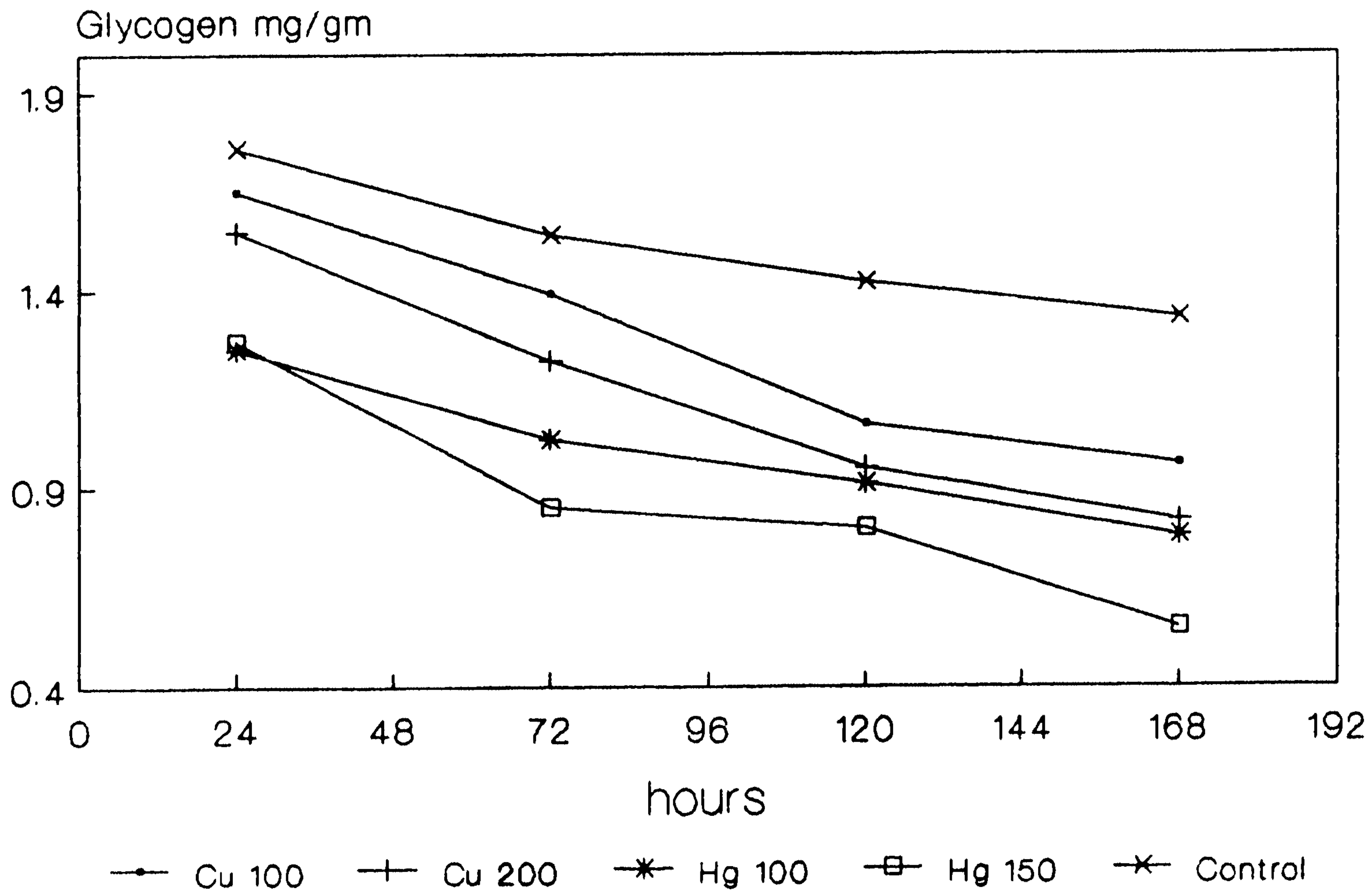


FIGURE 2. GLYCOGEN CONTENT OF THE MUSCLE OF <u>O. MOSSAMBICUS</u> EXPOSED TO COPPER AND MERCURY



with higher concentration of copper at 168 h. A similar change was observed in the fishes exposed to lower concentrations of mercury, the significant decrease occurred at 168 h. In the muscles of fishes treated with higher concentration of mercury the significant decrease ($P < 0.05$) in protein level started at 120 and continued upto 168 h.

DISCUSSION

In the present study there were significant depletion of glycogen in the liver and muscles of fishes exposed to copper and mercury. This indicates that the metals interfere with the carbohydrate utilisation in O. mossambicus. Reactions of the liver glycogen was more pronounced than that of the muscle. In the present study, similar to the observations by Dange (1986 a) the least extensive changes were seen in the copper-dosed fishes.

Significant decrease in the glycogen reserves of both liver and muscle has been reported in Heteropneustes fossilis in response to 25 and 50 ppm of mercury (Qayyum and Shaffi, 1977); in different fishes in response to cadmium, copper, lead and zinc (Shaffi, 1978 a, b; 1979 a, b; 1980 d); in rainbow trout in response to cadmium (Larsson and Haux, 1980); in Anabas scandens in response to zinc sulphate (Natarajan, 1982); in Heteropneustes fossilis in response to copper, cadmium and mercury (Srinivasthava, 1982); in Notopterus notopterus in response to mercury (Verma and Tonk, 1983); in rainbow trout in response to cadmium (Lowe-Jinde and Niimi, 1984); in Sarotherodon mossambicus in response to potassium dichromate (Ghosh and Chatterjee, 1985); in O. mossambicus in response to different pollutants (Dange, 1986 a) and in Barbus conchonius in response to lead (Tewari et al., 1987).

Apart from these, glycogen depletion in the liver is reported in H. fossilis in response to lead (Shaffi and Qayyum, 1979); in Puntius conchenius in response to mercury (Gill and Pant, 1981); in Channa punctatus in response to cadmium (Dubale and Shah, 1981); in C. punctatus in response to chromium (Sastry and Tyagi, 1982; Sastry and Sunita, 1983); in S. mossambicus in response to 1.5 ppm mercury (Naidu et al., 1984) and in Clarias batrachus in response to lithium administration (Goel et al., 1985).

Table 3. Protein content of the liver of *O. mossambicus* exposed to copper and mercury

Concentration of metals µg/l		Protein mg/gm wet weight $\bar{x} \pm$ S.D, (N = 10) Exposure time 24 h	72 h	120 h	168 h
Cu	100	91.93 ± 10.17	100.25 ± 8.73	96.77 ± 9.22	102.43 ± 10.05
	200	97.13 ± 11.61	104.65 ± 9.19	84.68* ± 8.33	80.95** ± 8.03
Hg	100	100.27 ± 8.52	94.70 ± 10.22	83.66* ± 9.58	81.00** ± 8.21
	150	94.14 ± 9.76	88.19* ± 9.36	76.07** ± 7.84	65.18** ± 9.32
Control		95.34 ± 7.21	98.94 ± 10.12	93.96 ± 8.69	96.34 ± 9.84

Table 4. Protein content of the muscle of *O. mossambicus* exposed to copper and mercury

Concentration of metals µg/l		Protein mg/gm wet weight $\bar{x} \pm$ S.D, (N = 10) Exposure time 24 h	72 h	120 h	168 h
Cu	100	124.83 ± 16.53	132.21 ± 12.66	123.46 ± 16.71	138.15 ± 16.87
	200	118.96 ± 13.88	122.75 ± 15.47	133.26 ± 17.39	116.68* ± 14.62
Hg	100	122.27 ± 11.42	135.07 ± 16.36	128.38 ± 13.93	118.16* ± 13.19
	150	131.19 ± 15.76	132.10 ± 14.66	112.74* ± 12.11	115.60* ± 15.37
Control		124.30 ± 13.78	131.96 ± 14.08	126.19 ± 14.43	132.76 ± 14.28

* $P < 0.05$ ** $P < 0.01$

FIGURE 3. PROTEIN CONTENT OF THE LIVER OF O. MOSSAMBICUS EXPOSED TO COPPER AND MERCURY

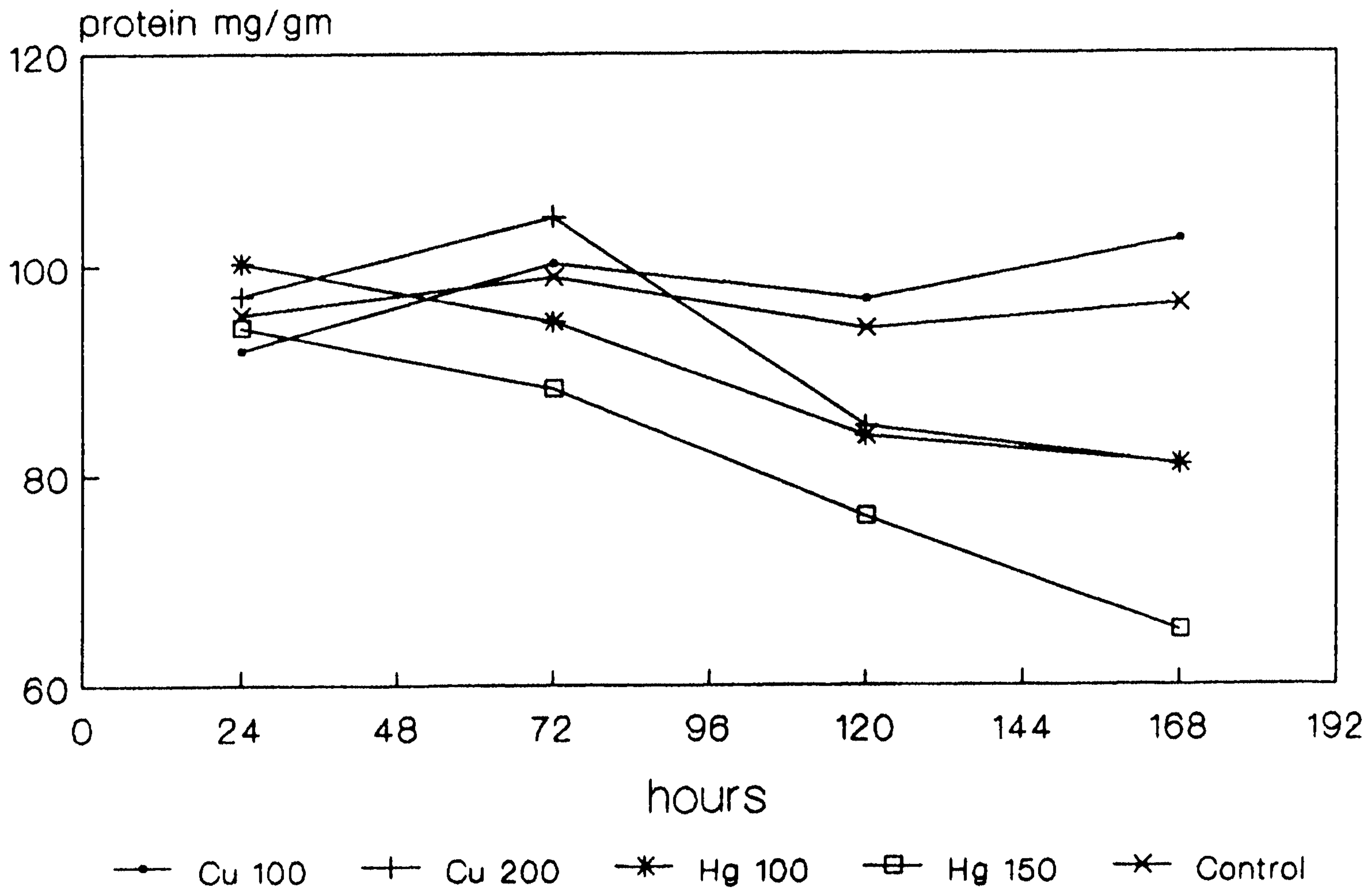
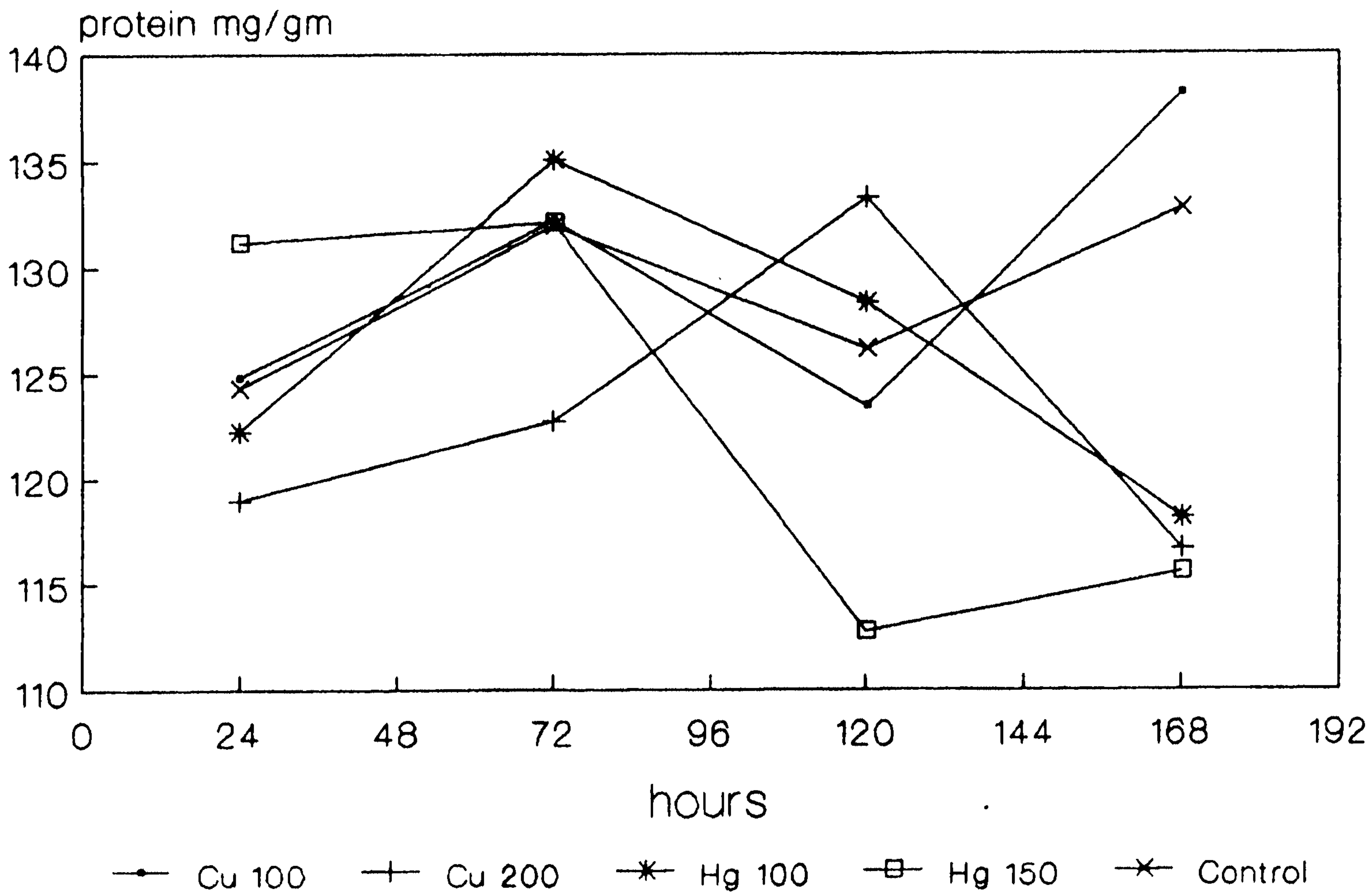


FIGURE 4. PROTEIN CONTENT OF THE MUSCLE OF O. MOSSAMBICUS EXPOSED TO COPPER AND MERCURY



However there are also reports that various toxicants caused an increase in the glycogen level of various tissues of different fishes (Grant and Mehrle, 1973; Buckley et al., 1979; Bakthavathsalam and Reddy, 1982 b; Anderson et al., 1987; Nath and Kumar, 1987).

But in some fishes, glycogen levels were not affected by mercury (Sastry and Rao, 1984) or copper (Tort et al., 1987).

Xenobiotics interfere in the various functions of the body. In the present study, it was found out that the fish became irritable after exposure to copper and mercury. They get irritated at the slightest provocation and were hyperactive and hyperactivity depletes the stored food materials present in the muscle and liver. Many toxicants are known to oxidise glutathione, Hb etc., damage cell membranes and organelles by lipid peroxidation, inhibit many enzymes and thus disrupt important physiological functions of the body. The body requires large quantities of energy to produce substances like glutathione, metallothionein, glucuronic acid and other substances to remove toxicants by activation, inactivation or conjugation etc. and to repair damaged organelles and replace lost cell constituents.

So, in fishes exposed to pollutants the energy demand is very high. This increased energy demand is met by utilizing the stored glycogen in different tissues. The increased metabolic activity of the liver after heavy metal intoxication was reported by Maynard and Loosli (1962). Muscle glycogen is rapidly depleted during intense activity and glucose is mobilized from the liver glycogen stores to supply both raw materials and rebuilding muscle glycogen. Both glycogenolysis and 'glycogenesis' involves a number of enzymes and is guided by hormones. Hormones increase Glycogenolysis during stress to meet the increased energy demand. The alterations in carbohydrate metabolism are produced indirectly by the environmental stresses through primary effect on the endocrine glands exciting them into releasing large amounts of hormones (Young and Chavin, 1965; Oguri and Nace, 1966; Fagerlund, 1967; Nakano and Tomlinson, 1967; Grant and Mehrle, 1973; Assem et al., 1978; Schreck and Lorz, 1978; Hille, 1982; Gluth and Hanke, 1984).

Hormones control this alterations of carbohydrate metabolism mainly by suppressing insulin production and increasing the glucocorticoids and catecholamine synthesis. Impaired secretion of insulin by β- cells of pancreatic islets tend

to decrease glycogen levels of the liver and muscle. Decreased insulin production after metal intoxication was observed in Caltus scarpius (Have, 1969) and rainbow trout (Wagner and McKeown, 1982). Eventhough, in fish there is no equivalent of mammalian adrenal gland, the chromaffin cells which are scattered in different organs (Mazeaud and Mazeaud, 1981) produce glucocorticoids and catecholamines. Glucocorticoids are known to influence intermediary metabolism (Selye, 1950). External toxic stimuli and stressors are known to elevate cortisol in fish (Schreck, 1981, Gluth and Hanke, 1984, 1985). The higher level of cortisol precedes the elevation of glucose and is one of the reasons for it. Hence stressful conditions release corticosteroids into the blood which is followed by an elevation of blood glucose (Hille, 1982). The other cause of glucose elevation is the release of catecholamines. In many fishes stress induced an elevation of catecholamines (Mazeaud et al., 1977). Regardless of the nature of stress, either adrenalin or noradrenalin are released into the blood and this increases blood glucose. Thus different kinds of stress induce an elevation of blood glucose (Hardisty et al., 1976). To keep the glucose level in the blood relatively high, despite metabolic process following the influence of stressors, glycogenolysis is essential. The elevation in blood glucose may form a part of restorative process in which glucose is mobilised from the liver glycogen stores (Wardle, 1978). It enters the muscle cells supplying both raw materials and energy for rebuilding muscle glycogen. There are reports that the blood sugar levels are elevated in fish during acute exposure to a variety of environmental alterations considered as stressful including exposure to toxicants (Holeton and Randall, 1967; Nakano and Tomlinson, 1967; Chavin and Young, 1970; Narasimhan and Sundararaj, 1971; Bashamohideen and Parvatheswara Rao, 1972; Hunn, 1972; McLeay et al., 1972; Wedemeyer, 1972; 1973; Grant and Mehrle, 1973; McLeay, 1973; 1977; Hattingh, 1976; Soivio and Oikari, 1976; Mazeaud et al., 1977; Banerjee et al., 1978; Shaffi, 1978 b, 1980 a, c; Assem and Hanke, 1979 a, b; Ito and Murata, 1980; Jorgensen and Mustafa, 1980; Larsson and Haux, 1980; Mukhopadhyay and Dehardrai, 1980; Gill and Pant, 1981; 1983; Srivasthava and Singh, 1981, 1982; Sastry and Siddiqui, 1982; Sastry and Tyagi, 1982; Wagner and McKeown, 1982; Mishra and Srivasthava, 1983, 1984; Srivastava, and Mishra, 1983; Cliff and Thurman, 1984; Gluth and Hanke, 1984; Goel et al., 1985; Sastry and Subhadra, 1985; Lal et al., 1986; Tewari et al., 1987).

Phosphorylase is a regulatory enzyme of glycogen breakdown. Bhaskar and Govindappa (1986) found that in the red muscle of Tilapia mossambica acclimated to alkaline medium, a stepped up glycogen breakdown and an increased phosphorylase activity in the red muscle. They suggested that the phosphorylase activity could be responsible for depleted glycogen content. The depletion of glycogen observed in the present study could be due to this phenomenon also.

Normally the glucose molecules undergo glycolysis and the pyruvic acid formed enter tricarboxylic acid cycle (TCA cycle) releasing energy. But when there is a deficiency of oxygen, the pyruvic acid gets reduced to lactic acid. During hypoxia or anoxia, the aerobic metabolism changes to anaerobic metabolism, as a result of which lactic acid is accumulated. So accumulation of lactic acid is a sign of anaerobic metabolism. Muscle glycogen is rapidly depleted during intense activity and appears to be the principal source of lactic acid during anaerobic metabolism in lower vertebrates (Bennet, 1978). Accumulation of lactic acid in fishes to many toxicants and stress were reported by Grant and Mehrle (1973); McLeay and Brown (1975); Shaffi (1979 a, b, 1980 a, c); Sastry and Siddiqui (1982, 1983); Srivasthava and Singh (1982); Sastry and Sunita, (1983); Cliff and Thurman (1984); Tort et al. (1984); Sastry and Subhadra (1985) and Bhaskar and Govindappa (1986). Thus lactic acid is produced in hypoxic tissue as a result of a switch from aerobic to anaerobic metabolism. This resulted in a decrease in the glycogen values of liver and muscle of fish.

Hypoxia or anoxia can result from the faulty gaseous exchange. Heavy metals are one class of pollutants which have a disruptive influence on the structural organization of the gill tissue. Gills of rainbow trout exposed to acute lethal concentration of zinc are damaged (Mathiesson and Brafield, 1973; Skidmore and Tovell, 1972). Burton et al. (1972) and Skidmore (1970) found that rainbow trout exposed to Zn^{2+} (40 ppm) die mainly through tissue hypoxia, a major factor being disruption of branchial respiratory epithelium (Skidmore and Tovell, 1972).

Hughes et al. (1979) have shown that exposure to pollutants causes a reduction in the morphological basis for diffusing capacity of the gills. The gill filaments have a key position in the bodies of fish because of their role in the transport of oxygen. In the secondary lamellae, the circulatory system

is separated from the surrounding medium only by a layer of epithelial cell, one or two cells thick, a basement membrane and a thin layer of cytoplasm lining the blood lacunae.

Lindahl and Hell (1970) demonstrated the effects of Phenylmercuric hydroxide (PMOH) on the gill tissue and found that the superficial layer of the gill filaments appear somewhat detached from deeper parts. This reduces the diffusing capacity and there is a fall in the oxygen supply to the tissues which becomes hypoxic. Lindahl and Hell (1970) found that O_2 consumption of gill is reduced by 30% after an exposure of the animal to PMOH. Davis (1973) found a decline in arterial oxygen tension in Sockeye salmon following pulpmill effluent exposure.

Diffusing capacity of the gills is further reduced following the irritating action of pollutants which causes a secretion of mucus over the gills (Shaffi, 1978 b). Interference with gas transfer will reduce oxygen levels within the blood circulating to the brain where responses are initiated by the respiratory centre. The respiratory centre may coordinate cardio-vascular changes and stimulate the hormonal system and erythropoietic tissue to take necessary steps to compensate for the decreased oxygen supply to the tissues. A decreased glycogen level of the body may be a step in that line.

Heavy metals are known to inhibit many enzymes (see chapter 3). It is known that structural changes of enzymes are induced by mercurials (Webb, 1966). So it might be possible that copper and mercury inhibited different enzymes of the TCA cycle, like pyruvic dehydrogenase, succinic dehydrogenase, malate dehydrogenase. The inhibition of these enzymes of TCA cycle can prevent the aerobic metabolism. Hence inhibition of the enzymes of TCA cycle indicate the impairment of aerobic metabolism. This also suggests a shift from aerobic to anaerobic metabolism in the fish under pollution stress. An inhibition of the enzymes of TCA cycle in fishes exposed to pollutants was observed by Siva Prasada Rao and Ramana Rao (1979); Sastry and Siddiqui (1983); Sastry and Sunita (1983); Balavenkatasubhaiah et al. (1984) and Naidu et al. (1984). In the present study even though no effort was made to find out the activities of TCA cycle enzymes, it would be reasonable to assume that the depletion of glycogen observed in O. mossambicus could have been due to such an inhibition also.

Increase of anaerobic metabolism has been shown to be a rapid and clear response against depletion of energy caused by lack of oxygen (Van den Thillart, 1982). These responses have also been observed after metal exposure (Burton et al., 1972; Hodson, 1976). Basha et al. (1984) suggested the prevalance of hypoxic condition in the tissue and a reduction in the rate of oxidative metabolism at the mitochondrial level in Tilapia mossambica exposed to toxicant. In the present study also, copper and mercury may create such a condition in the fish.

The significant decrease in the protein content of liver and muscles of metal dosed O. mossambicus occurred at the end of the exposure period, mainly at 120 and 168 h. This clearly indicates that the body utilises the glycogen stores first to meet the increased energy demand. When the glycogen stores were decreased, the body utilises the protein for energy production. This is manifested as a decrease in the protein content in different tissues and serum. There are many reports that the total protein in different tissues and serum of fish decreased after exposure to different toxicants. (Grant and Mehrle, 1973; Camp et al., 1974; McLeay and Brown, 1974; Venktachari, 1974; Sakaguchi and Hamaguchi, 1975; Oikari and Soivio, 1977; Goel and Garg, 1980; Panigrahi and Misra, 1980; Rath and Misra, 1980; Sharma and Davis, 1980; Dubale and Shah, 1981; Goel and Agrawal, 1981; Ramalingam and Ramalingam, 1982; Sastry and Siddiqui, 1983; Verma and Tonk, 1983; Awasthi et al., 1984; Gluth and Hanke, 1984; Naidu et al., 1984; Verma et al., 1984; Sashikala et al., 1985; Katti and Sathynesan, 1986; Kumar and Ansari, 1986; Yamawaki, et al., 1986; Reddy, 1987; Reddy and Bashamohideen, 1987 and Seshagiri Rao et al., 1987).

Many toxicants interfere with protein synthesis or utilises the protein to meet the extra energy demand due to pollutant exposure. So, this affects growth and there are reports that growth has been retarded in fish by these pollutants (Waiwood and Beamish, 1978; Marathe and Deshmukh, 1980; Buckley et al., 1982; Collvin, 1984). But there are instances in which total protein actually increased in different tissues of fishes dosed with toxicants (McKim et al., 1970; Calabrese et al., 1975; Helmy et al., 1979; Siva Prasada Rao and Ramana Rao, 1979; Ito and Murata, 1980; Sahib et al., 1984 and Hilmy et al., 1985). But McLeay and Brown (1979) and Tort et al. (1987) reported that there was no change in the protein content after exposure to pollutant.

The decline in the liver and muscle protein would suggest an intensive proteolysis which in turn could contribute to the increase of free amino acids to be fed into TCA cycle as Keto acids. Such a possibility is further strengthened by the investigation of Schafer (1967); Mehrle et al. (1971). Shakoori et al. (1976) revealed both qualitative and quantitative variations in the tissue amino acids of fishes exposed to toxicants. In addition, studies by Bell (1968); McKim et al. (1970); Lane and Scura (1970); Sakaguchi and Hamaguchi (1975) have also revealed marked variations in the activity of enzymes involved in transamination of fishes in similar situations. Sivaprasada Rao and Ramana Rao (1979) found that the decrease in glycogen is due to the immediate utilization in the tissues to meet the excess demands of the energy metabolism. They also suggested that high increase of amino acids they observed in T. mossambica treated with methyl parathion is utilised for gluconeogenesis through the transamination and transdeamination reactions to supply the necessary keto acids to act as precursors for the maintenance of carbohydrate metabolism to meet the energy requirements during stress. There are reports that transamination and transdeamination reactions are prominant under stress (Knox and Greengard, 1965; Harper et al., 1977).

Decreased protein content could possibly be due to protein breakdown which increased amino acid pool in the tissue. It is also reported that decreased protein moiety suggests damage to hepatic tissue and an intensive proteolysis (Rao, 1984) resulting in increased amounts of free amino acids to be fed into TCA cycle as keto acids. Loss of weight and elevation of nitrogenous compounds in tissues of fish exposed to benthiocarb were reported by Seshagiri Rao et al. (1983) in S. mossambicus. Seshagiri Rao et al. (1987) detected an increased protease activity, increased free amino acid content and decreased protein (soluble and insoluble) in liver, muscle, brain and gills of S. mossambicus.

The decrease in protein following exposure to Hg and Cu suggests their possible degradation by increased proteolysis. This increased proteolysis could be attributed to the damage caused to lysosomal membranes thus permitting the leakage of lysosomal enzyme into the cytosol (see Chapter 3).

The lack of alteration of protein level of liver and muscle of O. mossambicus exposed to copper and mercury at 24 and 72 h (except in

the liver of fishes exposed to higher concentration of Hg) could be that the body utilizes the glycogen of these tissues in the initial period of exposure (24 and 72 h). The depletion of glycogen in the tissues of O. mossambicus after metal-exposure prove this. These findings also support the concept of Fry (1971) that fishes tend to resist a changed situation for a specific period, but will eventually succumb as a result of their inability to adapt. According to Umminger (1970) carbohydrates represent the principal and immediate energy precursors for fishes exposed to stress conditions while proteins being the energy source to spare during chronic period of stress. Gluth and Hanke (1984) found that changes in plasma protein need time to occur and the reduction of protein can only be found after 70 h of exposure.

Radhaiah et al. (1987) observed that amino acids in the kidney increase along with a decrease in the protein values. This proves that intense proteolytic activity in the tissues can increase amino acids in the liver. Such an increase in amino acids after exposure to toxicants in different organisms were found by Girija (1984) and Rao (1984).

A defect in protein synthesis by the action of toxicants can also decrease the protein content in different tissues. An altered relationship between the ribosomes and the membranes of the endoplasmic reticulum may also produce a defect in protein synthesis. Rath and Misra (1980) examined the changes in nucleic acids and protein content in liver, muscle and brain of Tilapia mossambica exposed to the insecticide, dichlorovos. Post-exposure studies revealed a significant decline in DNA, RNA content of the liver, muscle and brain. They observed that the liver exhibited a greater loss of protein than the muscle. In the present study in O. mossambicus exposed to copper and mercury, also the liver showed a greater loss of protein than the muscle. Rath and Misra (1980) also found that the RNA/DNA ratio decreased in exposed fish and it showed a positive correlation with protein. Usually RNA, and RNA/DNA ratio of a tissue are considered to indicate the intensity of protein synthesis (Misra and Patnaik, 1974). It is possible that mercury and copper may have influenced the protein synthesis in O. mossambicus by inhibiting RNA synthesis.

