STUDIES ON LACTATE DEHYDROGENASE ISOZYMES IN *MUGIL CEPHALUS* AND *LIZA PARSIA*

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C E R T I F I C A T E

This is to certify that this dissertation is a bonafide record of work carried out by Shri. RAVI.N. under my supervision and that no part thereof has been presented before for any other degree.

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C O N T E N T S

P R E F A C E

Realising the importance of applying genetic principles in aquaculture programme in our country and the long term needs for preserving the natural genetic resources, fish genetics has been identified as a priority field for research and development.

Genetic studies using isozymes have made a substantial contribution towards efforts at preserving genetic resources of fish. They allow identification and description of the population genetic structure of species interest. They thus provide the basis for development of appropriate sampling and preservation procedures for particular species.

Lactate dehydrogenase in fishes has been used as a genetic marker for the investigation of several biological problems. This is partly because of its tetrameric structure, its ability to dissociate and hybridize without appreciable loss in enzyme activity. (Markert and Faulhaber, 1965). Also in most of the fishes, this enzyme has been found to be polymorphic.

The present work was aimed at standardizing the electrophoretic conditions to study the enzyme LDH in

detail and to study certain biochemical characteristics of the enzyme in the mullets Liza parsia and Mugil cephalus.

In the present study it has been possible to standardize experimental conditions like duration of tissue storage, polyacrylamide concentration and concentrations of substrate and cofactor in staining solution. The use of different combinations of acrylamide and bisacrylamide in this study has shown the scope for detecting a greater number of alleles. The different responses of the 5 LDH isozymes to varying concentrations of NAD and lithium lactate indicate that such studies can be extended for further individual biochemical characterization of the five isozyme as a function of activators, inhibitors, temperature, pH etc. The tissue specific expression of LDH observed in this study is distinct from that of other teleosts. Two different methods for estimating enzyme activity have been compared. Differences in enzyme activity between tissues and species have been demonstrated.

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INTRODUCTION

Lactate dehydrogenase is an essential respiratory enzyme. It serves to catalyse the reversible reactions of lactate in to pyruvate. Lactate dehydrogenase (LDH) exists in multimolecular forms, termed as isozymes. Vertebrates possess atleast two LDH Loci each encoding a different poly-peptide. In the tissues of the majority of fish(es) usually five tretrameric isozymes (A_4, A_3B_1, A_2B_2, A_1B_3 and B_4) are present which are the products of the combination of subunits A and B genetically controlled by two different loci (Markert, 1963). Lactate dehydrogenase isozymes are readily separable by electrophoresis and are useful gene markers for investigating gene action during embryogenesis and the mechanism of evolution of homologus genes.

Most Teleosts possess 3 genetic loci, coding for LDH subunits. The third loci is restricted to highly differentiated tissues like eye (LDH E_4) and the liver (LDH F_4). The retinal specific LDH (with kinetic properties suitable for aerobic conditions) is localized in the ellipsoidal region of the photroeceptor cells containing high concentrations of mitochondria (Whitt et. al., 1968). Immunochemical and phylogenetic data

suggest that the LDH E gene resulted from a duplication of the LDH B gene. The *Cypriniformes and Gadiformes* which lack the anodal retinal specific. LDH possess a cathodal specific, LDH isozyme (LDH F_4) predominantly synthesized in liver. The liver specific isozyme differs from the retinal specific isozyme in its electrophoretic mobility and its lability to heat denaturation (Whitt et. al., 1968). Besides the ubiquitous A and B loci, mammals and birds possess a third locus, the 'E' locus, which functions only in spermatocytes (Zinkham et. al., 1969). In Fundulus heterolitcus 3 tissue specific LDH loci, viz., A, B and C have been found (Whitt, 1969). Locus A predominated in white skeletal muscle, B locus predominated in heart, liver, and erthrocytes and C locus was found to be specific for eye tissue. Similarly in Gambusia affinis locus A was found principally in skeletal muscle, B locus in liver, but the C locus both in neural and retinal tissue. (Whitmore., 1978).

The LDH pattern in different size-classes of milk fish has been studied by Requintina et. al., (1981). The patterns were found to be tissue specific. They have identified 3 loci A, B and C and reported that the

subunits were present in all tissues (eye, liver, heart and skeletal muscle), but their relative concentration was tissue specific.

The distinctive isozyme synthesized in the nervous system was absent in the eyeless Mexican cave fish (Anoptichthys jordani) and other characins possessing normal eyes (Gregory et. al., 1970). In 23 species of freshwater teleosts other than A and B, two additional locus, E, specific of retina, lens and brain and L, specific of liver parenchyme, spleen and mid and hind gut were identified (Callegarini and Ricci, 1974).

The specificitity of LDH loci to specialized tissues could be attributed to factors like, physiological role of tissues, production of lactate etc. A negative corelation between the respiratory capacity and the occurence of heart type LDH was found by Gesser and Poupa (1973). They also found a positive corelation between the capacity of skeletal muscle to produce lactate and the 'H' type iso-enzymes in heart muscle.

In those fishes like salmon and carp which have a tetraploid chromosomes, 3 LDH loci have been duplicated.

In the tench (Tinca tinca), crucian carp (Carassius carassius) and carp (Cyprinus carpio) the isozyme patterns were tissue and species specific. In crucian carp, subunits with different electrophoretic mobility are present which are genetically controlled from the B^1B^2, A^1A^2 and C loci, while the set of loci in common carp is B^1B^2, A, C^1 and C^2. But in tench there are only one set of loci viz. A,B and C (Valenta et. al., 1977).

Lactate dehydrogenase was the first duplicated enzyme system in salmonids to be described. The diploid ancestor of the salmonids apparently possessed 3 LDH loci, a muscle specific locus, for LDH A, an eye specific locus, for LDH-C, and a third locus found in all tissues, for LDH B (Markert et. al., 1975). As many as eight LDH Loci have been suggested to be present in extant salmonids (Massaro and Markert, 1968). Ivaniva et. al., (1973) found that carp LDH isozyme spectrum contained 17 or even more bands grouped in several series - A^1 and B^1 A^2 and B^2, both in liver, E^1 and E^2 in eyes, heart and other organs and B^3 and E^1 (found everywhere). All the series except last had 5 or 6 isozymes.

Studies on the effect of storage on the resolution of isozyme pattern is very essential when the isozymes are

employed in genetic study. One may mistake the extra bands which appear due to storage for polymorphic phenotype. Hodgins et. al. (1969) found that freezing and thawing of sockeye salmon (Oncorhynchus nerka) sera, frozen for 6 years, didn't markedly alter LDH patterns. However they noted that discrete bands of LDH couldn't be resolved from a few of the long frozen sera and that two weak extra bands of LDH enzyme activity were frequently present anodal to LDH-1, particularly in older samples. The effect of freezing and thawing on LDH and esterase isozyme patterns of the gold fish Carassius auratus was demonstrated by Burch et. al. (1983) by means of isoelectric focussing.

Prolonged storage of samples would affect the activity of enzymes. A decrease in the activity of Adenosine monophosphate deaminase was observed by Cheuk et. al. (1979), during the early period of ice storage in pink and brown shrimp. They observed a complete loss of activity after 10 days in pink shrimp and after 16 days in brown shrimp. A sudden loss in the activity of Malate dehydrogenase and Lactate dehydrogenase was observed from the 16th day of storage in the fish Trachurus murphyi. An alteration in the band pattern

was observed from 32nd day onwards (Alay et.al., 1984).

The effect of storage on the products of different LDH loci have been found to vary. Kankova et. al. (1983) studied the changes in total LDH activity and the activity corresponding to subunit composition of LDH isozymes in the stored samples prepared from liver, white muscle and red muscle of tench (Tinca tinca). Samples had been stored at 25°C, 4°C and -20°C for 24, 48, 72 and 192 hrs. They found that isozymes composed of 'A' subunits were highly stable, while 'B' subunit were next to 'A' subunits in stability and 'C' subunits had the least stability.

In population genetics and other fields, where electrophoresis is used in a comparative way, one wishes to distinguish between electrophoretically homologus proteins. There is greater scope of detecting more number of alleles, when optimum concentrations of acrylamide and bisacrylamide (cross-linker) are standardized for the system concerned. Homologus proteins, which are known to differ from each other can be resolved by choosing the optimum cross-linker, concentrations. For example two forms (wild and modified) of Xanthine dehydrogenase (Xdh) of Drosophila melanogaster

are homologus proteins, but biochemically they are distinct. The two forms of Xdh have identical mobility at bisacrylamide concentration of 5%. But these proteins are readily resolved when cross-linker concentration is varied (Johnson, 1979). Parag (1984) found that the combination of 10% acrylamide (A) with 4% bisacrylamide (BA) resolves the protein system in the tissues of L. parsia in to more number of bands than other A and BA combinations.

The LDH pattern has been used to confirm or redefine taxonomical groupings. The LDH pattern in 33 species of Gadiform fishes, belonging to different genera and grouped under four suborders, have been studied by Shaklee and Whitt (1981). All species exhibited 3 homopolymers, LDH A_4, B_4 and C_4. Their electrophoretic mobility and tissue distrubution was species specific, with fishes belonging to each suborder having common pattern. Scholl and Herzberg (1972) have used the LDH isozymes of 17 species of South American cichlids to group them into six distinct groups, which do not correspond to the conventional grouping by morphological classification.

Species specifity of LDH isozymes has been extensively studied in fishes. LDH pattern in 5 different

Channa species have been studied by Chatterjee and Jyotidhar (1985) who found them to be species specific. Based on different Lactate dehydrogenase zymograms obtained for Tilapia species, Chen and Tsuyuki (1970) have suggested that, they could be used to differentiate the substrate spawners T. zilli and T. melanopleura from the other two mouth breeders, T. mossambica and T. hornorum. It is also possible to identify hybrids between species using LDH isozymes. The F_1 hybrids from salmon x sea trout expressed a classic hybrid isozyme pattern but the F_2 hybrids had the parental sea trout pattern. Extra bands in trout eye had been observed, which were heteropolymers and composed of subunit 'C' in various combinations with subunits A and B (Joyce et. al., 1973).

Ontological change in the expression of LDH system during different developmental stages has been observed in Liza parsia (Parag, 1984). In two species of cat fish, Trachurus haymela and T. muticus characters like the number, position and density of LDH Zones, have been found to be influenced by fish length (Wang and Yin, 1983).

Biochemical genetic data has served as a means for identifying discrete stocks and separating mixtures

into component stocks (Gharrett et..al., 1983). Since LDH is one of the highly polymorphic enzyme in fishes, this enzyme has been extensively used to separate fish stocks. The existence of 5 muscle LDH phenotype in megrim (Lepidorhombus whiff-iagonis) populations from the English channel has been reported by Dando (1970). He observed significant differences in phenotype distribution between megrim samples, caught in different areas of the channel in May and no significant difference in samples caught during Nov-Dec.

