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EFFECTS OF BUFFER, pH, IONIC STRENGTH AND TEMPERATURE ON LACTATE DEHYDROGENASE ISOZYMES

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SUMMARY

1. LDH 1* and LDH 5 were isolated from rat cardiac and skeletal muscle, respectively, and purified. LDH 1 and LDH 5 activities were affected by alterations in pH, ionic strength and buffer. These have been systematically examined over a small range. Alterations in K_m and v_{max} are produced by changes in pH and buffer.

2. Rat LDH 5 is much more thermolabile than rat LDH 1 at 39° in Tris buffer (pH 7.4), 0.05 M. The rate of decline of lactate dehydrogenase isozymes at physiologic temperatures depended on enzyme concentration. Thus, more rapid decline from plasma of intravenously administered LDH 5 than of LDH 1 may be attributable in warm-blooded vertebrates to heat instability as well as to more rapid rates of catabolism.

3. Turnover of the various isozymes should be determined according to techniques capable of yielding results which are not affected by differences among isozymes in thermolability or in activity at various pH's, ionic strengths and buffer concentrations. Measurement of the incorporation and decay of radioactive amino acids into intracellular isozymes is suggested as an alternative procedure.

INTRODUCTION

Two unresolved problems of major significance in the study of multiple molecular forms of enzymes (isozymes) concern the physiologic significance of tissue-specific patterns and the mechanisms that maintain such differences. In the case of lactate dehydrogenase (L-lactate: NAD^+ oxidoreductase, EC 1.1.1.27) the mechanism for maintenance of tissue-specific patterns has generally been assumed to be different rates of synthesis of the A and B subunits[1]. However, the intracellular rates of synthesis of the lactate dehydrogenase isozymes have not been determined in any tissue; and,

* The lactate dehydrogenase isozymes are abbreviated as LDH 1 to LDH 5 with the most rapidly migrating anodal form numbered 1.

as has been emphasized, distinctive tissue patterns could arise as well from different rates of subunit catabolism[2-4].

Differential rates of isozyme catabolism could account for the tissue-specific patterns, particularly if these rates changed from one tissue to another. Recent studies have suggested that the rates of catabolism of LDH 1 and LDH 5 are indeed markedly different[5,6]. These studies, however, should be interpreted with some caution because they are based on the disappearance from plasma of exogenously administered LDH 1 and LDH 5. Since LDH 5 is more thermolabile than LDH 1 *in vitro*, its more rapid disappearance from plasma *in vivo* may be attributable to instability at physiologic temperatures rather than to faster catabolism *in vivo*. However, *in vivo* the catabolic process may involve heat inactivation. Furthermore, the catabolic rate for each isozyme may be different in various tissues.

The present experiments illustrate some additional physicochemical differences between LDH 5 and LDH 1 that might influence their rates of disappearance from plasma *in vivo*.

METHODS

Lactate dehydrogenase. Lactate dehydrogenase activity was measured at 25° or 39° in a 2-ml reaction mixture consisting of 0.1 ml enzyme ($1.8 \cdot 10^{-9}$ M for LDH 1 and $1.0 \cdot 10^{-9}$ M for LDH 5), NADH ($12.5 \cdot 10^{-5}$ M) and pyruvate ($5 \cdot 10^{-4}$ M) in either Tris or phosphate buffer of different pH and molarity. The concentrations of NADH and pyruvate were 80% of the saturating values for LDH 1 and LDH 5. The change in absorbance at 340 mμ was followed in a Beckman DU spectrophotometer or in a Beckman DU spectrophotometer fitted with a Gilford multiple sample absorbance recorder Model 2000. Addition of enzyme solution to the mixture of coenzyme, substrate and buffer in a cuvette having a 1-cm light path initiated the reaction. The decrease in absorbance was measured at 15-sec intervals for 2 min. Lactate dehydrogenase activity was expressed in International Units.

To study loss of enzyme activity with time, enzyme solutions in a total volume of 1.0 ml of buffer or serum were maintained at 39° in a constant-temperature water bath and aliquots were removed at various intervals for assay. The activity was expressed as percentage of the maximum activity.

