Lactate Dehydrogenase Isozymes: Kinetic Properties at High Enzyme Concentrations

Abstract. *The kinetic properties of lactate dehydrogenase (LDH) isozymes have been determined at high enzyme concentrations. Spectrophotofluorometric assays revealed that the extent of substrate inhibition of LDH-1 and LDH-5 depends on enzyme concentration. At high enzyme concentrations, in the range of those that exist in most mammalian cells, no inhibition by pyruvate occurred. Pyruvate concentrations up to and including 20.0 millimoles per liter were used for each isozyme at 25° and 40°C at pH 7.0 and 7.4. These results suggest that substrate inhibition of LDH may not occur in vivo but only in vitro after appreciable dilution from physiologic enzyme concentrations. These experiments provide further evidence against the theory that substrate inhibition of LDH-1 in vivo accounts for the distribution of LDH isozymes within various tissues. They raise the possibility that, for other enzymes, kinetic properties determined at highly dilute concentrations in vitro may also be quite different from kinetic properties at the much higher concentrations that exist in vivo.*

Many enzymes exist intracellularly in such high concentrations that dilution of tissue homogenates several hundredfold is required before spectrophotometric assay (*1*). These assays are linear and most accurate when the absorbance change is less than 0.100 per minute (*2*). Therefore, the kinetics of many enzymes including lactate dehydrogenase (LDH) have been determined exclusively at highly dilute, and consequently unphysiologic, concentrations (*1*). The kinetic behavior of LDH isozymes is significantly affected by such environmental factors as temperature, *p*H, buffer, ionic strength, and addition of substances including nicotinamide-adenine dinucleotide (NAD) or lactate (*3*). Investigation of the kinetic behavior of LDH-1 and LDH-5 at near physiologic enzyme concentrations seemed important to determine whether the kinetics of LDH-1 and LDH-5 were as different at high, as they were at unphysiologically low, enzyme concentrations. A theory developed to explain the different distributions of LDH isozymes in various tissues is based on kinetic differences between LDH-1 and LDH-5 at low enzyme concentrations (*4*). General biological principles concerning the intracellular function of other enzymes have been deduced from their kinetic properties at dilute unphysiologic enzyme concentrations; therefore, the results of our study may have relevance to enzymes other than LDH.

Both commercial crystalline and partially purified LDH isozymes were used. Crystalline LDH-1 from pig heart and LDH-5 from rabbit muscle were purchased from Calbiochem, Inc; and LDH-1 and LDH-5 were purified from the kidney of adult male Sprague-Dawley rats as previously described (*5, 6*). Protein concentrations of crystalline LDH solutions were measured spectrophotometrically and calculated from their molar extinction coefficients at 280 nm (*7*). Estimation of the concentrations of partially purified LDH-1 and LDH-5 was based on the turnover numbers of the isozymes (*8*). The isozyme content of each LDH preparation was determined with a Millipore cellulose acetate (PhoroSlide) electrophoresis system.

Activities of LDH-1 and LDH-5 were measured over a range of pyruvate concentrations from 0.3 to 20.0 m*M*. Isozyme concentrations attaining 7.0×10^{-6} *M* (based on a molecular weight of 140,000) were used. The NADH (reduced NAD) concentration was 0.56 m*M*. Reagents and enzymes were made up either in 0.1*M* sodium phosphate buffer, *p*H 7.0, or in 0.02*M* tris(hydroxymethyl)aminomethane HCl buffer, *p*H 7.4; assays were performed at both 25° and 40°C. An Aminco-Bowman spectrophotofluorometer equipped with a stopped-flow apparatus (*9*) was used for measurement of LDH activity. The reaction was initiated by mixing a solution of pyruvate and a solution containing LDH and NADH in the 0.2-ml stopped-flow cuvette. The initial linear decrease in NADH fluorescence was followed at 460 nm (340-nm excitation) for 50 msec to 5 seconds. Reactions were recorded on a Hewlett-Packard storage oscilloscope.

At all concentrations, LDH-1 attained maximum activities at 0.6 to 1.0 m*M* pyruvate, whereas LDH-5 reached maximum activity only at 2.0 m*M* pyruvate (Fig. 1). This difference between LDH-1 and LDH-5 has been reported at dilute enzyme concentrations (*3, 4*). However, pyruvate inhibition did not occur at LDH-1 concentrations of 3.5×10^{-7}*M* or higher, nor at LDH-5 concentrations of 1.8×10^{-8}*M* or higher (Fig. 1). At these high, but near physiologic, enzyme concentrations, no loss of maximum LDH activity was detected, even with pyruvate concentrations of 20.0 m*M* (*10*). Even under highly anaerobic conditions, tissue concentrations of pyruvate do not exceed 2.0 m*M* (*11*), although concentrations at certain specific locations within the cell may be somewhat higher.

