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Effect of Heavy Metals on Lysosomal Enzyme Alkaline Phosphatase Activity of Bivalve L. marginalis…..

*Sandhya M. Sonawane.
Department of Zoology, JDMVPS ASC College, Jalgaon, Dist. Jalgaon. (M.S.) Mobile No. 9422977824
*Corresponding author: *Sandhya M. Sonawane*

Abstract: *Enzymes are referred as biological, catalysts which obey certain general rules. The literature shows investigative lacunae with regard to effects of heavy metals on Alkaline phosphatase activity of aquatic animals especially where fresh water molluscs are concerned. Hence an attempt has been made to study the effect of heavy metals $CuSO_4$, $HgCl_2$ and $CdCl_2$ on fresh water bivalve, Lamellidens Marginalis with respect to change in the level of alkaline phosphatase enzymes. The concentrations higher than those needed to prevent oxidative phosphorylation damage the mitochondrial network to a degree where the action enzyme involved in oxidative metabolism is blocked. Alkaline phosphatase showed a constant decrease after the heavy metal stress.*

Keywords: *Heavy metals $CuSO_4$, $HgCl_2$ and $CdCl_2$, L. marginalis, Alkaline phosphatase enzyme, acute, chronic.*

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I. INTRODUCTION

Enzymes are referred as biological, catalysts which obey certain general rules. The enzyme catalyzed reactions take place at physiologically low temperatures (37^0C) and require extremely small amounts of enzymes. Chemically, enzymes are complex protein molecules synthesized in the cells where they act as biocatalysts in carrying out various physic-chemical reactions. These proteins have their own specificity and kinetics. The enzymes help in attaining a reaction in a state of equilibrium. An enzyme recognizes its specific substrate and reacts with it to form products and gets regenerated at the end of the reaction.

The usual measure applied for the assessment of any environmental effect of a pollutant on animal is mortality. However, other effects which are gradual and are indicative of physiological change can be as detrimental as mortality to the animal's survival. Although instantaneous effects of heavy metal poisoning may be physical such as retraction of animal in the shell, loss of locomotory activity, changes is normal behaviour, reproductive disorders, suffocation, coating of respiratory surface with mucus, long term effect would be exclusively due to physiological alterations.

The most fundamental effects would be the study of changes in enzyme levels, since these organic cellular catalysts control the formation of biochemical intermediates which are indispensable to all normal physiological processes. Interactive effects of metal pollution and ocean acidification on physiology of marine organisms Anna V. Ivanina, Inna M. Sokolova, (2015)

Molluscs have exhibited ability to adapt themselves to life in so many different types of habitats. It is not surprising that they have learnt to feed in different ways. They show a variety of digestive patterns. The correlation between digestive enzymes and diet has been established but specific characterization of different enzymes of different animals presents many interesting and puzzling questions (Prosser 1973). Many workers (Mukherji and Kanungo, 1954) have investigated the digestive enzymes of pelecypods. They were reported from digestive diverticula and digestive gland of several lamellibranchs.

Heavy metals are pervasive components of the aquatic environment, including fresh waters. The gross effects of these components have been substantially documented in both marine and fresh water. Yet the extent of physiological damage resulting from high and low level heavy metal contamination of fresh water and the harm that is done to aquatic animals, has not been adequately described, though there are few studies which indicates the effect of heavy metals on aquatic organisms (Hewitt and Nicholas, 1963). A change in enzymatic pathway is another approach which though yet untried could potentially be used in toxicant analysis. Exposure to metals might in some way alter the enzyme and change its response in co-factors, temperature, pH and also its micheli's constant (Jackim, 1974).

Kreb's citric acid cycle is the final and common pathway for the oxidation of carbohydrates, proteins, lipids, since glucose, amino acids and fatty acids are metabolized to acetyl Co-A and then acetyl Co-A is oxidized to Co_2 and water through a series of metabolic steps. The oxidation of acetyl Co-A reduces equivalents in the form of electrons. These electrons are released due to activity of a group of specific enzymes known as

dehydrogenases. This dehydrogenase catalyses the formation of high energy phosphate bond through a process of oxidative phosphorylation (Campbell, 1973). The energy, thus generated is most important for the organism. Any disruption or alteration in the activity of these enzymes of the citric acid cycle, may therefore, disturb the entire physiological equilibrium resulting in complications of various nature.