In view of the significant correlation of RNA and protein, a deficient synthesis of any type of RNA should have its reflection in a corresponding failure of protein synthesis. Not only the reduction in the amount of RNA

but also a lesion of its functional capacity brings about such a failure (DeBruin, 1976a).

It has been demonstrated that in the cells of metal exposed organisms, Cd, Hg and Cu are able to reduce the protein synthesis (Viarengo et al., 1980) not only by reducing the rate of RNA synthesis, but also by influencing the attachment of polyribosomes to the rough endoplasmic reticulum and probably damaging the ribosomes themselves.

Hence a decreased protein synthesis and an increased proteolytic activity might have caused the decline in protein content in the liver and muscle of O. mossambicus exposed to the two metals.

CHAPTER 3

PHOSPHATASE ACTIVITY IN THE LIVER AND KIDNEY AFTER EXPOSURE TO COPPER AND MERCURY

The biotransformation and eventual excretion of the many biotransformed derivatives of toxicants, can be realized at different levels of functional complexity such as cellular, subcellular and molecular levels. In many cases the earliest detectable changes of primary events are associated with a particular type of subcellular organelles such as lysosomes, endoplasmic reticulum (ER) and mitochondria.

The lysosomal membrane is often a target of injury by xenobiotics or by their metabolites in addition to its role in sequesteration. Lysosomes are noted for their compartmentalization and accumulation of a wide variety of organic chemicals or metals (Allison, 1969; Sternlieb and Goldfischer, 1976; Moore, 1980). The role of lysosomes may be important as a detoxification system, particularly for metals (Viarengo et al., 1984; George, 1983). As with other detoxification systems, this process is effective until the storage capacity of the lysosome is overloaded or the lysosomes are damaged directly by the contaminant (Moore et al., 1985). Assessment of this type of injury has been confirmed as an index of cellular conditions (Moore, 1980, 1982; Moore et al., 1982, 1985). Cell membrane and the associated endoplasmic reticulum (ER) are also susceptible to the effect of pollutants as they bind the lipoprotein layer of the membrane and induce variation in the permeability which upset the whole cellular systems.

At the molecular level, the accumulation of inclusion bodies like lipofuschin granules, phosphate and carbonate granules and enzymes reflect the impact of xenobiotics. It appears that structural and other properties as well as activities of enzymes can be affected by exposure to pollutants, possibly leading to loss of cellular metabolic flexibility (Gould et al., 1976; Gould, 1977). Many metals have been reported to alter the activity of various enzymes in marine organisms (Webb, 1966; Moore and Stebbing, 1976).

Apart from this, the impact of xenobiotics on cellular and subcellular levels can also be perceived by a study at the molecular level ie., by the study of particular enzymes. Injury of lysosomes by the xenobiotics will result

in the destabilization of lysosomal membrane, resulting in the release of hydrolytic enzymes from the lysosomal compartment into the cytosol (Moore, 1976; Baccino, 1978) and such destabilization may also increase lysosomal fusion with other intracellular vacuoles, leading to the formation of pathologically enlarged lysosomes. The consequence of these lysosomal changes would be increased autolytic activity leading to the atrophy of the cells. This type of injury, resulting in destabilization of the lysosomal membrane bears a quantitative relationship to the magnitude of stress response (Bayne et al., 1979, 1982) and this presumably contributes to the intensity of catabolic or degradative effects as well as to the level of pathological change that results. Hence study of acid phosphatase (ACP) activity in the liver and kidney of fish exposed to heavy metals provides a measurement of the hydrolase latency and lysosomal membrane stability and furnish information on mechanisms involving molecular alterations in the lysosomal membranes which undoubtedly contribute to disturbances of the integration of cellular function (Slater, 1978).

In the present study the enzymes, acid phosphatase (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1) are selected to study the sublethal effects of copper and mercury on Oreochromis mossambicus. Acid phosphatase (ACP) is a lysosomal marker enzyme (De Duve and Wattiaux, 1966; Kendall and Hawkins, 1975). Gupta et al. (1975) reported that ACP is a good indicator of stress condition in biological systems. Racicot et al. (1975) established the diagnostic use of the enzyme by inducting carbon tetrachloride (CCl_4) toxicity and aeromonas infection in rainbow trout.

Intracellular alkaline phosphatase is associated with the plasma membrane and ER (Davison and Gregson, 1965; Bogitsh, 1974). It has been suggested that ALP is involved in membrane transport (Posen, 1967; Cornelius, 1971; Neville, 1974), conversion of NADP to NAD (Morton, 1955) and in several processes including food absorption and calcium deposition (McComb et al., 1979).

Hence a study was conducted to find out the effects of two sublethal concentrations of copper and mercury on the acid phosphatase and alkaline phosphatase activity in the liver and kidney of Oreochromis mossambicus.

MATERIAL AND METHODS

Collection of specimens, acclimatization and experimental set up were the same as described in Chapter 2.

Fishes were exposed to two concentrations of copper (100 µg/l and 200 µg/l) and mercury (100 µg/l and 150 µg/l) for 168 h. For the estimation of phosphatase activity, tissue samples from the liver and kidney were dissected out by immobilising the fish with a hard blow on the head at 24, 72, 120 and 168 h of exposure and then were homogenized in ice cold water (1:100 w/v). The homogenate was centrifuged in a refrigerated centrifuge at 12000 rpm for 15 minutes. The supernatent was collected and used for the analysis of phosphatases. Care was taken to maintain the temperature below 4°C during homogenization and collection of the supernatant.

Assay of acid phosphatase activity

Acid phosphatase activity was determined following the method given in the Sigma Technical Bulletin (No. 104) with slight modification. To 1 ml of 0.1 M citrate buffer of pH 4.5 containing 100 mM NaCl, 0.1 ml of the enzyme extract was added. This buffer enzyme mixture was kept in a water bath at 37°C, 0.1 ml of the substrate (2 mg of P-nitrophenyl phosphate (Merck) in 0.1 ml of distilled water) was added to start the reaction. After incubating for one hour at 37°C, the reaction was stopped by adding 2 ml of 0.25 N NaOH. The yellow colour of p-nitrophenol in the alkaline medium was read at 410 nm. The concentration of p-nitrophenol liberated by the action of the enzyme was found out from the calibration curve prepared. Simultaneously the protein content of 0.1 ml of the enzyme extract, was estimated following the method of Lowry et al. (1951). The specific activity of acid phosphatase as uM of p-nitrophenol liberated per mg of protein per hour was calculated. The results were analysed statistically. The significant difference between controls and experimental fishes was determined using student's 't' test (Zar, 1974).

Assay of alkaline phosphatase activity

To 1 ml of frozen 0.05 M glycine-NaOH buffer (pH 8.6), containing 100 mM NaCl and 0.1 mg of $MgCl_2$, 0.1 ml of the enzyme extract was added. The buffer-enzyme mixture was kept in a waterbath at 37°C and 0.1 ml of substrate

(2 mg of p-nitrophenyl phosphate in 0.1 ml of distilled water) was added to initiate the reaction. After incubating the mixture for one hour, the reaction was stopped by adding 2 ml of 0.25 N NaOH. The yellow colour of p-nitrophenol liberated by the action of alkaline phosphatase was read at 410 nm. After estimating the protein content of 0.1 ml of the enzyme extract following the method of Lowry et al. (1951), the specific activity of the alkaline phosphatase, as µM/mg protein/h was calculated and the results were analysed statistically (Zar, 1974).

RESULTS

Acid phosphatase activity in the liver

Acid phosphatase activity in the liver is represented in Table 5 and Fig. 5. When values of the ACP activity of the controls of different days were compared, no significant difference was observed. Similarly there was no significant difference in ACP activity between controls and 100 µg/l copper-dosed fishes throughout the experimental period. But fishes exposed to 200 µg/l copper showed a significant increase ($P < 0.01$) at 168 h when compared to controls.

In fishes exposed to 100 µg/l mercury a significant increase ($P < 0.05$) was observed at 168 h whereas significantly high values ($P < 0.01$) were observed at 120 and 168 h in fishes exposed to 150 µg/l mercury.

Acid phosphatase activity in the kidney

ACP activity in the kidney of controls did not vary significantly between days. ACP activity in fishes exposed to 100 µg/l copper showed a significant increase ($P < 0.05$) at 168 h when compared to controls. But fishes exposed to higher concentration of copper (200 µg/l) showed a significant increase at 72 h, 120 h ($P < 0.05$) and 168 h ($P < 0.01$). Fishes dosed with 100 µg/l mercury showed a significant ($P < 0.05$) increase in ACP activity at 168 h, from that of controls. But 150 µg/l mercury-dosed fishes showed an increase in ACP activity at 120 h ($P < 0.05$) and 168 h ($P < 0.01$). The values are given in Table 6 and Fig. 6.

Table 5. Acid phosphatase activity in the liver of *O. mossambicus* exposed to copper and mercury

Concentration µg/l		Specific activity (µM/mg protein/h) $\bar{x} \pm$ S.D (N = 10) Hours of Exposure 24 h	72 h	120 h	168 h
Cu	100	1.25 ± 0.39	1.09 ± 0.31	1.29 ± 0.41	1.30 ± 0.29
	200	1.18 ± 0.34	1.21 ± 0.27	1.49 ± 0.39	1.79** ± 0.37
Hg	100	1.29 ± 0.42	1.36 ± 0.30	1.38 ± 0.45	1.76* ± 0.35
	150	1.17 ± 0.26	1.09 ± 0.25	2.06** ± 0.45	2.10** ± 0.42
Control		1.35 ± 0.31	1.27 ± 0.22	1.38 ± 0.36	1.29 ± 0.36

Table 6. Acid phosphatase activity in the kidney of *O. mossambicus* exposed to copper and mercury

Concentration µg/l		Specific activity (µM/mg protein/h) x + S.D. (N = 10) Hours of exposure 24 h	72 h	120 h	168 h
Cu	100	1.10 ± 0.34	1.30 ± 0.28	1.42 ± 0.35	1.71* ± 0.37
	200	1.20 ± 0.28	1.52* ± 0.36	1.58* ± 0.27	1.86** ± 0.33
Hg	100	1.13 ± 0.31	1.36 ± 0.34	1.38 ± 0.30	1.62* ± 0.33
	150	1.35 ± 0.29	1.35 ± 0.31	1.57* ± 0.32	2.15** ± 0.39
Control		1.24 ± 0.25	1.18 ± 0.31	1.21 ± 0.28	1.28 ± 0.32

* $P < 0.05$ ** $P < 0.01$

FIGURE 5. ACID PHOSPHATASE ACTIVITY IN THE LIVER OF *O. MOSSAMBICUS* EXPOSED TO COPPER AND MERCURY

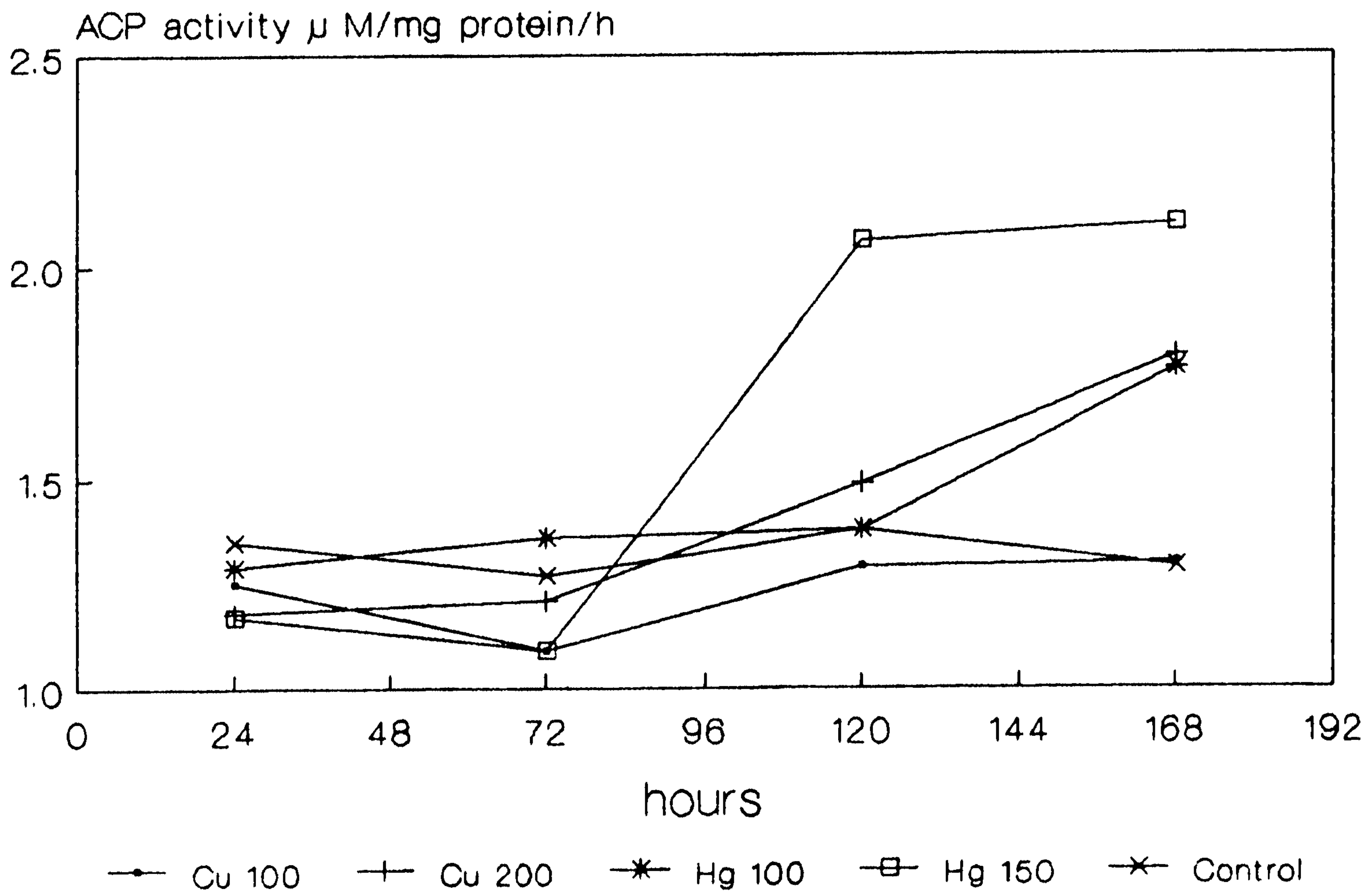
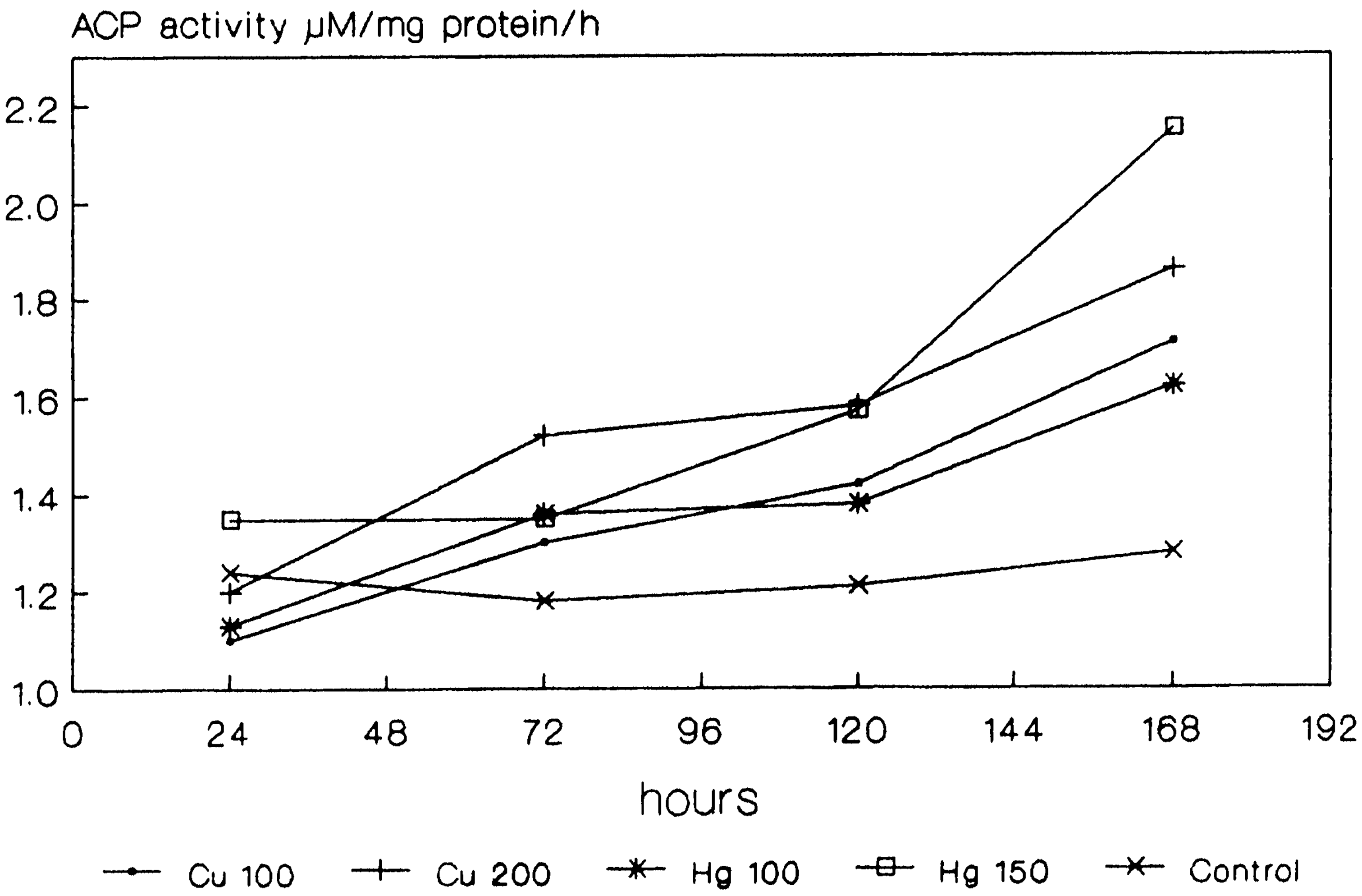


FIGURE 6. ACID PHOSPHATASE ACTIVITY IN THE KIDNEY OF *O. MOSSAMBICUS* EXPOSED TO COPPER AND MERCURY



Alkaline phosphatase activity in the liver

Alkaline phosphatase (ALP) activity in liver is represented in Table 7 and Fig. 7. No significant difference in ALP activity were observed among control fishes between days. ALP activity in the liver of 100 µg/l copper-dosed fishes showed no significant difference from that of controls. In 200 µg/l copper-dosed fishes ALP activity was significantly lower ($P < 0.01$) than that of the controls at 72 h and 120 h. ALP activity of 100 µg/l mercury-dosed fishes showed lower values at 72 h ($P < 0.01$) when compared to the ALP values of the controls. In the 150 µg/l mercury-dosed fishes, a decrease in ALP activity was found at 24 h ($P < 0.05$), 72 h ($P < 0.01$) and 120 h ($P < 0.01$).

Alkaline phosphatase activity in the kidney

There was no significant difference in ALP activity of the controls between days. ALP activity of 100 µg/l copper-dosed fishes showed higher values at 168 h ($P < 0.05$) when compared to controls, whereas 200 µg/l copper-dosed fishes showed higher values at 72 h, 120 h, ($P < 0.05$) and 168 h ($P < 0.01$). Similarly ALP activity of 100 µg/l mercury-dosed fishes showed higher values at 168 h ($P < 0.05$) when compared to controls. The 150 µg/l mercury-dosed fishes showed significantly higher values at 72 h, 120 h ($P < 0.05$) and 168 h ($P < 0.01$) (Table 8, Fig. 8).

DISCUSSION

The result of acid phosphatase (ACP) activity in the liver reveals that it is dose dependent, the higher concentration of heavy metals used eliciting a more significant increase in activity. It also appears that mercury-dosed fishes showed a more significant increase than copper-dosed fishes.

Increase in ACP activity in response to heavy metals and other toxicants have been reported earlier. Elevation of ACP activity in the liver, among other tissues has been reported in *Heteropneustes fossilis* in response to different organic pesticides (Thomas and Murthy, 1976); in *Ophiocephalus punctatus* in

Table 7. Alkaline phosphatase activity in the liver of *O. mossambicus* exposed to copper and mercury

Concentration µg/l		Specific activity (µM/mg protein/h) $\bar{x} \pm$ S.D. (N = 10) Hours of exposure 24 h	72 h	120 h	168 h
Cu	100	0.46 ± 0.04	0.39 ± 0.09	0.46 ± 0.07	0.44 ± 0.05
	200	0.40 ± 0.04	0.33** ± 0.05	0.39** ± 0.03	0.47 ± 0.05
Hg	100	0.40 ± 0.07	0.29** ± 0.05	0.47 ± 0.07	0.39 ± 0.08
	150	0.37* ± 0.07	0.27** ± 0.04	0.30** ± 0.06	0.46 ± 0.07
Control		0.45 ± 0.06	0.47 ± 0.09	0.44 ± 0.04	0.46 ± 0.07

Table 8. Alkaline phosphatase activity in the kidney of). *mossambicus* exposed to copper and mercury

Concentration µg/l		Specific activity (µM/mg protein/h) $\bar{x} \pm$ S.D. (N = 10) Hours of exposure 24 h	72 h	120 h	168 h
Cu	100	1.12 ± 0.32	1.18 ± 0.26	1.22 ± 0.37	1.50* ± 0.26
	200	1.01 ± 0.28	1.47* ± 0.31	1.51* ± 0.29	1.67** ± 0.33
Hg	100	0.99 ± 0.34	1.06 ± 0.29	1.28 ± 0.32	1.58* ± 0.35
	150	1.25 ± 0.36	1.38* ± 0.29	1.52* ± 0.28	1.71** ± 0.32
Control		1.08 ± 0.17	1.12 ± 0.21	1.18 ± 0.25	1.24 ± 0.23

* $P < 0.05$ ** $P < 0.01$

FIGURE 7. ALKALINE PHOSPHATASE ACTIVITY IN THE LIVER OF O. MOSSAMBICUS EXPOSED TO COPPER AND MERCURY

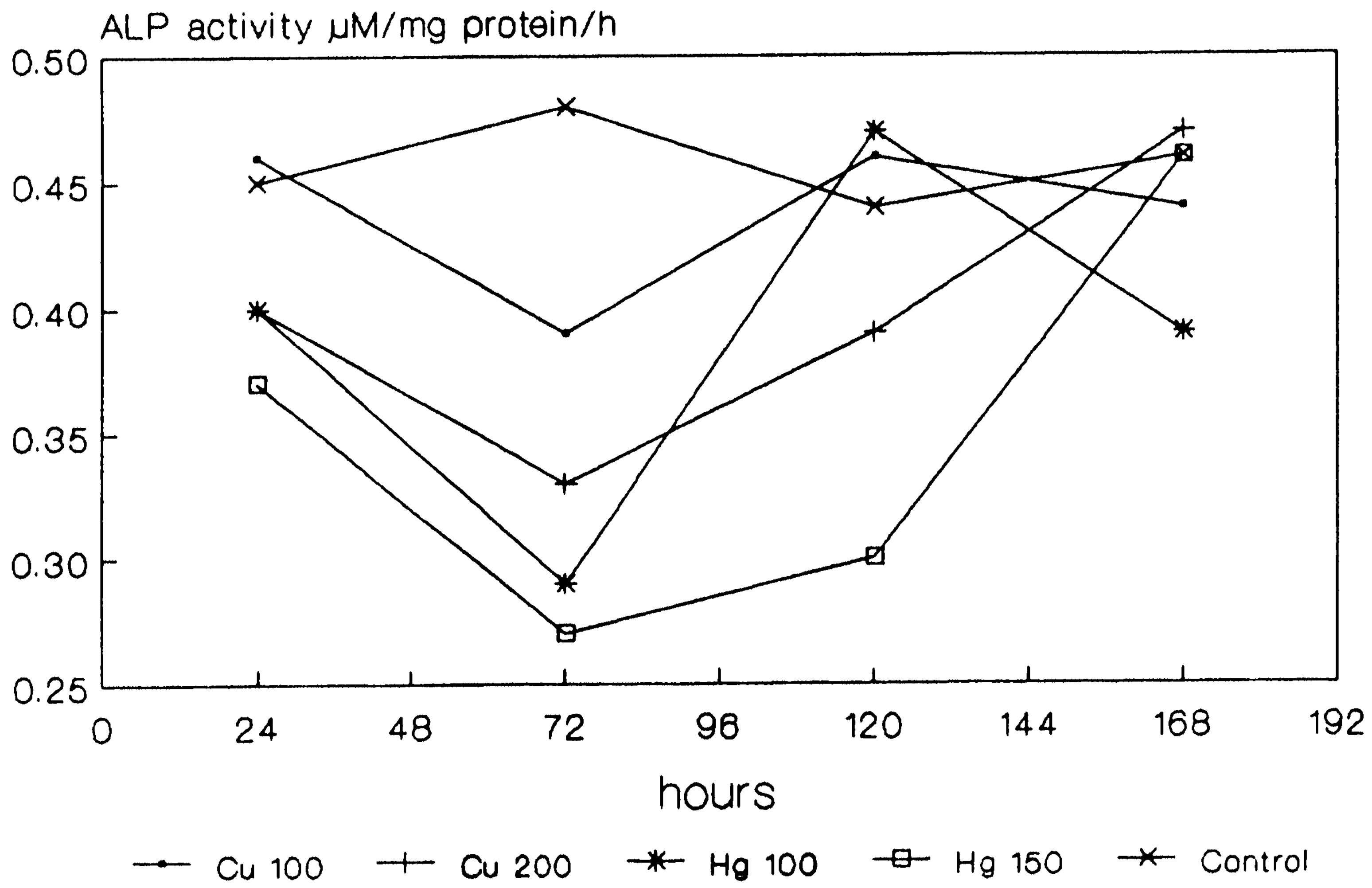
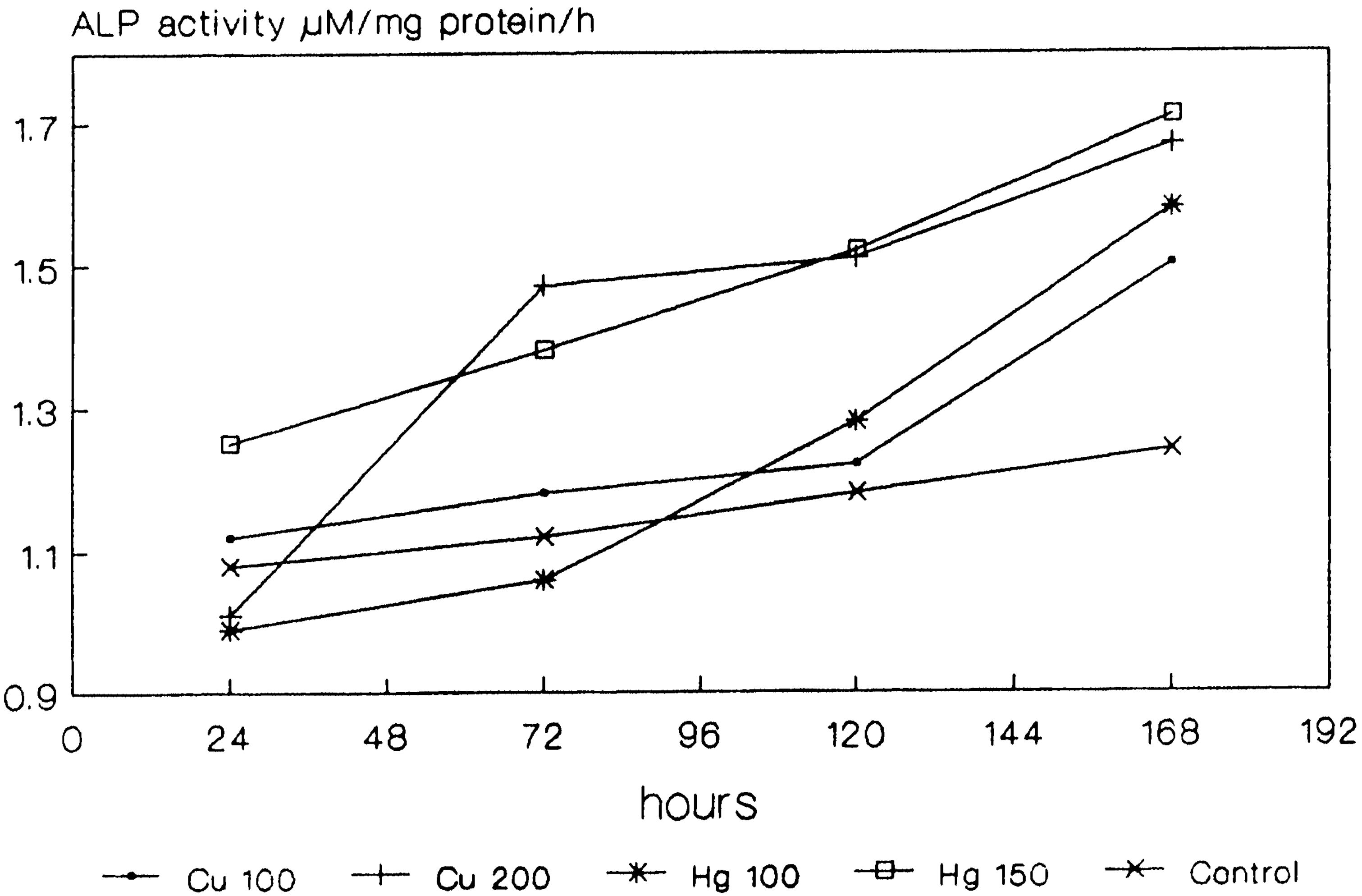


FIGURE 8. ALKALINE PHOSPHATASE ACTIVITY IN THE KIDNEY OF <u>O. MOSSAMBICUS</u> EXPOSED TO COPPER AND MERCURY



response to endrin (Sastry and Sharma, 1979); in carp in response to PCB (Ito et al., 1980); in Tilapia mossambica in response to monocrotophos (Joshi and Desai, 1981); in H. fossilis in response to mercuric chloride (Gupta and Sastry, 1981); in Clarias batrachus in response to lithium nitrate intoxication (Goel et al., 1985) and in Cyprinus carpio in response to cadmium chloride (Koyama et al., 1985). There are also many reports that the ACP activity increased in different other tissues on exposure to different metals (Gupta and Sastry, 1981; Hilmy et al., 1985; Sastry and Subhadra, 1985).

However, decreased ACP activity in the liver of fishes exposed to toxicants are also reported. These include a decrease in ACP activity in the liver of Fundulus heteroclitus in response to Cd, Cu, and Hg (Jackim et al., 1970); in C. batrachus in response to mercuric chloride (Sastry and Gupta, 1978 b) in Channa punctatus in response to mercury (Rana and Sharma, 1982); in Sarotherodon mossambicus in response to 1 ppm of mercury (Naidu et al., 1984); in different fishes in response to lead (Shaffi and Jeelani, 1985); in blue gill fish in response to cadmium (Versteeg and Giesy, 1985); in Mugil cephalus in response to cadmium (Hilmy et al., 1985); and in H. fossilis in response to cadmium (Sastry and Subhadra, 1985). It is also interesting to note that Sastry and Malik (1979) have reported no significant differences in ACP activity in the liver of C. punctatus in response to dimecron.

