BIOCHEMICAL AND PHYSIOLOGICAL PROPERTIES OF THE LACTATE DEHYDROGENASE ALLOZYMES OF THE BRAZILIAN TELEOST, *Leporinus friderici*, ANOSTOMIDAE, CYPRINIFORMES

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ABSTRACT - 1. Lactate dehydrogenase polymorphism of *Leporinus friderici* is determined by two loci, each one encoded by two alleles - Ldh-A, Ldh-A' and Ldh-B and Ldh-B'.

2. Biochemical characterization of the different LDH phenotypes was carried out. Substrate saturation curves show that extracts, where the LDH-B predominates, are much more sensitive to substrate inhibition and have higher thermostability than skeletal muscle extracts where the LDH-A predominates.

3. Pyruvate affinity parameters of the different LDH-B phenotypes show differencial values in all temperatures assayed. LDH-BB phenotypes presented always greater affinity values than the LDH-BB' and B'B' ones.

4. The different LDH-B phenotypes show diferential thermostability when tested at 65°C, so, the LDH-B'B' phenotype had higher stability than the LDH-BB' and LDH-BB phenotypes. This was corroborated with the isozymes partially purified.

5. It was also detected a variation in the LDH-B phenotype frequencies since the experiment was started in 1980.

6. All these features suggest that this polymorphism may have an adaptive significance in relation to environmental variability, i. e., temperature fluctuations and water pollution due to industrial waste that notably increased in these last years.

INTRODUCTION

As was reported in a previous paper (Panepucci *et al.*, 1984) genetic variants were detected at both LDH loci of *Leporinus friderici*, Anostomidae, Cypriniformes. The phenotip ic frequencies of the Ldh-BB' and Ldh-B'B' allotypes increased along the successive captures that started in 1980.

Although the precise metabolic function of differ ent LDH isozymes has long been an unsolved problem, the physio logical importance of the multiple forms has been inferred from their served tissue specific distributions and related with aer obic requirements of the tissues.

The significance of protein polymorphisms has con tinued to be a controversial subject. While some population bi ologists ("neutralists") maintain that most genetic variants of enzymes are selectively equivalent, others ("selectionists") be lieve that polymorphisms are functionally nonequivalent and are maintained by natural selection.

Combined biochemical and environmental studies have been succesful in helping to understand how selection works in particular cases. A number of works suggests that selection operates at a particular loci. Merritt (1972) demon strated different substrate affinities of LDH allozymes in *Pime phales promelas*, that also present a latitudinal cline related with temperature. Clarke (1975) provided strong evidence

that the polymorphism of alcohol dehydrogenase of *Drosophila melanogaster* is directly subject to selection. Brown (1977) reported the results of "in vivo" experiments on glycogen metabolism in the field mouse, *Apodemus sylvaticus*, of different genotypes at the phosphoglucomutase locus, which suggest that the genotype of an individual may be subject to natural selection. Klar *et al.* (1979) demonstrated that the physiological performance of rainbow trout, *Salmo gardnieri*, depends on sex as well as LDH phenotype. Place & Powers (1979), suggested that the differences in the temperature dependence and the pH dependence for pyruvate reduction found among the LDH-B allozymes of *Fundulus heteroclitus*, may reflect a selective adaptation. Powers *et al.* (1979) suggested a physiological correlation between the lactate dehydrogenase genotype of *Fundulus heteroclitus* and haemoglobin function. Hoffmann (1981), showed that the PGI alleles from *Metridium senile*, are functionally different, a requisite for the direct action of selection at the locus. Di Michele & Powers (1982a), related specific hatching times of *Fundulus heteroclitus* embryos, of different LDH-B phenotypes. Also, Di Michele & Powers (1982b), reported swimming endurance differences for different LDH-B phenotypes of *Fundulus heteroclitus*. Samollow & Soulé (1983), discovered a striking case of superior heterozygote survivorship among immature toads during the winter.

In order to study the adaptive nature of protein polymorphisms found in ectotherms and to provide suitable material for the detailed biochemical comparison of polymorphic vari

ants of LDH, the present report includes the partial purification in starch gel electrophoresis of LDH different phenotypes, their biochemical characterization and comparison of these alelic isozymes and a variation in phenotype frequencies that occurred since the experiment was started in 1980.

MATERIAL AND METHODS

Experimental Animals

Adult specimens of *Leporinus friderici*, Anostomidae, Cypriniformes were captured, by gill nets of two sizes(2.5 and 3.5 cm) in the Emas waterfall on the Mogi-Guaçú river(state of São Paulo, Brazil).

Preparation of tissues extracts, blood samples and electrophoresis

Fishes were kept in ice until they were brought to the laboratory, then the tissues were dissected and kept at or below -15°C until used. Skeletal muscle and heart muscle tissues were cut into small pieces and washed in ice-cold 0.25 M sucrose solution. Tissues were homogenized with 1 vol of ice-cold 0.071 M EBT buffer, pH 8.7 (Boyer *et al.*, 1963), for screening and partial purification purposes. For spectrophoto

metric assays 0.1 M potassium phosphate buffer pH 7.0 and pH 7.5 was used. Centrifugation was carried out for 30' min at 19.000 g and 4°C and the resulting supernatants were used for electrophoresis, for enzyme activity measurements, and for partial purification of LDH allozymes. Electrophoresis was carried out in horizontal gels containing 13% (w/v) corn starch prepared according to Val *et al.* (1981) using the Boyer *et al.* (1963) buffer system. A voltage of 8 v/cm was applied for 7hs at 4°C. 5.5% polyacrylamide disc electrophoresis according to Dietz & Lubrano (1967) was also used. The electrophoreses were carried out at 4°C and 2.5 mA/tube for about 45min. The gels were stained according to Shaklee *et al.* (1973) in 0.5 M phosphate buffer ph7.0 at 37°C in the dark.

