

**STUDIES ON CERTAIN ASPECTS OF BIOCHEMICAL
GENETICS IN SELECTED SPECIES OF FROGS
FROM NORTH-EASTERN REGION OF
INDIA**

ABSTRACT

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**DEPARTMENT OF ZOOLOGY
SCHOOL OF LIFE SCIENCES**



**THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENT OF
THE DEGREE OF
DOCTOR OF PHILOSOPHY**

To



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The transition from water to land is a very remarkable step in the phylogenetic history of vertebrates. This conquest of land was initiated by the primitive amphibians in the Devonian period. In the course of their evolutionary history amphibians have become extraordinarily modified to lead a dual life. Their life cycle involves a drastic metamorphosis. They are cold blooded and so hibernate during winter months.

They are world wide in distribution and as many as 2600 species of anurans, the largest group of amphibians have been reported. In India anurans are represented by about 165 species of frogs and toads belonging to 6 families. Out of the 55 species of anurans reported from North-Eastern region of India 40 belong to the hills of Meghalaya. In spite of this richness genetic studies with these anurans are very limited. The present investigation is thus carried out to evaluate the electrophoretic pattern of multilocus isozyme systems. Seven species of frogs belonging to three families were analysed from different populations to study the isozyme pattern. The results are recorded in chapter one. In chapter two the developmental genetic aspect of the isozymes during tadpole growth and metamorphosis has been studied for one commonly available species.

In each of these chapters we have included the pattern of gene expression of four dehydrogenases (viz. lactate dehydrogenase, Malate dehydrogenase, Alcohol dehydrogenase and Glucose-6-phosphate dehydrogenase). Seven tissues of adult frogs were analysed to study the isozyme pattern. Tadpole tail muscle, head region and liver tissues were used for similar study at different stages of development in chapter two. Related work has been recorded in introductory section of each chapter.

Live specimens were sacrificed in the laboratory and fresh tissues viz. gonad, kidney, heart, brain, eye, liver and skeletal muscle were used. Isozymes were separated on 7.5% polyacrylamide rod gel according to the standard disc-electrophoretic procedures. Gels were subjected to specific staining solution to visualize the isozyme bands.

Lactate dehydrogenase (LDH) is a tetrameric enzyme catalyzing the interconversion of lactate to pyruvate. In most vertebrates the enzyme is coded by two codominant loci (A and B). Random association between the products of these genes yield five electrophoretically distinguishable isozymes in vertebrates. In some vertebrates a third locus designated C codes for highly tissue specific LDH. The presently studied frogs revealed a similar pattern of the LDH isozyme phenotypes as observed in other vertebrates. The

pattern manifests itself in predominance of acidic isozymes of heart and predominance of basic ones in skeletal muscle. Activity of the locus A was found to be more than that of locus B. The expression of locus B showed a restriction in some of the tissues of certain species. The heteropolymeric isozyme A_1B_3 also showed a restricted assembly in certain species. Allelic variant of the locus A was detected in one species resulting into a twelve-banded heterozygotic expression of LDH. The observed number and their Hardy-Weinberg expectations for the polymorphic loci support the validity of the proposed model and indicate that the samples were collected from a single Mendelian population. Further in two species tissue specific expression of the isozyme in eye and liver were observed. The isozyme showed a different electrophoretic characteristics comparable to the C4 LDH observed in other vertebrates.

Malate dehydrogenase (MDH) is dimeric in nature and catalyzes the interconversion of malate to oxaloacetate in Krebs cycle. The cytosolic malate dehydrogenase (s-MDH) is encoded by two loci in most vertebrates. In the present study both the loci have been found to be active.

Alcohol dehydrogenase (ADH) in most vertebrates is the product of a single locus and is primarily a liver specific

enzyme. It catalyzes the interconversion of many alcohols to their corresponding aldehydes and ketones. A single banded pattern of the enzyme could be resolved in liver and kidney tissues of the frogs analysed here. However, additional bands of low staining intensity has also been observed in some species. These may be due to allelic variants in the population of the species.

Glucose-6-phosphate dehydrogenase (G6PDH) is a key enzyme in pentose phosphate shunt. It is dimeric in nature and exist in two forms (A and B), without exhibiting heterodimeric forms. A single form corresponding to the form A was resolved in most of the tissues of the frogs examined in the present study. However, liver, gonad and eye tissues revealed additional band corresponding to form B in some cases. Skeletal muscle tissue did not show any activity for G6PDH enzyme indicating little or no pentose phosphate shunt.

Analysis of the tadpole tissues at various stages of development showed a distinctive isozyme pattern. Tadpole tail, head region and liver tissues exhibited predominance of LDH polypeptides at the various developmental stages. Expression of the B locus showed a restriction. Malate dehydrogenase (MDH) showed a less complex enzyme pattern. Both the locus were equally active. However there was a

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restriction in the heteropolymeric assembly. A remarkable variation in the expression of alcohol dehydrogenase gene was noted in the liver tissue of the tadpoles. During early stages of development multiple of ADH bands were resolved. Subsequently there was reduction in the number of isozymes with the progression of development. Glucose-6-phosphate dehydrogenase (G6PDH) revealed a single banded phenotype in the tail, a two-banded phenotype corresponding to A and B form of the isozyme were observed in the head region. Liver tissue exhibited a differential repression activation phenomenon of the genes coding for G6PDH isozymes. During early metamorphic stages both the form showed higher concentration of the enzyme. Subsequently with the progression of growth and development the isozyme form A was predominantly synthesized.

The isozyme pattern obtained for the four dehydrogenases in the adult specimens of the seven species and the tadpole tissues of one frog have been correlated with the local ecological conditions as well as with the physiological states of the frogs. However, our presumption of the various correlates with the observed isozyme pattern need further research to draw a final conclusion.

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11th December, 1995.

This is to certify that the thesis entitled "Studies on certain aspects of biochemical genetics in selected species of frogs from North-Eastern region of India", submitted by Mr. Malay Dey for the degree of Doctor of Philosophy of the North-Eastern Hill University, Shillong (India), embodies the record of original investigations carried out under my supervision. He has been duly registered and the thesis presented is worthy of being considered for the award of Ph.D. Degree. This work has not been submitted for any Degree of any other University.

Forwarded

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Malay Dey 11.12.95

(M A L A Y D E Y)

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FOREWORD

The transition from water to land is a very remarkable step in the phylogenetic history of the vertebrates. This conquest of land was first initiated by the primitive amphibians in the *Devonian* period and was completed by the reptiles in course of time. Even though amphibians constitute a sort of transitional group, they are credited as the first land-dwellers. Radical changes have occurred in their biological organization in adapting themselves between the two opposing environments.

Biologically, amphibians have become quite extraordinarily modified to lead a dual life in land as well as in water. The life cycle involves a drastic metamorphosis. The young tadpoles of frogs and toads differ markedly from their parents not only being aquatic but also in their feeding habits. Amphibians are cold blooded and thus hibernate during winter months.

The importance of frogs and toads is known since times immemorial. They form a valuable component of our biosphere. For a long time the frog has been a favourite object for the study of animal structure and function. For more than a century, frogs have been used the world over, in educational institutes and research centres for demonstrating and elucidating vertebrate anatomy, physiology and development.

Perhaps no animal, except man, has been the subject of so many scientific investigations (Holmes, 1927). It indeed seems, as is often remarked that the frog is especially designed as a subject for biological research.

The value of frogs in food and medicine has been known since ages. They are eaten roasted or boiled by a number of tribal population in North-Eastern hills of India, as food as well as cure for certain endemic diseases. The frogs and toads have also been used to control insect pests in agricultural systems (Okada, 1927). Nevertheless, in the recent years there has been an increasing demand in many European nations, America and Japan for frog legs to be served in banquet. In this context India earn foreign exchange worth crores of rupees from export of frozen frog legs.

For all these purposes, whether academic or economic, millions of frogs are collected every year all over the country. This coupled with 'habitat destruction' due to man's developmental activities and poor 'conservation awareness' may lead to extinction of certain frog species. Thus, in order to achieve the goal of 'bio-diversity' it is essential to have strict conservation policies supported by sound scientific knowledge.

It is thus widely felt that both from conservation and evolutionary perspective there is a strong need for a detailed examination of the patterns of genetic diversity. In the management of both endangered species and wild life populations, a clear picture of the genetic structure of the population is a basic prerequisite for formulation of proper management programmes. Further, for a correct understanding of the genetic basis of evolutionary changes, the patterns of genetic diversity present in many species must be examined (Allendorf et al., 1997).

In view of the above facts we have undertaken a biochemical genetic study on a few selected species of frogs, commonly available in the North-Eastern India. The findings have been presented in two chapters of the thesis.

Chapter One presents the results of the general isozyme patterns from electrophoretic separation of selected tissue extracts. Chapter Two deals with detection of differential gene expression during developmental stages of one selected species.

CHAPTER - I
INTRODUCTION

There are as many as 2600 anuran species belonging to 250 genera grouped under 12 families (Heusser, 1974). They are world wide in distribution and about 165 species of frogs and toads belonging to 6 families have been reported from India (Tiwari, 1991). North-Eastern India is extremely interesting from biological point of view. Amphibian fauna of the region is intimately connected with geomorphological evolution of the area and one can be reminded that obliteration of Pre-tertiary Tethys Sea began in North-East India producing in its wake a land bridge between Indian Peninsula and main land of Asia to the North. This region served as a faunal gateway through which Indo-Chinese element of oriental fauna, and that of Palaearctic fauna spread to the Indian Sub-Continent. Located in the North-East angle of the Indian Sub-Continent criss-crossed by hill ranges flanking the Brahmaputra and Surma Valleys, the Constituent units of the region are Assam, Arunachal Pradesh, Meghalaya, Nagaland, Manipur, Mizoram and Tripura. The region offer a complex variety of ecosystems varying from the alpine meadows and semi temperate slopes of the eastern Himalayas, through the wet evergreen forests of North Assam and Arunachal Pradesh, to the mixed evergreen and moist deciduous forests of South and East Brahmaputra (Ghosh and Tiwari, 1984).

It is interesting to note (Table.1) that out of about 55 species reported from the region (Chanda, 1972) 40 belong to the hills of Meghalaya. As many as 24 species of **Ranidae** and 18 species of **Rhacophoridae** have been reported from the region, of which 19 species of **Ranidae** and 11 species of **Rhacophoridae** are found in Meghalaya. Tree frogs belonging to the family **Hylidae** have a restricted distribution in India and one species (viz. *Hyla annectens Jerdon*) has been reported from this region. The 4 species of **Bufo** found in the region have been reported from Meghalaya. Out of the 4 burrowing amphibians belonging to the family of **Microhylidae** available in the region two have been reported from Meghalaya. The family **Pelobatidae** is regarded to be intermediate between the advanced and primitive families. Of the four species reported from North-East India three belong to the hills of Meghalaya.

Besides, many amphibian are endemic to the region : as many as 15 species have been exclusively found in Meghalaya, 8 in Arunachal Pradesh, 3 in Assam, 1 in Manipur and 1 in Tripura. Amphibian fauna thus present one of the important components of faunal wealth, specially in view of increasing interest in utilisation of these animals in biological control of insect pest and could lead to an interesting aspect of economic Zoology in the region.

Palaeontological studies have revealed that the modern frogs are much similar to those living 90 million years ago (Estes, 1975). Thus it seems obvious that organismal evolution has been slow in frogs (Chatterjee and Prakash, 1990) yet at the molecular level evolution must have been rapid. Otherwise they would have eventually succumbed to the vagaries of the environment. Due to the existence of morphological plasticity among individuals, conventional morphological characters are often found to be insufficient to give the exact data to resolve species problem. In such cases, biochemical and physiological approaches are found to be potentially more useful adjustment to the conventional taxonomic criteria. Many recent taxonomic studies, have benefited greatly from the advent of biochemical procedures that have been used to quantify molecular differentiation and thus affinity among species (Avisé, 1974; Wake, 1981; Wilson et al., 1977). Molecular differentiation often is large and may be used, by considering patterns of genetic and evolutionary relationship among species.

The exploitation of biological events in terms of taxonomy mainly depends upon the presence of enormous specificity of macromolecules and metabolic processes at every taxonomic level which are basically same in the lowest and highest organisms (Dhar and Chatterjee, 1982). Moreover,

numerous fundamental questions concerning the species-structure and process of speciation seem capable of being answered by studies concentrated at the molecular level.

Phenotypic expressions of an organism have been manifested by sequential biochemical events which in turn have been controlled by differential activity of appropriate genes. The genetic information encoded in the nucleotide sequence of the DNA of structural genes is translated into a sequence of amino acids making up a protein.

Biochemical techniques designed to compare species on the basis of protein differences were started by Nuttal (1904). The analysis of proteins have been considered to be the simplest indirect approach to understand the structure and function of genetic material. Moreover, comparison of functionally homologous proteins from a variety of species provides valuable information on the phylogenetic relationships among these species. In recent years biochemical data have proven useful in determining systematic relationship between organisms. The degree of similarity between the proteins of organisms is directly proportional to their degree of relationship (Sibley, 1964). These proteins are either structural proteins or functional proteins. The functional proteins, called enzymes are direct

products of genes and variation in their expression is indicative to the variation in the corresponding gene (Markert and Ursprung, 1974).

The variation in the structure of proteins, leading to the surface charge can be detected by electrophoresis and this technique has been found to be useful in studying problems involving different taxonomic ranks. Protein characteristics as ascertained electrophoretically are often useful in elucidating patterns of relationship and genetic differentiation at and below the species level (Awise, 1974). The genetic relationships between two or more forms and even individual genetic variations can be revealed from the study of electrophoretic variants of enzymes. The variant molecules provide a natural 'label' which makes them readily detectable in small quantities. In addition, electrophoresis is used widely to detect a large portion of allelic variation at a specific locus, making it possible to calculate frequency of specific alleles in different populations (Case et al., 1975; Chatterjee and Prakash, 1970). The application of high resolution electrophoretic techniques to detect specific protein banding patterns showing signs of common ancestry instead of non-specific fraction of diverse origins are more meaningful. Individual variants or mutants have wide application as "genetic

markers" and in biochemical analysis of enzyme relationships, of the structure of complex enzymes, and of multiple substrate specificities.

Many enzymes even after crystallization have been found to be made up of mixture of closely related molecules. These forms are termed "isozymes" (Markert and Moller, 1959). According to the recommendations of the commission on Biochemical nomenclature of IUPAC - IUB (1977) isozymes are defined as multiple molecular forms of an enzyme occurring within a single species as a result of more than one structural gene. The multiple gene may be due to the presence of multiple gene loci or multiple alleles. Also included in this definition are those multiple forms of enzymes which arise by association of protein subunits that are themselves the product of distinct structural gene and excluding all multiple forms due to post-translational modifications.

Since their discovery a wealth of information on enzyme heterogeneity has occurred and it seemed likely that at least half of all enzymes exist as isozymes. This has been important in many areas of biological and medical sciences. Thus isozyme studies have provided the main experimental substance for neutral drift controversy in genetics and evolution. Their existence has made available a

multitude of highly sensitive "markers" for the study of differentiation and development, as well as providing indices of aberrant gene expression in carcinogenesis and other pathological processes. Isozymes are also being used increasingly in diagnostic clinical biochemistry.

The literature comprising genetic studies on amphibians using electrophoretic separation of isozymes as a tool are enormous and an effort to review all would always remain inadequate. A very comprehensive review is presented here, which may be treated as a representative one.

A wide spectrum of variations in proteins with respect to isozymes have been observed in amphibians. Blair (1962) reviewed the utility of nonmorphological techniques in studying anuran evolution. At that time chromatography was the biochemical technique used in anuran studies. However, gel electrophoresis of proteins was being perfected at that time and has since become extremely important in modern evolutionary studies. Wells (1964) developed a key to five anuran subspecies based upon electrophoretic patterns of plasma proteins. Wilson et al., (1964) detected differences in molecular expression of LDH isozymes amongst various amphibian species. Salthe (1965) studied the catalytic properties in the heart and muscle extracts for the LDH

isozymes obtained from 85 species of frogs from various populations and detected only heart Lactate dehydrogenase (LDH) to be highly polymorphic. Shöntz (1968) examined four species of salamander of the genus *Desmognathus* to determine the feasibility of distinguishing them by electrophoretic patterns of LDH and haemoglobin characteristic. The study suggested that the degree of intraspecific biochemical variation is correlated with the degree of variation in external morphology within each species. Dessauer and Nevo (1969) studied protein variation in cricket frogs throughout southern United States. Salthe (1969) analysed electrophoretic patterns of heart LDH and was able to distinguish ten isozymes at this locus within the frog *Rana pipiens* complex in the United States of America.

Guttman (1973) further reviewed the biochemical studies of anuran evolution and documented the emergence of electrophoretic technique as an important tool. Guttman and Wilson (1973) studied intrapopulation variation at protein loci of American toad *Bufo americanus*. Inger et al., (1974) were the first to investigate genetic variation and population ecology of some South-East Asian frogs and toads of the genus *Rana* and *Bufo* respectively. Maxson and Wilson (1974) found approximately equal numbers of heart and skeletal muscle LDH variants in *Hyla regilla*. Platz (1975)

stated that morphological traits are associated with some consistent electrophoretic protein differences. His analysis of isozymes from 653 Arizonan leopard frog (*Rana pipiens*) from 34 sites distinguished three well established distinct types. Vogel and Chen (1975) investigated the LDH isozyme patterns in *Rana esculanta* complex and observed that it has a genetic control mechanism. Guttman (1975) concluded his analysis of genetic variation in 25 *Bufo americanus* populations by noting that the high heterozygosity in *Bufo* could be linked to environmental heterogeneity. A comparison of four anuran species in Israel conducted by Nevo (1976) provided support for this hypothesis. Dessauer et al., (1977) utilising eletrophoretic comparison of isozymes from 13 species of New Guinea hylid frogs estimated the genetic variability within and among the species in concordance with morphological evidence and suggested the presence of two distinct lineages. Killer and Lyerla (1977) reported an initial study on LDH variants in the spring peeper, *Hyla crucifer*, and revealed polymorphism at the muscle type locus. Schwantes and Schwantes (1977) made a comparison of eletrophoretic pattern of nine isozymes between tetraploid and diploid species of *Odontophrynus americanus* and *O. cultripes*. Schwantes et al., (1977) discussed mobility, activity and number of glucose-6-phosphate dehydrogenase (G6PDH) electrophoretic band in the tetraploid frogs

Odontophrynus americanus from Brazil and concluded that though the number of genes coding for the enzyme has been duplicated only a single band was obtained electrophoretically indicating higher concentration of repressors reducing gene activity. Tabachnick (1977) analysed the serum albumin and tissue LDH of North American Newt to determine the genetic variation and suggested that even though the enzyme variants are physiologically neutral, are linked to loci that are under the influence of selection. Case (1978a) studied 11 isozymes and 2 serum proteins to assess levels of genetic variation and evolutionary relationship between *Rana boylei* and *R. muscosa* from California. Case (1978b), based on electrophoretic and immunological data presented the evolutionary relationship among North American frogs of the genus *Rana*. Faulhaber and Lange-Lyra (1978) examined the distribution and homologies of subunits in the LDH isozymes pattern of urodels from Germany. Pierce and Mitton (1980) assayed genetic variation in 2 subspecies of *Ambystoma* from United States and confirmed the nature of variation at 8 polymorphic isozyme loci. Gerhardt et al., (1980) made a detailed survey on the morphology, vocalization, electrophoretic analysis of natural hybrids between *Hyla cinerea* and *H. gratiosa*. Vonwyl and Fishberg (1980) studied LDH isozyme in *Xenopus* and

indicated a species-specific pattern. Daugherty et al., (1981) surveyed variation in the New Zealand frogs and discussed their implications as taxonomic tools. Dunlap and Platz (1981) examined the geographic variations of proteins and mating call in *Rana pipiens* from North Central United States. Miyamoto (1981) examined 19 isozyme loci among the Costa Rican frog species of the genus *Leptodactylus* to draw phylogenetic inferences. Pierce et al., (1981) assayed three morphotypes of tiger salamander from West Texas for genetic variation and concluded that in spite of considerable morphological and life history differences, there exist little genetic divergence in isozyme pattern. Danzmann and Bogart (1982) studied electrophoretic staining intensities at a polymorphic Malate dehydrogenase (MDH) locus in the tetraploid tree frog *Hyla versicolor* and diploid *H. charysocephala* and provided additional evidence that gene dosages can be accurately interpreted from staining intensities. Karlin and Means (1982) reported 13 of the 19 isozyme loci to be polymorphic from a comparison between 2 American frogs *Hyla andersonii* and *H. cinerea*. Gunther et al., (1983) surveyed LDH - B isozyme in water frogs from different parts of Europe and Central Asia and commented on its polymorphic nature among geographically separated populations. Lyster and Fournier (1983) examined tissue specific expression of Xanthine dehydrogenase (XDH) isozyme

activity in clawed frog, *Xenopus laevis* and concluded that only kidney tissue exhibit high levels of activity. Miyamoto (1983) analysed taxonomic status of Costa Rican frog *Eleutherodactylus bransfordii* based on electrophoretic comparison of proteins. Odendaal and Bull (1983) compared two morphologically identical species of the genus *Ranidella* in South Australia and from the observed isozyme variation concluded that the external morphological similarity between species could be the result of either convergent evolution or the maintenance of an ancestral morphology. Formas et al., (1983) examined biochemical variation in the South American Leptodactylid frog *Eupsophus roseus*. Miyamoto and Tennant (1984) were able to establish close relationship among three species of Central American rain frogs based on electrophoretic, myological and karyological investigations. Eight species of frogs of the genus *Rana*, comprising five endemic species from western North America were examined electrophoretically in order to ascertain their systematic relationships by Green (1986). Hedges (1986) analysed 33 isozyme loci in thirtyfive species of holarctic frogs from the United States and developed phylogenetic relationships among the species belonging to four genera. Shaffer et al., (1991), by comparison of isozymes analysis with morphological data, yielded points of both agreement and

conflict regarding phylogenetic relationships of all North American *Ambystoma*. Tyriakopoulou-Sklavounou et al., (1992) studied the morphometric and electrophoretic variation of two population of *Hyla arborea* in Greece. Tissue-specific expression, number of presumptive loci, and interlocus heteropolymer formation for numerous isozymes in eight tissues of the small mouth salamander, *Ambystoma texanum* was investigated by Titus (1994). A high degree of genetic variation in five populations of the green toad *Bufo viridis*, from northern Greece was revealed by Tyriakopoulou-Sklavounou and Karakousis (1995) based on studies of external morphology and isozyme analysis.

Biochemical genetic study on Indian amphibians was initiated by Lakshminaraj et al., (1980) when they demonstrated species-specific and tissue-specific patterns of LDH isozymes in three species of Indian frogs belonging to the genus *Rana*. However, inspite of the richness of amphibian fauna in North-Eastern India, no genetic studies was done until 1990. Chatterjee and Prakash (1990) based on isozyme data of nine population of *Rana limnocharis* from North-Eastern India concluded that protein polymorphism is adaptively important and maintained by natural selection.

From the foregoing review, it seems that considerable information is available on the genetics of dehydrogenase

specially Lactate dehydrogenase(LDH), Malate dehydrogenase (MDH), Alcohol dehydrogenase (ADH) and Glucose-6-phosphate dehydrogenase (G6PDH). It is also amply clear in comparison to the works done in the rest of the world, work in biochemical genetics on Indian frogs are scanty. The present work has, therefore, been undertaken to analyze the isozyme pattern of these dehydrogenases and subsequently to study their genetic pattern in seven species of frogs, viz :*Hyla annectans*, *Polypedates leucomystax*, *Rhacophorus maximus*, *Rhacophorus reinwardtii*, *Amolops afghanus*, *Rana cyanophlyctis*, and *Rana nicobariensis*.

TABLE : 1

Distribution of Frogs and Toads in N.E. India.

Family	Meghalaya		Assam	Arunachal Pradesh	Nagaland	Manipur	Mizoram	Tripura
Ranidae	24	19	12	7	4	7	7	4
Rhacophoridae	18	11	4	11	3	4	3	2
Hylidae	1	1	1	0	0	0	1	0
Bufoidea	4	4	1	2	1	1	2	1
Microhylidae	4	2	4	1	1	1	2	1
Pelobatidae	4	3	1	1	0	0	1	0
T o t a l	55	40	23	22	9	13	16	8

The numerals indicate the number of species available.

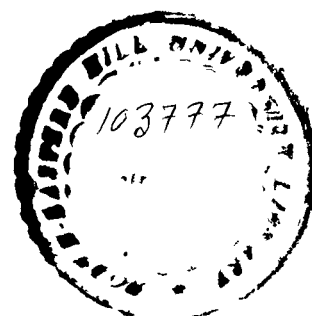


TABLE : 2

Total number of each species studied

Name of the species	Total number of specimen studied
<i>Hyla annectens</i> (Jerdon)	17
<i>Polypedates leucomystax</i> Gravenhorst	82
<i>Rhacophorus reinwardtii</i> (Boie)	26
<i>Rhacophorus maximus</i> Gunther	14
<i>Amolops afghanus</i> (Gunther)	65
<i>Rana cyanophlyctis</i> Schneider	54
<i>Rana nicobariensis</i> Stoliczka	18

TABLE : 3

Geographical location of the sites of collection of different species

Locality	Latitude	Longitude	Species collected
Shillong	25°36'	91°54'	<i>R. cyanophlyctis</i> , <i>A. afghanus</i> , <i>P. leucomystax</i>
Jowai	25°27'	92°12'	<i>R. cyanophlyctis</i> , <i>R. reinwardtii</i> , <i>R. maximus</i> , <i>P. leucomystax</i>
Williamnagar	25°32'	90°35'	<i>R. nicobariensis</i> , <i>R. cyanophlyctis</i> , <i>A. afghanus</i> , <i>P. leucomystax</i>
Mawhati	25°50'	92°5'	<i>R. nicobariensis</i> , <i>P. leucomystax</i> .
Mawkaiaw	25°32'	92°29'	<i>R. nicobariensis</i> , <i>A. afghanus</i> .
Nongkhlaw	25°42'	91°38'	<i>A. afghanus</i> .
Smit	25°35'	91°58'	<i>A. afghanus</i> , <i>H. annectens</i> , <i>R. reinwardtii</i> .
Mawnsynram	25°23'	91°34'	<i>A. afghanus</i> , <i>R. reinwardtii</i> .
Cherrapunji	25°18'	91°43'	<i>R. maximus</i> .
Mawphlang	25°28'	91°45'	<i>R. reinwardtii</i> , <i>H. annectens</i> .

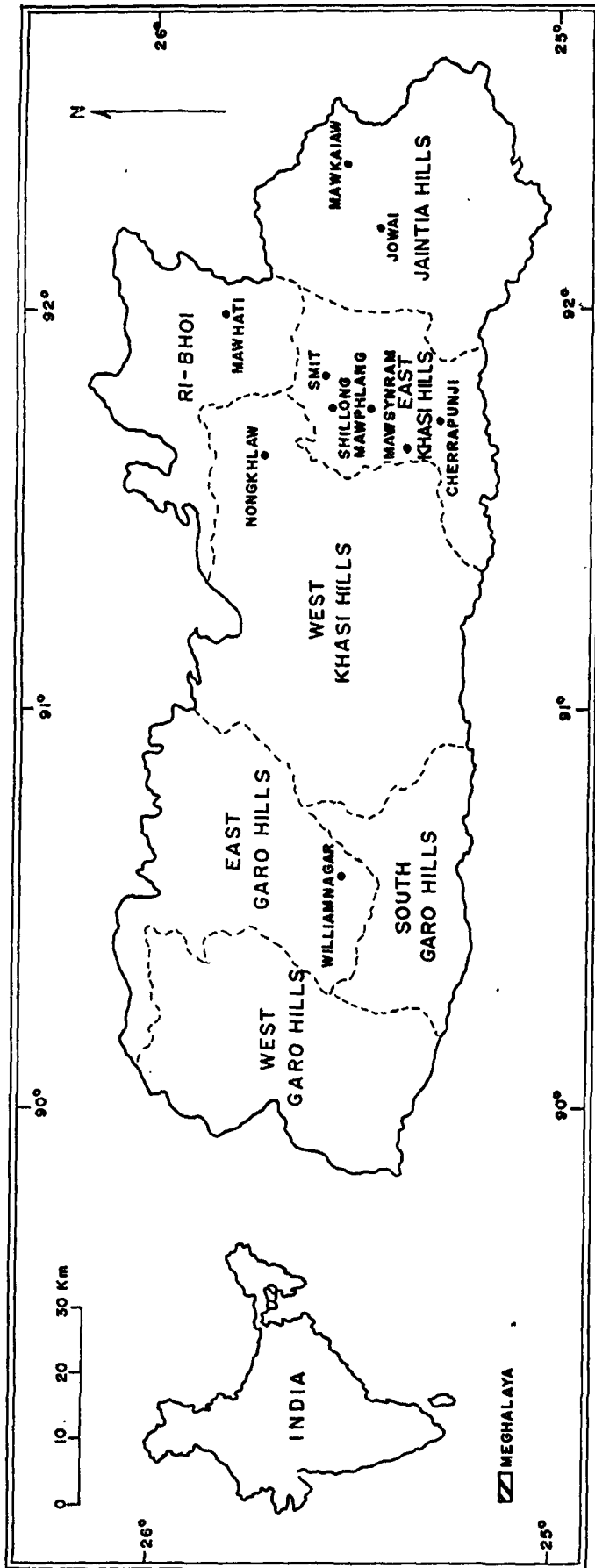


Figure : I Sites of Collection

CHAPTER-I
MATERIALS & METHODS

2.1 THE FROGS :

The materials for the present investigations comprise of seven species (Plates 1 to 8) belonging to three families of the order Anura.

- (a) *Hyla annectens* (Jerdon) : Family Hylidae.
- (b) *Polypedates leucomystax* Gravenhorst : Family Rhacophoridae.
- (c) *Rhacophorus reinwardtii* (Boie) : Family Rhacophoridae.
- (d) *Rhacophorus maximus* Gunther : Family Rhacophoridae.
- (e) *Amolops afgnanus* (Gunther) : Family Ranidae.
- (f) *Rana cyanophlyctis* Schneider : Family Ranidae.
- (g) *Rana nicobariensis* Stoliczka : Family Ranidae.

2.1.1 BRIEF DESCRIPTION, HABITAT AND DISTRIBUTION :

(a) *Hyla annectens* (Jerdon) : A small leafy green frog ; dorsal surface of the body is smooth and belly coarsely granular. A slate dark plate on both the lateral side of the body (Plate 1). Fingers and toes with large discs. They are nocturnal, arboreal frogs and live in hollow stems of trees during winter. They visit temporary water bodies in spring and summer for breeding. Distribution extends in Western China (Cochran, 1961), Thailand and Myanmar (Taylor, 1962), North-East India, Bangladesh (Heusser, 1974). In North-East India it is found in Assam, Meghalaya and Mizoram (Chanda,

1992). Interestingly this is the only species of *Hyla* found in India (Tiwari, 1991).

(b) *Polypedates leucomystax Gravenhorst* : A moderate sized frog. Dorsal skin colour variable from grey to bright yellowish brown with dark spotted dots or broken stripes (Plate 2). Belly white and coarsely granular. Fingers and toes webbed with large discs. They are nocturnal arboreal frogs. In summer and spring visit pools for breeding, hibernate under forest litters during winter. They exhibit amplexus and prepare foamy nests on twigs and leaves at the edges of water bodies. Its distribution extends in India, Malay Peninsula, Java (Annandale, 1912; Cochran, 1961 and Church, 1963), Thailand and through South-East Asia (Taylor, 1962). It is reported from all the North-East Indian states (Chanda, 1992).

(c) *Rhacophorus reinwardtii (Boie)*: A moderate sized, leafy green coloured frog. Belly rough yellowish white in colour and the dorsal skin is smooth (Plate 3). Fingers and toes webbed with large discs. Webs are bright orange in colour and 1-2 black circular spots on lateral armpit. An arboreal, nocturnal frog and hide in hollow trees in winter. During spring and summer female deposit eggs in a foamy nest at the edges of water bodies. Distribution extends in Java, Sumatra

(Gadow, 1901), Malayasia (Cochran, 1961) and Meghalaya, Arunachal Pradesh in North East India (Pillai and Chanda, 1976).