From the above, it can be inferred that there is no consistency in the ACP activity in the liver of fishes exposed to toxicants. This is equally true if we take the case of fishes exposed only to heavy metals as well. In the present study the liver of O. mossambicus registered an increase in activity in response to copper and mercury. Mercury induced high ACP activity than copper at both the concentrations employed.

As in the liver, the ACP activity in the kidney of fishes exposed to copper and mercury also showed an increase in activity and here also it is dose-dependent ie., the kidney of fishes exposed to higher concentration of copper and mercury registered higher ACP activity earlier and also for longer duration.

However unlike in liver it is to be noted that significantly higher ACP activity sets in earlier in the kidney and also its duration is longer in fishes exposed to higher concentration of copper than those exposed to higher concentration of mercury.

Increase in ACP activity in the kidney of fishes exposed to toxicants are also reported earlier. This include an increase in activity in the kidney of C. punctatus in response to mercury chloride (Sastry and Agrawal, 1979 a); in O. punctatus in response to endrin (Sastry and Sharma, 1979); in T. mossambica in response to monocrotophos (Joshi and Desai, 1981); in H. fossilis in response to cadmium (Sastry and Subhadra, 1985); in C. batrachus in response to lithium intoxication (Goel et al., 1985) and in the kidney of C. carpio dosed with cadmium chloride (Koyama et al., 1985). However, decrease in ACP activity in the kidney of H. fossilis exposed to lead nitrate (Sastry and Agrawal, 1979 b) and in the kidney of different fishes in response to lead (Shaffi and Jeelani, 1985) have also been reported.

The results of the present study indicate that the specific activity of ACP in both liver and kidney is influenced by copper and mercury. These metals caused an increase in ACP activity, but the time period of increase in activity varied with the concentration and is also dependent on the tissues.

In the present study the lower concentration of copper employed did not show any effect on the ACP activity in liver of O. mossambicus. But the lower concentration of mercury elicited an increase in the ACP activity at 168 h both in liver and kidney. Even though lower concentration of copper did not change the ACP activity significantly in the liver at 24, 72, 120 and 168 h, the ACP activity increased in kidney at 168 h. This shows a difference in behaviour of the two tissues towards the lower concentrations of these two metals. Hilmy et al. (1985) found that different tissues react differently in vivo exposure to cadmium. Such differences in the tissues behaviour towards toxicants were observed by Sastry and Subhadra (1985), Dalela et al. (1982),

Sharma and Sastry (1979), Sastry and Sharma (1979) and Sastry and Agrawal (1979 a).

ACP activity in response to higher concentration (200 µg/l) of copper showed an increase in liver at 168 h. But in kidney, higher concentrations of copper elicited an increase in ACP activity early at 72 h and after. ACP activity in response to higher concentration of mercury (150 µg/l) showed an increase both in liver and kidney at 120 and 168 h. These observations also indicate a difference in behaviour in response to copper and mercury.

Different concentrations of metals behaved differently in changing the ACP activity. Here the higher concentrations of copper evoked an increase in ACP activity, whereas there was no reaction in the liver of fishes exposed to lower concentration of copper. In mercury-dosed fishes the lower concentration showed an increase in ACP activity only at 168 h in the liver, whereas the higher concentration of mercury caused the effect at 120 h onwards. In the kidney, the effects of the higher concentrations of both metals had a greater effect on the ACP activity at 120 and 168 h. Thus the concentrations of toxicants are also important in causing a change in the activity of ACP in fishes. Changes in the ACP activity in fishes due to different concentrations of toxicants were reported by Hinton and Koenig (1975), Shaffi (1980 a), Dalela et al. (1982), Rana and Sharma (1982) and Arora and Kalshrestha (1985).

There are many ways in which structure and/or function of organelles can be disrupted by toxic contaminant. According to Slater (1978) the changes caused by contaminants can be classified into four major cateogires: (1) depletion or stimulation of metabolites or coenzymes (2) stimulation or inhibition of enzymes and other proteins, (3) activation of xenobiotic to a more toxic molecular species and (4) membrane disturbance. Many toxic substances or their metabolites result in cell injury by reacting primarily with biological membranes. Examples of membrane damage include changes in cellular compartmentalization, injury to lysosomes or mitochondria, changes in the content or activity of enzymes or other membrane components, lipid peroxidation giving rise to aldehydes and

ketones, changes in membrane fluidity leading to altered rates of fusion of cellular vesicles or alterations of enzyme interaction (Moore, 1985).

Lysosomes and the hydrolytic enzyme, acid phosphatase, present in the lysosome, play an important role in the detoxification process by compartmentalization and accumulation of metals that enter the cell (Allison, 1969; Viarengo, et al., 1981, 1984; George, 1983). This process is effective in containing the metals in the lysosome until the storage capacity of the lysosomes is overloaded or the lysosomes are damaged directly by the contaminant which is accumulated (Moore et al., 1985). But when the storage capacity of the lysosomes is exceeded, xenobiotics induce alterations in the bounding membrane which leads to destabilization of lysosome (Moore and Clarke, 1982; Moore and Lowe, 1985 and Moore et al., 1985). Destabilization may involve increased lysosomal fusion with other intracellular vacuoles, leading to the formation of pathologically enlarged lysosomes. Destabilization results release of degradative hydrolytic enzymes from lysosomal compartment into the cytosol (Moore, 1976; Baccino, 1978). This increases the acid phosphatase activity. Several mechanisms have been suggested for the release of ACP from the lysosomes. These are (1) alteration of osteoblasts resulting in more production and liberation of ACP (Cantarow and Schepartz, 1967), (2) proliferation of smooth ER in the parenchymatous cells that lead to more production and release of microsomal enzymes resulting in increased enzyme activity (Hart and Fouts, 1965), (3) peroxidation of lysosomal membrane leading to membrane breakdown and increasing permeability resulting in the release of ACP (Arstilla and Trump, 1968) and (4) degeneration and necrosis inducted in tissues and the resultant release of ACP (Trump and Arstilla, 1971). Any change in the activity, number, function of lysosomes or its damage could alter the activity of ACP. Verity and Reith (1967) observed that the lysosomes are structurally altered in response to toxic dosage of methyl mercury. Deung et al. (1978) reported an increase in the number of lysosomes in the liver cells of Carassius carassius exposed to mercuric chloride while Ferri and Macha (1980) observed a change in shape distribution and functional degree of lysosomes in the hepatic cells of Pimelodus maculatus exposed to cadmium. Assessment of this type of injury has been confirmed as an extremely sensitive general index of cellular conditions (Moore, 1980, 1982; Moore et al., 1982, 1985). Injury resulting in destabilization of the lysosomal membrane bears a quantitative

relationship to the magnitude of stress response (Bayne et al., 1979, 1982). Since release of ACP from the lysosome is associated to membrane injury due to xenobiotics, increased activity of ACP in the present study can be related to the lysosomal destabilization.

Failure of the lower concentration of copper to evoke an increase in the ACP activity in liver and also the absence of increase in ACP activity during the initial period of the present experiment may be due to the detoxification and consequent removal of copper and mercury before they could damage the lysosomes. But as the exposure time or the concentration of the metal is increased, the storage capacity of the lysosomes gets overloaded, altering the permeability and damaging the lysosomal membrane resulting in the increased leakage and activity of ACP. Such biphasic responses of ACP activity on exposure to different toxicants were also reported (Sastry and Gupta, 1978 b; Hinton and Koenig, 1975; Joshi and Desai, 1983; Arora and Kalshrestha, 1985). So depending on the concentration and period of exposure and type of tissue, ACP activity can vary from time to time and tissue to tissue and many of the reported stimulation of ACP activity by metals may be due to this phenomena described above.

The results of the present study indicated that the ALP activity of the liver of fishes exposed to lower concentration of copper did not show any significant difference from that of the control fishes, while those exposed to the lower concentration of mercury showed a significant decrease at 72 h. The liver of fishes exposed to higher concentration of copper showed a significant decrease at 72 and 120 h, whereas those exposed to higher concentration of mercury showed significant decrease at 24, 72 and 120 h. It is to be noted that the ALP activity returned to the control values at the later part of the experimental period. Decrease in ALP activity in the liver of F. heteroclitus exposed to beryllium and silver (Jackim, et al., 1970); in H. fossilis exposed to mercuric chloride (Gupta and Sastry, 1981); in the liver of C. punctatus dosed with mercury (Rana and Sharma, 1982); in S. mossambicus exposed to mercuric chloride (Naidu et al., 1984); in C. punctatus exposed to cythion (Narayan Ram and Sathyanesan, 1985) and in H. fossilis exposed to cadmium (Sastry and Subhadra, 1985) have been reported. However increase in ALP activity in the liver of F. heteroclitus exposed to mercury and lead

(Jackim et al., 1970) and no significant change in the ALP activity in F. heteroclitus exposed to copper and cadmium (Jackim et al., 1970) and in M. Cephalus exposed to cadmium (Hilmy et al., 1985) have also been reported.

Decrease in ALP activity in some other tissues of fishes dosed with different heavy metals have also been reported (Saleem and Alikhan, 1973; Koyama and Itazawa, 1977; Sastry and Gupta, 1978 a, b; Sastry and Agrawal, 1979 a, b; Koyama et al., 1985; Shaffi and Jeelani, 1985).

Intracellular ALP is associated with plasma membrane and endoplasmic reticulum (Davison and Gregson, 1965). Hence destabilization or damage to these membranes may affect the activity of ALP. It is probable that the membrane systems of the cell encounter the influx of heavy metal ions first and the latency of ALP associated with plasma membrane is lowered, thereby permitting leakage, away from the liver tissues. Moreover the heavy metal ions may also be inhibiting the enzymes directly. Inhibition of ALP activity in different tissues after exposure to metals have been reported (Hiwada and Wachsmuth, 1974; Cathala et al., 1975; Yokota, 1978). Sometimes heavy metals displace or replace metals of metalloenzyme. These changes alter the three dimensional configuration of the enzymes, so that substrate molecules no longer fit binding site (Friedberg, 1974) or splits enzymes into subunits (Gerhart and Schachman, 1965; Jovin et al., 1969) so that regulation of enzyme activity may be lost (White et al., 1968; Brown, 1977; Brown et al., 1977). These processes can give a reduction in the ALP activity in the liver tissue. The absence of any significant difference in the ALP activity of fishes exposed to lower concentration of copper also supports this view. Later, when the detoxifying process gain an upper hand, the ALP activity, returns to comparable values of ALP activity of the controls which may be through the hypersynthesis of this enzyme.

In the kidney of O. mossambicus exposed to copper and mercury, the ALP activity, showed a different trend. A significant increase in the ALP activity in the kidney of experimentals over the control values was obtained. The pattern of increased activity is similar between the concentration of copper and mercury but the duration of increase is greater in the kidney of fishes exposed to higher concentration of copper and mercury. Goel et al. (1985)

have reported an increase in the ALP activity in the kidney of C. batrachus in response to lithium. However a decrease in ALP activity in kidney was reported in C. punctatus exposed to mercuric chloride (Sastry and Agrawal, 1979 a); in H. fossilis exposed to lead nitrate (Sastry and Agrawal, 1979 b), and in H. fossilis exposed to cadmium (Sastry and Subhadra, 1985). Many workers have also reported increased ALP activity in different other tissues of fishes treated with metals (Cardeilhac and Hall, 1977; Sastry and Gupta, 1978 b, 1979; Banerjee et al., 1979; Saxena and Tyagi, 1979; Sastry and Sharma, 1980; Hilmy et al., 1985; Sastry and Subhadra, 1985).

In the present study the ALP activity in the liver of experimental fish showed a significant decrease over the controls, whereas in the kidney of experimental fish the ALP activity registered an increase over the controls indicating that the two tissues vary differently with respect to ALP activity in response to copper and mercury. Such differences in the ALP activity in different tissues exposed to same toxicants were reported by Sastry and Sharma (1979), Shaffi (1980 b), Hilmy et al. (1985), Narayan Ram and Sathyanesan (1985) and Sastry and Subhadra (1985). The observed increase in the ALP activity in the kidney could be associated with the functions of kidney.

ALP is reported to play an important role in the active transport of materials through phosphorylated intermediate (Goodman and Rothstein, 1957; Posen, 1967; Neville, 1974). The reports of George and Viarengo (1984) stating that some metals react with phosphate groups of lipid bilayer before being complexed by intracellular ligands supports the need of this enzyme in the transportation of heavy metal ions, by providing the phosphate group. In the kidney, ALP activity is thus important as it is reported that copper can be excreted in the urine, faeces and bile by complexing it with alpha globulin (Peisach et al., 1967). Enhanced urine flow in fishes exposed to heavy metal is also reported (Lock et al., 1981). Thus in the kidney it may be possible that there is hypersynthesis of ALP to facilitate transport and excretion of heavy metal ions resulting in the noted increase in the ALP activity.

CHAPTER 4

AMINOTRANSFERASE ACTIVITY IN THE LIVER AND KIDNEY AFTER EXPOSURE TO COPPER AND MERCURY

Aminotransferases or transaminases are a group of enzymes that catalyse the process of biological transamination. Transamination allows an interplay between carbohydrate, fat and protein metabolism (Cohen and Sallach, 1961), providing a source of keto acids for Krebs' cycle and gluconeogenesis. Weber (1963) states that this mechanism provides more energy to meet the increased demand under stress. The aminotransferases found in the tissues are mainly aspartate aminotransferase (L-Aspartate: 2-oxoglutarate aminotransferase (AAT) - EC 2.6.1.1) or glutamic oxaloacetic transaminase (GOT) and Alanine aminotransferase (L - Alanine : 2 - oxoglutarate aminotransferase (ALAT) - EC 2.6.1.2) or glutamic pyruvic transaminase (GPT). Wilson (1973 a) explained that the primary enzymes concerned with the amino acid metabolism and gluconeogenesis are glutamic dehydrogenase, aspartate aminotransferase (AAT) and alanine aminotransferase (ALAT). The substrates for AAT (GOT) and ALAT (GPT) are aspartic acid and alanine which serve as the two major glucogenic amino acids which give rise to glucose precursors (Lehninger, 1979). Since the highest activity of aminotransferases in most cases was observed in heart, liver and kidney tissues, these organs play a major role in amino acid metabolism and gluconeogenesis. So it is interesting to study the effects of pollutants on the activity of these enzymes in these specific tissues. There are two approaches (1) to study the serum enzyme activity and (2) to study the specific activity of the enzymes within the tissue.

The study of serum enzyme activity got its initial impetus when Karmen (1955) showed a direct link between serum AAT (GOT) levels and acute transmural myocardial infarction and in man it was later extended to both positive and differential clinical diagnosis (Agress, 1959; Bang et al., 1959). In mice infected with hepatic virus, the increase in serum transaminases has been shown to be roughly proportional to the degree of cell necrosis (Friend et al., 1955). Later Wroblewski et al. (1956) extended their work on serum AAT or serum GOT (SGOT) and they found that the acute and chronic hepatic diseases are associated with elevation of SGOT activity which are sufficiently characteristic to permit diagnostic differentiation. Zelman et al. (1959) in

a similar study of human subjects, found an excellent relationship between the extent of necrosis of liver cells and the rise in serum transaminases.

The rationale for using serum GOT and GPT or other serum enzymes was that elevated enzyme levels should simply reflect the amount of damage which had occurred in the enzyme rich tissue and this approach is used for the diagnosis of both the site and extent of organ injury (McKim et al., 1970; Schmidt and Schmidt, 1974; Racicot et al., 1975; Goel and Garg, 1980; Wotten and Williams, 1980).

In fishes, the use of transaminases (aminotransferases) in the diagnosis of tissue damage was investigated by Mollander et al. (1955), Wroblewski and La Due (1956), Wroblewski et al. (1956) and Bell (1968). Bell (1968) discussed the practical value of GOT estimation in serum of salmon for the detection (distinction) of apparently healthy fish from those treated with hepatic poison, bromobenzene or those affected by bacterial kidney disease. Apart from these earlier workers, serum GOT and GPT were frequently used by fish pathologists to diagnose sublethal insult or injury to liver by pollution (Mehrle and Bloomfield, 1974; Malevski et al., 1974; Racicot et al., 1975). In the field of environmental toxicology, serum and tissue analysis are becoming important for the detection of chemical pollutants. It is noted that toxic agents or factors which lead to chronic impairment of animal metabolism will cause changes in the activities of some enzymes (see Bell, 1968; McKim et al., 1970; Lockhart et al., 1972). Some of these responses are likely to be of a more general nature, indicating the organisms reaction to a situation of stress brought about by a general deterioration of water quality (Oikari and Soivio, 1977).

Eventhough it is suggested that a high level of GOT in the serum of a species is associated with high values in the liver, myocardium and kidney (Zimmerman et al., 1968), there are reports of finding no such association (Gaudet et al., 1975). Apart from these, studies are few dealing with the enzyme levels in specific tissues where the toxicants are accumulated or detoxified. Similarly, can we say that the serum activity is representative of different tissues? Is there any tissue differences in the activity of transaminases in response to toxicants? In pursuit of an answer to these

questions, the present study was conducted to examine the effects of copper and mercury on the activity of GOT and GPT in the liver and kidney of Oreochromis mossambicus.

MATERIAL AND METHODS

Collection of specimens, acclimatization and experimental setup were the same as described in Chapter 2. Preparation of the enzyme sample from the selected tissues was done as described in Chapter 3.

The activity of AAT (GOT) and ALAT (GPT) in the liver and kidney was determined following the method of Reitman and Frankel (1957) with slight modification. To 0.6 ml of 0.1 M frozen phosphate buffer (pH 7.5), 0.1 ml of enzyme preparation was added and again frozen until analysis. After the completion of the samplings, the frozen buffer-enzyme mixture was placed in a water bath at 37°C. When the mixture attained 37°C, 0.5 ml of the substrate (Prepared by dissolving 30 mg 2 - oxoglutarate and 1.57 g L-aspartate mono-sodium salt in 50 ml of distilled water for GOT estimation and by dissolving 30 mg 2 - oxoglutarate and 1.78 g DL - alanine in 50 ml of distilled water for GPT estimation) was added and incubated for 60 minutes at 37°C. After 60 minutes, the enzyme activity was stopped by the addition of 1 ml of 1 mM 2, 4, Dinitrophenyl hydrazine (Chromogen). The mixture was shaken well and allowed to stand for 20 minutes at room temperature. The reaction was stopped by adding 10 ml of 0.4 N NaOH. After 5 minutes, the hydrazone formed was measured spectrophotometrically at 546nm.

Enzyme activity was calculated from calibration curve prepared using known concentrations of sodium pyruvate. The amount of protein in each sample was calculated by following the method of Lowry et al. (1951). From this, the specific activity of the GOT and GPT (µM/mg protein/h) was calculated. The results were analysed using student's 't' test (Zar, 1974).

RESULTS

AAT (GOT) and ALAT (GPT) activity in the liver and kidney of the experimental and control fishes are presented in Tables 9, 10, 11, 12, 13 and 14 and Figs. 9, 10, 11, 12, 13 and 14 respectively.

AAT (GOT) activity in liver:

The liver of 100 µg/l copper-dosed fishes showed significantly higher GOT activity at 72 h ($P < 0.05$), 120 h ($P < 0.05$) and 168 h ($P < 0.01$) when compared with the controls. A similar increase also was observed ($P < 0.05$) in the fishes treated with higher concentration of copper at 72, 120 and 168 h. Liver of fishes dosed with 100 µg/l mercury showed significant increase at 72 h ($P < 0.05$) and 120 h ($P < 0.05$) whereas those dosed with 150 µg/l mercury showed significant increase at 72, 120 and 168 h ($P < 0.05$).

AAT (GOT) activity in kidney:

GOT activity in the kidney of fishes dosed 100 µg/l copper and mercury showed lower values at 72,120 and 168 h ($P < 0.01$) where as kidney of fishes dosed with 200 µg/l copper and 150 µg/l mercury showed significantly lower values at 24, 72, 120 and 168 h ($P < 0.01$) when compared with the controls.

ALAT (GPT) activity in liver:

Liver of fishes dosed with 100 µg/l copper showed significantly lower activity at 24 h ($P < 0.05$) and 72 h ($P < 0.01$) whereas liver of fishes dosed with 200 µg/l copper showed significantly lower values at 24, 72 ($P < 0.01$) and 120 h ($P < 0.05$). Liver of fishes dosed with 100 µg/l mercury showed significantly lower values at 24 ($P < 0.05$), 72 and 120 h ($P < 0.01$) and those dosed with 150 µg/l mercury showed significantly lower values at 24 ($P < 0.05$), 72, 120 ($P < 0.01$) and 168 h ($P < 0.05$) periods.

ALAT (GPT) activity in kidney:

GPT activity in the kidney of fishes exposed to 200 µg/l copper showed a significant decrease at 72 h ($P < 0.05$). GPT activity in the kidney of fishes exposed to 100 µg/l mercury showed significant decrease at 72 ($P < 0.01$) and 120 h ($P < 0.05$) whereas those exposed to 150 µg/l mercury showed significant decrease at 24 ($P < 0.05$), 72, 120 ($P < 0.01$) and 168 h ($P < 0.05$) when compared with the controls.

When the ratio of AAT/ALAT (GOT/GPT) in liver and kidney was

Table 9. Liver aspartate amino transferase (glutamic oxaloacetic transaminase) activity in *Oreochromis mossambicus* exposed to copper and mercury

Concentration µg/l		Specific activity µM pyruvate formed/mg protein/h $\bar{x} \pm$ S.D (N = 10) Hours of exposure			
		24 h	72 h	120 h	168 h
Cu	100	6.15 ± 1.22	8.44* ± 1.08	8.51* ± 1.03	8.62** ± 1.07
	200	6.85 ± 1.32	8.49* ± 1.21	8.60* ± 1.14	8.65* ± 1.29
Hg	100	7.12 ± 1.41	8.80* ± 1.13	8.72* ± 1.23	7.90 ± 1.15
	150	6.74 ± 1.15	8.58* ± 1.06	8.62* ± 1.31	8.70* ± 1.32
Control		6.95 ± 1.3	7.21 ± 1.23	7.30 ± 1.06	7.12 ± 1.11

Table 10. Kidney aspartate amino transferase (glutamic oxaloacetic transaminase) activity in *O. mossambicus* exposed to copper and mercury

Concentration µg/l		Specific activity µM pyruvate formed/mg protein/h $\bar{x} \pm$ S.D. (N = 10) Hours of exposure			
		24 h	72 h	120 h	168 h
Cu	100	10.02 ± 1.51	8.05** ± 1.37	6.76** ± 1.36	6.98** ± 1.48
	200	6.92** ± 1.45	6.69** ± 1.52	6.37** ± 1.44	6.32** ± 1.56
Hg	100	8.75 ± 1.41	7.85** ± 1.39	7.35** ± 1.61	6.52** ± 1.51
	150	7.96** ± 1.29	7.38** ± 1.45	4.12** ± 1.42	4.19** ± 1.47
Control		9.76 ± 1.34	9.92 ± 1.32	9.61 ± 1.43	9.75 ± 1.36

* $P < 0.05$ ** $P < 0.01$

Table 11 Liver alanine amino transferase (glutamic pyruvic transaminase) activity in *O. mossambicus* exposed to copper and mercury

Concentration µg/l		Specific activity µM pyruvate formed/mg protein/h $\bar{x} \pm$ S.D. (N = 10) Hours of exposure			
		24 h	72 h	120 h	168 h
Cu	100	9.08* ± 1.77	8.58** ± 2.11	9.86 ± 2.08	12.06 ± 2.82
	200	8.72** ± 2.13	8.24** ± 1.88	9.65* ± 1.97	11.65 ± 2.14
Hg	100	9.23* ± 2.08	7.16** ± 2.19	8.42** ± 2.12	9.12 ± 2.06
	150	9.14* ± 1.92	6.55** ± 2.27	7.88** ± 2.36	8.87* ± 2.14
Control		11.37 ± 1.62	12.14 ± 1.31	11.78 ± 1.92	11.12 ± 2.10

Table 12 Kidney alanine amino transferase (glutamic pyruvic transaminase) activity in *O. mossambicus* exposed to copper and mercury

Concentration µg/l		Specific activity µM pyruvate formed/mg protein/h $\bar{x} \pm$ S.D. (N = 10) Hours of exposure			
		24 h	72 h	120 h	168 h
Cu	100	12.58 ± 2.13	14.16 ± 2.19	15.33 ± 2.32	15.66 ± 2.41
	200	13.78 ± 2.52	11.61* ± 1.78	13.67 ± 2.11	14.32 ± 2.28
Hg	100	12.10 ± 1.82	10.96** ± 2.14	11.36* ± 2.07	13.39 ± 2.51
	150	11.07* ± 2.06	10.65** ± 2.01	10.98** ± 1.93	11.82* ± 2.12
Control		13.14 ± 1.84	13.87 ± 2.04	14.15 ± 2.33	14.73 ± 2.28

* $p < 0.05$ ** $P < 0.01$

FIGURE 9. LIVER ASPARTATE AMINO TRANSFERASE (GLUTAMIC OXALOACETIC TRANSAMINASE) ACTIVITY IN *O. MOSSAMBICUS* EXPOSED TO COPPER AND MERCURY

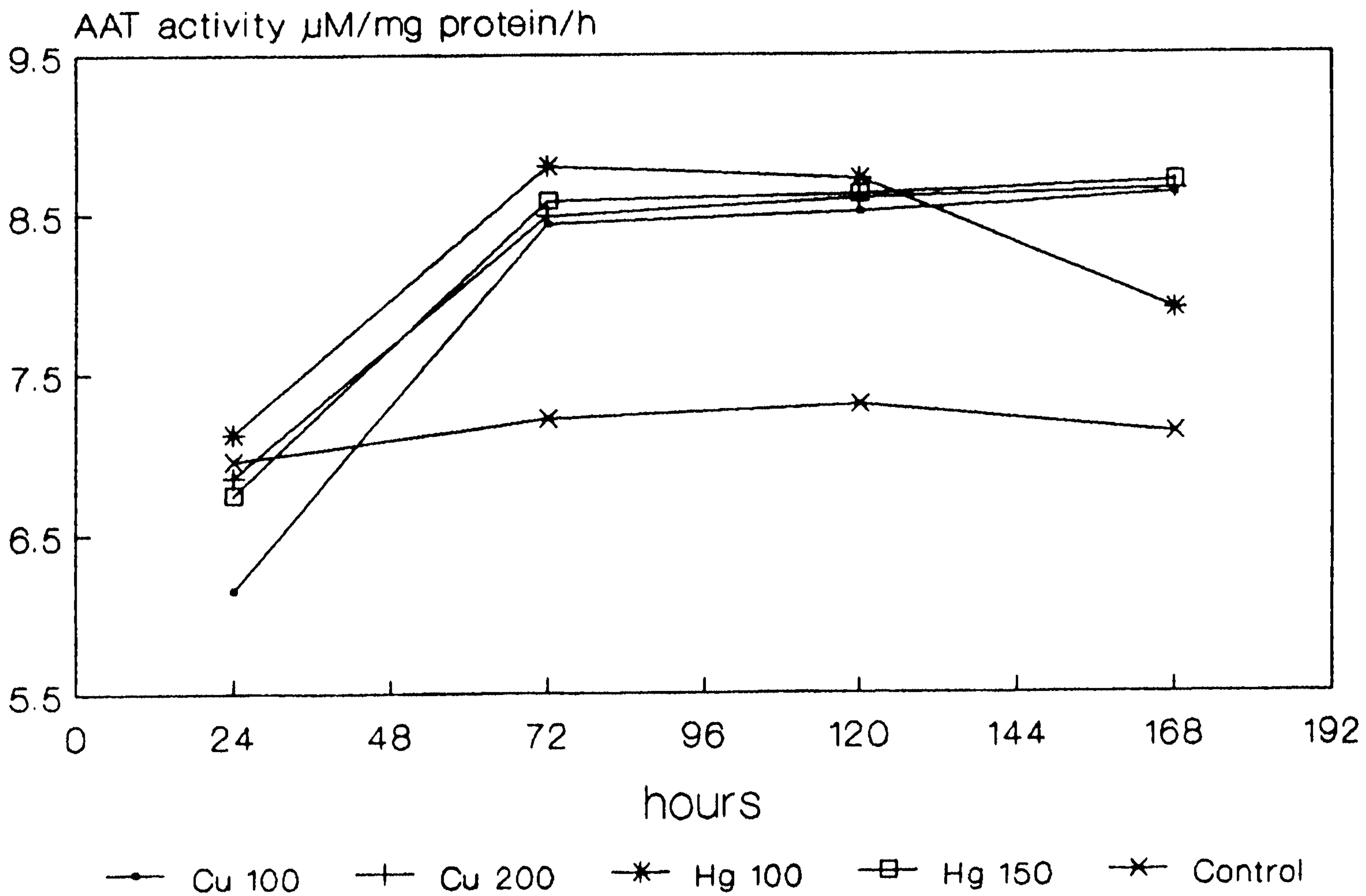


FIGURE 10. KIDNEY ASPARTATE AMINO TRANSFERASE (GLUTAMIC OXALOACETIC TRANSAMINASE) ACTIVITY IN *O. MOSSAMBICUS* EXPOSED TO COPPER AND MERCURY

