THE LACTATE DEHYDROGENASE ISOZYMES OF SIX SPECIES OF THE TELEOST FAMILY COTTIDAE

by

WILLIAM STEVEN GARLICK

A THESIS

Presented to the Department of Biology
and the Graduate School of the University of Oregon
in partial fulfillment
of the requirements for the degree of
Master of Arts

June 1974

APPROVED: Paul P. Rudy

Paul P. Rudy

VITA

NAME OF AUTHOR: William Steven Garlick

PLACE OF BIRTH: Cedar Rapids, Iowa

DATE OF BIRTH: January 15, 1947

UNDERGRADUATE AND GRADUATE SCHOOLS ATTENDED:

Linfield College
San Jose State College
University of Oregon

DEGREES AWARDED:

Bachelor of Arts, 1968, Linfield College

AREAS OF SPECIAL INTEREST:

Fish Physiology
Enzyme Chemistry

PROFESSIONAL EXPERIENCE:

Laboratory Instructor, Linfield College, McMinnville, Oregon, 1966-1967

Teaching Assistant, Department of Biology, University of Oregon, Eugene, 1972-1974

PUBLICATIONS:

McConnaughey _et al._, (1971) _Coos Bay Study: An Interdisciplinary Study of Man and the Estuary_. NSF Grant Number GY-9114.

ACKNOWLEDGMENTS

I would like to thank Dr. P. P. Rudy and Dr. R. C. Terwilliger for the use of the facilities of the Oregon Institute of Marine Biology, Charleston, Oregon. I would like to further thank Dr. R. C. Terwilliger for technical assistance.

TABLE OF CONTENTS

ILLUSTRATIONS

Figure Legends

INTRODUCTION

Lactate dehydrogenase (L-lactate: NAD^+ oxidoreductase, E.C. 1.1.1.27) is a zinc-bearing glycolytic enzyme which catalyzes the following reversible reaction:

$$\text{L-lactate} + NAD^+ \rightleftharpoons \text{pyruvate} + NADH + H^+$$

In vertebrates the enzyme exists as a tetramer. Each subunit has a molecular weight of 35,000, for a combined molecular weight of 140,000 for the tetrameric form (Dixon & Webb, 1964; Pesce et al., 1964; Pesce et al., 1967; Smith, 1968; Wold, 1971). The enzyme is specific for L-lactate among the vertebrates, and requires a pyridine nucleotide as a coenzyme (Wold, 1971). LDH shows a preference for NAD^+ over NADP. In reducing the coenzyme, LDH performs a stereo-specific hydrogen transfer reaction producing only one isomer of the reduced pyridine nucleotide (Wold, 1971).

LDH requires a free sulfhydryl group in each binding site (Di Sabato et al., 1963; Fondy et al., 1965; Allison, 1966; Wold, 1971). The enzyme binds four moles of substrate per mole of enzyme, with each subunit of the tetramer binding one mole of substrate (Allison, 1966; Wold, 1971).

Among the vertebrates whose LDH has been studied, at least two genetic loci code for two major polypeptides, A and B. These subunits readily associate at random _in vitro_ to form five possible permutations; A_4, A_3B, A_2B_2, AB_3, and B_4 (Cahn _et al_., 1962; Markert, 1963; Shaw & Barto, 1963; Vesell & Yielding, 1966; Markert & Holmes, 1969; Goldberg _et al_., 1969; Whitt, 1970a; Whitt & Booth, 1970; Baldwin _et al_., 1973; Schwantes, 1973; Shaklee _et al_., 1973). These multiple forms of the enzyme, called isozymes, are readily separable by electrophoresis, can be further differentiated by histochemical methods, and are immunochemically distinct (Cahn _et al_., 1962; Vesell & Yielding, 1966; Smith, 1968; Shaw & Prasad, 1969; Whitt & Booth, 1970; Baldwin _et al_., 1973; Schwantes, 1973).

The A_4 isozyme is found predominantly in the epaxial muscle or other tissues where the enzyme must perform under relatively anaerobic conditions (Pesce _et al_., 1964; Pesce _et al_., 1967; Wold, 1971). In these tissues A_4 operates primarily as a pyruvate reductase (Everse _et al_., 1970). In voluntary muscle where rapid and brief release of energy is essential, the enzyme has a high turnover rate under conditions of high pyruvate and is not inhibited by normal accumulations of pyruvate (Pesce _et al_., 1967; Dando, 1969; Everse _et al_., 1970). Lactate accumulates as an end product and must be transported to other tissues for oxidation to pyruvate, particularily in the heart and liver (Dando, 1969; Everse et al., 1970; Wold, 1971). A_4 binds four moles of coenzyme per mole of enzyme but has an anomalous binding curve that cannot be explained by any simple two substrate kinetic equation (Pesce et al., 1967; Wold, 1971).

The B_4 isozyme is found predominantly in aerobic tissues, such as heart muscle, where a continual supply of energy must be assured for sustained or rhythmic mechanical work (Pesce et al., 1964; Pesce et al., 1967; Wold, 1971). Pyruvate metabolism in the heart is in the direction of oxidation of lactate. Marked inhibition of the B_4 isozyme by high concentrations of pyruvate permits pyruvate metabolism to proceed through other pathways. In addition, this inhibition prevents the accumulation of deleterious amounts of lactate in aerobic tissues (Pesce et al., 1964; Vesell & Yielding, 1966; Pesce et al., 1967; Wold, 1971). The kinetic data for the B_4 isozyme can be applied to the Adair equation to demonstrate a maximum of four binding sites. The Hill coefficient for a typical B_4 isozyme is 1.2, indicating that the four sites are nearly independent (Wold, 1971).

These characteristic metabolic roles for the major subunits of LDH, as well as its tissue and temporal specialization, suggest the hypothesis that the relative distributions of A and B subunits and their resulting heterotetramers are determined by the availability of oxygen to the tissues (Whitt, 1970b; Gesser & Poupa, 1973). Thus, the complement of LDH in different tissues is in response to specific environmental pressures (Prosser, 1969; Wold, 1971; Wilson *et al*., 1973). The *in vitro* synthesis of LDH and the random association of subunits has been shown to follow a binomial distribution (Salthe *et al*., 1965; Clausen & Hustrulid, 1968; Ohno *et al*., 1968; Markert & Holmes, 1969; Whitt, 1970b; Schwantes, 1973). However, differential synthesis of the subunits can result in a skewed distribution of associated tetramers *in vivo* (Das & Prosser, 1967; Vesell *et al*., 1968). Recent studies of enzyme induction have shown that catabolism is also instrumental in determining a steady state of enzyme levels. Relative affinities between different subunit types may also vary between tissues. Hence, tissue-specific patterns may arise in spite of equal synthetic activity if the relative rates of A_4 and B_4 catabolism and subunit affinities vary from one tissue to another (Vesell & Yielding, 1966). Thus, the preponderance of A_4 isozyme in epaxial muscle and B_4 isozyme in heart muscle is generated and maintained. These tissue-specific patterns of isozymes are quite constant within a species (Cahn *et al.*, 1962; Markert and Holmes, 1969; Shaklee *et al.*, 1973).

In all higher vertebrates that have been examined, each tissue contains all five possible configurations of A and B subunits in a characteristic distribution. It has been postulated that only one or two isozymes are actually necessary for optimum function. The additional forms could exist as an environmental buffer allowing rapid response of the enzyme to drastic changes in the environment (Hochachka, 1965; Markert & Holmes, 1969; Prosser, 1969; Baldwin & Hochachka, 1970; Whitt & Horowitz, 1970). It is interesting to note that the intermediate heterotetramers of the A and B subunits have intermediate physical properties, such as heat lability and electrophoretic mobility (Hochachka, 1965; Whitt & Horowitz, 1970; Wold, 1971).

A further genetic locus has been discovered in endotherms which codes for a polypeptide called C, or LDH-X (Blanco & Zinkham, 1963; Goldberg, 1963; Markert & Faulhaber, 1965; Whitt, 1969; 1970a; Whitt & Booth, 1970; Baldwin *et al.*, 1973; Goldberg 1973; Shaklee *et al.*, 1973). This subunit exists as a homotetramer (Goldberg, 1973) and is quite specific spatially and temporally. In those organisms that possess a C_4 isozyme, it is found in sperm cells and in primary spermatocytes only at a time when the cells are functionally active (Blanco & Zinkham, 1963; Goldberg, 1963; Baldwin *et al.*, 1973; Goldberg, 1973). It apparently fulfills some unique metabolic requirement of the highly differentiated sperm cell. There appear to be no molecular restraints on *in vivo* combination of all possible A, B, and C tetramers, as demonstrated by recombination experiments *in vitro* (Markert, 1963; Salthe *et al.*, 1965; Clausen & Hustrulid, 1968; Goldberg, 1966; Goldberg *et al.*, 1969).