Poly·morphism of LDH loci have also been detected in a number of fishes. Hodgins et. al. (1969) observed 3 LDH phenotypes in sera of sockeye salmon (Oncorhynchus nerka). They suggested that random combinations of 3 types of subunits (B, B^1 and A) resulted in 9 isozyme bands. The same number of bands in heterozygotes was observed by Morrison and Wright (1966) in lake trout. Polymorphic loci have been identified in fishes belonging to Cyprinidae by Valenta (1978). He has reported that in bitterling locus B was polymorphic. In white bream, locus A was found to be polymorphic. In bream, rudd, silver, carp and barbel, polymorphism was found at C locus. The B locus of LDH in tench, the B^2 locus in crucian carp and the B^1C^1 and C^2 loci in carp were found to be polymorphic

each with two alleles (Valenta et. al., 1977). The absence of polymorphism at LDH loci has also been reported in fishes like, Gambusia affinis (Whitmore, 1978).

The detection of genetic variation by gel electrophoresis can also be applied to the problem of preserving genetic variation in fish species. Ryman (1980) analysed the distribution of genetic variation at 3 loci, coding for Creatinine phosphokinase, LDH and Glycerophosphate dehydrgoenase in hatchery stocks and natural populations which have been subjected to various degree of human manipulations. The genetic pattern observed in waters affected by human perturbation indicate, that the disturbance have drastically altered the distribution of genetic variation in those areas and that the genetic characteristics of previously existing populations have most likely been lost. Similarly Ivaniva et. al. (1973) found changes in allele frequency at locus A and B between cultured and wild carp.

Allele frequency data could be also used to study the migration pattern of populations. Varnavskaya (1984), identified two polymorphic loci ($LDH\text{-}B^1$, Pgm) in some spawning sockeye salmon. He observed that depending on the type of spawning grounds, the frequency of the $LDH\text{-}B^1$ allele changed from 0.1 to 0.9.

The occurrance of different alleles at LDH-loci is also reflective of physiological performance of the individual. Varnavskaya (1984) has reported that the adaptive significance of the LDH-B^1 allele in the spawning sockeye salmon seemed to be connected with higher swimming stamina, in conditions of high current velocity, low temperature and a high content of dissolved oxygen. Environmental parameters, like rapid water flow and tendency of rainbow trout to swim against current have been found to influence the phenotypic expression of LDH B^4 (Northcote et. al., 1970).

Isoenzymes study in the mullets is very limited. Some amount of work on the mullets available in the brackish water and estuaries of Cochin area has been carried out by Parag (1984) and Mary (1985). Parag(1984) studied the expression of general protein and Esterase enzyme system in liver, kidney, heart, eye, muscle and brain of Liza parsia and identified 3 groups of esterase system with a total of 6 bands. He analysed the LDH system in different tissues of Liza parsia, and noted that LDH pattern was tissue specific. Mary (1985) studied the expression of 7 enzymes in Liza parsia via., Esterase, Acid phosphatase, Alcohol dehydrogenase, lactate dehydrogenase, Tetrazolium oxidase, Malate dehydrogenase and Maleic enzyme, in muscle, liver, heart,

brain, kidney and stomach and found that enzyme patterns were tissue specific.

Isoenzyme patterns of mullets of Porto Novo was studied by Reddy et. al. (1979). They studied the isoenzymes (Succinate, Lactate, and Malate) in the mullets, L. parsia, L. macrolepis and Osteomugil cunnecius. More than 3 isoenzyme pattern have been observed in heart, liver, spleen, and gonadal tissues and a common slow moving LDH in heart, spleen and muscle tissues. All the above work were of general nature, dealing simultaneously with a number of enzymes.

The present work was aimed at studying the LDH system in detail in two culturably significant mullets, M. cephalus and L. parsia. An attempt was made to standardize the electrophoretic conditions with a view to study certain biochemical characteristics of the LDH system.

In view of this, studies on M. cephalus and L. parsia were carried out with the following three objects.

1. Standardization of electrophoretic conditions like
   a) Acrylamide concentration
   b) Concentrations of co-factor and substrate in staining of LDH Bands.

c) Duration of storage.

2. To study the genetic variation of the enzyme Lactate dehydrogenase in adult *Liza parsia*

3. To compare the enzyme activity in different tissues of adult and fingerlings of *L. parsia* and *M. cephalus*.

MATERIALS AND METHODS

In the electrophoretic study of any enzyme system, a number of variables need to be standardized to obtain better resolution. Some of the variables have already been standardized for the electrophoretic study of *Liza parsia* by previous workers, (Table 1).

In *Liza parsia*, Parag (1984) had standardized the polyacrylamide concentration for separation of proteins. He found that 10% Acrylamide with 4% Bis-acrylamide gave optimum results. He employed the same combination to study LDH. But clear resolution of a particular isoenzyme into its components also greatly depends on the polyacrylamide concentration which would be different from that of proteins. Once separated, using proper acrylamide concentration, then the visualisation of the bands require optimal concentrations of cofactor and substrate in staining solution.

In the present study keeping in mind the above factors the following variables have been standardized.

1.1. Quantity of sample

1.2. Polyacrylamide concentration

TABLE-1

Variables standardized for electrophoretic study of Liza parsia

No.	Variable	Range tested	Optimum	Tested for	Reference
1.	Tank buffer	Six buffers	Tris-Glycine p^H 8.9	LDH	Parag (1984)
2.	Amount of tissue mg/ml	30, 60, 80	60	Protein	Parag (1984)
3.	Extraction solvent	i) Double distilled water (DDW) ii) DDW with 0.001 mercaptoethamol iii) DDW with 0.07M sucrose iv) 0.2M Tris-Hcl buffer	DDW	LDH	Mary (1984)
4.	p^H of staining solution	7, 7.5, 8.5, 9.0	7.5	LDH	Parag(1984)
5.	Acrylamide concentration	Acrylamide -Bisacrylamide %A %B 7 2;2.5;3.5;4.5 9 2;2.5;3.5;4.5 10 2.5;3;4;5	7% A with 4% BA	Protein	Parag (1984)

1.3 Cofactor and substrate concentrations

1.4 Duration of storage.

Having standardized the electrophoretic conditions, the comparative study of M. cephalus and L. parsia were carried out to determine

2.1 Tissue LDH expression by electrophoretic method

2.2 Genetic variation of LDH locus by electrophoretic method

2.3 Enzyme activity by serial dilution method.

2.4 Enzyme activity by spectrophotometric method.

The different combinations and experimental conditions under which the present study was carried out is indicated in Tables(2 & 3).

Test species

1. Mugil cephalus is a euryhaline fish with katadromic tendencies. Locally it is designated as Thirutha' by the fisherman of Kerala. The grey mullet occurs in the estuaries and brackish waters. M. cephalus is caught in the Chinese dip nets at Cochin barmouth. It is widely

TABLE-2

Species and tissues tested in the present study

No.	Nature of study	Species: *Liza parsia*						*Mugil cephalus*	
		Tissue*: E	M	L	H	B	G	E	M
1	Quantity of sample	+	-	-	-	-	-	-	-
2	Standardization of acrylamide concentration	+	-	-	-	-	-	-	-
3	Standardization of concentrations of cofactor and substrate for staining LDH bands	+	-	-	-	-	-	-	-
4	Storage effect	+	+	-	-	-	-	-	-
5	Tissue expression	+	+	+	+	+	+	-	-
6	Genetic variation	+	-	-	-	-	-	-	-
7	Enzyme activity by serial dilution method	+	+	-	-	-	-	+	-
8	Enzyme activity by spectrophotometric method	+	+	-	-	-	-	+	+

* Tissues studied

E	Eye tissue	H	Heart tissue
M	Skeletal muscle	B	Brain tissue
L	Liver tissue	G	Gills
		-	not tested

TABLE-3

Experimental conditions and variables used in the present study

No.	Variable tested	Experimental conditions			
		Size class*	Acrylamide** A/BA	Stain*** LL/NAD	Storage
1.	Quantity of sample	Juveniles	7/2.5	***	Fresh
2.	Acrylamide concentration	Juveniles	-	*** 5/1	Fresh
3.	Cofactor and substrate concentrations for staining LDH bands	Juveniles	7/4	-	Fresh
4.	Storage effect	Juveniles	7/4	5/1	-
5.	Tissue expression	Adult	7/4	5/1	Fresh
6.	Genetic variation	Adult	7/4	5/1	Fresh
7.	Enzyme activity by serial dilution method	Juveniles	7/4	5/1	Fresh
8.	Enzyme activity by spectrophotometric method	Fingerlings	-	-	Fresh

** A/BA - Acrylamide/Bisacrylamide
7/4 - 7% Acrylamide with 2% Bisacrylamide

*** LL/NAD - Lithium lactate/NAD
5/1 - 5mg/ml Lithium lactate with 1mg/ml NAD

*** - - Stain concentrations not kept uniform For testing acrylamide, initially the stain concentration was not kept uniform; after optimising stain concentration, the acrylamide tests were repeated with LL/NAD of 5/1

Size Class*

	Total length (mm)
Fingerlings	30-55
Juveniles	65-90
Adult	100-160

cultivated in different countries like Japan, Hong Kong, China, Phillippines, Korea, India, Israel, Italy etc., It is generally cultured in brackish waters and occasionally in fresh waters as in China and India.

It feeds on a variety of materials like diatoms, blue green algae and detritus. It has been recorded to grow to a size of 400mm in 1 year and 2.27 - 2.72 kg. wt. in 2 years. (Pillay, 1949). Jhingran and Natarajan (1969) have reported that in Chilka lake the fish attains an average length of 309 mm in its 1 year. Fecundity of this fish varies from 1.28 to 2.8 million.

At the Cochin estuary the population of M. cephalus was found to spawn from November to February (Krishnan, Per. Comm.) The young ones are available in the brackish water creeks and lagoons and the period of availability varies with places.

2. Liza parsia. Commonly called the 'Kanambu' by the local fishermen of Kerala, is a euryhaline fish and constitutes a thriving fishery in the estuaries and back waters of India (Jhingran, 1982).

L. parisa grows to a maximum size of 400 mm. Males of this species attains maturity at a size of

120mm and females at a size of 129mm (Kurup & Samuel, 1983). From West Bengal, Sarojini (1957) has reported a feundity of 2 to 6 lakhs in this species. It has been found that feundity of L. parsia found in Cochin estuary varies from 64,000 to 3,90,000 (Kurup & Samuel, 1983).

L. parsia spawns in the Cochin estuary from October to May and exhibits peak spawning during December to April.

Sampling method:

Adult specimen of both Liza parsia and M. cephalus were purchased from the local Chinese dipnet fishermen (Plate.1) of the Vypeen barmouth region. Fingerlings of Liza parsia and Mugil cephalus were collected from the ponds of Kerala Agricultural University fisheries station at Puduvypeen. All samples were transported in polythene transportation bags. About 8-10 adults of average length 130mm were transported in a bag of 18 litre capacity. These bags were filled to three-fourth with the same brackish water, from where the specimen were collected. Aeration was provided by means of manual agitation of the water throughout the period of transportation. The duration of transportation was about 45 minutes.