(d) *Rhacophorus maximus Gunther* : A moderate sized leafy green coloured frog (Plate 4). Dorsal skin is smooth and folded near tympanum. Belly smooth and brownish in colour. Fingers and toes webbed with very large discs. The frog is nocturnal in habit and is found among dense foliage, grass and bushes. Colour change from bright green to dull green and finally to light brown have been observed during handling (Plate 5). Visit pools in spring, eggs are deposited in foamy nest on the sides of water bodies. Distribution extends in Himalayan forests (Gadow, 1901), North of Siam, Sumatra and Malay (Taylor 1962), Thailand, Malayasia, Kampuchea, Laos, Borneo (Heusser, 1974) and Meghalaya, Mizoram in North-East India (Chanda, 1992).

(e) *Amolops afghanus (Gunther)* : A stouter and larger frog. Dorsal skin smooth, dark olive spotted and marbled with black and grey. Belly light brown and finely granulated (Plate 6). Thighs with faint bars. Fingers and toes with large prominent discs. Toes webbed, fingers free. A nocturnal frog, live in cool streams near water falls and hide in crevices. Distribution extends from eastern Himalayas to Western Himalayas (Gadow, 1901 and Kripalani

1961). It is distributed in all the North Eastern states of India except Tripura (Chanda, 1992).

(f) *Rana cyanophlyctis Schneider* : A moderately small frog. Dorsal skin smooth olive brown with dark spots. Lower parts white and smooth. Fingers free, toes webbed (Plate 7). They are water frogs and do not hibernate. Distribution extends in South Arabia, Baluchistan, Afganistan, India, Srilanka, Northern Malay Peninsula (Boulenger, 1920; Satyamurthi, 1967) Thailand (Taylor, 1962) Pakistan (Minton, 1966; Khan, 1976). It is distributed in all the North-Eastern States of India (Chanda, 1992).

(g) *Rana nicobariensis Stoliczka* : A moderate frog. Dorsomedially the skin is brown with dark dots. Dorsolaterally green with broken yellow spots. Belly smooth and white. Limbs brown with darker stripes. Fingers and toes free (Plate 8). A nocturnal frog, live in warm damp places such as the base of banana plantations. Distribution extends in Nicobar islands (Stoliczka, 1870), Sumatra, Malayasia, Borneo, and Java (Boulenger, 1920). In India the species is widely distributed in West Bengal (Sarkar, et al., 1992), Tripura and Arunachal Pradesh (Sarkar and Ray, In Press). This is the first report of the species from Meghalaya.

The specimens have been collected from different places of Meghalaya during the months of April to October for a period of three years (1991 - 1993). To avoid ontogenic problems only fully grown adult specimens were analysed (please see table 2 for number of individuals analysed). The site of collection is indicated in Fig 1, table 3.

2.2 POLYACRYLAMIDE - GEL ELECTROPHORESIS :

Polyacrylamide gel electrophoresis of four isozymes, viz. Lactate dehydrogenase (LDH), Malate dehydrogenase (MDH), Alcohol dehydrogenase (ADH) and Glucose - 6 - Phosphate dehydrogenase (G6PDH) of the seven species of frogs were performed according to Davis (1964) with suitable improvisation.

2.2.1 PREPARATION OF TISSUE HOMOGENATES :

Frogs were cooled in ice and sacrificed to dissect out seven tissues viz. gonad, kidney, heart, brain, eye, liver and skeletal muscle. Dissected tissues were immediately wrapped in aluminium foil, collected in glass container and kept in an ice bath. Each tissue was then weighed accurately and taken in a glass homogenizer containing ice cold homogenization medium (0.25 M Sucrose solution). The tissues

were homogenized using an electric homogenizer with teflon pestle. During homogenization the tubes were always held in an ice bath to avoid denaturation of tissue proteins. A 10% homogenate was found to be suitable for all tissues. However, due to high protein content of liver and skeletal muscle a 5% solution proved satisfactory. The homogenates were immediately transferred into poly-propylene centrifuge tubes (15 ml) kept in an ice bath.

2.2.2 EXTRACTION OF TISSUE PROTEINS :

The structural elements were removed from the homogenates by differential centrifugation in a cooling - centrifuge (REMI-C-24), pre-adjusted to 4^o C. Centrifugation was carried out at 21,000 xg for 15 minutes. The resultant clear supernatant were decanted in test tubes and subjected to electrophoresis.

2.2.3 ELECTROPHORESIS : GENERAL PRINCIPLES :

When particle of effective charge (Q) is forced to migrate in a viscous medium (liquid or gel) by action of an electric field (potential gradient, E), the phenomenon is generally called as Electrophoresis (Maurer, 1971). The driving force

which acts upon the particle migrating with constant velocity is equal to the frictional resistance (F) which the particle must overcome in the medium, i.e.,

$$Q E = F$$

The electrophoretic mobility of a particle is defined as

$$m = \frac{d}{tE} \frac{V}{E} \frac{Q}{F} \left(\frac{\text{cm}^2}{\text{volt} \times \text{sec}} \right)$$

where 'd' is the migration distance of the particle in time 't', 'V' the velocity and 'F' is the frictional resistance.

The vertical disc electrophoresis system of Davis (1964) was employed. It is a discontinuous separating system with regard to pH value, buffer composition and gel pore size in which polyacrylamide gel serves as a matrix.

Disc electrophoresis is carried out with small columns of polyacrylamide gel consisting of three layers, in suitable container like cylindrical tubes. The three layers are : (i) a large pore spacer or stacking gel, (ii) a small pore separation or running gel in which the sample constituents are separated and (iii) a large pore sample gel containing sample solution. Electrophoresis is performed with a vertical column of gels attached to two different reservoirs : sample gel uppermost, attached to an upper reservoir and the lower end submerged in the buffer solution

of the lower reservoir. Electrodes are placed in each reservoir and polarity is set so that sample ions migrate towards the small pore gel. A voltage is applied for a specific time. The gel is then removed from the container and placed for a period of time in a solution of protein fixation and stained. Unbound dye is removed from the gel slowly by washing in 7% acetic acid and then the gel is preserved in a suitable solution.

2.2.3.1 REAGENTS :

Stock solution : To obtain a 7.7% polyacrylamide gel following stock solution were prepared and stored in dark bottles in a refrigerator. The shelf life of these solutions are two months.

Stock solution A : pH 8.9

1 N HCl : 48.00 ml

Tris (Hydroxymethyl methylamine) : 36.6 g

TEMED (N,N,N,N - Tetra methyl ethylenediamine) : 0.23 ml

The volume is made upto 100 ml with distilled water.

Stock solution B : pH 6.7

1 N HCl	:	48.00 ml
Tris	:	5.98 g
TEMED	:	0.46 ml
Distilled water	:	51.54 ml

Stock solution C :

Acrylamide	:	30.0 g
Bis (N-N- Methylene bis-acrylamide):	:	0.8 g
Distilled water	:	100.0 ml

Stock solution D :

Acrylamide	:	10.0 g
Bis	:	2.5 g
Distilled water	:	100.0 ml

Stock solution E :

Riboflavin	:	4.0 mg
Distilled water	:	100.0 ml

Stock solution F :

Sucrose	:	40.0 g
Distilled water	:	100.0 ml

Stock solution G :

Ammonium persulphate	:	0.14 g
Distilled water	:	100.0 ml

Stock buffer solution : pH 8.3

Tris	:	6.0 g
Glycine	:	28.8 g
Distilled water	:	1000.0 ml

For reservoir, 10% strength of this stock solution was used.

Indicator solution : 1% Bromophenolblue

Bromophenolblue	:	0.1 g
Distilled water	:	10.0 ml

Destaining solution : 7% Acetic acid.

Glacial acetic acid	:	70.0 ml
Distilled water	:	930.0 ml

2.2.3.2 Equipment used :

The size of the cylindrical gel tubes used were about 10 cm in length with an inner diameter of 5 mm. These tubes were fixed vertically in the electrophoresis running chamber, consisting primarily of the upper and lower chambers with platinum electrodes. The upper and lower chambers were the buffer reservoirs. Arrangements were there to hold the gel tubes vertically in the upper reservoir and to make a link with the lower reservoir. The additional equipment comprises of electrodes, cables and a power supply unit (Systronics 604), gel casting rack, fluorescent lamp, micropipette with

disposable tips, hypodermic syringe, needle, test tubes, incubator, etc.

2.2.3.3 GEL SYSTEMS AND THEIR COMPOSITION :

Separation gel was prepared just before use by mixing reagents in the following proportion (mixing ratio v/v) :

Stock solution	A	:	1 part
Stock solution	C	:	2 parts
Distilled water		:	1 part
Stock solution	G	:	4 parts

Spacer gel system was prepared by mixing the stock solution in the following proportion (v/v) :

Stock solution	B	:	1 part
Stock solution	D	:	2 parts
Stock solution	E	:	1 part
Stock solution	F	:	4 parts

2.2.3.4 PROCEDURE :

About 1.5 ml (upto 7 cm in length of the gel tubes) of the separation gel system was poured into the gel tubes held vertically on a casting rack. Care was taken not to allow any air bubble in the gel column. A few drops of distilled water were carefully layered above the separation gel

system and left undisturbed for about 3 hours to polymerize. After polymerization, the water from the top of the gel column was removed carefully and about 0.2 ml (about 0.5 cm in length of the gel tube) of the spacer gel system was poured carefully into the gel tube. A few drops of distilled water were again layered above this gel system and the gel tubes were left under fluorescent light for about 20 minutes to polymerize. After polymerization the layer of water was removed. The gel tubes were then fixed vertically as described by Davis (1964) in the disc electrophoresis chamber. The lower chamber of the apparatus was filled with 1 M Tris-glycine (pH 8.3). Measured quantity of fresh tissues extract was directly poured over the spacer gel system with the help of a micropipette. 25µl of extract was found to be suitable for separation of LDH, 100µl for ADH and G6PDH while 10-15µl extract was used for separation of MDH. The remaining vacant part of the gel tube was filled with the buffer used for the reservoir avoiding air bubbles. The upper buffer chamber was filled with Tris-glycine (pH 8.3). A drop of 1% Bromophenol blue solution was mixed in the upper buffer chamber in order to determine the time required for the separation of different isozyme. The upper buffer chamber was closed by placing the electrode lid.

The apparatus was then placed inside a refrigerator and the two electrodes were connected with a Systronic-604 power supply unit. The power supply switch was put on and a normal mode of current was allowed to flow. The voltage was set at 220 volt and a current of 1.5 mA/gel tube was allowed for the first 10 minutes following which current was doubled. After the requisite time for the separation of protein samples, the current supply was cut off and the gel tubes were removed quickly in a tray containing ice-cold distilled water. The gels were taken out promptly by flushing cold distilled water with the help of hypodermic syringe and a needle. the gels were immediately subjected to specific treatment to obtain different isozyme patterns.

2.2.3.5 VISUALISATION OF ISOZYME PATTERNS :

Gels were taken in different test tubes containing specific staining mixtures and were incubated for specific period. Gels incubated without substrate served as controls. After the reaction the gels were washed and preserved in 7% acetic acid to make genetic interpretation.

Four isozyme systems were investigated using the following staining recipes :

A. Lactate dehydrogenase (LDH; E.C. 1.1.1.27) :

For staining seven gels the following mixture was prepared:

1 M Tris-HCl	:	2.5 ml
1 N Lithium lactate	:	0.5 ml
NAD (β-nicotinamide adenine dinucleotide)	:	80.0 mg
NBT (P-Nitroblue Tetrazolium Chloride)	:	40.0 mg
PMS (Phenazine Methosulphate)	:	1.2 mg
Distilled water	:	47.0 ml

The gels were incubated in the above solution at 37°C for 15 minutes violet coloured LDH bands were visualised.

B. Malate dehydrogenase (MDH; E.C. 1.1.1.37) :

Only 0.5 ml of 1 N malic acid was used as a substrate instead of Lithium lactate, rest of the staining solution remained as for LDH.

C. Alcohol dehydrogenase (ADH; E.C. 1.1.1.1.) :

The solution remains as for LDH except that the substrate has been substituted for 0.5 ml of ethyl alcohol.

D. Glucose-6-phosphate dehydrogenase (G6PDH; E.C. 1.1.1.49)

0.05 M Tris.HCl (pH 8.0)	50.0 ml
Glucose-6-phosphate	40.0 mg
NADP	20.0 mg
PMS	0.6 mg
NBT	15.0 mg

The gels were incubated in the above mixture at 37° C for 15 minutes. The bands were visualised in violet colour.

2.2.4 HEAT INACTIVATION STUDIES OF ISOZYMES:

In order to distinguish between the different isozyme subunits, heat inactivation studies were performed for all the four dehydrogenases. The crude extracts after centrifugation were taken in test tubes and kept in water baths at variable temperature for different isozymes:

LDH	-	Temperature of water bathe 55°C - 65°C
MDH	-	Temperature of water bathe 40°C - 45°C
ADH	-	Temperature of water bathe 25°C - 30°C
G6PDH	-	Temperature of water balhe 60°C - 65°C .

Measured quantity of samples were taken after every 10 minutes, loaded in the polyacrylamide gel tubes and subjected to electrophoresis in the usual manner. The gels

were then stained for the specific isozyme. For convenience, tissues showing high activity for the specific isozyme were selected for this experiment. LDH isozymes were tested for cardiac muscle and skeletal muscle extract, for MDH all the seven tissues were examined, ADH was tested only in liver tissues and G6PDH was tested in all the tissues except skeletal muscle.

2.2.5 QUANTITATIVE ESTIMATION OF PROTEIN :

In order to determine the total protein content in different tissues of the frogs studied a preliminary estimation was made according to the procedure of Lowry *et al.*, (1951):
Principle : Protein when react with Folin-Ciocalteau, form a blue colour, the intensity of which at 750 nm is proportional to the amount of proteins present in a sample. The final colour reactions are the bi-uret reaction of phosphomolybdic phosphotungstic reagent by tyrosine and tryptophan present in the treated protein.

REAGENTS :

- 1) Stock solution A : 1% CuSO_4
- ii) Stock solution B : 2% K-Na-tartarate
- iii) Stock solution C : 2% Na_2CO_3 in 0.1 N NaOH.

iv) Folin-Ciocalteu reagent.

0.5 ml of stock solution A was mixed with 0.5 ml of the stock solution B and the volume was raised upto 50 ml with stock solution C just before use. Commercially available Folin-Ciocalteu reagent was diluted with equal volume of distilled water just before use.

Procedure : 5 ml of alkaline solution was added to 1 ml of crude protein extract, mixed thoroughly and allowed to stand at room temperature for 10 minutes. 0.5 ml of diluted F-C reagent was added and rapidly mixed. After 30 minutes, the extinction was read against an appropriate blank at 750 nm. in a colorimeter. Protein concentration was determined after preparing a standard curve using Bovine Serum albumin.

CHAPTER-I
RESULTS

3.1 The electrophoretic patterns of four isozyme systems viz. Lactate dehydrogenase, Malate dehydrogenase, Alcohol dehydrogenase and glucose-6-phosphate dehydrogenase in the seven species have been studied from different localities (Fig.1. Table.3) in Meghalaya.

3.1.1 *Lactate dehydrogenase* (Lactate : NAD - Oxidoreductase, E.C. 1.1.1.27):

(a) *Hyla annectens*: Specimens collected from two localities (Viz. Smit and Mawphlang) were studied. A classical five-banded LDH isozyme pattern was obtained in the Smit population (Fig.2a; Plate 9a). Of the seven tissues gonad, kidney and heart showed closely spaced five bands of LDH with identical electrophoretic mobility in all these tissues. Only four bands of LDH isozymes were resolved in brain and eye tissue. The homotetramer B_4 was not expressed in these tissues. Liver tissue exhibited a four-banded pattern represented by A_4, A_2B_2, A_1B_3 and B_4 isozymes. The skeletal muscle tissue extract showed a single intensely stained band of A_4 isozyme at the cathodal end. No other isozyme could be detected in this tissue.

Specimens examined from Mawphlang, however, possessed a single cathodal LDH band in all the seven tissues tested. (Fig.2b; Plate 9b). Comparison of relative mobility

indicates the band represents the A_4 isozyme.

(b) *Polypedates leucomystax*: Specimens were collected from four different localities (viz. Shillong, Jowai, Mawhati, and Williamnagar) for the present investigation. A five-banded LDH pattern was obtained for the species.

Specimens examined from Shillong possessed all the five isozymes (Fig.3a; Plate 10a,b) in kidney, brain, eye and liver tissues while gonad and heart presented only four bands, the homotetramer A_4 did not resolve in these tissues. Skeletal muscle tissue showed only A_4 and A_3B_1 isozyme bands. A five-banded LDH pattern was obtained in gonad, heart, (Fig.3g; Plate 10h) and liver (Fig.3h; Plate 10i) tissue of the specimens examined from Mawhati and in the heart and brain tissue of the specimens from Jowai (Fig.3i; Plate 10j). Less than five banded LDH pattern was observed in all the specimens examined from Williamnagar (Fig.3d,e; Plate 10e,f) where all the seven tissues showed either two or three bands of LDH. A similar pattern was obtained in some of the specimens examined from Mawhati (Fig.3f,g,h; Plate 10g,h,i).

Tissuewise, gonad was found to possess either three, four or five isozymes in different populations. However a two-banded pattern showing predominance of B_4 isozymes was

obtained in the specimens tested from Williamnagar (Fig.3d,e; Plate 10e,f) and Jowai (Fig.3i; Plate 10j). A single-banded pattern was also observed in some of the specimens examined from Mawhati. (Fig.3f; Plate 10g).

Kidney tissue LDH was resolved into a five-banded pattern in the specimens examined from Shillong (Fig.3a; Plate 10a,b), two to three bands in the specimen from Williamnagar (Fig.3d,e; Plate 10e,f), one to four bands in the specimens from Mawhati (Fig.3f,g,h; Plate 10g,h,i) and a two-banded pattern in the specimens from Jowai (Fig.3i; Plate 10j).

Cardiac muscle tissue showed a maximum of four LDH isozyme bands in the specimens examined from Shillong (Fig.3a; Plate 10a,b) Mawhati (Fig.3f,g,h; Plate 10g,h,i) and Jowai (Fig.3i; Plate 10j). Two to three bands were obtained in the specimens examined from Williamnagar (Fig.3d,e; Plate 10e,f) while some of the specimens from Mawhati showed either one or two bands (Fig.3f,g,h; Plate 10g,h,i).

A five-banded phenotype in the brain tissue extract was obtained for the specimens examined from Shillong (Fig.3a; Plate 10a,b) and Jowai (Fig.3i; Plate 10j). In the specimens examined from Williamnagar (Fig.3d,e; Plate 10e,f) and Mawhati (Fig.3g,h; Plate 10h,i) only A_4 , A_3B_1 and A_2B_2

isozymes were resolved. A single banded pattern was obtained in some of the specimens examined from Mawhati (Fig.3f; Plate 10g).

Eye tissue LDH resolved into a five-banded pattern in the specimens examined from Shillong (Fig.3a; Plate 10a,b), three to four bands in the specimens tested from Williamnagar (Fig.3d,e; Plate 10e,f) one to four bands in the specimens examined from Mawhati (Fig.3f,g,h; Plate 10g,h,i) and a two banded pattern in the specimens from Jowai (Fig.3i; Plate 10j).

Liver tissue showed a five banded isozyme pattern in the specimens examined from Shillong (Fig.3a; Plate 10a,b) and Mawhati (Fig.3g; Plate 10h). Three to four isozymes corresponding to A_4 , A_3B_1 , A_2B_2 and A_1B_3 were obtained in the specimens examined from Williamnagar (Fig.3d,e; Plate 10e,f) and Jowai (Fig.3i; Plate 10j). In some of the specimens examined from Mawhati (Fig.3f,h; Plate 10g,i) a four-banded or single-banded pattern have been recorded.

Skeletal muscle tissue LDH showed predominance of A_4 isozyme. A two-banded pattern corresponding to A_4 and A_3B_1 isozymes were obtained in the specimens examined from Shillong, Williamnagar and Mawhati (Fig.3a,d,e,g; Plate 10a,b,e,f,h). Specimens examined from Jowai, in addition

to A_4 and A_3B_1 also possessed A_2B_2 isozyme (Fig.3i; Plate 10j). Some of the specimens collected from Mawhati also showed a single banded pattern (Fig.3f,h; Plate 10h,i).

More than the usual five-banded isozyme pattern was obtained in some of the specimens examined from Shillong (Fig.3b,c; Plate 10c,d). All the seven tissues showed a multiple of LDH bands. Gonad tissue LDH was resolved into six to nine bands, kidney showed eleven bands, heart presented seven to nine bands, brain tissue showed nine to twelve bands, eye tissue possessed six to nine bands, liver tissue extract presented six to eleven bands and skeletal muscle tissue showed two to four bands of LDH isozymes.

c) *Rhacophorus reinwardtii*: Four populations (viz. Smit, Mawsynram, Jowai and Mawphlang) were studied to elucidate the LDH gene expression in the species. In general A_4 isozyme was found to be the most strongly active in all the specimens examined from the four localities. LDH isozyme in this species could be resolved into a maximum of four bands corresponding to A_4 , A_3B_1 , A_1B_3 and B_4 in the gonadal tissue of the specimens collected from Smit (Fig.4u, Plate 11d) and A_4 , A_3B_1 , A_2B_2 and B_4 in heart tissue of Mawsynram (Fig.4a; Plate 11a) specimens. In other populations as well as in these two population most of the tissues presented only three isozymes.

Tissuewise gonad showed three isozymes of A_4 , A_3B_1 and A_2B_2 in the specimens examined from Mawsynram and Jowai (Fig.4a,b; Plate 11a,b), a two-banded pattern of A_4 and B_4 in specimens from Mawphlang (Fig.4c; Plate 11c) and a four-banded pattern in the specimen from Smit (Fig.4d; Plate 11d).

Kidney tissue LDH was resolved into A_4 , A_3B_1 and A_2B_2 isozymes in the specimens from Mawsynram (Fig.4a; Plate 11a), A_4 , A_2B_2 and B_4 in the specimens from Jowai (Fig.4b; Plate 11b), A_4 and B_4 in the specimens from Mawphlang (Fig.4c; Plate 11c) and A_4 and A_2B_2 in the specimens from Smit (Fig.4d; Plate 11d).

Cardiac muscle tissue showed predominance of B_4 isozymes. A four-banded pattern with A_4 , A_3B_1 , A_2B_2 and B_4 isozymes was resolved in the specimens collected from Mawsynram (Fig.4a; Plate 11a) and a three banded pattern corresponding to A_4 , A_2B_2 and B_4 isozymes in the specimens examined from Jowai and Smit (Fig.4b,d; Plate 11b,d) while only A_4 and B_4 isozymes were expressed in the specimens from Mawphlang (Fig.4c; Plate 11c).

A three-banded isozyme pattern corresponding to A_4 , A_3B_1 and A_2B_2 was resolved in the brain tissue of the specimens tested from Mawsynram (Fig.4a; Plate 11a), A_4 ,

A_2B_2 and B_4 in the specimens examined from Jowai (Fig.4b; Plate 11b). Only two bands representing A_4 and B_4 in the specimens from Mawphlang (Fig.4c;Plate 11c) and A_4 and A_2B_2 , were resolved in the specimens from Smit (Fig.4d;Plate 11d).

In the eye tissue of specimens examined from Mawsynram (Fig. 4a;Plate 11a) a three-banded isozymes pattern of A_4 , A_3B_1 and A_2B_2 was resolved while only two bands of the isozymes corresponding to A_4 and B_4 and A_4 and A_2B_2 were obtained in the specimens examined from Mawphlang and Smit respectively (Fig.4c,d;Plate 11c,d). The specimen analysed from Jowai presented a four-banded phenotype (Fig.4b;Plate 11b). Of the four bands, three correspond to A_4 , A_2B_2 and B_4 while the fourth band showed a cathodal affinity and indicated a tissue-specific expression. Liver tissue LDH in the specimens examined from Mawsynram was resolved into A_4 , A_3B_1 and A_2B_2 isozymes (Fig.4a;Plate 11a), A_4 and A_2B_2 in the specimens from Jowai (Fig.4b;Plate 11b), A_4 , A_2B_2 and B_4 in the specimens from Mawphlang (Fig.4c;Plate 11c) and A_4 and A_2B_2 in the specimens from Smit (Fig.4d;Plate 11d).

Skeletal muscle tissue showed three isozymes viz A_4 , A_3B_1 and A_2B_2 in the specimens from Mawsynram (Fig.4a; Plate 11a), A_4 and A_2B_2 in the specimens from Jowai and Smit (Fig.4b,d; Plate 11b,d). Only A_4 isozyme was resolved in the

specimens from Mawphlang (Fig.4c; Plate 11c).

(d) *Rhacophorus maximus*: Frogs from different localities (viz. Jowai and Cherrapunji) were collected to carry out the investigation. The specimens showed a less complex LDH pattern and in most tissues only two bands of A_4 and B_4 isozymes were obtained (Fig.5; Plate 12).

Gonadal tissue LDH resolved into two intensely stained bands of A_4 and B_4 isozymes in the specimens from both the population (Fig.5a,b; Plate 12a,b). Minor activity of A_3B_1 isozyme was noted in the specimens collected from Cherrapunji.

Kidney tissue extract showed a two-banded pattern similar to that of gonadal isozyme in the specimens collected from Jowai (Fig.5a; Plate 12a), while a single band of A_4 isozyme was expressed in the specimens collected from Cherrapunji (Fig.5b; Plate 12b).

LDH isozymes in the cardiac muscle tissue of the specimens from both the population resolved into two major bands of A_4 and B_4 homotetramers (Fig.5a,b; Plate 12a,b). However, in the specimens examined from Cherrapunji the tissue also showed minor activity of A_3B_1 and A_1B_3 heterotetramers.

Brain tissue of the specimens from both the localities showed predominance of A_4 isozyme and only little activity of A_3B_1 heterotetrameric form in the specimens tested from Cherrapunji (Fig.5b;Plate 12b).

Eye tissue also possessed a single cathodal band of A_4 isozymes. The specimens collected from Jowai, however showed an additional lighter band representing B_4 activity (Fig.5a; Plate 12a).

Liver tissue LDH resolved into strongly stained A_4 and weakly stained B_4 homotetramers in the specimens collected from Jowai (Fig.5a;Plate 12a), while the specimens from Cherrapunji showed only the A_4 isozyme (Fig.5b;Plate 12b).

Skeletal muscle tissue presented a single-banded phenotype of A_4 isozyme in both the population. No other form of the isozyme could be detected in the tissue (Fig.5a,b; Plate 12a,b).

(e) *Amolops afghanus*: Six populations (viz. Nongkhlaw, Mawkaiaw, Williamnagar, Smit, Shillong and Mawsynram) were examined to depict the LDH gene expression in the specimens. Number of LDH bands in all the individuals studied from the said populations varied from 2-4 (Fig.6; Plate 13) homotetramers A_4 and B_4 showed the usual tissue specific

distribution in skeletal muscle and cardiac muscle respectively.

Specimens examined from Nongkhilaw, Mawkaiaaw and Shillong showed a three-banded LDH pattern in the gonadal tissue corresponding to A_4 , A_3B_1 and B_4 isozymes (Fig. 6a,b,c; Plate 13a,b,c). A four-banded phenotype corresponding to A_4 , A_3B_1 , A_2B_2 and B_4 was expressed in the specimens from Mawsynram (Fig. 6f; Plate 13f). Two-banded phenotypes corresponding to A_4 and B_4 isozymes in specimens from Smit (Fig. 6d; Plate 13d) and A_4 and A_3B_1 in the specimens from Williamnagar (Fig. 6c; Plate 13c) were also obtained.

Kidney tissue LDH resolved into a maximum of four bands in the specimens examined from Shillong and Mawsynram (Fig. 6e,f; Plate 13e,f) the B_4 isozyme being more intensely stained. A_4 , A_3B_1 isozymes were moderately stained while A_2B_2 took very light stain. Three bands of LDH corresponding to A_4 , A_3B_1 and B_4 isozymes were obtained in the specimens from Nongkhilaw and Mawkaiaaw (Fig. 6a,b; Plate 13a,b). Only two bands of strongly stained A_4 and weakly stained B_4 were resolved in the specimens from Smit (Fig. 6d; Plate 13d).

Cardiac muscle tissue showed a predominance of anodally

migrating isozymes. A four-banded pattern of A_4 , A_3B_1 , A_2B_2 and B_4 isozymes were obtained from the specimens analysed from Nongkhlaw, Williamnagar and Mawsynram (Fig. 6a,c,f; Plate 13a,c,f). Specimens examined from Mawkaiaw, Smit and Shillong possessed two to three bands corresponding to A_4 , A_3B_1 and B_4 isozymes (Fig. 6b,d,e; Plate 13b,d,e).

In the brain tissue LDH isozymes were resolved into three-banded pattern in the specimens analysed from Mawkaiaw, Shillong and Mawsynram (Fig. 6b,e,f; Plate 13b,e,f). These bands correspond to A_4 , A_3B_1 and B_4 isozymes. The specimens from Nongkhlaw showed the presence of A_2B_2 isozymes in addition to the above three bands (Fig. 6a; Plate 13a). Specimens examined from Williamnagar (Fig. 6c; Plate 13c) possessed A_4 and A_3B_1 isozymes while both A_4 and B_4 isozymes were expressed in the specimens examined from Smit. (Fig. 6d; Plate 13d).

Eye tissue LDH showed a similar electrophoretic pattern to that of brain tissue in the specimens of respective populations. However minor activity of A_3B_1 and A_2B_2 was observed in the population from Smit. (Fig. 6d; Plate 13d).

Liver tissue possessed only two isozymes corresponding to A_4 and A_3B_1 in all the specimens examined from Nongkhlaw,

Mawkaiaw, Williamnagar (Fig.6a,b,c; Plate 13a,b,c). A Three-banded LDH isozyme pattern was obtained for the other three populations. Specimens from Smit possessed A_4 , A_3B_1 and A_2B_2 isozymes (Fig.6d; Plate 13d), while specimens from Shillong showed A_4 , A_3B_1 and B_4 isozymes (Fig.6e; Plate 13e). Out of the three bands obtained in the specimens from Mawsynram, two bands correspond to A_4 and A_3B_1 respectively, whereas the third band showed a different electrophoretic mobility and resolved most cathodally, thereby indicating a tissue-specific expression (Fig.6f; Plate 13f).

LDH isozymes in skeletal muscle tissue showed a uniform pattern of two bands corresponding to A_4 and A_3B_1 isozymes in all the populations except Smit. Out of the four bands obtained in the specimens tested from Smit (Fig.6e; Plate 12e), the most cathodal band correspond to A_4 , two intermediate bands of low staining intensity correspond to A_3B_1 and A_2B_2 and the fourth most anodal band was the B_4 isozyme.

(f) *Rana cyanophlyctis*: Specimens collected from three localities (viz. Shillong, Jowai and Williamnagar) were examined to obtain the LDH gene expression. A typical five-banded LDH pattern was obtained for all the tissues except skeletal muscle.

Gonad tissue contained all the five isozymes. In comparison, the B_4 isozyme was most intensely stained followed by A_1B_3 isozymes. The isozymes A_4 , A_3B_1 and A_2B_2 showed similar staining intensity (Fig. 7a,b,c; Plate 14a,b,c).

Kidney tissue showed an LDH pattern of five isozymes similar to that of gonad. Both A_4 and B_4 isozymes showed identical staining intensity in the specimens tested from Shillong and Jowai (Fig. 7a,b; Plate 14a,b). The A_4 isozymes in the kidney tissue of specimens from Williamnagar was poorly stained (Fig.7c; Plate 14c).

All the five isozymes were expressed in cardiac muscle tissue, the B_4 isozyme being highly active towards the anode. The isozymes A_4 , A_3B_1 , A_2B_2 showed relatively less staining intensities in the specimens examined from Williamnagar (Fig.7c; Plate 14c).