Partial Purification

Partial purification was accomplished by a electro elution process. It consisted of a preparative electrophoresis prepared as follows: a 3mm wide slot was made in the starch gel and 2ml of the homogenate was mixtured thoroughly with the starch gel that had been taken out. After electrophoresis a small piece was stained to identify the different LDH bands. After the different allozymes and isozymes were identified, the bands were cut out of the starch gel and each one inserted in a dialysis bag with 3ml of the gel buffer. The different identified bags were submitted to the electroelution process. The bags were immersed in a 0.07l EBT buffer and 100 volts applied between the electrodes for 5 hours. In this way, the enzymes

would come out of the gel but still stay inside the bags. Afterwards, the buffer containing the enzymes was concentrated to half its volume with powdered silica gel.

Kinetics

LDH activity under several experimental conditions were assayed spectrophotometrically by measuring the initial rates of NADH/ min oxidation at different temperatures, 17°C, 25°C, and 35°C controlled with a Lauda circulating water bath. The change in optical density at 340nm was measured in a Beckman 25K spectrophotometer. The apparent Km values were calculated from Lineweaver-Burk plots (1/velocity versus 1/substrate concentration), with pyruvate or lactate as a substrate. The assays were carried out in 0.05M potassium phosphate buffer at pH 7.0 and 7.5. The measure of the sensitivity of the LDH partially purified isozymes to substrate inhibition by pyruvate is expressed as the ratio of enzyme activity at low pyruvate concentration (0.33 mM) to the activity at high pyruvate concentration (10 mM). This rate is designated L/H (Wilson *et al.*, 1963). A unit of LDH activity is the amount of enzyme necessary to catalize the transformation of 1μ mole of substrate per minute at 25°C. Following Guppy & Hochachka (1978) we tried to find diferential inhibition to 20 mM phosphocreatine for the different LDH phenotypes.

Thermal Inactivation

Values for thermal inactivation were determined for heart muscle and skeletal muscle extracts for the five different phenotypes and the Partially Purified iso and allozymes. This was done using a 65°C water bath. Samples were removed at five min intervals, immediately cooled on ice and then assayed for LDH activity. Thermostability was also measured by heating the extracts for 20min at various temperatures. The extracts were then quickly cooled and assayed for surviving enzimatic activity.

Oxigen Equilibria

Oxigen equilibria experiments were performed with whole blood and according to Mangun & Kodon (1975). Dissociation curves were recorded at five different pHs, 6.8, 7.4, 8.0, 8.6 and 9.2.

RESULTS

Fig. 1 shows the LDH phenotypes of *L. friderici* previously reported (Panepucci *et al.*, 1984). There are two loci for this enzyme, each one encoded by two alleles - Ldh-A, Ldh-A', and Ldh-B and Ldh-B'. Partial purification of the homotetramers can be seen in Fig. 2. This type of purification has proved to

be rapid and effective for a qualitative study.

Lactate Dehydrogenase-phosphocreatine interaction

According to Guppy & Hochachka (1978) phosphocreatine inhibits at least to some extent all the lactate dehydrogenase examined. Table 1 shows the values obtained before and after the addition of phosphocreatine with the different phenotypes of *L. friderici* LDH. We also used, a specimen of *Hoplias malabaricus* to compare our data with that of Guppy & Hochachka (1978) and French & Hochachka (1978). We did not observe inhibition by phosphocreatine, as they did, in any of the samples used except for skeletal muscle of *H. malabaricus* in which a 26% inhibition was detected.

pH Optimun

All the pH profiles carried out with the different LDH phenotypes in the direction of lactate formation showed their optima between pH 7.5 and 8.0. We used two pHs for our studies, pH 7.0 and pH 7.5.

Pyruvate Inhibition

Inhibitions of different phenotypes occurred in the same order in the pyruvate concentrations which were used, shown in Fig. 3 and Table 2. Inhibition registered ad different temperatures at 10mM pyruvate concentration showed differences between skeletal (30% inhibition) and heart muscle (70%

inhibition) but not between the different heart phenotypes. As is shown in Table 3, apparent Km differs significantly between heart muscle phenotypes. In general LDH-BB phenotype shows lower Km values which reveal a higher pyruvate affinity. The most dramatic differences occur at 17°C and 25°C, pH 7.0 and at 35°C pH 7.5, where there is a stair-like graduation in Km values from the lowest Km for LDH-BB and the highest for LDH-B'B'. The heterozigous phenotype LDH-BB', as expected, was always intermediary. Differences in apparent Km between skeletal muscle phenotypes (LDH-AA and LDH-AA') and heart muscle phenotypes (LDH-BB, LDH-BB' and LDH-B'B') are marked except for the values obtained at 17°C pH 7.0 and 7.5. In general Km values for pyruvate appear to increase with temperature, except for the heterozigous LDH-BB' that had a higher value at 25°C, pH 7.5. Apparent Km values for NADH did not show differences between the different heart phenotypes and varied from 0.013 mM to 0.023mM for heart muscle phenotypes and from 0.051 mM to 0.057 mM for skeletal muscle phenotypes.