The lysosomes which contain several acid hydrolases are believed to constitute an intracellular digestive pattern (Berhet, 1965; de Durve and Wattiaux 1966) and have been implicated in the digestion and ingested substances in various types of mammalian cells (Cohn and Hirsch, 1960; Straus, 1964 a, b). However, there is little evidence that intracellular digestion in mollusks is due to lysosomes. Acid and alkaline phosphatase are lysosomal enzymes. Summer (1969) demonstrated the presence of these enzymes in lysosomes and food vacuoles of the digestive gland of Mytilus edulis and Helix aspersa.

The acid phosphatase, a non specific monosterase and alkaline phosphatase, a nonspecific hydrolase are the two considerably important enzymes widely spread in the animal kingdom (Standtonan 1961). Acid phosphatase, is a lysosomal enzyme which hydrolyses the ester linkage of phosphate esters and helps in autolysis of the cell. Alkaline phosphatase catalyses the liberation of inorganic phosphatase from monophosphate esters in the alkaline medium and plays an active role in transport and metabolism of many biologically important compounds (Novikoff, 1961). These two enzymes are concerned with the presence of transphosphorylation and have an important role to play in the general energetic of an organism.

These two hydrolases have been studied by many workers – Bhattacharya et al. (1975) in the fish Clarius Batrachus exposed to endrin and Anees (1976) in the fish Channa punctatus noticed the activity of these enzymes in effect of heavy metals like Cu, Hg and Zn on these hydrolases in Viviparus bengalensis.

For going survey of literature showed that only a few workers have studied lysosomal enzymes of lamellibranch mollusks. The literature shows investigative lacunae with regard to effects of heavy metals on metabolic processes of aquatic animals especially where fresh water molluscs are concerned. Hence an attempt has been made to study the effect of heavy metals on fresh water bivalve, Lamellidens Marginalis with respect to change in the level of lysosomal enzyme alkaline phosphatase.

II. MATERIAL AND METHODS

The bivalves Lamellidens Marginalis were collected from the Godavari river at Paithan. After bringing the bivalves to the laboratory, they were cleaned thoroughly and placed in plastic troughs. They were acclimatized to the laboratory conditions for 5 to 6 days prior to subjecting them to experiments. The water in the troughs was changed every day. Only active and healthy animals were chosen for experiments. During chronic treatment the animals were fed on crushed fresh water algae and Hydrilla.

The bivalve were exposed to median lethal concentration and sub lethal concentration of pollutant as acute and chronic treatment respectively.

The acclimatized bivalves were divided into four groups, of ten each. The first groups of bivalves was kept as control. The remaining groups were exposed to 1.6 ppm $Cuso_4$, 0.6 ppm $HgCl_2$ and 3.9 ppm cadmium chloride for 72 hours, for acute treatment. The concentrations used for chronic exposure were 0.82 ppm copper sulphate, 0.32 ppm mercuric chloride and 1.95 ppm cadmium chloride. The chronic treatment was given upto 20 days. The control and treated bivalves were fed on freshwater crushed algae and Hydrilla during exposure period.

The digestive glands from five to ten mussels were separated and washed in distilled water. These digestive glands were then dried between the folds of muslin cloth, dehydrated and defatted by treatment with ice cold acetone (Summer and Summer, 1947). The material was ground in a clean ice chilled glass morter and repeatedly washed in acetone and filtered till and filtrate was colorless. The powder thus obtained was dried under fan and stored in a clean bottle in freezer, at 3 to 5^0C. In all the experiments, 1% homogenate of digestive gland prepared in glass distilled water was used. Half of this extract was boiled for half an hour to destroy the enzyme activity and this boiled extract was used as control for all the experiments.