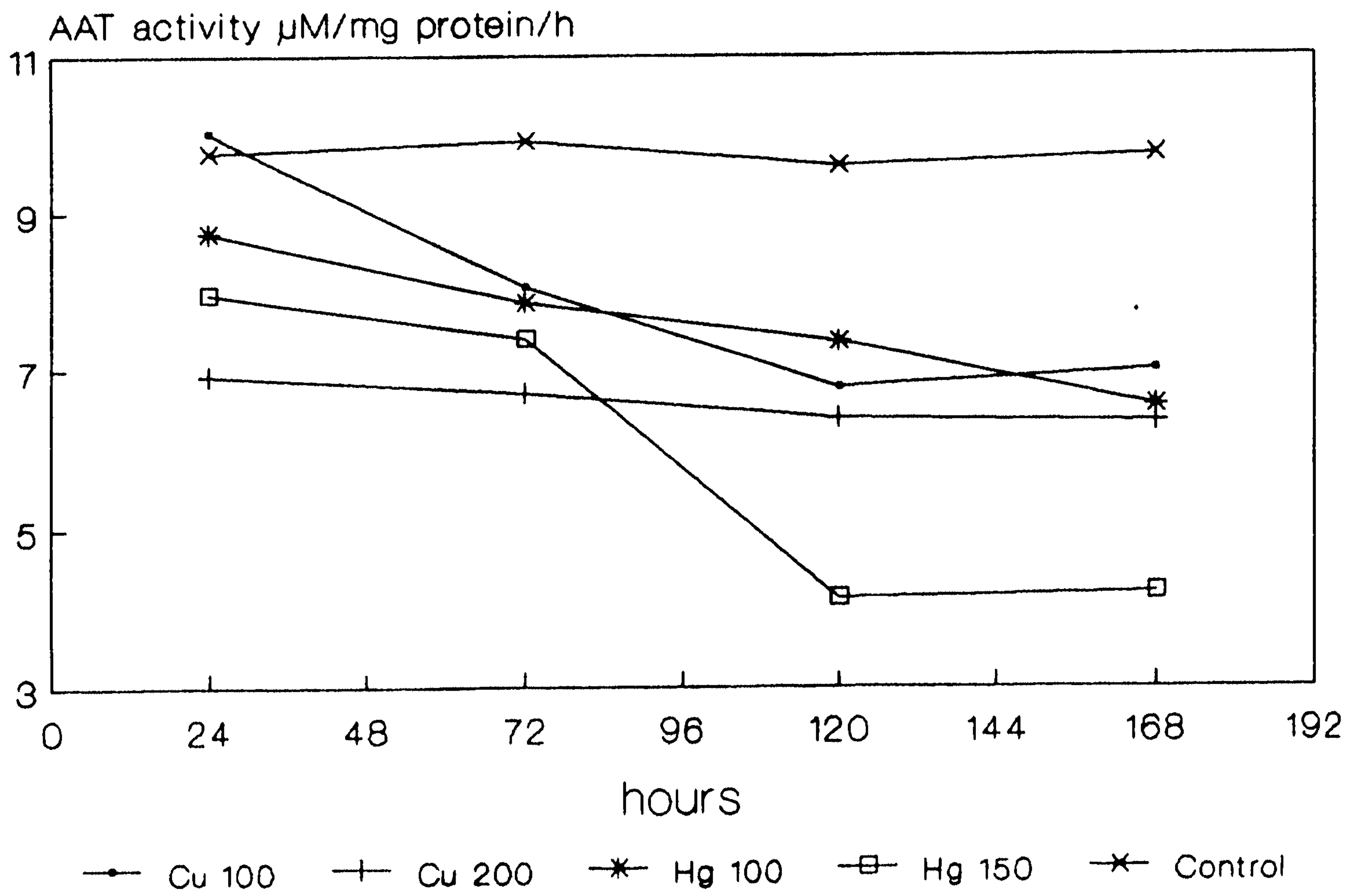


FIGURE 11. LIVER ALANINE AMINO TRANSFERASE (GLUTAMIC PYRUVIC TRANSAMINASE) ACTIVITY IN O. MOSSAMBICUS EXPOSED TO COPPER AND MERCURY

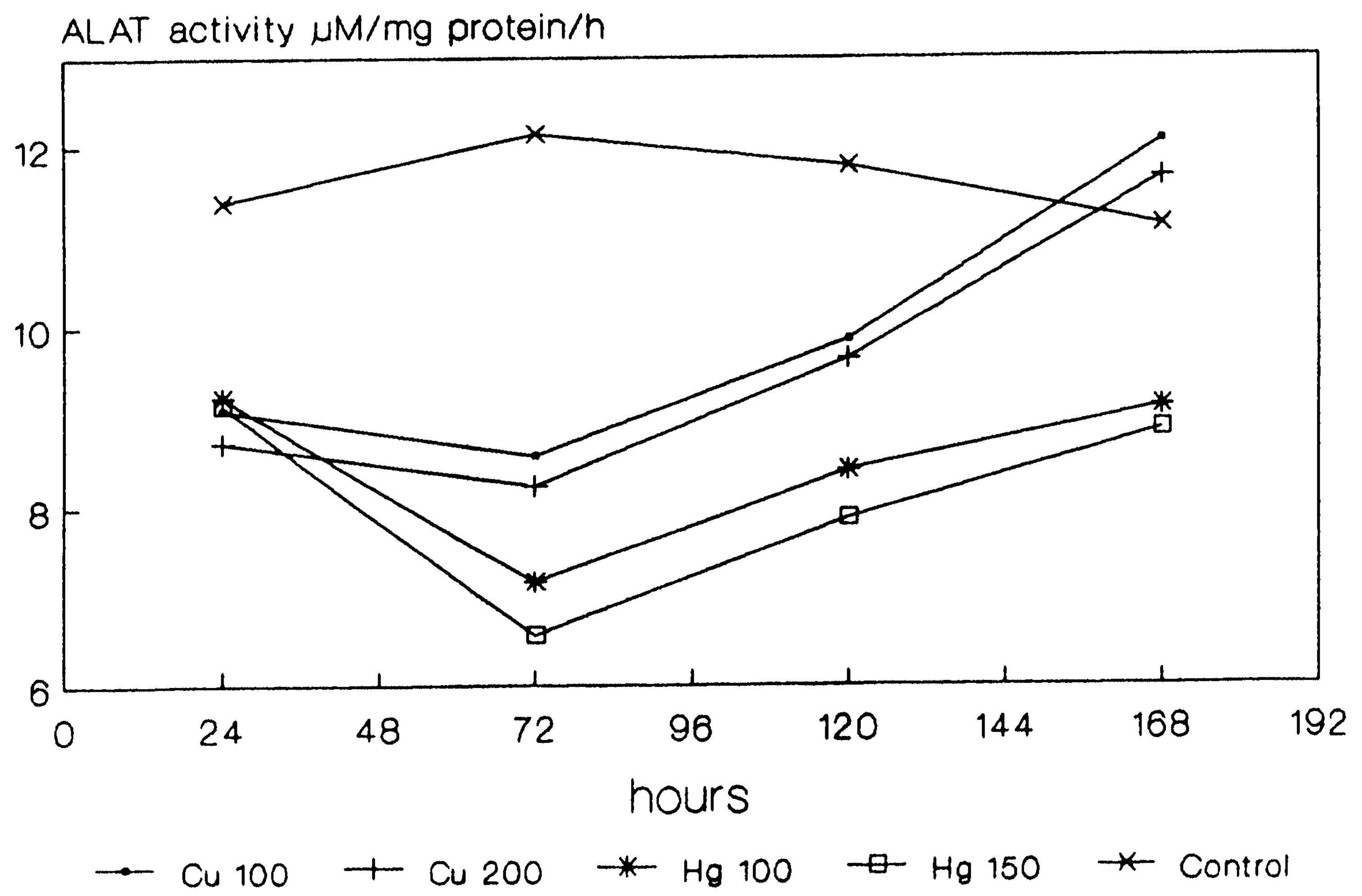
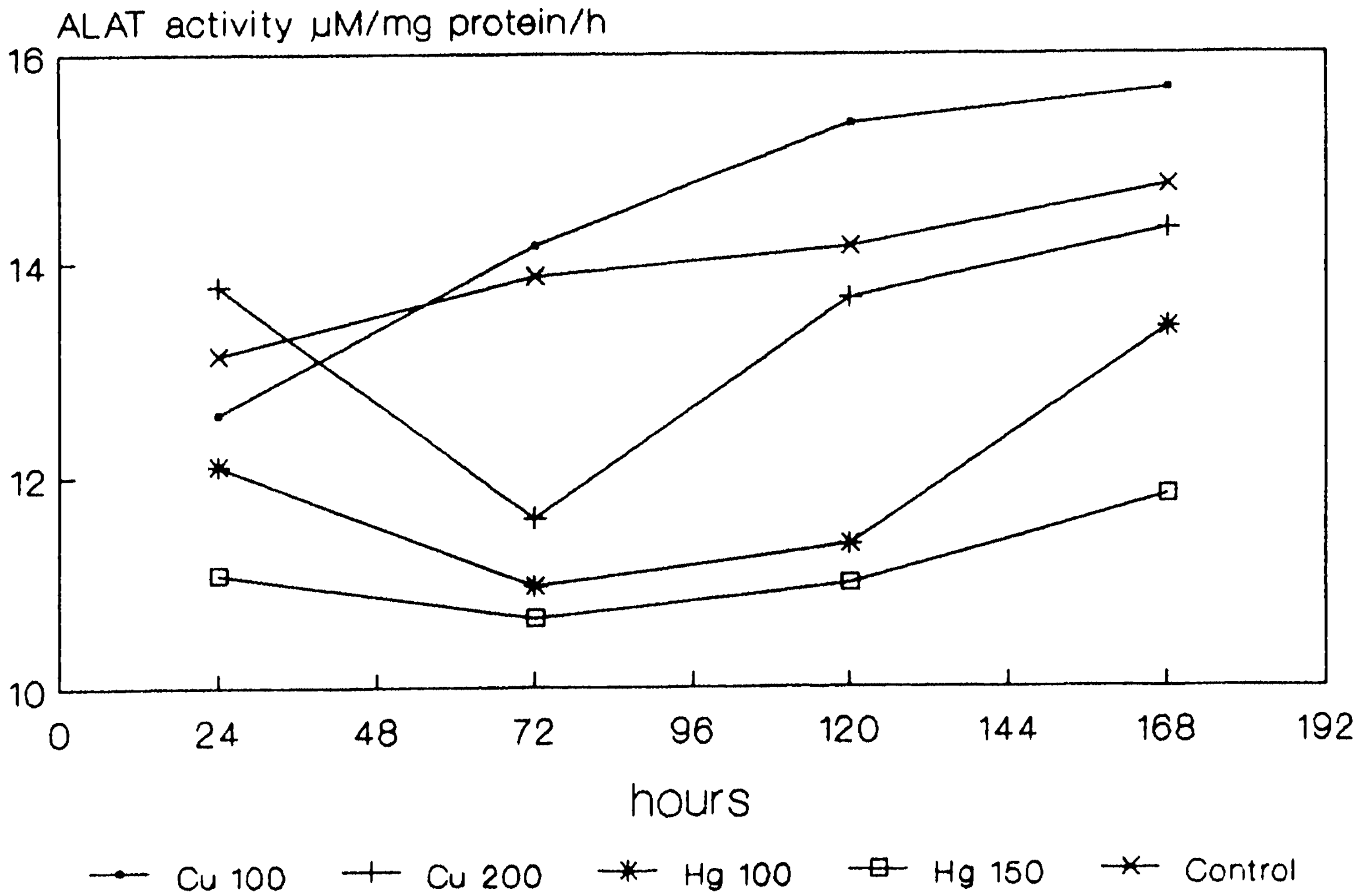


FIGURE 12. KIDNEY ALANINE AMINO TRANSFERASE (GLUTAMIC PYRUVIC TRANSAMINASE) ACTIVITY IN *O. MOSSAMBICUS* EXPOSED TO COPPER AND MERCURY



calculated, it showed different pattern in the liver and kidney. In the liver the AAT/ALAT ratio of the metal-dosed fishes increased from that of controls. But in kidney the AAT/ALAT ratio of the experimentals decreased from that of controls. The decrease of the AAT/ALAT ratio in kidney was neither dose nor exposure dependent. But in liver the rise in ratio was dose dependent.

DISCUSSION

The results indicate that there is a significant increase in the GOT activity in the liver of fishes exposed to copper and mercury. There is no significant difference in enzyme activity pattern between copper-dosed and mercury-dosed fishes. Similarly there is no significant difference in the pattern of enzyme activity between concentrations of the same metal employed for dosing. Variation of GOT activity in response to toxicants have been reported earlier. Increase in GOT activity in liver, among other tissues were reported in alevins of brook trout in response to methyl mercuric chloride and cadmium (Christensen, 1975); in Notopterus notopterus in response to phenol (Verma et al., 1982; Gupta and Dalela, 1985); in response to phenolic compounds (Gupta et al., 1983); in Porophrys vetulus in response to Carbon tetra chloride (CCl_4) (Cassilas et al., 1983); in Tilapia in response to methyl parathion (Prasada Rao and Ramana Rao, 1984); in Clarias batrachus in response to lithium (Goel et al., 1985); and in Oreochromis mossambicus in response to napthalene, toluene and phenol (Dange, 1986 b). An increase in the GOT (AAT) activity in the blood was observed in Poecilia latipinna in response to dieldrin (Lane and Scura, 1970); in brook trout in response to copper (McKim et al., 1970); in carp in response to PCB (Ito and Murata, 1980); in Channa punctatus in response to tri amino azobenzene (Goel and Garg, 1980); in rainbow trout in response to sewage (Wieser and Hinterleitner, 1980); in rainbow trout in response to copper and formalin (Wotten and Williams, 1980); in N. notopterus in response to various toxicants (Verma et al., 1981 b); in Aphanius dispar in response to mercury (Hilmy et al., 1981); in different fishes in response to copper sulphate (Nemcsok and Boross, 1982) and ammonia and paraquat (Nemcsok et al., 1982, 1985); in N. notopterus in response to mercury (Verma et al., 1986); and in carp in response to cadmium (Yamawaki et al., 1986).

However, no significant change in GOT activity in liver was observed

Table 13 Ratio of AAT/ALAT (GOT/GPT) in the liver of *O. mossambicus* exposed to copper and mercury

Concentration µg/l		Hours of exposure 24 h	72 h	120 h	168 h
Cu	100	0.68	0.98	0.86	0.71
	200	0.79	1.03	0.89	0.74
Hg	100	0.77	1.23	1.04	0.87
	150	0.74	1.31	1.10	0.98
Control		0.61	0.59	0.62	0.64

Table 14 Ratio of AAT/ALAT (GOT/GPT) in the kidney of *O. mossambicus* exposed to copper and mercury

Concentration µg/l		Hours of exposure 24 h	72 h	120 h	168 h
Cu	100	0.80	0.57	0.44	0.45
	200	0.50	0.58	0.47	0.44
Hg	100	0.72	0.72	0.65	0.49
	150	0.72	0.69	0.38	0.35
Control		0.74	0.72	0.68	0.66

FIGURE 13. RATIO OF AAT/ALAT (GOT/GPT) IN THE LIVER OF *O. MOSSAMBICUS* EXPOSED TO COPPER AND MERCURY

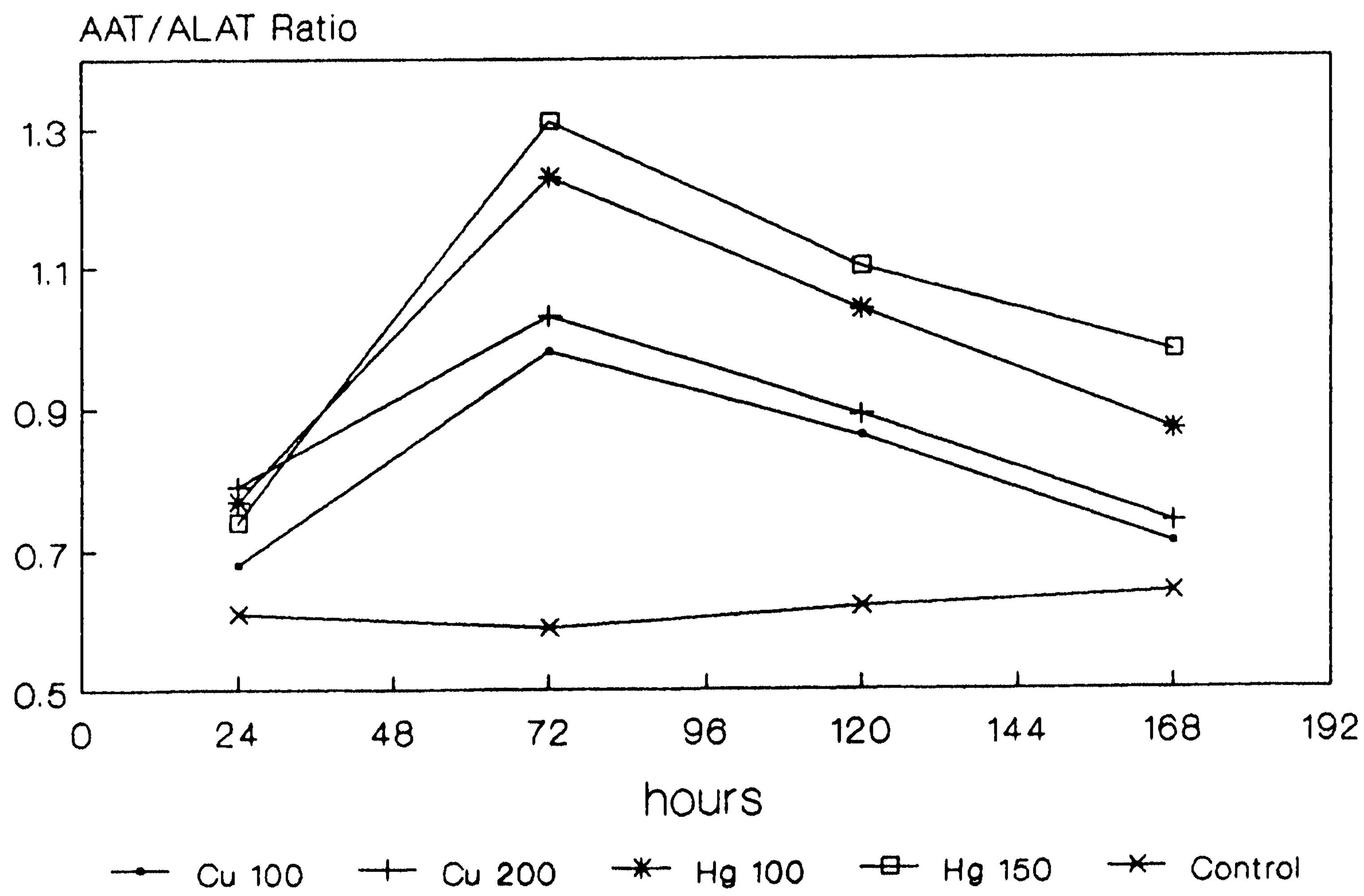
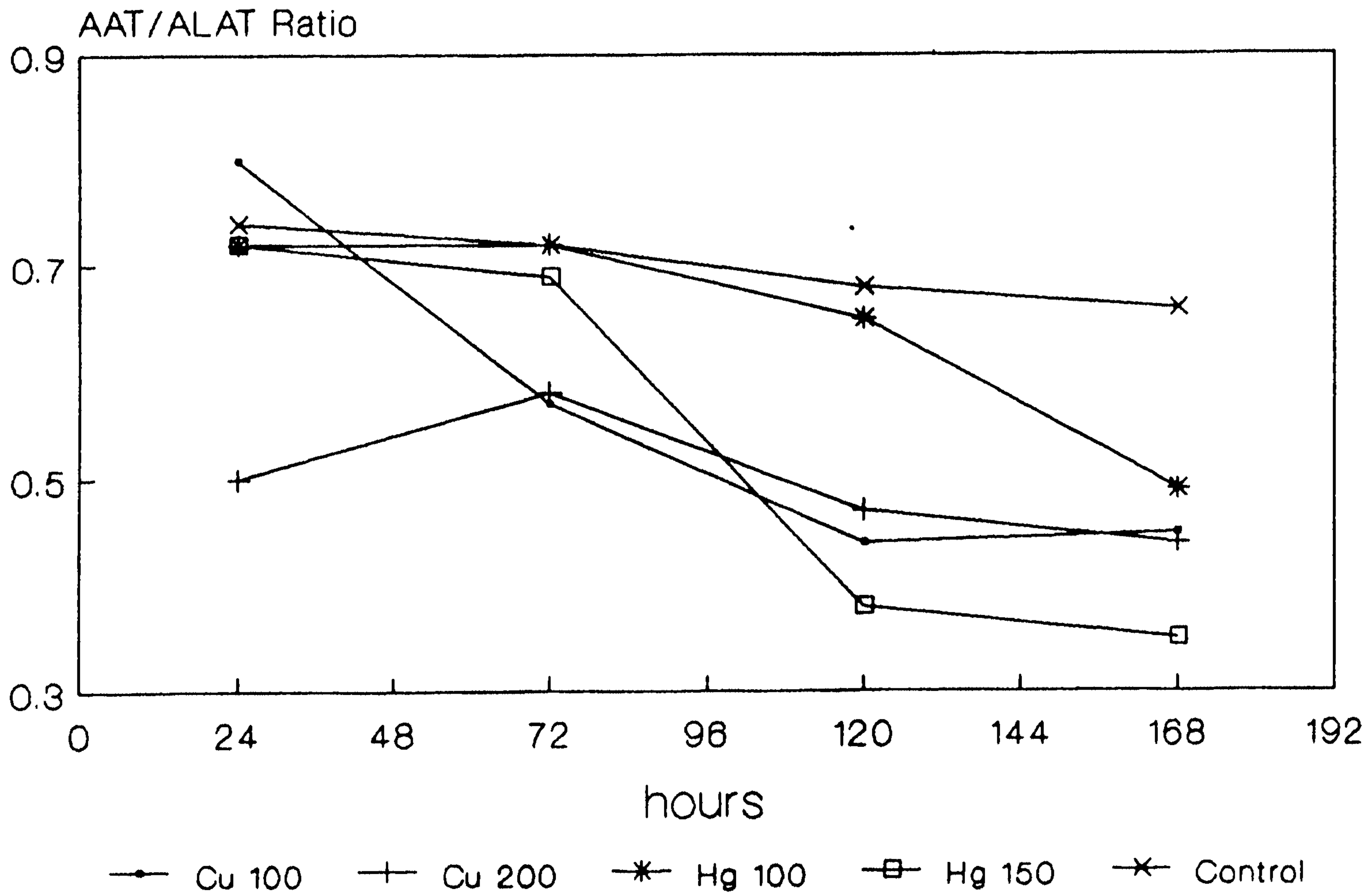


FIGURE 14. RATIO OF AAT/ALAT (GOT/GPT) IN THE KIDNEY OF *O. MOSSAMBICUS* EXPOSED TO COPPER AND MERCURY



in Anabas testudineus in response to lindane (Bakthavathsalam and Reddy, 1982 a) and in Nemachelius denisonii in response to a herbicide basalin (Rashatwar and Ilyas, 1983) whereas significantly lower GOT activity in liver was observed in Tantogolabrus adspersus in response to cadmium (Gould and Karolus, 1974; MacInnes et al., 1977); in embryos of brook trout in response to methyl mercury (Christensen, 1975); in striped bass in response to cadmium (Dawson et al., 1977); in Tilapia mossambica in response to ammonium toxicity (Chetty et al., 1980); in Cyprinus carpio in response to cadmium (Koyama et al., 1985); in C. batrachus in response to aflatoxin B (Parashari and Saxena, 1986). A decrease in GOT activity in the blood was observed in brook trout after copper exposure for 337 days (McKim et al., 1970); in brook trout in response to lead (Christensen et al., 1977); in Kuwait mullet in response to lead (Helmy et al., 1979).

It should be noted that the results of the present study varied markedly from a similar study using T. adspersus (Gould and Karolus, 1974; MacInnes et al., 1977); T. mossambica (Chetty et al., 1980) and Mugil cephalus (Hilmy et al., 1985) when dosed with cadmium. As reported earlier they observed a significantly lower GOT activity in the liver.

In the present study, in copper and mercury - dosed fishes, the GOT showed a significant increase in activity in the liver and a significant decrease in activity in the kidney. In the kidney also there is no significant difference in enzyme activity pattern between the copper-dosed and mercury-dosed fishes. However, the kidney of fishes exposed to higher concentrations of copper and mercury showed lower GOT activity levels for longer duration than the kidney of fishes exposed to lower concentrations of copper and mercury. Studies dealing with GOT activity in kidney in response to heavy metals are few. Significant decrease in GOT activity in the kidney of T. mossambica in response to ammonia toxicity (Chetty et al., 1980); in C. batrachus in response to aflatoxin B (Parashari and Saxena, 1986) and significant increase in GOT activity in the kidney, besides other tissues in N. notopterus in response to phenol (Verma et al., 1982; Gupta and Dalela, 1985); in response to phenolic compounds (Gupta et al., 1983); and in C. batrachus in response to lithium (Goel et al., 1985) have been reported.

In response to stress due to exposure to copper and mercury GOT activity

was different in the liver and kidney. In the liver when the GOT showed a significant increase in activity over the controls, GOT showed a significant decrease in activity in the kidney.

When the GOT showed a significant increase in activity in the liver of fishes exposed to copper and mercury, GPT showed a significantly lower activity. It appears that there is difference in the GPT activity pattern between copper-dosed and mercury-dosed fishes. Lower GPT activity lasted longer in the liver of mercury-dosed fishes. This type of longer duration of lower GPT activity is shown in the liver of fishes dosed with higher concentrations of copper and mercury, when compared with the GOT activity in the liver of fishes exposed to the respective lower concentrations copper and mercury. Significant decrease in GPT activity in liver in response to pollutants have been reported earlier. These include, the decreased GPT activity in the liver in rainbow trout in response to carbon tetrachloride (Racicot et al., 1975); in C. carpio dosed with cadmium (Koyama et al., 1985); in N. denisonii in response to the effect of herbicide basalin (Rashatwar and Ilyas, 1983); in M. cephalus in response to cadmium (Hilmy et al., 1985) and in C. batrachus in response to aflatoxin B (Parashari and Saxena, 1986). However increase of GPT activity in liver besides other tissues, was also reported as in N. notopterus in response to phenol (Verma et al., 1982; Gupta and Dalela, 1985); in response to phenolic compounds (Gupta et al., 1983); in Tilapia exposed to methyl parathion (Prasada Rao and Ramana Rao, 1984); in C. batrachus in response to lithium (Goel et al., 1985); and in O. mossambicus in response to naphthalene, toluene and phenol (Dange, 1986 b).

Based on the present study, it can be said that in the liver, the trend of increased GOT activity persists in the later period of study, whereas the trend of decreased GPT activity prevails in the early period of the study and this is more pronounced in those experimental groups dosed with lower concentrations of copper and mercury.

The results of the present study indicate that in the kidney of metal-dosed fishes, lower GPT activity is more pronounced in the mercury-dosed fishes. Unlike the GPT activity in the liver of the heavy metal dosed fishes, GPT activity in the kidney does not show a definite trend, the only comparison

being the lower GPT activity in the liver and kidney of the fishes dosed with higher concentration of mercury. Studies dealing with GPT activity in the kidney in response to heavy metals are few. Reports of GPT activity in the kidney of fishes in response to toxicants include a significant decrease in GPT activity in C. batrachus in response to aflatoxin B (Parashari and Saxena, 1986); increase in GPT activity in N. notopterus in response to phenol (Verma et al., 1982; Gupta and Dalela, 1985) and in response to phenolic compounds (Gupta et al., 1983); in C. batrachus in response to lithium intoxication. There are also reports that various toxicants cause an increase in serum GPT (Goel and Garg, 1980; Wieser and Hinterleitner, 1980; Wotten and Williams, 1980; Hilmy et al., 1981; Dalich et al., 1982; Nemcsok and Boross, 1982; Nemcsok et al., 1982, 1985; Cassilas et al., 1983; Verma et al., 1986; Yamawaki et al., 1986).

In fishes, processes like metal detoxification and repair of cellular damage in the tissues require greater energy demands. This shows that when the fishes are subjected to sufficiently high, but sublethal pollution stress, it could lead to abnormal exhaustion of the important energy stores (see chapter 2), while not actually causing death, this type of stress could still be responsible for some reduction in the amount of metabolic energy that can be invested in the growth and development of gonads, thereby affecting the reproductive success and consequently the chances of survival of the pollution as a whole (Dange, 1986 b).

Dange and Masurekar (1981, 1982, 1984) detected an increase in glycogenolysis in fishes exposed to different pollutants. Energy produced from glycolysis is augmented with energy from amino acid metabolism for which aminotransferases are necessary. In fishes, the transamination reaction probably play a significant role at some stage in the degradation of muscle protein (Siebert and Schmitt, 1965). It is probable that the pool of amino acids present in fish tissues is utilized for transamination to produce a variety of keto acids. Increased protein breakdown is probably mediated through increased secretion of cortisol in chronically stressed fish (Dange, 1986 b). Cortisol is known to stimulate the aminotransferase activities in fish tissues (Garbus et al., 1967; Storer, 1967; Freeman and Idler, 1973). Alanine and aspartic acid serve as two major glucogenic amino acids which through the activities of GOT (AAT) and GPT (ALAT) give rise to glucose precursors (Lehninger, 1979). GOT (AAT) and GPT (ALAT) are known to act as an important link between carbohydrate

and protein metabolism, providing a source for keto acids for Krebs' cycle and gluconeogenesis. An increase in GOT and GPT activity along with acceleration of glycogenolysis was observed in C. batrachus (Goel et al., 1985). Hence a stimulation of GOT (AAT) or GPT (ALAT) would alter the amino acid metabolism which in turn disturb all the metabolic processes of the body. So an increased GOT activity could supply more intermediate products for energy metabolism as the body requires more energy to meet the metal stress (Weber, 1963). In the present study, GOT (AAT) increases in the liver whereas GPT (ALAT) decreases in the liver on exposure to metals. But in the kidneys both the transaminases, decrease when dosed with both metals. The two tissues, liver and kidney show a differential response towards the metals. This could be due to their different functions.

Glutamic acid is one of the three amino acids required for the synthesis of glutathione, necessary for the detoxification of pollutants (see chapter 6). Hence, during metal stress, increased quantities of glutamic acid are required for this purpose. An increased transamination activity by GOT and GPT can synthesise glutamic acid from, α-keto glutaric acid. However, increased GPT activity also results in the increased production of pyruvic acid and during hypoxic conditions may be converted to lactic acid which may increase acidosis. There are many reports that metals increases the lactic acid production in fishes (see chapter 2). An increase in blood pyruvate, lactate etc. was observed during ammonia toxicity (Prior and Visek, 1972). Since GPT catalyses the formation of pyruvate from alanine, excess pyruvate formed may inhibit the GPT activity due to feed back mechanism. This may be reducing the synthesis of ALAT (GPT) which will be manifested as a decreased ALAT (GPT) activity. So product inhibition and reduced synthesis of GPT may be playing a key role in decreasing the GPT activity initially. When the pyruvate formed is used up for energy production, the GPT activity reverts to normalcy. In the present study also, GPT shows such a general trend in both liver and kidney and the GPT activity returns to control values at the end of the experiment.

Another role of glutamic acid lies in its ability to remove ammonia. Ammonia is constantly produced in liver due to the deamination process and during toxicant-mediated stress, ammonia production increases. Carbon tetrachloride is known to increase the production of a blood ammonia in fish

(d'Apollonia and Anderson, 1977). Many authors (McBean et al., 1966; Janicki and Lingis, 1970; Hochachka and Somero, 1973) have pointed out that GOT (AAT) and GPT (ALAT) play an important role in the production and detoxification of ammonia. The ammonia produced is thus removed from the circulation by the liver by converting it to glutamine or to urea. Formation of glutamine is catalysed by glutamine synthetase. Glutamic acid combines with ammonia in the presence of glutamine synthetase, ATP and Mg^{++} ions to form glutamine. Thus synthesis of glutamine from glutamic acid removes NH_3. The immediate source of glutamic acid for this purpose is α- keto glutaric acid. This would rapidly deplete the supply of citric acid cycle intermediates unless replaced (Harper and Rodwell, 1975). GOT can increase the supply of both oxaloacetic acid which is a citric acid cycle intermediate and glutamic acid which removes ammonia. Here, there is a switching over from alanine transamination to aspartate transamination. Tissues when subjected to trauma, switches over to aspartate transamination from alanine transamination (Malhotra et al., 1986). Such a switching over, increases the GOT levels and depress the GPT activity. In the liver of metal exposed fishes, such a switching over from alanine transamination to aspartate transamination may be occuring. It may be possible that observed increase in GOT (AAT) activity and decrease in GPT (ALAT) activity in the liver is the manifestation of this switching over.