Purification of LDH 1 from rat heart and of LDH 5 from rat skeletal muscle. Lactate dehydrogenase was extracted from minced rat cardiac and skeletal muscle into Tris buffer (pH 7.4), 0.1 M, in a Potter–Elvehjem motor driven homogenizer. Centrifuging for 10 min at 10 000 × *g* precipitated the particulate material and myosin, which were removed. The lactate dehydrogenase in the supernatant was precipitated by $(NH_4)_2SO_4$ between 35 and 65% saturation at 4°. The lactate dehydrogenase was further purified by passage through a BioGel P-150 column in Tris buffer (pH 7.4), 0.005 M. Electrophoretic homogeneity was obtained either by separation on a starch block prepared in barbital buffer (pH 8.6), 0.05 M, as previously described[7], or by chromatography on a DEAE-Sephadex column. The preparations of individual isozymes used in the experiments were demonstrated to be homogeneous on vertical starch gel electrophoresis both by enzymatic and protein staining[4].

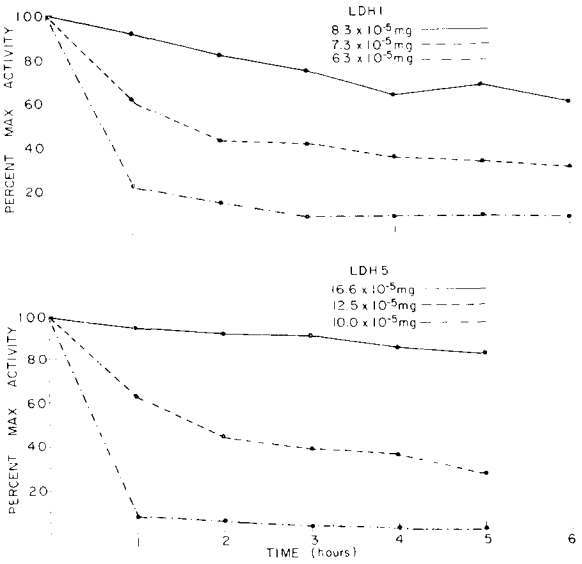

Fig. 1. Heat inactivation of electrophoretically homogeneous rat LDH 1 and LDH 5 at 39° in Tris buffer (pH 7.4), 0.05 M. The thermal inactivation is concentration dependent. Isozyme concentrations are expressed in mg/ml. Appropriate dilutions were made before assay.

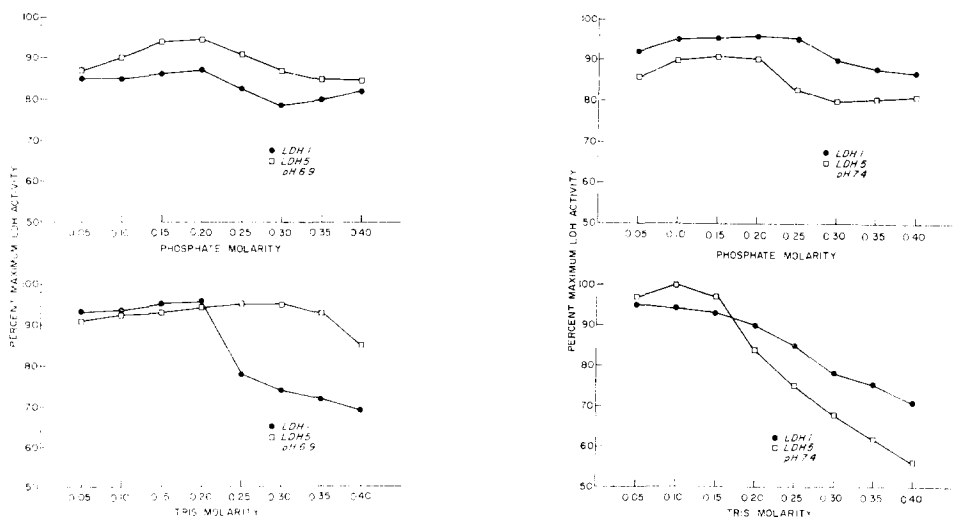

Fig. 2. Effect of phosphate and Tris concentrations at pH 6.9 on rat LDH 1 and LDH 5. For the LDH 1 assays the NADH concentration was $1.25 \cdot 10^{-5}$ M in the cuvette and the pyruvate concentration was $2.5 \cdot 10^{-5}$ M. For the LDH 5 assays the NADH concentration was $12.5 \cdot 10^{-5}$ M and the pyruvate concentration was $3.75 \cdot 10^{-5}$ M. These concentrations are approx. 80% of the saturating concentrations for each isozyme. The final concentration of the isozymes was approx. 10^{-9} M.