Lactate apparent Km values are shown in Table 4. There are no major differences between heart different phenotypes but they certainly occur between heart and skeletal muscle phenotypes.

It has been shown that higher vertebrate heart lactate dehydrogenase is generally more susceptible to inhibition by high pyruvate concentration than are their corresponding muscle isozymes (Bailey & Wilson, 1968). The L/H ratios

are obtained with partially purified extracts as shown in Table 5. The results show that heart isozymes, B_4 and B_4', are more subject to inhibition by high pyruvate concentration than is the muscle isozyme, A_4.

Thermostability

Susceptibility of the different phenotypes to inactivation by high temperature was examined. Results of the heart extracts submitted to 65°C are shown in Fig. 4. Differences in heat inactivation between the different heart phenotypes were maintained throughout the assay. These results are corroborated when the assays are performed with partially purified extracts, Fig. 5. When the samples were heated for 20 min to different temperatures, heart and muscle extracts responded differently(Fig. 6).

Oxygen Affinity

According to Powers *et al.* (1979), polymorphism for LDH-B in *Fundulus heteroclitus* might be related to different oxygen affinities in the different phenotypes. As a means of finding physiological differences we tried to determine oxygen affinities between the different LDH-B lactate dehydrogenase phenotypes. Oxygen equilibria curves for whole blood were determined for the different phenotypes, LDH-BB, LDH-BB' and LDH-B'B'. Because of the lack of differences, we calculated

the Bohr effect for the whole lot and it varied from -0.05 to -0.06.

Temporal Variation of the Gene Frequency

Table 6 shows the number of different LDH-B phenotypes related with the time of capture. Chi square test shows a dependency between time of capture and the different phenotype frequencies. Table 7 associates gene frequencies with the time of capture. Here also chi square values show that there is a significant difference and that genic frequency changes with the time of capture. Because variances were unequal and to ascertain whether the LDH-B' gene frequency increases with time a weighted regression was carried out which is shown in Fig. 7. To determine if the true slope estimated by b is different from zero, chi square tests were performed and the values obtained were:

Ldh-B $x = 7.43$ 1 d.f.

Ldh-B' $x = 5.56$ 1 d.f.

which shows that b is significantly different from zero.

DISCUSSION

The experiments with phosphocreatine did not

show inhibition except for *H. malabaricus* white muscle (26 % inhibition) as was expected according to Guppy & Hochachka (1978) and French & Hochachka (1978). This lack of inhibition is probably due to the fact that we are working with different fish species in the case of *L. friderici*, wehreas in the case of *H. malabaricus* we are probably working with a different population, hence with different metabolic requirements.

Previous reports indicate that typical values for muscle LDH pyruvate inhibition were between 10-20% at 10mM; in contrast, heart LDH isozymes are inhibited by 50-60% at this concentration (Gesser & Poupa, 1973; Baldwin & Gyuris, 1983). We found the same values in our experiments carried out at 25°C. As was shown by other authors when the experiments were performed at a lower temperature (17°C), the inhibition values were higher (Eby *et al.*, 1973; Enig *et al.*, 1976; Baldwin & Gyuris, 1983). At pH 7.0 there were interesting differences between the various LDH-B phenotypes that started at a concentration of 1 mM pyruvate. This was the first evidence that there was a small difference between the LDH-B different phenotypes. The next step was to compare Km values at different temperatures and pH. In general, Km values were similar to those found in the literature,, Table 8. Kinetic properties between heart and muscle. LDH isozymes showed differences, as in other vertebrates (Hochachka & Somero, 1973; Lim *et al.*, 1975; French & Hochachka, 1978; Place & Powers, 1979; Baldwin & Gyuris, 1983). Apparent Km values obtained with skeletal muscle extracts were a little lower

when the pH was lowered. Apparent Km values between LDH-AA and LDH-AA inverted their values when the pH was changed from pH7.0 to pH 7.5. Although at pH 7.5 (near the optimum pH) LDH-AA phenotype apparently has higher affinity than the LDH-AA' phenotype. According to Atkinson (1976), the pattern of substrate affinities of enzymes is one of the more fundamental adaptations necessary in a complex metabolism involving many reactions and many intermediates. We also found substrate - enzyme affinity diferences between the different LDH-B phenotypes for pyruvate. Except for the values obtained at 17°C pH 7.5, Km was always lower for the LDH-BB phenotype, which means a higher substrate affinity than for the LDH-B'B'. Apparent Km is intermediary for the heterozigous LDH-BB', which shows a relationship between the type of subunits and functional properties. Place & Powers (1979) in a similar study carried out at different temperatures and pH, found catalytic differences at 10°C when comparing LDH-B allozymes from *F. heteroclitus*. Differences obtained in apparent Km for lactate and NADH corroborate the hypothesis that we are working with allelic isozymes with quite different functional characteristics.

Thermostability tests show differences between LDH-A and LDH-B allozymes that are similar to those found by other authors in lower and higher vertebrates (Kaplan, 1964 ; Bailey & Wilson, 1968; Wuntch & Goldberg, 1970; Borgman & Moon, 1975). Although, there is no known relationship between the different physiological functions of LDH isozymes and their

thermostabilities, many authors believe that the increase in Km according temperature is the way nature has found to stabilize and regulate the enzymatic reaction rate (Hochachka & Somero, 1973; Somero, 1978; Richarson, 1983). The different Km values increased with temperature for both homozygous phenotypes. It is interesting to note that the differences between catalytic properties of LDH-B'B' and LDH-BB (Table 3) are in many cases, of the same order of magnitude as those reported for the A_4 and B_4 LDH of other vertebrates (Table 8). The differences found in thermostability between the different LDH-B phenotypes were also significant. Here the homozygous variant was the most susceptible to heat inactivation, and we obtained identical results with whole extracts and with extracts partially purified.

