Measurement of Total Lactate Dehydrogenase Activity

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ABSTRACT

Lactate dehydrogenase (LD: EC 1.1.1.27) is the most important clinically of several dehydrogenases occurring in human serum. Lactate dehydrogenase is cytoplasmic in its cellular location and in any one tissue is composed of one or two of five possible isoenzymes. While many of its clinical applications involve quantification of one or more specific serum isoenzymes, an estimate of total LD is required usually.

Lactate dehydrogenase catalyzes the reversible reaction: L-lactate + NAD\(^+\) ⇌ pyruvate + NADH. The bidirectional reaction is monitored spectrophotometrically by measuring either the increase in NADH at 340 nm produced in the lactate-to-pyruvate reaction (L → P) or by the decrease in NADH at 340 nm produced in the pyruvate-to-lactate (P → L) reaction. Kinetic assay systems for the measurement of the reaction system in both directions are comprehensively reviewed as well as the standardization efforts proposed to date.

Introduction

Dehydrogenases is the preferred name for the group of enzymes which belong to the oxidoreductase class of enzymes in which the substrate oxidized is regarded as a hydrogen donor and the usual acceptor is nicotinamide-adenine dinucleotide (NAD\(^+\)) or nicotinamide-adenine dinucleotide phosphate (NADP\(^+\)). Lactate dehydrogenase (LD: EC 1.1.1.27) (table I) is the most important dehydrogenase from the clinical viewpoint. Others measured much less frequently include malate dehydrogenase (MD), glutamate dehydrogenase (GLDH), isocitrate dehydrogenase (ICD), and sorbitol dehydrogenase (SDH).

Lactate dehydrogenase (LD) has a molecular weight of about 140,000 daltons and is cytoplasmic in its cellular location. It is composed of four peptide chains of two types, H and/or M subunits, thereby creating isoenzymes. LD 1, the heart type isoenzyme, consists of four H subunits, whereas LD 5, the muscle/liver type isoenzyme, consists of four M subunits with the intermediary forms being combinations of subunits.

Lactate dehydrogenase catalyzes the reversible reaction:

L-lactate + NAD\(^+\) ⇌ pyruvate + NADH.

The bidirectional reaction is monitored spectrophotometrically by measuring...
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One of these substrates, 2-oxobutyric acid, has been used "preferentially" to determine serum LD 1 (heart-type) activity. The reaction, known as B-hydroxybutyric dehydrogenase (HBDH), involves: $2\text{HBDH} + \text{2-oxobutyric acid} + \text{NADH} \rightarrow \text{2-hydroxybutyric acid} + \text{NAD}^+$ and is monitored by measuring the decrease in absorbance of NADH at 340 nm. The evidence indicates LD is the enzyme involved in the reduction of glyoxalate which has been found to be enzymatically reduced by human serum faster at 37°C than either pyruvate or 2-oxobutyrate.

However, the measurement of total LD activity in serum or other body fluids usually uses either lactate or pyruvate as substrate and is attributable to a mixture of isoenzymes which varies with an individual's physiological and pathological conditions.

### Pyruvate-to-Lactate Methodologies

The conditions for the primary continu-

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**TABLE I**

Abbreviation of Enzymes with Classification Numbers*

<table>
<thead>
<tr>
<th>Classification Numbers*</th>
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<tbody>
<tr>
<td>Dehydrogenases</td>
</tr>
<tr>
<td>Lactate dehydrogenase (LD)</td>
</tr>
<tr>
<td>Malate dehydrogenase (MD)</td>
</tr>
<tr>
<td>Glutamate dehydrogenase (GLDH)</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase (ICDH)</td>
</tr>
<tr>
<td>Sorbitol dehydrogenase</td>
</tr>
<tr>
<td>Others</td>
</tr>
<tr>
<td>Alkaline phosphatase (ALP)</td>
</tr>
<tr>
<td>Aspartate aminotransferase (AST)</td>
</tr>
</tbody>
</table>


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**TABLE II**

Lactate Dehydrogenase Kinetic Assay Methods (Pyruvate-to-Lactate)