Fishes also possess two genetic loci coding for the major subunit types, A and B (Hochachka, 1965; Markert & Faulhaber, 1965; Goldberg, 1966; Hochachka, 1966; Kusa, 1966; Morrison & Wright, 1966; Klose *et al.*, 1968; Clayton & Gee, 1969; Goldberg *et al.*, 1969; Lush *et al.*, 1969; Utter & Hodgins, 1969; Whitt, 1969; Baldwin & Hochachka, 1970; Whitt, 1970a; Whitt & Booth, 1970; Whitt & Horowitz, 1970; Scholl & Holzberg, 1972; Gesser & Poupa, 1973; Shaklee *et al.*, 1973; Wilson *et al.*, 1973). These subunits exhibit a definite homology to the A and B subunits of higher vertebrates, as exemplified by kinetic and immunochemical analyses (Morrison & Wright, 1966; Pesce *et al.*, 1967; Bailey & Wilson, 1968; Ohno *et al.*, 1968). However, fishes often exhibit tissue-specific isozyme patterns that are complex or are inexplicable in a physiological sense (Markert & Faulhaber, 1965; Hochachka, 1966; Goldberg *et al*., 1969; Lush *et al*., 1969; Markert & Holmes, 1969; Whitt, 1970a; 1970b; Whitt & Booth, 1970; Scholl & Holzberg, 1972). Fishes often lack the random associations of monomers which are observed in higher vertebrates. More often, specific tissues will exhibit only homotetramers or symmetric heterotetramers, such as A_2B_2 (Lush *et al*., 1969; Markert & Holmes, 1969; Whitt, 1970a; 1970b; Whitt & Booth, 1970). Apparently some genetic or epigenetic mechanism is restricting assembly in some fishes. The lack of asymmetric polymers could be due to genetically specific intrinsic properties in those fishes with restricted assembly, allowing some tetramers to form more easily than others (Whitt, 1970a). Alternatively, the constraint could be limited to cellular compartmentalization (Hochachka, 1966). The former alternative appears more likely and will be discussed more completely later in this thesis.

On the basis of karyotype, DNA content, and complexity of isozyme content, it has been postulated that many species of the orders Salmoniformes and Cypriniformes have a tetraploid origin (Klose *et al*., 1968; Ohno *et al*., 1968). Thus, they possess at least twice the number of genes coding for LDH subunits. Duplication at the A and B loci has resulted in considerable divergence, providing different amino acid compositions and immunochemical properties for the duplicate loci (Whitt, 1969). As many as five distinct subunits have been reported for members of these orders (Hochachka, 1966). Hence, members of these orders are capable of producing many different random assembly products and exhibit complex LDH zymograms (Hochachka, 1966; Klose _et_ _al_., 1968; Ohno _et_ _al_., 1968; Markert & Holmes, 1969; Whitt, 1969). It has been reported that some salmonid fishes possess as many as 20 different total isozymes in their tissues (Markert & Faulhaber, 1965).

In addition to the A and B loci, advanced fishes possess a third locus, E. This locus codes for a tissue-specific LDH found in only central nervous system tissue, primarily the retina of the eye (Markert & Faulhaber, 1965; Goldberg, 1966; Lush _et_ _al_., 1969; Whitt, 1969; 1970a; 1970b; Whitt & Booth, 1970; Whitt & Horowitz, 1970; Horowitz & Whitt, 1972; Scholl & Holzberg, 1972; Shaklee _et_ _al_., 1973; Baeyens _et_ _al_., 1974). The subunit has been found in only one non-nervous system tissue, the lens of the eye (Whitt, 1969; 1970a; 1970b).

The E gene is activated at the time of retinal differentiation (Whitt & Horowitz, 1970). It appears that it is instrumental in the regeneration of rhodopsin in the photoreceptor cells of the teleost retina. The oxidation of vitamin A to retinene is accomplished by retinol dehydrogenase. This reaction is a rate-limiting process in rhodopsin generation and is dependent upon coenzymes produced from retinal respiration and glycolysis (Whitt, 1970a; Whitt & Booth, 1970). The NAD^+ and NADH balance maintained in the retinal cell is dependent upon the lactate dehydrogenase equilibrium.

E_4 has a high affinity for pyruvate as well as lactate. However, it is susceptible to inhibition by lactate to prevent the accumulation of lactate in the photoreceptor cell. Since a low concentration of lactate is normally present in the cells, the affinity of the E_4 isozyme for lactate might allow efficient use of lactate as an energy source in the retina (Whitt & Booth, 1970).

The E subunit is capable of associating with either A or B subunit and may promote the association of these subunits (Whitt, 1969; 1970a; 1970b). The E locus is confined exclusively to the teleosts and is not found in more primitive fishes (Whitt & Horowitz, 1970; Horowitz & Whitt, 1972).

A further LDH locus in fishes is present in the liver tissue of some teleost species. This C locus has no homologies with the vertebrate LDH-X (Hochachka, 1966; Lush *et al.*, 1969; Markert & Holmes, 1969; Shaklee *et al.*, 1973). The locus has appeared in the gonads of a few fishes (Markert & Holmes, 1969). It has been suggested that the C and E subunits originate at the same locus. These subunits are quite similar in physical properties and never appear together in the same organism (Shaklee *et al.*, 1973).

The Family Cottidae was chosen to determine if tissue-specific isozymes of LDH are present in members of the family. The cottids inhabit different geographic ranges and a variety of niches on the eastern coast of the Pacific Ocean.

The six species of cottids chosen for examination exist in three various habitats. *O. maculosus* and *C. embryum* can exist in the upper littoral zone. Thus, they are presumably capable of tolerance to drastic diurnal and seasonal changes in physical factors imposed by dilution by fresh water runoff, evaporation, atmospheric temperature, and infrequent flooding of this zone with fresh sea water due to tidal change. *O. snyderi* and *S. marmoratus* exist lower in the intertidal zone and are subject to more frequent tidal flooding with sea water. Hence, these species are subject to less variability in physical factors. *L. armatus* and *E. bison* are estuarine inhabitants and are subject to the physical factors imposed by this situation, which are generally more constant than in the littoral situation.

The ability of a fish to modify its metabolic functions to conform to the physical demands of a specific habitat often determines whether the fish is able to exist there. Consequently, one could surmise that fishes with greater metabolic response to various physical parameters would be able to invade more diverse habitats than fishes with a more narrow ability to adapt. Also, one could expect some correlation between the physical factors of a habitat and the adaptability of fishes that permanently exist in that specific habitat.

Beyond conditions imposed by the special requirements of specific tissues, the metabolic adaptability of an organism is often determined by the responsiveness of its various enzyme systems. In the case of LDH, one might be able to predict that ability to adapt to a specific environmental situation is determined by the variety of isozymes present in critical tissues. Behavior of the organism could also be a factor of enzyme adaptability, since the organism must be able to recycle metabolic products with an efficiency related to its activity.

Thus, one might expect those fishes with the most variable physical parameters in their environment to possess the most economical and variable isozymes of LDH. A dominant isozyme could be present to perform the usual metabolic function of a specific tissue. However, accessory isozymes present could serve as a metabolic buffer against rapid environmental changes. These minor forms could provide more rapid and efficient accomodation of metabolism to the problems imposed by the changes until specific enzyme synthesis by the cells could satisfy the requirements. Alternatively, the accessory isozymes conceivably could negate the requirement for further enzyme synthesis by providing a solution to changes that are of a temporal nature, thus providing cellular economy.

Hence, the purpose of this thesis is to determine if these six closely related species of teleost fishes have developed variable tissue-specific isozymes of LDH in response to their particular habitat. It would also be desirable to determine if such responsive generation of LDH isozymes, if present, is predicatable or follows a pattern in its mode of response to specific habitats.

A further objective of this thesis is to determine if the forms of LDH possessed by the cottids are consistent with contemporary theories of teleost phylogeny.

MATERIALS AND METHODS

Experimental Animals

Six species of the teleost Family Cottidae were collected on the Oregon coast. *Oligocottus maculosus* (Girard, 1856), *O. snyderi* (Greeley, 1898), *Clinocottus embryum* (Jordan and Starks, 1895), and *Scorpaenichthyes marmoratus* (Girard, 1854) were collected in South Cove, Cape Arago, and near Coos Head, Charleston, Oregon. *Enophrys bison* (Girard, 1854) and *Leptocottus armatus* (Girard, 1854) (Bolin, 1944) were collected in Newport Bay, Newport, Oregon. The specimens were collected alive and frozen within two hours after capture. Subsequent examination showed that freezing after collection and thawing immediately prior to use had no detrimental effect upon enzyme activity. In fact, the freezing may fracture the cells, facilitating recovery of enzyme during tissue preparation. Samples from frozen tissues showed substantially more activity than those prepared from fresh material.

The experimental animals varied in length from 2.5 cm. for some *C. embryum* and *O. maculosus* to 22 cm. for *E. bison*.

The fact that all individuals were adults was evident since they were sexually mature and possessed ripe or recently spent gonads.

Preparation of the Homogenate

Tissue samples from epaxial muscle, heart muscle, liver, gill, gonad, eye, and brain were excised and minced with scissors. Care was taken to insure that no foreign tissue was included with the specific tissue in each sample. Sample weight was usually 0.4-1.0 g. Further homogenation with sand was performed with mortor and pestle on ice, in cold 0.1 M. Tris-HCl buffer, pH 7.0. Grinding was interrupted for 2 minutes at 1 minute intervals to insure that the tissue being homogenized did not overheat from friction. The sample was kept on ice at all times possible during tissue preparation. After being ground until homogeneous, the sample was usually diluted using 1 g. of tissue to 5 ml. buffer. However, in the case of heart muscle in some of the smaller cottids, a 1:20 dilution was necessary due to the small amount of this tissue obtainable. Dilution was initially performed with 0.1 M. Tris-HCl buffer, pH 7.0. If any further dilution was necessary, it was done with glass distilled water.

Following homogenation, the tissue was spun in a

Sorvall RC2-B refrigerated centrifuge using the SS-34 head for 1 hour at 3-5° C. at 20,000 x g. The supernatant was decanted and spun again under the same conditions for 30 minutes. Following this procedure, the sample was examined to insure clarity. Debris in the sample causes it to leave streaks in the gel after electrophoresis. Debris with enzyme attached cannot migrate as fast as the remainder of the sample in the gel but will still absorb stain, creating a streak.

Horizontal Starch Gel Electrophoresis

In all cases electrophoresis was performed horizontally on a 12.5% starch gel using the method of Smithies (1955). Hydrolyzed starch was obtained through Connaught Laboratories, Toronto, Canada. All starch used was taken from the same chemical lot to insure uniformity of hydrolysis. The starch was diluted to 100 ml. using the continuous buffer system of Markert and Faulhaber (1955).