Brain tissue LDH resolved into a five banded pattern of the major isozymes. The isozymes A_4 and A_3B_1 showed highest staining intensity followed by B_4 and A_2B_2 , the least being the A_1B_3 isozyme (Fig.7a,b,c; Plate 14a,b,c).

In the specimens from Shillong and Williamnagar eye tissue presented a similar pattern to that of brain, with

respect to number of bands and staining intensities. (Fig.7a,c; Plate 14a,c). All the five isozymes were faintly stained in the specimens tested from Jowai (Fig.7b; Plate 14b).

In the liver tissue all the homotetramers (A_4 and B_4), and heterotetramers (A_3B_1 , A_2B_2 and A_1B_3) were present. The specimens examined from Jowai (Fig.7b; Plate 14b) showed a six-banded LDH pattern. An intermediate band between A_4 and A_3B_1 was resolved in the specimens. Moreover the heterotetramers A_2B_2 and A_3B_1 were less intensely stained. The isozyme A_4 was found to be highly active in the tissue.

Skeletal muscle tissue presented a simple pattern of either two bands corresponding to A_4 and A_3B_1 (Fig.7a,c; Plate 14a,c) or a single band of A_4 isozyme (Fig.7b; Plate 14b) at the cathodal end. However a faint band of B_4 isozymes was observed in the specimens tested from Shillong (Fig.7a; Plate 14a).

(g) *Rana nicobariensis*: Individuals from three localities (viz. Mawkaiaw, Williamnagar, and Mawhati) showed a uniform LDH banding pattern with five isozymes (Fig. 8; Plate 15). Gonad LDH isozyme resolved into equally spaced five bands in the specimens analysed from Mawkaiaw and Williamnagar (Fig. 8a,b; Plate 15a,b). The homotetramer A_4 and

heterotetramers A_3B_1 , A_2B_2 and A_1B_3 were less intensely stained in the specimens tested from Mawhati (Fig.8c; Plate 15c).

Kidney tissue showed a similar pattern to that of gonad. The heterotetramers A_2B_2 and A_1B_3 were stained very lightly in the specimens examined from Williamnagar and Mawhati (Fig.8b,c; Plate 15b,c).

LDH isozymes in cardiac muscle resolved into a five-banded pattern, the B_4 isozyme being highly active. The A_4 isozyme could not be detected in the specimens examined from Mawhati (Fig.8c; Plate 15c).

An equally spaced five-banded phenotype was obtained in the brain tissue extract. Both the homotetramers A_4 and B_4 showed similar activity, of the heterotetramer A_3B_1 showed highest staining intensity (Fig.8a,b,c; Plate 15a,b,c).

Eye tissue presented a similar isozyme pattern to that of brain tissue. The isozymes A_4 and A_3B_1 being most intensely stained than the other isozymes (Fig. 8a,b,c; Plate 15a,b,c).

Liver showed a similar five-banded LDH pattern in the specimens tested from Williamnagar and Mawhati (Fig.8b; Plate 15b) while specimens collected from Mawkaiaaw showed

only three bands, heterotetramers A_2B_2 and A_1B_3 were not detectable (Fig. 8c; Plate 15c).

Skeletal muscle tissue extract presented a simple two banded LDH being composed of A_4 and A_3B_1 isozymes, in the specimens analysed from the three localities. (Fig. 8a,b,c; Plate 15a,b,c).

3.1.2. Malate dehydrogenase (L-malate NAD oxidoreductase, E.C.1.1.1.37).

(a) *Hyla annectens*: All the seven tissues of the specimens analysed from two populations (viz. Smit and Mawphlang) revealed only two bands of MDH with almost equal staining intensities (Fig. 9a; Plate 16a). The most cathodal band was designated as A_2 and the other as B_2 fraction respectively.

(b) *Polypedates leucomystax*: All the seven tissues of the specimens examined from four localities (viz. Shillong, Jowai, Mawhati, Williamnagar) showed similar staining intensities (Fig. 9b; Plate 16b). MDH was resolved into a less anodal relatively condensed band and a more anodal much diffused zone.

(c) *Rhacophorus reinwardtii*: Out of seven tissues of the specimens analysed from four populations (viz. Smit, Mawsynram, Jowai, and Mawhati) skeletal muscle and liver

showed a three-banded pattern (Fig.9c; Plate 16c), other tissues showed only two bands while gonad sMDH was resolved as single more anodal band.

(d) *Rhacophorus maximus*: A two-banded phenotype of s-MDH was observed in all the seven tissues of the specimens tested from Jowai and Cherrapunji. The bands showed similar staining intensities except liver tissue where both the A_2 and B_2 fractions took deep stain (Fig. 9d; Plate 16d).

(e) *Amolops afghanus*: Tissue s-MDH was resolved into 2-3 banded pattern. Gonad, brain, eye and skeletal muscle tissue s-MDH were found to be composed of two bands while kidney, heart and liver tissue s-MDH resolved into a three banded pattern (Fig. 9e; Plate 16e).

(f) *Rana cyanophlyctis*: A three - banded phenotype was observed in all the tissues of the specimens tested from different populations (Fig. 9f; Plate 16f). However the staining intensities of the isozyme was found to be very less in muscle tissue.

(g) *Rana nicobariensis*: All the tissues except kidney and liver showed a single-banded s-MDH pattern (Fig.9g; Plate 15g). In the kidney a very faint band close to the B_2 homodimer could be seen and liver tissue extract presented a

cathodal band in addition to the B₂ anodal band.

3.1.3. *Alcohol dehydrogenase* (Alcohol : NAD-Oxidoreductase, E.C.1.1.1.1.):

(a) *Hyla annectans*: Out of the seven tissues of the specimens collected from Smit and Mawphlang only liver and kidney tissues showed ADH activity (Fig.10a; Plate 17a). In the kidney tissue a single band of ADH with moderate staining intensity and slightly faster mobility towards anode was obtained. Liver tissue also showed a single ADH isozyme of high activity with less anodal migration in comparison to the kidney isozyme.

(b) *Polypedates leucomystax*: Kidney and liver tissue ADH of the specimens from all the four populations (viz. Shillong, Jowai, Mawhati and Williamnagar) presented a single banded isozyme pattern. The kidney isozyme showed anodal affinity and was faintly stained. In the liver tissue ADH was intensely stained and migrated slowly towards the anode (Fig.10b;Plate 17b). Other tissues did not show any activity for ADH.

(c) *Rhacophorus reinwardtii*: A similar pattern of ADH isozyme to that of *P.leucomystax* was found to occur in the species (Fig.10c;Plate 17c). ADH isozyme being resolved in the

kidney tissue as a faint anodally migrating band and a deeply stained less anodal band in the liver tissue of the species collected from all the four localities (viz. Smit, Mawsynram, Jowai and Mawphlang).

(d) *Rhacophorus maximus*: ADH isozyme of the specimens collected from Jowai and Cherrapunji presented identical pattern. ADH activity was observed only in kidney and liver tissues (Fig.10d;Plate 17d). A single ADH was resolved in the kidney tissue. Liver exhibited a two-banded isozyme pattern. Out of the two bands, one band was faintly stained and resolved cathodally while the other stained deeply and showed same mobility to that of the kidney isozyme.

(e) *Amolops afghanus*: ADH isozymes of the species collected from six different localities (viz. Nongthliaw, Mawkaraw, Williamnagar, Smit, Shillong and Mawsynram) showed a uniform pattern. Only kidney and liver tissue ADH could be resolved (Fig.10e;Plate 17e). Kidney showed two banded pattern, one band was very faintly stained and highly cathodal, while the other was moderately stained and showed a faster anodal migration. In the liver tissue only one intensely stained band of ADH was resolved. The liver ADH showed a slow anodal migration than that of the faster anodal ADH isozyme of kidney.

(f) *Rana cyanophlyctis*: All the specimens analysed from the three localities (viz. Shillong, Jowai, and Williamnagar) exhibited an identical ADH banding pattern. Except kidney and liver ADH activity was not observed in other tissues. Kidney showed a single isozyme band with very fast anodal migration. In the liver two bands of ADH was resolved. One of the band was resolved cathodally while the other moved faster towards the anode. However the faster anodally migrating liver ADH was not as fast as the kidney isozyme (Fig.10f;Plate 17f).

(g) *Rana nicobariensis*: Specimens collected from three localities (viz. Mawkaiaw, Mawhati, and Williamnagar) exhibited the same ADH pattern. In the kidney tissue ADH resolved into a single band of moderate staining intensity. Liver tissue exhibited a two banded phenotype. Out of the two bands a more cathodal band was intensely stained while the other less cathodal band was moderately stained. Comparison of the relative mobilities indicate the less cathodal isozyme of kidney and liver are same (Fig.10f;Plate 17f). ADH activity was not detected in other tissues.

3.1.4. *Glucose-6-phosphate dehydrogenase* (D-Glucose-6-phosphate : NADP-oxidoreductase, E.C. 1.1.1.49).

(a) *Hyla annectans*: Specimens collected from Smit and

Mawphlang presented the same banding pattern (Fig.11a;Plate 18a). Heart, kidney, brain and eye tissues possessed single G6PDH. In the gonad and liver two bands were observed. A faint band of the isozyme was resolved in these tissues cathodally in addition to the band observed in other tissues. Skeletal muscle tissue did not show any activity of the enzyme.

(b)*Polypedates leucomystax*: Four populations (viz. Shillong, Jowai, Mawhati, and Williamnagar) were analysed to study the G6PDH isozyme pattern of the species. Specimens showed identical results (Fig.11b; Plate 18b). A single-banded pattern of G6PDH was observed in kidney, heart, brain, eye and liver tissues. No activity was recorded in skeletal muscle. In the gonadal tissue two bands of G6PDH were resolved. Out of these one correspond to the isozyme found in other tissues while the other, more cathodal took deep stain.

(c)*Rhacophorus reinwardtii*: All the specimens analysed from four localities (viz. Smit, Mawsynram, Jowai and Mawphlang) exhibited single band of G6PDH in gonad, kidney, heart, brain tissues (Fig.11c;Plate 18c). In the eye tissues two bands were resolved, one band was faintly stained and resolved at the cathodal end. The other being less cathodal,

deeply stained and correspond to isozyme found in other tissues in its relative mobility. Liver also possessed two isozymes, of which one strongly stained band resolved cathodally corresponding to isozyme found in other tissues and a faint band moved faster towards anode. A similar faint band was also resolved anodally in the skeletal muscle of the species.

(d) *Rhacophorus maximus*: A single-banded G6PDH isozyme was observed in gonad, kidney, heart, brain and eye tissues of the specimens tested from Jowai and Cherrapunji. The band resolved cathodally. In the liver tissue the band was most intensely stained. In addition liver also showed a fast anodally migrating band similar to that of *R.reinwardtii*. This band was also found to occur in the skeletal muscle (Fig.11d; Plate 18d).

(e) *Amolops afghanus*: A single band of G6PDH isozyme was resolved in gonad, kidney, heart, brain, eye and liver tissues. Skeletal muscle did not show any activity of the isozyme (Fig.11e; Plate 18e). The activity was found to be highest in liver followed by kidney tissue. The pattern was uniform for all the specimens analysed from different populations (viz. Nongkhilaw, Mawkaiaw, Williamnagar, Smit, Shillong and Mawsynram).

(f) *Rana cyanophlyctis*: Specimens analysed from Shillong, Jowai and Williamnagar showed similar G6PDH isozyme pattern (Fig.11f; Plate 18f). Gonad, heart, brain and eye tissues possessed single isozyme. In the kidney and liver a two-banded phenotype was observed. Out of these two bands one band was deeply stained and correspond to the isozyme in other tissues. The second band was faintly stained and resolved more cathodally. In the skeletal muscle G6PDH could not be detected.

(g) *Rana nicobariensis*: Uniform result was obtained for specimens obtained from Mawkaiaw, Williamnagar and Mawhati (Fig.11g; Plate 18g). Single band of G6PDH was observed in gonad, heart, brain, eye and liver tissues. In the eye and liver tissues the band was intensely stained. Two bands of the isozyme were recorded in the kidney tissue, one corresponds to the isozyme found in other tissues and was intensely stained. A second faint band was resolved in the tissue more cathodally. Skeletal muscle did not show activity for G6PDH.

3.2. Thermostability studies :

3.2.1. *Lactate dehydrogenase*: Heart and skeletal muscle tissue sample incubated at 60°C for 10 minutes when analysed

electrophoretically showed no band at the cathodal end indicating the loss of A_4 isozyme. When samples were incubated at 65°C for 10 minutes, the anodal bands disappeared, which implied the denaturation of B_4 isozymes. Single banded phenotype obtained in some of the specimens of *P.leucomystax* (Fig.3f; Plate 10g) and *H.annectens* (Fig.2b; Plate 9b) when subjected to such test showed stability of the isozyme at the lower temperature and the activity starts declining at 60°C . This clearly indicated that the isozyme was A_4 .

3.2.2. *Malate dehydrogenase*: The heat inactivation tests applied to s-MDH isozymes in the tissue of seven species suggested, as commonly found in other vertebrates, that the A_2 dimer is thermostable than the B_2 fraction.

3.2.3. *Alcohol dehydrogenase*: Kidney and liver tissue sample incubated at 50°C for 10 minutes when analysed for ADH activity showed no bands indicating total denaturation of ADH.

3.2.4. *Glucose-6-phosphate dehydrogenase*: Tissue sample showed G6PDH activity upto 40°C . The activity diminished when the temperature was increased to 60°C .

3.3. TOTAL PROTEIN CONTENT ANALYSIS:

A 10% tissue extract analysed for the seven tissues revealed that the concentration of proteins was highest in the skeletal muscle tissue followed by liver tissue extract. Rest of the tissues showed a moderate concentration of protein, the least being the gonad.

CHAPTER-I
DISCUSSION

4.1 LACTATE DEHYDROGENASE (LDH; E.C.1.1.1.27)

Lactate dehydrogenase isozyme has proven to be extremely useful in the comparison and characterization of multilocus protein systems. The enzyme play important role in the carbohydrate metabolism, catalyzing the reactions of the lactate oxidation as well as pyruvate reduction. During anaerobic periods when the Krebs cycle is inoperative, pyruvate is converted to lactate by LDH with a concomitant production of NAD. This oxidation of NADH to NAD allows glycolysis to continue, which in turn permits the continued production of ATP for energy (Whitt *et al.*, 1973).

The isozyme has been reported to be of common occurrence in all groups of vertebrates (Markert and Ursprung, 1974). In vertebrates the ubiquitous enzyme has a tetrameric structure with a total molecular weight of 140,000 (Darnall and Klotz, 1975) and exists in several molecular forms.

The LDH polypeptide subunits in vertebrates are encoded in atleast two codominant loci (Markert, 1962; Shaw and Barto, 1963 and Cahn *et al.*, 1962). The two corresponding types of polypeptide subunits produced are designated A₄ and B₄ (Markert, 1962), which upon association generate five

tetrameric isozymes. These five functionally active molecular forms are designated as $LDH_1 = B_4$, $LDH_2 = A_1B_3$, $LDH_3 = A_2B_2$, $LDH_4 = A_3B_1$ and $LDH_5 = A_4$. These isozymes differ in net charge and thus can be separated by electrophoresis. In mammals, birds, reptiles and amphibians the B_4 homotetramer has the greatest net negative charge, and consequently migrates rapidly towards the anode. The same is true for most fishes, but a significant number showed a reverse mobility (Markert and Faulhaber, 1965; Chatterjee and Dhar, 1985). The A_4 isozyme predominate in tissues which undergo anaerobic glycolysis, such as the skeletal muscle whereas B_4 isozyme is the major form found in tissues with an aerobic metabolism like the heart (Wilson et al., 1963). Some vertebrates possess a third LDH, which in mammals and birds is called C-isozyme and is expressed only in spermatocytes (Goldberg, 1972, Zinkham et al., 1969). The C_4 isozyme have also been found in many bony fishes (Markert et al., 1975). The tissue specificity of LDH-C gene expression is very broad in lower bony fishes and is found in heart, gills, liver, spleen, kidney and gonads. In more advanced teleosts the gene has a very restricted expression to neural tissues such as the eye and brain, where it is designated as E_4 (Lush et al., 1969; Whitt, 1970). In *Gadida* the C_4 isozyme is predominantly synthesized in liver

designated as F_4 (Sensabaugh and Kaplan, 1972). Shaklee et al., (1973) have shown that the eye specific E_4 and the liver specific F_4 are coded by the same locus C which exhibits tissue specific expression. A kidney specific K_4 expression of the C gene has also been found in Indian catfishes (Chatterjee and Joseph, 1988). In electrophoretic separation the liver specific (F_4) and the kidney specific (K_4) LDH isozyme are found to be resolved cathodally while the eye specific (E_4) possess more net negative charge and migrates anodally.

Little is understood about the metabolic significance of C_4 isozyme and the heterotetramers (A_2B_2 , A_3B_1 and A_1B_3). However it has been postulated that the retinal E_4 is involved in the regeneration of rhodopsin in the photoreceptor cells (Nakano and Whiteley, 1965). One of the characteristics of the mammalian C-type LDH is their ability to utilize α -ketoglutarate as a major substrate (Schatz and Segal, 1969), this reflect a specialized need for the isozyme in the metabolism of mammalian spermatocytes. As regards the heterotetrameric hybrid forms of A_4 and B_4 types, it may be speculated that a tissue whose metabolism is intermediate between that of heart and muscle tissue may function best with a mixture of both types of lactate dehydrogenase.

A variety of LDH banding patterns has been observed in

the presently studied frogs. Each species presented a distinctive pattern with respect to tissue specificity and mobility of the isozymes on the polyacrylamide gel. The tissue specificity of the patterns manifests itself in the predominance of acidic isozymes of the heart, and the predominance of basic ones in skeletal muscle and liver in all the tested species. Among the three Ranid species, two (viz. *R. nicobariensis* and *R. cyanophlyctis*) were found to possess all the five isozymes in their tissues except skeletal muscle where the homotetramer A_4 and only one heterotetramer A_3B_1 were expressed. The other Ranid species (viz. *A. afghanus*) however showed a deviation from classical 5-banded pattern and a maximum of 4 bands could be resolved. The heterotetramer A_1B_3 was not detected in any of the tissue examined from the different population. The species however possessed a highly cathodal tissue specific band resolved in the liver (Fig. 6f; Plate 13f) tissue of the specimens examined from Mawsynram (East Khasi Hills), a high rainfall area. Studies on the Ranid species by earlier workers have also showed a similar pattern of 5-isozymes in various tissues. (Moyer et al., 1968; Case et al., 1975; 1978; Lakshmi pathi et al., 1980; Chatterjee and Prakash, 1990). Table 4 represents a compiled list of Ranid species examined for LDH isozyme pattern.

TABLE 4 : No. of LDH bands observed in Ranid species

Species	Maximum No. of bands	Reference
<i>Rana gryllio</i>	4	Wright et al., 1966
<i>R. temporaria</i>	2	Chen, 1968
<i>R. catesbiana</i>	8	Moyer et al., 1968
<i>R. clamitans</i>	4	''
<i>R. aurora</i>	3	''
<i>R. palustris</i>	4	''
<i>R. pipiens</i>	3	''
<i>R. septentrionalis</i>	6	''
<i>R. virgatipes</i>	25	''
<i>R. sylvatica</i>	18	''
<i>R. boylei</i>	5	Case, 1975
<i>R. esculanta</i>	5	''
<i>R. muscosa</i>	5	Case, 1978
<i>R. pretiosa</i>	4	''
<i>R. tetrahumaræ</i>	4	''
<i>R. cascadae</i>	3	''
<i>R. hexadactyla</i>	4	Lakshmipathi et al., (1980)
<i>R. tigrina</i>	5	''
<i>R. limnocharis</i>	5	Chatterjee and Prakash (1990)
<i>R. nicobariensis</i>	5	Present study
<i>R. cyanophlyctis</i>	5	''
<i>Amolops afghanus</i>	4	''

The situation in the Rhacophoridae seems to be more complicated. Various number of bands ranging from one to twelve have been found. In *R. reinwardtii* A_4 is predominantly expressed in all the tissues. The heterotetramer A_1B_3 showed no activity in any specimen except gonad in one population (Fig.4d; Plate 11d). Further the eye tissue presented a cathodal tissue-specific band in the specimens examined from Jowai (Fig.4b; Plate 11b).

Rhacophorus maximus, possessed only two homotetrameric A_4 and B_4 isozymes. No heterotetrameric form were found in any of the tissues of the species. the occurrence of only two bands (A_4 and B_4) have also been found in the urodales, *Iriturus* (Faulhaber and Lange-Lyra, 1978), *Desmoquathus* (Shontz, 1968) and in *Rana temporaria* (Glaender and Funz, 1966) with no hybridization between the polypeptides.

Polypedates leucomystax showed a great variation of LDH pattern from different populations and even within the same population. The specimens examined from Shillong presented a classical five-banded phenotype though some of the specimens possessed nine to twelve LDH bands in different tissues (Fig.3c,d; Plate 10c,d). Occurrence of such multiple bands than the usual five have been reported in *Xenopus laevis* by a number of workers (Funz and Hearn, 1967; Claycomb and Villet, 1971; Funz, 1973; Faulhaber and Lange-Lyra, 1978).

Hyla annectens, showed two types of LDH pattern from two different populations, one population (Smit) presented a typical closely spaced five bands (Fig.2a;Plate 9a) while the other showed only a single band (Fig.2b;Plate 9b) in all the tissues.

Similar studies in fishes have revealed that they may contain one, two, three, four, five, or many electrophoretically distinguishable isozymes of LDH (Markert and Faulhaber, 1965; Achaval, 1984). Although the A_4 and B_4 subunits have different amino-acid composition but are homologous, because they interact to form active tetramers *in vitro* and *in vivo* (Markert, 1963; Chilson et al., 1963, Markert et al., 1975). The binomial distribution of five isozymes have been reported in many vertebrates including frogs (Moyer et al., 1968; Vonwyl and Fischberg, 1980; Chatterjee and Prakash, 1990). In the present investigation an identical result was obtained for *Rana cyanophlyctis*, *Rana nicobariensis* and certain populations of *Polypedates leucomystax* (Fig.3a,b;Plate 10a,b) and *Hyla annectens*. (Fig.2a;Plate 9a). Further, in one population (Mawhati) of *P. leucomystax*, a five-banded pattern was obtained in heart and liver (Fig.3g,h;Plate 10h,i) while other tissues possessed one to three bands. The classical five-banded

pattern with identical electrophoretic mobility obtained for *Rana cyanophlyctis* and *Rana nicobariensis* in different populations reveals a homozygous state of the loci coding for LDH (Fig.7a,b,c;Plate 14a,b,c and Fig.8a,b,c;Plate 15a,b,c). Occurrence of all the five isozymes in most of the tissues (except skeletal muscle) of the specimens analysed from different localities indicate a greater demand of energy derivation utilizing both lactate and pyruvate as substrates. *Rana cyanophlyctis* is a non-hibernating, water frog and is commonly referred to as 'water skipper' because of its powerful diving habit. The capability of prolonged under water survival as well as occasional swim to the surface may be related to the tissue distribution of LDH of the species. *Rana nicobariensis*, on the other hand is a terrestrial frog available in warm humid climatic condition at lower altitude. During breeding season these frogs leap across the land in search of suitable water bodies. Further at lower altitude the environmental temperature is high and fluctuates diurnally. Thus at higher temperature under conditions of low ambient oxygen tension and high metabolic rate, as pyruvate levels increase, A_4 isozyme is most active, enabling the frogs to maintain a high level of anaerobic metabolism. At lower temperature oxygen availability probably increases and B_4 isozyme become active, allowing pyruvate to be aerobically oxidised.

Therefore the balanced catalytic requirement of A and B type of LDH subunits in the species could be enhanced by the thermal modulation.

A similar explanation to that of *R. nicobariensis* could be extended to the five banded LDH pattern observed in the specimens of *Polypedates leucomystax* (Fig.3a;Plate 10a,b) and *Hyla annectens* (Fig.2a;Plate 9a). These frogs are arboreal in habit and exhibits active migration locally during breeding season.

However, the binomial distribution of the five isozymes is not of universal occurrence in many vertebrates. This implies that the A and B subunits do not randomly associate in tetramers either because of the primary structure of the subunits or because epigenetic mechanisms that operate in order to restrict the association of subunits or because of the evolutionary divergence of subunits A and B (Markert and Faulhaber, 1965; Markert, 1968). A similar non-binomial distribution of the isozymes have been observed in some of our frogs viz. *A. afghanus*, *R. reinwardtii* and *R. maximus*. Less than the usual five LDH isozymes have also been reported in a number of frogs by numerous workers (Dessauer et al.,1977; Wright and Noyer, 1966; Lakshminpathi et al.,1980; Moyer et al., 1968; Case, 1978).

The two-banded pattern as observed in gonad, kidney and cardiac tissue of *R. maximus* was formed probably by the presence of two homopolymeric A_4 and B_4 isozymes and the absence of the expected heteropolymers.

This clearly indicates that both the loci coding for LDH were active in gonad, kidney and heart tissues of the specimens analysed from Jowai (Fig. 5a; Plate 12a) and gonad and heart tissues of the specimens from Cherrapunji (Fig. 5b; Plate 12b). Minor activity of B_4 isozyme was also noted in eye and liver tissues of the specimens from Cherrapunji in addition to the strongly expressed A_4 isozyme. Though Cherrapunji receives a higher rainfall compared to Jowai, the ecological conditions seems to be otherwise identical in both the places with large scale deforestation and mining of coal and limestone in the surrounding areas. Perhaps these factors coupled with their physiological adjustment resulted gonad, kidney, heart and to certain extent eye and liver tissues to utilise both lactate and pyruvate as substrates in energy metabolism. Other tissues, on the contrary are subjected to anaerobic glycolysis utilising only pyruvate (Holmes and Markert, 1969).

In *Rhacophorus reinwardtii* A_4 is predominantly

expressed in all the tissues. The heterotetramer A_1B_3 showed no activity in any tissues except gonad of the specimens analysed from Smit (Fig.4d;Plate 11d). Mawsynram receives the highest rainfall in the world, the biota consist of dense temperate forests with relatively less human influence. A similar condition prevails in Mawphlang, though the rainfall is not as high as Mawsynram. The specimens of *R. reinwardtii* collected from these two places as well as from Smit and Jowai showed predominance of A_4 isozyme followed by A_3B_1 and A_2B_2 in their tissues (Fig.4a,b,c,d; Plate 11a,b,c,d). However gonad and heart tissues of the specimens from Mawphlang and Smit also showed the activity of B_4 and A_2B_2 isozymes (Fig.4c,d;Plate 11c,d). The B_4 isozyme was also strongly expressed in the heart tissue of the specimens tested from Mawsynram (Fig.4a;Plate 11a). This restriction of B gene expression in all other tissues of the specimens reflect that they are dependant on anaerobic glycolysis for deriving energy (Markert and Holmes, 1969). The absence of the heterotetramers A_1B_3 in these frogs could be the result of restricted subunit assembly (Whitt, 1970) or an instability of the asymmetrical heteropolymer (Shaklee et al.,1973). Further the specimens analysed from Jowai, presented an additional cathodal band close to A_4 band in the eye tissue (Fig.4b; Plate 11b). From the ecological point of view, Jowai with its moderate rainfall, temperature

and subtropical forest cover constitutes an ideal habitat for amphibians . However the level of environmental pollution (No data available) seems to be high due to deforestation and unscientific means of coal mining activities in the surrounding areas. Thus in absence of the B gene expression this additional isozyme perhaps, plays a role in the visual physiology, as have been postulated for eye-specific LDH isozyme of C gene expression in fishes (Nakano and Whiteley,1965; Chatlerjee and Dhar,1985). The Kanid frog *Amolops afghanus* analysed from six different localities presented a maximum of three to four bands with restricted subunit assembly (Whitt, 1970). The heterotetramer A_1B_3 was not formed in any tissue of the specimens (Fig.6;Plate 13), while the isozyme A_2B_2 showed minor activity in certain populations (Fig.6a,c,e,f; Plate 13a,c,e,f). The liver tissue of the specimens collected from Mawsynram possessed three isozymes of LDH. Out of these, two bands correspond to A_4 and A_3B_1 while the third band resolved much cathodally and showed a tissue preference. Similar liver specific cathodal isozyme have been reported in fishes (Klose et al.,1969; Shaklee et al.,1973). The occurrence of this additional isozyme was probably of great physiological importance to the frogs inhabiting a highly humid niche. The B locus being inactive in the tissue,

predominance of A_4 isozyme along with the additional liver specific isozyme provided the frogs with greater metabolic precision.

Specimens of *P. leucomystax* analysed from Mawhati and Williamnagar having a higher ambient temperature, however exhibited a restriction in heteropolymer assembly in their tissues (Fig.3f,g,h; Plate 10g,h,i; and Fig.3d,e; Plate 10e,f). This restriction could be perhaps either due to a function of their physiological adjustment thereby eliminating the need of the isozyme or instability of heterotetramer (Shaklee et al.,1973).

Our observation of a single banded LDH pattern in one population of each of *P. leucomystax* and *H. annectens* is comparable to the most primitive form in lampreys where only A_4 isozyme occurs (Wilson et al.,1964; Whitt et al.,1975). Occurrence of a single band towards the cathodal end in these frogs suggests that the isozyme is a basic form of LDH (A_4). The single banded pattern of *Hyla annectens* (Fig.2b;Plate 9b) as confirmed by thermostability test as well as observation of relative mobility of the band to be composed of A_4 isozyme only. This raises a vital question regarding the functional significance of the isozyme in the specimens because the same species when tested from Smit showed all the five isozymes(Fig.2a;Plate 9a) in gonad,

kidney and heart tissues. The restriction of the B gene expression in the specimens from Mawphlang may be attributed to the local ecological conditions. The place is marked by its high rainfall and mixed evergreen "sacred grove". The average temperature remains constant in summer. The other locality (Smit) on the other hand lies at an higher elevation, receives low rainfall, high wind velocity and frequent temperature fluctuation. The single banded pattern of *P. leucomystax* (Fig-3f; Plate 10g) however was different than that of *H. annectens*. Heat inactivation studies revealed that the isozyme A_4 was resolved in kidney, eye, liver and skeletal muscle. However the relative mobility of the band in gonad, heart and brain showed a less cathodal affinity of the isozyme in these tissues. We presume that subunit composition of this isozyme in these tissues was A_3B_1 .

The occurrence of only A gene activity with a sharp reduction of the B gene expression have been reported in many advanced teleosts (Markert et al., 1975; Whitt et al., (1975) claimed that the LDH from American brook lamprey, possessed only A_4 isozyme. However Agata'Dell et al., (1988) based on electrophoretic and kinetic studies of LDH from *Lampetra planeri* suggested the occurrence of an intermediate form of LDH between the A and B types of polypeptides. They

further concluded that the occurrence of a single LDH type was probably due either through a gene inactivation or a gene loss.

Single banded pattern in one population of each of the above mentioned species in our study is probably due to a gene inactivation, since both the loci (A and B) have been found to be active in the specimens tested from other populations of the same species respectively. The reduction in B gene expression and wide spread expression of the A locus in these frogs perhaps may be linked to their niche condition. It could be suggested that this reduction in B gene expression and more particularly its absence in heart was a result of the frogs being subjected to periods of anaerobiasis (Holmes and Markert, 1969). As a consequence pyruvate level increase and place all the tested tissues, including heart, under conditions of oxygen shortage and accumulation of lactate. Thus A_4 isozyme would be expected to be better suited to those ecological conditions, it being less susceptible to substrate and product inhibition than the B_4 isozyme (Everse and Kaplan, 1970). This could explain the absence of B_4 isozyme in the specimens of above mentioned species.

A large proportion of the specimens of *P. leucomystax*

analysed from Shillong exhibited nine to twelve bands of LDH (Fig.3b,c; Plate 10c,d). Such complex LDH pattern have also been studied in many Anura (Salthe,1968; Salthe and Nevo,1969; Maxson and Wilson,1974; Vogel and Chen,1976) and Urodela (Balek and Snow,1967; Sherr,1968). Seven, nine, eleven and eighteen LDH isozymes have been reported for *Xenopus laevis* (Kunz and Hearn, 1967; Kunz,1973; Sasaki,1977; Lange-Lyra and Faulhaber,1978; Wall and Blackler,1974). It has been indicated in these studies that random association among the products of the alleles (A^1 and B^1) with the products of loci A and B in a heterozygous state produce such multiple bands. An identical heterozygotic genotype have been clearly reflected in the specimens of *P. leucomystax* analysed from Shillong.