Fig. 3. Effect of phosphate and Tris concentrations at pH 7.4 on rat LDH 1 and LDH 5. NADH, pyruvate and lactate dehydrogenase concentrations were as indicated in the legend to Fig. 2.

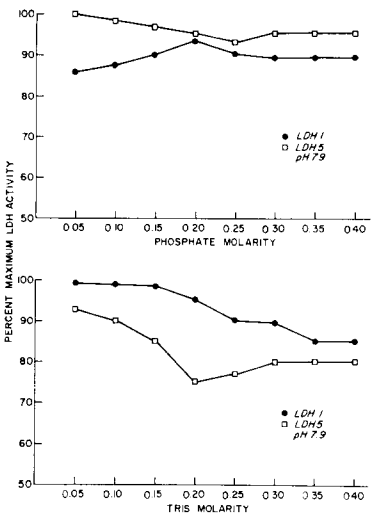

Fig. 4. Effect of phosphate and Tris concentrations at pH 7.9 on rat LDH 1 and LDH 5. NADH, pyruvate and lactate dehydrogenase concentrations were as indicated in the legend to Fig. 2.

RESULTS

Inactivation of lactate dehydrogenase isozymes in vitro at 39°. Differences in the rate of inactivation of rat LDH 1 and LDH 5 are illustrated in Fig. 1. Different concentrations of the isozymes were heated at 39° in Tris buffer (pH 7.4), 0.05 M; LDH 5 activity declined at concentrations in which LDH 1 was resistant. To demonstrate thermolability much lower concentrations of LDH 1 were required.

Effect of buffer molarity, pH, and type of buffer on LDH 1 and LDH 5. Generally alterations of buffer molarity at pH 6.9, pH 7.4 or pH 7.9 affected rat LDH 1 and LDH 5 activity to approximately similar extents (Figs. 2–4). However, in some instances LDH 1 and LDH 5 activities responded differently to changes of molarity: in Tris buffer (pH 6.9) (Fig. 2), LDH 5 activity was essentially unchanged from 0.05 to 0.35 M, whereas LDH 1 activity was maximum at 0.20 M and declined 20% at 0.25 M.

The effect of molarity on LDH 1 and LDH 5 activity is modified by the buffer and the pH. As the molarity increased, the activity of the two isozymes at all pH's declined more rapidly in Tris buffer than in phosphate buffer; however, LDH 5 was

TABLE I

v_{max} AND K_m OF RAT LDH 1 AND LDH 5 IN TRIS AND PHOSPHATE BUFFERS AT pH 6.9, 7.4 AND 7.9

Isozyme	*pH*	v_{max} *(M)*		K_m *(M)*	
		Tris	*Phosphate*	*Tris*	*Phosphate*
LDH 5	6.9	$9.4 \cdot 10^{-2}$	$5.3 \cdot 10^{-2}$	$1.4 \cdot 10^{-4}$	$0.69 \cdot 10^{-4}$
LDH 5	7.4	$7.6 \cdot 10^{-2}$	$3.9 \cdot 10^{-2}$	$2.3 \cdot 10^{-4}$	$0.65 \cdot 10^{-4}$
LDH 5	7.9	$5.3 \cdot 10^{-2}$	$5.3 \cdot 10^{-2}$	$7.7 \cdot 10^{-4}$	$3.8 \cdot 10^{-4}$
LDH 1	6.9	$1.5 \cdot 10^{-2}$	$1.5 \cdot 10^{-2}$	$0.80 \cdot 10^{-4}$	$2.1 \cdot 10^{-4}$
LDH 1	7.4	$1.7 \cdot 10^{-2}$	$1.3 \cdot 10^{-2}$	$1.1 \cdot 10^{-4}$	$0.58 \cdot 10^{-4}$
LDH 1	7.9	$2.7 \cdot 10^{-2}$	$1.3 \cdot 10^{-2}$	$4.8 \cdot 10^{-4}$	$1.1 \cdot 10^{-4}$