Three buffer systems were examined in electrophoresis including a Tris-citrate buffer, pH 7.0; Borate-LiOH, pH 8.1; and finally the Tris-Borate-EDTA system of Markert and Faulhaber mentioned above. The later provided the best results and was routinely used for all experiments. Laboratory stock buffer was prepared using 0.9 M. Tris, 0.02 M. EDTA, and 0.5 M. Boric acid. A 1:7 dilution was used in the anodal vessel, 1:5 in the cathodal vessel, and a 1:20 dilution for the starch solution. All dilutions were made with distilled water.

The starch gel was prepared by heating the starch solution over an open flame. A constant swirling motion was maintained to insure that the starch did not char. When the starch solution attained the appropriate viscosity and approached a boil, it was removed from the flame (Smith, 1968). The solution was then degassed with an aspirator for 20 seconds to insure a bubble-free gel. The degassing time was kept constant to insure that all gels were consistent in their moisture content. The solution was then poured into a plexiglas mold pre-greased with silicon stopcock grease and immediately covered. It was then allowed to cool for 2 hours at room temperature. The gel remained covered at all times to prevent desiccation.

A sample was introduced into the gel using a 3 mm. square wick of Whatman #3 filter paper. The paper wick was immersed in a sample and pushed into a pre-cut slit in the gel. Care was taken to insure that no excess sample remained on the surface of the gel, that the wick did not touch the bottom of the gel, and that no air bubbles were trapped in the slit with the sample.

The gel plate was placed above the anodal and cathodal chambers containing buffer. The gel was connected to these chambers with wicks made of 4 thicknesses of Whatman #1 filter paper. Ag/AgCl electrodes supplied electricity from a Heathkit Regulated H.V. Power Supply, Model IP-17, to the buffer tanks.

Electrophoresis was performed for either 12 or 18 hours at 300 Volts and 4 mA. with the 5 x 20 cm. gel. In the cases where the 10 x 20 cm. gel was used, the voltage was 250 at 5 mA. All operations were performed at 4° C. in a refrigerator. Preliminary trials showed that 12 hours was the optimum time for electrophoresis. The tissue-specific eye band migrated to the anode so rapidly that longer periods of time pulled the band off the end of the gel.

Staining Procedure

In all cases the following staining mixture was used (Smith, 1968):

NAD^+	66 mg.
NBT	35 mg.
PMS	2 mg.
1 M. Na-lactate	10 ml.
0.5 M. Tris-HCl, pH 7.0	10 ml.
Distilled water	80 ml.

NAD^+, nitro blue tetrazolium (NBT), and phenazine methosulphate (PMS) were obtained from Sigma Chemcial Company, St. Louis, Missouri. Na-lactate was prepared by mixing 10.6 ml. 85% lactic acid, 49 ml. 1 M. $Na_2CO_3 \cdot H_2O$, and brought to 100 ml. total with distilled water. After preparation, the stain was kept in the dark to insure retention of full activity.

After electrophoresis was terminated, the filter paper sample wicks were removed. The gel was sliced longitudinally with a thin wire and each half placed in a bath of the staining solution. The sample was then incubated for $1\frac{1}{2}$ to 3 hours in the dark at 37^{o} C. Tests showed the optimum staining time to be 2 hours. Since the isozymes probably were not present in equal concentrations in a given tissue, and because differences exist in activity between individual isozymes, the more active forms of the enzyme might be optimally stained while the weaker forms were still absorbing stain (Wilson et al., 1973). Therefore, care was taken to insure that incubation was long enough to show all the bands present but not so long that the bands were obscured through overstaining.

One half of the gel was stained in the staining mixture without the Na-lactate substrate. Previous reports have shown that a reaction called 'nothing dehydrogenase' sometimes results from activation of alcohol dehydrogenase by the staining mixture and subsequent stain absorption.

Staining without the substrate eliminates all of the bands actually due to LDH. Thus, any bands remaining could be attributed to a 'nothing' reaction (Smith, 1968; Schwantes, 1973).

Following incubation, the gels were rinsed free of stain and immediately photographed, since the stain is light sensitive. Measurements were then made of the various bands present and their relative migration in the gel.

Heat Denaturation

A sample was submerged in a water bath at $65^{\circ} \pm 0.5^{\circ}$ C. for the duration of each individual experiment. At various intervals an aliquot was withdrawn with a pipette after the sample was agitated slightly to stir the agglutinating proteins. Thus withdrawn, the sample was placed on a filter paper wick and introduced into the gel as previously described. Electrophoresis was then performed in the 10 x 20 cm. gel for 10 or 12 hours at 4° C. at 250 Volts and 5 mA.

Hybridization Experiments

Tissue samples from the epaxial muscle, heart muscle, and eye of *L. armatus* were prepared as previously described. These samples were added to the 10 x 20 cm. gel and electrophoresis was performed for 12 hours at 250 Volts and 5 mA. After the gel was sliced longitudinally only one half was stained. The portions required for this part of the experiment were excised from the unstained half of the gel, crushed, recentrifuged, and used as a source of purified sample. The relative locations of the isozymes required were determined by measuring the bands that appeared in the stained half of the gel and finding their location in the unstained half. One can expect a 40-fold purification in the samples if they are prepared in this manner (Hochachka, 1965).

These purified samples were combined as shown in the appropriate section of this thesis, diluted with an equal volume of 0.5 M. NaCl, and slowly frozen. The samples were later thawed slowly and simultaneously dialyzed against distilled water to remove the salt. Each sample was then subjected to electrophoresis for 12 hours in the 10 x 20 cm. gel as described.

RESULTS

Lactate Dehydrogenase Zymograms

Figures 1 through 3 represent lactate dehydrogenase electrophoretic zymograms of the six species of cottid fishes. Examination reveals a common feature in all cases. There is an extremely prominent band present in all of the tissues in all species. The most striking observation to be made is that this obvious band is the only one present in epaxial muscle, heart muscle, liver, gill, and gonad in five of the species. L. armatus is the exception, since it exhibits minor bands in epaxial and heart muscle. However, L. armatus possesses the prominent band mentioned as the major one in its heart and epaxial muscle. In all species the single band is most prominent in epaxial muscle when an equal quantity of each tissue is used.

With the exception of S. marmoratus the major band observed shows the same maximum anodal migration of 3.7 cm. under the experimental conditions described in the Materials and Methods section. S. marmoratus shows a maximum migration of 2.1 cm. in all five mentioned tissues.

Experimental artifact should not be considered in the case of S. marmoratus for the following reason. To eliminate the influence of discrepencies in gel consistency, variable voltage, and other factors that might influence speed or distance of migration, O. maculosus epaxial muscle was electrophoresed as a marker with each sample. The sample then could be compared with the band of O. maculosus, and any appropriate compensations could be made. In the case of S. marmoratus, its bands are clearly cathodal to those of O. maculosus.

The prominent band mentioned is also present in eye and brain tissues of all species. However, these tissues exhibit a variable array of bands that appears to be species-specific as well as tissue-specific. The number of bands in eye tissue varies from five (E. bison) to eleven (L. armatus), and the positions of these bands show considerable variation between species. The only exception is the band common to epaxial muscle and other tissues, which remains constant.

Brain tissue in all cases exhibits fewer bands than eye tissue. The bands that are observed are common to the few most cathodal bands of eye tissue.

The eye samples always exhibit a prominent band in the most anodal position. A similar band with this electrophoretic mobility has been observed previously in teleost fishes. It has been proved to be an eye-specific band, occasionally being present in the brain tissue (Whitt, 1969; 1970a; 1970b; Whitt & Horowitz, 1970; Horowitz & Whitt, 1972). The eye-specific band never appears in brain tissue in these species, even though brain tissue does exhibit a few bands common to eye tissue.

L. armatus is unique in that it is the only species examined that reveals bands cathodal to the prominent epaxial muscle band mentioned previously. The cathodal bands appear in eye, brain, and epaxial muscle. There are four bands in eye and brain, and three in muscle that are cathodal to the prominent band.

L. armatus is again unique in the fact that it shows more than one band in heart tissue. There are two bands anodal to the prominent band which have apparent complementary bands in eye and brain tissue of the same organism.

Heat Denaturation

Figures 4 and 5 diagrammatically demonstrate the effect of exposure to 65° C. for increasing durations upon epaxial muscle and eye lactate dehydrogenase samples from *L. armatus*. Figure 4 could also appropriately represent the heat denaturation of LDH from heart tissue.

65° C. was chosen as a standard temperature for heat denaturation experiments since most proteins denature, and subsequently lose activity, above 45^{o} C. (Dixon & Webb, 1964). The relative heat lability of a specific protein is a physical property of that protein. In this experiment this property is exploited as a means to identify the LDH subunits present in the sample.

After each exposure period, an aliquot was removed from the sample and introduced into the starch gel. Therefore, following electrophoresis, it is possible to observe a serial heat denaturation of the enzyme and make deductions about its heat lability.

Epaxial muscle lactate dehydrogenase remains stable for one minute. However, at two minutes duration a marked decrease in staining intensity is noted, demonstrating a corresponding decrease in enzyme activity. The intensity of the LDH band declines rapidly until it is barely visible at 12 minutes duration. At 17 minutes the band is entirely gone, representing a complete loss of activity between 12 and 17 minutes from the time of immersion in the 65^{o} C. bath.

The presumed identity of the prominent epaxial muscle band appears on the right of the zymogram in Figure 4. Reasons for this choice will be discussed later in this thesis.

Figure 5 represents the heat denaturation of lactate dehydrogenases of the eye tissue of L. armatus. Presumed identities of the major isozymes present are included on the right of the figure. The reasons for these choices will be discussed later. Their inclusion in Figure 5 is to facilitate discussion of the zymograms.

