*EFFECTS OF PH, IONIC STRENGTH, AND METABOLIC
INTERMEDIATES ON THE RATES OF HEAT INACTIVATION OF
LACTATE DEHYDROGENASE ISOZYMES*

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Various tissues of an organism possess different lactate dehydrogenase (LDH) isozyme patterns.^{1, 2} Control of these differences remains a fundamental problem involving graded phenotypic expression of two structural genes with wide variation in the same organism.^{3, 4} It has been proposed that the five main LDH isozymes may be formed *in vivo* from random recombination of two dissimilar subunits³ and that the two structural genes producing these subunits are regulated at different rates in various tissues.⁵ However, the rates of synthesis or catabolism of individual isozymes in tissues are unknown. Recent studies of enzyme "induction" demonstrate that, in addition to gene activity, catabolism is important in determining the steady state of enzyme levels in mammalian cells.⁶ Thus, regulation of enzyme concentrations *in vivo* occurs through synthesis, catabolism, or both. As previously suggested,⁷⁻⁹ therefore, different tissue isozyme patterns might arise in spite of equal synthetic activity at the *a* and *b* genetic loci if the relative rates of LDH 1 and LDH 5 catabolism vary from one tissue to another.

It is established¹⁰⁻¹³ that LDH 1 is more stable than LDH 5 at high¹⁰ as well as low¹⁴ temperatures, but extensive studies on the effects of pH, ionic strength, and commonly occurring metabolic intermediates have not been reported. In the present investigation these factors are shown to modify significantly the rates of heat inactivation of purified isozymes. The results suggest the possibility that differential effects of such variables on LDH 1 and LDH 5 *in vivo* may play a role in determining tissue isozyme patterns. Furthermore, the present study reveals that isozyme patterns in cold-blooded animals can be altered *in vivo* by changes in body temperature. These results, therefore, raise questions concerning the relationship of the stabilities of LDH 1 and LDH 5 to their tissue distributions and the relationship of the concentration of various metabolic intermediates in different tissues to rates of isozyme removal.

Materials and Methods.—Lactate dehydrogenase isozymes 1 and 5 from pig heart and rabbit muscle, respectively, were obtained from Boehringer. Figure 1 reveals that these preparations were of high purity containing only small amounts of other LDH isozymes. Nucleotides and substrates were obtained from commercial sources, and solutions were prepared fresh daily for use. Enzyme activity was measured at 25°C in a 3-ml mixture consisting of 0.0004 mg/ml LDH, 2.5×10^{-4} M pyruvate, 10^{-3} M NADH in 0.1 M NaPO₄ buffer, pH 7.4, by following absorbancy at 340 mμ in a Beckman spectrophotometer fitted with a Gilford multiple sample changer and recorder. To study loss of enzyme activity with time, enzyme solutions in a total volume of 1.0 ml of 0.1 M phosphate buffer were maintained at the indicated temperatures in a constant-temperature water bath and aliquots were removed at various intervals for assay. Solutions containing enzyme alone always

FIG. 1.—Photograph of a starch gel illustrating LDH isozymes from purified pig heart and rabbit skeletal muscle (Boehringer) in a concentration of 0.1 mg/ml.

assaying residual activity. Figure 3 shows that LDH 5 was more sensitive as the pH exceeded 7.5, whereas LDH 1 activity was affected to a smaller extent over the range from 6.5 to 8.2.

In contrast to differences in pH sensitivity of the two isozymes, both were approximately equally sensitized by lowering the buffer concentration (Fig. 4) and both were protected by addition of 2 *M* NaCl.

FIG. 2.—Rate of heat inactivation of LDH 5 and LDH 1 in a concentration of 0.05 mg/ml at 53°C and 63°C, respectively.

served as controls and were compared with solutions containing, in addition to enzyme, metabolic intermediates or other reagents.

Stock solutions of all reagents tested were adjusted to the same pH as the incubation mixtures. Since LDH 5 is more labile than LDH 1, the incubations for the two isozymes were carried out at 53°C and 63°C, respectively. Typical time courses for loss in activity are shown in Figure 2. This loss of LDH activity during heating appeared to follow first-order kinetics.