Our investigation reveals that the LDH-B isozymes not only differ in kinetic properties from the A containing isozymes, but also from each other.

The LDH-B locus has three electrophoretic phenotypes. The electromorphs encoded in alleles at this locus in natural populations of this species are more likely the result of the segregation of codominant alleles at a single locus, and not the result of post-translational modification. However, crosses were not performed to establish with certainty the genetic basis of the electrophoretic variants. The fact that the genotypic frequency changes with time of capture, resulting in an increase in the LDH-B'B' allotype, suggests that this polymorphism could be related to environmental factors.

One of the most polemic subjects in evolution, in late years, has been the adaptative nature of protein polymorphisms. In general, this discussion has been characterized by a polarization of opinion between the selectionists and the neutralists. We believe that our work, together with other ones carried out in this field, and having in mind organisms and environment as a whole, will stress the adaptive nature of protein polymorphisms. The change in genotypic frequency which we found to vary with the time of capture may well be related to the increasing quantity of industrial waste, especially that associated with alcohol production, which is speedily killing of all life in our rivers.

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Acknowledgements - This work was supported by CNPq - Conselho Nacional de Desenvolvimento Científico e Tecnológico - and by the OAS - Organization of American States. Lucia Panepucci was partially supported by a FAPESP fellowship. The assistance of Dr. E. Krieger in statistical analysis is gratefully acknowledged. The authors wish to thank Dra. Paula Ann Matvienko-Sikar who reviewed the manuscript.

FIGURES

Fig. 1: Electrophoretic patterns observed in different tissues from *Leporinus friderici* M.,skeletal muscle; H, heart muscle.

Fig. 2: Electrophoretic separation of LDH isozymes in 5.5% polyacrylamide gels; 1, 3 and 5 skeletal and heart(phenotypes LDH-BB and LDH-B'B') muscle extracts; 2, homotetramer A_4; 4, homotetramer B_4; 6, homotetramer B'_4 .

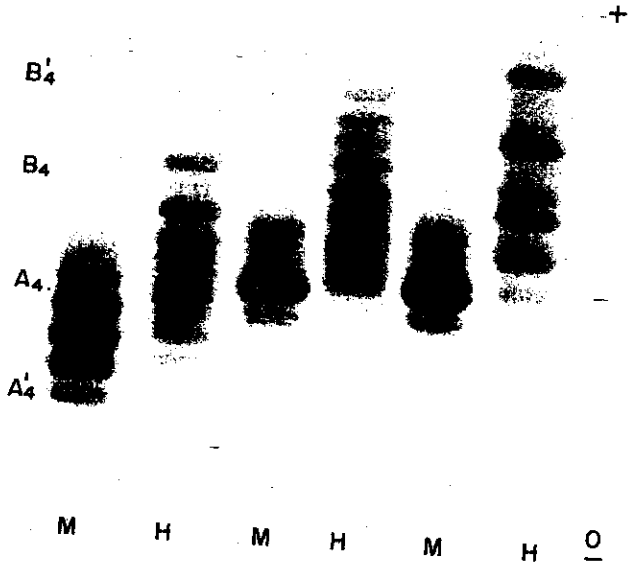
Fig. 3: The effect of pyruvate concentration on the rate of reaction of *L. friderici* LDH-B heart phenotypes. Reaction mixture (final concentration): pyruvate, as indicated; 0.13 NADH mM; 0.1 M potassium phosphate buffer pH 7.0; enzyme activity to give measurable reaction rate; Temperature, 17°C.

Fig. 4: Heat inactivation of heart LDH different phenotypes of *L. friderici*; 0.15 mM pyruvate, 0.10 mM NADH, 0.1 M potassium phosphate buffer, pH 7.0, 25°C.

Fig. 5: Heat inactivation of partially purified LDH homotetramers. 0.33 mM pyruvate, 0.13 mM NADH, 0.1 M potassium phosphate buffer pH 7.0, 25°C.

Fig. 6: Heat inactivation of LDH skeletal and heart muscle extracts from *L. friderici* heated for 20 min at various temperatures 0.15 mM pyruvate, 0.10 mM NADH, 0.1 M potassium phosphate buffer pH 7.5, 25°C.

Fig. 7: Weighted regression of Ldh-B and Ldh-B' gene frequencies versus the time of capture.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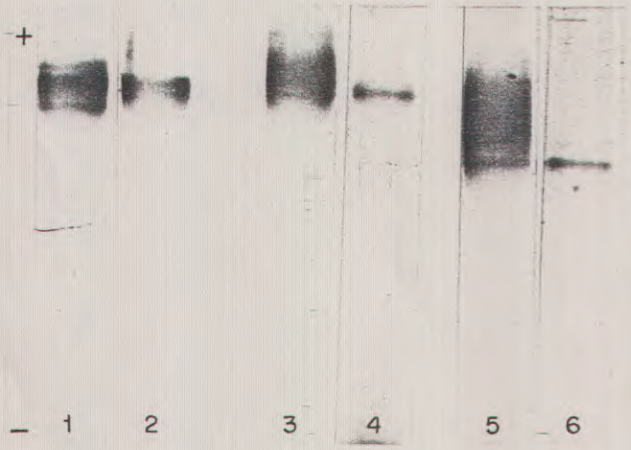

Fig. 2

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Fig. 3 Panepucci et al.