The prominent band marked A_4 occupies the same position in the zymogram as the prominent band present in epaxial muscle. It also demonstrates the identical heat denaturation time of 12-17 minutes.

In the two most cathodal bands the enzyme remains sufficiently active to absorb stain at the full duration of the experiment. The band labelled E_4, as well as the band immediately anodal to A_4, demonstrates a similar heat resistance. The remaining seven bands exhibit heat lability generally intermediate to the A_4 band and the bands mentioned above.

Hybridization

The hybridization experiments, as compiled in Figure 6, were performed in a further attempt to identify the LDH isozymes present. When purified enzymes from heart and epaxial muscle are hybridized, only one band is present in the resulting zymogram. If heart, epaxial muscle, and E_4 region of the eye zymogram are combined, only three bands appear. The middle band of these three is often quite wide and diffuse.

If the E_4 region from eye tissue is combined with enzyme from epaxial muscle, a zymogram similar to the one described above results. Hybridization of E_4 from eye, A_4 from epaxial muscle, and the region labelled B_4 on Figure 5, was attempted several times. The result was always variable with a diffuse staining area covering most of the zymogram. However, the A_4 and E_4 bands remained well resolved in all hybridizations.

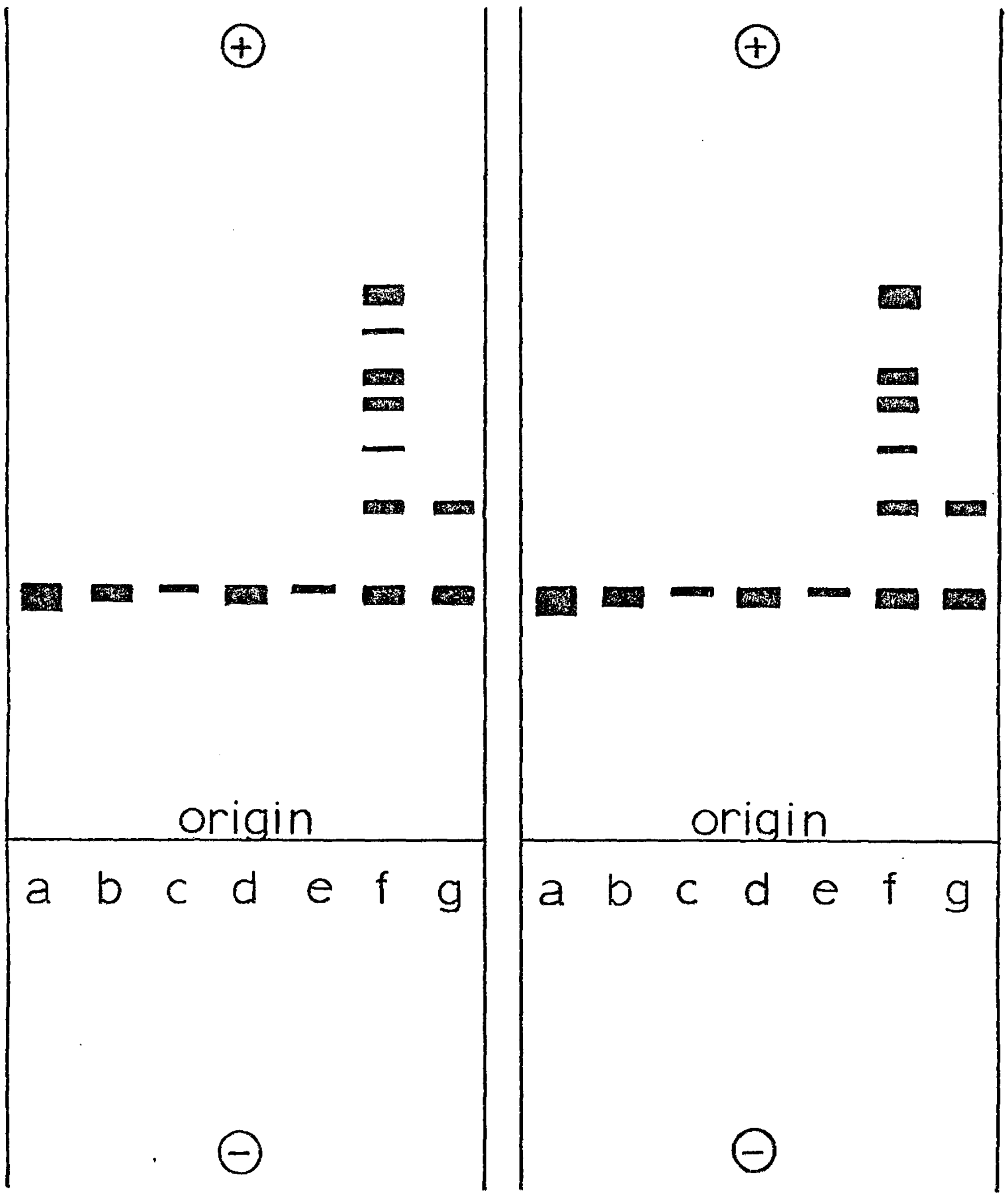

FIGURE 1

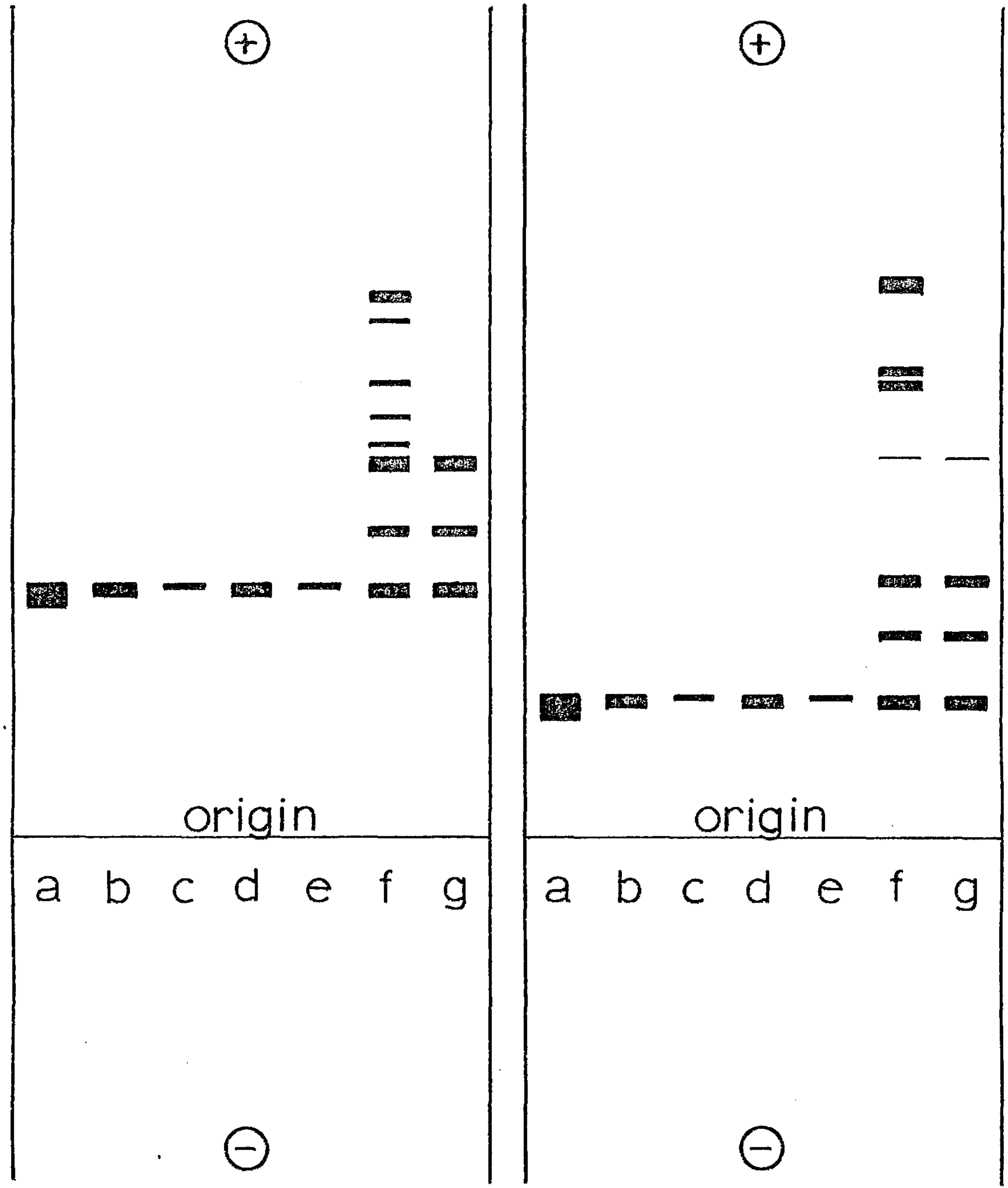

FIGURE 2

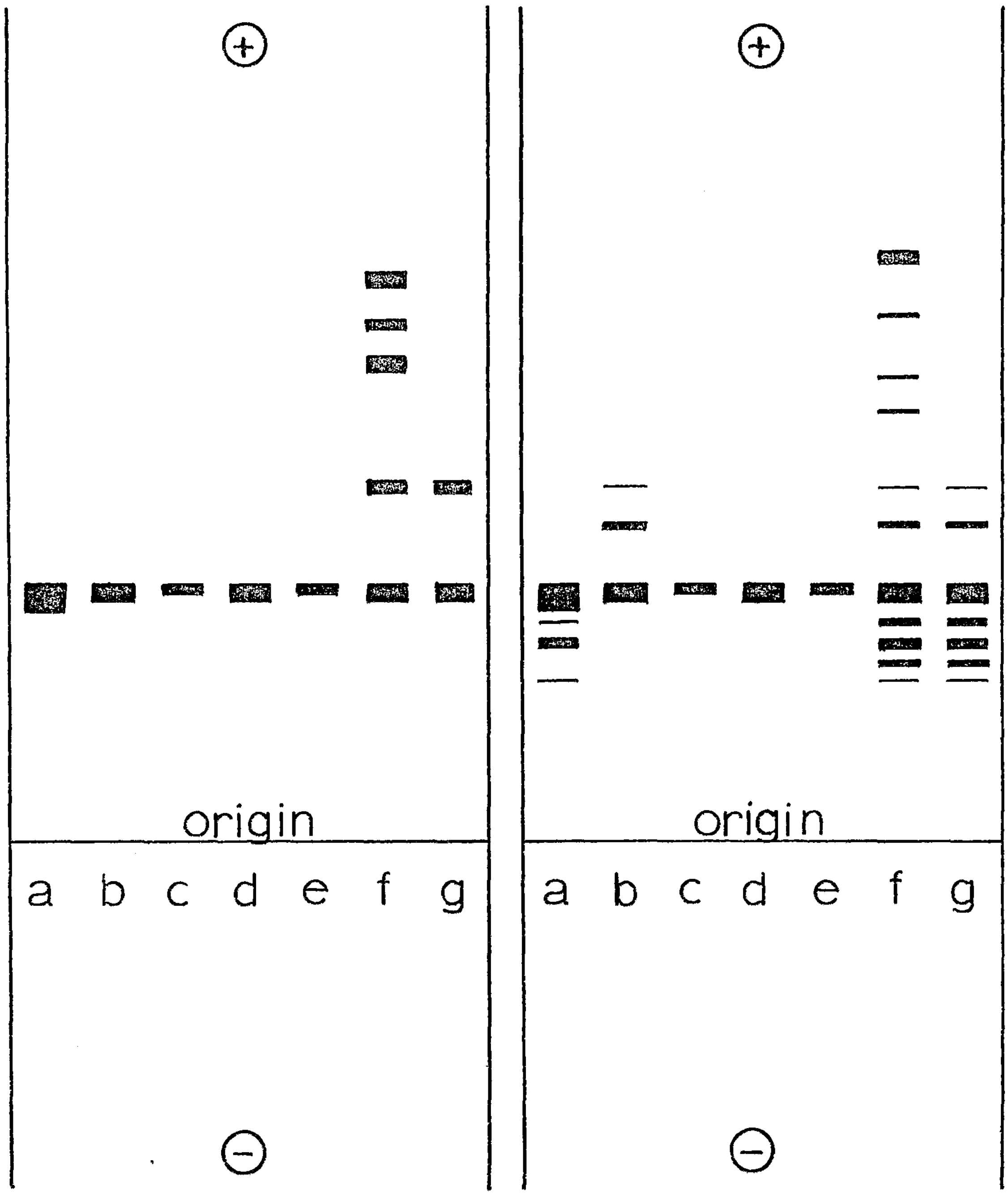

FIGURE 3

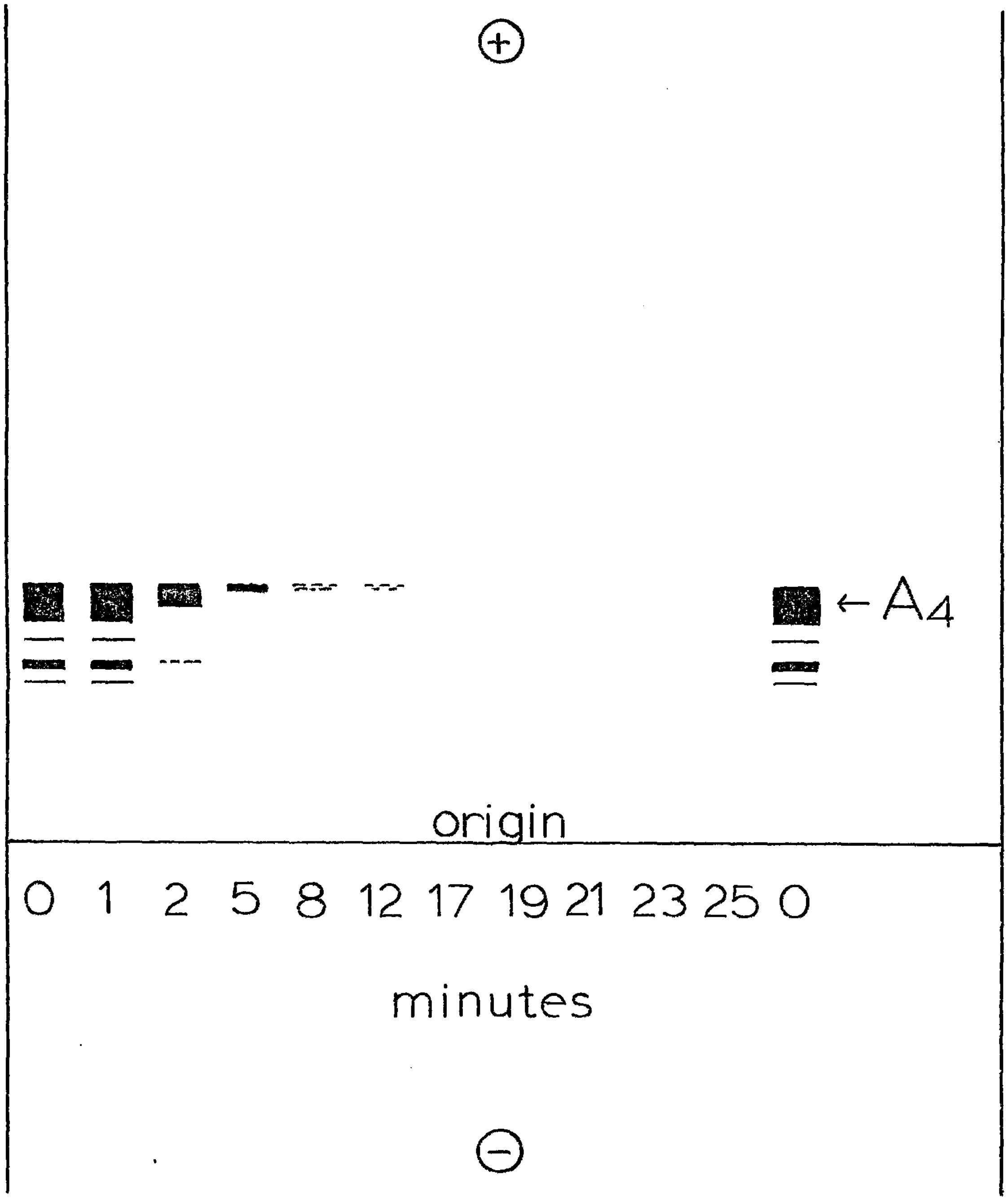

FIGURE 4

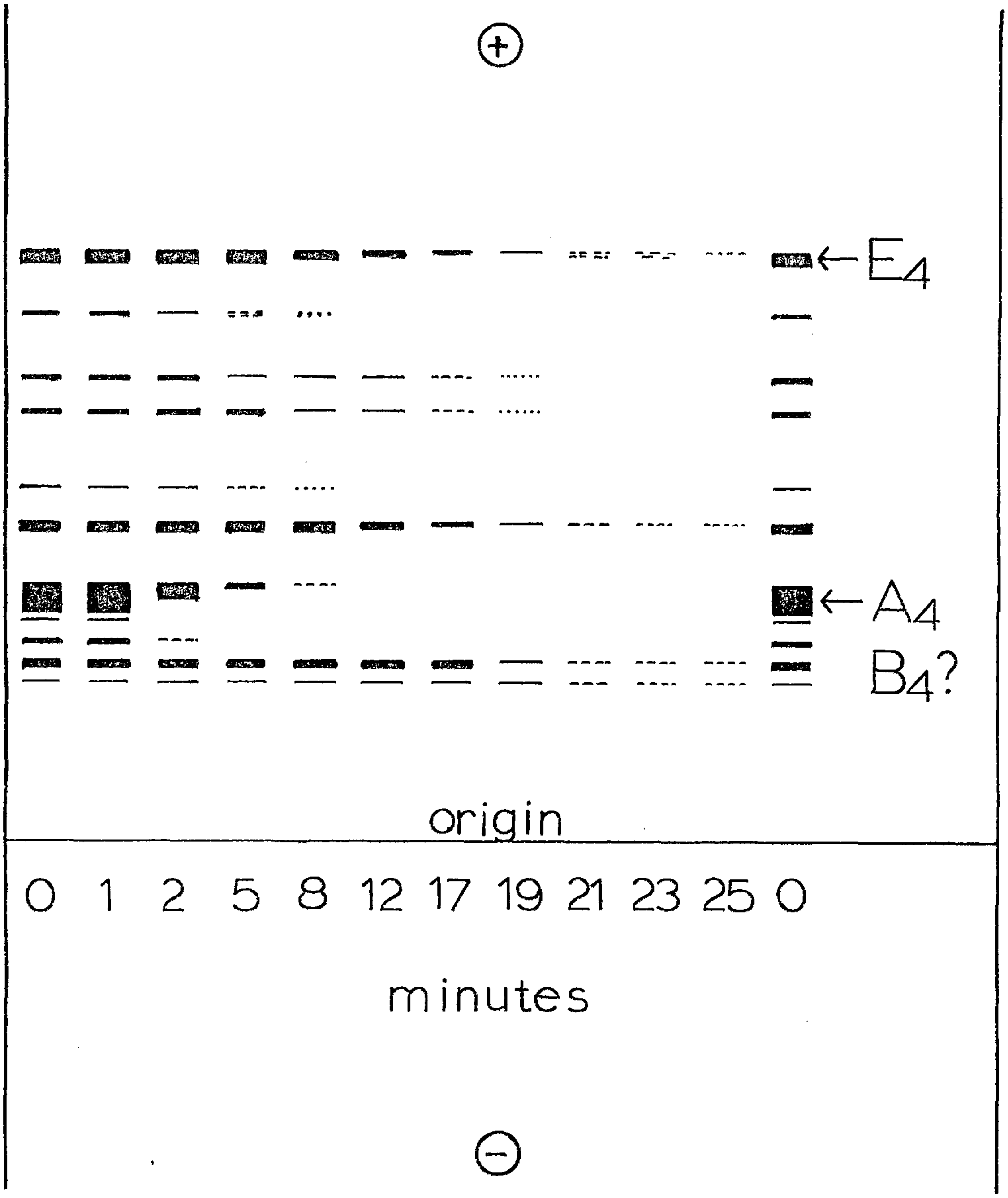
⊕
$\leftarrow E_4$
$\leftarrow A_4$
B_4?
origin
0 1 2 5 8 12 17 19 21 23 25 0
minutes
⊖