Plate. 1

Collection site of live specimens of
Mugil cephalus and Liza parsia
Chinese dip net at Vypeen Barmouth

About 15-20 fingerlings of average length 35mm were transported in a plastic bag of 10 litre capacity. One fourth of the bag was filled with the pond water then filled with oxygen. Maximum time taken for transportation was only two hours.

At the laboratory, the adult specimens were transferred to plastic lined circular pools of 1 ton capacity, filled with sea water of same salinity. The pool was provided with a biological filter and water circulation was maintained by an airlift pump. The fingerlings of L. parsia and M. cephalus were kept separately in fibre glass drums of 25 litre capacity, provided with aeration.

For population genetic study, whole eye tissue from freshly killed adult specimens was used. Live specimen were frozen in deep freezers for 48 hours. Whole eye tissue and skeletal muscle tissue, taken from just below the first dorsal fin of frozen specimens were used in the measurement of specific activity by serial dilution method. Eye and skeletal muscle tissues of freshly killed fingerlings of L. parsia and M. cephalus were used in the spectrophotometric estimation of lactate dehydrogenase activity.

Due to the restricted availability of M. cephalus specimens, all the studies carried out in L. parsia couldn't be repeated.

For both electrophoretic and enzyme activity studies, samples were homogenized with double distilled water (Mary, 1985) at the rate of 60mg of tissue/ml (Parag, 1984) in glass homogenizers. The homogenates were centrifuged at 4000 rpm for 10 minutes. All these procedures were carried out at lowered temperatures using ice.

Electrophoretic method:

Polyacrylamide dise gel electrophoresis, as described by Davis(1964)was followed. Tris-glycine tank buffer p^H 8.9 which was found to be optimum (Parag, 1984) for electrophoretic study of LDH was used.

Serial dilution method:

Specific activity of the enzyme lactate dehydrogenase in the eye and skeletal muscle tissues of juveniles of L. parsia and M. cephalus was estimated by serial dilution method as described by Klebe (1975). In this method, the enzyme sample was serially two fold

diluted, to a visual end point. The specific activity of lactate dehydrogenase was measured by (i) determining the isozyme titre (T), defined as mg protein/ml in the last visible band and (ii) determining the specific activity (S in units/mg protein) by applying the formula.

$$S = K/T$$

where $K = 1.6 \times 10^{-3}$ units/ml in the last visible band. The units/ml (U) in the starting material was calculated from the equation.

$$U = K\ (2)^{n-1}$$

where n = is the number of the tube producing the last visible band.

Protein concentrations were determined by the method of Lowry et. al., (1951) employing bovine serum albumin as a standard.

Measurement of Enzyme activity-Spectrophotmetric method

The activity of Lactate dehydrogenase was estimated in the eye and skeletal muscle tissues of fingerlings of L. parsia and M. cephalus by measuring the rate of appearance of DPNH at 340 mμ. The assay was carried out at p^H 10, since the reaction produced 1 equivalent of acid (Neilands, 1955).

Reagents:

1. 0.5M Lithium lactate - 4.801 gm lithium lactate dissolved in 100ml of double distilled water. p^H adjusted to 8.65 using 5N NaoH.

2. $2x10^{-}$"M DPN - 133 mg of NAD dissolved in 5ml of double distilled water. p^H adjusted to 6.0 with N, NaOH. Then the solution was diluted to 10ml. p^H of the final solution was 6.11.

3. 0.1M Glycine - 0.751 gms Glycine dissolved in 100ml. of double distilled water p^H adjusted to 10 with N NaOH.

Procedure

The activity measurements were carried out with a ECIL GS 865D UV/V senior model spectrophotometer at room temperature.

Exactly 1.8ml of glycine buffer, 0.1ml of lithium lactate solution, and 0.1ml of NAD solution were pipetted in to a 1cm quartz cell. The mixture was stirred with a smooth glass rod and the cell was placed in the holder. 0.02ml. of the crude extract was pipetted into the cell. The shutter was opened and the optical density change

at 340 mμ was measured at one minute interval for the first 15 minutes.

Optical density of the blank solution was measured at regular intervals of time and was found to be nil.

DPNH produced was calculated using the following formula.

$$C = \frac{O.D.}{(am)\ (1)}$$ (Segel, 1976)

where

C = Concentration of substrate produced.

am = Molar absorption Co-efficient

am for NADH = 6.22×10^3

l = Path length = 1 cm.

I. STANDARDIZATION OF METHODOLOGY

1.1 Quantity of sample

Different quantities of eye extract, 30μl, 40μl, 60μl were tested for lactate dehydrogenase system in Liza parsia to find out the optimum quantity of sample to get clear bands.

1.2 Polyacrylamide concentration

In the original method as described by Davis (1964) the following combination of acrylamide and bis acrylamide

have been used where bis-acryamide concentration was found to vary between acrylamide concentrations.

A% - 5	6.3	7	7.7
B% - 5	3.1	2.5	3.2

On the other hand Parag (1984) had used a fixed concentration of bis for all acrylamide concentrations (Table 1).

Acrylamide stock solution was prepared by dissolving 40 g of acrylamide in 100ml of double distilled water. Running gel solution (total volume 20ml) was prepared by mixing.

1. 1 part (5ml) of Tris-Hcl buffer pH 8.9
2. 1 part (5ml) of Ammonium per sulphate (catalyst-140mg./50ml distilled water)
3. 1 part (5ml) which contained respective volumes of acrylamide stock solution + double distilled water (Table 4).
4. 1 part (5ml) a combination of respective volumes of bis-acrylamide stock solution + double distilled water (Table 4).

TABLE - 4

Composition of the working gel solution (Total volume 20 ml)

No	Acrylamide		Bis-acrylamide		Double distilled water (ml)	Small pore buffer***	Ammonium per Sulphate****
	(%)	Solution A* (ml)	(%)	Solution B** (ml)			
1.	5	2.5	2	2	5.5	5	5
2.	5	2.5	3	3	4.5	5	5
3.	5	2.5	4	4	3.5	5	5
4.	5	2.5	5	5	2.5	5	5
5.	7	3.5	2	2	4.5	5	5
6.	7	3.5	3	3	3.5	5	5
7.	7	3.5	4	4	2.5	5	5
8.	7	3.5	5	5	1.5	5	5
9.	9	4.5	2	2	3.5	5	5
10.	9	4.5	3	3	2.5	5	5
11.	9	4.5	4	4	1.5	5	5
12.	9	4.5	5	5	0.5	5	5

No.	Acrylamide		Bis-acrylamide		Double distilled water (ml)	Small pore buffer***	Ammonium per Sulphate****
	(%)	Solution A* (ml)	(%)	Solution B** (ml)			
13.	10	5.0	2	2	3.0	5	5
14.	10	5.0	3	3	2.0	5	5
15.	10	5.0	4	4	1.0	5	5
16.	10	5.0	5	5	0.0	5	5

Solution A* - Acrylamide stock Solution 40 gms/100 ml DDW

Solution B** Bis-Acrylamide stock solution 2.1 gm/100 ml DDW

Small pore buffer***

Tris-Hcl-pH 8.9

Ammonium persulphate****

140 mg/50 ml distilled water.

The % of acrylamide in the running gel solution was calculated as follows:

$$\% \text{ of acrylamide} = \frac{\text{Quantity of Acrylamide(g)} \times 100}{20 \text{ ml.}}$$

(20 ml-Total volume of running gel solution).

Bis-acrylamide concentration was calculated by the formula.

$$\% \text{ of bis-acrylamide} = \frac{\text{Quantity of bis-acrylamide(g)}}{\text{Total acrylamide(g)}} \times 100$$

Total acrylamide(g) = Quantity of acrylamide + bis-acrylamide in 20 ml.

Stock solution of bis-acrylamide was prepared by dissolving 1.475 g. of bis-acrylamide in 100ml of double distilled water.

The preparation of various combinations of acrylamide and bis-acrylamide and their respective % in the final solution is explained in Table, 4. This procedure was adopted to avoid arriving at different final molar values of buffer as done with other studies involving different acrylamide concentrations (Bo Gahne et.al., 1977)

A spacer gel of 3% was used. The 3% acrylamide solution was prepared by dissolving 10 gms. of acrylamide and 2.5 gms of bis-acrylamide in 100 ml of double distilled water. Tris-Hcl buffer p^H 6.7 with TEMED (N,N,N',N'- tetra methylene diamine) was used in the spacer gel.

A total of 16 combinations of Acrylamide (A) and Bis-acrylamide (BA)(Table 5) were tested to determine the optimum A:B ratio for best resolution .

1.3 Cofactor and substrate concentrations:

The staining procedure described by Redfield and Salini (1980) was adopted. Tris-Hcl staining buffer p^H 7.5, which was found to be optimum for LDH staining by Parag (1984) was used. The composition of the staining solution, used by various authors for staining LDH bands is given in Table, 6.

In the present work the following concentrations of NAD and Lithium Lactate (LL) were used in combination to find the optimum concentrations of these factors per ml of the staining solution.

NAD - 0.05, 0.5, 1.0, 2.0, 3.0, 5.0
(mg/ml)

LL - 5 and 10
(mg/ml)

TABLE-5

Combinations of Acrylamide and Bis-acrylamide tested

No.	Acrylamide (%)	Bisacrylamide (%)
1	5	2
2	5	3
3	5	4
4	5	5
5	7	2
6	7	3
7	7	4
8	7	5
9	9	2
10	9	3
11	9	4
12	9	5
13	10	2
14	10	3
15	10	4
16	10	5

TABLE-6

Composition of the staining solution for LDH employed by various workers

No.	Staining buffer			Incubation		Concentration mg/ml				Tested for	Reference
	Composition	Strength (M)	pH	Period (min)	Temperature (°C)	LL*	NAD**	PMS***	NBT****		
1.	Tris-Hcl	0.1	8	30	Room	9.6	0.3	0.02	0.2	Rainbow trout	Morrison and Wright (1966)
2.	Tris-Hcl	0.1	8.3	20-60	30	9.9	0.3	0.10	0.2	Sockeye Salmon	Hodgins et. al. (1969)
3.	Tris-Hcl	-	7.5	-	37	19.2	0.1	0.07	0.17	Gadiform fishes	Shaklee and Whiff (1981)
4.	Tris-Hcl	0.2	7.5	30	37	5.0	1.0	0.06	0.9	Mullets	Present study (Optimum conditions)

LL* Lithium lactate

NAD** Nicotine amide adenine dinucleotide

PMS*** Phenozonium methosulphate

NBT**** Nitroblue tetrazolium.

\- Not indicated.

The concentrations of Nitroblue tetrazolium and Phenozonium methosulphate were held constant at 0.9 mg/ml and 0.06 mg/ml respectively.