Estimation of alkaline phosphatase :

Alkaline phosphatase activity was measured by the method described by King (1951). In a medium containing 2.0 ml of 0.1 M bicarbonate buffer (pH 10) and 2.0 ml of 0.1 M disodium phenyl phosphate, the reaction was initiated by adding 0.2 ml of 20% tissue homogenate and the reaction mixture was incubated at 37^0C for 15 minutes, 1.8 ml of folin ciocateu phenol reagent was added to stop the reaction. After centrifugation at 3000 rpm for 5 minutes, 4.0 ml of the supernatant was added to 2.0 ml of 15% sodium carbonate. Incubation was allowed for another 10 minutes after which the blue colour developed was read at 660 um in a colorimeter. A simultaneous blank was maintained with distilled water. For the calculation of alkaline phosphatase the following formula was used:

$$\frac{\text{Reading of (Test Control)}}{\text{Reading of (Standard - Blank)}} \text{ X Concentration of Standard x } \frac{6}{4} \text{ x } \frac{10}{\text{quality of tissue}}$$

= KA/ml/60 min at 37^0C.

III. OBSERVATIONS AND RESULTS

The activities of alkaline phosphatase were studied in the normally fed and pollutant (heavy metals) treated freshwater bivalve, Lamellidens marginalis. The experimental findings obtained are summarized in Table.

Alkaline Phosphatase :

The activity level of alkaline phosphatase in normal animals showed 0.989, 0.430 and 0.681 ug of Pi/mg protein/hr in digestive gland, foot and mantle respectively. Among the tested heavy metals $HgCl_2$ significantly decreased the activity of alkaline phosphatase compared to $CuSO_4$ and $CdCl_2$. The values of alkaline phosphatase were 0.392, 0.270 and 0.136 ug of Pi/mg protein/hr in digestive gland, foot and mantle respectively after 72 hours exposure. Similar decrease was also exhibited by $CuSO_4$ in chronic exposure. The values were 1.109, 0.408 and 0.603 ug of Pi/mg protein/hr in digestive gland, foot and mantle respectively after 20 days.

In acute exposure of $HgCl_2$ the value of alkaline phosphatase were 0.212, 0.150 and 0.087 ug of Pi/mg protein/hr in digestive gland, foot and mantle respectively after 72 hrs exposure. It was also observed that as the period increased, there was a significant decrease in the activity in $HgCl_2$. Similar trend was also observed in chronic exposure of $HgCl_2$.

$CdCl_2$ showed slightly increased activity in foot at 24 hrs. while in case of digestive gland and mantle the activity was decreased significantly. The decrease was more in digestive gland than mantle. The values were 0.571, 0.212 and 0.382 ug of Pi/mg protein/hr in digestive gland, foot and mantle respectively. In chronic treatment of $CdCl_2$ (Table 16) constant depletion in alkaline phosphatase activity was obtained. The values were 1.319, 0.458 and 0.555 ug of Pi/mg protein/hr in digestive gland, foot and mantle respectively after 20 days of exposure.

The maximum percentage decrease was upto 80.18% (72 hours of $HgCl_2$ stress in digestive gland), 88.28 (72 hrs of $HgCl_2$ stress mantle) 30.50% (20 days exposure of $HgCl_2$ in digestive gland). Acute stress of $HgCl_2$ showed drastic decrease in alkaline phosphatase activity in all tissues compared to chronic stress. All the values are statistically significant (in both acute and chronic treatment) at $P<0.05$, $P<0.05$ and $P<0.001$ levels.

Table - 1

Level of alkaline phosphatase activity after exposure of heavy metals, copper sulphate, Mercuric chloride and Cadmium chloride

