IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT)
e-ISSN: 2319-2402,p- ISSN: 2319-2399.Volume 10, Issue 11 Ver. I (Nov. 2016), PP 47-53
www.iosrjournals.org

Electrophoretic Variation of Creatine Kinase Isozymes as a Function of Methyl Parathion in the fish Labeo Rohita

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Abstract: *Serum samples were obtained from the control and experimental fish, Labeo rohita treated with 1/3rd of LC_{50} of methyl parathion. Creatine kinase (EC 2.7.3.2) isozyme and sub- bands (isoforms) were studied using clinical automatic electrophoresis and densitometer. CK isozymes and isoforms were found to display disparate electrophoretic mobility in fish when compared with CK patterns of human. It was interesting to observe that CK-MM was 90.1% in human while 75% in fish. CK-MB isozyme was present in human but absent in fish. Following the treatment of methyl parathion there was a marked increase in the CK- activity. The study reveals that methyl parathion exposure altered CK-B1 status in the brain due to induction of lipid peroxidation/ inhibition of acetylcholinesterase. The physiological and biochemical significance is reported herein for the first time.*

Keywords:*Serum CK isozymes, CK-MM, CK-MB, CK-BB, Enzyme- isoforms, Acetyl cholinesterase*

I. Introduction

Cells and tissues with intermittently high and fluctuating energy requirements such as skeletal, cardiac muscle and retina depend on the immediate availability of large amounts of energy. In these cells, the enzyme creatine kinase (CK; ATP;creatinephosphoryltransferase, EC 2.7.3.2), plays a key role in cellular energy metabolism [1] by replenishing ATP through the reversible transfer of the phosphoryl group from phosphocreatine (PCr) to ADP [2]. Creatinekinase constitute a family of different oligomericisozymes with tissue specific expression and isozyme specific sub-cellular localization. Three cytosolic isoforms, ubiquitous brain type CK-BB, sarcomeric muscle type CK-MM and CK-MB heterodimer [3] as well as two mitochondrial isoforms ubiquitous CK-Mi_a and CK-Mi_b are synthesized in a tissue specific manner [4, 5]. The cytosolic CK-M and CK-B subunits combine to form enzymatically active homo and heterodimer CK-MM, CK-MB and CK-BB whereas the mitochondrial protomer (CK-M_i) combines preferentially to homo-octamers[4]. In fully differentiated skeletal muscle CK-MM is the predominant isoform occurring in appreciable amount together with CK-M_{ib}. But CK-BB is the most widely ubiquitous isoform present in brain, smooth muscle, heart and a variety of other tissues [6]. All three cytosolic isoforms coexist together during myogenesis[7] and to some extent also in mammalian heart while CK-MB and CK-BB are undetectable in mature skeletal muscle [8].

Although ATP represents the universal energy currency in all organisms and cells, ATP levels are not simply upregulated in cells with high level and/ or intermittently fluctuating energy demand. Elevation of the intracellular ATP concentration [ATP], as an immediate energy reserve, followed by its hydrolysis would lead to massive accumulation of ADP plus P_i and also liberate H^+acidifying the cytosol. Since this would inhibit ATPase such as the myofibrillaracto-myosin ATPase and consequently muscle contraction and many other cellular processes, nature has evolved a means to deal with problems of the immediate replenishment of the ATP stores. The so called phosphagens evolved as high energy compounds that are "Metabolically Inert" and as such do not interfere with the primary metabolism. One of the phosphagens, PCr together with its corresponding kinase, CK first appeared at the dawn of eukaryotic evolution about one billion years ago [9].

Importantly, CK isozymes are localized differentially on the sub- cellular level and these specific locations are essential for the functioning of the CK network [1, 10]. The proposed CK/ PCr energy shuttle connect site of ATP production (glycolysis and mitochondrial oxidative phosphorylation) with sub- cellular site of ATP utilization (ATPase). The molecular basis of this spatial energy buffering is functionally sub- cellular CK- microcomponents at sites where ATP production and ATP consumption are tightly connected to CK/ PCr action.CK is specifically associated with those of glycolytic enzymes that are either involved in ATP generation such as Pyruvate kinase [11, 12] or regulatory enzyme of glycolysis, Phosphofructokinase (PFK) which is regulated by rising [ATP] via negative feedback mechanism of ATP or PFK [12].The CK- PFK interaction is pH dependent and stronger at lower pH than neutrality. This is physiologically relevant since under conditions of muscle activation and working glycolysis, the intracellular pH may drop, resulting in inhibition of glycolysis.