We presume a mutation of locus A resulted into an allele A^1 in the population. Thus the two homozygotes A/A and A^1/A^1 on random mating produced a hybrid A/A^1 with intermediate characteristics. The frequency of the three phenotypes among 52 studied frogs were : 9AA, 12 A^1A^1 and 31 AA^1 . The frequencies of two alleles A and A^1 were 0.47 and 0.53 respectively. The observed number of phenotypes and their Hardy-Weinberg expectations are given in table 5. Results showed that the differences between observed numbers and their Hardy-Weinberg expectations were not significant.

at $P < 0.05$ for the polymorphic locus examined here. This support the validity of the genetic model (Hardy-Weinberg) proposed for the polymorphism and indicate that the *P. leucomystax* samples were collected from a single Mendelian population. Our hypothesis was supported by good agreement between the observed and expected number of the three LDH phenotypes ($\chi^2_{1df} = 2$ and $P > 0.10$). Ofcourse, controlled inheritance experiments are needed to confirm our genetic interpretation. Thus in this heterozygous population of the frog, random association among the products of A, A' and B genes resulted into the observed pattern. We rule out the possibility of a B locus mutation in these frogs because the B₄ isozyme band was resolved as an isolated band in all the specimens with the same electrophoretic mobility (Plate 19). The appearance of the allele A' by a mutation might have a bearing to the general character of the habitat of the frog, due to man-modified environments. Forest environments are becoming increasingly constricted to islands with rapid expansion of urban activities, thereby subjecting the species into pressure of natural selection. Thus, while the homozygotes were able to form only five molecular forms of LDH, the heterozygotes produce a total of nine to twelve different molecular forms. From the observed numbers of heterozygotes (Table 5) in the population, it appears that they are yet to enjoy a definite selective advantage.

Since the hybrid contains three classes of polypeptides encoded by loci A and B and a codominant allele A', fifteen LDH isozymes were expected to be yielded in the specimens. The number of LDH bands observed in tissues of lower vertebrates is suggested to be reduced, presumably due to restriction in the types of monomeric associations that can give rise to enzymatically active tetramers. (Markert et al., 1975) or to similar electrophoretic mobilities of tetramers composed of different monomeric combinations (Maxson and Wilson, 1974).

In figure 12 we propose a hypothetical model from the precise count and measurement of relative mobilities of the LDH bands to explain the complex subunit composition in the heterozygotes. It may be proposed that the subunit composition of LDH isozymes in the individual heterozygotes with nine bands were due to heteropolymeric association between the products of alleles A and A' with that of B gene. Here we presume that no molecular hybridization between the products of A and A' occurred to produce functionally active isozymes. Alternatively it may be suggested that such hybridized products were either unstable or carry same net charge and thus co-migrated in the electrophoretic field. On the other hand individual heterozygotes showing twelve-banded LDH pattern may be

TABLE : 5 : ALLELE FREQUENCY AND CHI-SQUARE ANALYSIS FOR THE HETEROZYGOUS POPULATION OF SHILLONG.

GTYPE	PHENOTYPE	NO.OF INDIVIDUALS		ALLELE FREQUENCY	χ^2
		OBSERVED	EXPECTED		
A/A	AAAA	09	11.48	0.47 A	0.54
A/A'	AAA'A'	31	25.9	-	1.00
A'/A'	A'A'A'A'	12	14.6	0.53 A'	0.46
		N = 52			2.00
					1df and P>0.1

presumed to exhibit the hybridized polypeptides (viz. $A^2_3A_1$, $A^2_2A_2$, $A^2_1A_3$ etc.) there by accounting for the observed pattern.

Less than the expected fifteen isozyme bands for a locus polymorphic have been also reported in other anurans: Salthe (1969) analysed electrophoretic patterns of heart muscle lactate dehydrogenase (HLDH) and was able to distinguish ten isozymes at this locus within the *Rana pipiens* complex in United States. The LDH pattern of M variant of the frog *Hyla regilla* with twelve isozymes was investigated by Maxson and Wilson (1974); nine bands of LDH was observed in *Rana pipiens* (Platz, 1975) for polymorphism of B locus; the pattern of *Rana esculanta* with eleven isozymes organised by two H variant was revealed by Vogel and Chen (1976); seven bands of LDH was observed in *Hyla crucifer* for a variant LDH locus by Koller and Lyster (1977).

The phylogenetic relationships of the various vertebrate LDH isozymes appeared to have been well established (Holmes and Markert, 1969; Whitt, 1969; Holmes, 1972; Whitt et al., 1975). It has been postulated that the LDH A and B loci of vertebrates have evolved by duplication from a single ancestral gene. However, the locus A has been suggested to be the close representative of the ancestral gene and

considerable divergence between A and B loci has occurred in course of time. Further it has been presumed that the C locus in certain vertebrates arose from a duplication of B gene. This third gene is present in fishes, birds and mammals (Markert, 1984). A different evolutionary scheme for LDH genes (Li et al., 1983; Rehse and Davidson, 1986; Baldwin and Lake, 1987) has been proposed in which the C-gene is considered more ancestral like that of the A gene. Baldwin (1988) reviewing the subject, suggested that the ascidian single LDH type and teleost C subunit may represent the ancestral subunit type of vertebrate LDH.

In our experiment we have obtained at least one highly cathodal band other than the A_4 homotetramer in eye and liver tissues of *R. reinwardtii* and *A. afghanus* from two populations (viz. Jowai and Mawsynram) respectively. Similar tissue specific isozymes with different electrophoretic mobilities have also been observed by Andrew et al., 1977 in *Hyla crucifer*, which they stated to be comparable to the C isozymes seen in other vertebrates. Hornby et al., 1939, compared the extra band obtained in brain and eye tissue of *X. laevis* with C polypeptides of less advanced bony fishes. However, at the present state of our investigation it is difficult to ascertain whether or not these amphibians possess the C gene. Whitt (1969) suggested the C gene have

arisen after fish gave rise to the amphibians. Further to establish the presumption that C gene is present in these species, more rigorous experimental procedures are required (viz. purification of the protein, kinetic and immunological test and determination of primary structure) which are beyond the scope of the present study. Vonwyl and Fischberg (1980) observed a similar situation in the genus *Xenopus*. His analysis of heart and ovary tissue revealed an additional anodal band other than the B₄ isozyme. He substantiated the observation by the assumption of a B gene duplication resulting in the occurrence of the additional locus coding for the tissue specific form. We also propose a similar explanation for the occurrence of a cathodal band in eye and liver tissues of two species (viz. *R. reinwardtii* and *A. afghanus*) respectively, though in this case it may be a duplication of the A gene because of its cathodal location.

Salthe (1965) provided evidence based on substrate inhibition studies that amphibians living in standing water tend to be adapted for respiration under conditions of oxygen deprivation; these adaptations include a greater proportion of predominantly A₄ isozymes in their skeletal muscles. Terrestrial amphibians, living where oxygen is plentiful, have larger amount of B₄ isozymes in skeletal muscle than their aquatic relatives. Our collections include

only one standing water frog (viz. *R. cyanophlyctis*) and the rest are semiaquatic, either terrestrial or arboreal. We have not observed such varied expression of A_4 and B_4 isozymes in skeletal muscle of the tested species.

4.2 MALATE DEHYDROGENASE (MDH, E.C.1.1.1.37) :

Malate dehydrogenase (MDH) is a key enzyme of Krebs cycle in aerobic metabolism. The enzyme catalyzes the reversible oxidation of L-malate to oxaloacetate with the concomitant reduction of NAD. Malate dehydrogenase from mammalian (Siegel and England, 1962; Throne and Kaplan, 1963), avian (Kitto and Kaplan, 1966) and most eukaryotic organisms consist of two isozymes. One associated with the mitochondria designated m-MDH and the other with the cytosol, designated as s-MDH. In the present investigation attention have been paid only to the electrophoretic pattern of s-MDH. The cytosolic malate dehydrogenase is known to be dimeric in nature, being encoded in vertebrates at two gene loci, A and B (Banaszak and Bradshaw, 1975). The association of A and B polypeptides with molecular weights 30,000 - 35,000 results in the formation of three dimeric isozymes A_2 , A_1B_1 and B_2 .

The s-MDH electrophoretic pattern obtained (Fig.9;Plate

16) for the frogs in the present investigation is in agreement with those found in fishes (Bailey et al., 1970; Massaro, 1973) and amphibians (Schwantes and Schwantes, 1977). In most of the species two zones of the s-MDH activity were obtained on the gel: a relatively less anodal band and a more anodal broadly stained zone. An identical observation have been made in the anura *Bombina variegata*, where s-MDH isozymes resolved into a narrow band and a more anodal zone on polyacrylamide gel (Chen, 1968). Based upon electrophoretic mobility the less anodal band was designated as A_2 homodimer. Identification of the homodimer B_2 and heterodimer A_1B_1 creates difficulty from results where a broad zone of activity at the anodal end is obtained. However, the possible explanation which we presume applicable in the present case is that, in most of the frogs, both the loci A and B are equally active and code for the respective polypeptides to form A_2 and B_2 homodimers. The heterodimer A_1B_1 though formed, do not resolve separately into a distinct band during electrophoretic separation and closely follow the B_2 homodimers. So both B_2 and A_1B_1 appear together on gel as a broad zone of activity. This presumption draws support from the observation of similar nature in the teleost fish *Barbus* (Frankel and Wilson, 1984).

A different pattern of s-MDH expression was obtained in *R. nicobariensis*, (Fig.9g; Plate 16g) where only one locus was found to be active in all the seven tissues. Similar observation of a single banded s-MDH pattern have been made in anura and urodela (Chen, 1968; Fisher et al.,1980).

The thermostability tests performed in our experiment, agree with those obtained for other vertebrates (Schwantes and Schwantes, 1982). The isozyme A₂ shows itself to be more thermostable than B₂ isozyme.

The findings that A₂ and B₂ isozymes are related in chemical catalytic and immunological properties (Bailey et al.,1970) support the suggestion that these proteins are coded by duplicated genes, which have undergone limited evolutionary divergence unlike LDH isozymes. The occurrence of multiple forms of s-MDH in poikilotherm vertebrates raises the question as to whether the multiplicity is meaningful. It is possible that A₂ and B₂ may have different metabolic roles. It may therefore be that the s-MDH A and B loci have arisen from duplication (Karig and Wilson, 1971; Wheat and Whitt, 1971) and permitted very important functional specialization of the two forms. The presence of the A₂ isozyme, more thermostable in most tissues than B₂ suggests that this isozyme is correlated with temperature

(Basaglia, 1989). It has been suggested that at low temperature B_2 enters into oxidative muscular metabolism, and at high environmental temperature A_2 innervates in the reduction of malate favouring the glycolytic direction (Coppes, et al., 1987).

Allelic variants of s-MDH loci have been noted in a variety of teleost fishes (Frankel, 1985; Frankel and Wilson, 1984), nonteleostean fishes, amphibians (Bailey et al., 1970). Our investigation have not shown such variants.

4.3. Alcohol dehydrogenase (ADH, E.C.1.1.1.1.) :

Alcohol dehydrogenase (ADH) is an NAD (H) dependent enzyme that catalyzes the reversible interconversion of a vast number of aliphatic and aromatic alcohols to their respective aldehydes and ketones. The enzyme is universally distributed in living organisms, occurring in microorganisms, plants and animals. In vertebrates ADH activity is highest in liver (Vallee, 1985), but to a lesser extent it is also found in the kidney and stomach tissues of several species (Hitzeroth et al., 1968; Shaklee et al., 1974).

Alcohol dehydrogenase observed in several mammals, birds, reptiles, amphibians and teleost fishes behaves as a dimeric enzyme, and is apparently encoded in one gene locus

(Shaw and Koen, 1965; Beutler, 1967; Castro-Sierra and Ohno, 1968; Hitzeroth et al., 1968; Selander and Yang, 1969; Schwantes and Schwantes, 1977).

A variety of genetic models have been used to explain the formation of ADH phenotypic pattern. The activity of a single gene may exhibit either a single band or in case of an allelic variant three closely spaced bands on the gel. On the other hand when two genes are active then one may expect a three banded pattern with the bands relatively distant from each other. Occurrence of more than three bands can be attributed to the presence of allelic variant(s).

The genetics and biochemistry of ADH from *Drosophila melanogaster* has been extensively studied and this enzyme has served as a model system. ADH of the fly is encoded by a single structural locus (Grell et al., 1965). Moreover, four allelic variants of this isozyme have been distinguished (Thatcher, 1980).

Compared to other vertebrates the ADH isozymes in amphibians have not received much attention. Wesolowski and Lyerla (1983) obtained four isozymes in the clawed frog *Xenopus laevis*. The isozymes in the frog have been found to be coded by two structural genes designated as Adh-1 and Adh-2. Further Adh-1 possess three electrophoretically

separable isozyme variants capable of forming heterodimers with one another, the alleles being designated as adh-1^a, adh-1^b, and adh-1^c. Adh-2 codes for an isozyme that comigrates with adh-1^c and does not form heterodimers with products of Adh-1 and its alleles. A similar isozyme pattern has been detected in the adult frog of *Rana limnocharis* (Chatterjee and Prakash, 1990).

In the present investigation we have obtained a rather simple ADH isozyme pattern for the frog species tested and the model of Wesolowski and Lyerla (1983) does not hold good. ADH activity in all seven tested species was detected in the liver and kidney tissues. Tissues such as gonad, heart, brain, eye, and skeletal muscle tissues failed to show detectable ADH activity. A similar result of the occurrence of ADH isozymes only in kidney and liver tissues have also been observed in the frog *Odontophrynus americanus* (Schwantes and Schwantes, 1977).

The kidney tissue extract of all seven tested species revealed a single banded ADH phenotype (Fig.10; Plate 17) indicating a dimeric nature of the enzyme, being encoded at a single gene locus. However unlike fishes (Frankel, 1985) and some amphibians (Schwantes and Schwantes, 1977) where the isozyme was found to resolve towards the cathode, our

result showed a more anodal form of the isozyme in at least one species (viz. *R. cyanophlyctis*) (Fig.10f; Plate 17f). This we presume was due to the presence of an allelic variant of the locus coding for the enzyme having predominance of negatively charged aminoacid composition.

Liver tissue extract showed two electrophoretically detectable bands in the three Ranid species (viz. *R. cyanophlyctis*, *R. nicobariensis*, and *A. afghanus*) and in *Rhacophorus maximus* (Fig.10f,g,e,d;Plate 17f,g,e,d). Rest of the species (*R. reinwardtii*, *P. leucomystax* and *H. annectens*) possessed a single cathodal isozyme (Fig.10a,b,c; Plate 17a,b,c).

In *R. cyanophlyctis* and *A. afghanus*, out of the two bands one band showed a faster anodal mobility, indicating a close resemblance to that of the kidney isozyme. Thus it is presumed that the anodal isozyme of liver tissue and the kidney ADH isozyme are being encoded by a single gene locus. This locus may be identical to the ADH-2 locus of *X. laevis* (Wesolowski and Lyster 1983). An observation of the zymogram for liver-ADH isozyme of *R. nicobariensis* and *R. maximus* reveals that the two isozymes are similar in their electrophoretic characteristics. Being resolved at the cathodal end, of the two isozyme one may be presumed to be the product of the gene *Adh-1* and the other is the product

of an allele. Formation of a heterodimer between the gene products; however was not observed in our species.

4.4. GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PDH, E.C.1.1.1.49) :

Glucose-6-phosphate dehydrogenase (G6PDH) catalyzes the glucose-6-phosphate oxidation, providing NADPH for biosynthetic purposes, in the pentose phosphate pathway. It is the first step enzyme of the pentose phosphate shunt, its enzymatic properties and physiological functions have been extensively studied by numerous workers (Shatton, *et al.*, 1971; Cederbaum and Yoshida, 1976; Bautista, *et al.*, 1984). Further G6PDH has proved to be one of the accurate molecular clocks (Ohnishi, 1985).

Glucose-6-phosphate dehydrogenase isozyme is dimeric in vertebrates. (Shaw, 1966). Echinoderms and vertebrates have two isozymes of G6PDH. One designated as form A, is the G6PDH in a strict sense. It is the first step enzyme of the pentose phosphate shunt (E.C.1.1.1.49), and is highly specific for glucose-6-phosphate and NADP (Bautista, *et al.*, 1984) the other designated as form B is called glucose dehydrogenase or hexose-6-phosphate (H6PDH, E.C.1.1.2.47). This has a broad substrate specificity and catalyzes the oxidation of galactose-6-phosphate, 2-deoxyglucose-6-

phosphate as well as glucose-6-phosphate and accept NAD as cofactor (Bautista, *et al.*, 1984).

As regards to their evolution, Hori and his co-workers (1975) have predicted that H6PDH and G6PDH share a common ancestral molecule and their divergence was probably at the time of or before the echinoderm evolution. This prediction is mainly based on the similarity in their catalytic properties, and on the distribution of the two isozymes in animal kingdom (Hori, *et al.*, 1975; Yoshida, 1975). In addition G6PDH exists in all animals, while H6PDH occurs only in vertebrates and echinoderms (Mochizuki and Hori, 1973; 1976; Ohnishi and Hori, 1977).

A single form of G6PDH activity was resolved in gonad, kidney, heart, brain, eye, and liver tissue extracts of the three Ranid Species (*viz.* *R. cyanophlyctis*, *R. nicobariensis* and *A. afghanus*) tested in our investigation (Fig. 11f,g,e; Plate 19f,g,e). Of the various tissues the enzyme was stained more intensely in kidney and liver tissue extracts while skeletal muscle tissue failed to show any activity for the enzyme.

Two forms (A and B) of G6PDH isozyme were detected in the Rhacophorid species (*viz.* *R. reinwardtii*, *R. maximus* and *P. leucomystax*). kidney, heart, brain tissue extracts of all

the three species showed a single band corresponding to A form of isozyme (Fig. 11c,d,b; Plate 18c,d,b). Gonad tissue extract of *P. leucomystax* possessed two bands of G6PDH isozyme (Fig. 11c,d; Plate 17c,d). Eye tissue extract of *R. reinwardtii* showed a similar banding pattern of two G6PDH isozyme. Liver tissue extract of *R. reinwardtii* and *R. maximus* again found to possess both A and B form of isozyme. However, unlike gonad and kidney the Liver G6PDH-B showed an anodal affinity. G6PDH activity was not detected in skeletal muscle tissue extract of *P. leucomystax*, while *R. reinwardtii* and *R. maximus* showed minor activity for G6PDH-B corresponding to the anodally migrating band of liver tissue.

Hyla annectens showed a pattern of both A and B form of isozymes in gonad and liver tissue extracts (Fig. 11a; Plate 17a). Skeletal muscle tissue did not show any activity while a single band corresponding to A form was detected in kidney, heart, brain and eye tissue extract.

Similar studies on other amphibians have revealed an identical result. The total number of molecular form of G6PDH isozymes varied from four in *Bombina* to two in *Rana* and *Triturus* (Chen, 1966).

It is worth adding that little or no activity of G6PDH

in the skeletal muscle tissue of the frog species tested suggests that the generation of NADPH from glucose oxidation via the hexose monophosphate shunt does not play an essential role in this tissue. Similar observations have been made in teleosts (Fried, et al., 1969) and frogs (Chen, 1966).

A relatively high G6PDH activity (generally indicative of an active pentose shunt) in the liver tissue of these frogs suggest this tissue may be a major site of fatty acid synthesis at those phylogenetic levels preceding the development of adipose tissue or a synthetically competent site. In preliminary studies with amphibians (*Rana pipiens*) appreciable hepatic G6PDH activity has been encountered (Rosen, et al., 1968). Scholl and Anders (1973) have also reported the highest level of G6PDH activity in the liver tissue of *Xiphophorus*.

The genetic basis of G6PDH isozymes has been of particular interest. The mammalian G6PDH are encoded by two separate loci, one being X-chromosomally linked (Firkman and Hendrickson, 1963) and the other being autosomal (Shaw and Barto, 1965). The autosomally inherited isozyme, designated as H6PDH showed a higher Cathodal affinity in fishes (Scholl and Anders, 1973). Our observation of the cathodal isozyme

in eye, gonad and liver tissue extract of *R. reinwardtii*, *P. leucomystax* and *H. annectens* respectively is well in agreement with those found in fishes but specific substrate study demonstrates it to be G6PDH.

An increasing number of allelic variants of G6PDH have been reported in a number of vertebrates (Cederbaum and Yoshida, 1976; Diebig et al., 1976). Frequently the allelic products showed distinctive electrophoretic migration as have been observed in liver and skeletal muscle tissue extracts of our frogs (*R. reinwardtii* and *R. maximus*) with an anodal mobility.

The two forms G6PDH observed in our frogs are thus assumed to be homologous to those found in other vertebrates and the enzyme is dimeric in nature. There was however, no sex differential pattern observed in the frogs. This indicate that the influence of locus is uniform in both the sexes.

CHAPTER - I
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CHAPTER-I
FIGURES

Tissue LDH isozyme pattern:

G = Gonad, K = Kidney, H = Heart

B = Brain, F = Eye, L = Liver

M = Skeletal Muscle.

Fig 2: *H. annectens*

(a) Smit

(b) Hawphlang

Fig 3: *P. leucomystax*

(a) Shillong: 5-banded pattern

(b) Shillong: 9-banded pattern

(c) Shillong: 12-banded pattern

(d) Williamnagar: 3-banded pattern

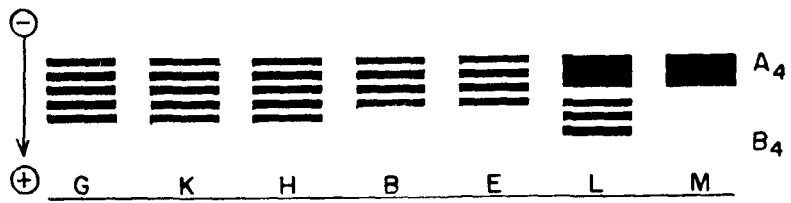


Fig. 2(a)

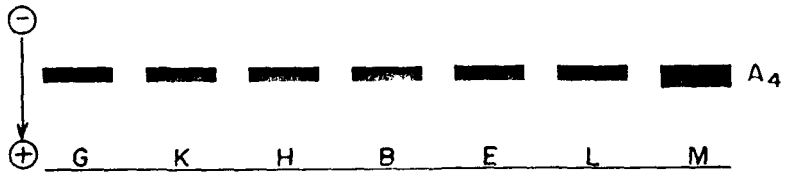


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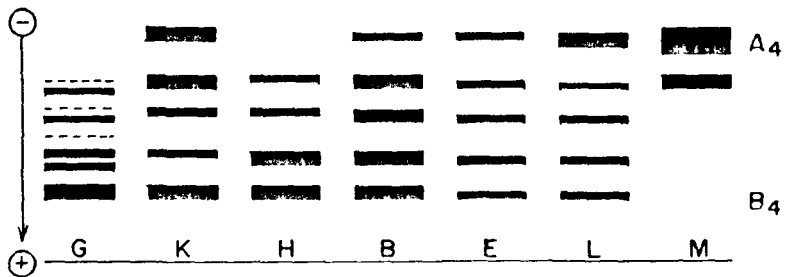


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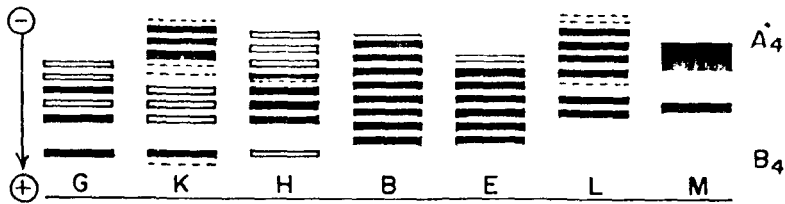


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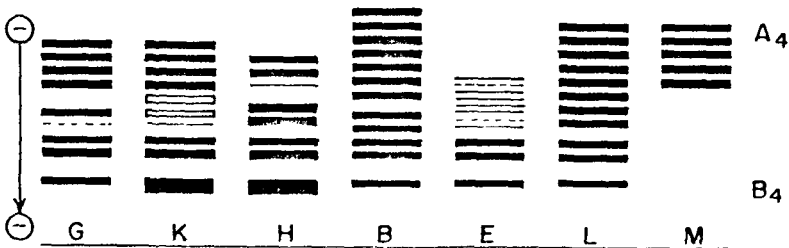


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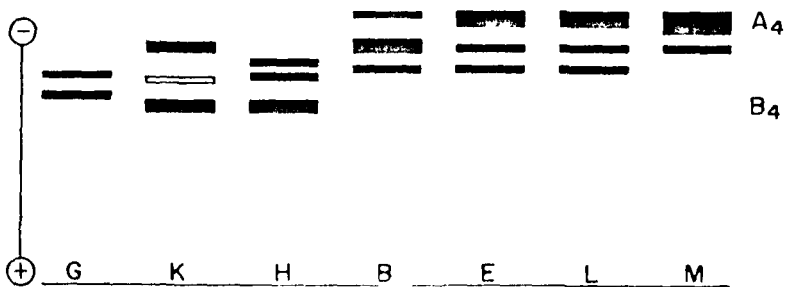


Fig. 3(d)

Tissue LDH isozyme pattern:

G = Gonad, K = Kidney, H = Heart,

B = Brain, E = Eye, L = Liver,

M = Skeletal muscle

Fig.3 *P. leucomystax*:

(e) Williamnagar: 4-banded pattern

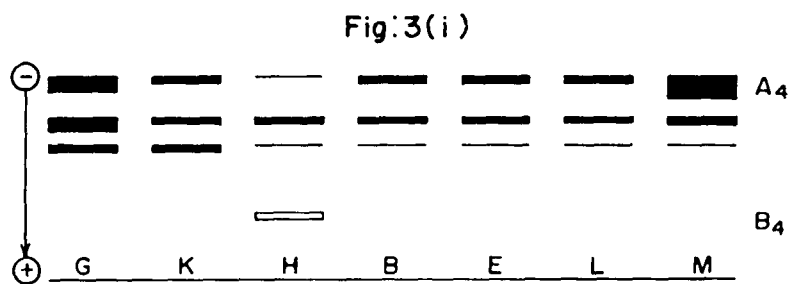
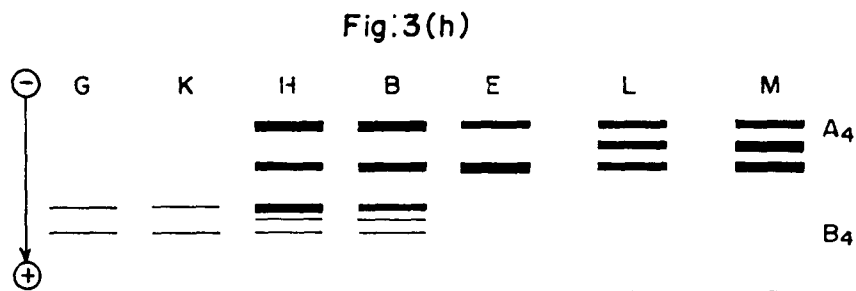
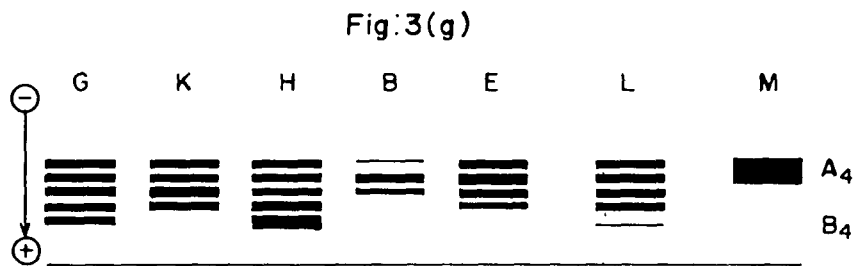
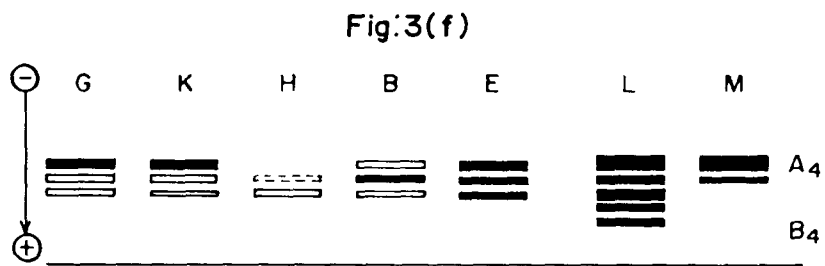
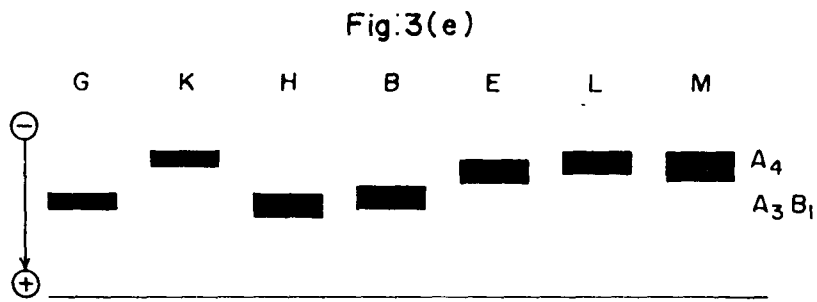
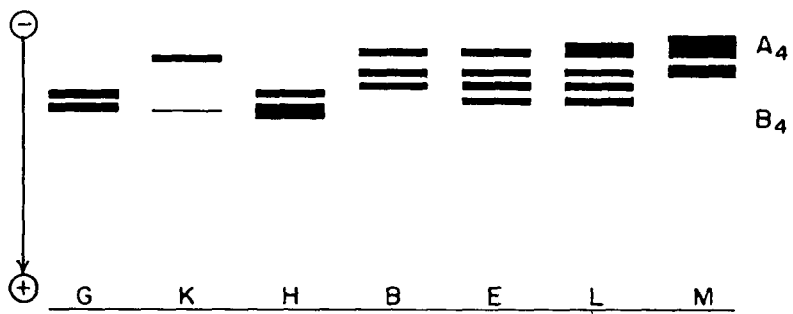
(f) Mawhati: 1-banded pattern

(g) Mawhati: 5-banded pattern

(h) Mawhati: 5-banded pattern

(i) Jowai: 5-banded pattern

Fig.4 (a) *R. reinwardtii*: Mawsynram



Tissue LDH isozyme pattern

G = Gonad, K = Kidney, H = Heart,

B = Brain, E = Eye, L = Liver,

M = Skeletal muscle.

