Lactate-to-Pyruvate or Pyruvate-to-Lactate Assay for Lactate Dehydrogenase: A Re-examination

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The pyruvate-to-lactate assay for determining lactate dehydrogenase (EC 1.1.1.27) can now yield linearity equal to or better than that obtained by the lactate-to-pyruvate assay. In addition, there are significant advantages to the pyruvate-to-lactate reaction: (a) a greater change in absorbance per unit time, which allows more accurate spectrophotometric readout; (b) lower reactant concentrations are required, which substantially reduces the cost per assay; (c) solid reactants are used to prepare the assay solution; and (d) reagent solutions are more stable. However, impurities present in commercial NADH preparations may substantially affect measured lactate dehydrogenase activities; therefore, a Standard Reference Material for NADH is being developed for issuance by the National Bureau of Standards.

In recent years, laboratories in the United States have increasingly shifted to measurement of lactate dehydrogenase (LDH, EC 1.1.1.27) in serum by use of the lactate-to-pyruvate (L → P) reaction. For example, in 1971, 56% of laboratories in upstate New York used the pyruvate-to-lactate (P → L) assay, but only 19% of them in 1975 (1). One reason for this shift has been increasing use of analysis with automated equipment that employs the L → P reaction.

Thiers and Vallee (2) studied the LDH assay from both the L → P and P → L directions. They criticized the P → L assay because they found that the absorbance change as a function of time depended greatly on the initial concentration of NADH. The relation was linear for initial NADH concentrations up to about 100 μmol/L; they attributed the nonlinearity with greater NADH concentrations to substrate inhibition by NADH. They also recognized substrate inhibition by pyruvate as an additional disadvantage of the P → L assay. Amador et al. (3) confirmed this poor linearity of the P → L reaction, but they found the L → P reaction to be linearly related to time over a wide range of substrate concentrations, when changes of less than 0.1 A/min were observed for as long as 5 min, and not dependent on NAD+ concentration.

Later, Gay et al. (4) found less substrate inhibition for NADH in the P → L reaction and also a better correlation between measurements made in the L → P and P → L directions for wide ranges of LDH activities than had Thiers and Vallee or Amador et al.

More recently, Sims (5) reported a failure to obtain linearity with the L → P reaction.

Buhl (6-8) studied both the L → P and the P → L reactions, using the separated human isoenzymes LDH-1 and LDH-5, and did not observe NADH substrate inhibition for NADH concentrations as high as 0.24 mmol/L.

In this paper, we show that with the currently available quality of commercial NADH no substrate inhibition is seen with NADH concentrations up to 0.5 mmol/L if the spectrophotometry is accurate. Furthermore, assays run in the P → L direction offer several significant advantages.

Materials and Methods

Materials

A specially purified preparation of NADH, not generally available commercially, was obtained from Boehringer-Mannheim Corp., New York, NY 10017. NADH was also obtained from Calbiochem, La Jolla, CA 92037; P-L Biochemicals, Inc., Milwaukee, WI 53205; and Worthington Biochemical Corp., Freehold, NJ 07728. Sodium pyruvate was obtained from Aldrich Chemical Co., Inc., Milwaukee, WI 53233; Boehringer-Mannheim; Calbiochem; Eastman Organic Chemicals, Rochester, NY 14650; and Fluka Chemische Fabrik, Buchs, Switzerland. NAD+ (Grades III and V), sodium pyruvate, and 2-amino-2-methyl-1-propanol were obtained from Sigma Chemical Co., St. Louis, MO 63178. Lactic acid, ACS reagent grade, was obtained from Fisher Scientific Co., Pittsburgh, PA 15219. Lyophilized human serum (Dade American Medical Hospital Supply Corp., Miami, FL 33152) was reconstituted and used without additional purification as the source of LDH activity for all rate measurements reported in this paper, unless otherwise specified. The isozyme content was found to be 22% LDH-1, 23% LDH-2, 29% LDH-3, 12% LDH-4, and 14% LDH-5 as measured on cellulose acetate plates with a Titan isozyme reagent set (Helena Laboratories, Beaumont, TX 77704). LDH-1 (pig heart) was obtained from Boehringer-Mannheim. Other reagents were of the

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1 In this report, in order to describe procedures adequately, it has occasionally been necessary to identify commercial products and equipment. In no case does such identification imply NBS recommendation or endorsement, nor does it imply that the item identified is necessarily the best available for the purpose.
highest quality commercially obtainable, and were used without further purification.