Creatine kinase isozymes are frequently used in man to assess the state of differentiation of muscle and neural tissue as well as serum diagnostic markers of acute myocardial infarction, skeletal muscle disease and neurological injury [13]. Changes in activity reflect pathological alterations in specific human organs. CK has also been studied in other species like dogs, rabbits, mice and chickens [14]. Serum enzyme activity reflects the

health conditions and their uses in diagnostics have been recommended [15, 16]have reported severe and degenerative myopathy in Atlantic salmon through elevated serum total CK level. Other authors have reported CK in fish brain, endocrine tissues and skeletal muscles. Electrophoretic variability of tissue CK has been used to demonstrate genetic variability between fish species [17].

However, not many reports are available on serum enzyme changes in fishes especially CK isozymes. CK in fish has always been the subject of much attention. Similarly, not much information is available with changes in the expression of CK isozymes on the toxicity of methyl parathion. The aim of the present paper is to evaluate the expression of CK isozymes in the serum of fish, *Labeo rohita* after the exposure to sub- lethal concentration of methyl parathion for 96h using a fully automatic clinical electrophoresis and densitometer.

II. Materials and Methods

Ethical Statement*:* Presently, we do not have any Ethical Committee in our University. But however, we have followed the ethical norms, which are being followed elsewhere which is evident in the Materials & Methods Section.

Maintenance of animals:*Labeo rohita*, a common carp was obtained from the local hatchery. Fishes were acclimated to laboratory conditions for about 5-7 days. They were kept in aquarium tank (250 L) and water was constantly aerated by a static system. During the acclimation period, they were given artificial (commercial) feed composed of ground shrimps available in the local market to avoid the possible effects of starvation. The feeding and maintenance of the fishes and physico-chemical characteristics of the aquaria water were measured (Tab. 1). Short-term test of acute toxicity over a period of 96h were performed on the fishes following the renewal of bioassay. The fishes were exposed intra-coelomatically with $1/3^{rd}$ of LC_{50} value of the pesticide methyl parathion. After 24, 48, 72 and 96 h of exposure fishes were processed for further investigations.

Determination of LC_{50}: The experiments were repeated several times and only arithmetic mean of the experiments at each concentration was taken to express the results. LC_{50} values were determined by EPA Probit analysis program [18]. The LC_{50} of methyl parathion for the fish *Labeo rohita* was 16.8 ppm.

Blood collection: The fishes were taken out of the aquarium water individually through fish net with a minimum possible disturbance. After preliminary investigations, the blood samples were collected from caudal fin as described by many authors. In the present study, the blood collection from caudal fin had to be abandoned because there was an unusual elevation in Lactate dehydrogenase (LDH) and Creatine phosphokinase (CPK) activities which were recorded due to leakage from the surrounding muscle tissues. Thus, cardiac sampling was the only suitable method available as an alternative to obtain blood under the present study. After the blood collection, the serum were separated and processed for enzymatic assays.

Determination of Acetyl cholinesterase activity: The serum and brain tissue acetyl cholinesterase activity were measured according to the method of Ellman*et al,* (1961) [19].

Determination of Serum Creatine Kinase Activity: The serum creatine kinase (CK) activity was measured according to the method of IFCC (1989) [20]. The activity of different isoforms of CK isozyme fractions were calculated from their respective percentage concentration.

Separation of CKisozymes by Fully Automatic Clinical Electrophoretic Unit:Fully automatic clinical electrophoretic unit is system software controlled electrophoretic separation unit having a voltage, current and run time control, operated by an inbuilt programme present in the driver software of the electrophoresis. Fully Automatic Clinical Electriphoresis Model- Interlab- Pretty was used for the present study having software namely 'Interlab'. The system constituted of an automatic dispensing system for samples, a Teflon coated Peltier Plate for placing previously casted agarose gel over sterile plastic plate having bar- code over it and valve- compressor system for pouring appropriate amount of staining, destaining and washing solutions over the gel plate after completion of the migration. About 30µl of previously separated non-hemolyzed serum were placed in the sample space and loaded over the gel plate by the automatic dispensing system to achieve single straight line of application. The temperature for migration was set at 29^0C, time for migration was 5 minutes, voltage for migration was 400V and after migration secondary treatment of the gel was needed which was done in another chamber with an incubation period of 30 minutes at 45^0C after addition of staining solution. The staining solution contained Creatine and ADP as substrate giving ATP which in presence of Hexokinase enzyme and glucose gives glucose-6-phosphate. Glucose-6-phosphate in presence of NAD^+ and glucose-6-phosphate dehydrogenase (G-6-PD) gives NADH. NADH reacts with NitroblueTetrazolium (NBT) and reduceit toformazanin presence of PMS (Phenazinemethosulphate). After the treatment with the staining solution, which stained the CKisozyme bands, fixation of the band was achieved in the above mentioned external chamber. After staining and fixing the bands, the gel plate was re-placed into the electrophoretic chamber for repeated destaining with destaining solution comprising of acetic acid of strength 5% (v/v). Then washing was done with a surfactant solution for a number of times previously fed in the driver software. After completion of these steps, scanning of the gel plate was done in the densitometer with a driver software 'Scanlab' provided by the manufacturer.