Table 1. Approximate concentrations of LDH isozymes in rat renal medulla, heart muscle, and skeletal muscle.

Enzyme	Tissue concentrations of LDH isozymes	
	pmole/g	$\times 10^{-7}$ mole/liter*
	Renal medulla	
LDH-1	750	7.50
LDH-2	640	6.40
LDH-3	337	3.37
LDH-4	383	3.83
LDH-5	630	6.30
	Heart muscle	
LDH-1	2340	23.40
LDH-2	970	9.70
LDH-3	532	5.32
LDH-4	77.0	0.77
LDH-5	39.0	0.39
	Skeletal muscle	
LDH-1	52.1	0.521
LDH-2	50.0	0.500
LDH-3	31.5	0.315
LDH-4	214	2.14
LDH-5	1720	17.20

* See text for assumptions made in these calculations.

Pyruvate inhibition appeared at enzyme concentrations of 1.8×10^{-7}*M* for LDH-1 and of 9.0×10^{-9}*M* for LDH-5. Pyruvate inhibition increased progressively as the isozymes were diluted (Fig. 1). The degree of pyruvate inhibition with increasing enzyme dilution was greater for LDH-1 than for LDH-5 (Fig. 1). With high concentrations of LDH-1 or LDH-5, similar results were obtained under more physiologic conditions of *p*H (7.4) and temperature (40°C). With these conditions, high pyruvate concentrations caused no enzyme inhibition. Elevation of the temperature from 25° to 40°C approximately doubled the reaction rate which was unaffected by alterations in *p*H. Identical results were obtained for commercial isozymes and isozymes purified from rat kidney.

To determine where, in relation to the curves in Fig. 1, physiological concentrations of LDH-1 and LDH-5 would lie, we have attempted to calculate the concentrations of these isozymes in vivo. Such calculations are based on assumptions about the volume and mass of various cells and, there-

fore, must be regarded only as approximations. In addition, intracellular concentrations of LDH isozymes and their substrates are compartmentalized rather than uniform (*12*), and our calculations neglect these intracellular differential distributions. Mammalian cell volumes have been reported to be in the range of 10^{-13} to 10^{-11} liter (*13*). These measurements include the nucleus, intracellular spaces, and inclusions such as mitochondria. Because the volume of cytoplasm which contains most of the soluble LDH is substantially less than the total cell volume, our calculations of LDH concentrations based on this figure are underestimations. We assumed an "average" cell to have a volume of 10^{-12} liter and a mass of 10^{-9} g (*14*) in our calculations of the approximate molar concentrations of LDH isozymes in rat renal medulla, heart muscle, and skeletal muscle. Quantities of LDH isozymes in the renal medulla and skeletal and heart muscle were determined by the method of Fritz *et al.* (*15*). Due to differences of cell volume and mass, the molar concentrations given in Table 1 may vary within a range of several fold. Nevertheless, calculations of the approximate intracellular LDH isozyme concentrations are useful and relevant to the discussion. Our estimated value of 7.5×10^{-7} mole/liter for the concentration of LDH-1 in rat renal medulla lies on a curve in Fig. 1 that exhibits no substrate inhibition. A high value (6.4×10^{-7} mole/liter) was also calculated for the tissue level of renal medulla LDH-2. In the heart, cellular concentrations of 23.4×10^{-7} and 9.7×10^{-7} mole/liter were determined for LDH-1 and LDH-2, respectively. Since LDH-2 is similar in kinetic behavior to LDH-1 (*8, 16*), the effective concentration of each isozyme for purposes of resisting substrate inhibition is probably the sum of both. However, even considering separately from LDH-1 the calculated values for tissue concentrations of LDH-2 in renal medulla and in heart muscle, they are on curves that reveal no substrate inhibition (Fig. 1). Tissue concentrations of LDH-5 in rat muscle are also above those concentrations where pyruvate inhibition occurs (Fig. 1). On the basis of these theoretical calculations, pyruvate inhibition of LDH-1 and LDH-5 is probably not of physiological significance.