Rana catesbeiana (males) from Lemberger Company (Oshkosh, Wisc.) were divided into groups of six; each group was kept for 3 weeks at 4, 12, 25, and 37°C. They were then sacrificed and their livers homogenized at 4°C in a Potter-Elvehjem homogenizer, 1 part to 6 parts Tris buffer pH 8.9, 0.1 *M*. The homogenates were centrifuged at 10,000 *g* for 1 hr and the supernatants subjected to vertical starch gel electrophoresis at 12 v/cm for 5 hr at 4°C in the discontinuous buffer system of Poulik.¹⁵ The gels were then sliced and LDH activity was identified as described previously.¹⁶

Results.—Effects of pH on heat inactivation of LDH 1 and LDH 5 were determined by heating at 63°C and 53°C, respectively, for 20 min at various pH's and

Addition of NADH to the LDH isozymes had a pronounced effect on their thermal stability (Fig. 5). In the range of 10^{-5} *M* to 10^{-4} *M*, both isozymes were stabilized, while at 10^{-3} *M* both were completely inactivated in the 30-min time period. NAD failed to exhibit any effect on enzyme stability, and at 2×10^{-2} *M* it did not influence the NADH effect (Table 1).

Table 2 summarizes a number of different experiments in which the influence of various metabolic intermediates on the rates of heat inactivation of the two isozymes was tested. Oxalacetate and, to a somewhat less extent,

FIG. 3.—Effect of pH on LDH 5 and LDH 1 (0.05 mg/ml) at 53°C and 63°C, respectively. Aliquots were removed at 0 time and after 20 min of heating.

FIG. 4.—Effect of molarity on rate of heat inactivation of LDH 5 and LDH 1. Incubations were performed at 53°C for LDH 5 and 63°C for LDH 1 in phosphate buffer, pH 7.0 in the concentrations indicated.

malate protected LDH 5, but not LDH 1. In contrast, fructose-1,6-diphosphate stabilized both enzymes.

In final concentrations of 10^{-2} *M* to 10^{-6} *M*, α -ketoglutarate, glutamate, fumarate, isocitrate, phosphoenolpyruvate, 3-phosphoglycerate, glucose, glucose-6-phosphate, 6-phosphogluconate, urea, ADP, ATP, 3',5'-AMP, NADP, NADPH, coenzyme A, L-alanine, L-glycine, and albumin failed to protect LDH 1 or LDH 5 from heat inactivation. In a recent report, histones in low concentrations apparently stimulated LDH synthesis by embryonic chick brain tissue in culture, whereas in high concentrations they inhibited LDH synthesis.¹⁷ In the present studies neither lysine-rich histone (Worthington) nor arginine-rich histone (Sigma) in concentrations of 0.1 mg/ml to 8 mg/ml stabilized LDH 1 or LDH 5. However, lysine-rich histone in concentrations above 4 mg/ml accelerated heat inactivation of LDH 1 and LDH 5; this effect diminished when the lysine-rich histone was dialyzed prior to addition to the isozymes.

FIG. 5.—Effect of various concentrations of NADH on rates of heat inactivation of LDH 5 at 53°C and LDH 1 at 63°C.

To explore whether the stability of an enzyme *in vitro* might be related to its properties *in vivo*, groups of six frogs (*Rana catesbeiana*) were maintained at constant temperatures of 4, 12, 25, or 37°C for 3 weeks, and their LDH isozymes from liver were compared. In this organism, the isozymes migrating most rapidly toward the anode were most labile when heated at 63°C for 30 min *in vitro*. These

TABLE 1
EFFECT OF NAD ON NADH PROTECTION OF LDH 1 AND LDH 5 FROM
HEAT INACTIVATION

	Activity (%) after 30 Min LDH 5 (53°C)	LDH 1 (68°C)
Enzyme alone	30	14
Enzyme in 2×10^{-4} M NADH	71	77
Enzyme in 2×10^{-4} M NAD	22	26
Enzyme in 2×10^{-4} M NADH and 2×10^{-4} M NAD	80	72
Enzyme in 2×10^{-4} M NADH and 2×10^{-2} M NAD	77	65