<table>
<thead>
<tr>
<th>Method*</th>
<th>T (°C)</th>
<th>Pyruvate (mmol/l)</th>
<th>NADH (mmol/l)</th>
<th>Buffer/conc. (mmol/l)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wroblewski and LaDue (1955)</td>
<td>25-27</td>
<td>0.74</td>
<td>0.12</td>
<td>Pi†</td>
<td>7.4 (7.2-7.6)</td>
</tr>
<tr>
<td>Henry, Chiamori, Golub, and Berkman (1960)</td>
<td>32</td>
<td>0.6</td>
<td>0.187</td>
<td>Pi‡</td>
<td>7.4</td>
</tr>
<tr>
<td>Bowers (1963)</td>
<td>37</td>
<td>1.2</td>
<td>0.21</td>
<td>Tris 100</td>
<td>7.35 at 37°C</td>
</tr>
<tr>
<td>Gay, McComb, and Bowers (1960)</td>
<td>30</td>
<td>0.9‡</td>
<td>0.22</td>
<td>Tris 100</td>
<td>7.30 at 30°C</td>
</tr>
<tr>
<td>German Society (1972)</td>
<td>25</td>
<td>0.6</td>
<td>0.18</td>
<td>Pi 50</td>
<td>7.5</td>
</tr>
<tr>
<td>McQueen (1972)</td>
<td>37</td>
<td>2.5</td>
<td>0.12</td>
<td>Pi 67</td>
<td>7.18 at 37°C</td>
</tr>
<tr>
<td>Buhl, Jackson, and Graffunder (1978)</td>
<td>29§</td>
<td>1.0</td>
<td>0.15</td>
<td>TEA 100</td>
<td>7.0 at 25°C</td>
</tr>
<tr>
<td>30§</td>
<td>1.5</td>
<td>0.15</td>
<td>0.15</td>
<td>TEA 100</td>
<td>7.0 at 30°C</td>
</tr>
<tr>
<td>37§</td>
<td>1.5</td>
<td>0.22</td>
<td>0.22</td>
<td>TEA 100</td>
<td>7.0 at 30°C</td>
</tr>
<tr>
<td>Société France de Biologie Clinique (1981)</td>
<td>30</td>
<td>1.6</td>
<td>0.20</td>
<td>Tris 80</td>
<td>7.2 at 30°C</td>
</tr>
</tbody>
</table>

*Sample volume fraction of 1/30 except as noted.
†Pi designates phosphate.
‡Used 0.9 mmol/l in their study but found 1.2 mmol/l to be optimal for mixed isoenzyme sample.
§Sample volume fraction 1/60
¶pH adjustment at temperature designated.
§§Buffer contained 5 mmol/l ethylenediaminetetraacetic acid (EDTA).
### Table Continued

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uously monitored rate (kinetic) measurement of LD in the pyruvate-to-lactate (P → L) direction are summarized in Table II. In 1955, Wroblewski and LaDue developed the first continuously monitored spectrophotometric (kinetic) assay method for LD using pyruvate, reduced NADH and serum in phosphate (Pi) buffer at pH 7.4. The reaction does not have to be forced because the equilibrium lies in the direction of the formation of lactate. In 1960 Henry, Chiamori, Golub, and Berkman, in their classical publication on revised spectrophotometric methods, took a major stride by examining and “revising” the conditions for three major clinical laboratory enzyme measurements including LD in the P → L direction. They were the first to: optimize substrates, emphasize temperature control, and stress pH control.

A very complete investigation of LD assay conditions was carried out by Gay, McComb, and Bowers in 1968 utilizing crudely extracted preparations of human heart and liver LD for investigation of the optimal conditions for both the P → L and lactate-to-pyruvate (L → P) reactions. For the P → L reaction Gay et al recommended Tris (hydroxymethyl) aminomethane (Tris) buffer which system has been adopted with modifications by the Scandinavian Society for Clinical Chemistry and Clinical Physiology. On the other hand, the German Society for Clinical Chemistry recommendations are for a modification of the Henry et al method which utilizes phosphate buffer. While Bowers reported that phosphate buffer inhibited LD activity assayed in the P → L system, Buhl, Jackson, and Graffunder found phosphate up to 125 mmol per l and, to a small extent, Tris, were stimulatory for human LD 1 and 5 at pH values less than 7.5.

