Optimal Conditions for Assaying Human Lactate Dehydrogenase by the Lactate-to-Pyruvate Reaction: Arrhenius Relationships for Lactate Dehydrogenase Isoenzymes 1 and 5

Steven N. Buhl, Kathryn Y. Jackson, Rita Lubinski, and Raymond E. Vanderlinde

Optimal reaction conditions to assay human lactate dehydrogenase (lactate-to-pyruvate) were established for isoenzymes 1 and 5 at 25, 30, and 37 °C in diethanolamine and 2-amino-2-methyl-1,3-propanediol. Different substrate concentrations are required at each temperature. The conditions permit measurement of lactate dehydrogenase 1 and 5 with the lowest substrate concentrations that allow for the highest equal sustainable efficiency in measuring both isoenzymes. About 95% of each isoenzyme activity is measured if the assay is performed within the first minute after the reaction is initiated even for activities as high as triple the upper limit of normal. The Arrhenius relationship is different for each isoenzyme, but results obtained for each at one temperature can be compared with results at another temperature by use of simple conversion equations. Assays at 25 and 30 °C are more economical and less variable than assays at 37 °C.

Additional Keyphrases: activation energies for LD 1 and 5 enzyme activity, optimal conditions

Our previous studies have shown that the several methods for determining lactate dehydrogenase (LD) by the lactate-to-pyruvate reaction do not give equivalent results for highly purified human LD or for serum samples (1, 2). Examination of the reaction conditions showed that one commercial lactic acid gave low LD activities (1), and Baboon and Arndt had found that not all commercial NAD+ preparations gave the same results for LD (3).1

In addition, there are physical and chemical difficulties with use of the four most frequently used buffers (2). Increasing concentrations of pyrophosphate or glycine are inhibitory to LD 1 activity. If tris(hydroxymethyl)methylamine or glycine is used under conditions optimal for both LD 1 and LD 5, the pKa of the buffer is so far removed from the optimal reaction pH for either isoenzyme, that there would be little buffering capacity at the reaction pH. The difficulties with the use of 2-amino-2-methyl-1-propanol are that (a) the pH optima for LD 1 and LD 5 are so greatly separated that no one pH will enable quantitative assays of both isoenzymes; (b) 2-amino-2-methyl-1-propanol is highly stimulatory to LD 1 activity; (c) interaction of 2-amino-2-methyl-1-propanol with the substrates NAD+ and lactate during reagent preparation lead to diminished LD 5 activity.

Our studies did show that two buffers met stringent chemical and physical criteria for use in LD determinations: diethanolamine and 2-amino-2-methyl-1,3-propanediol (2). We recommended using either buffer at 200 mmol/liter at pH 8.7, although we preferred diethanolamine because it is cheaper.

In this report we extend our observations on diethanolamine and 2-amino-2-methyl-1,3-propanediol by presenting substrate saturation curves for both lactate and NAD+; optimal reaction conditions at 25, 30, and 37 °C for purified preparations of human LD 1 and LD 5; and the Arrhenius relationships for these two isoenzymes assayed in diethanolamine, 2-amino-2-methyl-1,3-propanediol, 2-amino-2-methyl-1-propanol, tris(hydroxymethyl)methylamine, and pyrophosphate.

Materials and Methods

β-NAD+ (Grade III and Grade V), Li L(+)-lactate, and tris(hydroxymethyl)methylamine were purchased from Sigma Chemical Co., St. Louis, Mo. 63178; β-NAD+ (Grade I) from Boehringer Mannheim (now called Bio-Dynamics/bmc), Indianapolis, Ind. 46250; pyrophosphate and diethanolamine from J. T. Baker Chemical Co., Phillipsburg, N.J. 08865; and 2-amino-2-methyl-1-propanol and 2-amino-2-methyl-1,3-propanediol from Aldrich Chemical Co., Milwaukee, Wis. 53233.

1 Nonstandard abbreviations used: LD, lactate dehydrogenase (l-lactate:NAD+ oxidoreductase, EC 1.1.1.27); and ΔA, change of absorbance.