very stable in Tris buffer at pH 6.9 (Fig. 2). Figs. 2, 3 and 4 show that the maximum activity of an isozyme at constant pH varies with molarity. Maximal LDH 1 activity occurs in 0.20 M phosphate buffer at pH 6.9, pH 7.4 and pH 7.9. LDH 5 activity is maximal in this molarity of phosphate buffer at pH 6.9 and pH 7.4, but in 0.05 M phosphate buffer at pH 7.9. Maximal LDH 1 and LDH 5 activities were observed in Tris buffer 0.05 M and 0.10 M at pH 7.4 and pH 7.9, but in higher molarities at pH 6.9 (Table I). Alterations of pH from 6.9 to 7.4 to 7.9 had little effect on the activity of LDH 1 and LDH 5 in the presence of increasing concentrations of phosphate buffer (Figs. 2–4). In increasing concentrations of Tris buffer LDH 1 and LDH 5 activities were greatest at pH 7.9 and pH 6.9, respectively, and were least at pH 7.4.

Appreciable losses in activity of both isozymes occurred at high concentrations of Tris buffer (pH 7.4) (Fig. 3). Marked effects of pH on the kinetics of the individual LDH isozymes have been described recently[10,11]. Figs. 5–7 illustrate kinetic changes of rat LDH 1 and LDH 5 plotted according to LINEWEAVER AND BURK[12]. The K_m and v_{max} for LDH 1 and LDH 5 derived from these plots are given in Table I. The conditions for the assay are the same as noted above except that the temperature was 25°.

DISCUSSION

Heating rat LDH 1 and LDH 5 at 39° in Tris buffer (pH 7.4), 0.05 M, caused differential inactivation of LDH 5. The extent of this inactivation depended on enzyme concentration. Lower concentrations were required for thermal inactivation of LDH 1 under these conditions than for thermal inactivation of LDH 5. This experiment confirms the well-documented thermolability of LDH 5 (ref. 13); it was performed to serve as an illustration of one type of denaturation that might occur following intravenous administration of LDH 5 and LDH 1 to warm-blooded vertebrates. More rapid inactivation of LDH 5 in such experiments may be attributable to greater thermolability of LDH 5 rather than to differences between LDH 1 and LDH 5 in rates of catabolism. To what extent thermolability contributes to intracellular LDH catabolism is unknown. The degree of thermolability of LDH 5 depends on isozyme concentration; furthermore, isozyme thermolability is affected by the presence of such protectors as NADH, oxalacetate and fructose-1,6-diphosphate[3]. In light of the dependence of thermolability of isozymes on their concentrations, it should be emphasized that the concentration of lactate dehydrogenase isozymes in plasma is in the order of one thousandth of that existing in tissues. This relationship renders even more hazardous extrapolation from lactate dehydrogenase isozyme decay in plasma to lactate dehydrogenase catabolism within tissues.

During investigations of lactate dehydrogenase isozyme inactivation *in vitro* BOYD observed variations in the sensitivity of LDH 5: he found negligible inactivation of purified sheep lactate dehydrogenase isozymes when incubated in phosphate buffer (pH 7.4), 0.1 M, at 39° for 48 h, but complete inactivation of LDH 5 in less than 1 day when the isozyme was incubated in undialyzed sheep plasma[6]. BOYD attributed this inactivation to an elevation of pH from 7.4 to 7.8.

Effects of buffer, pH and molarity on the activities of purified rat LDH 1 and LDH 5 *in vitro* have been described in the present experiments; alterations in some of these parameters affected LDH 1 and LDH 5 activities and kinetics to varying extents (Figs. 2–7). Previous studies indicated that alterations in pH affected lactate dehydro-

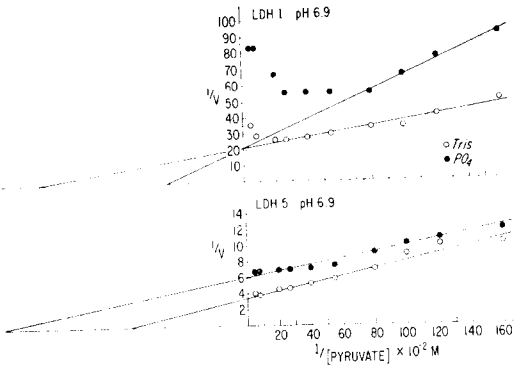

Fig. 5. Activities of rat LDH 1 and LDH 5 as affected by pyruvate concentration at pH 6.9 and 25° plotted according to the method of LINEWEAVER AND BURK[12]. The concentration of NADH was $12.5 \cdot 10^{-5}$ M; the concentration of LDH 1 was $1.8 \cdot 10^{-9}$ M; and the concentration of LDH 5 was $1.0 \cdot 10^{-9}$ M.

genase kinetics[14]. Recent experiments on the binding of *p*-hydroxymercuribenzoate to beef heart lactate dehydrogenase in the presence of various ions have suggested that phosphate and Tris ions, as well as nitrate and arsenate ions, have different effects on the conformation of the lactate dehydrogenase molecule[15], and that Tris inhibits recombination of A and B subunits following freezing[16].

