Optimal Conditions and Comparison of Lactate Dehydrogenase Catalysis of the Lactate-to-Pyruvate and Pyruvate-to-Lactate Reactions in Human Serum at 25, 30, and 37 °C

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We report optimal conditions for assaying highly purified human lactate dehydrogenase isoenzymes with the lactate-to-pyruvate and pyruvate-to-lactate reactions, as they apply to human serum. Interconversion of results between reactions is not practicable. Measurements of lactate dehydrogenase in either reaction direction at 25, 30, or 37 °C can be equally reliable if the volume fraction and the resulting ΔA/min is small. However, for interinstrument and interlaboratory comparisons, results from the lactate-to-pyruvate reaction are more reliable.

Lactate dehydrogenase (LD; EC 1.1.1.27) can be assayed by the change in either lactate or pyruvate, by monitoring the change in absorbance that accompanies NADH formation or oxidation. Numerous "optimal" methods for assaying LD activity have been proposed; the definition of an optimal method has varied from that in which activity in normal serum is greatest to that in which the activity is measured in the same volume of a mixture of isoenzymes.

We have previously examined purified human isoenzymes LD 1 and LD 5 to determine the optimal conditions for assaying mixtures of LD isoenzymes at 25, 30, and 37 °C (1, 2). Two difficulties in choosing optimal substrate concentrations were (a) finding the substrate concentrations that would saturate one isoenzyme while not inhibiting the other and (b) finding the combination of substrate concentrations that would sustain the activities of both isoenzymes with equal efficiency after the reaction was initiated.

Here, we show the optimal conditions determined from our studies on the purified isoenzymes apply to human serum.

Materials and Methods

Purified human LD 1 and LD 5 were prepared and assays were performed as previously described (1, 2), except that in some experiments absorbance was read at 3-s intervals with a Model 250 spectrophotometer with a Model 533 enzyme programmer (Gilford Instruments, Oberlin, Ohio 44074).

Serum pools were made by combining normal sera collected a day earlier. We simulated pathological sera by trebling the activity by adding LD 1 or LD 5 or both. Reactions were initiated by adding pyruvate or enzyme for the pyruvate-to-lactate (P → L) or enzyme for the lactate-to-pyruvate (L → P) reactions. The ratio of serum to total reaction volume was usually 1/20. The pH of the buffers was adjusted or measured at the reaction temperatures. Serum LD isoenzyme patterns were visualized after electrophoresis and staining with the Microzone System (Beckman Instruments Inc., Fullerton, Calif. 92634). The serum pool was considered normal when the activities were <150 U/liter at 30 °C and when the electrolytic pattern showed LD 2 to be predominant.

Results

Buffer effects. Human sera were assayed at 30 and 37 °C with five buffers: imidazole, triethanolamine hydrochloride (TEA), diethanolamine (DEA), 2-amino-2-methyl-1,3-propanediol (AMPdiol), and 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]aminoethanesulfonic acid (TES). Activities varied slightly (<10%) at both temperatures with increasing concentrations of imidazole and TEA when assayed P → L and with concentrations of DEA and AMPdiol > 30 mmol/liter when assayed L → P (Figure 1). Increasing concentrations of TES inhibited the P → L reaction. Results were similar in identical experiments on six different pools of human sera. Either DEA or AMPdiol and TEA or imidazole is suitable for assaying LD in the L → P or P → L direction, respectively. However, in previous studies slight advantages were found with DEA and imidazole (1, 2).

pH effects. The pH optimum of an LD mixture depends on the isoenzyme content (1, 2). The pH optimum of normal serum is 7.2 for the P → L reaction and >9 for the L → P reaction (Figure 2). Adding LD 5 to the serum pools decreased the pH optima. The recommended pH optima had been chosen such that each isoenzyme would be measured with equal efficiency.

Rate of loss of reaction. Our previously recommended optimal concentrations (Table 1) and other likely substrate concentrations were used to assay normal serum and simulated pools of pathological serum, to determine whether LD activity would be sustained (Figure 3). The optimal conditions, designed to measure reliably activities up to triple the upper limit of normal, did apply to both the normal and simulated pathological human sera. As previously reported, however, the higher activities, whether assayed P → L or L → P were lower (P ≤ 0.05) 1 min after reaction initiation than within the first 22 s (1, 2).

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1 Nonstandard abbreviations used: LD, lactate dehydrogenase (L-lactate:NAD+ oxidoreductase, EC 1.1.1.27); ΔA, change in absorbance; TEA, triethanolamine; DEA, diethanolamine; AMPdiol, 2-amino-2-methyl-1,3-propanediol; P → L, pyruvate to lactate; and L → P, lactate to pyruvate.

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The observed activity decreased more rapidly when assayed at 30 °C than when assayed at 25, 30, or 37 °C. When equal amounts of serum were used for each assay at all three temperatures, the slopes of the lines at 25, 30, and 37 °C were for L → P = 0.025, -0.045, and -0.050, respectively, and for P → L (curve A) = -0.14, -0.49, and -0.81 (these slopes are the averages from both buffers at each temperature).

When amounts of serum were adjusted so that the ΔA/min of the P → L reaction equaled that of the L → P reaction at 30 and 37 °C, the slopes of the P → L reaction with the lower ΔA/min (Figure 4) at 30 and 37 °C were -0.19 and -0.18, respectively (the slope of P → L at 25 °C was 0.00).