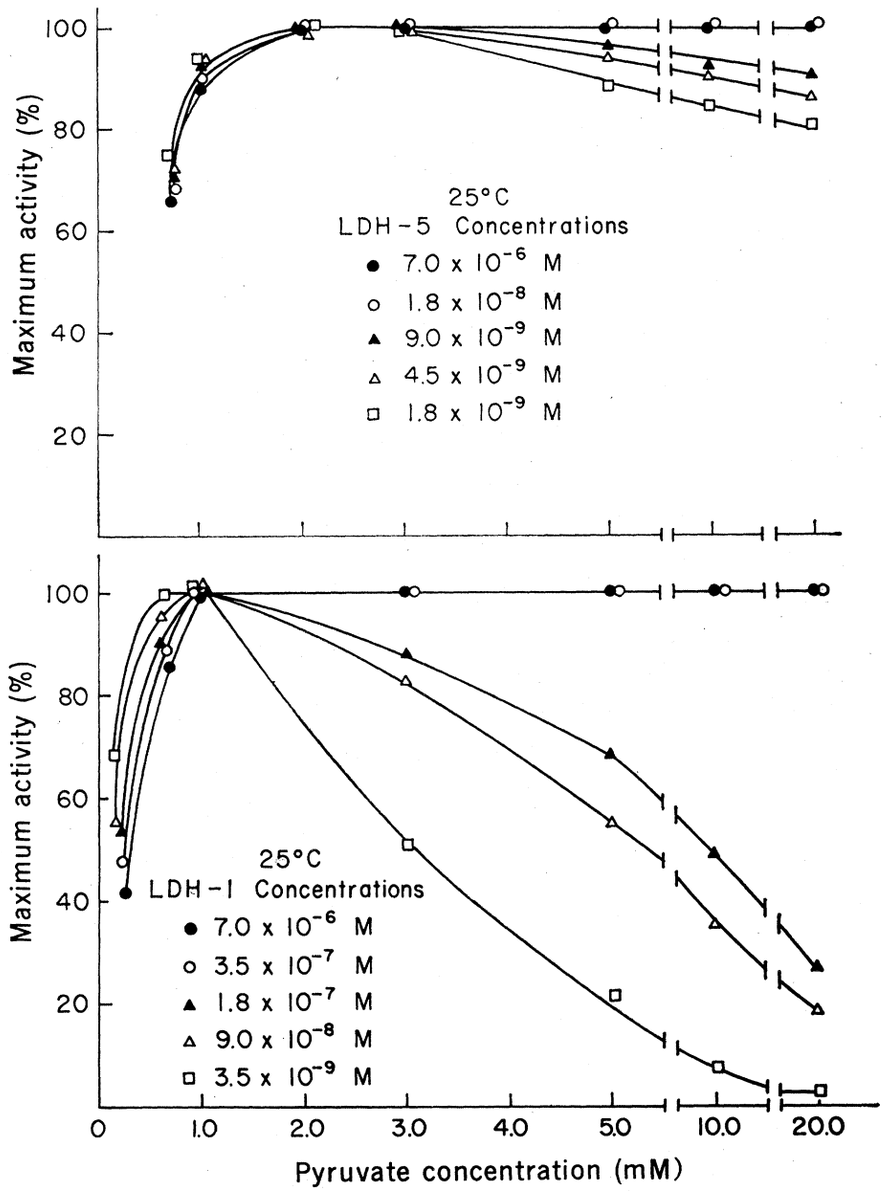

Fig. 1. Effect of increasing pyruvate concentration on the activity of several concentrations of LDH-1 and LDH-5 purified from rat kidney. All reagents were prepared in 0.1*M* sodium phosphate buffer, *p*H 7.0, and the final NADH concentration was 0.56 m*M*. Molar concentrations of partially purified LDH-1 and LDH-5 were calculated from turnover members (*8*). The LDH-1 was purified 130-fold, and LDH-5 was purified 98-fold. The maximum specific activity remained constant over the range of enzyme concentrations examined. With 1.0 m*M* pyruvate, approximately 3.2 μmole of NADH were oxidized per second per milligram of LDH-1, and approximately 4.5 μmole of NADH were oxidized per second per milligram of LDH-5 with 2.0 m*M* pyruvate.

These results demonstrate that LDH kinetics at highly dilute and at physiologic enzyme concentrations are markedly different. Substrate inhibition, which is so prominent in assays with low LDH-1 concentrations, is not detected at physiologic enzyme concentrations, even at pyruvate concentrations of 20.0 m*M*. Substrate inhibition increases progressively as LDH-1 is diluted. These experiments raise objections to the hypothesis that LDH-1 and LDH-5 are distributed in tissues solely according to the extent of their intracellular inhibition by pyruvate (*4*). The following additional criticisms of this theory have previously been raised. (i) In mammals, the liver is a highly aerobic tissue, exhibiting almost exclusively LDH-5; according to the theory, liver, as an aerobic tissue, should contain mainly LDH-1. (ii) The theory predicts that concentrations of pyruvate or lactate sufficiently high to inhibit LDH-1 exist in many so-called anaerobic tissues (*4*). However, pyruvate and lactate concentrations have been investigated in vivo; even in the most anaerobic environments, they apparently do not attain concentrations

required to inhibit even highly dilute solutions of LDH-1 (*11*). Concentrations of NADH, rather than of pyruvate, are rate-limiting for the LDH reaction because under basal and particularly under anaerobic conditions NADH concentrations are appreciably lower than pyruvate concentrations. (iii) Inhibition of LDH-1 by pyruvate, though marked at 6° and 25°C, is greatly diminished at the physiological temperatures of many mammals (*3*, *11*, *17*).