III. Results and Discussion

Table-I. Physico-chemical characteristics of aquaria water

Sl no.	Parameter	Value
1.	Temperature	(24±2) ^{0}C
2.	pH	7.1± 0.2 at 24^{0}C
3.	Dissolved Oxygen	8.5 ±0.5 mg/L
4.	Total Hardness	23.4± 3.4mg $CaCO_3$/L
5.	Conductivity	<10 µs/cm

Table-II. Methyl parathion treated *L. rohita*

Sl no	Parameter	Hours of Treatment				
		Control	24h	48h	72h	96h
1	AchE_Brain_Activity (µM/min)	53.18	21.1	18.33	15.22	12.43
2	CK_BB1_Activity (IU/L)	7.72	4.76	6.48	4.97	4.14

Table-III.CK- Isozyme fractions in Methyl parathion treated *L. rohita* and Human

	Lane: 7	Lane: 8	Lane: 2	Lane: 4	Lane: 9	Lane: 13
CK-Isozyme	Human	Control	24h treated	48h treated	72h treated	96h treated
Unknown band/ New Band	.....	0.7%	1.0%	1.2%	3.5%	1.0%
CK- MM 1	90.1%	75.0%	34.0%	33.5%	31.9%	29.4%
CK- MM 2	.....	.....	40.8%	17.2%	28.4%	31.9%
CK- MM3	.....	.....	.....	23.1%	11.1%	17.6%
CK- MB	8.6%	.....	.....	.....	.....	.....
CK- BB 1	1.3%	23.5%	12.0%	14.0%	11.1%	10.1%
CK- BB 2	.....	0.8%	12.2%	11.0%	11.0%	10.0%

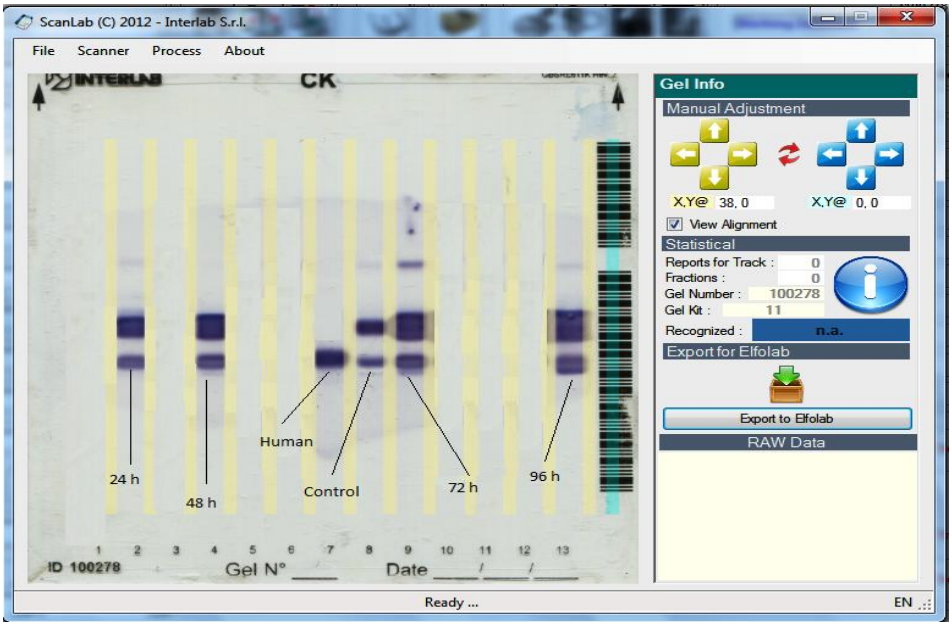

Figure- IA. Plate-I showing the lanes of migration of CK isozymes of fish and human