TABLE 2
EFFECTS OF VARIOUS COMPOUNDS ON THE PERCENTAGE ACTIVITY REMAINING AFTER
LDH 1 AND LDH 5 ARE HEATED FOR 30 MIN AT 63°C AND 53°C, RESPECTIVELY

Molarity	LDH 1 (%)					
	Fructose-1, 6-diphosphate	Oxalacetate	Malate	Pyruvate	Lactate	NaCl
10^{-2} M	68	41	37	32	14	30
10^{-3} M	69	18	22	19	11	28
10^{-4} M	25	15	18	16	12	26
10^{-5} M	17	16	17	16	10	30
10^{-6} M	11	13	17	16	12	29
Enzyme alone	17	18	14	18	18	29
Molarity	LDH 5 (%)					
	Fructose-1, 6-diphosphate	Oxalacetate	Malate	Pyruvate	Lactate	NaCl
10^{-2} M	90	100	51	14	19	35
10^{-3} M	59	55	36	10	21	26
10^{-4} M	53	47	16	10	20	14
10^{-5} M	33	40	18	10	18	20
10^{-6} M	29	39	12	10	18	19
Enzyme alone	21	13	16	13	13	23

FIG. 6.—Photograph of LDH isozymes from livers of frogs kept at temperatures indicated for 3 weeks. The LDH activity of each liver homogenate was 2000 IU prior to application on the starch gel.

isozymes exhibit greatly diminished activity on starch gels of liver homogenates from animals kept at 37°C for 3 weeks prior to sacrifice (Fig. 6).

In addition to measurement of loss of isozyme activity, the effects of heating were determined by spectrophotometric alterations at 500 m μ during heating. Figure 7 illustrates increases in the optical density of LDH 5 with time at 53°C. These increases are diminished by addition of NADH (2×10^{-4} M), oxalacetate (10^{-2} M), or fructose-1,6-diphosphate (10^{-2} M). LDH 1 exhibits no elevations of optical density at 500 m μ during 60 min of heating at 63°C, whereas increased turbidity of LDH 5 is visually apparent after 15 min at 53°C.

Discussion.—These experiments reveal that a variety of conditions alter the rates of heat inactivation of LDH 5 and LDH 1. Rates at which LDH 5 and LDH 1 activities decline on heating may be accelerated or retarded by changes in pH and ionic strength, and by addition of certain common metabolic intermediates such as NADH, fructose-1,6-diphosphate, oxalacetate, and malate. When the pH exceeds 7.5, heat inactivation of LDH 5 and, to a smaller extent, of LDH 1, is accelerated. Addition

FIG. 7.—Increases in optical density at 500 m μ during heating of LDH 5 (0.05 mg/ml) with (▲) and without (●) oxalacetate (10^{-2} M).

of 2 *M* NaCl protects both LDH 5 and LDH 1 from heat inactivation, whereas reduction from 0.1 *M* to 0.05 *M* accelerates heat inactivation of both enzymes. NADH (2×10^{-4} *M* to 5×10^{-4} *M*) protects LDH 5 and LDH 1 from heat inactivation, but at higher concentrations it increases the rate of heat inactivation. Fructose-1,6-diphosphate protects both isozymes, whereas oxalacetate and malate protect only LDH 5.

If, as is generally maintained, loss of activity on heating is secondary to a change in isozyme conformation, then alterations in pH, ionic strength, and concentration of certain metabolites may exert their effects by either stabilizing or weakening the native conformation of the isozyme. This assumption was strengthened by the observation that NADH, oxalacetate, and fructose-1,6-diphosphate reduced heat-induced turbidity of LDH 5 (Fig. 7). In this connection, Fritz showed that oxalacetate affects the activity of LDH 5 and proposed an allosteric site on this isozyme.¹⁸ Furthermore, Wolin demonstrated a requirement for fructose-1,6-diphosphate by the LDH from several species of *Streptococcus* and suggested that in these streptococcal LDH's fructose-1,6-diphosphate acts as an allosteric effector.¹⁹ Two distinct, but interacting, binding sites for pyruvate have been described on an LDH from *Escherichia coli*.²⁰