FIGURE 5

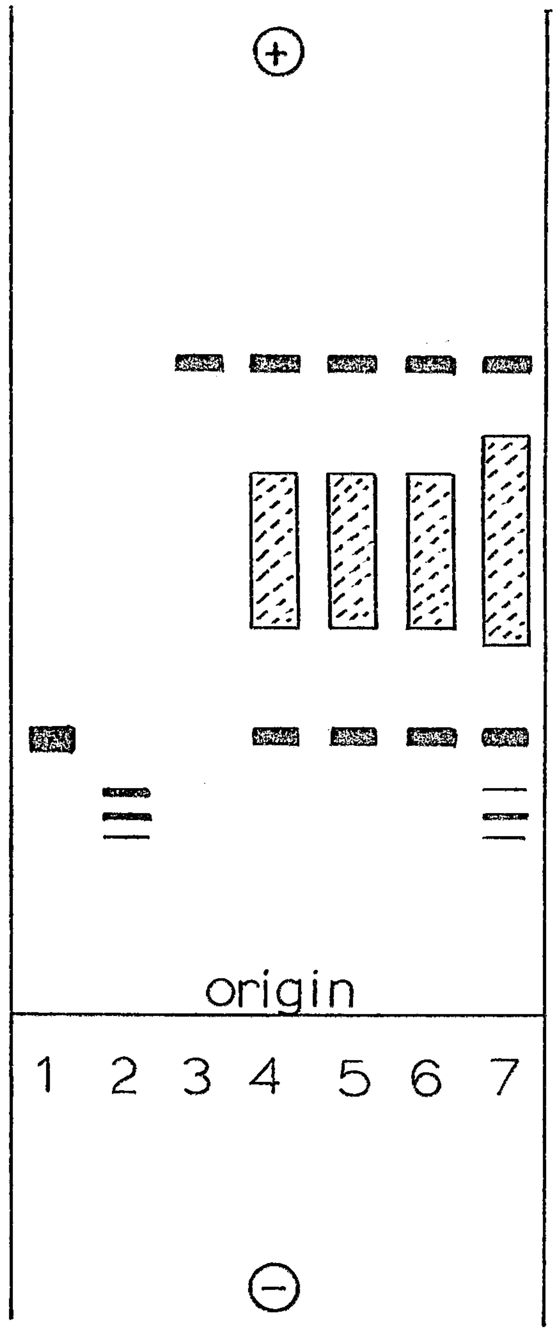

FIGURE 6

DISCUSSION

The zymograms presented in Figures 1 through 3 demonstrate that only one band of LDH appears electrophoretically in all tissues, except eye and brain, in most of the cottid species examined. *L. armatus* is the exception, possessing minor bands in heart and epaxial muscle tissue.

The prominent band of LDH found in heart and epaxial muscle was subsequently subjected to heat denaturation and hybridization experiments. These experiments were performed to determine if the LDH observed in both heart and epaxial muscle represents the LDH isozyme present. It has been shown that the A_4 tetramer is the most heat labile LDH isozyme, while E_4 is the most heat resistant. The B_4 isozyme occupies an intermediate position, more similar to the E_4 isozyme than A_4 (Vesell & Yielding, 1966; Vesell *et al.*, 1968; Whitt, 1969).

The progress of heat denaturation, as depicted in Figure 4, demonstrates that the LDH extracted from both heart and epaxial muscle shows simultaneous and relatively rapid heat denaturation when exposed to 65° C. This simultaneous heat denaturation demonstrates a physical property common to both LDH's. It is unlikely that another isozyme has a coincident electrophoretic mobility in these tissues. If this were the case, then an interrupted or variable heat denaturation would be evident.

The prominent band in heart and skeletal muscle is further shown to be distinct and common to both tissues by molecular hybridization. Uniform results were obtained when the band from skeletal muscle or heart tissue was hybridized with the anodal band in eye tissue, presumed to represent the E subunit.

In general, the A and B subunits are capable of hybridizing _in vivo_ to form heteropolymers. Usually only the symmetric heteropolymer A_2B_2 is formed. However, asymmetric heteropolymers are occasionally formed. If the A and B subunits are prevented from hybridizing _in vivo_ due to some molecular constraint, it has been demonstrated that addition of E subunit will promote the formation of a series of heteropolymers _in vitro_ (Whitt, 1969; 1970a; 1970b; Whitt & Booth, 1970). Whitt (1970a) has suggested that the E subunit does not merely prevent the interaction of A and B subunits by spatially separating them during assembly, since X-ray data has shown that LDH exists as a tetrahedron and that all subunits are in intimate contact. The action of the E subunit might rather be analogous to the modification of polypeptide subunits observed for interallelic complementation, where one subunit corrects a misfolding in another. During polymer formation, the affinity for like, is greater than between unlike subunits. E exerts its effect by changing the conformation of any subunits to which it binds, or by changing the conformation of the EA or EB dimer to broaden its capacity to combine with other dimers. In other words, it removes the conformational barrier between the combination of A and B subunits. B cannot bind to A, but E, closely related to B, is able to bind to A subunit (Whitt, 1969; 1970a; Shaklee et al., 1973).

Hence, the uniform results obtained when the prominent band of heart or epaxial muscle tissue is hybridized with the E subunit support the postulate that one similar isozyme is present in both tissues. The electrophoretic mobility and heat denaturation provide additional evidence. The data also suggests that this isozyme contains predominantly A subunits and that it is most likely the homotetramer, A_4. Similar electrophoretic mobilities of the single bands in liver, gill, gonad, and the prominent band in the same location in eye and brain, would suggest that these are the same A_4 isozyme.

Eye and brain tissues present a more complex series of isozymes. The most rapidly migrating anodal band found in all 6 species of cottids in eye tissue appears similar in properties to the E subunit reported for many teleost fishes (Markert & Faulhaber, 1965; Whitt, 1969; 1970a; 1970b; Whitt & Horowitz, 1970; Horowitz & Whitt, 1970). It demonstrates more heat resistance to 65° C. than the A_4 band. The bands progressively more cathodal from this E_4 band probably represent heteropolymers between E, A, and B subunits.

The A_4 subunit also appears in the eye and brain zymogram. In the case of <u>L. armatus</u> a series of four bands is noted cathodal to the A_4 band. The heat resistant characteristics of these bands suggest that they are of B subunit origin. However, the evidence is not conclusive enough to verify this assumption. If a B subunit does indeed exist, it exhibits an electrophoretic mobility reversed from that of the normal vertebrate B subunit. Usually B and the heteropolymers richest in this subunit are more anodal than those isozymes containing mostly A subunit, indicating that B is more electronegative than A. This reversed situation, where the A subunit is more electronegative than B, has been noted in other fishes (Markert & Holmes, 1969; Whitt, 1969; 1970a). However, reversal of the relative mobility of the A and B subunits does not change their other physical properties (Whitt, 1969; 1970a). In addition, neither E_4 nor A_4 is shifted in mobility if the B subunit mutates to change its electronegativity and thus its mobility (Whitt, 1970a).

It is interesting to note that if B subunit is indeed present in cottid fishes, L. armatus is the only one investigated that demonstrates this apparent reversed mobility. The other five species show no bands cathodal to the prominent A_4 isozyme in the tissues examined.

A conclusion that can be presented at this time is that the eye zymogram contains at least three subunit types in all cottids. Five is the maximum number of permutations when two subunits assemble to tetramers. With three types of subunits up to 15 isozymes are possible. The presence of five to eleven isozymes in eye tissue of the six cottids examined supports this conclusion.

The E subunit is not found in brain tissue in any cottid examined. It has been found in mackeral brain, usually in those portions involved in eye function (Whitt, 1970a). The optic nerve was often included in brain sample as an artifact of dissection. From the absence of the E_4 band in brain tissue, one could assume that the E subunit is absent from the optic nerve.

Thus, it can be assumed that the cottids possess at least three subunit types assembling to form tetramers of LDH. These subunits form tissue-specific and species-specific distributions only in the eye and brain tissues of the six species of cottids examined. Several questions are immediately appropriate. Is the B subunit synthesized in tissues other than eye and brain? Are the LDH isozymes present in the tissues influenced by the physical factors of the environment of the animals? Do the LDH complements of each cottid species determine their ability to adapt and inhabit a variety of environments? What evolutionary implications do the LDH isozymes of cottids present?

It would be difficult to discover any correlations between environment and the LDH isozymes exhibited by the cottids that were examined. One would expect that the variety of isozymes present in a species would vary as a function of the conditions imposed by the particular habitat of that species. As a function of cellular economy and adaptability, superfluous isozymes would have been eliminated by selective pressures upon that organism. Therefore, one could expect that the species subject to the harshest or most variable environment would have the most extensive complement of LDH isozymes.