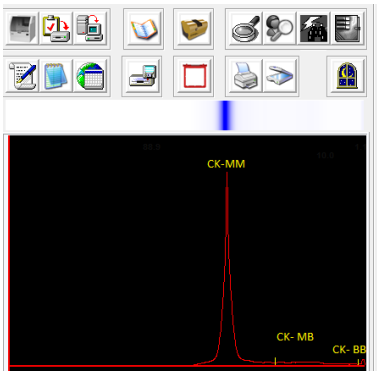

Figure- IB. Human CK-Peaks

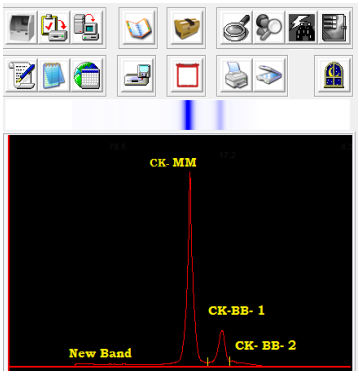

Figure- IC. Control Fish CK-Peaks

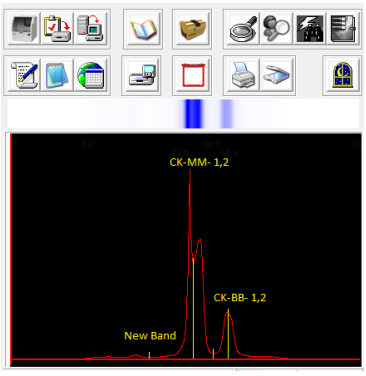

Figure- ID. 24h Fish CK-Peaks

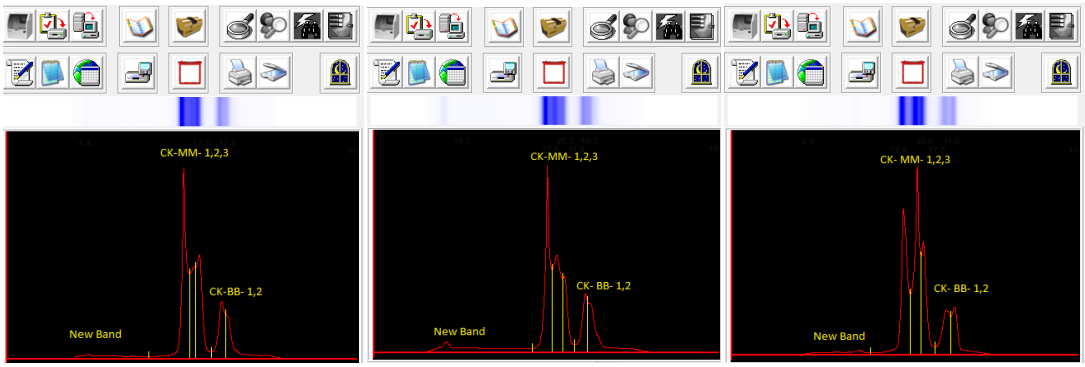

Figure- IE. 48h CK-Peaks **Figure- IF. 72h** Fish CK-Peaks **Figure- IG.** 96h Fish CK-Peaks

Figure- I A is showing the bands developed over the gel plate after electrophoretic separation of the CK- isozymes in the respective lanes.Figure- I B to G showing the different peaks of CK isozyme fractions where CK- MM is confined to skeletal muscle, CK- MB is confined to cardiac muscle and CK- BB is confined to brain. The numbers 1, 2 etc. show the corresponding isoforms of the isozymes.