Fig.4. *R.reinwardtii*

(b) Jowai

(c) Mawphlang

(d) Smit

Fig.5. *R.maximus*

(a) Jowai

(b) Cherrapunjee

Fig.6.(a) *A.afghanus*: Nonqkhlaw

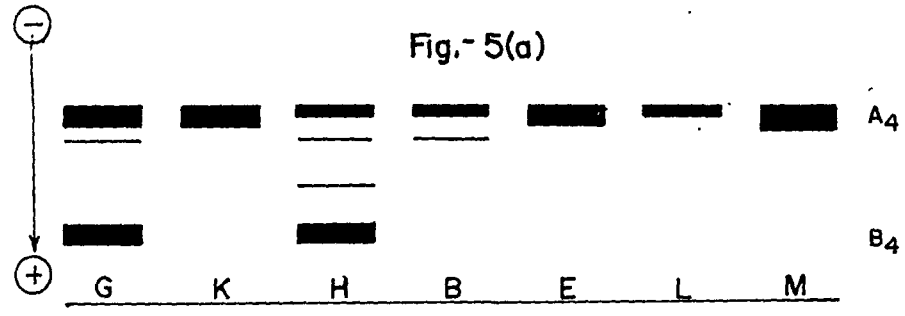
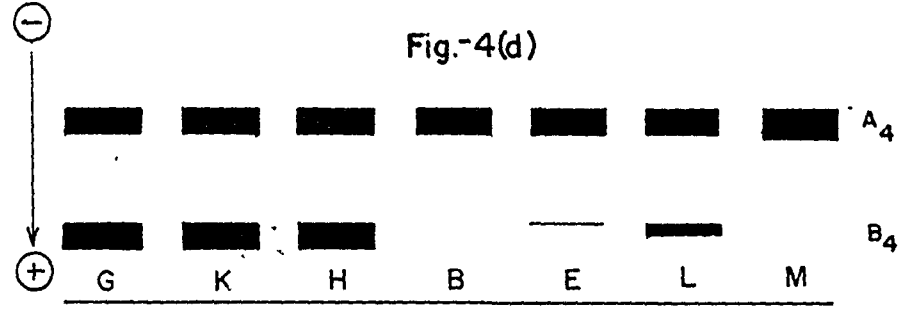
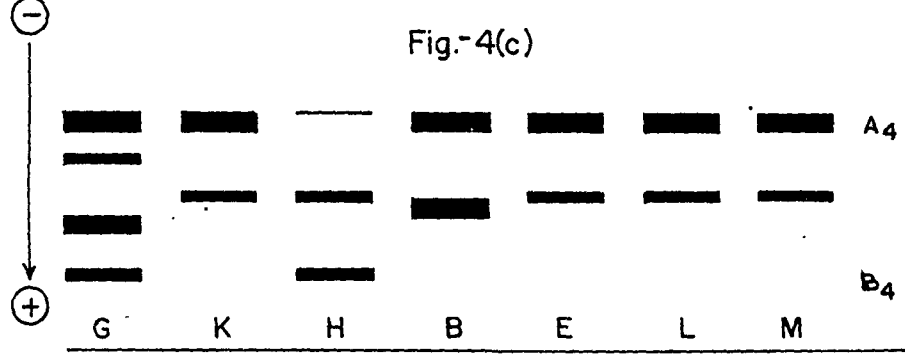
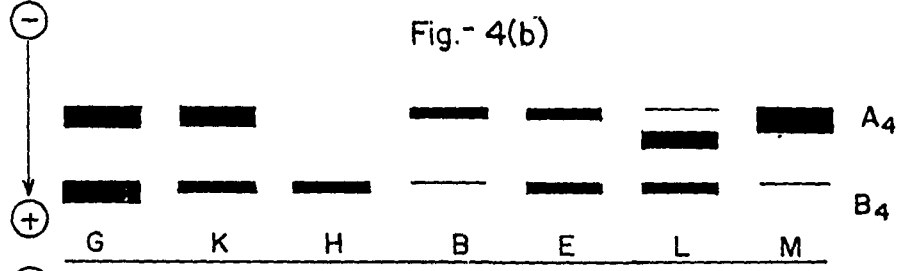
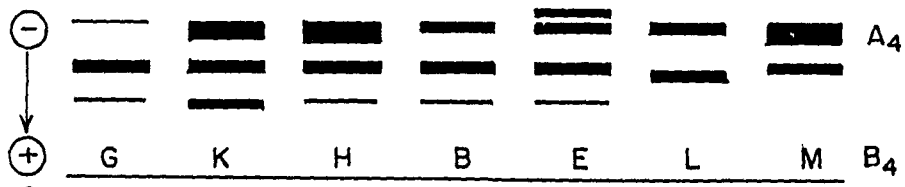


Fig. 6(a)

Tissue LDH isozyme pattern

G = Gonad, K = Kidney, H = Heart,

B = Brain, E = Eye, L = Liver,

M = Skeletal muscle.

Fig-6: *A. afghanus*

(b) Mawkairaw

(c) Williamnagar

(d) Smit

(e) Shillong

(f) Mawsynram

Fig-7: *R. cyanophlyctis*

(a) Shillong

Tissue LDH isozyme pattern

G = Gonad, K = Kidney, H = Heart,

B = Brain, E = Eye, L = Liver,

M = Skeletal muscle.

Fig.7. *R. cyanophlyctis*

(b) Jowai

(c) Williamnagar

Fig.8. *R. nicobariensis*

(a) Mawkaiaw

(b) Williamnagar

(c) Mawhati

Fig.9. Tissue MDH isozyme pattern

(a) *H. annectens*

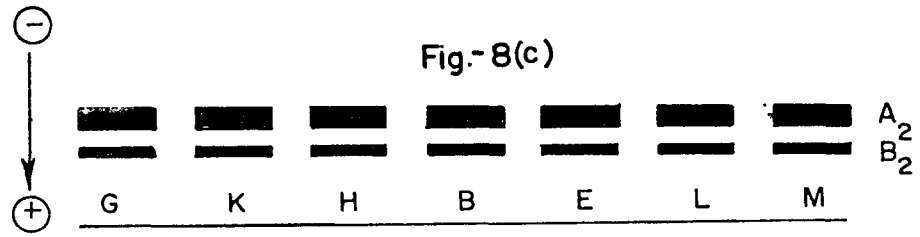
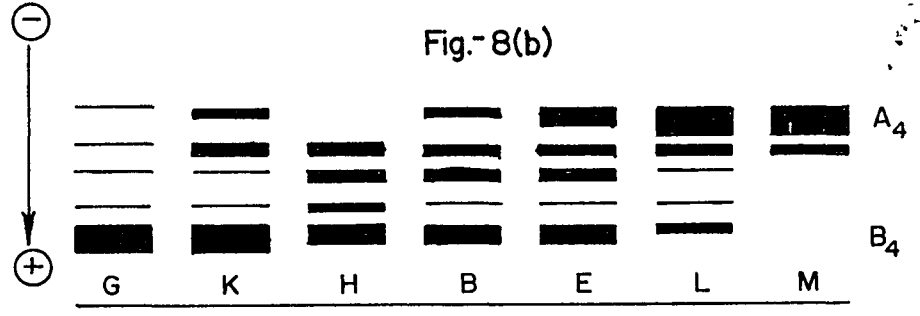
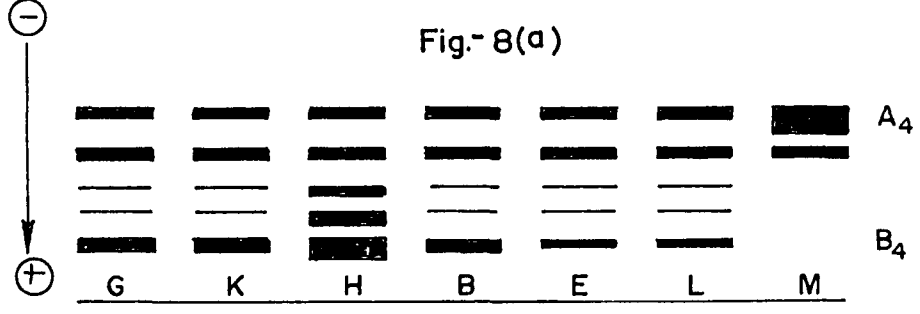
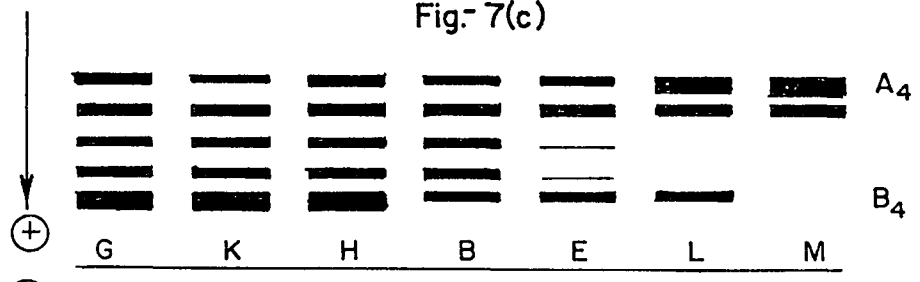
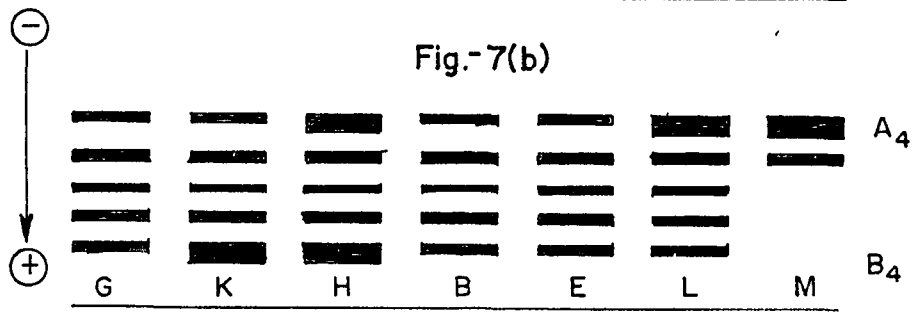
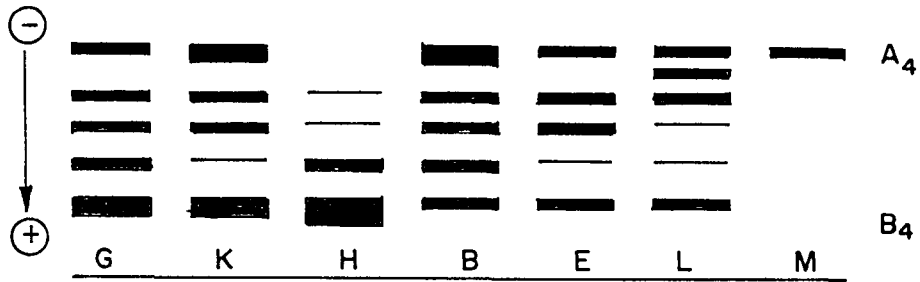


Fig.9. Tissue MDH isozyme pattern

Fig.9. (b) *P. leucomystax*

(c) *R. reinwardtii*

(d) *R. maximus*

(e) *A. afghanus*

(f) *R. cyanophlyctis*

(g) *R. nicobariensis*

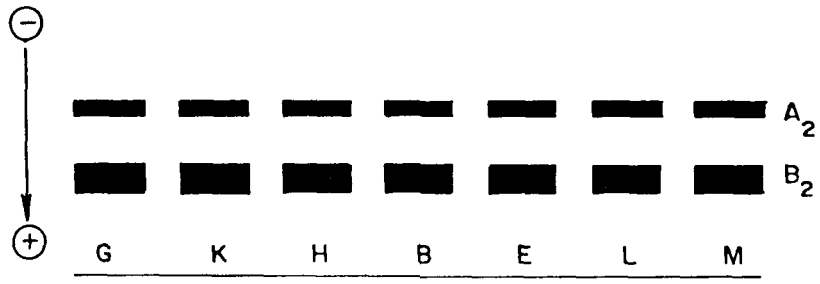


Fig.- 9(b)

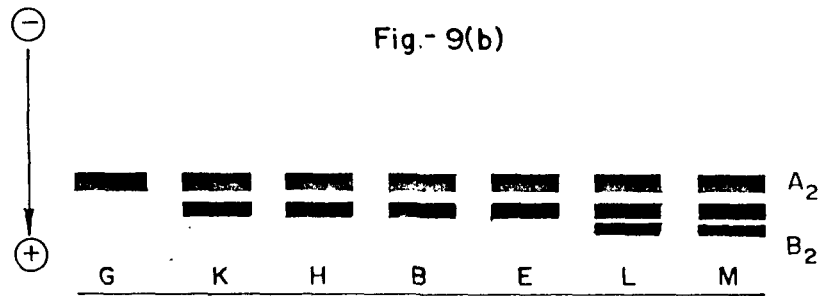


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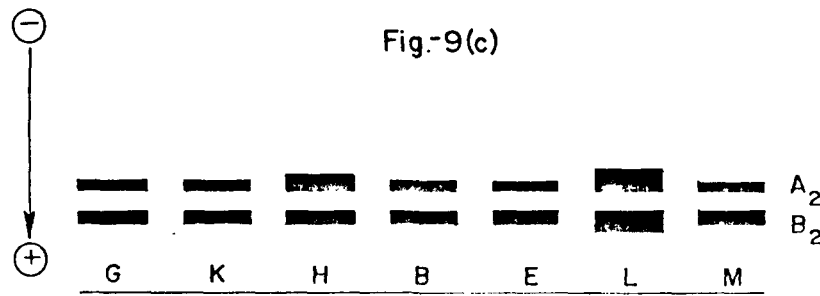


Fig-9(d)

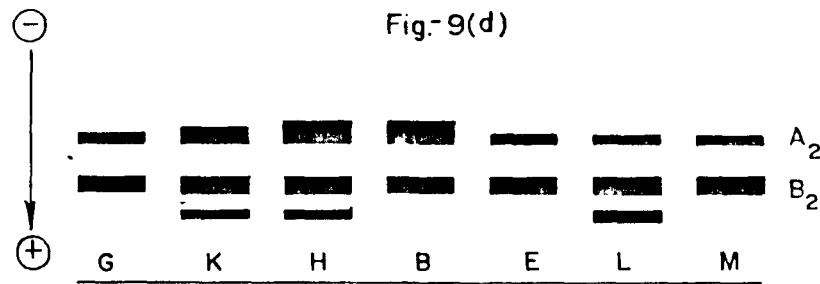


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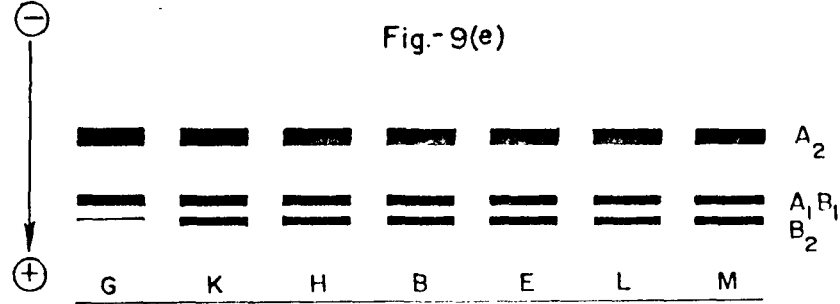


Fig-9(f)

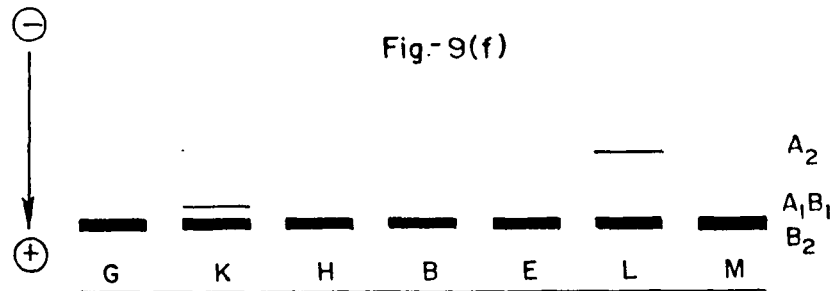
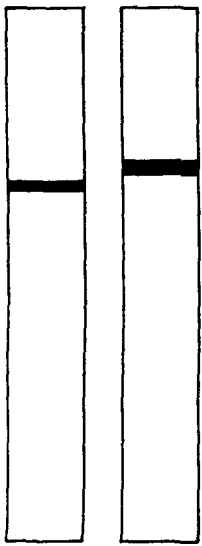


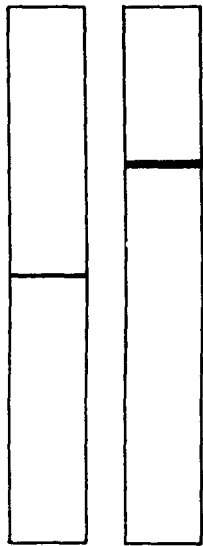
Fig-9(g)

Fig.10. Tissue ADH isozyme pattern

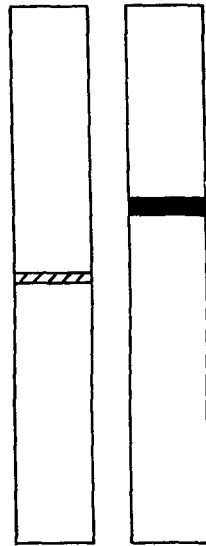
- (a) *H. annectens*
- (b) *P. leucomystax*
- (c) *R. reinwardtii*
- (d) *R. maximus*
- (e) *A. afghanus*
- (f) *R. cyanophlyctis*
- (g) *R. nicobariensis*



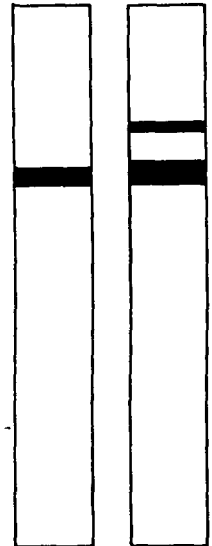
K L
Fig:10(a)



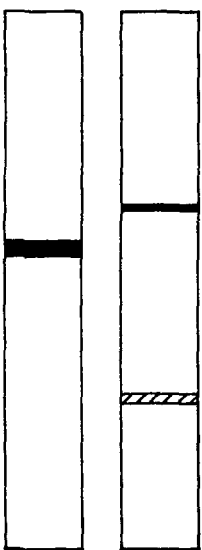
K L
Fig:10(b)



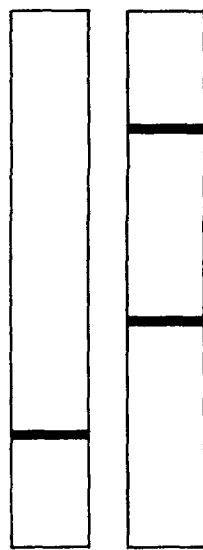
K L
Fig:10(c)



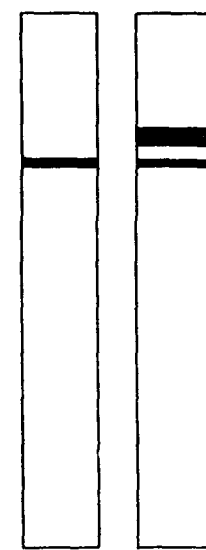
K L
Fig:10(d)



K L
Fig:10(e)



K L
Fig:10(f)



K L
Fig:10(g)

Fig.11. Tissue G6PDH isozyme pattern

- (a) *H. annectens*
- (b) *P. leucomystax*
- (c) *R. reinwardtii*
- (d) *R. maximus*
- (e) *A. afghanus*
- (f) *R. cyanophlyctis*
- (g) *R. nicobariensis*

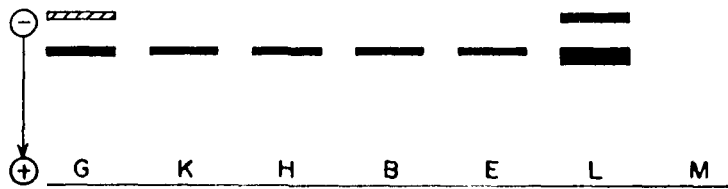


Fig:II(a)

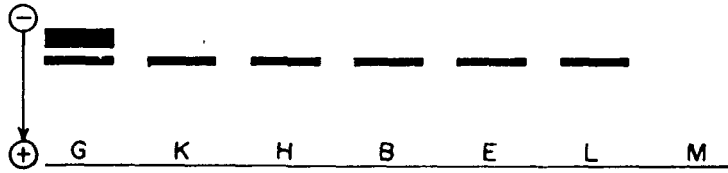


Fig:II(b)

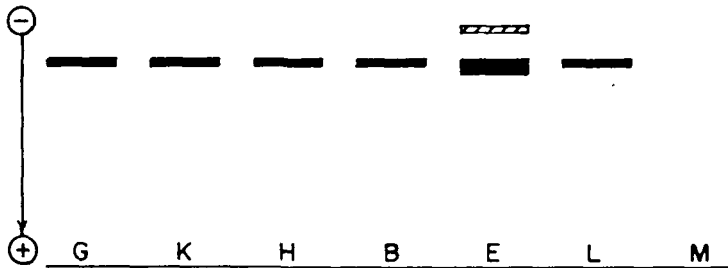


Fig:II(c)

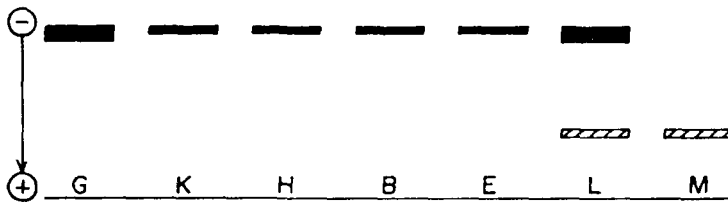


Fig:II(d)

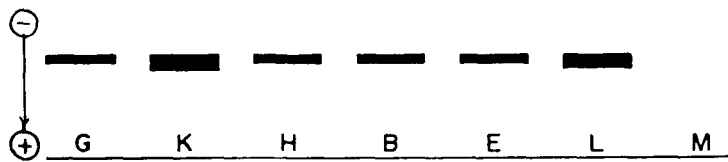


Fig:II(e)



Fig:II(f)

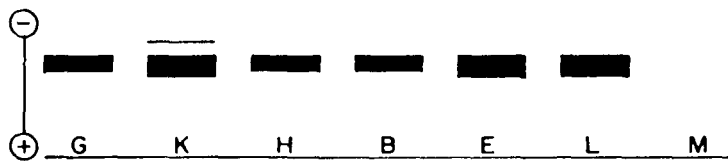


Fig:II(g)

Fig.12. Model suggesting submit composition of
LDH phenotype in heterozygous *P. leucomystax*
population (Shilong)

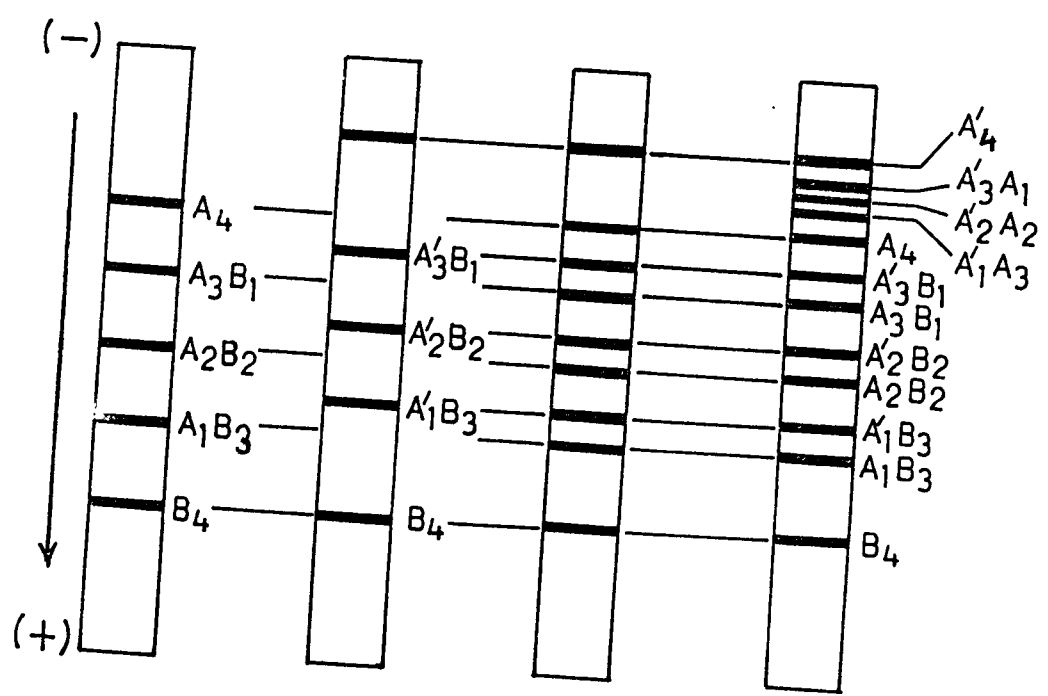
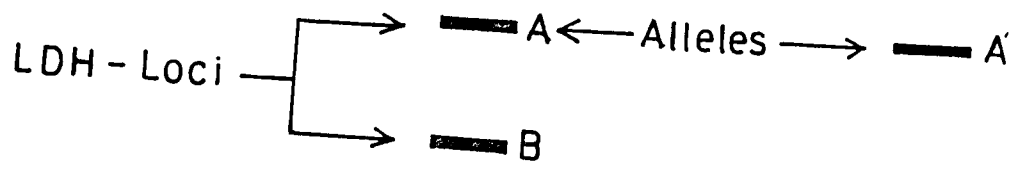


Fig:12

CHAPTER-I
PLATES

Plate 1 : *H. annectens*

Plate 2 : *P. leucomystax*



1



2

Plate 3 : *R. reinwardtii*

Plate 4 : *R. maximus* (undisturbed)



3



4

Plate 5 : *R. maximus*
(colour change due to disturbance)

Plate 6 : *A. afghanus*



5



6

Plate 7 : *R. cyanophlyctis*

Plate 8 : *R. nicobariensis*



7



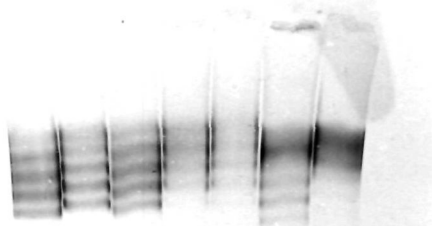
8

Plate 9(a) *H. annectens* : LDH (Smit)

Plate 9(b) *H. annectens* : LDH (Nawphiang)

Plate 10(a) *P. leucomystax* : LDH (Shillong, 5-banded)

Plate 10(b) *P. leucomystax* : LDH (Shillong, 5-banded)



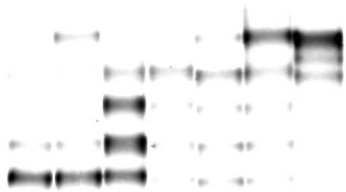
G K H B E L M

9a



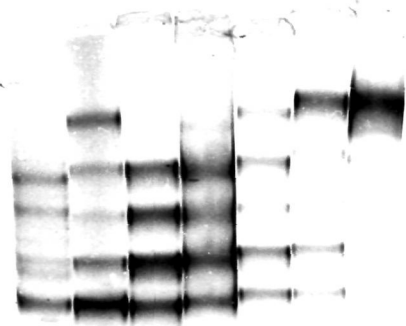
G K H B E L M

9b



G K H B E L M

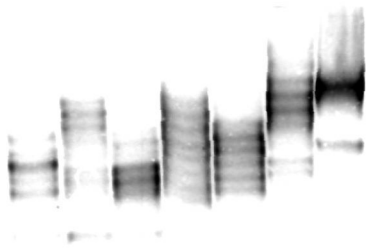
10a



G K H B E L M

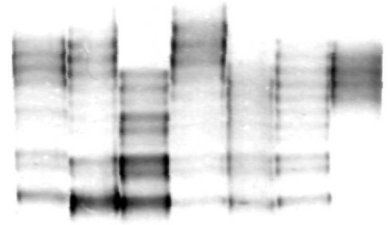
10b

Plate 10(c) *P. leucomystax* : LDH (Shillong, 9-banded)
Plate 10(d) *P. leucomystax* : LDH (Shillong, 12-banded)
Plate 10(e) *P. leucomystax* : LDH (Williamnagar, 3-6-banded)
Plate 10(f) *P. leucomystax* : LDH (Williamnagar, 4-banded)



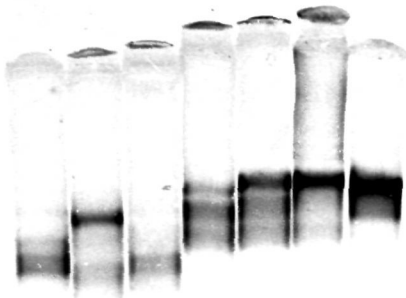
G K H B E L M

10c



G K H B E L M

10d



G K H B E L M

10e



G K H B E L M

10f

Plate 10(η) *P. leucomystax* : LDH (Mawhat1, 1-banded)
Plate 10(h) *P. leucomystax* : LDH (Mawhat1, 5-banded)
Plate 10(i) *P. leucomystax* : LDH (Mawhat1, 5-banded)
Plate 10(j) *P. leucomystax* : LDH (Jowai)



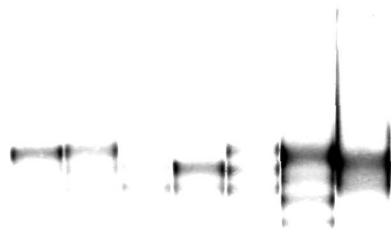
G K H B E L M

10g



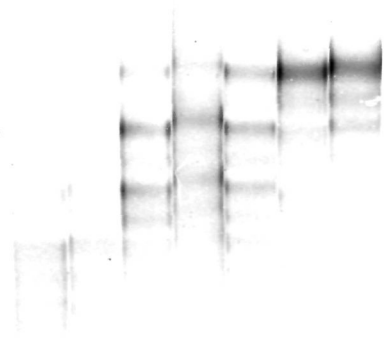
G K H B E L M

10h



G K H B E L M

10i



G k H B E L M

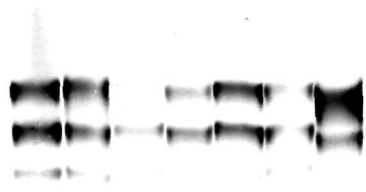
10j

Plate 11(a) *R. reinwardtii* : LDH (Mawsynram)

Plate 11(b) *R. reinwardtii* : LDH (Jowai)

Plate 11(c) *R. reinwardtii* : LDH (Mawphlang)

Plate 11(d) *R. reinwardtii* : LDH (Smit)



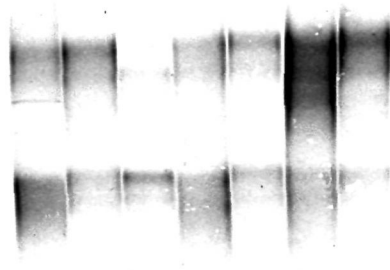
G K H B E L M

IIa



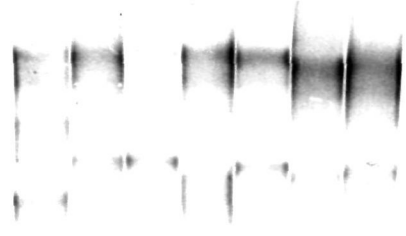
G K H B E L M

IIb



G K H B E L M

IIc



G K H B E L M

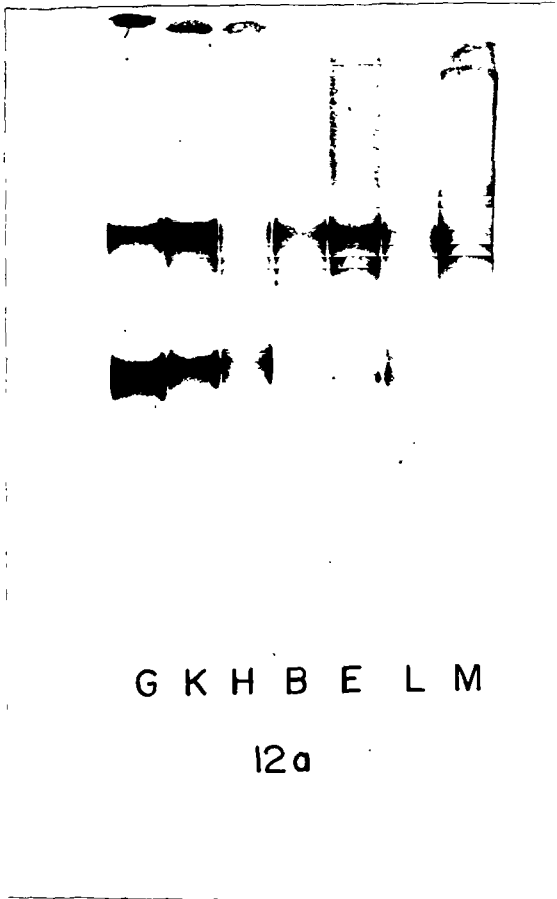
II d

Plate 12(a) *R. maximus* : LDH (Jowai)