Equipment

Rate measurements were made with a Model 240 spectrophotometer (Gilford Instruments Laboratories, Inc., Oberlin, OH 44074), which gave wavelength readings within 1 nm of the expected value when checked with a holmium oxide filter. Linearity was checked at 373 nm with use of serial dilutions of potassium chromate solution in 0.05 mol/L KOH (λmax = 373 nm). Instrument response was 2% greater than the linear value at an absorbance of 4, but there was no detectable deviation from linearity at an absorbance of 3. In a cell with a 0.1-cm pathlength, NADH solutions ranging from 0 to 0.500 mmol/L showed no discernible deviation from linearity. Rate measurements were also made with a MS 2 kinetic analyzer (Micromedic Systems, Inc., Philadelphia, PA 19105). The pipetter-diluter used was manufactured by Micromedic Systems. Scans for isoenzyme determination were made with a spectro-densitometer (Model SD3000; Schoeffel Instrument Corp., Westwood, NJ 07675).

Methods

Unless otherwise stated, final concentrations, per liter, used for L → P assay were: 72 mmol of L(+)-lactic acid, 5.5 mmol of NAD+, and 890 mmol of 2-amino-2-methyl-1-propanol buffer (pH 9). Assay temperature was 30 °C. Volumes of reconstituted serum used ranged from 0.1 to 0.5 mL in 3.0 mL of reaction mixture. These conditions are nearly those recommended by Henry (9) for assay in the L → P direction. Final concentrations, per liter, used for the measurement of LDH activity in the P → L direction were: 150 μmol of NADH, 0.1 mmol of pyruvate, 0.1 mmol of human serum per 3 mL of reaction mixture, and 50 mmol phosphate buffer (pH 7.4). The assay temperature was 30 °C unless otherwise stated.

Results and Discussion

Figure 1 illustrates the effect of increasing NADH concentration on the reaction rate of the P → L reaction. The rate relations shown in Figure 1 were verified by repeating the rate measurements in cuvettes with a 0.1-cm pathlength. The rate dependence on NADH concentration was similar to that shown in Figure 1, where a 1-cm pathlength was used. For both sets of experiments there was little or no decrease in rate for NADH concentrations ranging from 100 to 500 μmol/L.

Although the reaction rates were considerably different with each of the four NADH preparations used, none showed the previously reported (2–4) marked decreases (substrate inhibition) when NADH concentrations exceeded 100 μmol/L. Even the poorest-quality commercial NADH that we tested showed a uniform rate over a wide concentration range. It seems probable, therefore, that many currently available commercial preparations contain no substances such as adenosine nucleotides (10) that produce substrate inhibition.

Spectrophotometric inaccuracy at the very high absorbances produced by the high NADH concentrations used might provide data that could be misinterpreted as substrate inhibition. Our failure to detect the previously reported substrate inhibition may be attributed to the quality of the commercially available NADH used for these measurements and to accurate spectrophotometry.

As shown in Figure 2, the reaction rate (enzyme activity) in the P → L direction deviates from linearity by only about 20% with sera having up to 10-fold normal LDH activity, if 100 U/L is taken to be the average normal value for LDH activity. This range of linearity is comparable to that found by Amador et al. (3) and Buhl et al. (6–8) for measurements in the L → P direction.

The problem of inhibition by pyruvate, which results in
nonlinearity in the $P \rightarrow L$ assay, is especially pronounced with LDH from heart tissue (11) and imposes a limitation on the concentration of pyruvate that can be present in the reactant solutions. The inhibition by pyruvate is much larger than that by lactate, and appears to be inherent in the mechanism of reaction. For the scheme

$$R + E \rightleftharpoons E \cdot R \rightleftharpoons E \cdot R \cdot P \rightleftharpoons E \cdot O \cdot L$$

(1)

$$(\text{L})$$

$$E \cdot R \cdot L$$

$$E \cdot O \rightleftharpoons E + O$$

(2)

$$E \cdot O \cdot P$$

(where the symbols R, E, P, O, and L represent, respectively, molar concentrations of NADH, LDH, pyruvate, NAD$^+$, and lactate), accumulations of the “dead-end” complexes $E \cdot O \cdot P$ and $E \cdot R \cdot L$ are intrinsic with build-up of reaction products.

The smaller dissociation constant for $E \cdot O \cdot P$ ($2 \times 10^{-5}$ mol/L, 12) compared with that for $E \cdot R \cdot L$ (0.075 mol/L, 13) explains the greater substrate inhibition observed at high concentrations of pyruvate. [Kaplan (11) has suggested that these complexes function as regulators of LDH activity.]