For enzyme activities above the normal range, the loss of observed activity for equal amounts of enzyme was greater after 1 min at 37 °C than at 30 or 25 °C, owing solely to increased activities at the higher temperature (1, 2); that is to say, for a similar change in absorbance (ΔA) with time the loss was similar at 25 and 37 °C. The slope of curve A at 25 °C is -0.14 and the slope of the lower P → L curve at 37 °C is -0.18. The ΔA of the serum is -0.140 per minute.

### Table 1. Optimal Conditions for Assaying Human LD

<table>
<thead>
<tr>
<th>Reaction and temperature, °C</th>
<th>Substrate concn, mmol/liter</th>
<th>β-NAD⁺</th>
<th>Li lactate</th>
<th>β-NAD⁻</th>
<th>Pyruvate</th>
<th>Pyruvate-to-lactate</th>
<th>pH</th>
<th>M/min</th>
<th>Activity</th>
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<tbody>
<tr>
<td>Lactate-to-pyruvate³</td>
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<td></td>
<td>3</td>
<td>40</td>
<td></td>
<td>7</td>
<td>70</td>
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<td>7</td>
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<tr>
<td>Pyruvate-to-lactate</td>
<td></td>
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<td>0.15</td>
<td>1.5</td>
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<tr>
<td>25</td>
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<td>0.15</td>
<td>1.0</td>
<td></td>
<td>0.22</td>
<td>1.5</td>
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<tr>
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<td>0.22</td>
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</tr>
</tbody>
</table>

³ Ref. 1. Buffers: DEA or AMPdiol (200 mmol/liter, pH 8.7).

⁴ Ref. 2. Buffers: imidazole or TEA (100 mmol/liter, pH 7.0).
Analytical recovery. For serum pools supplemented with LD 1 or LD 5 we could account identically and nearly completely for each isoenzyme, whether assayed L → P or P → L under the suggested optimal conditions (Figure 5).

Interconversion. Finally we examined the feasibility of interconverting P → L with L → P results. Unfortunately the relationship between the L → P and P → L reactions differs for each isoenzyme (P < 0.05) (Table 2). Unless the proportion of each isoenzyme in a mixture is known, it would not be practicable to interconvert L → P and P → L results.

Discussion

Reaction conditions determined with highly purified human LD isoenzymes to measure each isoenzyme with most nearly equal efficiency evidently apply to both normal and simulated pathological human sera.

The advantages of our recommended optimal conditions are that the buffers chosen do not inhibit or stimulate enzyme activity and their \( pK_a \) are near the reaction pH. Thus the buffer does not enter into the reaction as an effector, and buffering capacity is maximum. The substrate concentrations and pH chosen favor neither LD 1 or LD 5, but do measure each activity with >95% efficiency near the start of the reaction. Hence these conditions will adequately quantitate any isoenzyme mixture or effluents from column or electrophoretic separations.

Choice of reaction temperature. The optimal conditions and enzyme response vary with reaction temperature. Less substrate is required at lower temperatures. This can mean substantial savings in reagent costs for large laboratories, though in the short run these may be offset by required modifications or purchase of equipment. Most present instruments are predominately intended for use with 37 or 30 °C.

In general, the results appear to be more reliable at 25 than at 30 or 37 °C because the \( \Delta A/\text{min} \) is more nearly constant at the lower temperature, solely because enzyme activities are lower at the lower temperatures. Regardless of which temperature is chosen, the results will be most reliable when the
volume fraction is reduced to give smaller $\Delta A$/min and the measuring interval is short (1, 2, and Figures 3 and 4).

Choice of measuring interval. The nonlinearity of the reaction response indicates that there will be difficulties in comparing assay results from instruments with which either different measuring times or different volume fractions are used. The $P \rightarrow L$ reaction should be measured as soon as possible after the start of the reaction, and the measuring interval should be reasonably short (<10 s). The $L \rightarrow P$ reaction should be measured within the first 40 s.

Any future reference method or selected method should include a recommendation for the time and duration of the measuring interval after the reaction is initiated. The most logical variables to be standardized are $\Delta A$/min and the allowable per cent decrease in $\Delta A$/min as a function of time.

Choice of reaction. Neither the $P \rightarrow L$ nor the $L \rightarrow P$ reaction response is linear. However, at equal $\Delta A$/min the activity of the $L \rightarrow P$ reaction does not decrease as rapidly as the activity of the $P \rightarrow L$ reaction (Figure 4). Thus it is probably more reliable and will give less variability in interinstrument and interlaboratory comparisons (1, 2).

Two other problems should also be considered in the choice of reaction for the LD assay. Some available preparations and improperly stored $\beta$-NADH contain inhibitors of LD (3, 4), whereas many $\beta$-NAD$^+$ preparations are free of LD inhibitors. The mode of reaction initiation affects the results of the $L \rightarrow P$ reaction, but not of the $P \rightarrow L$ reaction (5).

Reliable results for assays of LD can be obtained with either the $P \rightarrow L$ or $L \rightarrow P$ reaction at 25, 30, or 37 °C. The choice of reaction and temperature depends on the instrumentation and volume fraction. Instruments with short measuring times immediately (<20 s) after initiation and capable of using small volume fractions can use the $P \rightarrow L$ reaction. Instruments with longer measuring times and large volume fractions should use the $L \rightarrow P$ reaction. The higher temperatures should not be used unless the volume fraction and the resulting $\Delta A$/min is small.

References