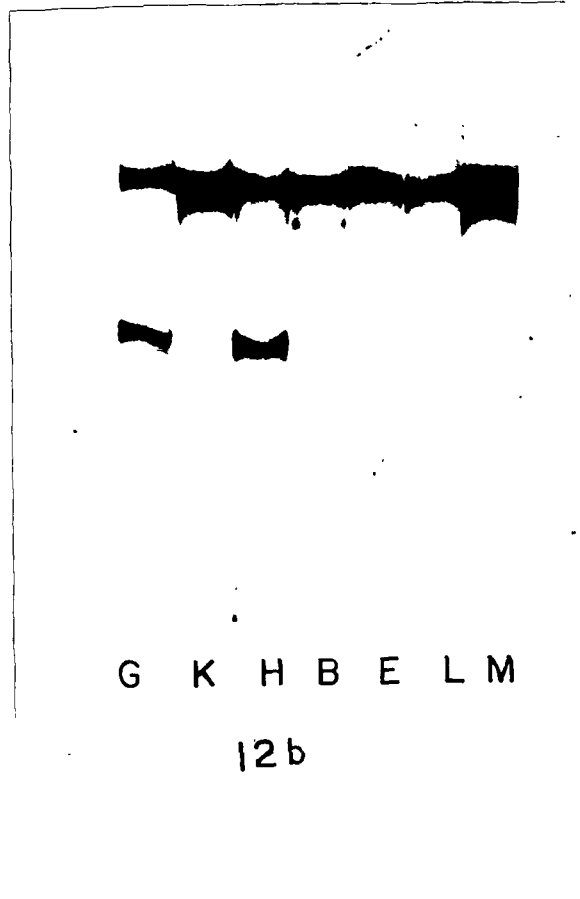
Plate 12(b) *R. maximus* : LDH (Cherrapunji)

Plate 13(a) *A. atghanus* : LDH (Nongkhlaw)

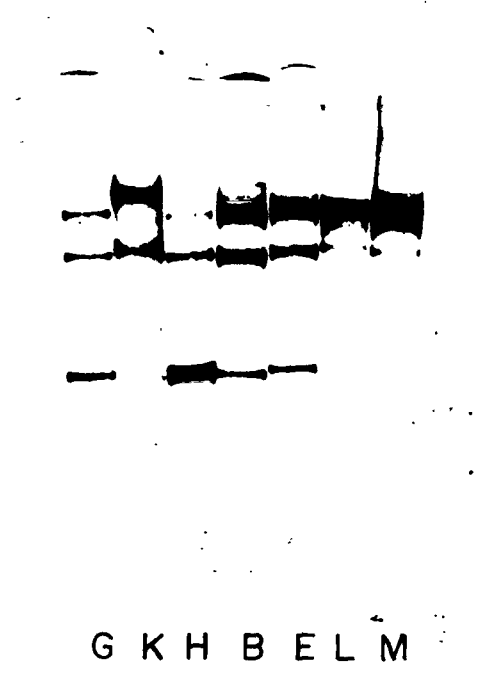
Plate 13(b) *A. atghanus* : LDH (Mawkaiaw)



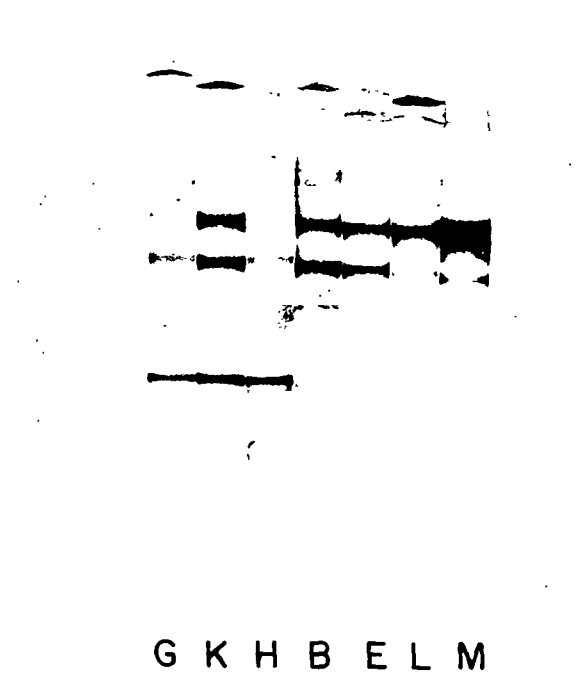
12a



12b



13a



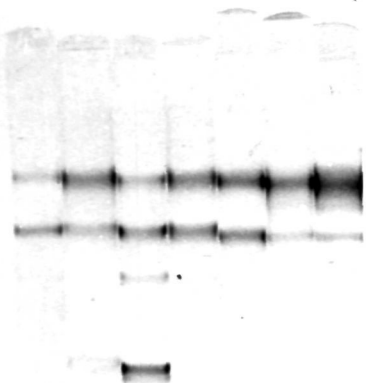
13b

Plate 13(c) *A. afghanus* : LDH (Williamnagar)

Plate 13(d) *A. afghanus* : LDH (Smit)

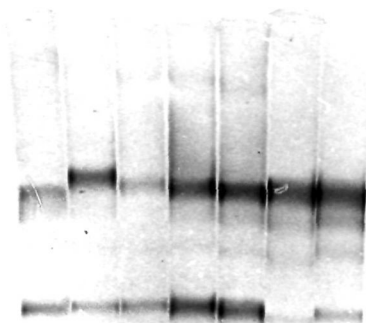
Plate 13(e) *A. afghanus* : LDH (Shillong)

Plate 13(f) *A. afghanus* : LDH (Mawsynram)



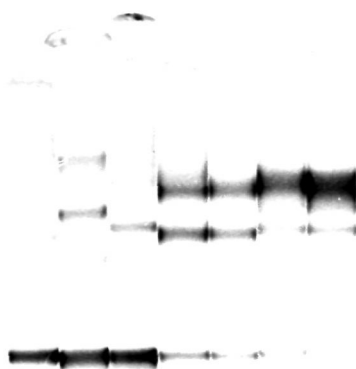
G K H B E L M

13c



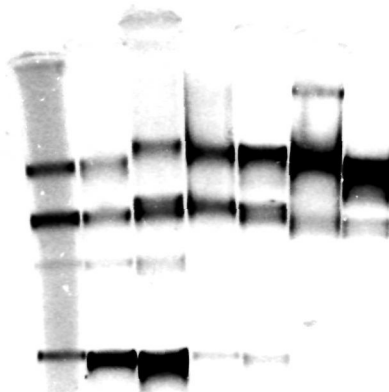
G K H B E L M

13d



G K H B E L M

13e



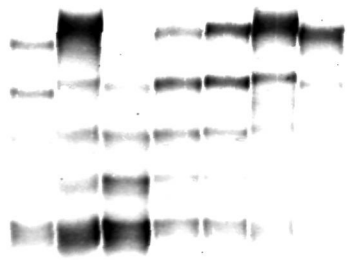
G K H B E L M

13f

Plate 14(a) *R. cyanophlyctis*, LDH (Shillong)

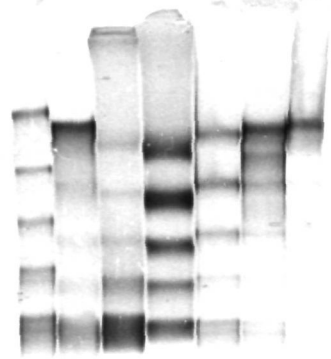
Plate 14(b) *R. cyanophlyctis*, LDH (Jowai)

Plate 14(c) *R. cyanophlyctis*, LDH (Williamnagar)



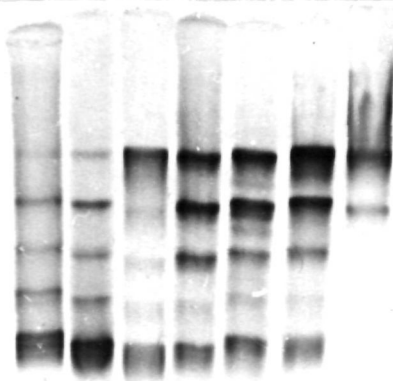
G K H B E L M

14a



G K H B E L M

14b



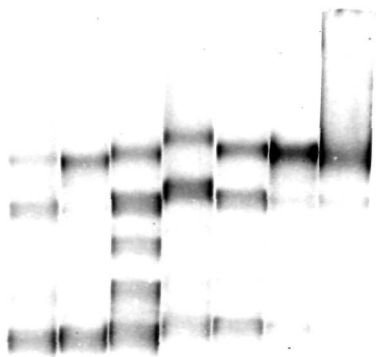
G K H B E L M

14c

Plate 15(a) *R. nicobariensis* : LDH (Mawkaiaw)

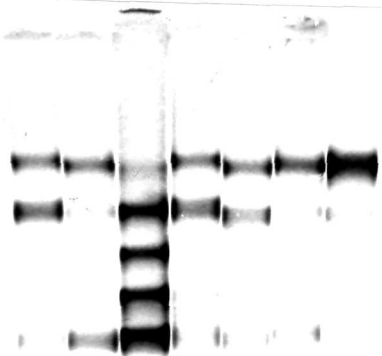
Plate 15(b) *R. nicobariensis* : LDH (Williamnagar)

Plate 15(c) *R. nicobariensis* : LDH (Mawhati)



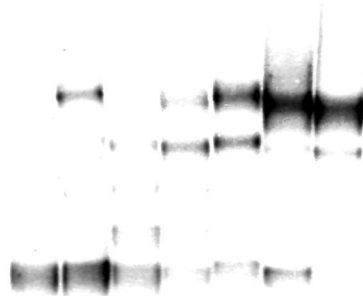
G K H B E L M

15a



G K H B E L M

15b



G K H B E L M

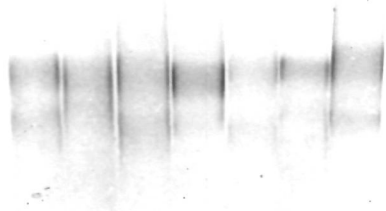
15c

Plate 16(a) *H. appetens* : MDH

Plate 16(b) *P. leucomystax* : MDH

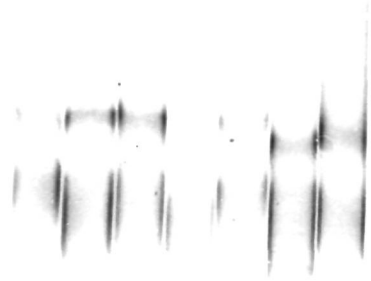
Plate 16(c) *R. reinwardtii* : MDH

Plate 16(d) *R. maximus* : MDH



G K H B E L M

16a



G K H B E L M

16b



G K H B E L M

16c



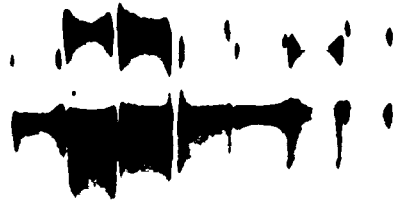
G K H B E L M

16d

Plate 16(e) *A. atqhanus* : MDH

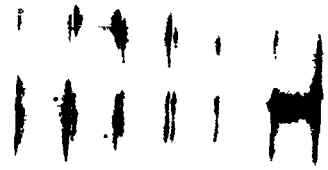
Plate 16(f) *R. cyanophlyctis* : MDH

Plate 16(g) *R. nicobariensis* : MDH



G K H B E L M

16 e



G K H B E L M

16 f



G K H B E L M

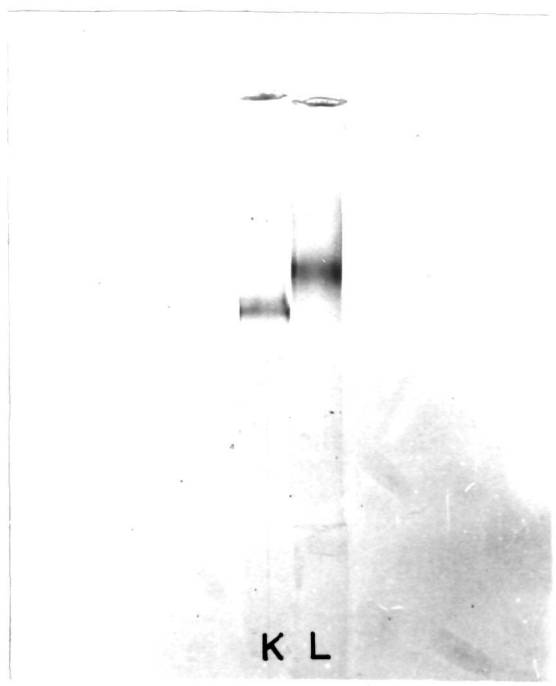
16 g

Plate 17(a) *H. annectens* : ADH

Plate 17(b) *P. leucomystax* : ADH

Plate 17(c) *R. reinwardtii* : ADH

Plate 17(d) *R. maximus* : ADH



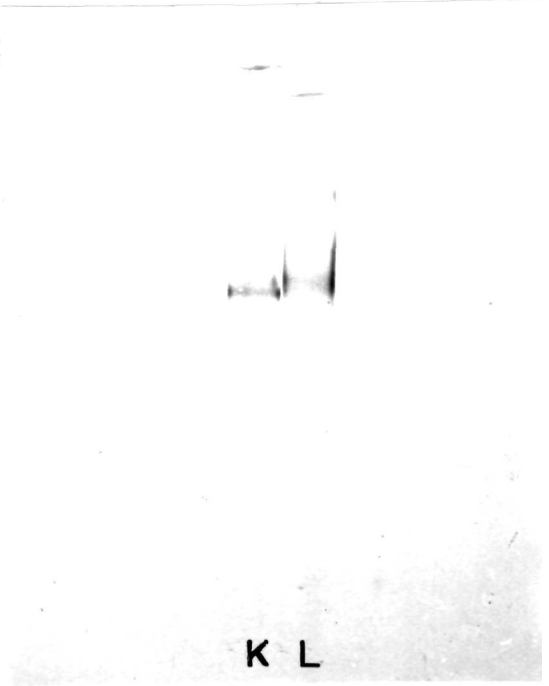
K L
17 a



K L
17 b



K L
17 c



K L
17 d

Plate 17(e) *A. afghanus* : ADH

Plate 17(f) *R. cyanophlyctis* : ADH

Plate 17(g) *R. nicobariensis* : ADH



K L

17e



K L

17f



K L

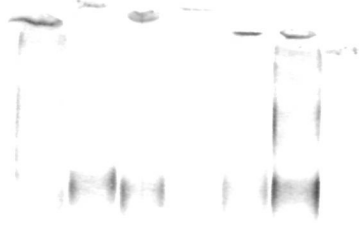
17g

Plate 18(a) *H. annectens* : G6PDH

Plate 18(b) *P. leucomystax* : G6PDH

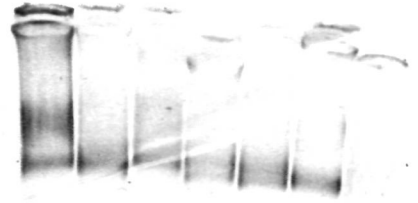
Plate 18(c) *R. reinwardtii* : G6PDH

Plate 18(d) *R. maximus* : G6PDH



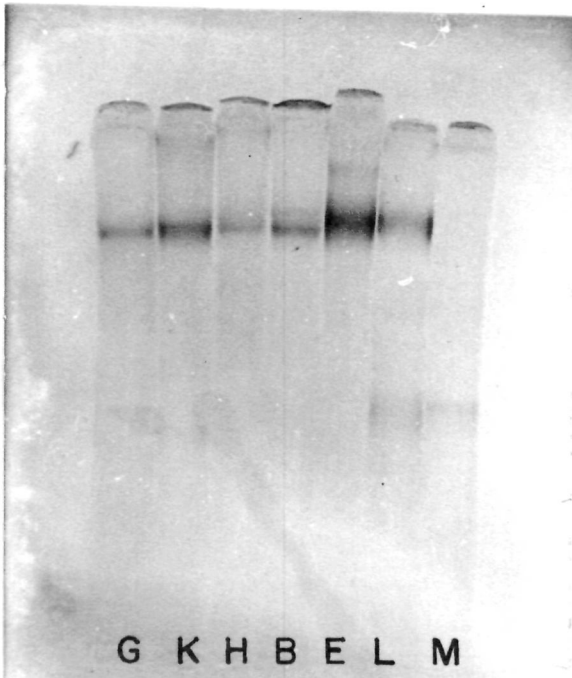
G K H B E L M

18a



G K H B E L M

18b



G K H B E L M

18c



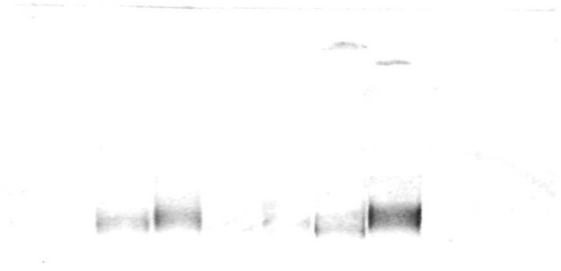
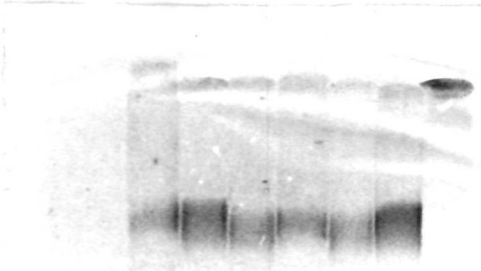
G K H B E L M

18d

Plate 18(e) *A. afghanus* : G6PDH

Plate 18(f) *R. cyanophlyctis* : G6PDH

Plate 18(q) *R. nicobariensis* : G6PDH

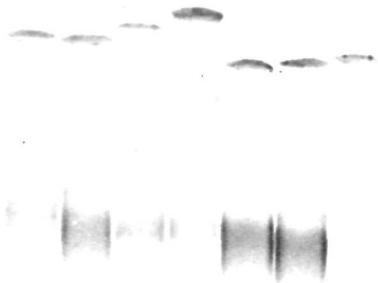


G K H B E L M

18e

G K H B E L M

18f



G K H B E L M

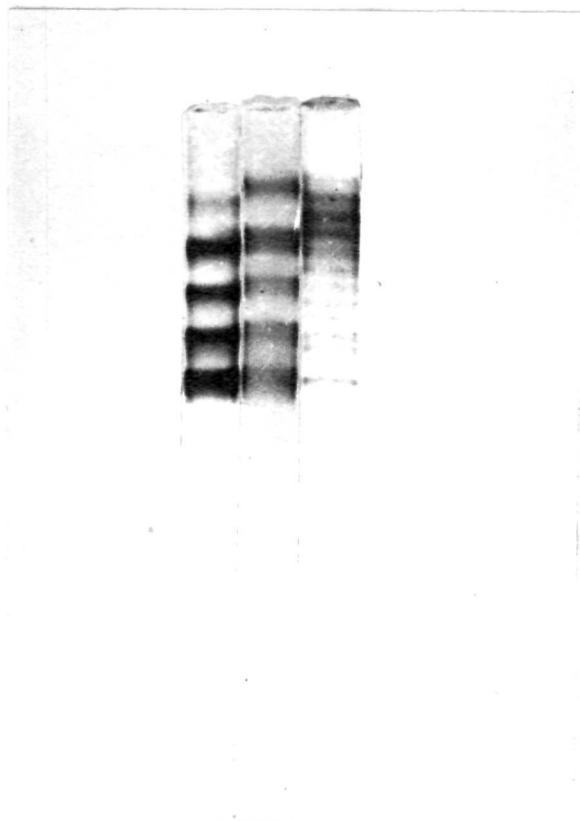
18g

Plate 19. Homozygous (AA and A'A') and heterozygous (AA')

phenotypic LDH pattern in *P. leucomystax* population

(Shillong).

A,B = Homozygous, C = Heterozygous



A B C

PLATE : 19

PLATE : 19

CHAPTER-I I
INTRODUCTION

Many major taxa have evolved complex life histories featuring a rapid transformation, or metamorphosis, between successive forms. Some of the best-known and most spectacular examples can be found among the holometabolous insects and the anurans. The extensive morphological reorganization that occurs during metamorphosis poses profound questions at cell levels of biological organisation and has stimulated a large body of literature on the physiological and developmental processes involved (Gilbert and Frieden, 1981). However, little is understood about the genetic and evolutionary significance of complex life histories.

Amphibians are an excellent group for the comparative study of the evolution of complex life histories; they have invaded nearly all major land habitats, and they exhibit a corresponding diversity of life histories and reproductive modes. The striking variety of life histories represented in the amphibians highlights the broader issues of the adaptive properties, the course of evolution, and maintenance of complex life cycles. Such life cycles have persisted over long periods of evolutionary time in amphibians.

A remarkable series of changes takes place when an aquatic fish like tadpole is transformed into a land-dwelling frog.

Throughout the embryonic and larval life the development of the frog has been almost entirely a gradual, unfolding process of growth and differentiation, but during the metamorphic period growth is almost halted and differentiation and degeneration occur simultaneously with dramatic suddenness. Etkin(1934) regards Anuran metamorphosis as consisting of three phases. The first postembryonic period is characterized by much growth with very little change in form. This is designated as premetamorphic period. Prometamorphosis is marked by the rapid elongation of hindlimbs. The appearance of forelimbs marks the beginning of metamorphic climax.

In the theoretical scheme proposed by evolutionary genetics, development is the function that maps the genotype onto the phenotypes. It is well known that, even at the lowest level of protein transcription, the relationship genotype-phenotype is not one to one. At higher levels of interactions, such as morphological traits, the genotype-phenotype is more complex. The interactions at the molecular, cellular and tissue levels give a structure to developmental systems that may have important evolutionary consequences.

Genes control developmental processes, which in turn

generate form. Genes make proteins that either regulate the expression of other genes, or in the case of products of the so-called morphogenetic genes (Edelman, 1988), determine morphogenetic properties. Thus morphogenesis is the result of complex physio-chemical interactions at this level.

A major theme of development is that the specialization of cells is the result of and or the impetus for, differential gene activity. The appearance of specific proteins during embryogenesis reflects both biochemical and metabolic changes associated with development and therefore, functional and morphological states of differentiation.

In a continuing survey of some of the biochemical changes which occur during amphibian metamorphosis, Frieden and Herner (1957) noted significant changes in both the amount and the distribution of the proteins.

In applying the principles of modern genetics it must be appreciated that the tadpole has all the genetic machinery to become a frog and that represents a change in genetic expression. Great emphasis has been placed in changes in protein that accompany metamorphosis (Frieden, 1961). The genetic control of information transcription and translation is expected to be untimely reflected in terms of the synthesis of specific proteins. During development, it is

believed that many hundreds or perhaps thousands, of genes are selectively turned on and off in a complex concerted fashion (Britten and Davidson, 1969) or that an integration of a large number of simple regulatory events is involved (Rutter *et al.*, 1968).

In this respect multilocus isozyme systems have proven to be ideally suited to studies designed to characterize the tissue specificity and ontogenic patterns of gene expression. It is believed that the appearance of particular isozymes at specific stages of development reflects metabolic and, thus, biochemical changes in the developing embryo. Therefore, in addition to being able to characterize the temporal changes in isozyme activity during development, it is possible to correlate certain biochemical changes with morphological or functional states of differentiation.

Studies investigating the ontogenic expression of isozymes have been undertaken by many workers in a variety of vertebrates. Markert and Ursprung (1962) examined the ontogenic isozyme patterns of lactate dehydrogenase in mouse and concluded that embryonic tissues possess only one form of LDH and the tissue - specific distribution develops as the tissue differentiates.

Similar observations have also been made in chickens

(Cahn et al., 1962). Lactate dehydrogenase ontology, paternal gene activation and tetramer assembly in embryo of brook trout, lake trout and their hybrids have revealed repression or delay of either paternal or maternal alleles (Goldberg et al., 1963).

The time of gene expression has been determined for G6PDH in quail (Ohno et al., 1968), ADH and LDH in fish (Hitzeroth et al., 1960; Goldberg et al., 1969; Tolse et al., 1969).

Among amphibians, anuran larvae (tadpoles) are especially noted for dramatic changes, which occur in their form and mode of existence during metamorphosis. In connection with the problem of cellular differentiation the isozymes of amphibians have attracted interest of many investigators. Grainer and Kunz (1966) recorded the changes in isozymes of LDH and MDH during the development of the frog *Rana temporaria*. The ontogenic patterns in both normal and hybrid frog embryo have been analysed by Wright and Moyer (1966). Kunz and Hearn (1967) demonstrated distinct morphogenetic changes in the pattern of LDH in *Xenopus laevis*. Chen (1968) analysed the isozyme patterns of dehydrogenase (LDH, MDH, GDH, and ADH) in both embryonic and adult tissues of three frogs. Claycomb and Villet (1971) studied the electrophoretic pattern of LDH isozyme in

tissues from adult organs and embryo of *Xenopus laevis*. Soluble proteins from liver, serum and tail muscles of *Alytes* tadpole were electrophoretically analysed by Guyotant(1977). Activity and electrophoretic profiles of MDH during metamorphosis in *Rana pipiens* was studied by Long et al.,(1983). The expression of LDH genes throughout early development of different *Xenopus* species and interspecies hybrid were compared by Vonwyl(1983). LDH-electrophoretographs of tail-base, tail-tip, tail-middle and heart from *X.laevis* and *R.temporaria* were compared by Hornby et al.,(1989) to look for any differential distribution of LDH isozymes correlating with muscle function and differentiation as reported for mammalian muscle.

The above review clearly indicates that a large volume of literature is available on the isozyme pattern in the developmental stages of amphibians. However, little or no attention has been paid to use Indian frogs for similar studies. In view of this the present investigation has been undertaken to evaluate the isozyme pattern of post embryonic tadpoles of *Rana limnocharis* Weigmann at four progressive stages:premetamorphic, prometamorphic, metamorphic climax and postmetamorphic juvenile.

CHAPTER-I I
MATERIALS & METHODS

2.1. THE FROG : *Rana limnocharis* Weigmann. (Plate 1) the widely distributed species of the frog in India and whole of South-East Asia (Satyamurti, 1967). The breeding season of the frog extends from April to September. Breeding occurs in temporary as well as permanent bodies of water. Its life cycle is completed in approximately five weeks and the post-embryonic period is relatively shorter than the embryonic period (Roy and Khare, 1978).

2.2 Methods: Electrophoretic analysis was carried out to depict isozyme pattern in tadpoles at different stages of metamorphosis. Adult tissue pattern of the isozymes was analysed to compare with the developmental pattern.

(a) REARING OF TADPOLES : Egg masses in a jelly coat of *R. limnocharis*, were collected from paddy fields beside the Umkhrak river, Shillong (Lat. $15^{\circ}36'$, Lon. $91^{\circ}54'$), Meghalaya. The egg masses were maintained in laboratory aquaria containing pond water, and at room temperature of 25° - 30° C. Some algae and water plants were added to the aquaria. The tadpoles hatched out in about a week and started feeding on the added algae. The aquaria was cleaned on every alternate day and freshly collected pond water added.

(b) **STAGING OF TADPOLES:** The stages of tadpoles were established from external visible characteristics according to the "Normal table of *Rana limnocharis*" (Roy and Khare, 1978):

(i) Premetamorphosis :

Stage 25 : Eyes with well developed lens and retina, coiled intestine, tail long, starts feeding on algae (Plate 2a,b,c).

Stage 26 : Hind limb bud stage. Appearance of hind limbs at the groove between the base of the tail and belly wall (Plate 3).

(ii) Prometamorphosis :

Stage 27 : Tadpoles with well developed diving hind limbs and halted tail growth (Plate 4).

Stage 28 : Tadpoles with developing forelimbs (Plate 5).

(iii) Metamorphic climax :

Stage 29 : Tadpoles with both limbs developed. From this stage onwards, the tadpoles undergo metamorphosis. Tail darker and less transparent (Plate 6).

Stage 30 : Tail starts resorbing and shortens gradually (Plate 7).

Stage 31 : Tail resorption continue and become a

small triangular dark coloured stub (Plate 8a,b)

(iv) Post-metamorphic juvenile :

Stage 32 : Fully metamorphosed miniature frog with all anuran characters (Plate 9).

(c) **TISSUES:** tadpoles of known stages (Plate 2-9) were placed in ice, the alimentary canal was removed, head, tail and liver were separately used for preparation of crude extract.

(d) **CONTROLS:** Adult specimens of *R. limnocharis* were placed in ice and gonad, kidney, heart, brain, eye, liver and skeletal muscle tissues were dissected out to prepare extract for control experiment.

(e) **EXTRACTION :** Tissues collected at different stages of development as well as adult tissues were weighed accurately and homogenised in ice cold sucrose (0.25M) solution. The homogenised tissues were centrifuged for 15 minutes at 21,000xg. The temperature of the centrifuge machine (REMI-C24) during operations was maintained at 4°C. After centrifugation the supernatant was collected in test tubes and measured volumes of the samples were subjected to electrophoresis for separation of isozymes.

(f) **ELECTROPHORESIS :** Vertical disc-electrophoresis system (Davis, 1964) was employed using 7.5% polyacrylamide gel (For

procedural detail please refer chapter one, (section 2.2.3).

(g) **STAINING OF ISOZYMES:** Four isozyme (viz. LDH, MDH, ADH and G6PDH) systems were stained in the gel using the staining protocol described in section 2.2.3 of chapter one. The stained gels were washed and preserved in 7% acetic acid.

CHAPTER-I I
RESULTS

3.1 The electrophoretic pattern of Lactate dehydrogenase (LDH), Malate dehydrogenase (MDH), Alcohol dehydrogenase (ADH) and Glucose-6-phosphate dehydrogenase (G6PDH) were analysed from tadpole tail, head and liver tissues at the various developmental stages (Stage 25 to stage 31) and head and liver from young froglet (Stage 32). Adult tissues of the frog were analysed for comparison with the isozyme pattern of tadpoles.

3.1.1 LACTATE DEHYDROGENASE (Lactate:NAD-Oxidoreductase, E.C.1.1.1.27)

(a) Adult tissue LDH pattern : Seven tissues, viz. gonad, kidney, heart, brain, eye, liver and skeletal muscle were analysed (Fig.1; Plate 10).

Gonad tissue LDH was resolved into four-bands. The most cathodal A_4 showed highest activity. A_3B_1 , A_2B_2 were relatively less intensely stained than A_4 isozyme while A_1B_3 was faintly stained and B_4 isozymes was not resolved.

Kidney presented similar four-banded pattern to that of gonad with high staining intensity of A_4 isozyme. The isozymes A_3B_1 , A_2B_2 and A_1B_3 were faintly stained and showed identical mobility to that of gonad isozymes.

LDH isozymes in the heart tissue was resolved into a

five-banded pattern. The A_4 isozyme was faintly stained, A_3B_1 , A_2B_2 and A_1B_3 heterotetramers showed highest staining intensity while the homotetramer B_4 was moderately stained.

Brain tissue LDH showed a four-banded phenotype of A_4 , A_3B_1 , A_2B_2 and A_1B_3 isozymes. The heterotetramers A_3B_1 and A_2B_2 showed a relatively higher staining intensity than that of gonad and kidney.

Eye tissue LDH pattern was identical to that of brain. However the homotetramer A_4 was more strongly stained than in brain. The heterotetramers A_3B_1 , A_2B_2 and A_1B_3 showed similar staining intensity and electrophoretic mobility.

Four-banded isozyme pattern resolved in liver tissue showed predominance of A_4 isozyme while heterotetramers A_3B_1 , A_2B_2 were moderately stained and A_1B_3 was faintly stained.

A two-banded phenotype with a very high activity of A_4 isozyme and a minor activity of A_2B_2 was resolved in skeletal muscle of the frog.

(b) Tadpole tail LDH pattern: The tail muscle LDH at different stages of development (Stage 25 to stage 31) resolved into a two-banded phenotype. The relative

mobilities of the bands from the cathodal end were similar in all the stages (Fig.2; Plate 11). Out of the two bands the more cathodal band was more strongly stained than the other band. Comparison of the relative mobilities of the two bands with the adult tissue LDH pattern indicated that these bands correspond to the homotetramer A_4 and A_3B_1 heterotetramer.