However, this assumption is not appropriate, since isozyme number appears to vary in a haphazard fashion. L. armatus exhibits the highest number of LDH isozymes found in the six species that were examined, but it has a narrower tolerance to salinity and temperature than some cottids (Morris, 1961). The remaining five species demonstrate a variable number of isozymes apparently not related to their habitats.

One perplexing observation is that only one major isozyme, A_4, exists in both heart and skeletal muscle. Only one other group of fishes has been discovered with a similar genetic expression of LDH isozymes, the flatfishes, or Order Heterosomata (Markert & Faulhaber, 1965; Lush et al., 1969; Markert & Holmes, 1969). Members of both of these groups seem to be inconsistent with the generalization that since heart and skeletal muscle have different metabolic requirements, they should possess at least two different LDH isozymes with different catalytic properties. One could speculate that the presence of a single isozyme with the kinetic properties of A_4 in the heart would be injurious to heart cells. The typical A_4 isozyme is not inhibited by high lactate concentrations. Hence, an accumulation of lactate in the heart would be possible. However, the heart is under the direct influence of lactate formed in the skeletal muscles due to the supply of venous blood and its serial position in the circulatory system (Dando, 1969). Dando has shown that blood lactate values can reach quite high levels for comparatively long periods of time. The cardiac cells of fishes are under low pO_2 due to the single circulation fed by lacunary blood directed from the consumption site to the oxygenation site (Gesser & Poupa, 1973). Hence, in these fishes the selection for a preponderance of A subunits in the heart would appear physiologically sound, due to low pO_2 plus the presence of high concentrations of lactate in the cardiac cells.

The genetic basis for both A and B subunits exists in the flatfishes (Lush _et_ _al_., 1969; Markert & Holmes, 1969) and apparently exists in the cottids. Thus, it would appear that a physiological basis for some selection for the A subunit in cardiac tissue is inherent.

The presence of a relatively large number of isozymes in eye tissue is accountable. The E subunit operates optimally more aerobically than the B subunit. It is thus responsive to the conditions in the eye, since the vertebrate eye is one of the most aerobic tissues (Baeyens _et_ _al_., 1974). E_4 is also inhibited by accumulating lactate, thus preventing lactate damage to the eye. The additional isozymes could exist as a buffer to protect the eye from sudden environmental change, or to accomodate response to changes in concentration of metabolities coming in the blood. For these reasons, a greater number of isozymes in the eye tissue would have a selective advantage. Future investigations into the teleost visual system might provide additional insight into the evolution of a tissue-specific E locus of LDH.

The A locus of LDH is believed to be the ancestral gene. Initially, this gene governed the synthesis of a protein capable of aggregating to the single homotetrameric form of the enzyme. Later, gene duplication through an initial tetraploidization and gradual divergence, resulted in two forms of the enzyme (Markert & Faulhaber, 1965; Ohno et al., 1968). This event transpired sometime prior to the emergence of fish-like vertebrates. Studies reveal that even the most primitive of the fishes, representing the jawless state of vertebrate evolution, is endowed with two separate gene loci for LDH (Klose et al., 1968; Onho et al., 1968).

During the mid-Triassic, the teleosts began to successfully compete with the fresh water Holosteans. They then underwent adaptive radiation in both fresh and salt water. The E gene probably arose prior to this event, since it is present in both fresh and salt water forms of fishes. The E gene probably arose through a duplication of the B gene and underwent subsequent divergence until the forms were unique, although similar. The E locus is found only in teleostean fishes, which suggests that it may have arisen after fish gave rise to amphibians (Whitt, 1969; Whitt & Horowitz, 1970).

Greenwood et al. (1966), postulate that teleostean evolution is polyphyletic with three main stems. Division

III gives rise to the Superorder Acanthopterygii, to which the Orders Scorpeniformes, Perciformes, and Heterosomata, among others, belong. The LDH gene products in the Acanthopterygii are quite variable. Some members of the Order Perciformes lack an E gene, while the majority possess the gene. The Heterosomata are a Perciformes derivative and generally lack the B subunit in aerobic tissues, whereas the majority of the Perciformes does not (Markert & Faulhaber, 1965; Lush et al., 1969; Markert & Holmes, 1969; Whitt & Horowitz, 1970).

Although evidence is scant for the Order Scorpaeniformes, one can presume that at least the Family Cottidae presents a variety of expression of LDH genes. The cottids appear to be following the advanced teleost trend in possessing three distinct loci for LDH production. In this sense their inclusion as an advanced teleost form is justifiable.

At the time of gene duplication, merely a redundancy is created. This redundant gene could confer immediate selective advantage upon the organism. If not, the locus would drift freely, acquiring numerous point mutations. As long as the essential or active sites remain intact, the enzyme will retain its functional benefit. If function is lost, the locus will degenerate as the organism selects for cellular economy.

In higher vertebrates it would appear that as the two LDH subunits diverged, they retained an almost constant affinity for each other. An infinite affinity between subunits would be a strong selective advantage. Each somatic tissue could exploit this property by producing A and B subunits in varying amounts to generate the optimum LDH complement for that specific tissue. Equal A and B production would result in a 1:4:6:4:1 ratio for the five subunits by a binomial distribution. It follows that a 4A:1B ratio of production would result in a 256:256:64:16:1 ratio. Hence, it is possible to establish tissue-specific LDH patterns (Ohno et al., 1968).

In the case of the flatfishes and cottids, it could be argued that the genetic drift of the LDH A and B genes has progressed to the point where the subunit affinities no longer exist. However, A and B heteropolymers are evident in neural tissue. Also, the autotetramers A_4 and B_4 are still formed, and the B_4 tetramer is not evident in all tissues except eye and brain.

The B gene exists in all tissues, since they have a common genetic complement. However, if a modulator allowed a preponderance of A subunit synthesis in all tissues except neural, even an infinite affinity between A and B subunits would result in few tetramers containing B subunits. These B-containing tetramers could represent such a small activity that their presence might remain undetected by experimental methods.

Another postulate is that the B gene function could have been secondarily lost as a response to some environmental pressure.

A final postulate is that perhaps temperature-sensitive forms of the enzyme exist which function at specific physiological temperatures. It is possible that temperature could release a cofactor or metabolic intermediate from an allosteric binding site, thus allowing the enzyme to function (Vesell & Yielding, 1966).

Hence, it can be proposed that the loss of the influence of B subunits in aerobic tissues represents a response to an unique environmental situation. It can be further postulated that the lifestyle of cottids, and perhaps flatfishes, allows them to take advantage of a more efficient mode of eradicating lactate accumulations. The accumulation of blood lactate, coupled with a low pO_2, can be efficiently neutralized in all tissues, including aerobic tissues, if a preponderance of A subunits is produced.

It is inconceivable that a system such as the one proposed above could function in fishes exhibiting an active existence. The rapid accumulation of lactate from anaerobic glycolysis in epaxial muscle would be deleterious to aerobic tissues by causing extensive product inhibition. However, the cottids, as well as flatfishes, lead a relatively sedentary existence. Their muscular activity is limited to brief bursts to escape predators or to capture prey. Usually the presence of B-containing tetramers is essential in the conversion of lactate to pyruvate rapidly to prevent fatigue. However, the rapid fatigue associated with such a system lacking B subunits would be of little consequence, since muscular activity of these fishes is limited.

Hopefully, further examination of the kinetic properties of the cottid LDH isozymes will clarify the applicability of the above proposed scheme for subunit adaption to function.

BIBLIOGRAPHY

AGASTONI A., VERGANI C. & VILLA L. (1966) Intracellular distribution of the different forms of lactic dehydrogenase. Nature 209, 1024-1025.

ALLISON W. S. (1966) Structure and evolution of triose phosphate and lactate dehydrogenases. Ann. N. Y. Acad. Sci. 151, 180-189.

BAEYENS D. A., HOFFERT J. R. & FROMM P. O. (1974) A comparative study of the influence of oxygen on lactate dehydrogenase. Comp. Biochem. Physiol. 47B, 1-7.

BAILEY G. S. & WILSON. A. C. (1968) Homologies between isozymes of fish and those of higher vertebrates: evidence for multiple H_4 LDH in trout. J. Biol. Chem. 243, 5843-5853.

BALDWIN J. & HOCHACHKA P. W. (1970) Functional significance of isozymes in thermal acclimation. Acetylcholinesterase from trout brain. Biochem J. 116, 883-887.

BALDWIN J. & TEMPLE-SMITH P. (1973) Distribution of LDH-X in mammals: presence in marsupials and absence in the monotremes Platypus and Echidna. Comp. Biochem. Physiol. 46B, 805-811.

BLANCO A. & ZINKHAM W. H. (1963) Lactate dehydrogenase in human testes. Science 139, 601-602.

BOLIN R. L. (1944) A review of the marine cottid fishes of California. Stanford Ichth. Bull. 3, 1-135.

CAHN R. D., KAPLAN N. O., LEVINE L. & ZWILLING E. (1962) Nature and development of lactic dehydrogenases. Science 136, 962-969.

CLAUSEN J. & HUSTRULID R. (1968) Factors affecting recombination of lactate dehydrogenase isoenzyme subunits. Biochim. Biophys. Acta 167, 221-226.

CLAYTON J. W. & GEE J. H. (1969) Lactate dehydrogenase isozymes in longnose and blacknose dace (Rhinichthys cataractae and R. atratulus) and their hybrid. J. Fish. Res. Bd. Canada 26, 3049-3053.

CRICK F. H. C. & ORGEL L. E. (1964) The theory of interallelic complementation. J. Mol. Biol. 8, 161-165.

DANDO P. R. (1969) Lactate metabolism in fish. J. Mar. Biol. Ass. U. K. 49, 209-223.