In the kidney, which is the major excretory organ for nitrogenous waste products, glutamine is converted back to glutamic acid catalysed by glutaminase releasing ammonia. The ammonia liberated is removed through urine. Increased urine flow after exposure to toxicants was postulated by Lock et al. (1981). The excretion into the urine of the ammonia produced by renal tubular cells constitute a far more significant aspect of renal ammonia metabolism. Ammonia production forms part of the renal tubular mechanism for regulation of acid-base balance as well as conservation of cations. (see Harper and Rodwell, 1975). Ammonia production by the kidney is markedly increase in metabolic acidosis and is derived particularly from glutamine catalysed by glutaminase which converts glutamine to glutamic acid. The glutamic acid concentration increases in the kidney which may inhibit both the transminases, GOT and GPT in the kidney. The observed decrease of GOT and GPT activity in the kidney could be due to this phenomenon. A differential response by GOT

and GPT was found in the liver of rainbow trout dosed with CCl_4 (Racicot et al., 1975). A drop in GPT activity at the 3rd day after CCl_4 injection was observed by Racicot et al. (1975), but there was no change in the GOT activity in the liver of fish dosed with CCl_4. The GPT activity returned to control levels at the 10th day. Hilmy et al. (1985) also observed a differential response of AAT (GOT) and ALAT (GPT) in the liver of M. cephalus exposed to cadmium upto 48 h, where the AAT (GOT) increased but GPT decreased. However, the AAT (GOT) activity later decreased whereas inhibition of ALAT (GPT) activity continued throughout the experiment. There are many other reports in fishes in which the GPT activity decreased while GOT activity either increases or does not change, so that there is a rise in the GOT/GPT ratio (see Tiedge et al., 1986; Racicot et al., 1975; Verma et al., 1982; Rashatwar and Ilyas, 1983). However, these authors did not give the actual GOT/GPT ratios. Eventhough Dange (1986 b) found that both aspartate aminotransferase (AAT or GOT) and alanine aminotransferase (ALAT or GPT) increased in the liver of O. mossambicus exposed to toluene, napthalene and phenol, if the liver AAT/ALAT (GOT/GPT) ratio was calculated in controls and fishes exposed to lower concentration of napthalene and toluene for 10 weeks, there was an increase in the AAT/ALAT ratio in the dosed fishes from that of the controls. Since there are many reports about increase, decrease or no change in the activities of GOT or GPT, it would be better to calculate the GOT/GPT ratios in different tissues to understand the exact role played by these enzymes.

In the present study also the pattern of activity of GOT and GPT in the liver were different from that of kidney. It is pertinent to observe the findings of Malhotra et al. (1986) that tissues when subjected to trauma switches over to aspartate transamination from alanine transamination which increases the GOT levels and depress the GPT activity. According to his observation, regions of higher GOT levels tend to show lower GPT levels. The GOT and GPT activities obtained in the present study tends to support this observation. If this is true a ratio of GOT/GPT will be a better estimate of tissue reaction towards xenobiotics, as this will take into consideration both the enzyme activity pattern.

In the present study, the ratio of GOT/GPT in the liver and kidney vary slightly. The kidney show a higher GOT/GPT ratio than the liver of control

fishes. In the liver GOT/GPT ratio of the metal dosed fish shows an increase from that of controls in all days. The ratio increases to its highest level at 72 h and show a tendency to fall. In the kidney, a totally different trend was seen. The GOT/GPT ratio began to drop in the kidney of O. mossambicus dosed with copper and mercury. The decrease began at 72 h in the kidney of copper treated fish and a maximum decrease was observed at 168 h. But in mercury-dosed fishes, the GOT/GPT ratio fell at 120 h onwards.

However, it is to be noted that Goel and Garg (1980) reported a GOT/GPT ratio of less than 1.0 in the serum of toxicant treated fish and a ratio of more than 1.0 for the normal fish. In the present investigation, the GOT/GPT ratio of the metal treated fishes was more than that of the controls in the liver and less than that of the controls in the kidney. It is felt that GOT/GPT ratio may be a better estimate of the stress reaction in different tissues of fish exposed to toxicant. A high or low GOT/GPT ratio from the normal may indicate a stress response in this fish. However more research work is necessary before fixing arbitrary values for measuring the stress response in O. mossambicus exposed to heavy metals.

CHAPTER 5

EFFECT OF COPPER AND MERCURY ON THE HAEMOGLOBIN, HAEMATOCRIT AND MEAN CELL HAEMOGLOBIN VALUES

Fishes are susceptible to any changes that may occur in the environment. It is expected that these changes would be reflected in the physiology of fish, particularly in the values of haematological parameters and haematology has been used as an index of health status of a number of fish species (Blaxhall, 1972).

Blood takes part directly and indirectly in almost all the activities of fish and thus it can be a good indicator of stress conditions. The use of haematological parameters as indicators of sublethal stress can provide information on the physiological responses that the fish make to a changing environment. This is the result of the close association of the circulatory system with the external environment and with tissues. When values are obtained under abnormal conditions it should be possible to monitor the changes in the quality of water (Mawdesley-Thomas, 1971). Since haematological tests have been an important diagnostic tool in medicine for many years, it is speculated that they may be an equally valuable indicator of stress or disease condition of fish (Larsson,1975).

Haematological changes have been detected in response to diseases, pollutants, surgical procedures, hypoxia etc. (Eisler and Edmunds, 1966; DeWilde and Houston, 1967; Gardner and Yevich, 1969; McKim et al., 1970; Soivio and Oikari, 1976; Duthie and Tort, 1985). Blood alterations or damage to the haemopoietic organs in these organisms may also be associated with pathological conditions related to water-borne pollutants (Reichenbach-Klinke, 1966; Gardner and Yevich, 1969; Saad et al., 1973).

One of the important functions of blood is the transportation of oxygen and carbondioxide in the body. The immature red blood corpuscles (RBC) is uniquely concerned with the synthesis of Hb; it is otherwise comparable to many other cells in its metabolic activity. When its maturation is complete the RBC functions primarily in the transportation of haemoglobin (Hb).

The haemoglibin present in the red blood cell enables the blood to carry

adequate amount of the gases to different tissues as the capacity of the Hb to carry these gases is very high. Hence an estimation of the Hb in the blood provide us information about the physiological status of the body. The increase or decrease of the Hb content and RBC, variation of the packed cell volume (PCV) or haematocrit (Hct), mean cell haemoglobin concentration (MCHC) etc. indirectly indicates the oxygen carrying capacity of the blood. Alterations of the haematological parameters can be due to factors like retention of metabolites, metabolic problems, oxidation of Hb, increased or decreased erythropoiesis, haemodilution or haemoconcentration.

RBC count is a long cumbersome procedure which nowadays is replaced by haematocrit determination which express PCV (Packed Cell Volume) as the percentage of the whole blood volume. Haematocrit provides a rapid approximation of the volume of circulating RBC and is used as a routine method for haematological diagnosis of fish health in field studies. This method has the advantage of speed and simplicity and is suitable for the capillary blood.

Heavy metals are one class of pollutants which have a disruptive influence on the structural organisation of the gill tissues because the gills are intimately associated with ionic regulation and it is predictable that heavy metals will influence aspects of osmotic and ionic regulation in fish which may influence the composition of blood.

McKim et al. (1970) compared data for Hb, Hct, RBC count etc of blood from male and female of fishes exposed to copper and found that no significant differences at 95% level. Bell (1968) also did not find any difference in the Hct values between male and female fishes.

In the present study also both the male and female fishes were used for the experiment. In this chapter the effects of the metals, copper and mercury on the Hb, Hct, and MCHC values of Oreochromis mossambicus are described.

MATERIAL AND METHODS

Specimens of O. mossambicus were collected and acclimatized in the laboratory for a month as described in Chapter 2. Later they were transferred to five large experimental tanks containing 200 l dechlorinated tap water. Calculated volumes of 1000 mg/l solutions of copper sulphate and mercuric chloride were separately added to different tanks to give concentrations of 100 µg/l and 200 µg/l for copper and 150 µg/l and 100 µg/l for mercury. The last tank without any toxicant, served as the control. The test medium was renewed every 24 h. The temperature in the tanks was maintained at 28 $\pm$ 1°C. Fishes were not fed during the experiment. Samples of blood were collected at 24, 72, 120 and 168 h. The fishes were caught and immobilised with a hard blow on the head. Immediately the caudal peduncle of the fish was cut and the blood was collected in tubes rinsed with heparin. From this, the Hb and Hct were determined.

Estimation of Hb

Haemoglobin was determined by the cyanmethaemoglobin method (Drabkin, 1946). To 5 ml of Drabkin's reagent, 0.02 ml of blood was added and mixed thoroughly. The potassium ferricyanide present in the reagent converts the Hb iron from ferrous to ferric state to form methaemoglobin. The methaemoglobin thus formed combines with potassium cyanide of the Drabkin's reagent to produce a stable pigment, cyanmethaemoglobin. This represents the sum of oxyhaemoglobin, carboxyhaemoglobin and methaemoglobin. The cyanmethaemoglobin formed is measured colorimetrically at 540nm.

Haematocrit values

Haematocrit values of the samples were determined, following the procedure described by Hesser (1960). Heparinized microhaematocrit capillary tubes were filled with blood and one end was sealed with plastic clay. Blood-filled capillary tubes were centrifuged at 12000 rpm for 5 minutes and haematocrit values were measured with a Spiracrit reader.

Mean corpuscular haemoglobin concentration (MCHC) of the different blood samples was calculated from the Hb and Hct values.

$$MCHC = \frac{Hb}{Hct} \times 100$$

RESULTS

The results are presented in Tables 15, 16 and 17 and Figs. 15, 16 and 17.

Haemoglobin values

There was no significant variation in the Hb values in the control fishes between days eventhough the Hb values declined by the end of the experiment. The Hb values of the copper-dosed fishes were significantly lower than that of the control fish at 24 h. But mercury-dosed fishes did not show any significant difference in the Hb content. However at 72 h the copper-exposed fishes showed a different trend in Hb values. A highly significant increase in the Hb content of copper-treated fishes was seen at 72 h. The Hb content increased significantly in the copper and mercury-dosed fishes at 120 and 168 h.

Haematocrit values

In spite of a slight decrease in Hct values, the controls did not show any significant variation in Hct values. Haematocrit values did not alter significantly from that of controls in the copper and mercury-dosed fishes at 24 h. But at 72 h the copper-treated fishes and fishes exposed to the higher concnetration (150 µg/l) of mercury showed a significant increase in the haematocrit values over the controls. And at 120 and 168 h the haematocrit values increased in fishes exposed to all concentrations of copper and mercury.

The MCHC values in the metal-dosed fishes did not vary significantly from that of the controls.

DISCUSSION

In the present study, a distinct difference in Hb values between copper-dosed and mercury-dosed fishes in the initial stage (24 h) of the experiment was observed. Unlike fishes exposed to mercury, the copper-dosed fishes showed

Table 15. Haemoglobin values in *O. mossambicus* exposed to copper and mercury

Concnetration µg/l		Hb g/100 mL blood, $\bar{x} \pm$ S.D. (N = 22) 24 h	72 h	120 h	168 h
Cu	100	8.59* ± 0.55	9.25** ± 0.69	9.92** ± 0.69	9.94** ± 0.63
	200	8.28** ± 0.69	9.43** ± 0.45	10.19** ± 0.79	10.27** ± 0.71
Hg	100	8.88 ± 0.67	8.57 ± 0.88	9.15** ± 0.58	9.56** ± 0.67
	150	8.76 ± 0.51	8.51 ± 0.73	9.34** ± 0.62	9.70** ± 0.57
Control		9.01 ± 0.74	8.52 ± 0.84	8.19 ± 0.76	8.05 ± 0.53

Table 16. Haematocrit values in *O. mossambicus* exposed to copper and mercury

Concentration µg/l		Haematocrit percentage, $\bar{x} \pm$ S.D. (N = 12) 24 h	72 h	120 h	168 h
Cu	100	34 ± 2.6	34* ± 2.9	35** ± 2.6	35** ± 3.1
	200	34 ± 2.6	34* ± 3.4	38** ± 2.3	39** ± 2.7
Hg	100	34 ± 2.2	33 ± 2.9	34* ± 2.8	35** ± 2.9
	150	33 ± 2.4	34* ± 2.8	36** ± 2.4	37** ± 2.3
Control		33 ± 2.8	31 ± 2.7	31 ± 2.9	30 ± 2.4

* $P < 0.05$ ** $P < 0.01$

Table 17. Mean corpuscular haemoglobin concentration in _O. mossambicus_ exposed to copper and mercury

Concentration $\mu g/l$		MCHC g/100 ml packed RBC $\bar{x} \pm$ S.D. (N = 12) Exposure time			
		24 h	72 h	120 h	168 h
Cu	100	26.30 ± 3.1	27.11 ± 2.4	26.25 ± 2.2	27.14 ± 2.5
	200	25.49 ± 3.4	27.63 ± 3.3	27.93 ± 3.7	28.06 ± 2.6
Hg	100	27.12 ± 2.5	26.10 ± 3.9	26.73 ± 2.3	26.79 ± 2.4
	150	26.19 ± 3.7	25.38 ± 2.7	25.92 ± 2.5	27.14 ± 3.1
Control		27.55 ± 2.2	26.72 ± 2.4	26.70 ± 3.6	27.71 ± 2.2

The values are not significantly different from that of controls.

FIGURE 15. HAEMOGLOBIN VALUES IN O. MOSSAMBICUS EXPOSED TO COPPER AND MERCURY

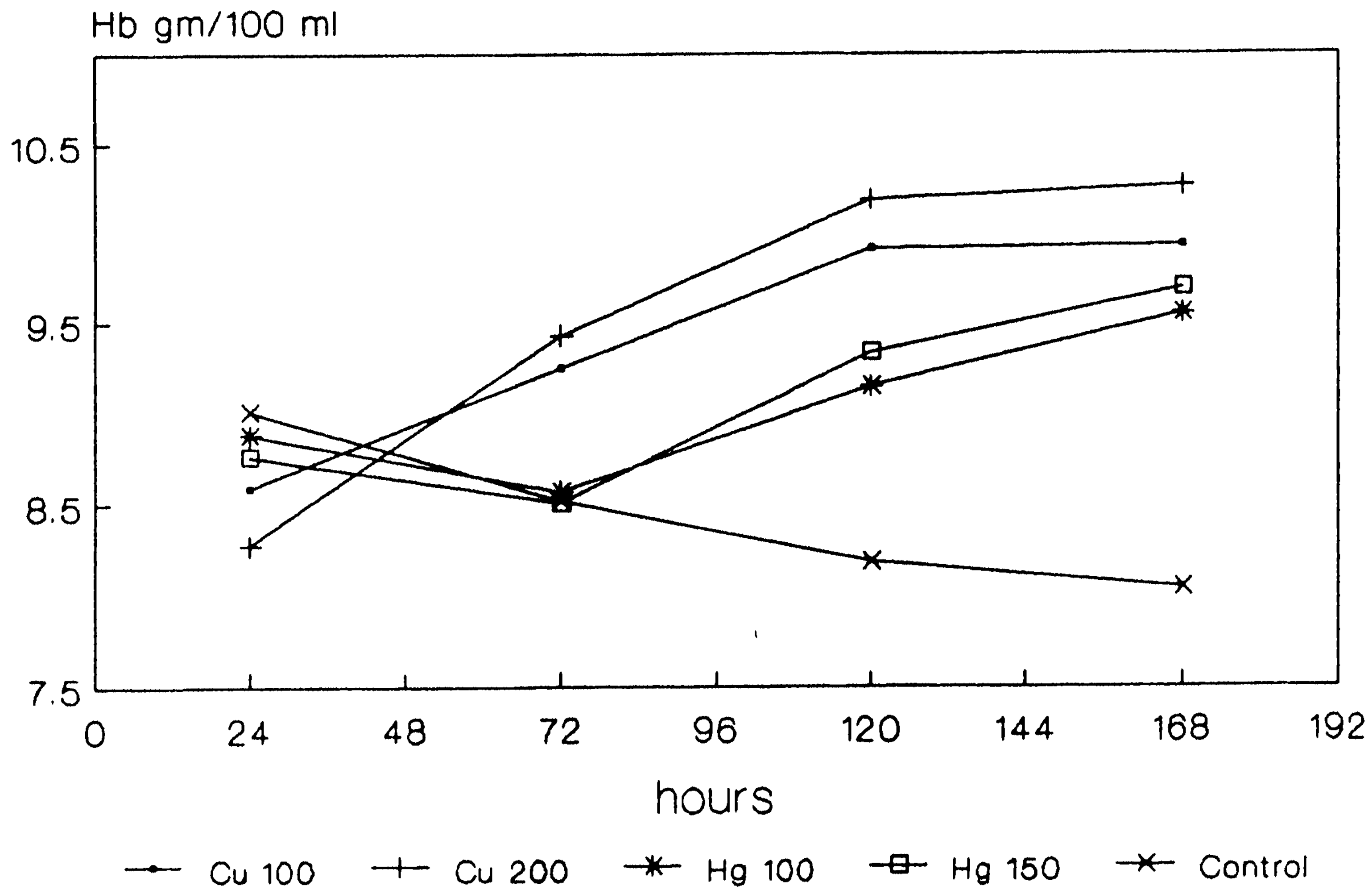


FIGURE 16. HAEMATOCRIT VALUES IN *O. MOSSAMBICUS* EXPOSED TO COPPER AND MERCURY

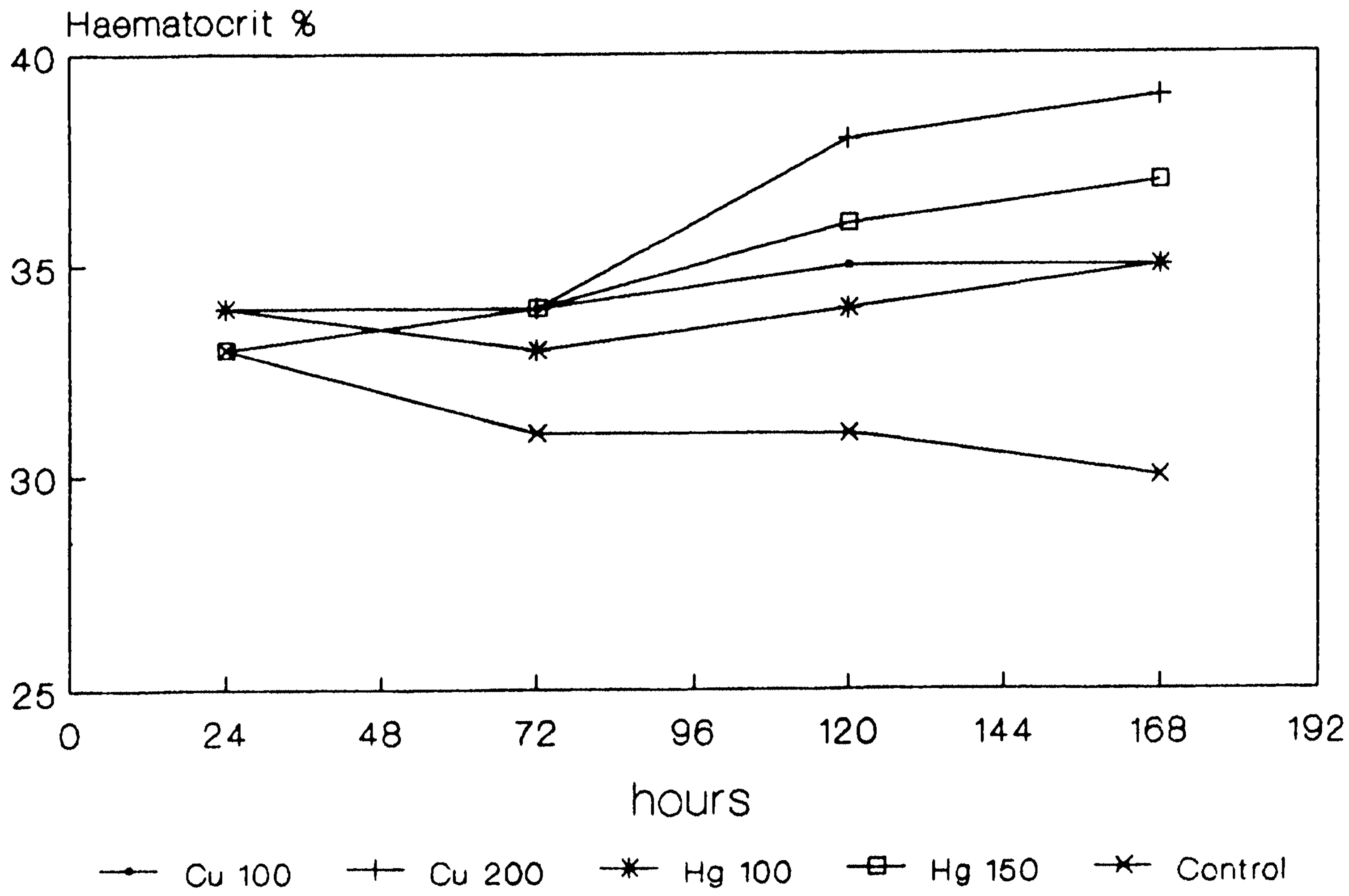
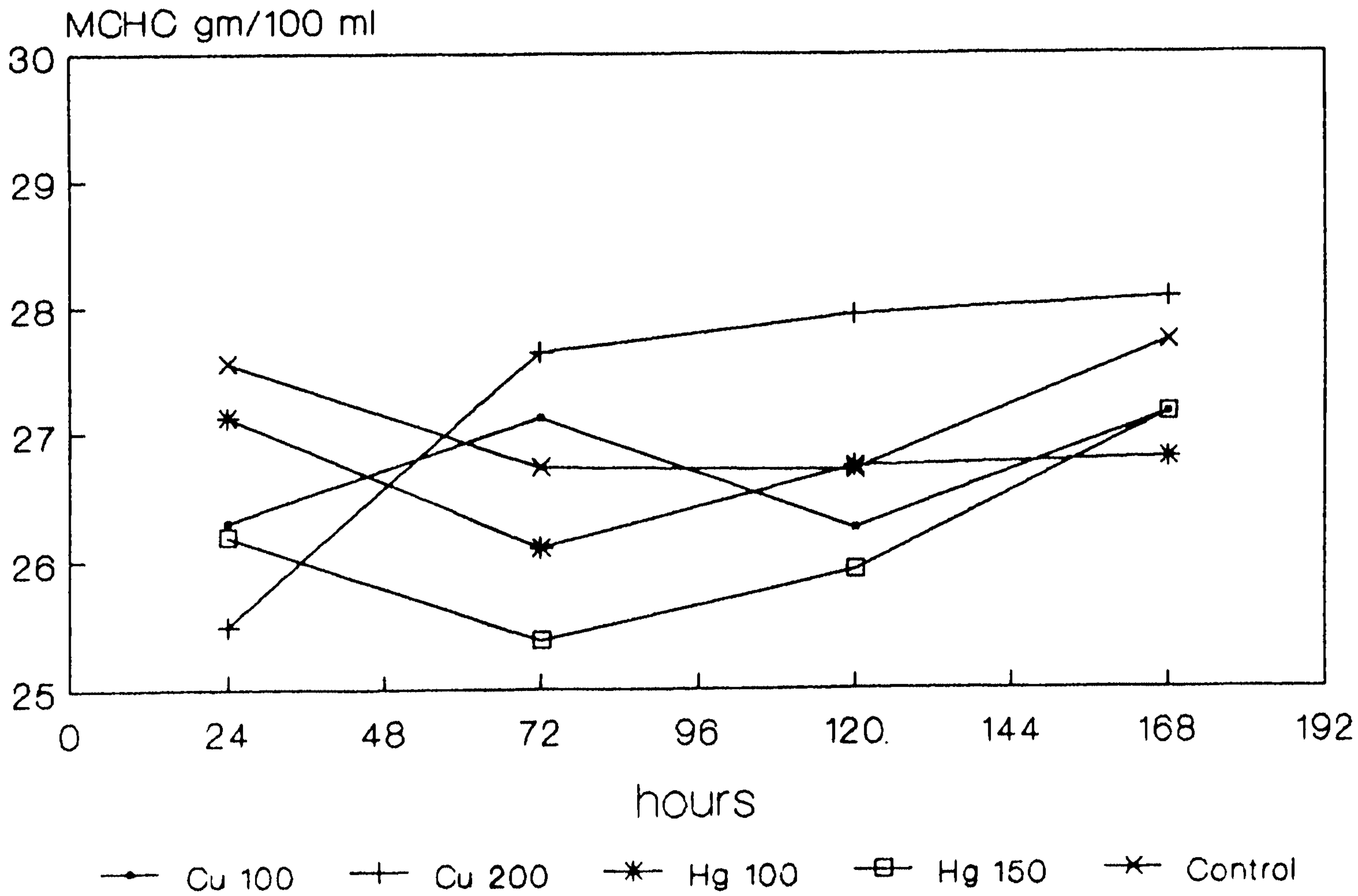


FIGURE 17. MEAN CORPUSCULAR HAEMOGLOBIN CONCENTRATION IN _O. MOSSAMBICUS_ EXPOSED TO COPPER AND MERCURY



a significant decrease in the Hb content at 24 h, but there was no change in the Hct values of these fishes. This shows a haemodilution and the resultant swelling of the erythrocytes combined with the release of erythrocytes from the erythropoietic organs.

Haemodilution has been interpreted as a mechanism which reduces the concentration of an irritating factor in the circulatory system (Smit et al., 1979). Decreased osmoregulation is the consequence of copper toxicity in fish (Leland and Kuwabara, 1985). Haemodilution has been observed in Colisa fasciatus exposed to zinc by Mishra and Srivastava (1979, 1980). One of the consequences of haemodilution is the decrease in plasma osmotic pressure as observed in Ictalurus punctatus in response to zinc and copper (Lewis and Lewis, 1971) which would result in the swelling of RBC as reported in the dogfish Scylorhinus canicula in response to copper (Tort et al., 1987). Such an increase in the erythrocyte size is generally considered as a response against stress. The swelling would also be a consequence of factors like high pCO_2 high lactate concentration or low pO_2 in the blood, leading to low ATP concentration, which would increase the oxygen affinity of blood (Soivio and Nikinmaa, 1981). Since metals produce changes in blood gases and lactate, the swelling of red blood cells could be involved in the response of fish against heavy metal pollution (Tort et al., 1987). A decrease in Hct value and an increased RBC count were observed by Tort and Torres (1988) in the dogfish S. canicula exposed to cadmium. Abrahamsson and Nilsson (1975) observed that the contraction of spleen of cod exposed to a stress would release blood cells into the blood stream. A similar pattern has been detected in Cyprinus carpio after cadmium exposure (Koyama and Ozaki, 1984), in which haematocrit decrease, maintenance of RBC count and an increase of circulating reticulocytes were recorded.

Helmy et al. (1978) reported a decrease in RBC count, Hb, and Hct in Kuwait mullet exposed to copper and mercury. Similar effects were detected in flounder exposed to cadmium (Johansson-Sjobeck and Larsson, 1978); in winter flounder exposed to mercury (Dawson, 1979); in C. fasciatus exposed to lead (Srivasthava and Mishra, 1979); in marine teleost, Aphanius dispar (Hilmy et al., 1980); and in C. carpio exposed to cadmium and mercury (Beena and Viswaranjan, 1987) and in Sarotherodon mossambicus treated with cadmium (Ruparelia et al.,

1987). Panigrahi and Misra (1978, 1980) reported low Hb and RBC count associated with reduced respiratory rate in the freshwater fish Anabus scandens and Tilapia mossambica dosed with mercury.

Decrease in Hb was observed in perch in response to cadmium (Larsson, 1975); in Pleuronectes flesus exposed to cadmium (Larsson et al., 1976); in brooktrout exposed to lead (Christensen et al., 1977); in A. scandens dosed with mercury (Panigrahi, 1977); in Labeo umbratus treated with various pollutants (van Vuren, 1986); in S. mossambicus exposed to mercury (Aruna and Gopal, 1987); in Clarias lazera intoxicated with copper (El-Domiaty, 1987) and in the dogfish S. canicula exposed to copper (Tort et al., 1987).

There are reports that various other chemical substances, also cause a decrease in the Hb and Hct values in different fishes (McLeay, 1973; Buckley, 1976; Buckley et al., 1976, 1979; Oikari and Soivio, 1977; Anees, 1978; Koundinya and Ramamurthy, 1979; Dalela et al., 1981; Natarajan, 1981; Pandey et al., 1981; Verma et al., 1981 c; Goel et al., 1982; Mishra and Srivastava, 1983, 1984; Madhyastha and Nayak, 1984; Scarano et al., 1984; Torres et al., 1986).

At 72 h the copper-dosed fishes showed a reverse trend. There was a significant increase in Hb content and a corresponding increase in the haematocrit values. The body of the copper exposed fish might have adapted to the metal stress by this time. Haemodilution could be an initial reaction of the body to stress. Afterwards the living system rectified the imbalance by removing water from the blood. This could result in haemoconcentration. Hilmy et al. (1980) reported that values of Hct, Hb and RBC count returned to control levels after an initial decrease in marine teleost A. dispar in response to mercury toxicity. Buckley (1976) also observed a partial recovery of Hb after a decline in coho salmon exposed to treated water containing total residual chlorine ($TRCl_2$). He suggested (1) decreased haemolysis as a result of elimination of susceptible cells and decreased sensitivity of younger cells to oxidants and (2) compensatory erythropoiesis with the establishment of a balance between cell destruction and formation resulting in reduced number of circulating cells. But Tort and Torres (1988) ruled out the haemolysis or RBC destruction as the RBC count increased in the fish after cadmium exposure. They postulated that the RBC count elevation was due to consequence of blood cell reserve

release combined with cell shrinkage, probably due to osmotic alterations of blood by the action of the metal. In addition, haemoglobin measurements by Tort and Torres (1988) in plasma showed no increase of extracellular haemoglobin. Hence the alteration in the haematological parameters observed in copper-treated fishes at 24 and 72 h could be due to changes in blood water content, that is, a change from haemodilution to haemoconcentration. Torres et al. (1986) found that in fish subjected to confined stress condition, zinc treatment significantly decreased Hct and RBC count and the decrease was identical. Gluth and Hanke (1985) postulated a biphasic response to pollutants in C. carpio consisting of water loss followed by a water gain in the blood. But in the present study the biphasic response observed in the copper-dosed fish was just the reverse, that is, water gain followed by a water loss. Gill and Pant (1981) also obtained a biphasic response similar to the findings in the present study. They observed a fall in Hb, RBC following 1-3 weeks exposure to sublethal concentrations of mercury in the teleost Puntius conchonius but recorded an increase in Hb and RBC count after 8 weeks of exposure. Gill and Pant (1981) ascribed the initial fall in Hb to haemolysis by mercury whilst subsequent recovery and the rise to enhanced erythropoiesis was triggered by stress.

During the entire experimental period there was no significant variation in the mean corpuscular haemoglobin concentration (MCHC) in the fishes exposed to metals. Svobodova (1982) in C. carpio treated with copper and Gill and Pant (1985) in P. conchonius dosed with cadmium did not find any significant difference in MCHC values from that of control values. Because MCHC is the ratio of blood Hb concentration to the Hct, it is not dependent on the blood volume or on the number of red cells per unit volume. This clearly indicates that the decrease of Hb noted in the present study was not due to haemolysis or unusual RBC destruction but caused by haemodilution. Similarly the increase in Hb and a corresponding increase in Hct was due to either haemoconcentration or increased erythropoiesis or both.