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LD 1 was prepared from outdated human erythrocytes and LD 5 was purified from human liver by a modification of the Burd and Usategui-Gomez procedure (4). The enzyme assays were done with either an LKB 8600 (LKB Instruments, Rockville, Md. 20852) or a Gilford 2000 (Gilford Instruments, Oberlin, Ohio 44074). Change of absorbance per minute (ΔA/min) at 340 nm was calculated from two absorbance readings taken on the LKB 8600 at 6-s intervals, with 2 s between adjacent measuring intervals. All reactions were started by adding enzyme in a volume equal to 0.083 of the total reaction volume.

Results

When LD activity is assayed in diethanolamine or 2-amino-2-methyl-1,3-propanediol at saturating concentrations of NAD⁺, the amount of lactate required for saturation increases with increasing temperature (Figure 1). LD 1 becomes saturated at lower lactate concentrations than LD 5. At high concentrations of lactate LD 1 becomes substrate-inhibited, but LD 5 does not. LD 5 appears to be temperature-labile.

When LD activity is assayed in the same buffers at saturating concentrations of lactate, the amount of NAD⁺ required for saturation also increases with increasing temperature (Figure 2). In contrast to the lactate profiles, there is no substrate inhibition, and LD 1 and LD 5 have similar responses.

Three different grades of commercial NAD⁺ were examined for any effects on LD activity, two grades from Sigma and one from Boehringer Mannheim. No differences were found for LD 1 and LD 5 assayed in diethanolamine, 2-amino-2-methyl-1,3-propanediol, or tris(hydroxymethyl)methylamine at NAD⁺ concentrations ranging from 1 to 5 mmol/liter.

We then attempted to select the optimal concentrations of lactate and NAD⁺ for the LD lactate-to-pyruvate assay. Three criteria were established: Each must be the lowest saturating concentration that (a) allows for the highest equal efficiency in measuring both isoenzymes, (b) maintains constant product formation (ΔA) over time for 1 min, and (c) gives a linear regression for serial dilutions of activities ranging from midnormal to about triple the upper limit of normal.

Unfortunately, no combination of substrate concentrations gave a constant ΔA for 60–90 s for either LD 1 or LD 5 at activities near and at about triple the upper limit of normal (Figures 3 and 4). The plots were curvilinear, rising to the maximum ΔA/min at about 20 s, and decreasing thereafter. The apparent increase before 20 s probably reflects deficiencies in instrumental response and experimental design, rather than any chemical attributes of the reaction. The decrease has two components, one of which was affected by adding substrate and the other not. The loss in ΔA/min associated with substrate depletion was more apparent for LD 5 than for LD 1. With increasing lactate concentration we saw both substrate inhibition and a decrease in ΔA/min with LD 1, implying that the decrease in ΔA/min does not result from substrate depletion. At 25 and 30 °C the decrease in ΔA/min was similar for LD 1 and LD 5, whereas at 37 °C the decrease in ΔA/min
was greater for LD 5, making it more difficult to measure each isoenzyme with the same efficiency at 37 °C than at 25 or 30 °C.

We then tested the reaction conditions that most nearly met our definition of optimal (Table 1), to determine whether they gave results that were linearly related to serial dilutions of LD activities. There was a linear relation at each temperature for activities from midnormal to three times the upper normal range (Figure 5), and activities were similar in both buffers.

The Arrhenius relationships for LD 1 with diethanolamine, 2-amino-2-methyl-1,3-propanediol, and three other buffers are linear between 25 and 45 °C (Figure 6), and the regression equations are essentially the same (Table 2). However, 2-amino-2-methyl-1-propanol is a stimulant and pyrophosphate an inhibitor of LD 1. These phenomena are more evident at the lower temperatures and are reflected in the differences in the equations. The equation of the line for all the data points is: log U/liter = -3.4(1/TK) + 13.4 (TK is temperature Kelvin).

The Arrhenius relationships for LD 5 with the same buffers are linear between 25 and 40 °C, but at temperatures above 45 °C the activities are lower than the regression predicts. The regression equations for each of the five buffers are similar. The equation for all the data below 41 °C is: log U/liter = -2.43 (1/TK) + 10.1.