Pollutant	**Exposure Period (in hrs)**	**Digestive gland**		**Foot**		**Mantle**	
1	**2**	**3**		**4**		**5**	
Control		0.989±0.018		0.400±0.009		0.681±0.0020	
$CuSO_4$	24 hrs	0.732±**0.007** **P<0.001**	-40.30	0.290±**0.005** **P<0.001**	-40.58	0.521±**0.0016** **P<0.05**	-60.13
	48 hrs	0.572±**0.006** **P<0.005**	-57.20	0.300±**0.008** **P<0.001**	-20.70	0.230±**0.0018** **P<0.001**	-67.21
	72 hrs	0.392±**0.007** **P<0.001**	-72.80	0.270±**0.006** **P<0.001**	-50.13	0.136±**0.0021** **P<0.05**	-75.19
$HgCl_2$	24 hrs	0.630±**0.010** **P<0.05**	-47.30	0.156±**0.004** **P<0.001**	-30.17	0.313±**0.0025** **P<0.05**	-52.13
	48 hrs	0.580±**0.009** **P<0.05**	-50.60	0.189±**0.006** **P<0.05**	-42.93	0.124±**0.003** **P<0.001**	-84.32
	72 hrs	0.212±**0.008** **P<0.05**	-80.18	0.150±**0.005** **P<0.001**	-80.13	0.087±**0.0016** **P<0.05**	-88.28
$CdCl_2$	24 hrs	0.902±**0.007** **P<0.001**	-15.25	0.371±**0.005** **P<0.01**	-5.23	0.597±**0.0072** **P<0.05**	-15.40

	48 hrs	0.653±**0.009** **P<0.001**	-37.17	0.282±**0.006** **P<0.001**	-20.45	0.668±**0.0032** **P<0.001**	-3.70
	72 hrs	0.571±**0.009** **P<0.05**	-49.19	0.212±**0.005** **P<0.05**	-60.80	0.382±**0.0042** **P<0.05**	-50.11

Alkaline phosphatase activity is expressed as ug of Pi/liberated/mg protein/hr.
Each value is the mean of five observations
Values are significant at P<0.001, P<0.05 level
-ve sign indicates % inhibition.

Table - 2

Level of Alkaline phosphatase activity in the tissue of L.marginalis after chronic exposure of heavy metals, copper sulphate, Mercuric chloride and Cadmium chloride

Pollutant	**Exposure Period (in Days)**	**Digestive gland**		**Foot**		**Mantle**	
1	**2**	**3**		**4**		**5**	
Control	5 10 15 20	1.339±0.0027 1.336±0.0025 1.334±0.0021 1.337±0.0028		0.512±0.0021 0.508±0.0027 0.509±0.0023		0.085±0.0043 0.882±0.0042 0.883±0.0040	
$CuSO_4$	5	1.224±**0.0024** **P<0.05**	-18.11	0.432±**0.0027** **P<0.001**	-21.70	0.627±**0.0034** **P<0.001**	-20.91
	10	1.220±**0.0025** **P<0.05**	-7.15	0.430±**0.009** **P<0.05**	-19.20	0.620±**0.0032** **P<0.05**	-12.70
	15	1.118±**0.0018** **P<0.001**	-19.30	0.422±**0.0028** **P<0.05**	-32.90	0.615±**0.0014** **P<0.05**	-17.30
	20	1.109±**0.0020** **P<0.05**	-15.30	0.418±**0.0016** **P<0.05**	-34.13	0.603±**0.0035** **P<0.05**	-18.13
$HgCl_2$	5	1.180±**0.0028** **P<0.05**	-22.20	0.399±**0.0027** **P<0.05**	-30.31	0.580±**0.0032** **P<0.05**	-38.42
	10	1.175±**0.0029** **P<0.05**	-20.90	0.388±**0.0017** **P<0.001**	-32.41	0.578±**0.0030** **P<0.05**	-32.40
	15	1.160±**0.0020** **P<0.05**	-27.59	0.372±**0.0027** **P<0.05**	-40.70	0.572±**0.0019** **P<0.05**	-33.51
	20	1.152±**0.0021** **P<0.001**	-30.50	0.363±**0.009** **P<0.05**	-50.70	0.565±**0.0015** **P<0.05**	-35.30
$CdCl_2$	5	1.329±**0.0036** **P<0.05**	-5.69	0.480±**0.0033** **P<0.001**	-5.50	0.653±**0.0033** **P<0.001**	-22.40
	10	1.327±**0.0025** **P<0.05**	-7.99	0.478±**0.0014** **P<0.05**	-15.60	0.650±**0.0032** **P<0.05**	-12.70
	15	1.325±**0.0016** **P<0.05**	-6.70	0.468±**0.0022** **P<0.05**	-20.58	0.643±**0.0024** **P<0.05**	-17.50
	20	1.319±**0.0023** **P<0.001**	-18.20	0.458±**0.0023** **P<0.05**	-30.21	0.555±**0.0052** **P<0.05**	-19.30

Alkaline phosphatase activity is expressed as ug of Pi/mg protein/hr.
Each value is a mean of five observations.
Values are significant at P<0.001, P<0.05 level or NS = Not Significant
-ve sign indicates % inhibition.