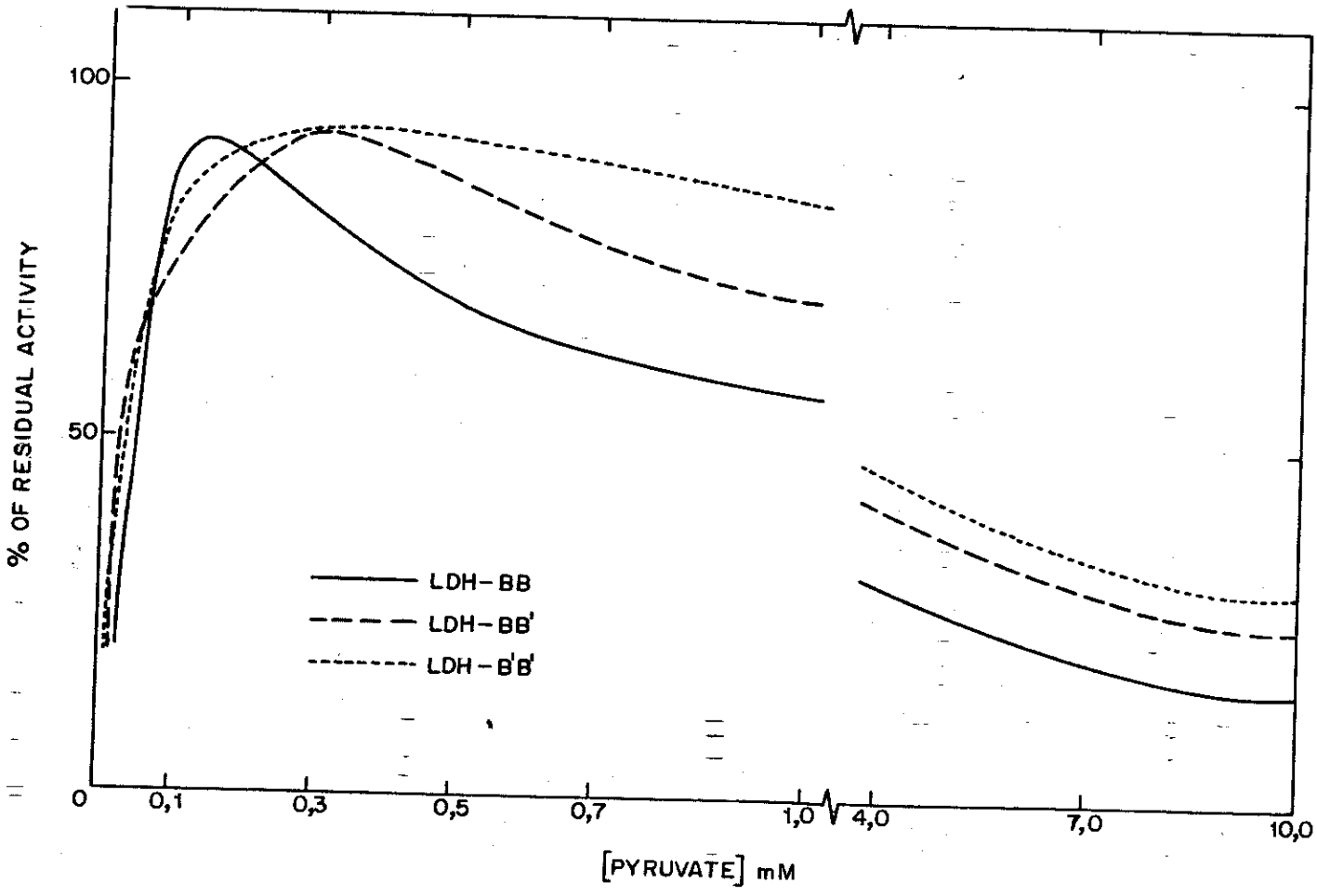

Fig. 4
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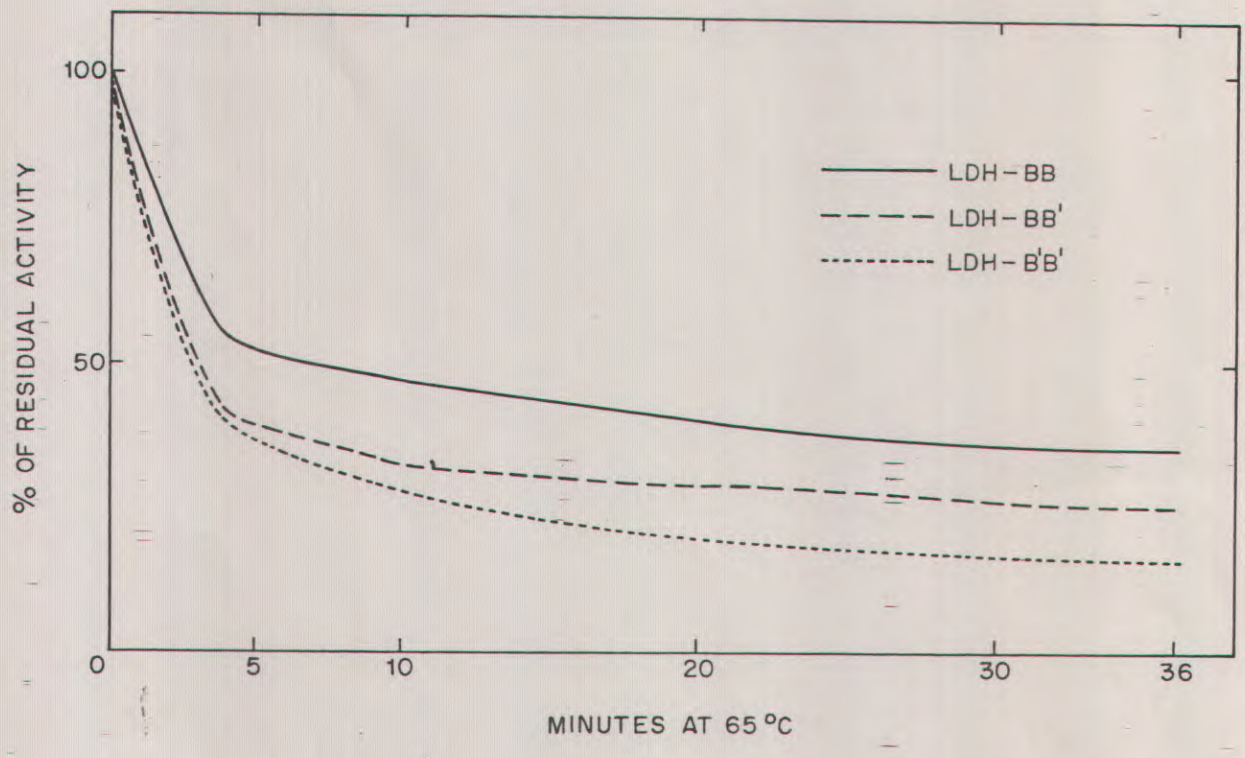