1.4 Duration of storage (at -4°C)

The present study was also carried out to find out the storage effect on the resolution of lactate dehydrogenase system in the eye and skeletal muscle tissues of Liza parsia. Following were the variables in the storage effect study.

a. Tissues

(i) eye

(ii) Skeletal muscle

b. Nature of tissue

(i) as tissue

(ii) as extract.

c. Hours of storage

d. Number of thawing.

Terminology.

Band		Homo/Hetero Polymer		Polarity
I	-	A_4 Homopolymer	-	Cathodal
II	-	A_1B_3 Heteropolymer		
III	-	A_2B_2 Heteropolymer		
IV	-	A_1B_3 Heteropolymer		
V	-	B_4 Homopolymer	-	Anodal

R E S U L T S

I. STANDARDIZATION OF METHODOLOGY.

1.1 Quantity of Sample.

The quantity of sample used was standardized and samples of 30 µl, 40 µl and 60 ul were tested for Lactate dehydrogenase system. While sample concentrations of 30 µl gave light bands, 60 µl samples gave diffused bands. But clear band with distnict margins were obtained with 40 µl sample. For the rest of the study 40 µl sample was used.

1.2 Polyacryalmide concentration.

With varying combinations of Acrylamide (A) and Bisacrylanide (BA), the intensity, thickness and inter space between bands have been observed to change (Table, 7 Fig.1). The zymograms (Fig.1) reveal the differences in the resolution of eye LDH of Liza parsia in 16 varying combinations of acrylamide and bis-acrylamide.

It had been observed that:

a. The number of bands was constant at 5% and 7% 'A' levels, for all the tested concentrations of BA%.

TABLE- 7

Differences in the resolution of Lactate dehydrogenase system in the eye of Liza parsia in gels having different percentages of Acrylamide (A) and Bis-acrylamide (BA) in 16 combinations (i to xvi)

No.	Gel(%)		No. of bands	Intensity of bands*					Thickness of Ist band(mm)	Interspace (mm)				Separation**	Diffusion***
	A	BA		I	II	III	IV	V		Origin to I band	I-V	II-III	III-IV		
i	5	2	5	4x	x	x	x	4x	2.0	14	15	8	3	+	+
ii	5	3	5	4x	x	x	x	4x	2.0	12	16	5	2	+	+
iii	5	4	5	4x	x	x	x	4x	2.0	12	16	6	3	+	+
iv	5	5	5	4x	x	x	x	4x	2.0	12	15	5	2	+	+
v	7	2	5	4x	2x	2x	2x	4x	2.0	11	12	3	2	+	+
vi	7	3	5	4x	2x	2x	2x	4x	1.5	9	10	3	2	+	+
vii	7	4	5	4x	2x	2x	2x	4x	2.0	9	10	2	2	+	+
viii	7	5	5	4x	2x	2x	2x	4x	1.0	7	9	1.5	2	+	+
ix	9	2	5	4x	x	½x	2x	4x	1.0	7	8	1.5	2	+	+
x	9	3	4	4x	x	-	2x	4x	0.5	5	7	-	-	(I↔II), (IV↔V)	IV
xi	9	4	4	4x	x	-	2x	4x	0.5	5	6	-	-	(I↔II), (IV↔V)	V
xii	9	5	4	4x	x	-	2x	4x	0.5	3.5	5	-	-	(I↔II), (IV↔V)	V

Table Contd.....

No.	Gel(%)		No.of bands	Intensity of bands*					Thickness of Ist band(mm)	Interspace(mm)				Separation**	Diffusion***
	A	BA		I	II	III	IV	V		Origin to I band	I-V	II-III	III-IV		
xiii	10	2	5	4x	x	x	x	4x	0.5	6	6	1	0.5	(III↔IV)	+
xiv	10	3	3	4x	-	2x	-	4x	0.5	5	5	-	-	+	+
xv	10	4	3	4x	-	2x	-	4x	0.5	4	4	-	-	+	I III
xvi	10	5	3	3x	-	x	-	3x	0.5	3	3	-	-	+	III

Intensity of bands*

4x	dark
3x	medium
2x	light
x	faint
½x	trace
-	absence of band

Separation**

+ -	Clearly separated
(I↔II)	Ist and IInd bands closely associated
(III↔IV)	IIIrd and IVth bands closely associated
(IV↔V)	IVth and Vth bands closely associated

Diffusion***

+	no diffusion
I	Ist band diffused
III	IIIrd band diffused
IV	IVth band diffused
V	Vth band diffused.

Fig. 1. Eye LDH of *Liza parsia* resolved in varying combinations of acrylamide and bisacrylamide.

Arrow indicates the direction of migration.

No. 1-5 denotes bands.

dark

medium

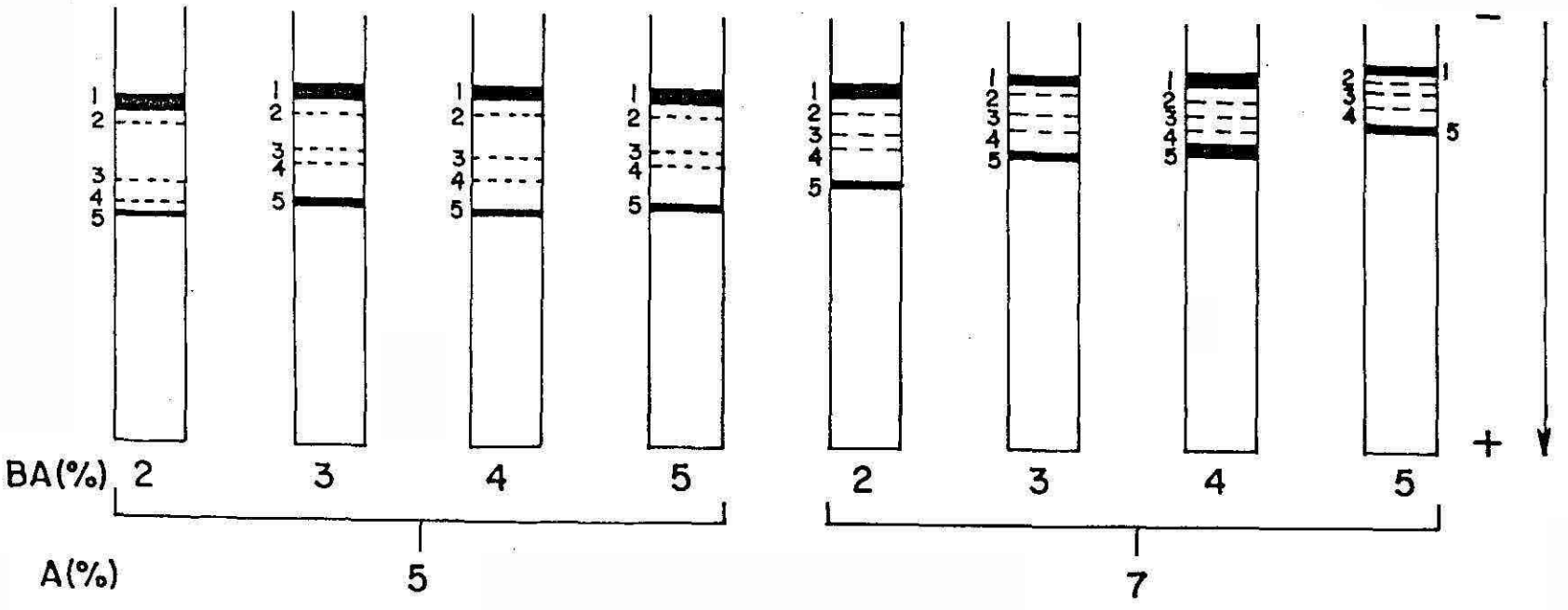
light

faint

trace

Fig. 1.

EFFECT OF ACRYLAMIDE AND BISACRYLAMIDE COMBINATIONS IN THE RESOLUTION OF EYE LACTATE DEHYDROGENASE OF LIZA PARSIA



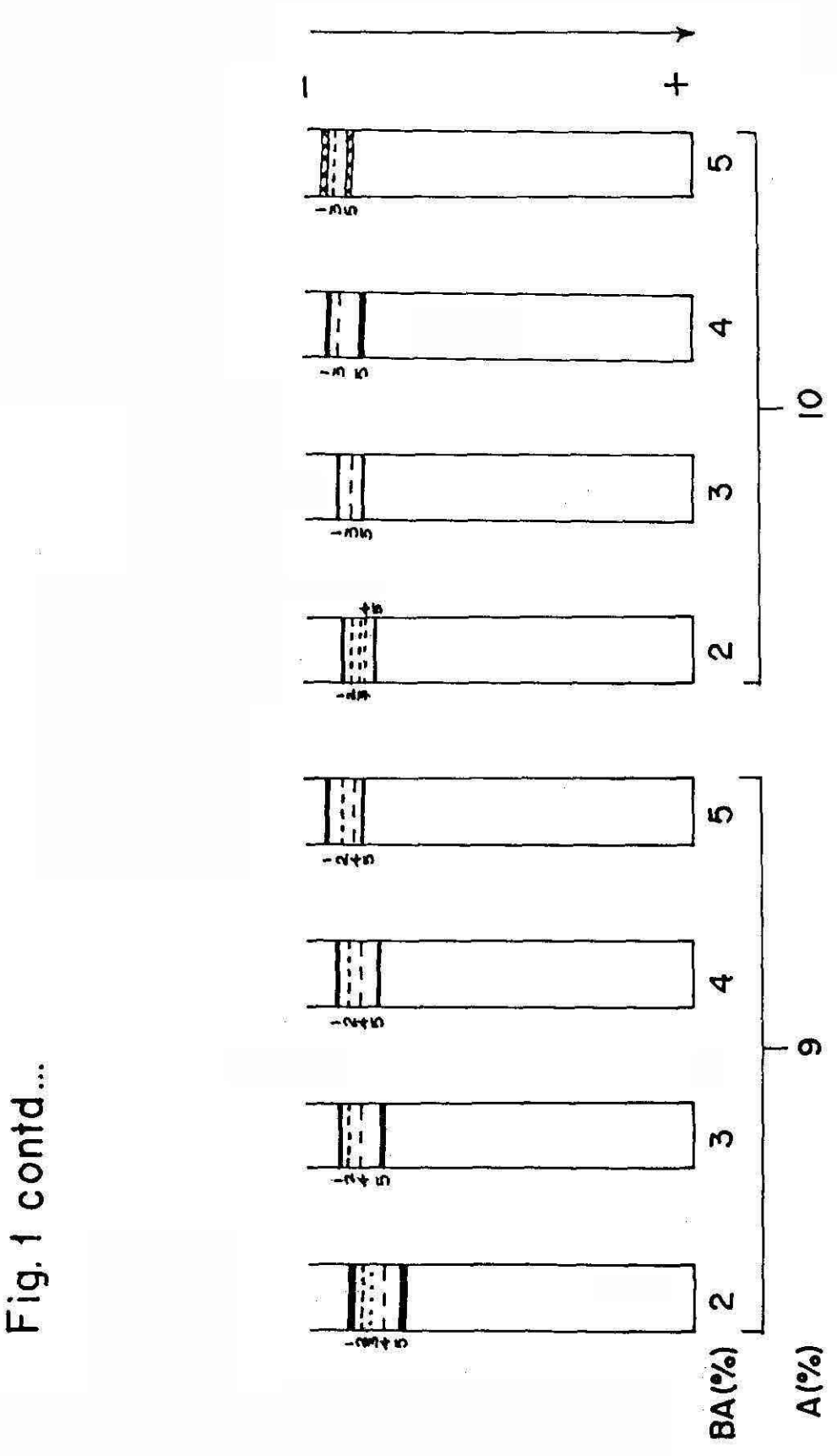


Fig. 1 contd...