The Arrhenius relationships for LD 1 and LD 5 are different. The activation energy of LD 1 is 15.55 kcal/mol; that of LD 5 is 11.11 kcal/mol.

Because LD is commonly assayed at any of three temperatures, it is frequently convenient to interconvert the data. Conversion equations were determined (Table 3) for the data from Figure 5, which was determined with the recommended optimal conditions. The exis-
Fig. 3. Change of absorbance per minute as a function of time for LD 1 and LD 5 assayed in diethanolamine at 25, 30, or 37 °C. Diethanolamine was at 200 mmol/liter, pH 8.7. NAD⁺ and lactate were at the concentrations indicated (mmol/liter). Each point is a mean of eight assays. (To obtain U/liter values, ΔA/min should be multiplied by 1.938). Two levels of enzyme activity were examined at each temperature.

Fig. 4. Change of absorbance per minute as a function of time for LD 1 and LD 5 assayed in 2-amino-2-methyl-1,3-propanediol at 25, 30, or 37 °C. All conditions were as shown above and as described in Figure 3.
Table 1. Optimal Reaction Conditions for Assaying Human LD (Lactate-to-Pyruvate) in Diethanolamine or 2-Amino-2-methyl-1,3-propanediol (200 mmol/liter) at pH 8.7

<table>
<thead>
<tr>
<th>Temperature, °C</th>
<th>NAD⁺</th>
<th>l Lactate mmol/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>3</td>
<td>40</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>37</td>
<td>7</td>
<td>70</td>
</tr>
</tbody>
</table>

Table 2. Arrhenius Relationships for Human LD Assayed with Five Buffers (y = mx + b)

<table>
<thead>
<tr>
<th>Buffer</th>
<th>LD 1</th>
<th>LD 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diethanolamine</td>
<td>y = -3.26x + 12.9</td>
<td>y = -2.39x + 9.9</td>
</tr>
<tr>
<td>2-Amino-2-methyl-1,3-propanediol</td>
<td>y = -3.29x + 13.0</td>
<td>y = -2.27x + 9.5</td>
</tr>
<tr>
<td>Tris(hydroxymethyl)methylamine</td>
<td>y = -3.35x + 13.4</td>
<td>y = -2.40x + 10.2</td>
</tr>
<tr>
<td>2-Amino-2-methyl-1-propanol</td>
<td>y = -3.03x + 12.2</td>
<td>y = -2.39x + 10.0</td>
</tr>
<tr>
<td>Pyrophosphate</td>
<td>y = -3.49x + 13.6</td>
<td>y = -2.42x + 9.9</td>
</tr>
</tbody>
</table>

* log U/liter = (slope)(1/Tₐ) + intercept.

Table 3. Interconversion of Results for LD, Obtained at 25, 30, and 37 °C with Use of Diethanolamine or 2-Amino-2-methyl-1,3-propanediol Buffer (y = mx + b)

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Temperature (°C)</th>
<th>LD 1</th>
<th>LD 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diethanolamine</td>
<td>25 30</td>
<td>y = 1.43x + 1.8</td>
<td>y = 1.47x + 4.8</td>
</tr>
<tr>
<td></td>
<td>25 37</td>
<td>y = 2.13x + 0.6</td>
<td>y = 2.33x + 1.3</td>
</tr>
<tr>
<td></td>
<td>37 30</td>
<td>y = 0.62x + 1.2</td>
<td>y = 0.63x + 4.9</td>
</tr>
<tr>
<td>2-Amino-2-methyl-1,3-propanediol</td>
<td>25 30</td>
<td>y = 1.4x + 6.9</td>
<td>y = 1.44x + 1.8</td>
</tr>
<tr>
<td></td>
<td>25 37</td>
<td>y = 2.53x + 13.4</td>
<td>y = 2.31x + 0.6</td>
</tr>
<tr>
<td></td>
<td>37 30</td>
<td>y = 0.55x + 0.2</td>
<td>y = 0.62x - 1.2</td>
</tr>
</tbody>
</table>

Combined data for both isoenzymes and both buffers

<table>
<thead>
<tr>
<th></th>
<th>Temperature (°C)</th>
<th>LD 1</th>
<th>LD 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 30</td>
<td>y = 1.46x + 1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 37</td>
<td>y = 2.5x + 0.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37 30</td>
<td>y = 0.58x + 2.5</td>
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</table>

Fig. 5. LD activity at 25, 30, or 37 °C as a function of enzyme dilution
Each point shown is an average of eight determinations. Open symbols, diethanolamine; closed symbols, 2-amino-2-methyl-1,3-propanol

The presence of intercepts in the equations probably indicates that the deviation from linearity of product formation is different for each temperature.