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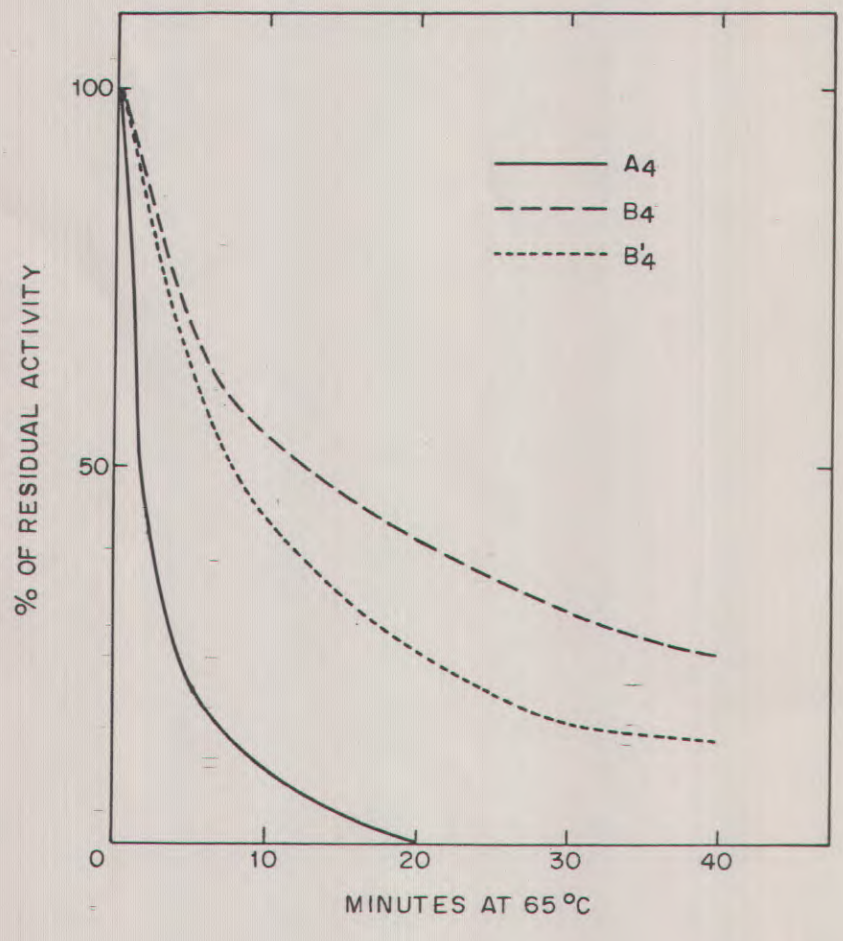

Fig. 6 Pamplucci et al.