But at 9 and 10% A level, there was a reduction in the number of bands to 4 and 3 respectively when BA% was increased to above 2%.

b. The intensity of the homopolymer (A_4, B_4) was constant (at 4x) for all the tested A and BA% levels, except at 10% A and 5% BA when it decreases to 3x.

c. The intensity of heteropolymer (A_3B_1, A_2B_2, A_1B_3) were maximum (2x) at 7% A and above or below this level of A% it decreased.

d. The thickness of Ist band decreased from 2 to 0.5 mm when A was increased from 5 to 10%.

e. The inter space between bands become reduced both when BA% was held constant and BA% increased or when BA% was held constant and A% increased.

f. The separation of bands was better at 5 and 7% A levels for all BA levels. At 9% A, for BA levels 3, 4 and 5, the bands (I II) and (IV V) were closely associated. In other BA% levels bands II and IV were absent.

g. There was no diffusion of bands at 5% and 7% A levels. But at 9%, increase in BA level above 2% causes diffusion of bands IV and V. At 10% 'A' level increase in BA above 3% causes diffusion of Ist and IIIrd bands.

It is obvious both from figure 1 and the above conclusions that 7% acrylamide with 4% bix-acrylamide gives the best resolution. In this combination the LDH system has been resolved clearly into 5 isozymes with equal spacing of A_3B_1 and A_1B_3 heteropolymers on either side of A_2B_2. The homopolymers (A_4 & B_4) has been resolved with equal thickness and intensity. Also the 3 heteropolymers (A_3B_1, A_2B_2 and A_1B_3) have equal thickness and intensity.

The same combination of acrylamide and bis-acrylamide was used to resolve LDH system in different tissues tested in all further studies.

1.3 Concentration of Co-factor(NAD) and substrate (Lithium Lactate)

It had been observed that the number, intensity and thickness of the bands were greatly affected by varying

combinations of NAD and lithium lactate (LL) (Table 8, Fig.2, Plate 2).

From the zymograms (Fig.2) it could be observed that at both levels of lithium lactate (5 & 10 mg) there is a increase in the number of bands with increasing NAD concentrations. At 5mg/ml LL, the intensity and thickness of bands also increasing until a NAD concentration of 1 mg, above which there is a reduction in the intensity of bands. The heteropolymers A_3B_1 and A_1B_3 were affected much, by the increasing concentrations of NAD. Also, there is a reduction in the thickness of homopolymers with increasing concentrations of NAD, beyond the level of 1 mg/ml.

When substrate concentration was held constant at 10 mg/ml, increase in NAD concentration had no effect on the thickness or intensity of homopolymers. But at this level of substrate, the homopolymers were better expressed even at lower concentrations of NAD, when compared with lower NAD concentrations at 10 mg LL. But increase in substrate concentration causes suppression, in the intensity of all heteropolymers, A_2B_2 being the worst affected.

TABLE- 8

Effect of varying combinations of Lithium lactate and Nicotineamide adenine dinucleotide (NAD) on the resolution of Lactate dehydrogenase system in the eye of Liza parsia

No.	Concentration mg/ml		No. of bands	Band Characteristics*					Distinct** Margin	Diffusion***
	Lithium Lactate	NAD		I	II	III	IV	V		
1.	5	0.05	2	2x –	0	0	0	2x	II, IV	+
2.	5	0.50	4	3 –	0	½x –	½x –	3x –	+	+
3.	5	1.00	5	4x (2mm)	2x –	2x –	2x –	4x (2mm)	+	+
4.	5	2.00	5	4x (1.5mm	½x –	2x –	2x –	4x (1mm)	II	II
5.	5	3.00	5	4x (1.5mm)	½x –	2x –	½x –	4x (1mm)	II, IV	+
6.	5	5.00	5	4x (1.5mm)	½x –	2x –	½x –	4x (1mm)	I,II,IV,V	I,II,IV,V
7.	10	0.05	4	4x (1mm)	½x –	½x –	0	4x (1mm)	III, IV	+
8.	10	0.50	5	4x (1mm)	½x –	x –	½x –	4x (1mm)	I	I

Table Contd.....

No.	Concentration mg/ml		No. of bands	Band Characteristics*					Distinct** Margin	Diffusion***
	Lithium Lactate	NAD		I	II	III	IV	V		
9.	10	1.0	5	4x (1mm)	½x -	½x -	½x -	4x (1mm)	I	I
10.	10	2.0	5	4x (1mm)	½x -	½x -	½x -	4x (1mm)	I, III	I, III
11.	10	3.0	5	4x (1mm)	½x -	x -	x -	4x (1mm)	I, II	I, II
12.	10	5.0	5	4x (1.5mm)	½x -	x -	x -	4x (1mm)	I, II	I, II

Band characteristics*

Intensity of bands

4 x	dark
3x	medium
2x	light
x	faint
½x	trace

Band thickness

( )	thickness of band
-	thickness below 0.5mm
0	absence of band

Distinct margin**

+	all bands with distinct margin
I	first band without distinct margin
II	second band without distinct margin
III	third band without distinct margin
IV	fourth band without distinct margin
V	fifth band without distinct margin

Diffusion***

+	no diffusion
I	first band diffused
II	second band diffused
IV	fourth band diffused
V	fifth band diffused

Fig.2. Differential expression of eye LDH of Liza parsia with varying combinations of substrate and cofactor.
Shaded portion above first band indicates trailing
Arrow indicates the direction of migration. No. 1-5 denotes bands.

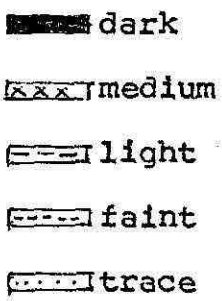


Fig. 2.

EFFECT OF NAD AND LITHIUM LACTATE (LL) ON THE EYE LDH OF LIZA PARSIA

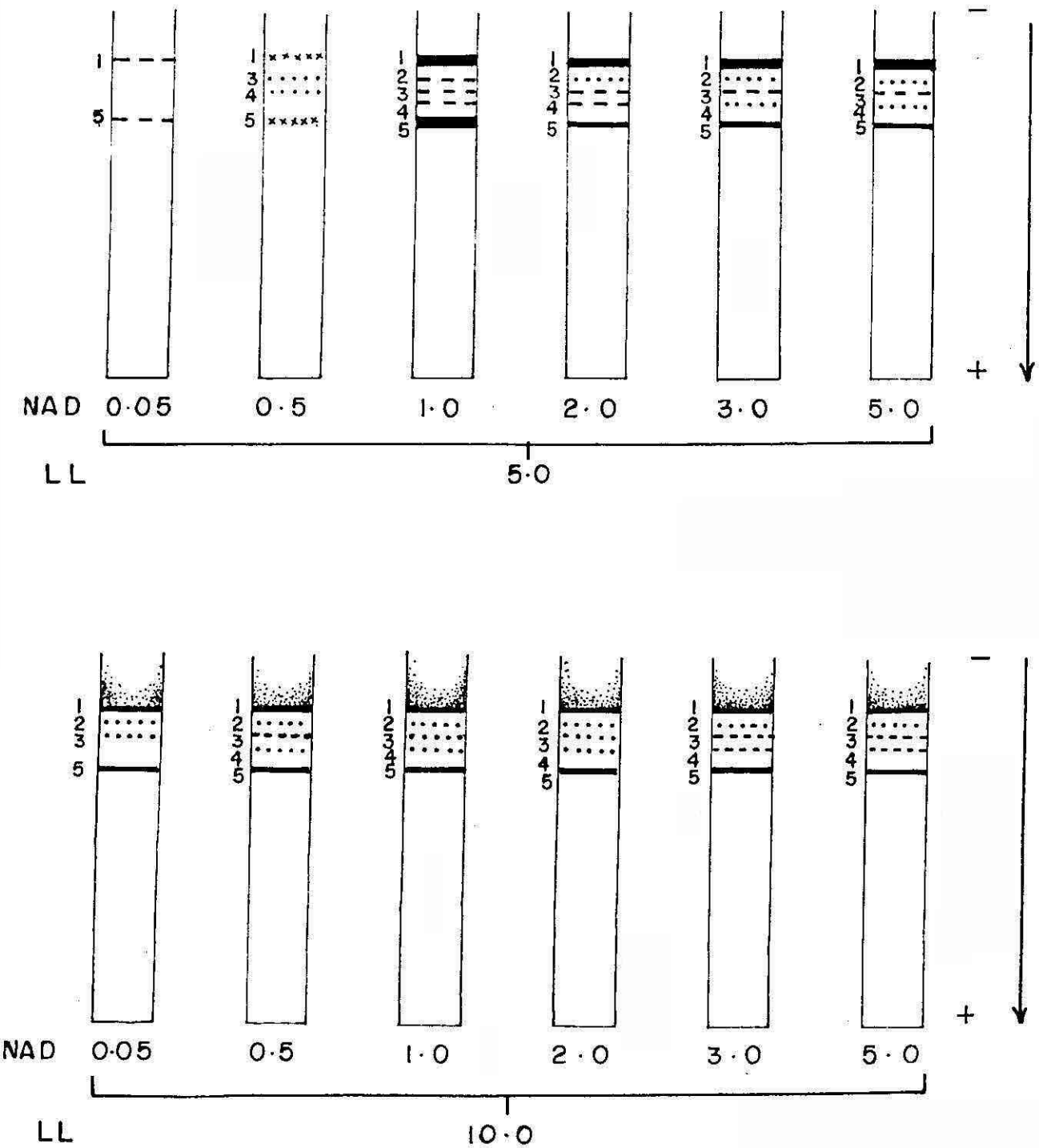


Plate.2

NAD (mg ml^{-1})	0.05	0.5	1.0	2.0	3.0	5.0
LL (mg ml^{-1})	5.0	5.0	5.0	5.0	5.0	5.0

Differential expression of LDH with varying combinations of substrate and co-factor.

The heteropolymers, A_3B_1 and A_1B_3 were found diffused at 3 different levels of NAD, 0.05, 3.00 and 5.00, when substrate concentration was held constant at 5 mg. Both the homopolymers were without distinct margin and diffused only when both substrate and NAD concentrations were held at 5 mg level. At 10 mg substrate level, A_4 homopolymer was found diffused without distinct margin, when NAD was increased beyond 0.05 mg/ml (Fig. 2). At 10 mg substrate level, A_3B_1 was found diffused at NAD levels of 3 and 5.