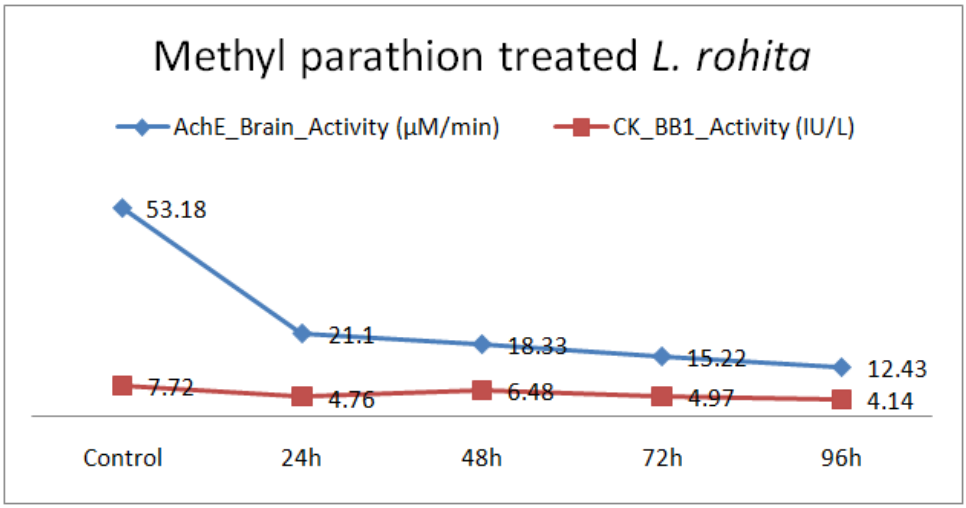

Figure- II. Line- diagram representation of relationship between CK_BB-1 and Acetyl cholinesterase activities

Sensitivity of lactate dehydrogenase, creatine phosphokinase and other enzymes to environmental adaptation is an area of current interest. Temperature and oxygen availability are among the predominant environmental determinants of evolution as well as physiological and biochemical adaptation in teleost fishes [21]. LDH activity or LDH isoenzymes are one of the best indicators of switch over to anaerobic metabolism by which teleosts survive during periods of oxygen unavailability [22, 23]. Creatine kinase and lactate dehydrogenase are primarily used as indicators of stress. Changes in the serum enzyme activity are used as indicators of tissue damage, environmental stress. Earlier the authors have reported changes in LDH isozymes following the treatment of methyl parathion in the fish, *Labeo rohita*. In the present study, electrophoretic separation and quantification of CK- isozymes in the fish, *Labeo rohita* is being reported and compared with the human serum CK isozymes because the later has been well studied. The differences between banding pattern of the fish, *Labeo rohita* (Plate I, lane 1 and Fig. I) and that of control human sample (Plate 1, lane 7 and Fig. I), demonstrating CK-MM, CK-MB and CK-BB clearly visible. CK- MM mobility is more towards anode in the fish than human which implies the presence of greater negative charges on the isozyme which may be caused by loss of positively charged amino acids resulting in the increased mobility towards the anode. CK-BB shows two peaks i.e. CK-B1 and CK-B2 in fish (Fig. I). This result is in marked contrast to human CK-BB. The CK-BB in fish contributes 24.3% of the total CK activity. CK-BB has been demonstrated in the brain of the rainbow trout [24]. Creatine may act as a reservoir of energy in these areas and augment their capabilities to secrete stress hormone like cortisol and TSH, T_3, T_4 and other hormones[25]. Its appearance in the serum may reflect normal leakage from the brain. Serum CK-MB in fish was not found in the present study. Similar observation have been reported by Perriard*et al*(1972) [26] in rainbow trout. In human serum CK-MB is 8.6% of the total CK (Fig. II) an important isozyme in the diagnosis of myocardial infarction have been extensively reported.

In human serum, CK-MM is 90.1% and CK-BB is 1-3% whereas in control fish CK-MM is 75% and CK-BB is 24.3% (Tab. II). Following the treatment of methyl parathion, there are two peaks in 24h namely CK-M1 (40.8%) and CK-M2 (32%) whereas in 48h, there are three peaks CK-M1, CK-M2 and CK-M3 with 33.5%, 17.2% and 23.1% respectively. In 72h CK-M1 is 31.9% showing almost no change but CK-M2 increases to 28.4% CK-M3 decreases to 11.1% as compared to 48h following the treatment of methyl parathion. In 96h there are also three peaks with CK- M1 (29.4%), CK-M2 (31.9%) and CK-M3 (17.6%) (Tab. III).

Results also show that CK-BB in the control fish has two peaks i.e. CK-B1 (23.5%) and CK-B2 (0.8%). But following the treatment of methyl parathion in 24h CK-B1 is (12.0%) and CK-B2 (12.2%), in 48h while CK-B1 is (14%) and CK-B2 (11.0%), in 72h CK-B2 (11.1%) and CK-B2 (11.0%) and in 96h CK-B1 is 16.2% and CK-B2 (1.5%) (Tab. III).The results obtained in the activities of CK and its isozymes are indicative of the fact that methyl parathion caused distortions in the cell organelles especially the excitable tissues particularly the brain and muscles. The differential percentage of increase or decrease of different kinds of CK-isozymes in the treated fish during different periods may be due to the differential mode of action of methyl parathion in different tissues. Secondly, gene duplication as well as post- translational processing has been proposed as important factor in modulating tissue specific enzymes [27, 23]. This increase and decrease or absence of some CK- isozymes could be interpreted as a result of mutational events that would have occurred in the regulatory genes which could lead to inhibition, alteration or constitutive gene expression. This could be the reason that some bands of isozymes become more intense or faint (Fig. I, Plate 1). The differences in the distribution of different CK-isozymes in the serum reflect differences in the bioenergetic activity.