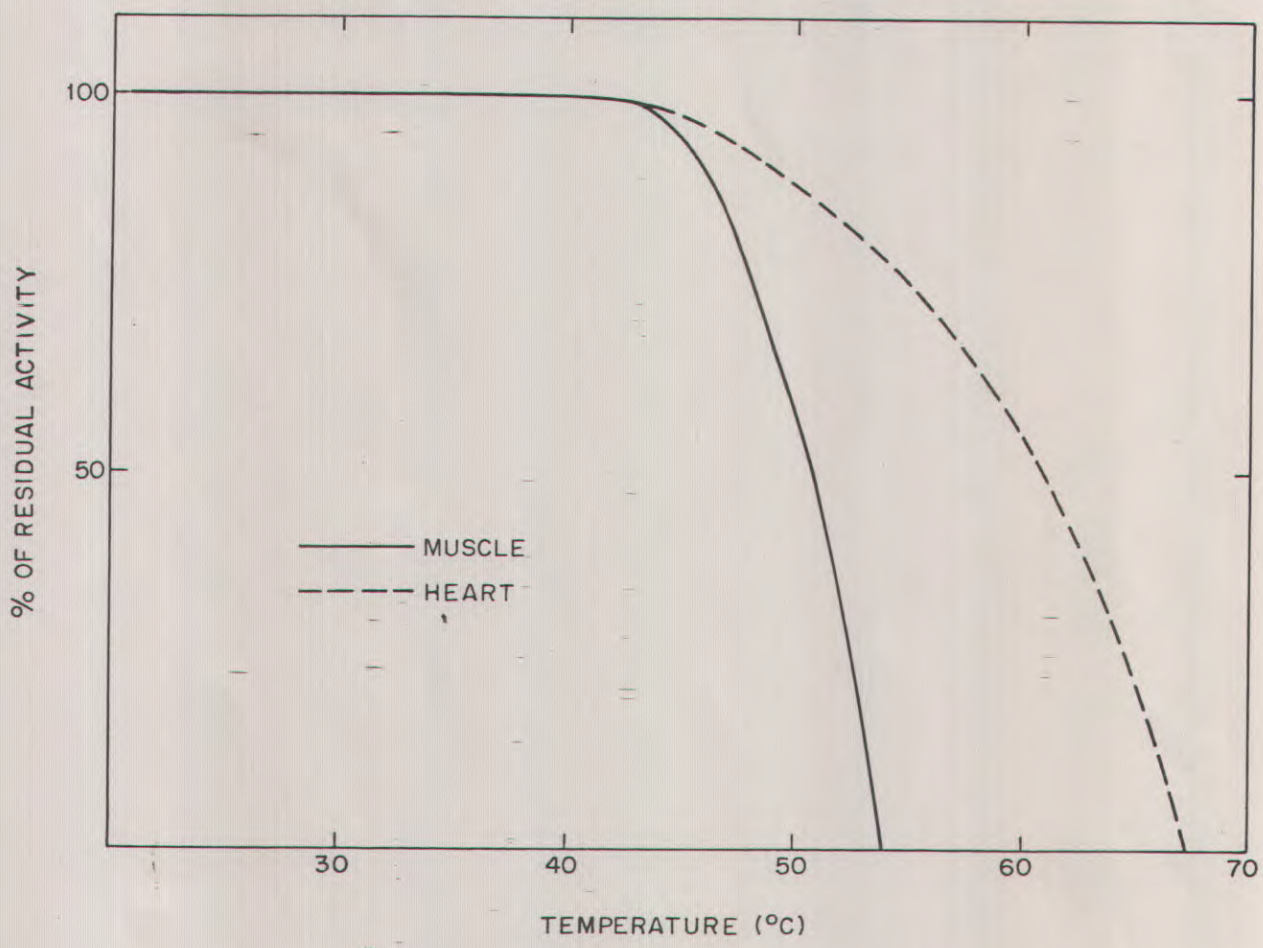

Fig. 7 Parapucci et al.

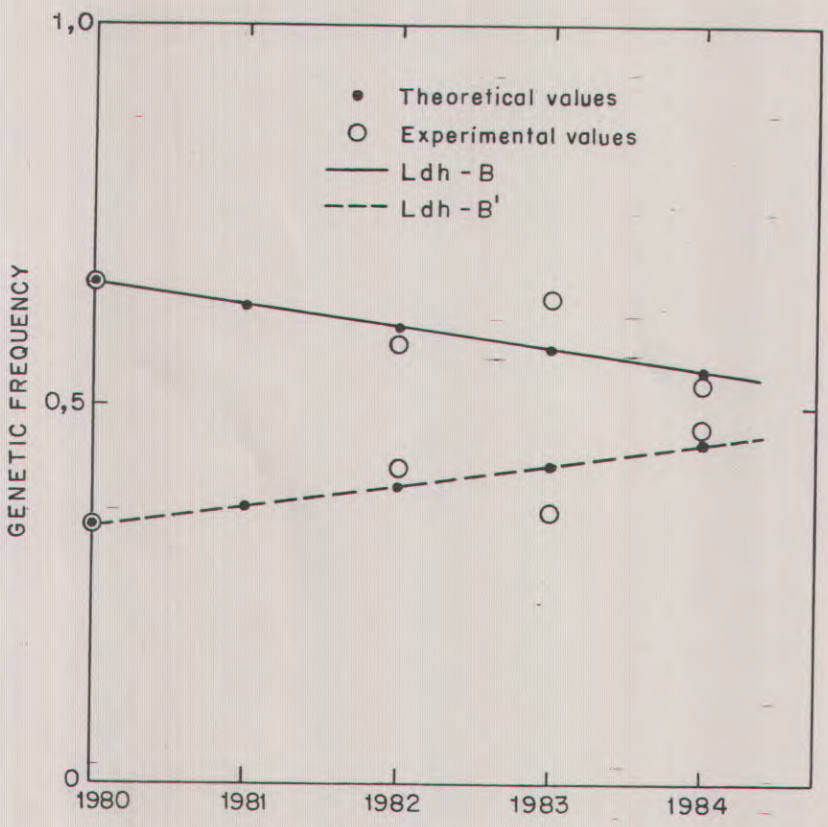

TABLE 1: Lactate dehydrogenase activity of different phenotypes submitted to a 20 mM phosphocreatine concentration. Assays conditions: 0.33 mM pyruvate, 0.13 mM NADH, potassium phosphate buffer pH 7.0, 25°C.