In accord with these observations, protection of LDH isozymes from heat inactivation in the present experiments by addition of fructose-1,6-diphosphate, oxalacetate, and malate may be mediated through allosteric sites. Moreover, occupation of allosteric sites by these or similar compounds might possibly alter the rates of catabolism of certain LDH isozymes *in vivo*. Through such allosteric sites, intracellular metabolic conditions might regulate the proportion of LDH isozymes observed in various tissues. Rates of intracellular isozyme catabolism would then be determined in part by the relative availability of allosteric effectors in different tissues and in various subcellular regions. Protection against heat inactivation afforded both LDH 5 and LDH 1 only at certain NADH concentrations and accelerated inactivation occurring at other concentrations may reflect occupation of several sites by the coenzymes. A ternary complex of enzyme, coenzyme, and substrate probably occurs at the active site,²¹ but the coenzyme in a reduced form might also bind at an allosteric site. Inability of NAD to protect isozymes from heat inactivation or to diminish protection by NADH may indicate failure of the oxidized form of the coenzyme to bind either at an allosteric site or, in the absence of substrate, at the active site. Alternatively, NAD may bind at a site whose occupation does not affect the rate of heat inactivation of LDH isozymes.

At present, the intracellular rates of catabolism of LDH 1 and LDH 5 are unknown. A detailed model for the intracellular degradation of LDH, or any other enzyme, has not yet been proposed. Only recently has it been demonstrated that intracellular enzyme levels are dependent on rates of catabolism as well as on rates of synthesis.⁶ Indeed, since certain compounds classically employed to inhibit protein synthesis also can block protein catabolism,²² it has been proposed that protein synthesis is necessary for the continuation of protein breakdown.²² Therefore, actinomycin blockade of estrogen-induced LDH 5 elevations in rat uterus²³ may result from effects on isozyme catabolism as well as synthesis.

The present experiments illustrate a variety of conditions *in vitro* that affect a single type of degradative process, heat inactivation. They are not intended to

account for the more complex processes of catabolism *in vivo*. However, the experiments with *R. catesbeiana* showing loss *in vivo* of heat-labile enzymes at elevated temperature suggest that heat stability *in vitro* may indeed be correlated with enzyme stability *in vivo*.

The five commonly encountered LDH isozymes differ from each other in numerous ways, although it has been proposed that, like the α and β chains of human hemoglobin, they all originally arose from a single gene which during evolution became duplicated and that subsequently each gene underwent multiple mutations.¹⁶ Thus, LDH 1 and LDH 5, tetramers of four B and four A subunits, respectively,^{3, 4} exhibit different total amino acid compositions,²⁴ Michaelis-Menten constants,¹⁰ and behavior toward inhibitors.²⁵ In view of the physicochemical similarity of homologous LDH's from various vertebrates as contrasted to the differences between the LDH 1 and LDH 5 of any given species,²⁶ use of two species to obtain purified LDH 1 and LDH 5 appeared justified for the purposes of the present study. This assumption was validated by use of electrophoretically purified LDH 5 from pig skeletal muscle and LDH 1 from rabbit heart. With respect to the effects of NADH, oxalacetate, malate, and fructose-1,6-diphosphate on heat inactivation of isozymes, pig LDH 5 behaved like rabbit LDH 5; and pig LDH 1 resembled rabbit LDH 1. Since the five LDH isozymes are distinguishable in so many other respects, differences in rates of catabolism might be anticipated and are, indeed, suggested by differential loss of LDH 5 activity during maturation *in vivo* of erythroblasts, megakaryocytes, and lens fibers.⁸

Summary.—NADH, oxalacetate, malate, and fructose-1,6-diphosphate as well as alterations in pH and ionic strength significantly affected the rates of heat inactivation of LDH 1 and LDH 5. Oxalacetate and malate differentially protected LDH 5 from heat inactivation, whereas NADH and fructose-1,6-diphosphate protected both LDH 1 and LDH 5. In *R. catesbeiana*, temperature changes *in vivo* produced alterations of liver LDH isozyme patterns; frogs kept at 37°C lost heat-labile isozymes (LDH 1 and LDH 2) conspicuous at lower temperatures.

The possibilities that differential rates of catabolism play a role in regulating tissue isozyme patterns and that allosteric effectors may protect certain isozymes from catabolism are discussed.

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