Figs. 5 to 7 reveal that at 25° high substrate concentrations inhibit LDH 1 but not LDH 5. The data show that substrate inhibition of LDH 1 is greater in phosphate

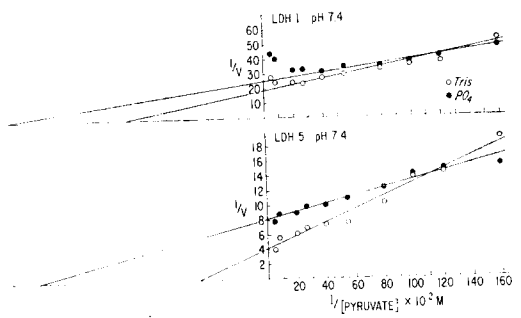

Fig. 6. Activities of rat LDH 1 and LDH 5 as affected by pyruvate concentration at pH 7.4 and 25° plotted according to the method of LINEWEAVER AND BURK[12]. The concentration of NADH was $12.5 \cdot 10^{-5}$ M; the concentration of LDH 1 was $1.8 \cdot 10^{-9}$ M; and the concentration of LDH 5 was $1.0 \cdot 10^{-9}$ M.

than in Tris buffer. The extent of inhibition decreased as the pH was raised from 6.9 to 7.4 and to 7.9. Therefore, such factors as pH and type of buffer alter the extent of substrate inhibition of LDH 1. Previously, it was shown that differences in substrate inhibition between LDH 1 and LDH 5 were appreciably diminished when the kinetics of the reaction were studied at the physiologic temperature of warm-blooded vertebrates, rather than at 25° (refs. 17, 18). Apparently it is hazardous to draw conclusions

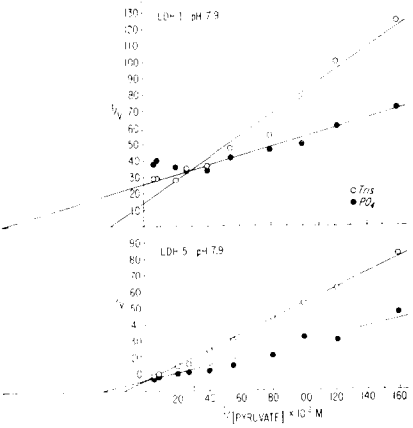

Fig. 7. Activities of rat LDH 1 and LDH 5 as affected by pyruvate concentration at pH 7.9 and 25° plotted according to LINEWEAVER AND BURK[12]. The concentration of NADH was $12.5 \cdot 10^{-5}$ M; the concentration of LDH 1 was $1.8 \cdot 10^{-9}$ M, and the concentration of LDH 5 was $1.0 \cdot 10^{-9}$ M.

about the function of the isozymes *in vivo* based on kinetics performed at 25°, in a single buffer system, at one pH and with unphysiologic concentrations of enzyme and coenzyme.

The pH, ionic strength, composition and temperature of blood change *in vivo* over the period during which turnover studies are conducted. Therefore, caution should be observed in interpreting alterations of lactate dehydrogenase activity in blood following intravenous administration of exogenous isozymes. BOYD has recently shown that ^{14}C-labeled LDH 5 injected intravenously disappears rapidly from sheep plasma[19]; the mechanism of this disappearance remains unclear; and therefore it should not be assumed that LDH 5 disappears from plasma by mechanisms similar to those responsible for its intracellular degradation[20]. Such observations will eventually be compared to turnover studies on intracellular lactate dehydrogenase isozymes based on rates of incorporation and decay of radioactive amino acids in the isozymes. Unfortunately, reports on the turnover of labeled intracellular lactate dehydrogenase isozymes have not been published. Such data are needed to determine whether tissue differences in the synthetic and catabolic rates of individual isozymes are responsible for tissue variations in isozyme pattern.

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