From the results it is clear that the LDH system was better expressed when the substrate and cofactor concentrations were 5 mg and 1 mg respectively. At this level of lithium lactate and NAD the LDH system was expressed with maximum number of bands; the homopolymers with equal intensity and thickness. All the three heteropolymers were also expressed with equal intensity. Hence this combination was used in vizualizing LDH bands in all further studies.

1.4 Duration of Storage.

Band characteristics such as intensity, thickness and interspace have been observed to change with storage time and frequency of thawing (Table 9).

TABLE-9

Effect of storage on the Lactate dehydrogenase system in the eye and skeletal muscle tissues of Liza parsia

No.	Tissue and number of thawing	Storage (hrs)	Band characteristics*					Dist-inct** margin	Interspace(mm)		
			I	II	III	IV	V		I-V	II-III	III-IV
	A. Fresh										
1.	Eye tissue	0	4x (2mm)	2x	2x	2x	4x (2mm)	+	10	2	2
2.	Skeletal muscle	0	3x (1mm)	2x –	0	0	x –	+	8	–	–
	B. Once thawed										
1.	Eye tissue	24	4x (1.5mm)	½x –	x –	x –	4x (1mm)	+	10	2	3
2.	Eye tissue	48	3x (1.5mm)	½x –	x –	½x –	4x (0.5mm)	+	10	2	3
3.	Eye tissue	72	3x (1.0mm)	½x –	x –	½x –	3x (0.5mm)	+	10	2	3
4.	Eye tissue	96	3x (1mm)	½x –	½x –	½x –	3x (0.5mm)	II	10	2	3
5.	Skeletal muscle	24	3x (1mm)	0	0	0	x –	+	8	–	–
6.	Skeletal muscle	48	3x (0.5mm)	0	0	0	½x –	V	8	–	–

Table Contd.....

No.	Tissue and number of thawing	Storage (hrs)	Band characteristics*					Distinct** margin	Interspace (mm)		
			I	II	III	IV	V		I-V	II-III	III-IV
7.	Skeletal muscle	72	4x (1mm)	x –	0	0	½x	V	8	-	-
8.	Skeletal muscle	96	2x (0.5mm)	½x –	0	0	½x –	II, V	8	-	-
9.	Eye extract	24	4x (1mm)	½x –	x –	x –	4x (1mm)	+	10	2	2
10.	Eye extract	48	4x (1mm)	½x –	x –	½x –	4x (1mm)	II, IV	10	2	3
11.	Eye extract	72	3x (1mm)	½x –	½x –	½x –	3x (1mm)	II, IV	10	2	3
12.	Eye extract	96	3x (0.5mm)	½x –	½x –	½x –	3x (0.5mm)	II, III, IV	10	2	1
	C. Twice thawed										
1.	Skeletal muscle	48	2x (0.5mm)	0	0	0	½x –	V	8	-	-
2.	Skeletal muscle extract	48	3x (1mm)	0	0	0	½x –	V	8	-	-
3.	Eye extract	48	4x (1mm)	x –	x –	x –	4x (1mm)	+	10	2	2

Table Contd.....

No.	Tissue and number of thawing	Storage (hrs)	Band characteristics*					Dist-inct** margin	Interspace (mm)		
			I	II	III	IV	V		I-V	II-III	III-IV
	D. Thrice thawed										
1.	Skeletal muscle	72	2x -	x -	0	0	0	V	-	-	-
2.	Skeletal muscle extract	72	2x (0.5mm)	0	0	0	½x -	V	8	-	-
3.	Eye extract	72	4x (1mm)	½x -	x -	½x -	4x (1mm)	+	10	2	2
	E. Four times thawed										
1.	Skeletal muscle	96	2x -	½x -	0	0	0	V	-	-	-
2.	Skeletal muscle extract	96	2x -	x -	0	0	½x -	V	8	-	-
3.	Eye extract	96	4x (1mm)	½x -	½x -	½x -	4x (1mm)	+	10	2	2
	F. Six times thawed										
1.	Skeletal muscle	144	3x (0.5mm)	0	0	0	0	II,V	-	-	-
2.	Skeletal muscle extract	144	3x -	½x -	0	0	0	V	-	-	-
3.	Eye extract	144	3x (0.5mm)	0	0	0	3x (1mm)	*	10	-	-

Table Contd.....

No.	Tissue and number of thawing	Storage (hrs)	Band characteristics*					Distinct** margin	Interspace(mm)		
			I	II	III	IV	V		I-V	II-III	III-IV
	G. Seven times thawed										
1.	Skeletal muscle	264	2x –	2x –	0	0	0	+	–	–	–
2.	Skeletal muscle extract	264	3x –	3x –	0	0	0	II	–	–	–
3.	Eye extract	264	2x –	0	0	0	3x –	I	10	–	–
	H. Eight times thawed										
1.	Skeletal muscle	408	3x (1mm)	x –	0	0	0	+	–	–	–
2.	Skeletal muscle extract	408	2x –	2x –	0	0	0	II	–	–	–
3.	Eye extract	408	2x –	0	0	0	0	+	–	–	–
	I. Nine times thawed										
1.	Skeletal muscle	840	3x –	½x –	0	0	0	II	–	–	–
2.	Skeletal muscle extract	840	2x –	2x –	0	0	0	II	–	–	–
3.	Eye extract	840	½x –	0	0	0	0	I	–	–	–

Table Contd.....

Band characteristics

Intensity of Bands		Band thickness		** Distinct margin	
4x	dark	( )	band thickness	+	all bands with distinct margin
3x	medium	-	thickness below 0.5mm	I	first band diffused
2x	light	0	absence of band	II	second band diffused
x	faint			III	third band diffused
½x	trace			IV	fourth band diffused
				V	fifth band diffused

(i) Once thawed:

Eye tissue: Even with 24 hrs of storage the distance migrated by the IVth band from origin increased from 15mm to 16mm. A gradual reduction in the intensity and thickness of homopolymers was observed with increase in storage time. The B_4 homopolymers showed more reduction in thickness, when compared with A_4 homopolymers. At the end of 96 hrs of storage, both A_4 and B_4 homopolymers had equal intensity, but differed in thickness. All the heteropolymers had equal intensity at the end of 96 hrs of storage, but the second band was without distinct margin.

Skeletal muscle.

At 72 hrs of storage A_4 homopolymer was expressed with increased intensity and thickness. Except for this, a gradual reduction in thickness and intensity of A_4 homopolymers was observed with increased hours of storage. After 24 hours, the intensity of B_4 homopolymer was reduced to traces, but it was observed even after 96 hrs of storage as a trace. The heteropolymer A_3B_1 which

disappeared at 24 hrs at storage, reappeared at 72 hrs of storage.

Eye extract.

With increasing hrs of storage both homopolymers showed the same rate of reduction in the intensity and thickness. Reduction in the intensities of A_2B_2 heteropolymer was observed only after 48 hrs of storage.

(ii) Thawed more than once:

Skeletal muscle.

Intensity and thickness of A_4 & B_4 homopolymers of twice thawed skeletal muscle was the same as that of once thawed skeletal muscle. B_4 homopolymer disappeared after third thawing. An increase in the intensity and thickness of A_4 homopolymer was observed after the 6th thawing. A_3B_1 heteropolymer reappeared after third thawing and disappeared after 6th thawing.

Skeletal muscle extract.

Traces of B_4 homopolymer was observed even upto the fifth thawing. A gradual reduction in the intensity

and thickness of A_4 homopolymer was observed upto fourth thawing. Then a sudden increase in intensity was observed upto the 7th thawing, beyond which there was reduction in the intensity. A_3B_1 heteropolymer reappeared after fourth thawing and traces of that band was observed after sixth thawing. Then there was a sudden increase in the intensity after 7th thawing. With further thawing a decrease in intensity was observed.

Eye extract.

A_4 and B_4 homopolymer of twice thawed eye extract had the same intensity and thickness as that of once thawed eye extract. Those bands had the same intensity and thickness even after fourth thawing. B_4 homopolymer disappeared after eigth thawing. Traces of A_4 homopolymer was observed after 9th thawing. A reduction in the intensity of heteropolymers was observed upto fourth thawing, after which they disappeared.

Taking into consideration all the results of the storage study certain general conclusions can be arrived.

a. Even 24 hrs of storage alters the expression of LDH system.

b. The effect of storage is more on muscle tissue LDH than on eye LDH.

c. The homopolymer A_4 is more stable than B_4. The homopolymer B_4 is more stable in extract than in the tissue.

d. The heteropolymer A_3B_1 of Skeletal muscle tissue is reassembled after it has completely disappeared at 264 hrs in muscle tissue and at 96 hrs in muscle extract.

e. Storage affects the mobility of heteropolymer A_2B_3 even by 24 hrs.

f. The distance migrated by the homopolymers is not altered by long duration of storage (upto 264 hrs) or number of thawings or even when the extract is tested.

g. Repeated thawings have a pronounced effect on the thickness of bands than on intensity of bands Table (10).

TABLE-10

Variation in the intensity and thickness of Homopolymers (A_4 & B_4) from that of fresh tissue expressed as Percentage*

Frequency of thawing	Tissue/extract	Intensity of homopolymers					Thickness of homopolymers				
		Storage: (Hrs) 24	48	72	96	96**	24	48	72	96	96**
Once											
	Eye tissue	100	88	75	75	-	63	50	38	38	-
	Eye extract	100	100	75	75	-	50	50	50	25	-
	Sketetal muscle	100	88	113	63	-	100	50	100	50	-
More than once	Eye extract	-	100	100	100	43	-	50	50	50	38
	Skeletal muscle	-	63	50	50	69	-	50	0	0	75
	Skeletal muscle extract	-	88	63	63	63	-	100	50	0	0

* The values (intensity/thickness)of Homopolymers of fresh tissue (Table 9) were added and fixed as 100%. The values for storage hrs 24, 48, 72, 96 and 96 were expressed as a percentage of the fresh tissue.

** This represents the average of 144, 264, 408 and 840 hrs of storage or average of those hrs above 96 hrs in which the respective parameters were expressed.

\- Not tested.

From the above observations it is seen that both duration and frequency of thawing affect the expression of isozyme bands.

II. OBSERVED VARIABLES.

2.1 Expression of Lactate dehydrogerase in different tissues.

LDH expression was found to be tissue specific (Table 11, Fig.3, Plate 3)

In the whole eye two loci Ldh-A and Ldh-B were identified with a total of 5 isoenzymes (I, II, III, IV, V)(Fig.4). Bands II, III and IV were lighter. Eye tissue expect lens (Fig.4) revealed all the 5 isoenzymes, identical in all respects as that of whole eye tissue. In lens alone (Fig.4) only 3 bands I, II and V were observed. The A_4 homopolymer was identical in terms of intensity and thickness with that of full eye. Though the intensity of B_4 homopolymer was same as that of full eye, it had only half the thickness as seen in full eye. The A_3B_1 heteropolymer were identical with that of full eye.