The lack of decrease in Hb in mercury-treated fishes could be due to increased production of urine which might remove the excess water present in the blood as a result of haemodilution. Lock et al. (1981) observed that increased water uptake by the gills did not result in the decreased Haematocrit

values of mercury-treated rainbow trout and instead there was an increase in the haematocrit values. He suggested that the inflow of water is offset by an enhanced urine flow. The enzyme Na, K-ATPase appears to be involved in osmoregulatory transepithelial electrolyte transport in the gills, intestine and urinary bladder as well as in active sodium-potassium exchange across all cell membranes (Schmidt-Nielsen, 1974). In a wide variety of tissues this enzyme is sensitive to mercurials and other sulphydryl reagents (Schwartz et al., 1975). In fact, previous studies (Renfro et al., 1974) demonstrated mercurial inhibition of both active sodium transport and Na, K-ATPase in seawater flounder urinary bladder in vitro and of sodium transport by freshwater killifish gills in vivo. Hence mercury may prevent the reabsorption of water across kidney tubules, resulting in the increased urine flow and hence haemoconcentration.

The Hb content in both the copper and mercury-dosed fishes increased at 120 and 168 h. There was a corresponding increase in the haematocrit values as well. The effect of mercury in fish was not felt at 24 h. The significant increase of Hb and Hct observed in copper and mercury-treated fishes could have been due to an increased production of RBC by the erythropoietic organs along with haemoconcentration. McKim et al. (1970) in brook trout Salvelinus fontinalis and Svobodova (1982) in C. carpio exposed to copper reported a significant increase in RBC, Hct and Hb. The mean cell volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) in C. carpio remained without change. This indicates that the increase in Hb and Hct is due to an increase in RBC number. Svobodova (1982) explained the changes in the haematological parameters in the intoxicated carp as disorders in the oxidation process in the fish.

There are many reports that both Hb and Hct in fishes increase after exposure to metals. An increase in Hb, Hct values were observed in S. fontinalis in response to copper (McKim et al., 1970); in Ictalurus nebulosus exposed to copper (Christensen et al., 1972); in C. fasciatus treated with nickel (Agrawal et al., 1979); in rainbow trout dosed with copper (Wotten and Williams, 1980) and in C. carpio exposed to copper (Svobodova, 1982).

Many other substances also elevate both Hb and Hct in fishes (Agrawal et al., 1978; Juelich, 1979; Natarajan, 1984; Ghosh and Chatterjee, 1986; Beevi

and Radhakrishnan, 1987; Pant et al., 1987).

Haemoglobin levels were elevated in response to copper in brook trout (McFadden, 1965); in S. fontinalis in response to methyl mercury (Christensen et al., 1977); in Channa punctatus in response to mercury (Chitra and Ramanarao, 1986) and in dogfish in response to cadmium (Tort and Torres, 1988). Similarly Hct values increased in rainbow trout exposed to methyl mercury and in Mystus vitatus in response to copper and zinc (Singh and Singh, 1982).

The increase in Hb and Hct observed in the present study in metal-dosed fishes may be an attempt by the body to counteract the low oxygen content of the blood. The low oxygen content may be due to the low oxygen carrying capacity of the blood or faulty gaseous exchange caused by damage to the gills.

It has been widely reported that many pollutants enter the RBC and either oxidise or denature the Hb by inhibiting the glycolysis or metabolism of the hexose monophosphate shunt (HMPS). Buckley (1976) found degenerative changes including formation of Heinz bodies in the erythrocytes of cocho salmon Oncorhynchus kisutch exposed to chlorinated waste water containing total residual chlorine ($TRCl_2$). Fairbanks (1967) showed that copper penetrates the intact erythrocyte, inhibiting glycolysis, denaturing Hb and oxidising glutathione. Chlorine also seemed to diffuse readily through gills oxidising Hb to methaemoglobin and disrupting erythrocyte membrane resulting in haemolysis (Zeitoun, 1977). In a study on the mechanism of acute toxicity of monochloramine to fathead minnows (Pimephales promelas), Grothe and Eaton (1975) found a methaemoglobin (MHb) level of 30% of total Hb. Formation of methaemoglobin reduces the oxygen carrying capacity of the blood. Therefore, methaemoglobinemia and the resulting anoxia were considered as the basis for the toxicity of monochloramine under test conditions (Buckley, 1976). Scarano et al. (1984) observed a decrease in Hb and increase in methaemoglobin in seabass exposed to nitrite.

Asano and Hokari (1987) stated that toxic concentration of copper may cause a cytotoxicity by its oxidant action and can affect the functions of

erythrocytic enzymes leading to oxidation of Hb, a disulphide formation of the membrane proteins and a decrease in the intracellular concentration of glutathione. Boulard et al. (1972) also postulated that copper might inhibit erythrocytic enzymes. Cupric ions can bind to membrane protein sulphydryl groups and cause a disulphide formation of neighbouring sulphydryls (Salhany et al., 1978).

Johansson-Sjobeck and Larsson (1978) in Pleuronectes flesus detected a significant reduction in Hct, Hb and RBC count accompanied by a significant increase of aminolevulinic acid dehydratase, the enzyme necessary for erythropoiesis in renal tissue, indicating a compensatory stimulation of erythropoiesis so that the oxygen carrying capacity is increased.

Hodson et al. (1980) studied the effects of waterborne selenium on rainbow trout and found that eventhough the blood parameters decreased from the control levels by 30%, the fish appeared to be compensating for these changes by increased erythropoiesis. Sahib et al. (1981) found that the exposure of fish to a sublethal concentration of malathion showed a consistent increase in the oxygen consumption up to 24 h and later declined to 48% suggesting a reduction of oxidative metabolism at the end of 48 h. A decrease in the rate of oxygen consumption of the fish Labeo rohita was observed with an increase in the concentration of effluents (Hingorani et al., 1979). Panigrahi and Misra (1980) found that the uptake of oxygen decreased 27% in Tilapia mossambica exposed to mercury. Similarly chlorine produced oxidants (CPO) reduced oxygen carrying capacity of the fish Leiostomus xanthurus (Middaugh et al., 1980).

A decrease in the oxygen carrying capacity may stimulate erythropoiesis in fish so that blood carries enough oxygen to meet the requirements of the body. The increase in Hb and Hct in metal-exposed fishes of the present study may be due to this phenomenon. An increased erythropoiesis may result in an increase in RBC count, Hb and Hct. An increase in RBC count or polycythemia in fishes after exposure to various toxicants were reported by many authors (Buckley et al., 1976, 1979; Agrawal et al., 1979; Juelich, 1979; Verma et al., 1981 c; Singh and Singh, 1982; Juneja and Mahajan, 1983; Lal et al., 1986; Haniffa et al., 1986; and Pant et al., 1987). Increased

erythropoiesis to compensate for the inhibition of Hb by water-borne and dietary lead was recorded by Hodson et al. (1978). Along with stimulation of erythropoiesis, a reduction in plasma volume and a mobilization of new erythrocytes into circulation could also have contributed to the increase in Hb and Hct. Milligan and Wood (1982) observed a reduction in plasma volume and a mobilization of new RBC in rainbow trout associated with low pH. Plasma volume reduction reflected a general redistribution of body water from extracellular compartments in response to ionic disturbances. Erythrocyte recruitment was associated with depletion of splenic RBC reserves which may be reflected in the erythrocyte count (Milligan and Wood, 1982). Lal et al. (1986) found that increase in RBC count was followed by a reduction in spleeno-somatic index indicating a release of RBC from the spleen. Larsson (1973) also suggested that contracting and partly emptying spleen of RBC as a cause of polycythemia. Buckley (1976) and Buckley et al. (1979) had shown that there is an increase in the number of circulating immature erythrocytes when fishes were exposed to different pollutants. Overt increase of circulating immature erythrocytes can be used in monitoring lead poisoning in fish (Srivasthava and Mishra, 1979). Buckley (1976) postulated that increased number of RBC in the circulatory system was an attempt by the body to meet the elevated demands for O_2 or CO_2 transport as a result of increased metabolic activity during stress or by a destruction of gill membrane causing faulty gaseous exchange. Nayak and Madhyastha (1980) found an erythropoietic response as evidenced by a significant increase in the number of immature RBC.

Pollutants can influence the functioning of all parts of respiratory chain. Pollutants may not only restrict gas transfer, but their irritant effect can also interfere with ventilation (Hughes, 1981). Lindahl and Hell (1970) found that the gills from fishes exposed to mercurials show clear tissue injuries. The layer of epithelial cells is detached from the deeper layers. This causes faulty gaseous exchange. When gill from fish exposed to phenylmercurial were studied, a decrease in the circulation of blood was observed in the secondary lamellae. They also found that the oxygen content of blood collected from poisoned fish is greatly reduced. This may either be the effect of decreased circulation of the blood in the secondary lamellae or diminished exchange between water and blood in the secondary gill filaments or structural

change in the Hb molecule due to binding of phenylmercury ions. Diffusing capacity of the gill is reduced, following the action of pollutants and consequently there is a fall in oxygen supply to the tissues which become hypoxic. Pant et al. (1987) suggested that the polycythemia observed in Barbus conchonius exposed to aldicarb was related to enhanced erythropoiesis, and it is hypoxemia that triggers an exodus of erythrocytes from haemopoietic organs in an attempt to compensate for the reduced oxygen carrying capacity of the blood. Davis (1973) found an increase in the oxygen uptake, ventilatory water flow, cough, and buccal pressure in sockeye salmon (Oncorhyncus nerka) exposed to Bleached Kraft Pulp Mill Effluent (BKME). He also found that arterial oxygen tension decreases rapidly when exposed to BKME. On the average, this decline represented a 20% decrease in oxygen saturation of the blood. Increase in Hb content could be a mechanism by which the body tries to absorb more O_2 from the surrounding medium to meet the increased demand. Haniffa et al. (1986) observed that in fishes exposed to paper mill effluents the increase in RBC number was more in nonaerated fish than in aerated fish, indicating an association between RBC number and oxygen content of the blood. Interference with gas transfer will reduce oxygen levels within the blood circulating the brain where responses are initiated by the diffusely located respiratory centre (Ballintijn and Bamford, 1975).

Wedemeyer (1971) explained the increased pituitary activity in formalin treated rainbow trout on the basis of a chemical adversely affecting gill function. Such an interference with gill function can be expected to reduce its respiratory role so that the Hb was increased in the treated fish to compensate the loss. This sort of compensatory reaction is known to occur in fishes infected by certain parasites (Kabata, 1970).

So a faulty gaseous exchange of gases as a result of damage to the gills by the action of metals or oxidation of Hb to MHb by various toxicants lowers the oxygen carrying capacity of the blood. Reaction to such a situation would be by stimulating the erythropoietic tissue and increasing the Hb content of the blood. The increased Hb and Hct values observed in the metal treated fishes of the present study could be due to the increased erythropoiesis and Hb synthesis.

CHAPTER 6

GLUTATHIONE CONTENT IN THE BLOOD OF FISH EXPOSED TO COPPER AND MERCURY

Many environmental toxicants act through free radical or related oxidant-mediated reactions (Bus and Gibson, 1979; Mason, 1982; Mason et al., 1982). Redox cycling of cellular reductants (such as NADPH, glutathione etc) with a xenobiotic results in a xenobiotic free radical. This radical species is then able to reduce molecular oxygen to oxygen based free radicals while regenerating the original xenobiotic moiety which is capable of redox cycling again. It is well established that high concentrations of molecular oxygen and oxygen based free radicals are toxic to animals. It has been postulated that oxygen toxicity is due to (a) oxidation of -SH groups (Barron, 1955), (b) lipid peroxidation of poly unsaturated acids (Roubal and Tappel, 1966), (c) oxidation of cellular reductants and consequent alteration of cell redox state (Balin et al., 1977; Halliwell, 1981 a), and (d) augmentation of the production of superoxide free radicals and hydrogen peroxide (Fridovich, 1978; Chance et al., 1979). The enzyme superoxide dismutase (SOD) catalyses the conversion of superoxide radical (O_2^-) formed by the monovalent pathway of biological reduction of oxygen to hydrogen peroxide, and molecular-oxygen (McCord and Fridovich, 1969). The superoxide (O_2^-) and hydrogen peroxide (H_2O_2) if not scavenged efficiently are known to give rise to potential intermediates namely hydroxyl radical and singlet oxygen which are believed to be involved in the pathogenesis of inflammation and formation of lipid peroxides. Lipid peroxidation of the polyunsaturated acids by the toxic free oxygen radicals can affect membrane permeability, ion transport and other essential membrane functions (Freeman and Crapo, 1982). There are reports that metal ions produce lipid peroxidation in liposomal systems and natural membranes (Bus and Gibson, 1979; Hochstein et al., 1980; Florence and Stauber, 1986).

Several antioxidants and free radical scavengers are known to be present in biological systems to protect the cells from the above mentioned oxygen based free radicals. Some of these are enzymes (catalase, peroxidase, glutathione peroxidase), whereas others are vitamins (tocopherols, ascorbic acid) and peptides like glutathione (De Lucia et al., 1975). The components

of the antioxidant defence cycle decrease lipid peroxidation and maintain membrane stability (Ghosh et al., 1988). Reduced glutathione (GSH) together with glutathione dependent enzymes, glutathione peroxidase (GPX), glutathione-s-transferase (GST) and the catalase-superoxide dismutase couple efficiently scavenge toxic free radicals. Glutathione peroxidase (GPX) and glutathione-s-transferase (GST) are GSH consuming enzymes and are stimulated during toxic situation. GST catalyses conjugation with a second substrate before they can bind to cellular organelles and exert their toxic or mutagenic effects (Chasseaud, 1976). GPX protects biological membranes from lipid peroxidation by catalysing lipid peroxide to stable alcohol.

Reduced glutathione (L-r-glutamyl-L-cysteinyl-glycine) is a key cellular reducing agent. Oxidation of reduced glutathione affects proper functioning of the cell, GSH levels should be optimal. It contributes largely to the reducing potential of the cell. GSH, reduced through hexose monophosphate shunt (HMPS) activity, plays an important role in the protection of susceptible sulphydryl groups including those of globins from oxidation (Allen and Jandl, 1961), and subsequent haemolysis (Fegler, 1952). Hexokinase, glutathione reductase, glucose-6 - phosphate dehydrogenase (G6PDH) are inactivated under conditions of oxidation of GSH. The inactivation of these enzymes leads further oxidation of GSH. The same chain of events would occur when one or a number of enzymatic, self-stabilizing chains of GSH metabolism is missing. Since the chain of -SH enzymes comprise of hexokinase as well as key enzymes of oxidative breakdown of glucose and glutathione reduction, the oxidation of glutathione might also lead to a decline in the metabolism of glucose and diminution of the formation of ATP with the consequent breakdown of cell structure and haemolysis.

Mature erythrocyte functions primarily in the transportation of Hb. This task requires firstly the functional integrity of Hb molecules. Maintenance of Hb function depends largely upon intracellular reducing mechanisms. In erythrocyte the preferential substrate for oxidation by H_2O_2 is haemoglobin (Hb) which is oxidised to methaemoglobin (MHb). In blood, elevated concentrations of MHb are indicative of oxidative stress. MHb is then reduced again to Hb via GSH system.

GSH is involved in a number of physiological and detoxification process (Kosower and Kosower, 1978; Moron et al., 1979; Hazelton and Lang, 1980; Reed and Beatty, 1980). GSH is one of the most potent biological molecules that affect scavenging functions of the body. Active participation by GSH in the deactivation of intermediates formed during metabolism of xenobiotics is also reported by Oduah et al. (1985). In addition, it conjugates with exogenous toxicants aiding in their excretion. GSH is a sulphydryl containing major non-protein thiol involved in the detoxification of metals. GSH interacts with metals in several ways. The cysteine and GSH content in mammals influence uptake, distribution and toxicity of mercury (Congui, et al., 1978; Alexander and Aaseth, 1982; Johnson, 1982). Methyl mercury glutathione is a major methyl mercury derivative excreted in rat bile (Hirata and Takahashi, 1981; Ballatori and Clarkson, 1982). GSH may also serve as cysteine reservoir for metallothionein synthesis (Higashi et al., 1977).

Because of the unusually high content of unsaturated fatty acids present in fish tissues (Love, 1970), protection against free radical damage is particularly important. In biological materials, toxic organic peroxides can be formed as a result of the oxidation of unsaturated lipids (Tappel, 1972). Lipid peroxides inactivate certain oxidative enzymes (Wills, 1971) and oxidise thiol groups in amino acids and proteins (Roubal and Tappel, 1966).

Fish tissues contain considerable amounts of GSH (Dalich and Larson, 1980), GST (Grover and Sims, 1964; James et al., 1979), GPX (Aksnes and Njaa, 1981) and glutathione reductase (Muramatsu et al., 1980). Hence it is presumed that GSH is indeed an essentially active agent in the cellular protection of fish. Dierickx and Vanderwielen (1986) postulated that GSH system can play an important role in the detoxification of aquatic pollutants.

Erythrocytes are extremely vulnerable to oxidative damage. The GSH accounts for 90% of the non-protein Sulphydryl compounds present in erythrocyte. It is well known that GSH plays an important role in protecting the -SH groups of haemoglobin, structural proteins of the erythrocyte membrane and several enzymes of the erythrocytes.

A review of the previous work shows that despite the growing recognition that GSH is intimately involved in the detoxification, conjugation and excretion

of xenobiotics, there are not many reports on the GSH status of blood in fishes exposed to pollutants. Hence it was decided to undertake a study in the GSH content of the blood of Oreochromis mossambicus exposed separately to copper and mercury.

MATERIAL AND METHODS

Collection and acclimatization of fish and experimental set up were similar as described in chapter 2. Fishes were exposed separately to copper (100 µg/l and 200 µg/l) and mercury (100 µg/l and 150 µg/l) for 168 h with suitable controls. Blood samples were collected by severing the caudal peduncle at 24, 72, 120, and 168 h.

Glutathione in the blood was estimated following the method adopted by Patterson and Lazarow (1954). Glutathione reacts with alloxan producing a substance with an absorption maximum at 305 nm.

0.5 ml of blood sample was diluted with 3.5 ml of distilled water. After completion of the haemolysis, 1 ml of 25% of metaphosphoric acid (MPA) was added to the haemolysate, shaken well and was then centrifuged for 10 minutes at 3000 rpm. 0.5 ml of the supernatant was transferred to two test tubes and the reagents were added in the order indicated.

Test tubes	Sample		Standard	
	A	AO	B	BO
Blood centrifugate	0.5	0.5	0	0
5% MPA	0	0	0.5	0.5
Distilled water	0	0.5	0	0.5
0.1 M alloxan	0.5	0	0.5	0
0.5 M phosphate buffer pH 7.5	0.5	0.5	0.5	0.5
Equivalent NaOH	0.5	0.5	0.5	0.5
Time of standing (minutes)	6	6	6	6
1 N NaOH	0.5	0.5	0.5	0.5
Total volume (ml)	2.5	2.5	2.5	2.5

Equivalent NaOh solution was prepared by titrating a mixture of 5% metaphosphoric acid and 0.1 M alloxan solution (in equal volume) with 0.5 N NaOH solution to an end point at pH 7.5 so that 10 ml of NaOH, solution would be equivalent to a mixture of 10 ml of 5% metaphosphoric acid plus 10 ml of 0.1 M alloxan solution.

The addition of 1 N NaOH stops the reaction and stabilizes the reaction product.

In determining the absorption of tube A, tube AO is used as blank. This corrects for the nonspecific absorption of blood centrifugate and the reagents used in the reaction. The correction for the absorption of the decomposition product of alloxan is determined by measuring the absorption of tube B, using tube BO as a blank. This value is substracted from the reading obtained with the reaction mixture.

A standard curve is prepared with glutathione and the amount of glutathione in the extract is determined by comparing the corrected optical density obtained for the extract with the standard curve.

RESULTS

The GSH content of the blood of controls did not vary significantly between days throughout the experiment.

In fishes exposed to 100 µg/l copper, there was no significant difference in the GSH content of the blood at 24 and 72 h when compared to the controls. But at 120 h onwards the GSH in the blood increased significantly ($P < 0.01$) from that of controls.

But in fishes exposed to higher concentrations of copper (200 µg/l), there was a significant decrease initially at 24 h ($P < 0.01$) followed by a recovery to control levels at 72 h and at 120 and 168 h the GSH content increased significantly ($P < 0.01$) when compared to controls.

Both the concentration of mercury showed a similar trend. Eventhough there was no significant change in the blood GSH content at 24 h in the fishes exposed to both concentration initially, significant increase in GSH

content were observed at 72 ($P < 0.05$), 120 and 168 h ($P < 0.01$).

From 120 h onwards all metal-treated fishes showed significant increase in the blood GSH content when compared to controls.

The results are presented in table 18 and fig. 18.

DISCUSSION

The net GSH concentration is dependent upon its metabolism, transport, participation in physiological functions and excretion (Griffith and Meister, 1979). The important functions of the GSH in the body includes maintaining reduction/oxidation (Redox) potential of the cell together with NADH and NADPH and forming the reducing system of the cell; scavenging the toxic free radicals generated by the xenobiotics acting as a substrate for the important enzymes involved in the detoxification of xenobiotics and conjugation of xenobiotics and making them less toxic so that they can be trnasported and excreted.

In the present study fishes exposed to copper showed a significant increase in GSH from 120 h onwards whereas fishes exposed to mercury showed a significant increase in GSH content from 72 h onwards indicating a difference in the animal's reaction towards copper and mercury.

Increase in GSH in response to exposure to various toxicants are reported earlier. These include the increase in blood GSH of Salmo gairdneri during exposure to chlorine toxicity (Zeitoun, 1977); in Mugil cephalus exposed to cadmium (Thomas et al., 1982 b; Wofford and Thomas, 1984); in S. gairdneri on exposure to chlorothalonil (Davies, 1985 a; Davies and White, 1985); in Atlantic croaker in response to lead (Thomas and Jeudes, 1985); in plaice after exposure to cadmium (George et al., 1985) and in S. gairdneri in response to copper (Lauren and McDonald, 1987). Thomas and Wofford (1984) found that acid soluble thiol content of the liver increased in M. cephalus exposed to a variety of chemicals including cadmium and mercury. GSH forms a major part of the acid soluble thiol of the body (Thomas and Wofford, 1984).

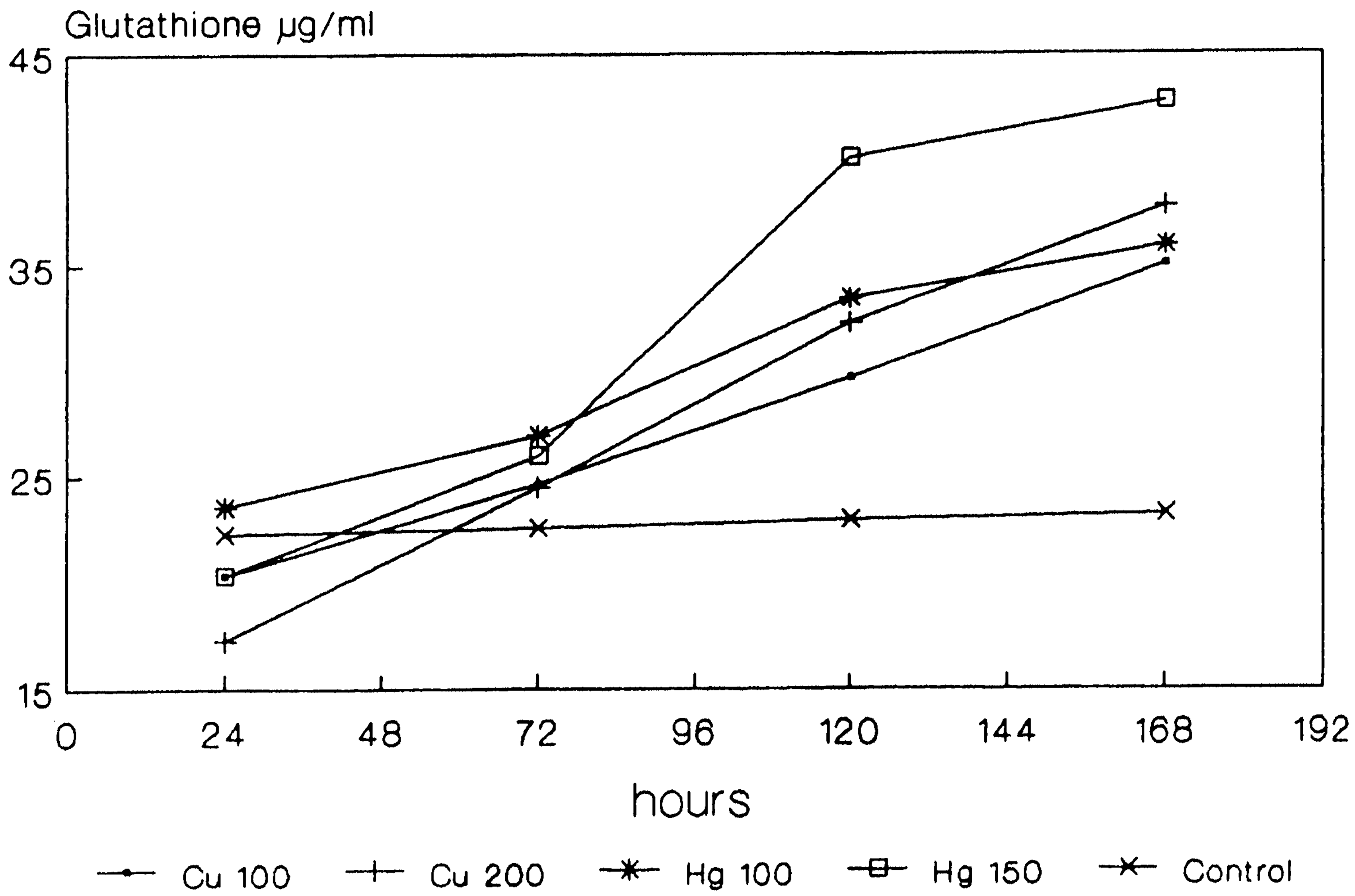
Such increases in GSH values were also reported in other animals exposed to various chemicals (Congui et al., 1978; Sasame and Boyd, 1978; Hsu, 1981;

Table 18 Blood glutathione content in *Oreochromis mossambicus* exposed to copper and mercury

Concentration µg/l		GSH µg/ml blood $\bar{x} \pm$ S.D (N = 10) Exposure period			
		24 h	72 h	120 h	168 h
Cu	100	20.36 ± 2.97	24.66 ± 2.68	29.63** ± 2.88	35.05** ± 2.81
	200	17.32** ± 1.91	24.49 ± 2.83	32.27** ± 2.80	37.82** ± 2.69
Hg	100	23.59 ± 2.74	26.90* ± 3.72	33.44** ± 2.72	35.92** ± 2.92
	150	20.35 ± 3.08	25.96* ± 3.01	40.11** ± 2.94	42.78** ± 3.01
Control		22.31 ± 3.07	22.60 ± 2.61	22.97 ± 2.85	23.25 ± 2.78

* $P < 0.05$ ** $P < 0.01$

FIGURE 18. BLOOD GLUTATHIONE CONTENT IN *O. MOSSAMBICUS* EXPOSED TO COPPER AND MERCURY



Allen et al., 1984 a, b, 1985; Chattopadhyay et al., 1987).

However, decrease in GSH concentration in different tissues of fishes exposed to different chemicals and stress factors are also reported. Huillet et al. (1970) found that the tissues GSH levels of the scorpion fish decreased under conditions of stress. Gingerich et al. (1978) showed a depletion of GSH in rainbow trout treated with carbon tetrachloride, whereas Dalich and Larson (1980, 1985) found a lower GSH content in rainbow trout dosed with monochlorobenzene. Buckley (1981, 1982) detected a decrease of GSH in the erythrocytes of S. gairdneri treated with monochloramine.

Diverse array of compounds can undergo redox cycling in cells. The activated oxygen species such as superoxide anion (O_2^-), hydrogen peroxide and hydroxyl radicals (OH^-) associated with redox cycling activity are often important in the toxicity of these compounds (Hassan and Fridovich, 1979; Bus, 1982; Mason and Chignell, 1982). Copper can enter reduction/oxidation cycle (Rothstein, 1959) and catalyse the formation of cytotoxic free radicals from membrane phospholipids (Hochstein et al., 1980; Florence and Stauber, 1986).

Both GSH and NADPH play an important role in maintaining optimal redox state (Chance et al., 1979). It has been suggested that the alteration of cellular redox state constitutes one of the manifestations of oxidative stress (Nishiki et al., 1976). Allen et al. (1984 b) postulated that GSH concentration may play a role in stabilizing redox state of the cells and reduced glutathione (GSH) changes into oxidised glutathione (GSSG). Glutathione reductase (GR) which converts the oxidised form of glutathione (GSSG) to its reduced form has been isolated from the fish (Muramatsu et al., 1980). Hence an inhibition of GR can cause a change in the redox potential as the oxidised glutathione (GSSG) cannot be reduced to GSH. Alternately an increase in the GR activity can increase the GSH concentration by the reduction of the GSSG. The importance of reduced glutathione and the role of GR in maintaining adequate GSH levels were reported by Sandy et al. (1986).

Hexose monophosphate shunt activity is also critical for maintaining glutathione in the reduced state (Allen and Jandl, 1961; Eaton et al., 1973).

Marked stimulation in the activity of glucose-6-phosphate dehydrogenase (G6PDH) increases the HMPS pathway (Chattopadhay et al., 1987). This may have a role in ultimately increasing the activity of glutathione reductase (GR) which depends on the supply of NADPH (Muramatsu et al., 1980).

Hyperglycemia usually associated with exposure to toxicants may be aiding in the restoration of GSH. The following observations support this contention. Meister (1982) reported that GSH levels are reduced during starvation, and starvation results in hypoglycemia. GSH is also known to increase in diabetic animals where hyperglycemia is found (Price and Jollow, 1982). Again Davies (1985 b) observed that feeding helped to restore GSH levels in S. gairdneri exposed to chlorothalonil. He also observed significant decrease in liver thiol levels of starved fish exposed to chlorothalonil. He postulated that lack of nutritional input in the GSH caused a failure to restore GSH after depletion. In vitro studies also support the role of glucose in the restoration of GSH levels (Buckley, 1981).

Reduced glutathione protects the body from many xenobiotics. Smith and Nunn (1986) had shown that GSH protected erythrocytes from haemolysis and oxyhaemoglobin from oxidation to methaemoglobin from the action of t-butylhydro peroxide. Buckley (1981) found that monochloramine treated erythrocytes underwent rapid dose - dependent oxidation of haemoglobin to methaemoglobin and depletion of GSH. Depletion of GSH might have caused oxidation. Tillman et al. (1973) attributed the fungitoxin action of chlorothalonil to its ability to deplete cellular GSH reserves and thus loses the protection of GSH against toxicant.

The presence of a detoxification enzyme system in fish has been well established (Khan et al., 1979). This includes GSH and GSH - related enzyme system. GSH is a substrate for GPX and GST. GPX performs a vital function in protecting the cell from oxidative processes particularly by inhibiting the lipid peroxidation of biological membranes (Flohe et al., 1976) by xenobiotics and their metabolites (Wendel et al., 1978). GPX is also reported to decrease the toxic effects of methylmercury by securing the intergrity of the cell components by antioxidation (Chang and Suber, 1982).