For five of the six temperature/buffer pairs, the equations for LD 1 and LD 5 differ by less than 10% (the difference is 13% for 2-amino-2-methyl-1,3-propanediol at 30 vs. 37 °C). Because of the need to retain simplicity in converting results, we ignored this difference and combined the data from both isoenzymes and
both buffers to calculate the general interconversion equations for all three temperature pairs (Table 3).

**Discussion**

Choosing the optimal concentration of lactate for each temperature was difficult, because excess lactate inhibits LD 1, whereas LD 5 requires large amounts (relative to LD 1) of lactate for saturation. The amounts of lactate recommended as optimal permit each isoenzyme to be determined with equal efficiency (>95%) if assayed within the first minute after the reaction is initiated for activities up to about three times the upper limit of normal.

LD 1 and LD 5 have nearly identical NAD+ saturation curves at each temperature. The most difficult criterion to fill was the determination of the lowest concentration of NAD+ which would maintain linearity at higher LD activities. The lowest concentration of reagents is desirable to make the assay as economical as possible.

The lack of linearity in product formation (constant ΔA/min) has several components, which have not been separated in previous studies (5–7). The initial increase (≤20 s) is probably due to experimental design or deficiencies in instrument response. The decrease after 20–30 s has more than one component, including substrate depletion and also probably inhibition by the product. Substrate depletion is overcome by simply adding additional substrate (5). Attempts to remove the product formed, by reaction with semicarbazide, have met with mixed success (6, 7), probably because of the influence of unrecognized additional factors. Any new studies on this problem should be done with isolated isoenzymes and with attention to specific contributing factors.

The apparent $K_m$ for lactate and NAD+ for nonhuman LD increase with temperature (8–10). Our findings that increased amounts of lactate and NAD+ are required for saturation with increasing temperature is consistent with these observations.

We have established reaction conditions under which LD 1 and LD 5 are measured with equal efficiency. Assuming that the other three LD isoenzymes are measured equally well, these conditions should be appropriate for determining total LD activity in both normal and pathological sera, as well as individual isoenzyme determinations from column procedures.

We recommend that all LD measurements be done by use of the lactate-to-pyruvate reaction be made within 60 s after the reaction is started, or as quickly as the instrument allows. Longer measuring times can be used if the volume fraction of enzyme is decreased.

We also recommend that these assays be performed at 25 or 30 °C because at these lower temperatures (a) the reaction response is linear for a longer time, (b) less reagents are needed, and (c) less interlaboratory and intralaboratory variation will be encountered on samples measured with various instruments and on different isoenzymic mixtures having similar total LD activity.

Although it would seem more precise to have higher assay values, our results show this not to be true. Be-
cause of an appreciably different decrease in $\Delta A$/min for LD 1 and LD 5 at 37 °C but not at 30 or 25 °C, it is more difficult to measure each isoenzyme at 37 °C with equal efficiency, and the difference in efficiency becomes greater with time after the reaction is started. Consequently, any assay of an isoenzymic mixture with supranormal LD activity will have a large interinstrument variability. More intra-instrument variability will also be encountered at 37 °C than at 30 or 25 °C in samples with the same total LD activity but differing isoenzymic mixtures. This single-instrument variation will be greatest for instruments which measure LD activity 1 min or more after initiation.

Unless the measuring-time interval is standardized, perhaps relative to a constant $\Delta A$/min for some reasonable enzyme activity, assays of LD, measured lactate-to-pyruvate, will show less variation attributable to instrument and isoenzyme content at 25 or 30 °C than at 37 °C.

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References