(c) Tadpole head region LDH pattern: Head region of the tadpoles analysed during the various developmental stages (Stage 15 to stage 32) of tadpoles revealed a distinctive LDH pattern (Fig.3; Plate 12). A two-banded pattern of A_4 and A_3B_1 were resolved at the premetamorphic stages 25 and 26. The A_4 isozyme was more intensely stained than A_3B_1 . A very faint band of A_4 activity was also observed at stage 25. A total of five bands were resolved at stage 27. The most cathodal isozyme band A_4 showed highest activity and appeared as a compact zone. The band A_3B_1 was moderately stained while A_2B_2 , A_1B_3 and B_4 bands were faintly stained. During the late prometamorphosis (Stage 28) the homotetramer B_4 showed highest activity and was most intensely stained while the isozymes A_3B_1 , A_2B_2 and A_1B_3 took a moderate stain. At the onset metamorphosis (Stage 29) the isozymes A_4 , A_3B_1 and A_1B_3 were moderately stained. The heterotetramers A_2B_2 showed highest activity while the homotetramer B_4 was not expressed. An identical pattern was observed at stage

30. In the late metamorphic period (stage 31) the homotetramer A_4 was again highly active and resolved as a strongly stained band. The heterotetramers A_3B_1 , A_2B_2 and A_1B_3 were moderately stained while the homotetramer B_4 was not expressed. In the juvenile frog a four banded phenotype was obtained of which the homotetramers A_4 and B_4 were moderately stained while the heterotetramers A_2B_2 and A_1B_3 were faintly stained.

(d) Tadpole liver tissue LDH pattern: Liver tissue LDH analysed at early premetamorphosis (stage 25) revealed a four banded phenotype being resolved into A_4 , A_3B_1 , A_2B_2 and B_4 isozymes (Fig.4;Plate 13). Out of these the homotetramer A_4 was most intensely stained. The homotetramer B_4 and the heterotetramer A_3B_1 were moderately stained while A_2B_2 took a faint stain. In stage 26 and 27 only two bands of LDH were resolved, the A_4 isozyme was resolved cathodally as a dark band. The B_4 isozyme did not show any activity while of the heterotetramer only A_2B_2 band was faintly stained. A three-banded LDH pattern of A_4 , A_3B_1 and A_2B_2 was obtained at stage 28. The isozyme band A_4 was most intensely stained and the least being the A_3B_1 band. Four bands of the isozyme consisting of A_4 , A_3B_1 , A_2B_2 and A_1B_3 were observed in stages 29 and 31 respectively while at stage 30 A_4 , A_3B_1 , A_2B_2 and B_4 isozymes were resolved.

Out of these A_4 isozyme was most intensely stained in the three stages (29,30 and 31), the heterotetramer A_3B_1 showed an identical moderate staining intensity in these stages, A_2B_2 showed a higher concentration at stage 29 and 31 while it was not resolved at stage 30. The B_4 isozyme observed at stage 30 was moderately stained. The young froglet (stage 32) liver LDH was resolved into a three-banded phenotype representing A_4 , A_3B_1 and A_2B_2 isozymes, of these the homotetramer A_4 was most strongly stained, A_3B_1 showed a moderate staining intensity and A_2B_2 was faintly stained.

3.1.2. MALATE DEHYDROGENASE (L-malate:NAD-Oxidoreductase, E.C.1.1.1.37)

(a) Adult tissue MDH pattern: Seven tissues (viz.gonad, kidney, heart, brain, eye, liver and muscle) of adult frog analysed electrophoretically showed similar MDH isozyme (Fig.5;Plate 14) pattern. A three-banded phenotype of the isozyme was resolved in all the tissues of which the more cathodal band presumably the homodimer A_2 was very faintly stained in all the tissues. However the band was relatively more intensely stained in the skeletal muscle than in other tissues. The homodimer B_2 and the heterodimer A_1B_1 were resolved as a zone of activity. The zone was faintly stained in all the tissues except liver and skeletal muscle where

this showed highest staining intensity.

(b) Tadpole tail MDH pattern: Tail muscle of the tadpole analysed at different developmental stages revealed an identical MDH pattern (Fig.6; Plate 15). The isozyme was resolved into a more cathodal condensed band corresponding to A_2 homodimer and a less cathodal much diffused zone corresponding to A_1B_1 heterodimer and B_2 homodimer. All the three isozymes showed highest staining intensity at stage 25. In the following stages (Stage 26 and stage 32) a gradual decrease in the staining intensity of the bands was noteworthy.

(c) Tadpole head region MDH pattern: Malate dehydrogenase isozyme was resolved into a single band corresponding to B_2 homodimer (Fig.7; Plate 16) at stage 25. No other isozyme was resolved at this stage. Three bands of the isozyme corresponding to A_2 , A_1B_1 and B_2 were resolved at stage 26. In the subsequent stages (stage 27 and stage 32) a single band of the isozyme corresponding to A_2 homodimer was resolved. Staining intensity of the band was found to decrease considerably with the progression of development.

(d) Tadpole liver tissue MDH pattern : A distinctive pattern of MDH isozyme was obtained in liver tissue of the tadpoles. During the premetamorphic stages (25 and 26) the isozyme

could not be resolved in liver tissue. A broad zone of cathodal isozyme presumably composed of A_2 and A_1B_1 was resolved at stage 27 (Fig.8;Plate 17). A single band of the isozyme corresponding to A_2 homodimer was detected at stage 28 while both the homodimers A_2 and B_2 were resolved at stage 29. The heterodimer A_1B_1 could not be detected at this stage. At stage 30 a single band of MDH isozyme corresponding to A_2 homodimer was obtained. A faint band of A_2 isozyme was detected at stage 31. The juvenile frog (stage 32) liver MDH could be resolved into homodimers A_2 and B_2 .

3.1.3. ALCOHOL DEHYDROGENASE (Alcohol NAD-Oxidoreductase, E.C.1.1.1.1)

(a) Adult tissue ADH pattern : Out of the seven tissues of the adult frog analysed only liver and kidney (Fig.9;Plate 1B) showed ADH activity. A single band ADH was resolved in both the tissues. Liver ADH showed higher staining intensity than that of kidney. In both the tissues the enzyme showed identical electrophoretic mobility.

(b) Tadpole liver tissue ADH pattern : Alcohol dehydrogenase(ADH) isozyme pattern was studied only in liver tissue of the tadpole at the different stages of development. During early developmental stages (stage 25,26

and 27) ADH isozyme could not be resolved in the liver tissue. Tail muscle and head region of the tadpole also did not show any activity for ADH.

Liver tissue of the tadpole analysed at stage 28 showed a maximum of six ADH isozyme bands (Fig.10;Plate 19). Out of these the most cathodal band was faintly stained (Designated band-1). Bands 2,3 and 4 showed higher activity and were closely spaced to each other. These bands were however spaced further away from the cathodal end. The fifth ADH isozyme was resolved still further towards the anode as a highly active condensed band. Band-6 showed a faint staining intensity and was resolved anodally.

Only two bands of ADH isozyme could be resolved in stages 29 and 30. Out of these one cathodal isozyme showed higher staining intensity and correspond to the band-1 of stage 28 in electrophoretic mobility (Fig.10;Plate 19). The other band was resolved anodally, exhibited less staining intensity and correspond to the sixth ADH band of stage 28.

A three-banded phenotype of ADH isozyme was exhibited in the liver tissue of the tadpoles at stage 31. Out of these the intensely stained cathodal band correspond to band-2 of stage 28 and a faintly stained band correspond to band-4 in relative mobility. The third isozyme band with

higher staining intensity was resolved anodally with identical electrophoretic mobility to that of the band-6 of stage 28.

ADH isozyme was resolved into a three banded pattern in liver tissue^{of} young frog. Two faintly stained bands showing minimum activity resolved cathodally and correspond to band-1 and band-4 respectively of stage 28. The third ADH band with highest activity was resolved away from the cathode and showed identical mobility to that of the band-5 of stage 28. This band also correspond to the single isozyme obtained in adult liver and kidney (Fig.9;Plate 18).

3.1.4. GLUCOSE-6-PHOSPHATE DEHYDROGENASE (D-Glucose-6-phosphate : NADP-Oxidoreductase, E.C.1.1.1.49):

(a) Adult tissue G6PDH pattern : A two-banded phenotype of G6PDH isozyme was obtained in gonad, kidney, heart, brain, eye and liver tissues of the adult frog (Fig.11;Plate 20). Both the bands were resolved cathodally of which one band exhibited strong staining intensity while the other band was faintly stained. Tissue wise gonad, kidney, eye and liver showed a higher concentration of the isozyme. In these tissues the isozyme was most intensely stained than in heart and brain. No activity of the isozyme could be detected in

skeletal muscle.

(b) Tadpole tail muscle G6PDH pattern : A characteristic pattern of G6PDH isozyme was obtained in the tail muscle of the tadpole at different stages of development (Fig.12;Plate 21). An identical two-banded pattern was observed in stages 25 and 26. Both the bands showed higher staining intensity at stage 26 than at stage 25. A single band of the isozyme with a strong staining intensity was resolved at stage 27. This band showed an identical electrophoretic mobility with that of the second cathodal band of G6PDH observed at stage 25 and 26. A single G6PDH band with slightly faster electrophoretic mobility was obtained in stages 28, 29, 30 and 31. At stage 28 the band was most strongly stained and exhibited a reduction in the staining intensity in the subsequent stages (29, 30 and 31).

(c) Tadpole head region G6PDH pattern : An identical two-banded G6PDH isozyme pattern was observed in the head region of the tadpoles at the different developmental stages (Fig.13;Plate 22). Both the bands were resolved cathodally and were closely spaced to each other. The first cathodal band was more strongly stained than the second band.

(d) Tadpole liver tissue G6PDH pattern : Liver tissue G6PDH exhibited a distinctive phenotypic pattern at the specific

stages of development (Fig.14;Plate 23). A three-banded pattern of the isozyme was resolved at stage 25, of these one most cathodal band showed highest staining intensity while the other two bands showed faster anodal migration and were moderately stained. At stage 26 a single isozyme corresponding to the fastest anodal band of stage 25 was obtained. In comparison the band was more intensely stained at stage 26 than that of at stage 25. A single band of G6PDH corresponding to the cathodal band of stage 25 was resolved at stage 27. The band showed a higher staining intensity than at stage 25. Some sub-bands were also observed at this stage. Only one band of G6PDH with identical electrophoretic mobility to that of the fastest anodal band of stage 25 was resolved at stages 28 and 29. The band showed a higher staining intensity at stage 29 than at stage 28. A two-banded pattern of the isozyme was observed at stage 30, of these one most strongly stained band resolved cathodally while the other band with a very faint intensity moved faster and showed identical mobility to that of the fastest anodal band observed at stage 25. Single G6PDH band was resolved cathodally at stage 31. The juvenile presented a two-banded highly cathodal phenotypic pattern identical in mobility to that of the adult G6PDH isozyme (Fig.11;Plate 20).

CHAPTER-I I
DISCUSSION

4.1. Development is the process by which a programme of selective gene expression, operating on a constant pool of genetic information produces a complex adult organism from a single fertilised egg (Kider and Taylor, 1980). The primary objective in the area of ontogenic research is to gain insight into the mechanism underlying differential gene expression. This also provides an understanding of the evolution of the regulatory specificity of gene loci during development.

Isozymes, as specific gene products, are efficient markers of cell type, enabling to define a cell in terms of its molecular composition (Markert and Moller, 1959). It has been found that there is a gradual transformation of the pattern of isozymes during the ontogeny of a variety of tissues (Markert and Ursprung, 1962; Kunz and Hearn, 1967; Chen, 1968; Hornby *et al.*, 1989), and that an abrupt change to the pattern characteristic of the adult tissues occur just before or just after hatching or parturition (Masters and Holmes, 1972; Takasu and Hughes, 1969a,b). In the present investigation we have observed a tissue specific gene expression of the four isozyme systems (viz. LDH, MDH, ADH, G6PDH) during the postembryonic developmental stages of the frog *Rana limnocharis*.

(a) LACTATE DEHYDROGENASE (LDH; E.C.1.1.1.27) :

Lactate dehydrogenase isozyme system has been the focus of much interest in isozyme changes in ontogeny, beginning with the pioneering observation of Markert and Moller (1959). In most vertebrates the tetrameric isozyme is coded by two loci (A and B) and form five different molecular forms (A_4 , A_3B_1 , A_2B_2 , A_1B_3 and B_4). The total concentration of LDH and the proportion of the five isozymes vary from tissue to tissue within an individual and within a given tissue at different developmental stages thus reflecting differential gene expression (Coffin and Hall, 1974).

Ontogenic pattern of mouse lactate dehydrogenase was investigated by Markert and Ursprung (1962). This study indicated that all embryonic tissues first exhibit a predominance of A_4 isozyme. The pattern gradually shifted towards B_4 with developmental progression. The extent of this shift however varied enormously in different tissues. In skeletal muscle, very little change in the pattern occurred, nearly all LDH activity being in A_4 , both in the embryo and in the adult. Heart muscle exhibited quite another picture. In this tissue enzyme activity was progressively shifted from A_4 toward B_4 . On the contrary Cahn et al., (1962) reported a contrasting result of LDH gene

expression at various stages of chick development. Their analysis showed that B subunit appears first during development, even in breast muscle. However, at a later stage there was a progressive shift to A subunit activity in the breast muscle. Roy and Chatterjee(1993) reported similar pattern of B gene activation in early cellular differentiation of chick embryo. A similar observation was also made in the ontogenic pattern of LDH gene expression in fishes (Frankel and Hart,1977; Frankel,1985). Comparison of the LDH isozyme pattern obtained from embryo at various stages of development of several species of frogs and newts showed an identical result (Moyer *et al.*,1968) to that of chick. Chen(1968) while investigating the pattern of soluble proteins and multiple form of dehydrogenase in amphibian development, obtained contrasting result in three different species. The anura *Bombina variegata* and the urodela *Triturus alpestris* possessed both A_4 and B_4 isozymes in their embryo, while *Rana temporaria* embryo possessed only B_4 isozyme. The electrophoretic pattern of LDH during early embryonic development of *Xenopus laevis* indicated that the B_4 isozyme was predominant form until the initial muscular response in tadpoles. At this stage there was an abrupt appearance of A_4 isozyme (Claycomb and Villedie,1971). Vonwyl(1983) analysed the LDH pattern in early embryonic and tadpole development of *Xenopus l.victorianus* and *X.borealis*.

The analysis revealed that the gene coding for A subunit was activated first followed by the gene coding for B subunit. The foregoing account reveal, that the activation of LDH gene loci during development is highly species-specific and thus shows a diversity of isozyme pattern. In our experiment we have also encountered a species specific developmental pattern.

The tail muscle of the tadpoles revealed a characteristic two banded pattern at all the developmental stages (Fig.2; Plate 11). The pattern did not alter throughout different stages of development. A comparison of relative mobilities of the bands with that of adult tissue LDH pattern (Fig.1; Plate 10) revealed that the subunit composition of tadpole tail muscle LDH were A_4 and A_3B_1 . The high concentration of the A_4 isozyme suggests that the gene coding for A polypeptides is highly active in the tadpole tail muscle. Anuran tadpole tail has a thick white muscle with relatively few mitochondria (Muntz et al., 1988). This muscle is probably used in short bursts of rapid swimming and may perhaps is subjected to hypoxic condition resulting in accumulation of pyruvate. The isozyme A_4 being less susceptible to substrate inhibition was most helpful in the tail to maintain a steady generation of energy.

In an attractive analysis of tadpole tail muscle LDH of *X.laevis* and *R.temporaria*, it has been found that in both the species B_4 isozyme was the embryonic form and expressed most strongly at the tadpole tail tip. However in tail base A_4 isozyme predominated (Hornby *et al.*, 1989). It was concluded that the tail base being rich in white skeletal muscle showed predominance of A_4 isozymes while the tail tip contains some red skeletal muscle and possessed B_4 isozymes.

The electrophoretic pattern of LDH obtained in the head region of tadpoles during developmental stages (Fig.3; Plate 11) indicated the predominance of A subunit activity. However B_4 isozyme was most active form during late prometamorphosis (Stage 28). The subunit also exhibited moderate activity at stage 25,27 during development and in the juvenile (Stage 32). Heteropolymeric association between A and B subunits was allowed in all the stages, though the type and concentration of the assembly varied considerably from stage to stage.

The observed pattern indicated that there was a differential gene expression of the isozyme correlated with the progressive cellular specialization (Champion and Whitt, 1976; Philipp *et al.*, 1979; Whitt 1981). Activation of the B locus was not allowed in the tadpoles until the prometamorphosis (Stage 27). Perhaps, at this stage there

was a sudden increase in energy demand of the tadpoles and both glycolytic and TCA cycles were operative to fulfill the need.

During metamorphosis, the amphibian liver undergoes a major metabolic reorganisation (Brown, 1964). Bilewicz (1938) has demonstrated an increase in glycogen content of the liver in *R. temporaria* during premetamorphosis. At the metamorphic climax there is a reversal and a pronounced utilization of glycogen occurs, as reflected in a drastic decrease in liver glycogen. In a study of the carbohydrate of several species, Faraggiana (1933) and Urbani (1957) both noted a decline in these constituents during premetamorphosis in contrast to the findings of Bilewicz. The decrease was more rapid at the beginning of the metamorphic climax. These studies suggest that glycogen reserves in the liver acquire importance as a source of energy nourishment during the period of metamorphosis when dramatic changes occur in the digestive system and the animal stops feeding.

In our study we have obtained a distinctive LDH pattern characteristic of specific developmental stages. The earliest premetamorphic stage 25 was characterized by beginning of muscular movement and feeding (Roy and

Khare, 1978). It was observed that at this stage both the gene loci encoding A_4 and B_4 isozymes were active (Fig. 4; Plate 13). However A_4 being more strongly expressed may be indicative of a higher dependence of liver on glycolytic energy metabolism. Following this there was a sudden repression of the B locus. A strikingly identical pattern of only A_4 and A_2B_2 isozymes were observed during the stages 26 to 28. This perhaps may be correlated to a temporary hypoxic state of the liver during the period. In the beginning of metamorphosis (stage 29) synthesis of B polypeptides was probably stepped up and the heterotetramer A_1B_3 assembly occurred for the first time in the course of development. The process of metamorphosis reached a climax at stage 30, marked by resorption of tail and frequent use of the limbs by the tadpoles.

The animals rehearse their performance occasionally in the adjoining terrestrial habitat. The B_4 isozyme was again observed at this stage. All the major isozymes were expressed at this stage though A_4 showed highest activity. The pattern remained unaltered till the end of metamorphosis. The young froglet liver did not show any activity of B_4 isozyme and was strikingly identical to that of the adult LDH pattern (Fig. 1; Plate 10).

From the physiological point of view it may be presumed

that all along the tadpole development liver tissue primarily utilizes pyruvate as an energy deriving substrate. This may directly or indirectly influence the liver tissue to maintain a higher concentration of A subunit of LDH. However for a brief period during development (stage 25,30 and 31) both lactate and pyruvate were utilized to meet the increased energy demand.

Looking at the overall picture of LDH isozyme pattern in the three tissues it appears that the expression of the loci A and B at the various stages of development show a high tissue specificity. Activation/repression of the genes were accurately programmed. However, what is the molecular mechanism in the synthesis of the different subunits remain unclear. The regulating mechanisms, which distinguishes between the two genes at a specific time may be related either to the level of oxygen or to intermediates of oxidative metabolism (Lindy and Rajasalmi,1966).

(b) MALATE DEHYDROGENASE (MDH; E.C.1.1.1.37) :

Malate dehydrogenase in most anurans is a dimer, constituted by the combination of two types of subunits, A and B (Danzmann and Bogart,1982). The enzyme is encoded by two separate genes, Mdh-A and Mdh-B (Schwantes and

Schwantes,1977). MDH isozyme pattern during development has been studied in several species of fishes (Champion and Whitt,1976; Philipp *et al.*,1979; Arai,1984; Padhi and Khudabuksh,1989) and amphibians (Kim *et al.*,1965; Schwantes and Schwantes,1977; Long *et al.*,1983).

In the present investigation adult tissue s-MDH was resolved into three bands (Fig.5; Plate 14). The subunit composition of the isozymes was designated on the basis of tissue specificity and electrophoretic mobility. The presence of three forms of the enzyme indicated that the frog possesses two loci A and B encoding the cytosolic MDH. Electrophoretic mobility of the homodimer A_2 and B_2 are in general agreement with the observed pattern in other anurans (Schwantes and Schwantes,1977). Out of the seven tissues analysed liver and skeletal muscle exhibited a higher concentration of the isozyme. This may be attributed to a greater metabolic need of the isozyme in these tissues.

Tadpole tail muscle analysed during the developmental stages (stage 25 to stage 31) a characteristic pattern. Both the loci A and B were active producing a three banded pattern (Fig.6; Plate 15). The isozyme showed a decreasing trend in activity with the progression of development. In the head region only one isozyme corresponding to B_2

homodimer was resolved at stage 25, both the loci seemed to be active at stage 26 while only one s-MDH corresponding to A_2 homodimer was expressed in the rest of the developmental stages (Fig.6; Plate 16).

Liver tissue of the tadpole exhibited a higher concentration of s-MDH isozymes (Fig.7; Plate 17). The gene expression showed a characteristic developmental pattern. The s-MDH isozyme did not show any detectable activity during premetamorphic growth stages (25 and 26). This may be either due to total inactivation of the MDH loci or a very low concentration of the enzyme in liver of the tadpole in these stages. During premetamorphic growth phase (stage 27 and 28) only the locus A encoding A_2 homodimer was found to be active. At the beginning of metamorphosis (Stage 29) both the loci were active. In the next two stages (30 and 31) the tadpoles showed pronounced growth of their limbs and resorption of tail. The isozyme in these stages are represented by a single band corresponding to A_2 homodimer. Both the isozymes A_2 and B_2 were again detected in the juvenile frog (stage 32).

Malate dehydrogenase is one of the key enzyme in the tricarboxylic acid (TCA) cycle. The enzyme catalyzes the reversible interconversion of oxaloacetate to malate using NAD as coenzyme. Enzymes of TCA cycle generally have been

found to decrease in activity during metamorphosis (Long et al., 1983; Yamamoto, 1960). MDH activity in normal and thyroxine treated tadpoles of *Rana pipiens* has been found to decrease remarkably during development (Long et al., 1983).

The decreasing trend of MDH activity observed in tail and head region along with the progression of development of tadpoles may be ascribed to the fact that these tissues contain enough white skeletal muscle and perhaps anaerobic breakdown of carbohydrate is the predominant pathway of energy metabolism. It may be presumed that the TCA cycle was operative in these tissues. It has been reported (Salmon & Leqname et al., 1971) that in *Bufo arenarum* development, TCA cycle was incompletely operative in certain tissues.

MDH activity decline may also result from deficiencies in energy-rich compounds, such as TCA cycle substrates (Long et al., 1983). If regulation of MDH amounts present at any given time is controlled at the "transcriptional level", then the amount of active enzyme may reflect the amount of substrate available within the cell at that period.

Liver tissue of the tadpoles exhibited highest concentration of s-MDH isozymes. The expression of isozyme varied considerably during development. This may perhaps be related to the feeding habit of the tadpoles at the various

stages. In feeding tadpoles (stage 27,28,30, and 31) only one locus(A) was activated while in the nonfeeding stages (stage 29 and 32) both the loci (A and B) showed equal activity.

(a)ALCOHOL DEHYDROGENASE (ADH, E.C.1.1.1.1) :

ADH isozyme was analysed in liver and kidney tissues of the adult frog and liver tissues of the tadpoles. Other tissues (viz. gonad, brain, eye, heart and muscle) of adult and tadpoles (viz. tail muscle and head region) did not show ADH activity. Our results are thus in conformity with the observation made in other vertebrates (Raiha *et al.*,1967; Markovik *et al.*,1971), where it has been regarded as a liver specific enzyme. The isozyme is known to be dimer (Rossman *et al.*,1975) and encoded at a single locus in majority of vertebrates (Hitzeroth *et al.*,1968; Shaklee *et al.*,1974; Vallee,1985; Feung *et al.*,1989).

Ontogenic pattern of ADH isozymes have been investigated in a number of vertebrates (Raiha *et al.*,1967; Hitzeroth *et al.*,1968; Smith *et al.*,1971; Shaklee *et al.*,1974; Lavine and Hadley,1975; Frankel,1980) including amphibians (Chen,1963; Wesolowski and Lyerla,1979).

The ADH isozyme pattern of liver and kidney tissues of adult frog investigated here revealed a single banded pattern (Fig.9; Plate 18). A greater activity of ADH was noted in the liver tissue than in kidney. The single banded pattern with identical electrophoretic mobility in both the tissues revealed that ADH being coded by the same gene locus in these tissues.

The ADH isozymes observed in tadpole liver at various stages of development showed a unique pattern of gene expression (Fig.10;Plate 19). Premetamorphic (stage 25,26) as well as early prometamorphic (stage 27) tadpole liver did not show ADH activity. This may be presumed either due to repression of the ADH gene or absence of specific substrate. Nevertheless, a distinctive pattern of the isozyme was observed at stage 28. The liver tissue ADH at this stage was resolved into as many as six isozymes. Similar multiple bands of ADH has been observed previously in the embryo of *Bombina variegata* (Chen,1968) and *Xenopus laevis* adult (Wesolowski and Lyerla,1983). This phenomenon may be explained by assuming that there are at least three structural genes A, B and A', which are coding for the ADH dimers. Genes A and A' may be allelic in expression. The possible subunit composition of isozymes therefore may be designated as A_2 , A_1B_1 , $A_1A'_1$, B_2 , A'_1B_1 , A'_2 (Fig.9). The

sudden activation of all the structural genes at this stage may have relation with the feeding habit of the tadpoles. Additional genes were probably activated in response to accumulation of alcoholic substrates in liver as a byproduct of other pathways of metabolism.

A sharp reduction in the isozymes have been observed during the later period of development (stages 29, 30 and 31) which may be indicative of lesser metabolic requirement and repression of the additional genes. However a detailed study on the expression of ADH isozymes with ecological correlates is needed to draw any concluding remark.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PDH, E.C.1.1.1.49) :

Glucose-6-phosphate dehydrogenase catalyzes the oxidation of glucose-6-phosphate, providing NADPH for biosynthetic purposes in the pentose phosphate pathway. G6PDH isozymes in amphibians is apparently dimeric in nature (Chen,1968; Schwantes *et al.*,1969; Chatterjee and Prakash,1990). In the present investigation a two banded phenotype of the isozyme was observed in the tissues of the adult frog (Fig.11;Plate 20). Both the bands were resolved cathodally, of which the most cathodal band was heavily stained than the other. Similar results were observed in fishes (Scholl and

Anders,1973). Out of the two bands of G6PDH, one designated A_2 is highly specific for glucose-6-phosphate and NADP, whereas the other called B_2 has a broad substrate specificity (Bautista *et al.*,1984). However, the molecular and genetic basis governing the expression of G6PDH isozymes in amphibians has not yet been resolved. Little or no activity of G6PDH in skeletal muscle of the frog may suggest that the generation of NADPH from glucose oxidation via hexose monophosphate shunt does not play an essential role in the tissue (Fried *et al.*,1969).

In contrast the white skeletal muscle of tadpole tail exhibited significant G6PDH activity (Fig.12; Plate 21). During premetamorphosis (stage 25 and 26) and early prometamorphosis (stage 27) only A_2 form was most intensely expressed. The pattern altered in the subsequent stages of development and only B_2 was expressed.. This pattern was maintained in the tail muscle through entire metamorphic period. However a gradual decrease in the staining intensity of the isozyme was noted in tail muscle with the progression of development. This reduction in concentration of the enzyme with the progressive developmental activities and resorption of tadpole tail may be correlated with the metabolic switch over from hexose monophosphate shunt to an alternative pathway of NADPH generation. Alternatively, it

may be suggested that during metamorphosis as the tail is resorbed biosynthetic activities diminishes resulting in lower demand of NADPH.

G6PDH isozymes analysed in the head region of the tadpoles during development (Fig.13; Plate 22) showed a striking similarity with that of adult tissue G6PDH pattern (Fig.11; Plate 20). Both the form (A_2 and B_2) of the enzyme were expressed. The isozyme showed a progressive increase in concentration from premetamorphosis to metamorphosis. This may imply greater biosynthetic activities using NADPH in the tissue with progression of development.

Liver receives a mixture of free monosaccharides resulting from the digestion of oligosaccharides and polysaccharides. About two-thirds of the free glucose coming to the liver enters its cells and is phosphorylated to glucose-6-phosphate by hexokinase (Lehninger,1975). Monosaccharides other than glucose such as D-fructose, D-galactose and D-mannose are also phosphorylated in the liver and further transformed into glucose-6-phosphate. About half of the glucose that undergo degradation in liver enters the hexose monophosphate shunt generating NADPH (Axelrod,1967).

Our analysis of the tadpole liver G6PDH during development revealed that both the isozymes A_2 and B_2 were

catalytically active. The pattern however varied considerably with the progression of development. Both the forms were expressed at stage 25. A single enzyme representing A_2 homodimer was found to be highly active in stages 27, 30 and 31 (Fig.14; Plate 23) while B_2 in stages 26, 28 and 29. It may be presumed that the feeding habit of the tadpoles directly or indirectly influence this differential pattern of the isozymes. During late premetamorphosis and late prometamorphosis feeding activities decrease while in the beginning of metamorphosis feeding virtually ceases. Perhaps in these stages liver was constrained with glucose availability and only B_2 form of the isozyme was synthesized. In the feeding stages of the tadpoles (stages 25, 27, 30 and 31) A_2 homodimer was the most active form of the enzyme. This shift of enzyme activity has been regarded to be the inherent property of liver tissue (Lehninger,1975). Thus it has been suggested that the expression of the gene coding for specific enzymes in liver is a highly regulated process.

The overall pattern of G6PDH observed in this frog suggest that the isozymes may be encoded by two separate gene loci A and B. The differential activity of the loci observed during the tadpole development may have specific functional significance at various stages. The expression of the loci may be related to the alteration of metabolic pathways in accordance with the availability of substrates.

CHAPTER - I I
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CHAPTER - I I
FIGURES

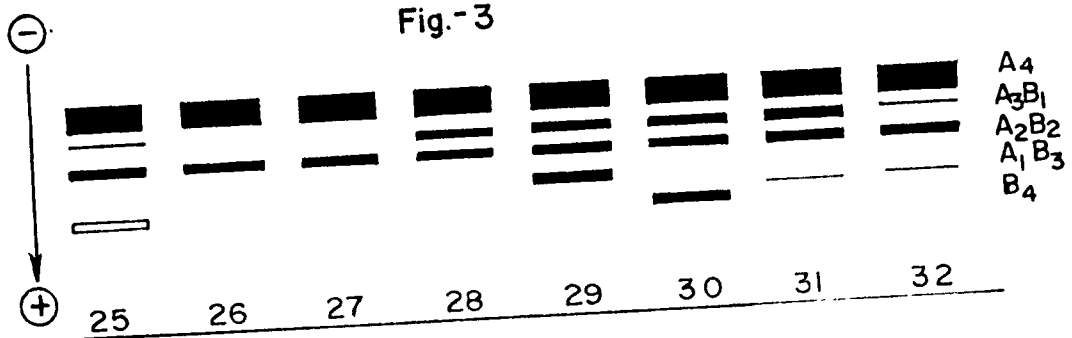
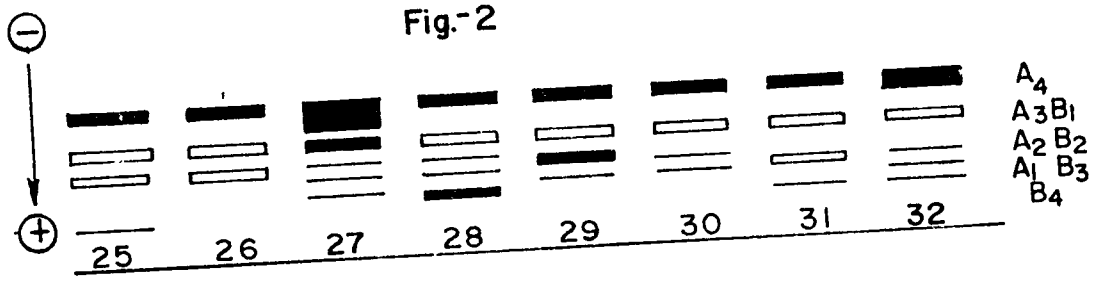
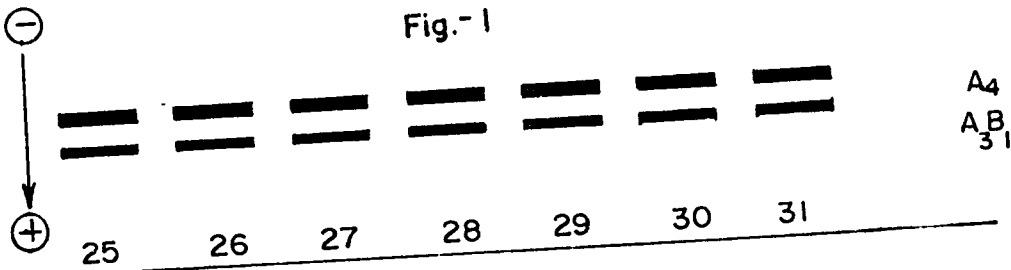
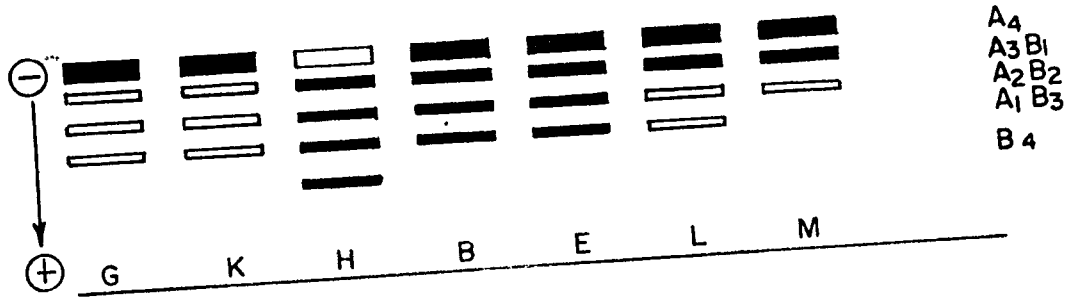
Tissue LDH isozyme pattern.