Brain tissue revealed six bands. Among these, the position of bands I, II & III was the same as observed

TABLE-11

Expression of Lactate dehydrogenase in different tissues of adult *Liza parsia*

No.	Tissues	No. of bands	Intensity of bands*					
			I	II	III	IV	V	VI
1	Brain	6	4x	3x	x	1/2x	1/2x	1/2x
2	Eye	5	4x	2x	2x	2x	4x	-
3	Muscle	3	3x	2x	x	-	-	-
4	Gills	2	4x	-	-	-	1/2x	-
5	Heart	2	4x	-	-	-	1/2x	-
6	Liver	1	4x	-	-	-	-	-

*Intensity of bands

4x	dark	x	faint
3x	medium	1/2x	trace
2x	light	-	not present

Fig. 3. Zymograms of LDH in different tissues of *Liza parsia*.

Arrow indicates the direction of migration.

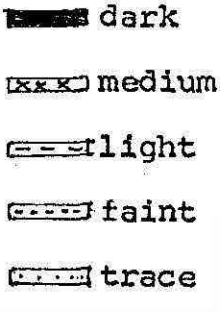
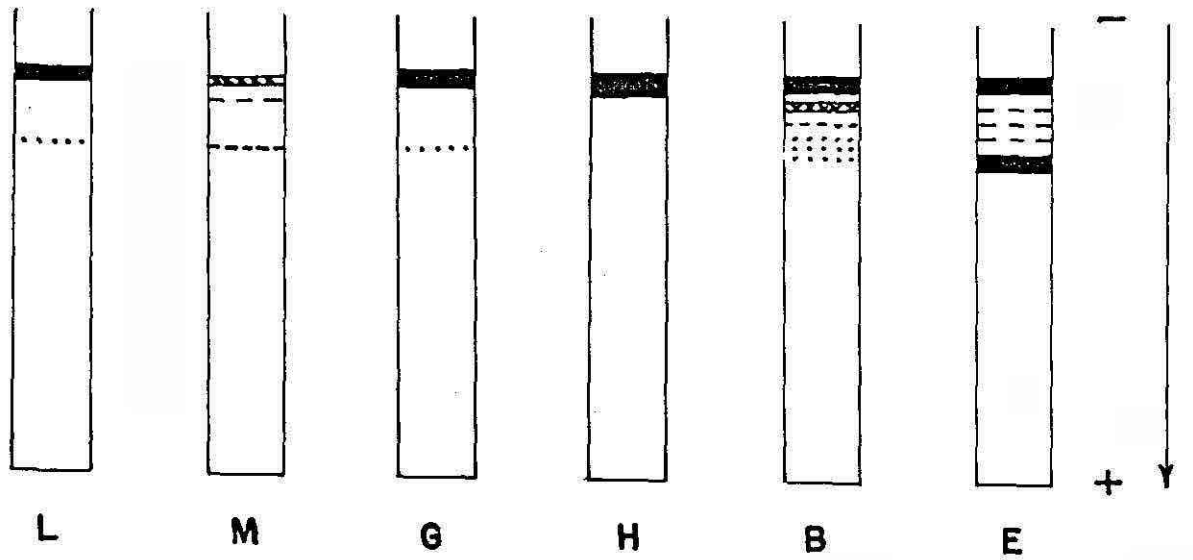


Fig. 3.

LACTATE DEHYDROGENASE EXPRESSION IN DIFFERENT TISSUES OF LIZA PARSIA



L - Liver

M - Skeletal muscle

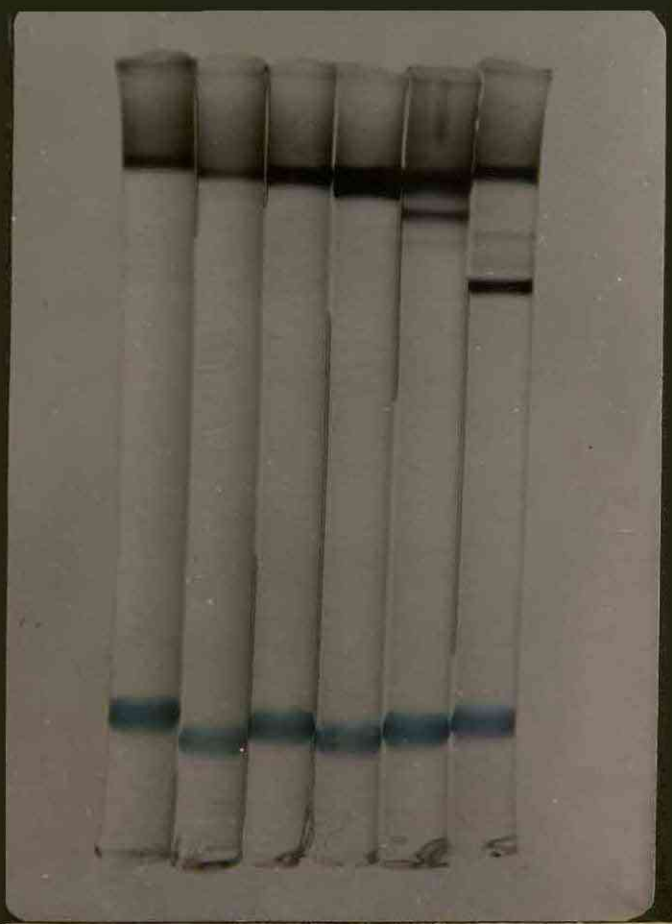
G - Gills

H - Heart

B - Brain

E - Eye

Plate.3



L M G H B E

Expression of LDH in different tissues of *Liza parsia*

L- Liver, M- Skeletal Muscle,
G- Gills, H- Heart, B- Brain,
E- Eye.

Fig. 4. Zymograms of LDH in different parts of the eye tissue of Liza parsia.

Arrow indicates the direction of migration

No. 1-5 denotes bands.

dark

medium

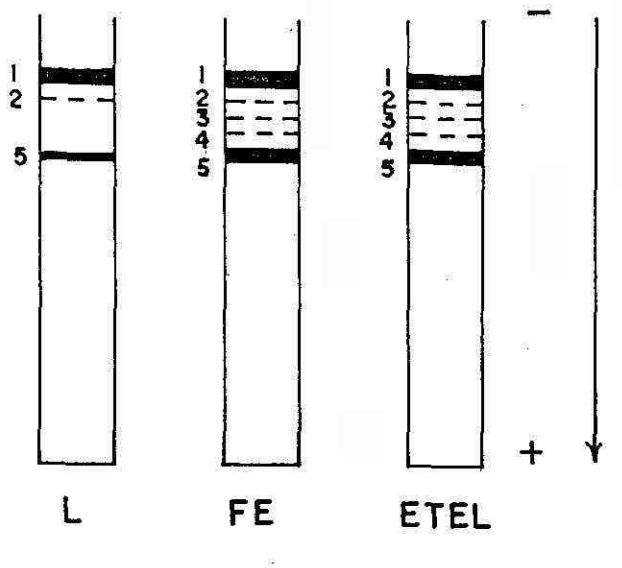
light

faint

trace

Fig. 4.

LACTATE DEHYDROGENASE EXPRESSION IN DIFFERENT PARTS OF THE EYE TISSUE OF LIZA PARSIA



L - Lens
FE - Full eye
ETEL - Eye tissue except lens

in the case of eye tissue, but they differed in intensity. Band I was darker, than the normal A_4 homopolymer. Band II had medium intensity. Band III was faint. Other 3 bands (4, 5 & 6) were faintly expressed.

Gill and liver revealed the expression of A_4 homopolymer with faint expression of the homopolymer B_4.

Three bands (I, II & V) were observed in the skeletal muscle. Among the three, band I was darkely stained, but when compared to A_4 homopolymer of eye tissue, it is lighter and less (1mm) thick. The heteropolymer (A_3B_1) was observed as a light band, whereas B_4 homopolymer was faintly expressed.

Parag (1984) observed all the three heteropolymers of eye tissue, as bands of different intensity. In the liver he observed the heteropolymer A_1B_3, a faint expression of the B_4 homopolymer in the heart tissue and a faint expression of the A_2B_2 heteropolymer in the skeletal muscle.

Mary (1985) identified all the isozymes of LDH in the skletal muscle she found that the B_4 homopolymer, and

the heteropolymers A_3B_1 and A_1B_3 were of equal intensity. She observed a faint expression of the B_4 homopolymer in the liver tissue.

The above observed differences could be attributed to different acrylamide concentration and staining parameters used by these workers.

2.2 Genetic variation:

No significant variation was observed among the 34 specimen tested for the LDH locus. Minor variation in the Rf values(Table 12) were observed, which are within the experimental error.

2.3 Enzyme activity by serial dilution method:

There was differences in the enzyme activity between tissues of an individual; between individuals of the same species and between tissues of two different species (Table 13).

In the case of L. parsia, for the eye, the last visible band was observed in the 9th tube and for muscle the last visible band was observed in the 6th tube. For eye tissue of M. cephalus, the last visible band was observed in the 12th tube.

TABLE-12.

Variation in the Rf values ($\bar{x} \pm$) of the homo and hetero-polymers (bands 1 to V) of the eye LDH in Liza parsia.

No.	Band	Rf Value	Mean($\bar{x}$)	S.D.($\pm$)
1	I	16.4-17.40	16.90	0.50
2	II	20.0-23.20	22.60	0.60
3	III	23.30-27.10	26.10	0.96
4	IV	26.60-31.00	30.10	0.82
5	V	34.70-36.80	35.60	1.01

TABLE-13

Variation in the activity of Lactate dehydrogenase in the juveniles of Mugil cephalus and Liza parsia

Sp. No.	Species and tissue	Units$x10^{-2}$/ml	Specific activity		
			Units$x10^{-2}$/mg protein Individual values	Mean	S.D.
	Liza parsia.				
	I. Eye tissue.				
1.		40.96	32.00	$34.5x10^{-2}$	0.33
2.		40.96	40.00		
3.		40.96	34.00		
4.		40.96	32.00		
	II. Skeletal muscle				
1.		5.12	4.57		
2.		5.12	4.20		
3.		5.12	4.80	$4.4x10^{-2}$	0.23
4.		5.12	4.40		
5.		5.12	4.10		
	Mugil cephalus. Eye tissue.				
1.		327.68	4.00		
2.		327.68	5.00		
3.		327.68	3.20	$3.925x10^{-2}$	0.68
4.		327.68	3.50		

Specific activity of LDH in the skeletal muscle tissue of L. parsia was less, when compared to that in the eye tissue of L. parsia. Butween individuals of the same species not much difference in the specific activity had been observed. The specific activity of LDH in the eye tissue of L. parsia was greater than that in the eye tissue of M. cephalus.