GST utilises GSH for the detoxification of xenobiotics and its metabolites. GST plays a key role in the detoxification of a large number of chemicals (Jakoby, 1978; Chasseaud, 1979). Davies (1985 b) found that GST mediated GSH conjugation reaction plays a protective role by decreasing toxicants available for protein binding. Metals such as mercury, silver, lead, cadmium etc., have high reactivity towards sulphydryl groups of functionally important proteins, peptides, coenzymes and amino acids (De Bruin, 1976 b). GSH is a sulphydryl containing non-protein thiol which can combine with metals and this decreases the toxic nature of the metals. GSH can reverse the inhibitory effects of mercury on lysosomal enzyme (Verity and Reith, 1967). GSH appear to offer protection against mercury toxicity possibly by complexing with it and thereby transforming mercury ions into some nontoxic form or by providing a system with additional thiol groups. GSH is reported to offer protection against heavy metals which are known to destroy thiol groups (Knox, 1960). Chandy and Patel (1985) found that GSH offers effective protection against mercury and blocked the entry of mercury.

Conjugation of xenobiotics with GSH is a biotransformation process that generally results in the formation of less toxic products. One way of detoxifying foreign compounds is to convert them to N - acetyl cysteine conjugates (mercapturic acids) which are then excreted in the faeces or urine (Boyland and Chasseaud, 1969; Kaplowitz et al., 1975; Baars et al., 1978; Meister, 1981 b). The first step in the mercapturic acid formation is the conjugation with GSH and is catalysed by GSI (Jakoby, 1978). The r-glutamyl group is subsequently removed by r-glutamyl transpeptidase and the glycyl group by a peptidase. Finally the cysteine conjugate is acetylated by N-acetyl transferase (Boyland and Chasseaud, 1969). The initial reaction is thought to occur predominantly in the liver and the remaining ones in the kidney (Hughey et al., 1978; Meister, 1981 b).

In mammals, bile is recognised as the primary excretory site of GSH (La Du et al., 1972). The existence of in vivo production of mono and di glutathione conjugates of chlorothalonil as the prime metabolites in chlorothalonil dosed S. gairdneri is confirmed by Davies (1985 a). Parker et al. (1980, 1981) described the occurrence of glutathione conjugate of acetaminophen in isolated trout hepatocytes and their breakdown by kidney homogenate. Bauermeister

et al. (1983) described the GST activity, GSH and r - glutamyl-transpeptidase activity in rainbow trout and postulated that a mercapturate metabolic pathway exists in the species. A rapid degradation of GSH conjugation resulting in a complex mixture of compounds in bile has been reported by Hirata and Takahashi (1981). They suggested that biliary enzymes contributed to the metabolic breakdown process. Conjugation results in the formation of highly polar glutathione and mercapturate metabolites is regarded as less toxic and easily excretable. Here GSH is acting in a protective detoxification role. Methylmercury glutathione is a major methylmercury derivative excreted in rat bile and its rate of excretion is related to the ability of the liver to excrete GSH (Hirata and Takahashi, 1981; Ballatori and Clarkson, 1982). Mercapturic acids which are formed by GSH conjugation have been detected in fish (Roubal et al., 1977; Lay and Menn, 1979).

GSH is the only nonenzymatic antioxidant whose levels are dynamically monitored by cells under the condition of oxidative stress. Augmentation of the level of GSH following inhibition of the enzymatic antioxidant defences suggest the existence of a dynamic equilibrium between the enzymatic and non-enzymatic defences. Under normal conditions GSH regulates its own synthesis from cysteine by the enzyme r-glutamyl cysteine synthetase (Richman and Meister, 1975; Meister, 1981 a). Thomas et al. (1982 b) found that cadmium could prevent GSH from exerting a feed back on synthetase activity by forming GSH conjugate so that GSH concentration would increase. The activity of r-glutamyl cysteine synthetase, the enzyme that regulates GSH content is dependent on the GSH concentration (Meister, 1981 a). The binding of metals to GSH reduces the amount of free active GSH, which could excrete a feed back on the synthetase activity, thus enhancing the GSH content.

Studies by Hsu (1981) on the effect of lead on GSH metabolism in rats suggested that heavy metals can increase GSH concentration by stimulating r-glutamyl cysteine synthetase activity. Hence an increase in GSH could be due to the stimulation of r-glutamyl cysteine synthetase activity (Hsu, 1981) or increased production of this enzyme (Sasame and Boyd, 1978).

In the present study the observed significant decrease in GSH concentration at 24 h in fishes exposed to 200 µg/l copper may be due to the utilization of GSH in the conjugation of copper. However, the body compensates for

the loss of GSH by increaseing the GSH synthesis and GSH levels returns to control levels at 72 h. This increased GSH synthesis continues to meet the influx of metal ions, so that the metal ions are complexed and detoxified. The elevated GSH content detected in metal dosed O. mossambicus towards the end of experiment clearly demonstrates the increased synthesis of GSH on metal exposure. In mercury-dosed fishes there was no decrease at any time during the exposure. In such fishes the depletion of GSH might have occurred very early and by 24 h the body might have restored the GSH status to the control levels so that a decrease was not observed. There are also enzymatic antidefence mechanisms which might have been activated very early in mercury-treated fishes so that GSH stores were not affected initially. Indeed, such a phenomenon was observed in mercury-treated fishes (see chpater 7). When catalase activity increased at 24 h in fishes exposed to mercury, copper-treated fishes did not show any elevation in catalase activity at 24 h.

In the fishes exposed to lower concentration of copper also there was no significant change of GSH at 24 h. This might be due to the very low concentration of copper which might not evoke any harmful effects by 24 h.

The GSH status of the cell is thus closely related to the accumulation and excretion of heavy metals as reported by Cherian and Vostal (1977); and Congui et al. (1978). When the fish is exposed to heavy metals, the body utilises the GSH stored in the body for the detoxification, conjugation and excretion of various heavy metals. These processes naturally deplete the reduced glutathione of the body; a continued depletion of GSH is harmful to the body and can cause death. However, the body prevents a sharp decline in the GSH content by increasing the production of GSH. The increased GSH concentration observed towards the end of the present study shows that the body of the fish has successfully adapted to the metal exposure by increasing the GSH synthesis, so as to combat the toxic effects of the metal.

CHAPTER 7

EFFECT OF COPPER AND MERCURY ON THE BLOOD CATALASE ACTIVITY

Catalase (hydrogen peroxide : hydrogen peroxide oxidoreductase, EC 1.11.1.6) is a special type of peroxidase enzyme that uses hydrogen peroxide (H_2O_2) as a substrate and acceptor at the same time.

Although most of the oxygen consumed by respiring cells is utilized by cytochrome oxidase which reduces oxygen to water without releasing either superoxide (O_2^-) radical or hydrogen peroxide, superoxide is produced by the monovalent pathway of biological reduction of oxygen in respiring cells in different occasions. In the body, complete reduction of a molecule of oxygen to water requires four electrons and is a sequential univalent process in which several intermediates like superoxide anion radical, hydrogen peroxide and hydroxyl (OH^-) radicals are encountered.

Superoxide anions are generated by a variety of metabolic pathways and physical agents (Misra and Fridovich, 1971; Autor, 1974; Drath and Karnofsky, 1975; Bus and Gibson, 1979; Haugaard, 1965; Menzel, 1970). The detoxification and removal of the toxicants from the body involves two phases by a series of cellular enzymes. Phase I reaction involves insertion of a polar reactive group into the toxicant molecule through oxidation, reduction and hydrolysis and usually these reactions prepare the toxicant for subsequent phase II reaction. Although the major objective of the phase I and phase II reactions is to convert the toxicants into compounds with lower lipophilicity to be conjugated subsequently with moities like glutathione, amino acids, glucuronic acid etc., leading towards detoxification, in some cases reaction products after phase I reaction may make the compounds more toxic. In addition to the formation of reactive metabolites, phase I reaction may also catalyse electron transfer reactions between endogenous electron sources, toxicants and molecular oxygen. The product of this type of reaction is O_2^- which may form extremely toxic oxygen species such as OH^-. The autoxidation of hydroquinones, leukoflavins, catecholamines, thiols, tetrahydropterins and reduced ferredoxins have all been shown to generate superoxide radical (Fridovich, 1978). Haemoglobins and myoglobins slowly liberate superoxide radicals as they are converted to methaemoglobin and metmyoglobin.

Fluxes of O_2^- generated induce lipid peroxidation, damage membranes and kill cells. There are indications that O_2^- is not by itself the species that causes these effects but is a precursor of a more potent oxidant whose generation depends on the simultaneous presence of H_2O_2. Moreover O_2^- and H_2O_2 together cooperate and produce an oxidant more potent than O_2^- and H_2O_2 which then attacks DNA, membrane lipids and other cell components. The mechanism of paraquat poisoning in animals is generally thought to be an oxygen free radical-mediated toxicity (Halliwell and Gutteridge, 1985). Many environmental toxicants function through free radicals or related oxidant mediated reactions (Bus and Gibson, 1979; Mason, 1982; Mason et al., 1982) and activated oxygen species (O_2^-, H_2O_2, OH^-) of these reactions are associated with toxic activity of these compounds (Hassan and Fridovich, 1979; Bus, 1982; Mason and Chignell, 1982). There are reports that free radicals can oxidise -SH groups (Barron, 1955), initiates lipid peroxidation (Roubal and Tappel, 1966) and can alter cell redox state (Halliwel, 1981 a).

Tissues of many fishes are very rich in fat (Eschmeyer and Philips, 1965; Malins et al., 1965; Mazeaud et al., 1979) and fish lipids are unusually rich in polyunsaturated fatty acids (Love, 1970). Superoxide and other forms of "active oxygen" induce violent lipid peroxidation which is a chain reaction involving polyunsaturated lipid and oxygen. In view of the unusually high content of unsaturated fatty acids present in the fish, protection against free radical damage is very important. The occurrence of free radical induced lipid peroxidation causes considerable changes in the structural organization and functions of cell membranes (Mead, 1976) and makes the membrane leaky. Peroxidation of membrane lipids has been associated with a number of pathological phenomena such as increased membrane rigidity, decreased cellular deformability, reduced erythrocyte survival and lipid fluidity. H_2O_2 is capable of introducing peroxide groups by reacting with unsaturated alkyl side chains (Selvam and Anuradha, 1988). It is also known that H_2O_2 and O_2^- are involved in the oxidative degradation of haemoglobin, oxidation of -SH groups and lipid peroxidation. Lipid peroxides inactivate certain oxidative and respiratory enzymes (Wills, 1971), and oxidise thiol groups, aminoacids, and proteins (Desai and Tappel, 1963; Roubal and Tappel, 1966).

Generation of free radicals does not always result in the toxic injury to the cell, since several defence mechanisms have been identified which detoxify these reactive oxygen products. Cells contain a variety of enzymatic antioxidant defences such as superoxide dismutase (SOD), catalase, and peroxidase as well as non-enzymatic free radical quenchers such as β -carotene, α –tocopherol , ascorbate, urate and glutathione. The primary defence is provided by enzymes that catalytically scavenge the intermediates of oxygen reductions. The superoxide radical is eliminated by superoxide dismutase (SOD) which catalyses its conversion to H_2O_2 (McCord and Fridovich, 1968; Fridovich, 1974) and H_2O_2 thus formed is removed by catalase (Rapoport and Müller, 1974) which converts it into water and oxygen. H_2O_2, though not a free radical, is a related oxidant. The efficient removal of the O_2^- and H_2O_2 will prevent the formation of OH^- and OH^- radical reacts avidly with many substances (Dorfman and Adams, 1973).

In the erythrocytes, catalase, which is a haemoprotein, is one of three enzymes responsible for dimnishing the toxic effects of H_2O_2 formed by the dismutation of O_2^- (McCord and Fridovich, 1969). Eventhough, catalase is mainly particle bound in tissues, it exists in a soluble state in erythrocytes. The catalase activity of the blood is practically due to the erythrocytes. Since SOD and catalyse are inducible enzymes (Fridovich, 1974) which react with activated oxygen species both in the cytosol and subcellular organelles (Frank and Massaro, 1980), it is likely that the activities of these enzymes may be affected by toxicant exposure. Paraquat was shown to increase the activities of antioxidant enzymes in the erythrocytes of fish (Matkovics et al., 1984; Gabryelak and Klekot, 1985).

The net effect of catalase is to greatly lower the steady state of the level of H_2O_2 while the SOD do the same for O_2^-. The likelihood that O_2^- and H_2O_2 will participate in deleterious reactions with other cell components is dimnished in proportions to the decrease in their concentration (Fridovich, 1978). These defensive enzymes are thus likely to exert a synergestic effect in protecting cells against consequences of the production of O_2^- and H_2O_2 (Fridovich, 1978). It has been observed that in some in vitro systems, SOD and catalase inhibit the rapid process of lipid peroxidation (Kellogg and Fridovich, 1975, 1977; Penderson and Aust, 1975; Gutteridge, 1977).

Relatively little work has been done on the enzymatic antioxidant defence mechanism in fishes in response to heavy metals. Hence an investigation on the activities of the catalase in Oreochromis mossambicus exposed to copper and mercury was carried out.

MATERIAL AND METHODS

Collection and acclimatization of fish and experimental set up were similar as described in chapter 2. Fishes were exposed separately to copper (100 µg/l and 200 µg/l) and mercury (100 µg/l and 150 µg/l) for 168 h with suitable controls. Capillary blood was collected by cutting the caudal peduncle of the fish at 24 h, 72 h, 120 h and 168 h. 0.025 ml of the blood was haemolysed with 20 ml of distilled water. The catalase activity in the haemolysate sample was analysed following the method of Beers and Sizer (1952). Catalase decomposes H_2O_2 into water and oxygen. The decomposition of hydrogen peroxide could be followed directly by the decrease in extinction at 240 nm, the difference in extinction per unit time is a measure of the catalase activity.

1 ml of 50 mM phosphate buffer (pH 7.0) and 2 ml of haemolysate was transferred to the reference cuvette. Into the test cuvette 2 ml of the sample (haemolysate) and 1 ml of 30 mM hydrogen peroxide (prepared by diluting 0.34 ml 30% H_2O_2 to 100 ml with phosphate buffer) solution was added. The test solution was mixed well using a plastic paddle. The decrease in extinction of the test solution at 240 nm was monitored using a spectrophotometer at 5 seconds interval. Care was taken that the values for E 240/15 sec. was in the range of 0.1 and 0.02.

To find out the catalase activity per ml of blood the rate constant of the first order reaction (k), as described by Aebi (1974) was used. The results were analysed statistically.

RESULTS

The results are presented in Table 19 and Fig. 19. At 24 h the catalase activity of mercury-dosed fishes increased significantly from that of the controls. But there was no change in the catalase activity in the copper-treated fishes.

The catalase activity of the fishes exposed to higher concentration of copper (200 µg/l) and mercury-treated fishes showed a significant increase at 72 h. All metal-treated fishes showed a significant increase in the catalase activity at 120 h. But at 168 h, the catalase activity reverted to control levels, except in fish exposed to higher concentration of copper.

The two metals, copper and mercury, elicit different reactions in the blood catalase activity initially. While mercury causes an elevation in the activity from 24 h onwards, the copper-treated fishes showed a belated response by increasing the activity from 72 h in the copper-treated fish (200 µg/l). In fish exposed to lower concentration of copper, the significant increase in activity occured at 120 h only. But this increase in catalase activity appears to be a temporary phenomenon as the activity tends to revert to control levels by the end of the experiment at 168 h. However is the fishes exposed to the higher concentration of copper the increase persisted even at 168 h.

DISCUSSION

From the results, it is evident that metals do have an impact on the catalase activity. The activity of the catalase increased after exposure to these metals. In mercury-dosed fishes the increase in catalase activity was observed from 24 h onwards whereas in copper-treated fishes the increase in catalase activity was observed later.

Apart from the present study, increase in the catalase activity in response to different toxicants are reported in other fishes. Thomas and Murthy (1978) reported an increase in hepatic catalase in response to Endrin toxicity and an increase in renal catalase activity in response to sevin in Heteropneustes fossilis. Matkovics et al. (1984) and Gabryelak and Klekot (1985) also reported an increase in the catalase activity in carp exposed to paraquat.

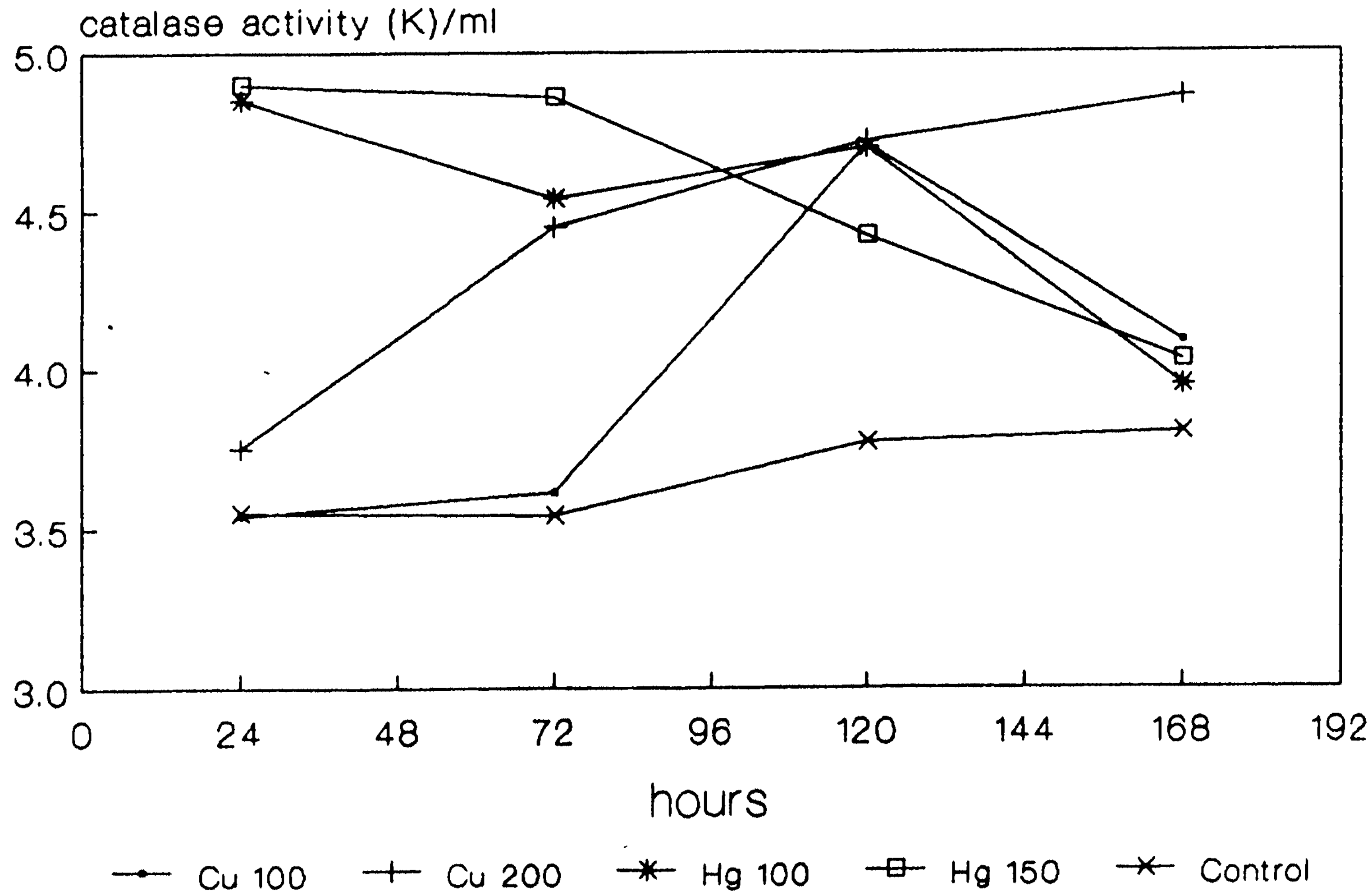
However, there are also reports that catalase activity actually decreases in the liver and kidney of Clarias batrachus exposed to phosphemidon (Thomas and Murthy, 1974); in the renal tissues of H. fossilis exposed to endrin and the hepatic tissue of H. fossilis exposed to sevin (Thomas and Murthy, 1978); in the Fundulus heteroclitus exposed to cadmium (Pruell and Engelhardt, 1980); in the liver of Sarotherodon mossambicus exposed in vitro to many metals

Table 19. Catalase activity in *O. mossambicus* exposed to copper and mercury

Metal concentration µg/l		Catalase activity (K)/ml $\bar{x} \pm$ S.D (N = 10) Exposure time			
		24 h	72 h	120 h	168 h
Cu	100	3.54 ± 0.61	3.61 ± 0.68	4.71** ± 0.50	4.09 ± 0.67
	200	3.75 ± 0.97	4.45** ± 0.49	4.72** ± 0.52	4.86** ± 0.63
Hg	100	4.85** ± 0.62	4.54** ± 0.39	4.70** ± 0.58	3.95 ± 0.68
	150	4.90** ± 0.47	4.86** ± 0.44	4.42** ± 0.39	4.03 ± 0.61
Control		3.55 ± 0.53	3.54 ± 0.48	3.77 ± 0.38	3.80 ± 0.53

* $P < 0.05$ ** $P < 0.01$

FIGURE 19. CATALASE ACTIVITY IN *O. MOSSAMBICUS* EXPOSED TO COPPER AND MERCURY



(Singh and Sivalingam, 1982); and in different tissues of C. batrachus exposed to mercury (Sahana and Jana, 1985).

It is interesting to note that H_2O_2 content in the body increases in response to toxicants. Increase in H_2O_2 content in C. batrachus in response to mercury has been reported by Sahana and Jana (1985). This increase in H_2O_2 may be because of the decrease in catalase activity mentioned earlier.

Reports stating that H_2O_2 cause haemolysis accompanied by lipid peroxidation (Krishnamurthy et al., 1984) and that H_2O_2 and O_2^- together cooperate and produce an oxidant more potent than themselves which then attacks DNA and other cell components, and are involved in the pathogenesis of inflammation and formation of lipid peroxidation, gives a clear picture of the need of its removal from the body.

From kinetic consideration hydrogen peroxide would be expected to be preferentially metabolized by GPX because of its lower Km for hydrogen peroxide than catalase as shown by Cohen and Hochstein (1963) in mammalian erythrocyte. However, catalase has a greater affinity for hydrogen peroxide at higher concentration of the oxidant (Chance et al., 1979). Thus cells may avoid GSH depletion and GSSG accumulation by depending more on catalase during increased rates of cellular hydrogen production.

Increased activity of catalase under the toxic situation can effectively counteract the lipoperoxidation process. Any hydrogen peroxide or organic hydroperoxides accumulating in the system is effectively destroyed by these scavenging enzymes, thus depressing lipid peroxidation and protecting the cell from injury (Matters and Scandalios, 1986).

It is reported that increased catalase activity protects the cells. Agar Nihal et al. (1986) observed that although nucleated cells (L 1210 murine leukemia) are readily killed by an enzymically generated flux of superoxide (and therefore hydrogen peroxide), the addition of human and murine erythrocytes blocked lethal damage to the target cells. Inhibition of erythrocyte catalase abrogated the protective effect and the addition of purified catalase restored it. Furthermore erythrocytes derived from a hypocatalase mice (in which other antioxidant systems are intact) do not protect L 1210 cells.

There are also reports showing a protective action of catalase by preventing the lipid peroxidation and accumulation of H_2O_2 in the body (Allen et al., 1984 a, b; Kirshnamurthy et al., 1984; Atalla et al., 1985; Garbyelak and Klekot, 1985; Albro Philip et al., 1986; Rossi et al., 1986; Toth et al., 1986 a, b; Yoshikawa et al., 1986). When the catalase activity decreases in the body, lipid peroxidation increases (Addya et al., 1986; Bhuyan et al., 1986; Nadkarni and D'Souza, 1986).

The increase in catalase activity observed in the present study could thus be due to the increased production of hydrogen peroxide as a result of metal exposure. Since catalase can convert hydrogen peroxide into oxygen and water, the body faces the new situation by increasing the synthesis of catalase, thereby protecting the cells from the action of hydrogen peroxide.

In the present study there is a distinct difference in the activity of catalase in the copper-dosed and mercury-dosed fishes in the initial stage of the experiment (at 24 h). Unlike copper-treated fishes, the mercury-treated fishes showed a significant increase in the activity of catalase at 24 h. For the lack of increase in the catalase in the copper-dosed fishes in the initial period, there are three possibilities. It could be due to the haemodilution observed in these fishes (see chapter 5) exposed to copper at 24 h. An influx of water into the blood can dilute the blood and can mask the increase in the blood catalase values as catalase activity is represented per ml of blood. However, such a haemodilution was not observed in the mercury-treated fishes (see chapter 5). Hence the increase in the catalase activity was not masked by haemodilution. It was found that the haemodilution observed in the initial period in copper-dosed fishes did not continue after 24 h (see chapter 5). This could have increased the blood catalase values in the copper-dosed fishes later.

Another possibility is that in the copper-treated fishes, the increased production of catalase was delayed as the body did not recognise copper, which is a micro-nutrient, as a xenobiotic, till the concentration in the body becomes higher. Hence the belated response. It is also possible that the copper-treated fishes may be using other nonenzymatic substances like GSH, initially to scavange the oxygen-free radicals, till catalase takes over the function. Actually there

was a sharp decline in the GSH content in the fish exposed to 200 µg/l copper at 24 h (see chapter 6).

However 72 h onwards, in all fishes exposed to copper and mercury (except in fishes exposed to 100 µg/l copper which showed a definite increase in catalase activity only at 120 h) there was a significant increase in the catalase activity. This clearly demonstrates that these two metals, copper and mercury, initiates the production of catalase to scavenge the increased production of hydrogen peroxide.

By 168 h the catalase activity of the experimentals returns to control levels (except in 100 µg/l copper-treated fishes). The return of catalase activity to the control levels indicate that catalase which is an enzymatic antioxidant substance, is used by the body only during the initial period. It is only a first line defence arising out of a sudden exposure to xenobiotics. The initial increase in catalase activity provides the body with sufficient time to synthesise other nonenzymatic antioxidant substances like GSH (see chapter 6). Addya et al. (1986) found that in rats exposed to $HgCl_2$, prolonged exposure to vitamin E, which is also a powerful scavenger, lowered catalase and SOD significantly. Thus the system may be adapting by lowering the levels of scavenging enzymes in the presence of alternate scavengers. In the present study also GSH increases in fishes exposed to these two metals in the latter part of the experiment. (see chapter 6). Similar increases in GSH after exposure to pollutants for long hours were reported by many workers (see chapter 6). Allen et al. (1983) observed that an inhibition of catalase in the adult housefly induced a compensatory increase in the concentration of reduced glutathione. Hence increased catalase activity could be a stop-gap arrangement in the antioxidant defence mechanism till other antioxidants are produced.

CHAPTER 8

EFFECT OF COPPER AND MERCURY ON THE ASCORBIC ACID CONTENT IN THE LIVER, KIDNEY AND BLOOD

Although many vertebrates synthesise ascorbic acid (ASA), most of the fishes lack the ability to synthesise it. Studies of fish nutrition suggest that dietary deficiency of ascorbic acid results in anorexia, lethargy, high mortality rate, skeletal deformities such as fracture and dislocation of spine, lordosis and scoliosis, distorted hyperplastic gill cartilage and mottled sclerotic cartilage in the eye as well as haemorrhage, bizarre distortion of support cartilage, impaired collagen formation, loss of scales, delayed wound healing, capillary fragility, cellular atrophy, damage to nerve cell etc. (Kitamura et al., 1965; Poston, 1966; Halver et al., 1969; 1975; Kitamura, 1969; Halver, 1972; Lovell, 1973; Wilson, 1973 b; Wilson and Poe, 1973; Ashley et al., 1975; Andrews and Murai, 1975; Sulkin and Sulkin, 1975; Hilton et al., 1978 a, b; Lim and lovell, 1978; Mahajan and Agrawal, 1980 a, b; and Durve and Lovell, 1982).

Ascorbic acid (Vitamin C) is a cofactor for the hydroxylase which hydroxylate proline and lysine in the collagen molecule (Barnes, 1975). The role of ASA in the synthesis of collagen has been reported by a number of workers in fishes (Halver et al., 1975; Lim and Lovell, 1978; Mauck et al., 1978).

Yoshinaka et al. (1978) observed that not only the hydroxylation of collagen proline but also the collagen protein was impaired in the skin of ASA deficient fish. ASA has two roles in the formation of collagen hydroxyproline. (1) It act as a cofactor for prolyl hydroxylase and (2) it is involved in the conversion of a precursor of prolyl hydroxylase to active enzyme. As a result of a lack of ASA, the formation of collagen hydroxyproline has been shown to be impaired in the skin of scorbutic channel catfish, Ictalurus punctatus. Sato et al. (1978) also indicated impaired hydroxylation of collagen protein in vivo in the skin and bone of ASA deficient rainbow trout.

The presence of this vitamin in the heart may be related to its direct and indirect role in preventing deposits of insoluble complexes of calcium/ phosphorus cholesterol in the vascular system by lowering the serum surface tension and removal of Na^+ and toxic cations (Lewin, 1974). In rainbow trout

ASA was noted to function in the iron metabolism suggesting either a redistribution of iron stores through impaired release of iron from reticuloendothelial cells or an increase in the efficiency of intestinal absorption (Hilton et al., 1978 a).

Ascorbic acid is also known to increase the immune response against pathogenic bacterial infection (Durve and Lovell, 1982; Li and Lovell, 1985) and fishes require higher than normal dietary level of ASA for certain physiological functions in response to bacterial infection. The protective function of ASA was pointed out by Agrawal et al. (1978) and Verma et al. (1981 d) against pesticides and by Fox and Fry (1970), and Suzuki and Yoshida (1978) against heavy metal poisoning.

Fishes have a system of storing a chemically stable form of ASA which acts differently from other vitamin C requiring organisms (Tucker and Halver, 1984). Halver et al. (1975) suggested that rainbow trout can store ascorbic acid in the form of ascorbate-2-sulphate.

Many ASA - dependent biochemical reactions are known to occur in liver viz., NADH dependent hepatic redox reaction, microsomal drug metabolising enzyme system, hepatic transfer of iron through the transferrin-ferritin mechanism, cholesterol and bile metabolism. This explains the high level of ASA in the liver tissue. In the kidney ASA is excreted in glomerular filtration, most of which is retained by tubular reabsorption (Wilson, 1974) which may account for the high concentration of ASA in this organ; ASA concentration in liver (Hilton et al., 1977) and in kidney (Halver et al., 1975) can be used as an index of ASA status in the body. ASA is not only an indicator of nutritional status but a decrease from the normal is a sign of physiological stress (Wedemeyer, 1969); pollution (Chatterjee and Pal, 1975; Mauck et al., 1978), infection (Lewin, 1974) or diseases (Wilson, 1974).

It is known that dietary requirement of ASA increases during adverse environmental conditions (Thomas and Neff, 1984; Mahajan and Agrawal, 1980 a, b). There are reports that different pollutants like pesticide, oil and heavy metals cause a decline in the ASA reserves of the body (Yamamoto et al., 1977, 1981; Mayer et al., 1978; Thomas et al., 1982 a).