Fig.1. Adult tissue LDH

Fig.2. Tadpole tail muscle LDH

Fig.3. Tadpole head region LDH

Fig.4. Tadpole liver LDH



Tissue MDH isozyme pattern

Fig.5. Adult tissue MDH

Fig.6. Tadpole tail muscle MDH

Fig.7. Tadpole head region MDH

Fig.8. Tadpole liver MDH

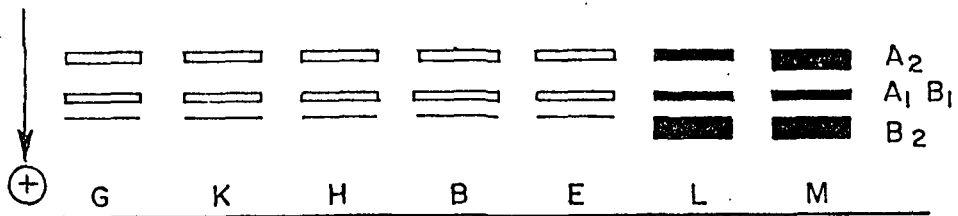


Fig.-5

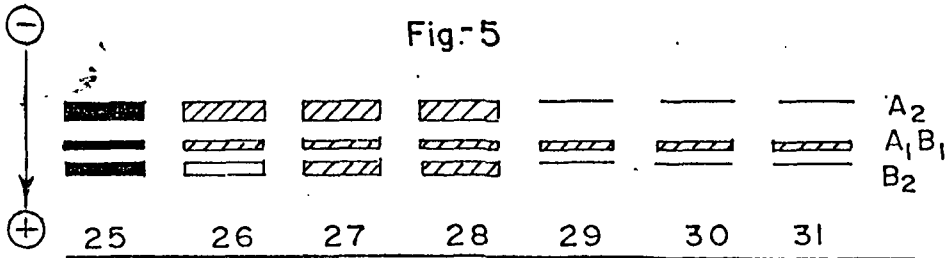


Fig.-6

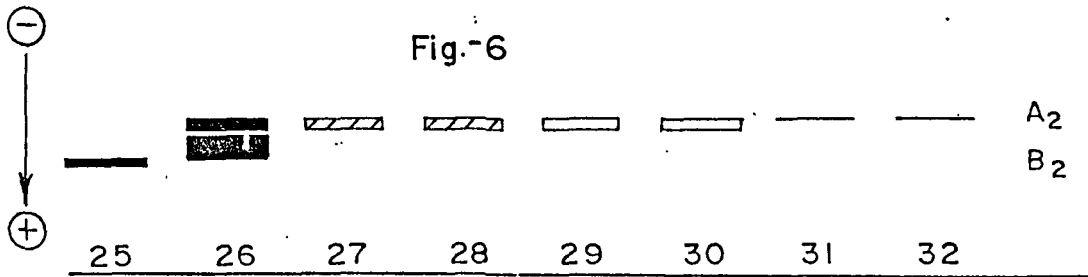


Fig.-7

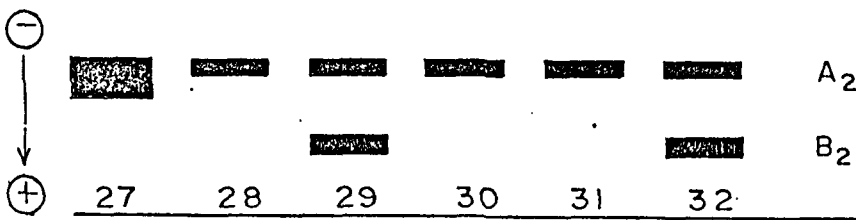


Fig.-8

Tissue ADH isozyme pattern

Fig.9. Adult liver and kidney ADH

Fig.10. Tadpole liver ADH

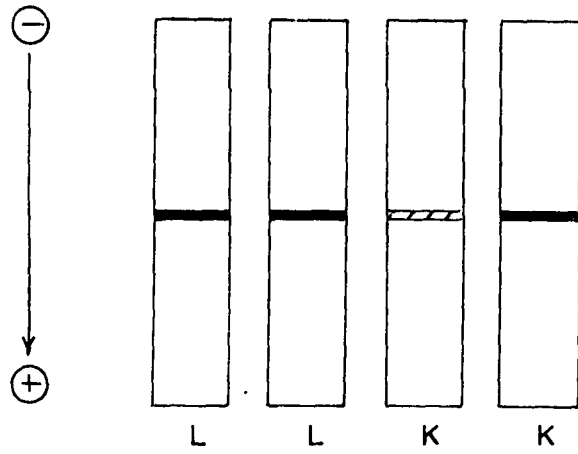


Fig.- 9

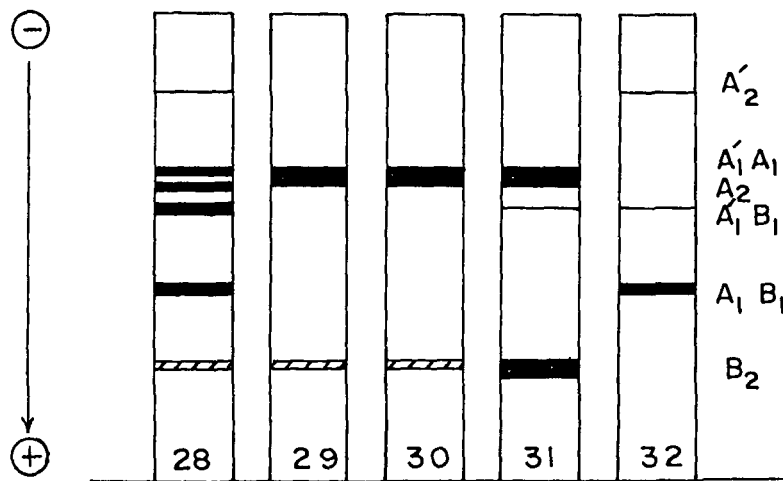


Fig- 10

Tissue G6PDH isozyme pattern

Fig.11. Adult tissue G6PDH

Fig.12. Tadpole tail G6PDH

Fig.13. Tadpole head region G6PDH

Fig.14. Tadpole liver G6PDH

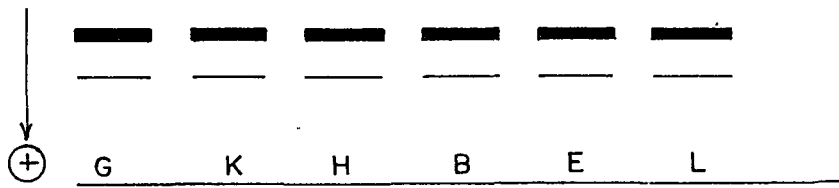


Fig.- 11

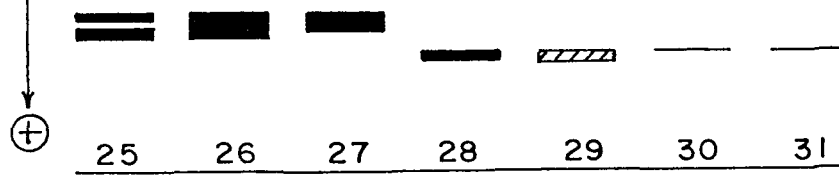


Fig.- 12

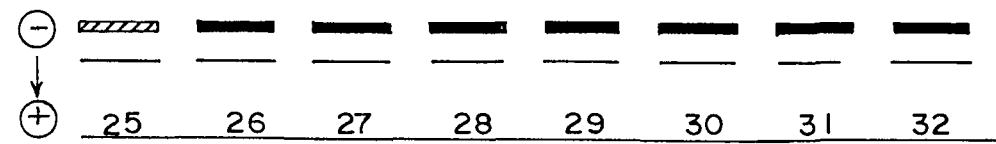


Fig.- 13

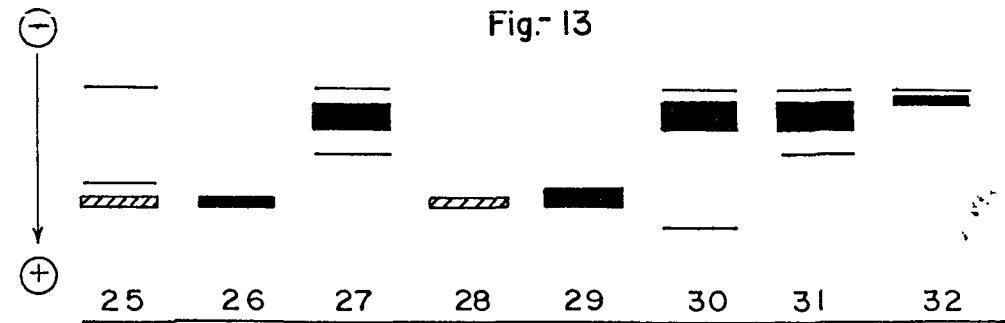


Fig.- 14

CHAPTER-I I
PLATES

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Plate:1. Adult frog : *Rana limnocharis*



1

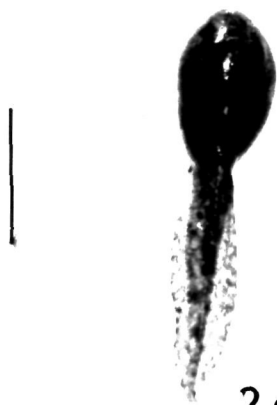
Plate:2(a-c) and 3: Premetamorphic tadpoles
Bar = 5mm



2 a



2 b



2 c



3

Plate:4 and 5: Prometamorphic tadpoles

Plate:6 and 7: Metamorphic tadpoles

Bar = 5 mm



4



5



6



7

Plate:8(a,b): Metamorphic stages

Plate:9: Juvenile frog
Bar = 5 mm



8a



8b



9

Plate:10: Tissue LDH isozyme pattern of adult frog.

Plate:11: Tadpole tail region LDH pattern

Plate:12: Tadpole head region LDH pattern

Plate:13: Tadpole liver LDH pattern

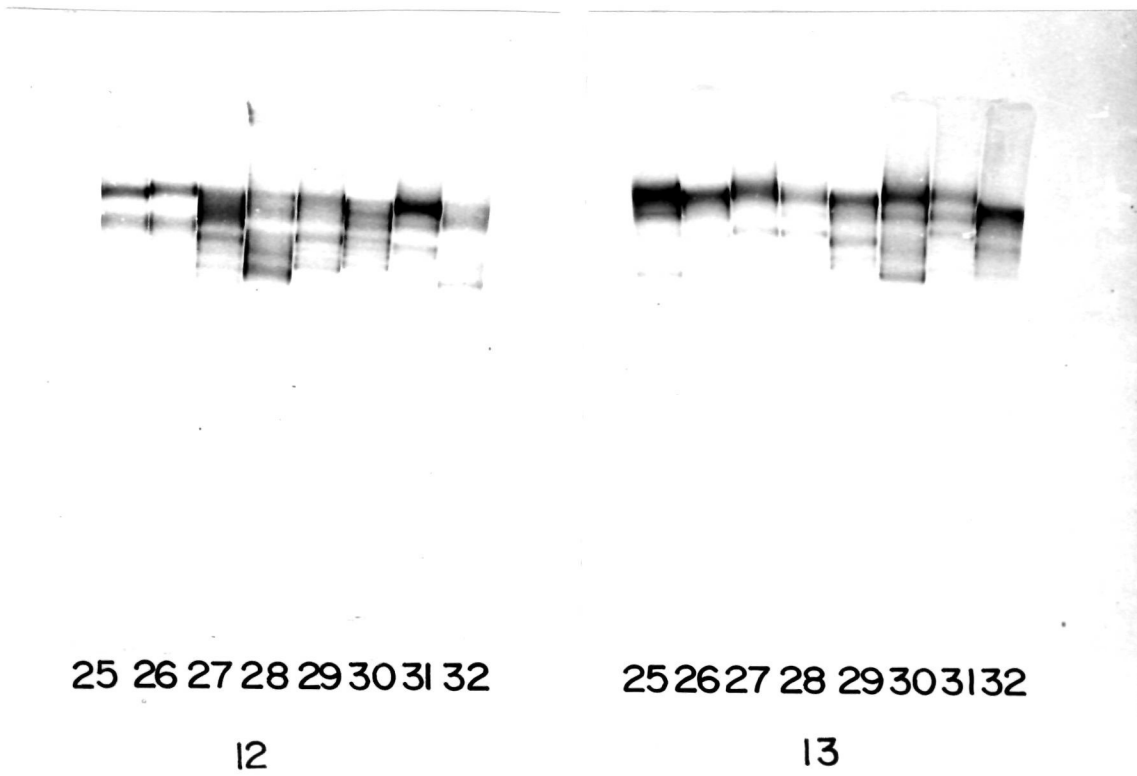
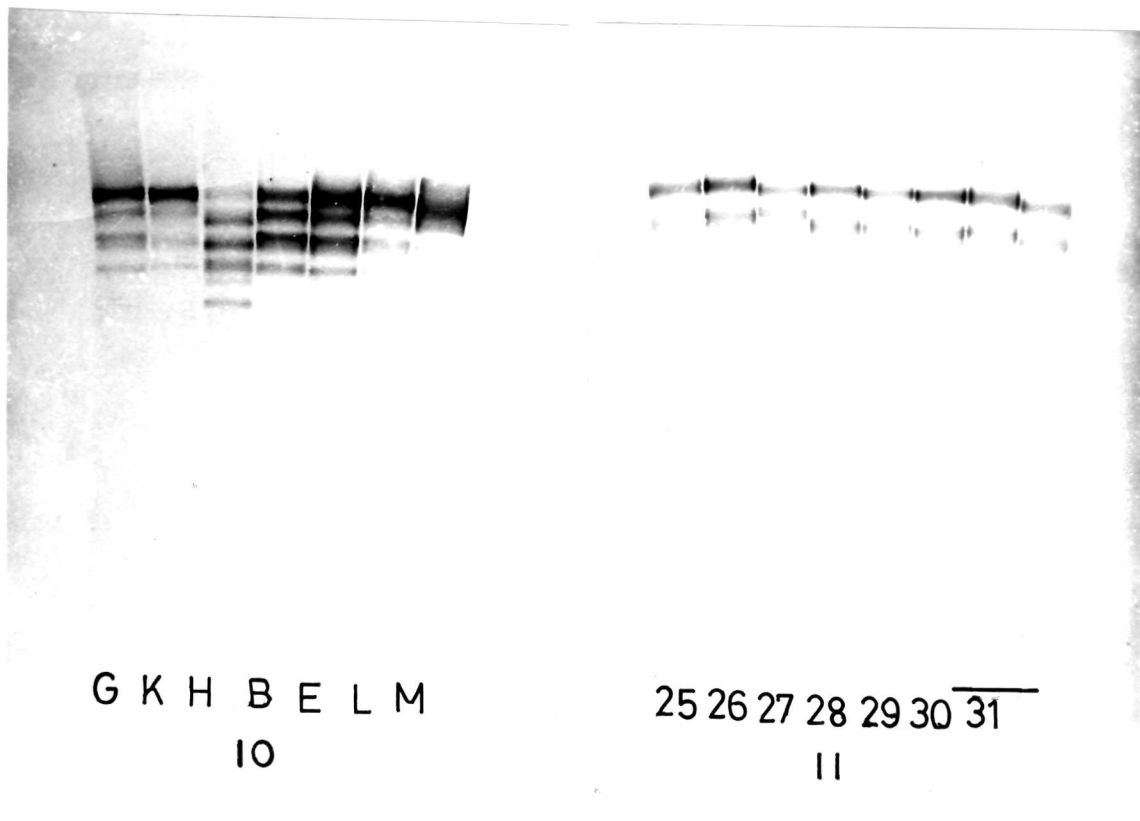


Plate:14: Tissue MDH isozyme pattern of adult frog

Plate:15: Tadpole tail muscle MDH pattern

Plate:16: Tadpole head region MDH pattern

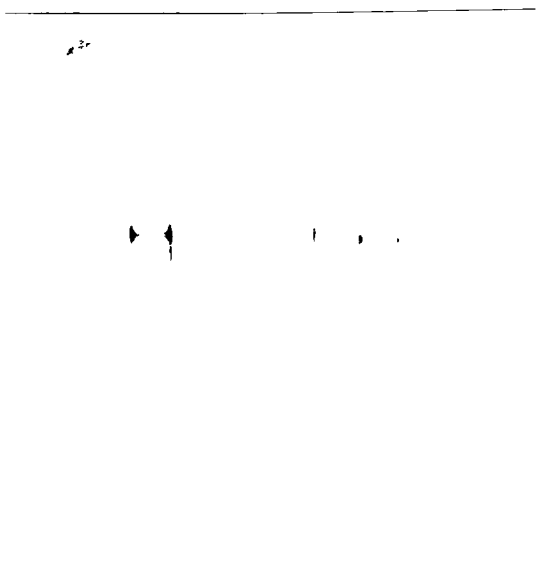
Plate:17: Tadpole liver MDH pattern



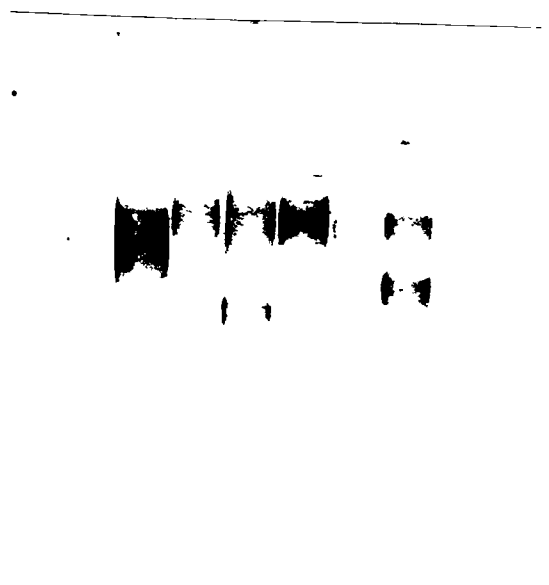
G K H B E L M
14



25 26 27 28 29 30 30^{''}
15



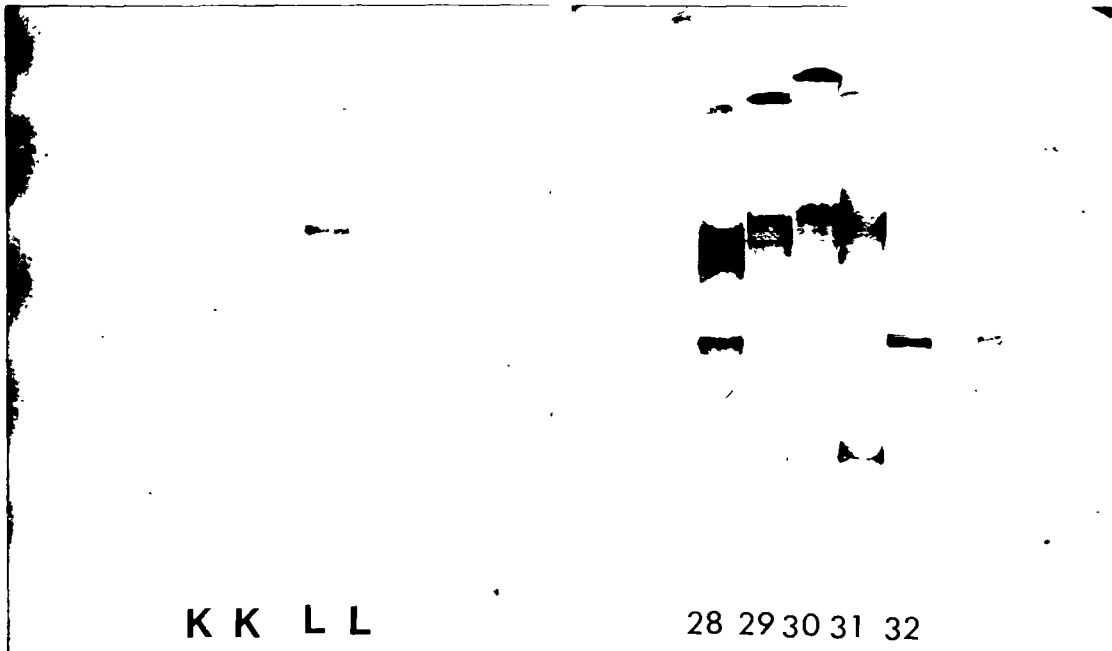
25 26 27 28 29 30 31 32
16



27 28 29 30 31 32
17

Plate:18: Liver and kidney tissue of ADH
pattern of adult frog .

Plate:19: Tadpole liver ADH pattern



K K L L

18

28 29 30 31 32

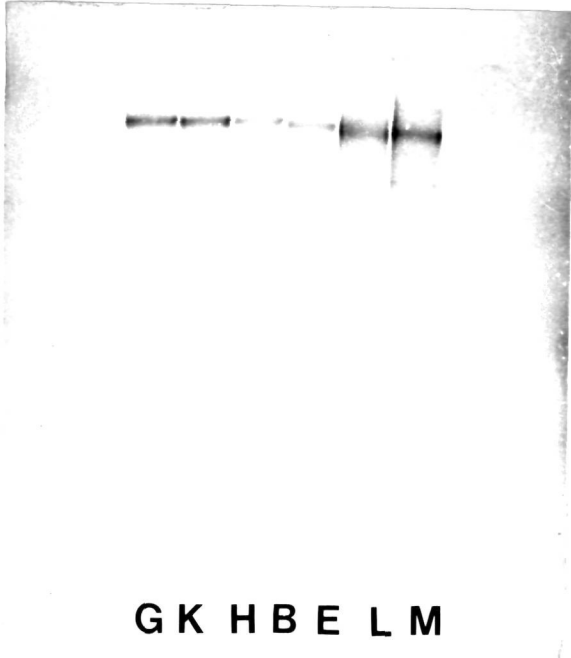
19

Plate:20: Tissue G6PDH isozyme pattern
of adult frog

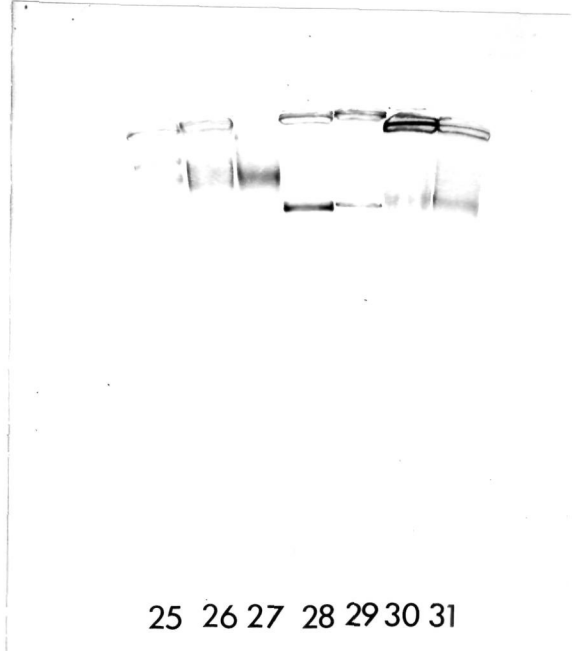
Plate:21: Tadpole tail muscle G6PDH pattern

Plate:22: Tadpole head region G6PDH pattern

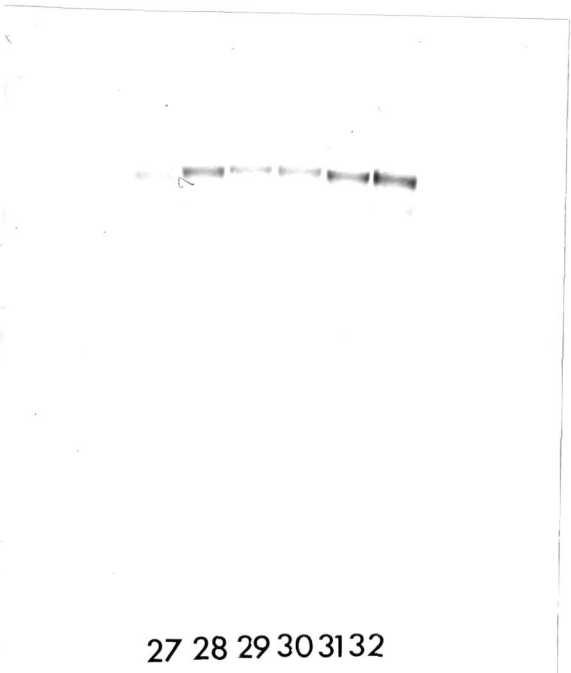
Plate:23: Tadpole liver G6PDH pattern



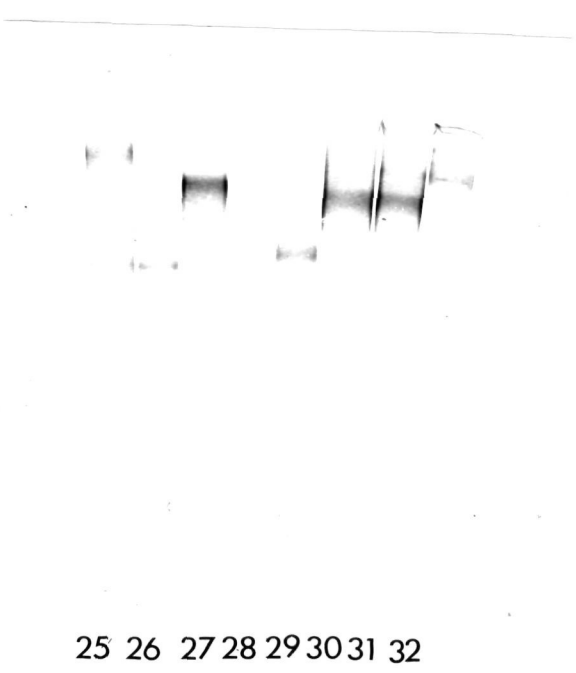
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CONCLUDING REMARKS

Most of the studies on enzyme electrophoresis have been designed primarily to assess allelic or allozymic (Case, 1978; Chatterjee and Prakash 1990) variation among populations and species. The number of tissues incorporated in these studies generally is small, usually restricted to one or two tissues. Studies involving multiple tissue may be used to assess isozyme characteristics, such as tissue-specificity, heteropolymer assembly and number of loci coding for a single enzyme (Buth, 1984). For multilocus enzymes, the pattern of tissue-specific expression for each locus is often evolutionarily conserved, and tissue-specific expression may be important in determining locus homologies among taxa (Whitt, 1983).

Our analysis of LDH gene expression involving multiple tissues of the seven species supports the earlier findings that in most vertebrates there are mainly two loci coding for these isozymes. However, the observed individual variation from different populations as well as inter-species variability of the isozyme pattern was remarkable. We have tried to correlate these variations with the suspected macro-environmental factors (viz. deforestation, mining, urban activities, etc.) as well as presumed physiological state of the frogs. Neither a specific environmental factor nor any physiological criteria was

measured within the framework of our study. Incorporation of these parameters to the presently evaluated isozyme pattern may reveal more accurate biological information.

It is claimed that the third gene locus C encoding tissue specific LDH in birds and mammals (Zinkham et al., 1969; Goldberg, 1972) as well as in bony fishes (Markert et al., 1975) is absent in amphibians (Whitt 1969). However, the possibility of the occurrence of the gene in frogs cannot be ruled out totally. Our observation of an additional isozyme in liver and eye tissues in two species (viz. *A.afghanus* and *R.reinwardtii*) arises some questions in this regard. A confirmation to this observation is possible only when an extensive analysis involving purification of the isozyme to homogeneity, immunological test, determination of primary sequence of the protein, etc. are undertaken. However, such analysis were beyond the objective of the present study. Thus this may provide a clue for further investigation.

Moreover, we have detected a highly heterozygous population of *P.leucomystax*. Though the size of our sample was small even then we were able to record variability. This of course needs further research involving analysis of larger sample size and controlled breeding experiments. This

data can be more meaningful if substantiated with environmental correlates.

The pattern of s-MDH isozymes in these frogs revealed a considerable homology among them. The isozyme in these frogs seems to be encoded by two loci (A and B) and are under the similar regulatory mechanism. Our results are in conformity with the pattern in previously studied amphibians (Chen, 1968; Fisher et al., 1980; Schwantes and Schwantes, 1982).

Alcohol dehydrogenase in these frogs seems to be dimeric in nature and is being coded at a single locus. However, additional bands of the enzyme have also been observed in some species (*R. cyanophlyctis* and *R. nicobariensis*). Such additional bands may arise due to allelic variation (Wesolowski, 1983). We propose a more extensive investigation with larger samples to score the alleles from different populations.

A uniform pattern of G6PDH observed in the frogs may be indicative of identical gene expression. We could not detect any activity of G6PDH in skeletal muscle tissue of the frogs. We tried to substantiate this with a low pentose-phosphate shunt of glucose metabolism. This speculation may be more meaningful with the incorporation of the elaborate

biochemical analytical data.

In the second chapter of the thesis we have analysed the isozyme pattern of *Rana limnocharis* during tadpole metamorphosis. The observed pattern provided additional evidence of differential gene activity and usefulness of isozymes as 'gene markers' in genetic studies.

The differential pattern of isozymes observed during the growth and metamorphosis of tadpoles implied an accurately programmed activation/repression phenomenon of structural genes at specific developmental stage. However, the molecular genetic mechanism of the events remains unclear though we have tried to explain this with physiological correlates. Moreover the simultaneous activation of all the ADH genes during early stages of development and a gradual repression of one or more genes with the progression of development clearly reflected a differential gene action. It has been suggested that the degree of dissimilarity between paternal and maternal alleles may affect their synchronous activation (Numachi, 1972). Thus in order to determine the paternal and maternal gene effect during individual development of the frog further experiments may be designed involving controlled breeding as well as ecological and physiological measurements.

The present investigation was one of the first step analysis involving these frogs. We have observed that in most of the species LDH gene locus A was highly active. The locus showed almost identical gene expression in all the tissues (except gonad and heart in certain cases) of the frogs. Further, the locus also exhibited a considerable amount of variability (viz. *P. leucomystax*). Nevertheless the locus showed equally high activity even during the tadpole metamorphosis as evident in the second chapter. Thus it may be presumed that the LDH gene locus A play some adaptive role. However with the limitation of our study it was not possible to measure such aspect. Further investigation may enlighten us about the adaptive role of the locus in the frogs.

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