If separate metabolic roles for LDH isozymes do exist, they are apparently based upon factors other than differential inhibition of LDH-1 and LDH-5 by pyruvate. Other metabolic roles recently suggested for LDH isozymes include (i) a regulatory function for LDH-5 which Fritz reported to be an allosteric protein (*18*); (ii) an association of isozymes in different proportions with various subcellular particles, in particular LDH-5 within the nucleus (*19*); (iii) predominance of LDH-5 in rapidly dividing cells or in tissues capable of rapid cell proliferation (*20*); and (iv) a conservative metabolic role in which one isozyme would be required to maintain critical enzymatic function in a tissue where another isozyme was rapidly degraded (*15*).

Our calculation of the LDH concentration in rat kidney reveals that in vivo LDH exists in concentrations several orders of magnitude above those customarily employed for the spectrophotometric assays in which pyruvate inhibition occurs. Those who have assayed LDH activity in mammalian tissue homogenates are acquainted with the requirement of a several hundredfold dilution before spectrophotometric measurement of the rate of NADH oxidation. We have previously suggested that an abortive ternary complex composed of LDH, pyruvate, and NAD may be responsible for the pyruvate inhibition of dilute concentrations of LDH (*6*). At low enzyme concentrations the abortive ternary complex forms rapidly, but it forms very slowly at physiologic LDH concentrations (*6*). This may explain why we did not detect substrate inhibition with high LDH concentrations and why substrate inhibition became pronounced at lower enzyme concentrations.

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10. The initial velocities measured were calculated to involve more than one catalytic turnover even at the highest LDH concentrations examined. In the 0.2-ml reaction mixture $7.0 \times 10^{-9}M$ LDH is equivalent to 1.4×10^{-6} mmole of LDH, which combine with 5.6×10^{-6} mmole of NADH, since each LDH molecule contains four binding sites for the coenzyme. At this concentration of LDH-5, the linear change in relative fluorescence intensity was 0.4 during the initial 50 msec of the reaction. This change corresponds to 4.5×10^{-5} mmole of NADH oxidized and indicates that eight catalytic turnovers have occurred within the 50-msec. interval.
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Membrane Potential as the Sum of Ionic and Metabolic Components

Abstract. *The resting potential of a molluscan neuron can be separated experimentally into two components: one which depends on ionic gradients and permeabilities in accordance with the Goldman equation, and a second which depends on the electrogenicity of active sodium transport.*

According to the ionic hypothesis (*1*), the neuronal resting potential results from ionic concentration gradients across a selectively permeable cell membrane. For some excitable cells, the potassium concentration gradient is primarily responsible for this potential, and the deviations of the membrane from the behavior of a simple potassium electrode have been accounted for by permeability of the membrane to other ions, such as sodium and chloride (*2*, *3*). However, to describe the resting potential of many excitable cells, different mechanisms of electrogenesis must be invoked or the ionic hypothesis modified (*4*, *5*). It has been hypothesized that the resting potential is generated by multiple mechanisms in a heterogeneous membrane (*6*). The questions may be asked whether a resting potential that is not predicted by the ionic hypothesis can be separated experimentally into components and whether a component exists that can be predicted by the ionic hypothesis. We report that the resting potential of a neuronal membrane is separable into at least two components: one which shows a classical dependence on ionic gradients and permeabilities, and a second which depends on temperature and metabolic activity.

Experiments were performed on the giant cell of the gastroesophageal ganglion of the marine mollusk *Anisodoris nobilis* (MacFarland) (*7*). This animal is generally found at water temperatures of 10° to 18°C, but may also live outside this range (*8*). The isolated ganglion was mounted in a linear chamber filled with flowing artificial seawater (478 m*M* NaCl, 10 m*M* KCl, 10.5 m*M* $CaCl_2$, 26 m*M* $MgCl_2$, 29 m*M* $MgSO_4$, and 2.5 m*M* $NaHCO_3$) which approximates the blood of the animal (*9*). Solutions were changed in less than 10 seconds. Test solutions were prepared by exchanging equivalent amounts of sodium and potassium, or by replacing sodium or chlo-