In any cell, creatine kinase isozymes are especially localized at strategic sites of ATP consumption to efficiently regenerate ATP generation to build up phosphocreatine pool. Accordingly, the creatine kinase/phosphocreatine system plays a key role in the cellular energy buffering and energy transport particularly in cells with high and fluctuating energy requirements like skeletal muscle and neural tissues. Earlier, the authors have reported that the cortisol, a stress hormone increased within one hour and goes on increasing till 6h following the treatment of methyl parathion [25]. Similarly, LDH increases in the serum exponentially in 24h and 48h following the treatment of methyl parathion in the fish, *Labeo rohita*[28]. The other evidence of oxidative stress we have observed in our laboratory that when the fish is exposed to methyl parathion, the experimental fish flock around the aerator[23]. Oxidative stress is an important event that has been related to the physiological and behavioural conditions during which CK plays an important role. Creatine kinase expression may be an earlier indicator of oxidative stress.

Oxidative stress has been an important event related to the pathogenesis of the disease affecting the CNS (Central Nervous System). This is quite logical because brain tissue is highly sensitive to oxidative stress due to its high oxygen consumption and high lipid content especially polyunsaturated fatty acids and low activity of antioxidant defence [29]. Methyl parathion induces free radicals and membrane lipids are attacked leading to increased permeability and altered fluidity of the membrane. The perturbation in the structural and functional integrity of the membrane could have resulted in the release of the enzyme in circulation.

The ATP binding domain of the creatine kinase-BB contains essential amino acid residues of arginine, histidine, lysine and cysteine that may be targeted by ROS (Reactive Oxygen Species). It may be one of the reasons for decreased creatine kinase activity in the brain of methyl parathion exposed fish (Tab. II). Oxidative inactivation of creatine Kinase-B1 may involve the direct free radical mediated oxidation of these amino acids in the ATP binding site of CK-B1 to carbonyl containing derivatives. Studies show conversion of amino acid side chains into carbonyl containing derivatives cause activity loss of many enzymes [30].

CK-B1 activity follows approximately a similar pattern to that of AchE in the brain (Fig. II). CK-B1 isozyme may reflect a selective adaptation because of physiological correlation of B1 to AchE. Adaptive nature of CK- enzyme polymorphism displayed in the fish *Labeo rohita* provides a biochemical comparison of the polymorphic variants of CK. One of the most polemic subjects in evolution in the recent years has been the adaptive nature of protein polymorphism. The authors in the present study believe that organisms and the environment as a whole will stress the adaptive nature of enzyme/ protein polymorphism because the adaptation is dependent on the isozymes/ isoforms. This study has revealed that because the fish lives in diverse environmental conditions and as such to meet the challenge of the varying conditions, adaptive nature of the protein/ enzyme polymorphism is essential. A wide range of functional demands of an enzyme cannot be met by a single enzyme/ protein form in many cases and as such isozymes/isoforms have evolved that may differ in kinetic properties, sensitivity to effectors, susceptibility to covalent modification, binding, interactions with other macromolecules, sub-cellular location, tissue distribution and so forth.Polymorphism can be considered as an optimal strategy in a highly unpredictable environmental situation such as fish habitat and therefore concordant changes result in the relative proportions of the polymorphs.Differences in net electric charge, as a function of amino acid substitution and detectable by electrophoresis are indicative of the differences in the genetics of isoallelic proteins.

IV. Conclusion

It is concluded that a wide range of functional demands by an enzyme or protein cannot always be met by single enzyme or protein form and therefore, isozymes/ isoforms have evolved that may differ in kinetic properties, sensitivity to effectors. The variation in the electrophoretic mobility of CK- isozymes provides much information on the genetic variation at the molecular level. Isozymes reflect variations in the amino acid sequences of the genes encoding them which lead to a difference in the isozyme's overall ionic charge.The CK- enzyme/ protein polymorphism could be influenced by methyl parathion for biochemical adaptation eventually leading to survival of the fish. Authors firmly believe that the manifestation of isozymes/ or isoforms appear for a biochemical adaptation.

Acknowledgement

The authors thank the Head of the Department of Zoology, Patna University, Patna for providing necessary laboratory facilities.

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