Source of LDH	Phenotype	U/ml	
		Control	With Phosphocreatine
Muscle	AA	22.35	23.10
Muscle	AA'	23.60	26.70
Heart	BB	27.30	27.45
Heart	BB'	24.05	23.95
Heart	B'B'	26.50	26.90
Partially purified	A_4	16.35	17.48
Partially purified	B_4	0.73	0.73
Partially purified	B'_4	0.75	0.80

TABLE 2: Percentage of inhibition of lactate dehydrogenase by 1 mM and 10 mM pyruvate concentration. 0.13 mM NADH, 0.1 M potassium phosphate buffer pH 7.0, 17°C.

Phenotypes	*Pyruvate Concentration*	
	1.0 mM	10.0 mM
LDH-AA	-	65
LDH-AA'	-	65
LDH-BB	43	85
LDH-BB'	30	74
LDH-B'B'	15	70

TABLE 3: Apparent Km (mM) values for pyruvate of *L. friderici* LDH different phenotypes. 0.13 mM NADH, 0.1 M potassium phosphate buffer.

Temp. °C	pH	LDH AA	LDH AA'	LDH BB	LDH BB'	LDH B'B'
17	7.0	0.107	0.071	0.023	0.033	0.051
	7.5	0.133	0.148	0.090	0.119	0.077
25	7.0	0.310	0.197	0.134	0.045	0.084
	7.5	0.347	0.669	0.137	0.190	0.196
35	7.5	0.393	0.545	0.150	0.172	0.295

TABLE 4: Apparent Km (mM) values for lactate with *L. friderici* skeletal and heart muscle extracts. 1mM NADH, 0.1 M phosphate buffer pH 7.0, 25 °C.

Phenotype	*Km (mM)*
LDH-AA	1.38
LDH-AA'	2.31
LDH-BB	0.79
LDH-BB'	0.80
LDH-B'B'	0.91

TABLE 5: Inhibition of *L. friderici* partially purified LDH isozymes by high concentration of pyruvate. 0.13 mM NADH, 0.1 M potassium phosphate buffer, pH 7.0, 25°C.

Isozymes	*L/H ratio*
A_4	1.73
B_4	3.92
B'_4	4.33

TABLE 6: Time of capture and distribution of the LDH phenotypes obtained from *L. friderici*.

Collects	LDH-BB	LDH-BB'	LDH-B'B'	Total
1980-OBS.	31	40	6	77
EXP.	28,49	33,91	14,60	
1982-OBS.	24	23	14	61
EXP.	22,57	26,86	11,57	
1983-OBS.	28	24	11	63
EXP.	23,31	27,74	11,94	
1984-OBS.	38	57	31	126
EXP.	46,62	55,49	23,89	
TOTAL	121	144	62	327

$X^2 = 12,81$ 6d.f

TABLE 7: Distribution of the Ldh-B gene frequencies obtained from *L. friderici* with time of capture.

Collects	*LDH-B*	*LDH-B'*	*Total*
1980-OBS.	102	52	154
EXP.	90,89	63,11	
1982-OBS.	71	51	122
EXP.	72,01	49,99	
1983-OBS.	80	46	126
EXP.	74,37	51,63	
1984-OBS.	133	119	252
EXP.	148,73	103,27	
TOTAL	386	268	654

$X^2 = 8,45$ 3d.f

TABLE 8: Km values for pyruvate of *L. friderici* and representative vertebrates.

Species	Assay Temperature	Km (mM) Heart	Muscle	Reference
Hoplias	30	0,33	0,70	1
Hoplerythrinus	30	0,40	1,30	1
Arapaima	30	0,15	1,00	1
Osteoglossum	30	0,25	--	1
Salvelinus fontinalis	25	0,083	4,80*	2
S. namaycush	25	0,077	3,85*	2
Hybrid splake trout	25	0,091	4,00*	2
Carassius auratus	30	0,097*	0,048*	3
Gillichthys mirabilis	15	--	0,138	4
Salmo salar	25	0,048*	0,40*	5
Fundulus heteroclitus	10	B_4^b 0,038*	--	6
	10	B_4^{ab} 0,046*	--	6
	10	B_4^a 0,083*	--	6
	25	--	0,404*	6
Melanogramus aeglefinus	25	0,037*	0,33*	7
Pseudopleuronectes americanus	25	0,084*	0,56	8
Oncorhynchus tschwgtscha	25	B 0,037*	0,65	9
	25	B_4' 0,075*	--	9
Mixine glutinosa	25	0,45*	0,53*	10
Leporinus friderici	25	BB 0,034AA'	0,197	11
	25	BB' 0,045AA	0,310	11
	25	B'B' 0,084	--	11

* Values obtained with partially purified isozymes.

1. French & Hochachka (1978); 2. Wuntch & Goldberg (1970); 3. Yamawaki & Tsukuda (1979); 4. Walsh & Somero (1982); 5. Gesser & Poupa (1973); 6. Place & Powers (1979); 7. Sensabaugh & Kaplan (1972); B. Markert & Holmes (1969); 9. Lim et al. (1975); 10. Sidell & Beland (1980); 11. This study.