Hence in this chapter, changes on the ASA reserves in the liver, kidney and blood of Oreochromis mossambicus exposed to two sublethal concentrations of mercury and copper are reported.

MATERIAL AND METHODS

Specimens of O. mossambicus were collected and acclimatized as described in chapter 2.

Specimens selected for the experiment were transferred to the experimental tanks. The fishes were exposed separately to two concentrations of copper (100 µg/l and 200 µg/l) and mercury (100 µg/l and 150 µg/l) for 168 h. Controls were run concurrently. Water in the tanks was changed daily. The fishes were not fed during the experiment. The temperature of the tanks was maintained at 28 ± 1°C. Samples were collected at 24, 72, 120 and 168 h.

The animals were caught and immobilized with a hard blow on the head. Blood was collected by severing the caudal peduncle. The body was cut open to remove the liver and kidney. The ASA content of the samples was estimated by following the procedure described by Roe (1954).

Liver and kidney samples were weighed accurately and homogenised with 6 ml of 4% TCA. After the extraction, 100 mg of acid washed norit was added to the extract and shaken well. The extract was filtered using a Whatman No. 12 filter paper.

To 2 ml of the filtrate, 0.5 ml of freshly prepared 2, 4-dinitrophenyl hydrazine-thiourea reagent (prepared by adding 2 gm of 2, 4-dinitrophenyl hydrazine and 4 gm of thiourea in 100 ml of 9 N sulphuric acid) was added. To the test tube marked blank, 2 ml of the filtrate was added. The samples and the blank were incubated in a waterbath at 37°C for 3 h. After the incubation the tubes were transferred to a beaker containing ice water containing sufficient quantities of ice. To each tube, 2.5 ml of 85% sulphuric acid was added drop by drop to avoid a sudden rise in the temperature. The tubes were shaken well. To the blank tube 0.5 ml of 2, 4 DNPH-thiourea reagent was added. After 30 minutes the tubes were brought to the room temperature. The colour developed was read at 540 nm.

The amount of ascorbic acid present in the representative obtained from the calibration curve prepared.

Estimation of ASA in blood

Aliquots of 0.5 ml of blood was added to 2 ml of 5% mixture was centrifuged for 10 minutes at 3000 rpm. 1.5 ml of th was transferred to another tube. To this 0.5 ml of dinitroph thiourea-copper sulphate was prepared by adding 2 g 2, 4-dinitroph 0.25 g of thiourea and 0.03 g copper sulphate in 100 ml of acid. The contents of the tubes were mixed well and incubate at 37°C. To each tube 2.5 ml of ice-cold 65% sulphuric acid drop by drop and mixed thoroughly. After 30 minutes, the o was read at 520 nm. For preparing the blank, 1.5 ml of 5% T to a tube followed by 2.5 ml ice cold 65% sulphuric acid a DNPH - thiourea-copper sulphate reagent. The amount of present in the blood sample was determined from the calibration cu

RESULTS

The ASA content of the liver, kidney and blood of the not show any significant changes though there was a gradual values with the progress of time; it may be noted that th not fed during the experimental period.

ASA content in the liver

The ASA content of the liver of fishes exposed to the two of mercury and higher concentration of copper (200 µg/l) showed decrease ($P < 0.01$) when compared to controls throughout the period. The ASA content of liver of fishes exposed to lower copper (100 µg/l) showed a significant decline from 72 h only. an apparent dose-dependent reduction in ASA values and the r fishes showed a greater decrease of ASA content.

ASA content in the kidney

The kidney of fishes exposed to mercury showed a significant decrease in the ASA content throughout the experimental period, when compared with controls. The ASA content of the kidney of fishes exposed to copper showed a significant decrease from 72 h only when compared with the respective controls.

ASA content of the blood

ASA content of blood of fishes exposed to mercury and copper showed a significant decrease from that of the controls during the entire experimental period. A greater decrease was noticed in the blood of fishes exposed to mercury.

The results are presented in Tables 20, 21 and 22 and Figs. 20, 21, and 22.

DISCUSSION

Malformation of bones in fishes exposed to different heavy metals have been reported earlier. Exposure to cadmium causes vertebral anomalies in carp (Muramoto et al., 1972; Muramoto, 1979), in fathead minnows (Pickering and Gast, 1972), in bluegill (Eaton, 1974), in guppies (Fujimagari, et al., 1974), in dace (Nakmura, 1975) and in *Cyprinus carpio* (Muramoto, 1981). Malformation of bones were also reported in minnows exposed to zinc (Bengtsson, 1974), in rainbow trout (Davies et al., 1975) and in brook trout (Holcombe et al., 1976) exposed to lead. Muramoto (1979) reported that occurrence of anomalous metabolism in fish with vertebral abnormalities was due to heavy metals. Many pesticides also caused abnormalities in the skeletal system of fishes (Halver et al., 1969; McCann and Jasper, 1972; Wilson and Poe, 1973; Mehrle and Mayer, 1975 a, b; Mayer et al., 1978).

Malformation of bones, skeletal deformities such as fracture and dislocation of spines etc., impaired collagen synthesis were reported in fishes due to ascorbic acid deficiency also. Doimi et al. (1983) concluded that the anomalous presence of heavy metals in the feed could produce symptoms similar to

Table 20. Ascorbic acid content in the liver of O. mossambicus exposed to copper and mercury

Concentration of metals µg/l		Ascorbic acid µg/g $\bar{x} \pm$ S.D. (N = 10) Exposure time 24 h	72 h	120 h	168 h
Cu	100	54.13* ± 5.15	40.02 ± 5.46	25.75 ± 4.43	23.52 ± 4.48
	200	36.02 ± 5.32	26.32 ± 4.75	23.50 ± 4.61	20.89 ± 4.66
Hg	100	28.09 ± 4.91	24.18 ± 4.57	19.40 ± 4.22	18.83 ± 4.36
	150	26.89 ± 5.32	23.58 ± 4.71	17.84 ± 4.45	11.21 ± 3.84
Control		56.34 ± 5.04	52.01 ± 5.80	46.98 ± 6.70	43.71 ± 5.68

Table 21. Ascorbic acid content in the kidney of O. mossambicus exposed to copper and mercury

Concentration of metals µg/l		Ascorbic acid µg/g x + S.D. (N = 10) Exposure time 24 h	72 h	120 h	168 h
Cu	100	37.54* ± 3.78	26.02 ± 3.89	18.87 ± 3.30	20.43 ± 3.28
	200	34.23* ± 3.80	23.60 ± 3.84	17.31 ± 3.22	19.68 ± 3.87
Hg	100	28.68 ± 3.75	24.40 ± 3.50	20.09 ± 3.45	17.36 ± 3.28
	150	29.39 ± 3.02	22.92 ± 3.80	18.11 ± 3.38	15.73 ± 3.32
Control		37.84 ± 3.51	36.85 ± 3.46	35.56 ± 3.66	34.55 ± 3.76

* non significant. All other values are significantly different from $(P < 0.01)$ that of controls.

Table 22. Ascorbic acid content in the blood of O. mossambicus exposed to copper and mercury

Concentration of metals µg/l		Ascorbic acid µg/ml x + S.D. (N = 10) Exposure time			
		24 h	72 h	120 h	168 h
Cu	100	14.60 + 1.61	12.49 + 1.45	9.58 + 1.38	7.69 + 1.05
	200	13.42 + 1.75	10.55 + 1.54	9.95 + 1.57	7.79 + 1.24
Hg	100	9.84 + 1.58	8.65 + 1.25	7.84 + 1.14	6.65 + 1.18
	150	10.06 + 1.56	8.45 + 1.31	7.53 + 1.11	6.12 + 0.94
Control		17.42 + 1.87	15.98 + 1.49	15.35 + 1.43	14.98 + 1.191

All values are significantly ($P < 0.01$) different from that of controls.

FIGURE 20. ASCORBIC ACID CONTENT IN THE LIVER . *O. MOSSAMBICUS* EXPOSED TO COPPER AND MERCURY

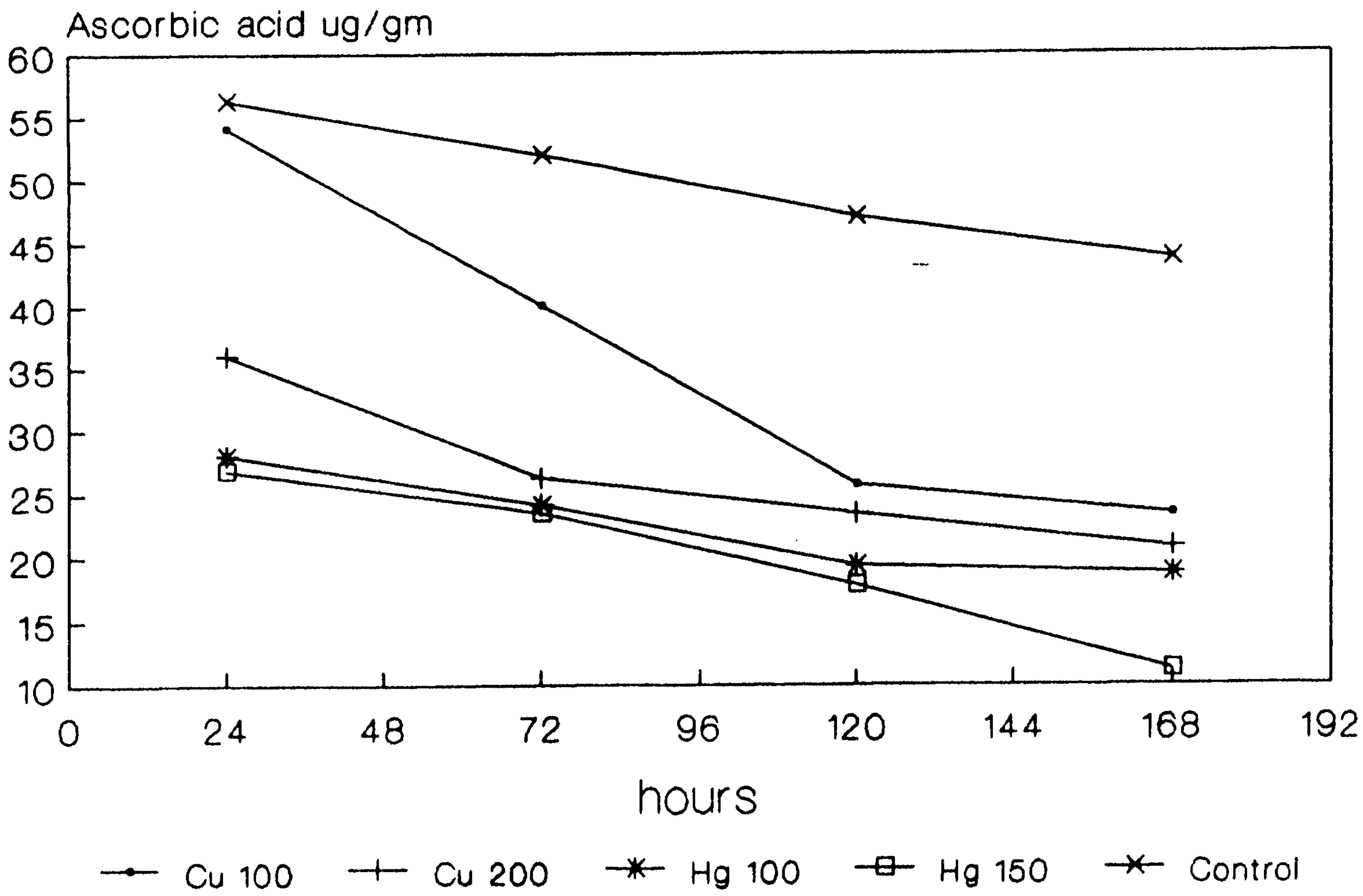


FIGURE 21. ASCORBIC ACID CONTENT IN THE KIDNEY OF *O. MOSSAMBICUS* EXPOSED TO COPPER AND MERCURY

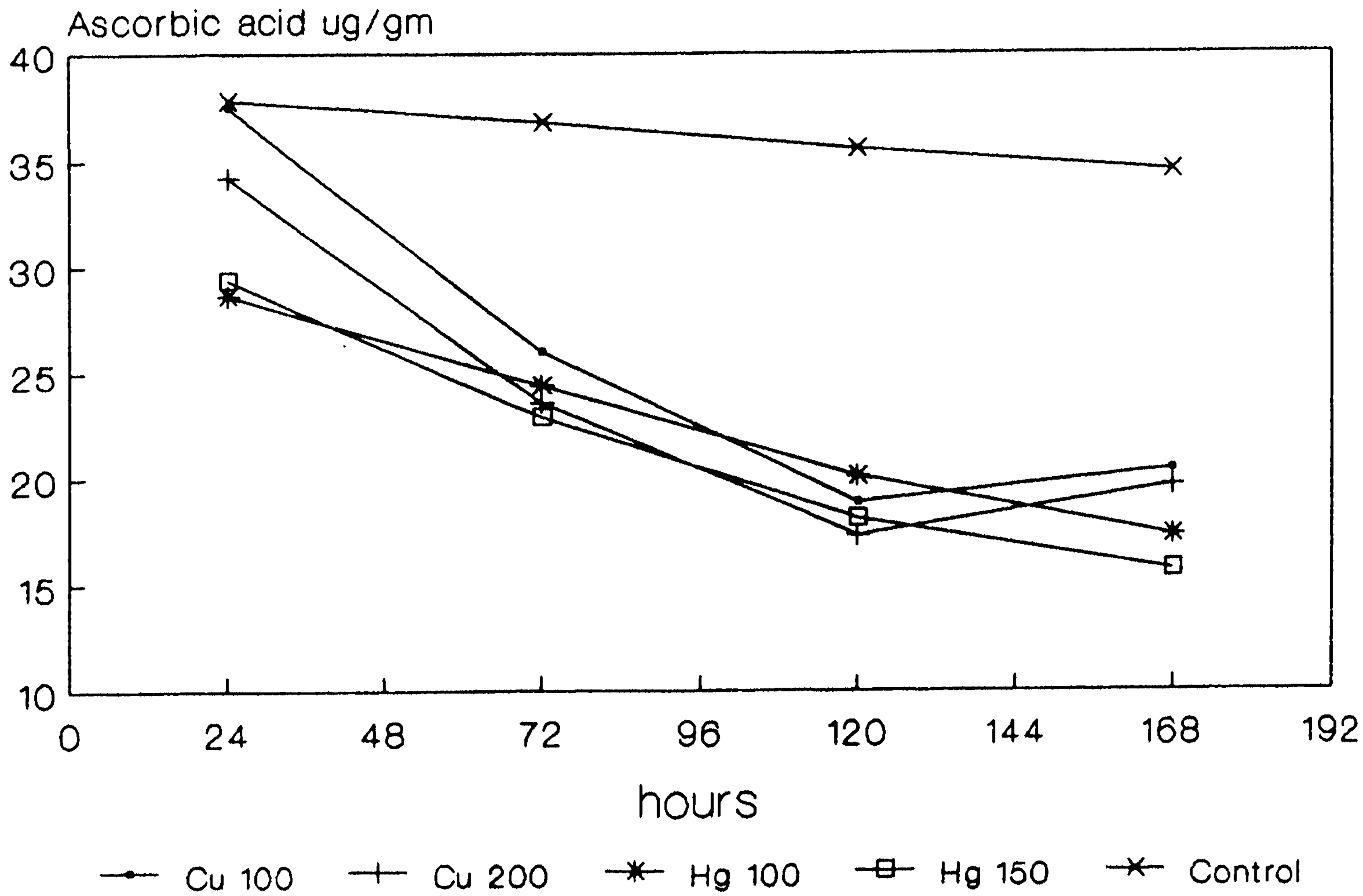
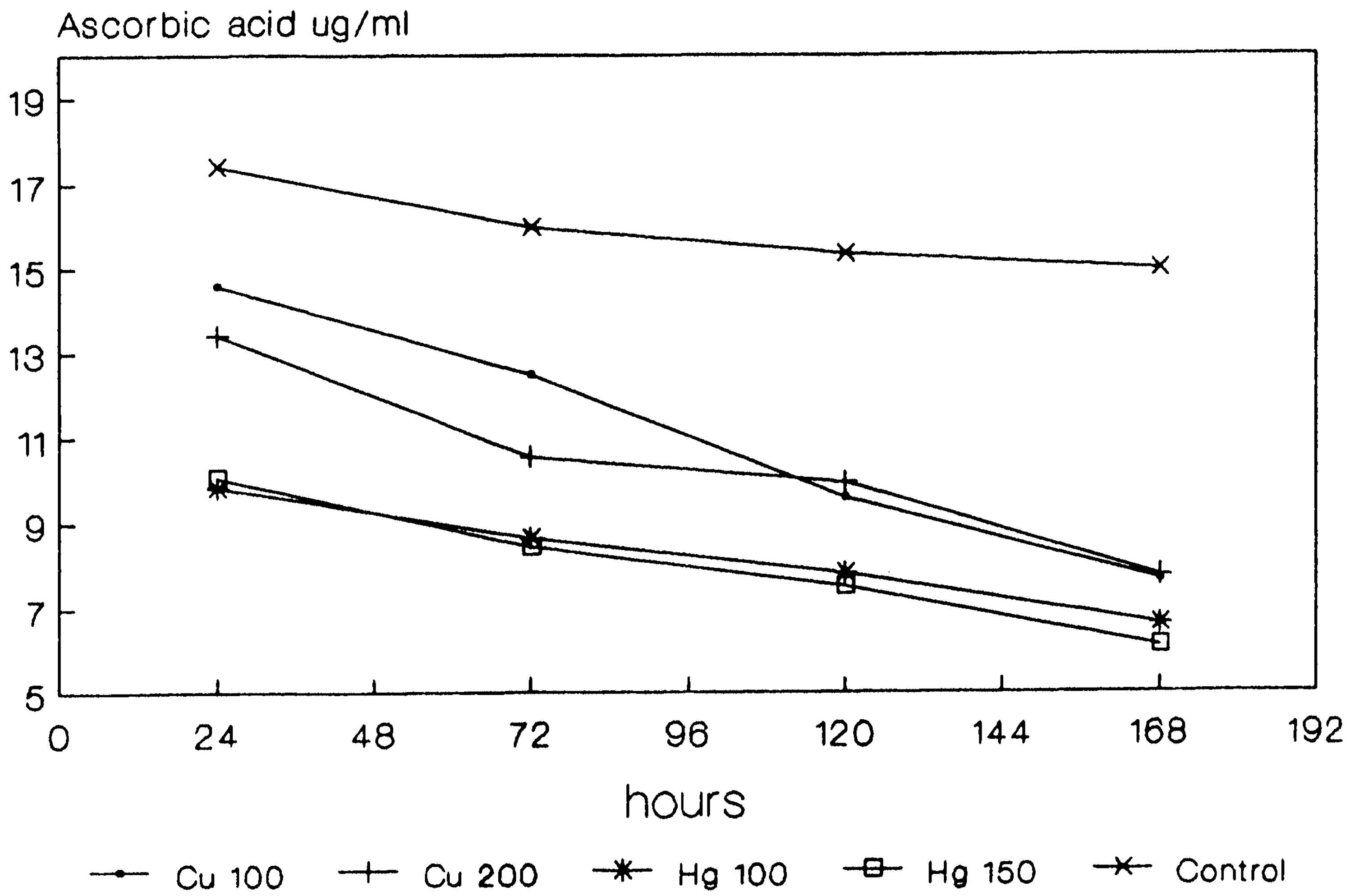


FIGURE 22. ASCORBIC ACID CONTENT IN THE BLOOD OF *O. MOSSAMBICUS* EXPOSED TO COPPER AND MERCURY



scurvy and hypovitaminosis indicating that heavy metals depletS vitamin C from the body. Stickney et al. (1984) reported that Tilapia aurea cannot synthesise vitamin C. In young fishes the gross clinical signs of scurvy are manifested as lordosis and scoliosis which are by underhydroxylated collagen in the vertebrae (Sato et al., 1982).

Vitamin C is essential for collagen synthesis. The role of ASA in the synthesis of collagen has been reported by a number of workers in fishes (Halver et al., 1975; Lim and Lovell, 1978; Mauck et al., 1978; Yamamoto et al., 1977, 1981; Mayer et al., 1978; Thomas et al., 1982 a).

A marked decline in the ASA reserves, in rainbow trout exposed to water-borne copper (Yamamoto et al., 1977), in Mugil cephalus dosed with cadmium (Thomas et al., 1982 a), in Clarias batrachus exposed to cadmium and lead (Katti and Sathyanesan, 1984) and in M. cephalus exposed to oil (Thomas and Neff, 1984; Thomas, 1987) were also reported. Many pesticides also cause a decrease in the ASA concentration in different fishes (Mehrle and Mayer, 1975 a, b; Mayer et al., 1978; Mauck et al., 1978).

With the studies of the effects of toxaphene on growth and backbone composition of fathead minnows (Pimephales promelas) Mehrle and Mayer (1975 a) postulated that toxaphene could alter several intermediary metabolic pathways associated with collagen and bone metabolism and suggested that toxaphene might have altered the vitamin C content which resulted in the impaired synthesis of collagen. This contention is supported by the findings that the deformities of the bones formed after toxaphene - exposure can be corrected by appropriate doses of vitamin C in the diet (Mayer et al., 1978). Addition of vitamin C in the diet significantly reduced whole body residues of toxaphene and increased tolerance of fish to chronic effects of the insecticide. Toxaphene also produced symptoms like decreased collagen, increased mineral collagen ration and bone density in the vertebral column in channel catfish and such symptoms were also produced in these fishes subjected to a diet deficient in vitamin C (Hamilton et al., 1981). Vitamin C and hydroxyproline concentration in sac fry of the brook trout were decreased after exposure to Aroclor (Mauck et al., 1978) which were similar to effects induced by toxaphene in brook trout (Mayer et al., 1975) and fathead minnows

and channel catfish (Mayer et al., 1977; Mehrle and Mayer, 1975 a). A massive dose of ASA considerably neutralised the toxic effects of Aldrin in Channa punctatus. A 10 fold decrease in mortality and significantly lowered haematological responses like polycythemia was observed in this fish after the administration of massive doses of vitamin C (Agrawal et al., 1978). A reduction in the toxicity of mercuric chloride and a significant degradation of methylmercury to inorganic mercury in goldfish was reported by Sharma et al. (1982). ASA plays a protective role in preventing the alteration in blood parameters in the freshwater fish Saccobranchus fossilis exposed to pesticide (Verma et al., 1981 d). Dietary ASA supplementation is known to decrease copper toxicity and accumulation in carp (Yamamoto et al., 1977). The protective action of ASA and dihydroascorbic acid on acute cadmium toxicity in rainbow trout were observed by Yamamoto and Inoue, (1985). Large amounts of dietary ASA or dihydroascorbic acid reduced cadmium toxicity in this fish.

From the above reports we can infer that the contention put forward by Mehrle and Mayer (1975 a) and Mayer et al. (1978) is applicable in the case of heavy metal toxicity. The involvement of ASA in the nonenzymatic detoxification of free oxygen radical is well reported (Amdur, 1980; Anderson et al., 1980; Milne and Omaye 1980; Cort, 1982; Barclay et al., 1983). ASA which is an active redox agent is also involved in the functioning of a great number of biochemical systems (Koeing, 1984). The experiments of Hamilton et al. (1981) in channel catfish proved that body uses vitamin C to neutralise the action of toxicants. Hilton et al. (1977) postulated that environmental toxicants in water and feed could markedly shorten the time periods for appearing overt deficiency symptoms in rainbow trout fed unsupplemented diets by increasing the ASA requirement.

Exposure to pollutants induce a competition between developmental and detoxification processes for the use of vitamin C and causes a decline in the ASA concentration in the body resulting in the functional deficiency of vitamin C. The rate of such ASA depletion is a process affecting growth (Barnes, 1975), primarily as a result of ascorbate function in collagen formation. Wagstaff and Street (1971) showed that vitamin C was necessary for the functioning of microsomal hydroxylative enzymes necessary for the detoxification

of xenobiotics. Mayer et al. (1978) showed that vitamin C is involved as a cofactor in detoxification mechanism of fish. Since utilization of vitamin C for detoxification of metal is necessary for survival, the vitamin C stores of the different tissues are depleted and a functional deficiency of vitamin C develops. Chronic deficiency of vitamin C will cause impaired collagen synthesis resulting in the malformation of bones, reported earlier in response to heavy metal toxicity.

Apart from impaired collagen synthesis, functional ASA deficiency will result in decreased uptake of calcium by the gill which is a major site of calcium absorption in fishes (Mahajan and Agrawal, 1980 b). This can also contribute to the skeletal abnormalities.

As fishes lack the ability to synthesise ASA and cannot meet the increased ASA requirement for the detoxification of metals, they utilise the stored ASA of the body and this causes a depletion of vitamin C. Hence it can be safely inferred that the decrease of ASA in the liver, kidney and blood observed in the present study was due to its utilization in the detoxification of metal ions. It is interesting to note that decrease of ASA was more in fishes exposed to mercury indicating a differential response. Such a differential response by different environmental variables and pollutants were reported by Thomas and Neff (1984).

From these observations it is clear that ASA has an important role in protecting the body from the deleterious effects of metals. ASA is used up by the body to detoxify the metals accumulated in the different organs. ASA also scavenges free oxygen radicals generated on metal exposure. Hence exposure to metals increases the ASA requirement and depletes ASA content in the liver, kidney and blood of O. mossambicus.

CHAPTER 9

SUMMARY

The present study embraces investigations on the sublethal effects of copper and mercury on the fish, Oreochromis mossambicus (Peters). The fishes were exposed to two sublethal concentrations of each metal and the changes in glycogen, protein, haemoglobin, haematocrit, some selected enzymes, glutathione and ascorbic acid in selected tissues, during metal-exposure were monitored.

Chapter 1: In this chapter, a broad outline of heavy metal pollution, heavy metal uptake, requirements of a suitable bio-monitoring organism, criteria for a standard test fish, and the suitability of O. mossambicus for the toxicological study are given.

Chapter 2: The glycogen and protein contents of liver and muscle after exposure to copper and mercury were studied. There was a significant decrease of glycogen in the liver and muscle of metal-treated fishes. A decrease in protein content in the liver and muscles was observed during the latter period of the experiment. During metal-intoxication, the energy requirement goes up owing to the detoxification and tissue repair processes. This increased energy demand and the hypoxic condition usually associated with metal exposure, result in the depletion of glycogen reserves. When the glycogen reserves decrease, the proteins in the tissues are utilized for energy production as there is a decrease in the protein content towards the end of the experimental period.

Chapter 3: A study of acid phosphatase (a lysosomal marker enzyme) and alkaline phosphatase (a plasma membrane marker enzyme) activity pattern in the liver and kidney of the fish exposed to the two sublethal concentrations of copper and mercury is presented. The variation of the enzyme activities is interpreted with reference to destabilization of the membrane system and hypersynthesis of these enzymes during metal stress. A significant increase in the acid phosphatase activity in the liver and kidney of fishes exposed to the two metals was observed. The increased acid phosphatase

activity could be due to the destabilization of lysosomal membrane and consequent leakage of the enzyme into the cytosol. The observed decrease in alkaline phosphatase activity in the liver is explained as due to the inhibition of the enzyme by the influx of metal ions, the destabilization of the plasma membrane and the resultant depletion of the enzyme away from the liver tissues. Hypersynthesis of alkaline phosphatase in kidney may be responsible for the elevation of alkaline phosphatase activity in the kidney. This may increase the active transport and excretion of heavy metal ions across kidney tubules.

Chpater 4: A differential response was seen in the liver and kidney in the aspartate aminotransferase activity (GOT) of the metal treated fish. In the liver, the aspartate aminotransferase (GOT) increased during metal intoxication, whereas the enzyme activity was much lower in the kidney. But the alanine amino transferase (GPT) activity was lower in the liver and kidney. In the liver, there may be a switching over from alanine transamination to aspartate transamination resulting in the increased aspartate aminotransferase synthesis and decreased alanine amino transferase synthesis. In the kidney, the increased occurence of glutamic acid may reduce the synthesis of AAT (GOT) and ALAT (GPT) which is manifested as decreased enzyme activity in the fish.

Chapter 5: The effect of copper and mercury on the haemoglobin, haematrocrit and mean cell haemoglobin concentration was studied. The two metals elicited different responses initially on the haemoglobin content. There was a decrease of haemoglobin in copper-dosed fishes. But there was no such decrease in the mercury-treated fish, possibly due to the increased removal of water through urine. However, later there was a significant increase in the haemoglobin and haematocrit values in both copper and mercury-treated fishes. This increase might be an attempt of the body to increase the oxygen carrying capacity of the blood as hypoxia generally occurs in metal intoxicated fishes. The lack of significant change in the mean cell haemoglobin concentration indicated that the increase in the haemoglobin and haematocrit values was due to increased erythropoiesis to compensate for the reduced oxygen carrying capacity of the blood.

Chapter 6: There was an increase in the blood glutathione content after exposure to copper and mercury towards the end of the experimental period. The important functions of glutathione include maintaining redox potential of the cell, scavenging the toxic free radicals, acting as substrate for the enzymes involved in the detoxification of xenobiotics and finally conjugation of xenobiotics so that they can be excreted easily. The increased blood glutathione observed in fishes exposed to copper and mercury could be due to the hypersynthesis of glutathione which is necessary for the detoxification, conjugation and excretion of heavy metals and scavenging of toxicant-mediated free radicals.

Chapter 7: This chapter deals with the studies on the blood catalase activity after metal exposure. Copper and mercury evoked a differential response initially. There was an increased catalase activity, in the mercury-treated fishes at 24 h, but copper-treated fishes showed no such increase. But later there was an increase in the catalase activity of all metal-treated fishes. But by the end of the experimental period, catalase activity of the metal dosed fishes showed a tendency to return to control values. The increased catalase activity under toxic condition may be for counteracting lipid peroxidation and removing toxic hydrogen peroxide or other organic hydroperoxides formed during metal exposure, till other antioxidant defence mechanisms are initiated towards the end of the experiment.

Chapter 8: In this chapter, the effect of copper and mercury on the ascorbic acid content of the liver, kidney and blood is presented. A significant decrease in the ascorbic acid level was observed in all tissues studied. Since ascorbic acid is used for the detoxification of heavy metals and for the protection of the body during metal exposure, these metals induce a competition between developmental and detoxification processes for the use of ascorbic acid. Since most of the fishes cannot synthesise ascorbic acid, exposure to copper and mercury causes a functional deficiency of ascorbic acid and depletes ascorbic acid reserves of the tissues.

In the present study it was observed that eventhough copper and mercury evoked different responses initially with respect to some parameters studied,

there was no such difference towards the end of the experimental period. This study also indicated that different tissues responded differently towards these two metals.

It is felt that the analysis of phosphorylase and amino acid content of the liver and muscle would be helpful for a better interpretation of the utilization of glycogen and protein during exposure to heavy metals. The analysis of serum phosphatases also would help in a better understanding of its activity in the tissues studied. Similarly a study of the superoxide dismutase and glutathione peroxidase might provide a better knowledge of the detoxification system operating during metal exposure. Further studies on these parameters may provide a new insight into the functioning of the fish body during metal exposure.

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