DAS A. B. & PROSSER C. L. (1967) Biochemical changes in tissue of goldfish acclimated to high and low temperatures. 1. Protein synthesis. Comp. Biochem. Physiol. 21, 449-467.

DI SABATO G., PESCE A. & KAPLAN N. O. (1963) The sulfhydryl content of some lactate dehydrogenases. Biochim. Biophys. Acta 77, 135-138.

DIXON M. & WEBB E. C. (1964) Enzymes; Academic Press, New York.

EVERSE J., BERGER R. L. & KAPLAN N. O. (1970) Physiological concentrations of lactate dehydrogenases and substrate inhibition. Science 168, 1236-1238.

FONDY T. P., EVERSE J., DRISCOLL G. A., CASTILLO F., SOLZENBACH F. E. & KAPLAN N. O. (1965) The comparative enzymology of lactic dehydrogenases. IV. Function of sulfhydryl groups in lactic dehydrogenases and the sequence around the essential group. J. Biol. Chem. 240, 4219-4234.

GESSER H. & POUPA O. (1973) The lactate dehydrogenase system in the heart and skeletal muscle of fish: a comparative study. Comp. Biochem. Physiol. 46B, 683-690.

GOLDBERG E. (1963) Lactic and malic dehydrogenases in human spermatozoa. Science 139, 602-603.

GOLDBERG E. (1966) Lactate dehydrogenase of trout: hybridization in vivo and in vitro. Science 151, 1091-1093.

GOLDBERG E. (1973) Molecular basis for multiple forms of LDH-X. J. Exp. Zool. 186, 273-278.

GOLDBERG E., CUERRIER J. P. & WARD J. C. (1969) Lactate dehydrogenase ontogeny, parental gene activation, and tetramer assembly in embryos of brook trout, lake trout, and their hybrids. Biochem. Genet. 2, 335-350.

GOSLINE W. A. (1965) Teleostean phylogeny. Copeia 2, 186-194.

GREENWOOD P. H., ROSEN D. E., WEITZMAN S. H. & MYERS G. S. (1966) Phyletic studies of teleostean fishes with a provisional classification of living forms. Bull. Amer. Mus. Nat. Hist. 131, 339-456.

HITZEROTH H., KLOSE J., OHNO S. & WOLF U. (1968) Asynchronous activation of parental alleles at the tissue-specific gene loci observed on hybrid trout during early development. Biochem. Genet. 1, 287-300.

HOCHACHKA P. W. (1965) Isoenzymes in metabolic adaptation of a poikilotherm: subunit relationships in lactic dehydrogenases of goldfish. Arch. Biochem. Biophys. 111, 96-103.

HOCHACHKA P. W. (1966) Lactate dehydrogenase in poikilotherms: definition of a complex isozyme system. Comp. Biochem. Physiol. 18, 261-269.
HOCHACHKA P. W. & SOMERO G. N. (1968) The adaptation of enzymes to temperature. Comp. Biochem. Physiol. 27, 659-668.
HOROWITZ J. J. & WHITT G. S. (1972) Evolution of a nervous system specific lactate dehydrogenase isozyme in fish. J. Exp. Zool. 180, 12-32.
HUNTER R. L. & MARKERT C. L. (1957) Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. Science 125, 1294-1295.
KAPLAN N. O., CIOTTI M. M., HAMOLSKY M. & BIEBER R. E. (1960) Molecular heterogeneity and evolution of enzymes. Coenzyme analogs are useful for studying the evolution, classification, and differentiation of enzymes. Science 131, 392-397.
KLOSE J., WOLF U., HITZEROTH H., RITTER H., ATKIN N. B. & OHNO S. (1968) Duplication of the LDH gene loci by polyploidization in the fish Order Clupeiformes. Humangenetik 5, 190-196.
KUSA M. (1966) Lactate dehydrogenase isozyme patterns of the stickleback, Pungitius pungitius. Proc. Jap. Acad. 42, 146-150.
LUSH I. E., COWEY C. B. & KNOX D. (1969) The lactate dehydrogenase isozymes of twelve species of flatfish (Heterosomata). J. Exp. Zool. 171, 105-118.
MARKERT C. L. (1963) Lactate dehydrogenase isozymes: dissociation and recombination of subunits. Science 140, 1329-1330.
MARKERT C. L. & FAULHABER I. (1965) Lactate dehydrogenase isozyme patterns of fish. J. Exp. Zool. 159, 319-332.
MARKERT C. L. & HOLMES R. S. (1969) Lactate dehydrogenase isozymes of the flatfish, Pleuronectiformes: kinetic, molecular and immunochemical analysis. J. Exp. Zool. 171, 85-104.
MARKERT C. L. & WHITT G. S. (1968) Molecular varieties of isozymes. Experimentia 24, 977-991.
MASSARO F. J. & BOOKE H. E. (1970) Photoregulation of the expression of lactate dehydrogenase isozymes in Fundulus heteroclitus (Linnaeus). Comp. Biochem. Physiol. 38B, 327-332.
MORRIS R. W. (1960) Temperature, salinity, and southern limits of three species of Pacific cottid fishes. Limnology and Oceanography 5, 175-179.
MORRIS R. W. (1961) Distribution and temperature sensitivity of some eastern Pacific cottid fishes. Physiol. Zool. 34, 217-227.

MORRISON W. J. & WRIGHT J. E. (1966) Genetic analysis of three lactate dehydrogenase isozyme systems in trout: evidence for linkage of genes coding subunits A and B. J. Exp. Zool. 163, 259-270.

OHNO S. U., WOLF U. & ATKIN N. B. (1968) Evolution from fish to mammals by gene duplication. Hereditas 59, 169-187.

PESCE A., MCKAY R. H., STOLZENBACH F., CAHN R. D. & KAPLAN N. O. (1964) The comparative enzymology of lactic dehydrogenase. I. Properties of the crystalline beef and chicken enzymes. J. Biol. Chem. 239, 1753-1761.

PESCE A., FONDY T. P., STOLZENBACH F., CASTILLO F. & KAPLAN N. O. (1967) The comparative enzymology of lactic dehydrogenase. III. Properties of the H_4 and M_4 enzymes from a number of vertebrates. J. Biol. Chem. 242, 2151-2167.

PROSSER C. L. (1969) Principles and general concepts of adaptation. Environ. Res. 2, 404-416.

SALTHE S. N., CHILSON O. P. & KAPLAN N. O. (1965) Hybridization of lactic dehydrogenase *in vivo* and *in vitro*. Nature 207, 723-726.

SCHOLL A. & HOLZBERG S. (1972) Zone electrophoresis studies on lactate dehydrogenase isoenzymes in South American cichlids (Teleostei, Percomorphi). Experimentia 29, 489-491.

SCHWANTES M. L. (1973) Lactate dehydrogenase isozyme patterns of thirteen species of snakes. J. Exp. Zool. 185, 311-316.

SHAKLEE J. B., KEPES K. L. & WHITT G. S. (1973) Specialized lactate dehydrogenase isozymes: the molecular and genetic basis for the unique eye and liver LDH's of teleost fishes. J. Exp. Zool. 185, 217-240.

SHAW C. R. & BARTO E. (1963) Genetic evidence for the subunit structure of lactate dehydrogenase isozymes. Proc. Natl. Acad. Sci. U. S. 50, 211-214.

SHAW C. R. & PRASAD R. (1969) Starch gel electrophoresis of enzymes: a compilation of recipes. Biochem. Genet. 4, 297-320.

SMITH I. (1968) *Chromatography and Electrophoresis of Enzymes*; Interscience Publishers, New York.

SMITHIES O. (1955) Zone electrophoresis in starch gels: group variations in the serum proteins of normal human adults. Biochem J. 61, 629-641.

UTTER F. M. & HODGINS H. O. (1969) Lactate dehydrogenase isozymes of Pacific hake (*Merluccius productus*). J. Exp. Zool. 172, 59-68.

VESELL E. S., FRITZ P. J. & WHITE E. L. (1968) Effects of buffer, pH, ionic strength, and temperature on lactate dehydrogenase isozymes. Biochem. Biophys. Acta 159, 236-243.

VESELL E. S. & YIELDING K. L. (1966) Effects of pH, ionic strength, and metabolic intermediates on the rate of heat inactivation of lactate dehydrogenase isozymes. Proc. Natl. Acad. Sci. U. S. 56, 1317-1323.

WHITT G. S. (1969) Homology of LDH genes: E gene function in the teleost nervous system. Science 166, 1156-1158.

WHITT G. S. (1970a) Developmental genetics of the lactate dehydrogenase isozymes of fish. J. Exp. Zool. 175, 1-36.

WHITT G. S. (1970b) Directed assembly of polypeptides of the isozymes of lactate dehydrogenase. Arch. Biochem. Biophys. 138, 352-354.

WHITT G. S. & BOOTH G. M. (1970) Localization of lactate dehydrogenase activity in the cells of the fish (Xiphophorus helleri) eye. J. Exp. Zool. 174, 215-224.

WHITT G. S. & HOROWITZ J. J. (1970) Evolution of a retinal specific lactate dehydrogenase in teleosts. Experimentia 26, 1302-1304.

WILSON A. C., KAPLAN N. O., LEVINE L., PESCE A., REICHLIN M. & ALLISON W. S. (1964) Evolution of lactic dehydrogenases. Federation Proc. 23, 1258-1266.

WILSON F. R., WHITT G. S. & PROSSER C. L. (1973) Lactate dehydrogenase and malate dehydrogenase isozyme patterns in tissues of temperature-acclimated goldfish (Carassius auratus L.) Comp. Biochem. Physiol. 46B, 105-116.

WOLD F. (1971) Macromolecules: Structure and Function; Prentice-Hall, Inc., Englewood Cliffs, New Jersey.

Typed by

Karen O. Garlick