2.4 Enzyme activity by spectrophotometric method.

Eye LDH of Liza parsia and Mugil cephalus exhibited almost identical rate of reaction (Fig.5). A gradual reduction in the rate of production of DPNH was observed in both the cases.

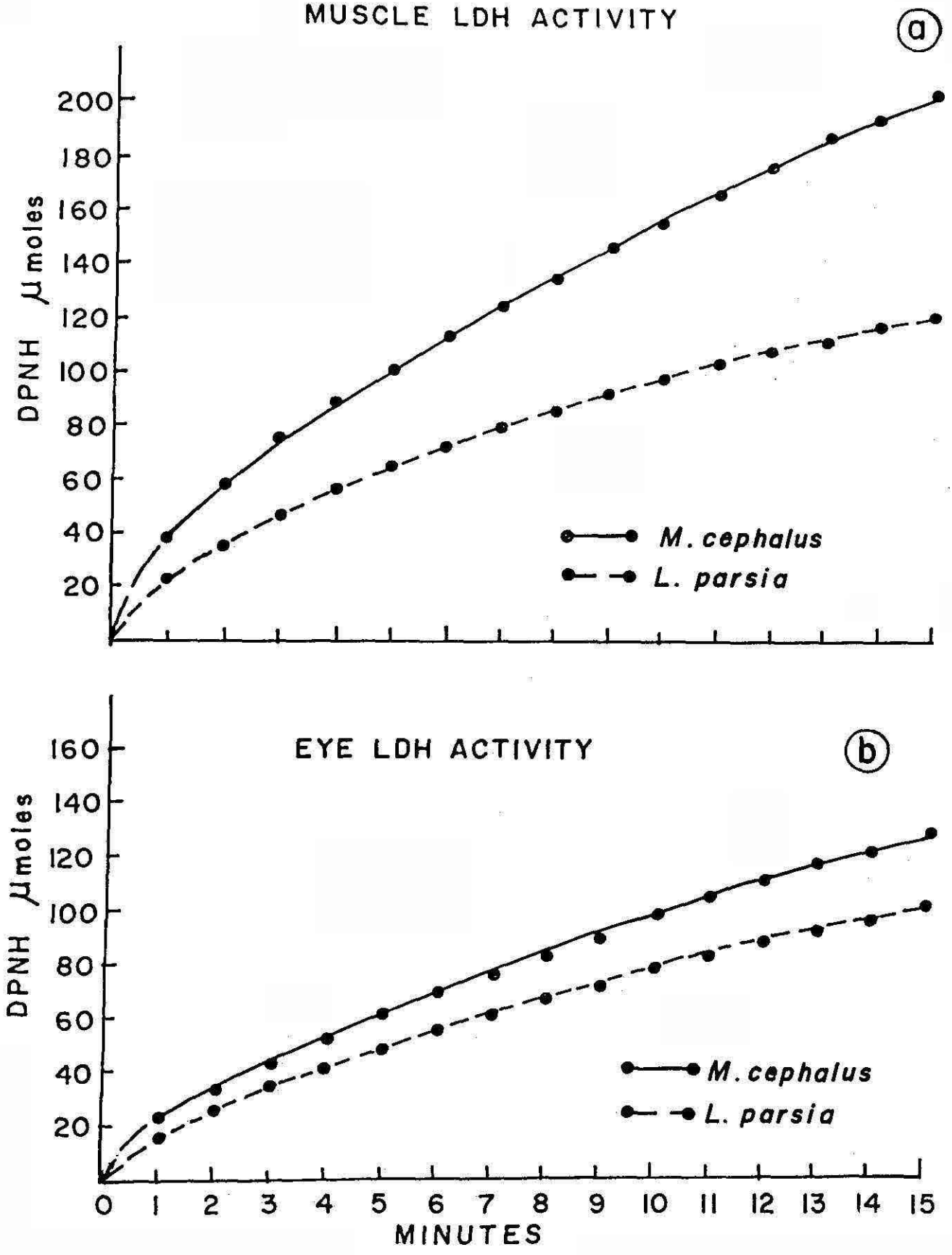
The activity of skeletal muscle LDH of both species was not comparable. M. cephalus exhibited a higher rate of production of DPNH during the intiial period of the reaction. When compared to L. Parsia the rate of production of DPNH was nearly uniform from 7th to 12th minute of the course of reaction in the case of M. cephalus. In L. parsia the decrease in the rate of production of DPNH was uniform from the sixth minute onwards.

In both species, skeletal muscle LDH was superior to eye LDH in activity.

Fig. 5. a. LDH activity (Cumulative DPNH(μ mol) produced in the muscle of Mugil cephalus and Liza parsia as a function of time of incubation (0-15 min).

b. LDH activity (Cumulative DPNH (μ mol) produced in the eye of Mugil cephalus and Liza parsia as a function of time of incubation (0-15 min).

Fig. 5.


MUSCLE LDH ACTIVITY
a
DPNH µmoles
200
180
160
140
120
100
80
60
40
20
M. cephalus
L. parsia
EYE LDH ACTIVITY
b
DPNH µmoles
160
140
120
100
80
60
40
20
M. cephalus
L. parsia
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
MINUTES

D I S C U S S I O N

The major advantages of electrophoretic methodlogy for genetic studies of populations are (i) the relative ease of application and efficiency of the techniques and (2) the direct relationship between electrophoretically detected protein variants and genetic variation.

The amount of genetic variation detected can be enhanced by the use of different concentrations of bisacrylamide (Johnson, 1979) and pH of gel buffer (Beaumont and Beveridge, 1983). Jhonson (1979) resolved the homologus forms of the protein Xanthine dehydrogenase of Drosophila melanogaster in to two distinct bands by varying cross linker concentrations. This was not possible at 5% bisacrylamide. Beaumont and Beveridge (1983) have reported differences in the number of phosphoglucomutase alleles depending on the pH of the tris-maleic electrode buffer used. In the present study a reduction in the number, change in electrophoretic mobility and thickness of Lactate dehydrogenase isozymes have been observed when bisacrylamide concentration was increased above 2%. The above findings in L. parsia offers scope for detecting more number of alleles by employing various bisacrylamide concentrations. Also when

polymorphism is detected one can choose the acrylamide concentration which would help in increasing the differences in relative mobility of the observed alleles and also increase or decrease the thickness of polymorphic isozyme as required.

In the present study it has been observed that the homo and heteropolymers of locus A and B react differently to changing concentrations of NAD and Lithium lactate. Also the response to increased NAD concentration was found to depend on the level of substract. Unless the enzyme is purified and separated into isozymes, it is not possible to get such kind of informations. The approach used in this study can be extended to study the effect of activators, inhibitors, pH, temperature etc.

In biochemical genetic study one may wish to detect a rare variant. The occurence of extra bands due to storage, will affect the interpretation of genetic data. Hence it is essential to know how long samples could be stored for studies concerning genetic variation and enzyme activity. Hodgins et. al. (1969) observed extra bands of LDH enzyme activity in sera of sockeye salmon stored for long duration. But in the present study no new extra

bands were observed. Only the disappearance of bands, the conversion of A_4 homopolymer to A_3B_1 heteropolymer was seen. The result of the study indicate that for estimating genetic variation, eye and muscle samples stored for 72 and 48 hrs respectively can be used. In Trachurus murphyi a sudden decrease in the activity of malate dehydrogenase had been observed by Alay et. al. (1984) after 16 days of storage. But in the present study a reduction in intensity and thickness of bands at even 24 hrs of storage indicate that the decline commences immediately after the tissue is stored.

The effect of storage is different on the subunits of LDH isoenzymes. In tench LDH isoenzymes composed of A_4 subunits had been found to be more stable than subunits composed of B or C polypeptides. (Kankova et. al., 1983) A similar picture was observed in the present study, where traces of A_4 homopolymer of eye LDH of Liza parsia was observed even after 840 hrs of storage, where as, B_4 homopolymer disappeared after 264 hrs of storage.

The tissue specificity of LDH observed in the present study is similar in some aspects to those found by many workers in other fishes. But the retinal specific C or E loci is not observed in mullets. Callegarini and

Ricci (1974) have identified retinal specific E locus in 23 species of fresh water teleosts. But like the mullets observed in the present study, eye specific isozyme of LDH was found to be absent in the nervous system of eyeless Mexican cave fish (Anoptichthys jordani) and other characins possessing normal eyes. (Gregory et. al., 1970). A comparison of tissue expression of LDH in both species of mullet reveal that B locus is more active in eye than in other tissue. There is a possibility that this locus might have taken the function of C or E locus observed in other fishes. Another deviation from the picture, observed in other fishes, is the difference in locus specifity between brain and eye. In the present study a difference in the intensity of homopolymers of brain LDH, from that of eye was observed. In other fishes (Callegarini and Ricci, 1974) the same pattern has been observed in the eye and nervous systems.

In Liza parsia fingerlings of Puduvypeen area, the locus B in eye had been found to be polymorphic with two codominant alleles by Parag (1984). But in the present study, no polymorphism has been observed in both loci of the eye tissue of adult L. parsia collected from Vypeen bar mouth area. This may be attributed to differences in the population and size class studied.

While there is no much difference between species in the tissue specifity of LDH bands, there is variation with regard to activity. Mugil cephalus exhibits higher rate of activity for both eye and muscle. But that of muscle tissue is more pronounced. A comparison of the two methods employed in the present study indicate that by serial dilution method it is not possible to detect significant variation between individuals of the same species as shown by the spectrophotometric method.

Though in the eye two locus are present in both species, the muscle LDH activity is higher than that of eye LDH, indicating that total enzyme activity is influenced by physiological role of tissues.

In the present work a combined study of genetic make up and biochemical characteristics of Lactate dehydrogenase isozyme has been attempted. It is hoped that above findings will find wider application in biochemical genetic study.

S U M M A R Y

1. The polyacrylamide concentration for electrophoretic separation of Lactate dehydrogenase system was standardized. The use of optimum acryalmide concentration in isoenzyme studies enhances the scope of detecting rare variants in a wide population.

2. Optimum concentrations of co-factor (NAD) and substrate (Lithium lactate) for staining LDH bands were obtained. The differential response of LDH isozymes to varying concentrations of substrate and cofactor would facilitate the selective study of various forms of an isozyme.

3. The effect of duration of storage and frequency of thawing on the resolution of LDH system in the eye and skeletal muscle tissues of Liza parisa was studied.

4. Lactate dehydrogenase expression was obtained in 6 tissues viz., Brain, Eye, Skeletal muscle, Liver, Gills and Heart.

5. No genetic variation was observed in the adult species of Liza parsia.

6. The activity of Lactate dehydrogenase enzyme in the skeletal muscle and eye tissue of Liza parsia and in the eye tissue of Mugil cephalus was determined by serial dilution method and the differences in the activity between species and tissue discussed.

7. In the fingerlings of Liza parsia and Mugil cephalus the activity of muscle and eye LDH was determined by spectrophotometric method.

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