Figure 3 shows changes in the reaction rates for the $L \rightarrow P$ (a and b) and $P \rightarrow L$ (c) reactions with time for substrate and serum concentrations commonly recommended for clinical assay. The absorbance changes at 340 nm with time found for the $P \rightarrow L$ reaction at commonly recommended substrate concentrations is 0.1 absorbance unit (A) per min, whereas that for $L \rightarrow P$ was seen to be only 0.01 A/min. The larger rate of change in absorbance accompanying the $P \rightarrow L$ reaction allows for a substantially more accurate spectrophotometric readout. In either direction, for the first 3 min, the reaction rate is seen to be nearly linear, after which there are substantial deviations from linearity. In the $P \rightarrow L$ direction the deviation from linearity occurs as equilibrium is approached. In the $L \rightarrow P$ direction, the reason for the initial rate increase is not known. A small initial rate increase with time has been repeatedly observed in our measurements when we used the conditions ordinarily used for clinical assay. This increase was greater with 2-amino-2-methyl-1-propanol than with phosphate buffer under similar conditions. A rate increase within the first 20 s of the $L \rightarrow P$ reaction has also been reported by Buhl (6). For the $P \rightarrow L$ assay, the observed deviations from linearity with time indicate that rate measurements are best made as soon as temperature equilibrium is attained (after 30 s for many instruments) and within the first 2 min.

Figure 4 illustrates the dependence of NAD$^+$ reduction rate on NAD$^+$ concentration. Here, the highest-quality NAD$^+$ commercially obtainable was used. This study shows a small amount of substrate inhibition at the highest concentrations of NAD$^+$ used. Substrate inhibition was observed for both phosphate and bicarbonate buffers (pH 9) at 10 and 20 mmol
of lactate per liter. Additional experiments showed that measured reaction rates decreased steadily as NAD$^+$ concentrations are increased to higher values than those shown in Figure 4.

Large amounts of substrate are required for the $L \rightarrow P$ assays because of the highly unfavorable equilibrium (14).

$$\frac{[O][L]}{[R][P][H^+]^2} = 2.7 \times 10^{11}$$

With the required large concentrations of lactic acid (143 mmol of the racemic mixture per liter) and of NAD$^+$ (5.5 mmol/L), the expense per test is clearly greater than that for the tests run in the $P \rightarrow L$ direction, for which $150$ μmol of NADH and $1.2$ mmol of pyruvate per liter are often used.

Accurate rate measurement by the $P \rightarrow L$ assay requires the use of quality reagents. Our tests on commercially available sodium pyruvate indicate that reagent-grade material is of adequate quality for $P \rightarrow L$ assay. NADH stability during prolonged storage has been questioned. Therefore, we tested high-quality NADH by storing it for more than a year, protected from oxygen and moisture, and found kinetically insignificant deterioration. Although deterioration of NADH in solution appears to be autocatalytic, we have also found that solutions of good-quality NADH change little in kinetic properties when stored for as long as a week at pH 7.9 and 4 °C. In contrast, our results, as well as those of Lowry et al. (15) and Gallati (16), show that at pH 9, the pH recommended for $L \rightarrow P$ assay, NAD$^+$ undergoes appreciable degradation during 8 h.

The principal problem with the $P \rightarrow L$ assay is that commercial preparations of NADH may contain inhibitors. We have found that commercially available NADH preparations frequently differ in activity by 15%, so that a reference NADH is needed for normalization of NADH activity if LDH determinations are to be accurate. Workers at NBS plan to issue NADH as a Standard Reference Material in the near future. However, inhibitors have also been reported in commercial preparations of NAD$^+$ (17), so that careful attention to reagent quality is essential for LDH assays performed with the reaction going in either direction.

The chief objection to the $P \rightarrow L$ method has been the nonlinear relation between reaction rate and serum LDH activity for measurements made in that direction (3). In our measurements we found the linearity of the relationship between reaction rate and LDH activity in serum for the $L \rightarrow P$ reaction to be equal to or better than that for the $L \rightarrow P$ reaction.

Use of the $P \rightarrow L$ reaction offers important advantages. These include (a) better measurement accuracy because of the greater rate of change in absorbance, (b) lower reagent cost because of the smaller concentrations required, (c) the use of solid reagents for preparation of assay solutions, and (d) greater stability of the working reagents once they are placed in solution.

References