IV. DISCUSSION

Digestion is a sequential process which renders food absorbable through the gut wall by breaking the food into simple molecular components through enzymatic action, such enzymes are secreted by specialized cells at digestive tract. Digestion although dependent on enzymatic action is determined by the functional organization and the structure of digestive system.

Out of the many devices which can be applied to investigate the physiological alterations made by the pesticidal and heavy metal treatment, the most fundamental one would be the study of changes in the enzyme

activities, since these organic cellular catalysts control the formation of biochemical intermediates which are indispensible to all the normal physiological processes.

In mollusks (Owen, 1968) digestion takes place extracellularly in the gut lumen by the action of digestive enzymes secreted by salivary glands and the enzymes juice that may flow from hepatic channels into the gut lumen. Gut epithelium is, however, poorly supplied with secretory cells. They are chiefly mucous secretory cells. The spheres that are to be phagocytized are first coated with the mucous layer so that they can easily be taken by the digestive cells of digestive gland. The relation importance of the initial extra cellular digestion, however, varies considerably.

There is no doubt that the effect of certain metals is profound on the enzyme activity in aquatic organisms though the mode of action of these heavy metals has not been clearly outlined with regard to their mechanism of action in certain key enzymes which are responsible for the general energetic of animals. There is a considerable amount of literature devoted to the study of organic pesticides concerning the enzyme system of various animals (Yap et al., 1975; Koundinya and Ramamurthi, 1978; Natrajan, 1981; Shastry and Malik, 1981 and Dalela et al., 1982). However, information regarding the effect of heavy metals on the enzyme action is restricted (Hewitt and Nicholas 1963 and Jackim, 1974).

Rees and Sinha (1969) have suggested that damaged organs produce augmented quantity of enzyme. Novikoff (1961) and De Duve (1968) have also suggested that increased lysosomal activity occurs as a part of the prenecrotic changes. Increase in lysosomal activity ultimately results in elevated acid phosphatase activity. These observations support the present findings. During all acute and chronic exposures the activity of acid phosphatase was enhanced indicating constant cell necrosis which was highest in $HgCl_2$ followed by $CuSO_4$ and $CdCl_2$. Similar findings were also reported by Alam (1984) in Viviparous bengalensis when exposed to heavy metals. A significant depletion in the activity of alkaline phosphatase was observed in both acute and chronic (Table 1 and 2) exposures which might be due to the decrease in the rate of transphosphorylation.

Loomis and Lipman (1948) and Desaiah (1978) suggested that uncoupling of oxidative phosphorylation is the cause for inhibition of phosphatases. Dalela et al. (1980) are also of the opinion that uncoupling of oxidative phosphorylation has been the main reason for inhibition of acid and alkaline phosphatases. Similar decrease in acid and alkaline phosphatase has been reported in the snail, V. bengalensis by Alam (1984).

Simon (1953) reported that the concentrations higher than those needed to prevent oxidative phosphorylation damage the mitochondrial network to a degree where the action enzyme involved in oxidative metabolism is blocked. According to pressman (1963) uncouplers promote conductivity of protons within the mitochondrial membrane and subsequently present the formation of gradient across the membrane. It is generally supposed that alterations in mitochondrial mechanisms are reflected in their morphological changes and that normal metabolic profiles are dependent on the continuous supply of energy rich interference of uncouplers. These pathways are blocked finally affecting the activity of phosphatase.

In the present investigation the mode of action of heavy metals might be the same as that of pesticides.

V. SUMMARY

The influence of different heavy metals, copper sulphate, mercuric chloride and cadmium chloride on the enzymatic activity of the bivalve, Lamellidens Marginalis was observed. The important lysosomal enzyme alkaline phosphatase was studied in relation to heavy metal stress. Alkaline phosphatase showed a constant decrease after the heavy metal stress.

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