Krause and Lott have demonstrated the use of the simplex method to optimize analytical conditions in clinical chemistry by applying it to the P → L reaction for LD. Optimization of pH and substrates was carried out in order to achieve the maximum rate for Versatol-E.* No application to clinical specimens was included.

In 1972 McQueen developed an optimal assay for the P → L reaction at 37°C based upon normal and pathological sera as a source of LD activity. Subsequently, Tuckerman and Henderson evaluated the Henry assay carried out at 37°C instead of 32°C, the McQueen assay, and an optimized commercial assay kit, all P → L assays at 37°C. The McQueen assay, which uses a high pyruvate concentration, gave significantly less LD activity owing to substrate inhibition, than the other two methods which appeared to be equivalent.

Szasz investigated the effect of temperature on enzyme activity for the various enzyme methods recommended by the German Society for Clinical Chemistry. He showed both “fast” LD (LD 1 and 2) and “slow” LD (LD 5) to deviate from linearity when measured at temperatures greater than 30°C under the German optimized conditions recommended for 25°C. Szasz concluded that almost all of the kinetic factors relevant to optimization are highly dependent on assay temperature. Thus, McQueen documented the true Arrhenius relationships of human LD 1 and 5 extracts in the P → L reaction at 25°, 30°, 35°, 40°, 45°, and 50° and showed clearly for the first time the increasing pyruvate and NADH concentrations required at higher temperatures with different isoenzymes.

In 1978 Buhl et al, in a follow up to earlier LD studies, investigated the pyruvate-to-lactate reaction for optimal reaction conditions at 25, 30, and 37°C. Nine buffers were investigated as well as the non-linearity of the reaction response. Highly purified LD 1 from

* General Diagnostics Co., Morris Plains, NJ.
human erythrocytes and LD 5 from human liver were utilized as the sources of enzyme activity. Optimal substrate concentrations for the measurement of the P → L reaction in imidazole, TEA (triethanolamine) or TES (N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid) at pH 7.0 are shown in table II. Imidazole, TEA, and TES were chosen as suitable reaction buffers because their pKₐ's are near the recommended reaction pH of 7.0 and because they exerted no stimulatory or inhibitory effects. Tris was not evaluated thoroughly but was found to be stimulatory, as previously reported by Bowers. Also, Buhl found phosphate to be stimulatory for the human LD 1 and LD 5 isoenzymes in the P → L direction only. His findings that buffer effects are pH and substrate dependent suggest a source of variation in commercial reagent kit systems. Buhl has recommended that all pyruvate-to-lactate measurements be made as soon as possible after the reaction is initiated and be performed at 25°C or, if necessary, 30°C because at 37°C there is a different rate of decrease in absorbance for LD 1 and LD 5. His studies suggest that the measuring time interval at each temperature should be standardized relative both to the time after the reaction is initiated and to its duration. The activity of human LD 1 and LD 5 assays, based on the P → L reaction, were not affected by the manner of reaction initiation since triggering with pyruvate, enzyme or NADH gave equivalent results.

Volume 9 of Selected Methods in Clinical Chemistry entitled Selected Methods for the Small Chemistry Laboratory includes the measurement of LD by the P → L reaction in imidazole buffer at 37°C by the NADH to NAD⁺ change at 340 nm. This kinetic method is felt to be achievable in small laboratories by the author and the editors.

Very recently, Vassault, Maire, Seville, Bozon, and Lalegerie have proposed an official recommendation by the Société Française de Biologie Clinique entitled An Improvement for the Determination of Total Lactate Dehydrogenase Catalytic Activity Whatever the Isoenzymes. It is well known that the reaction catalyzed by LDH is inhibited in the presence of high levels of substrate. This inhibition is observed in both directions and with both types of enzymes, although the inhibition is much more pronounced with LD 1 than with LD 5 and with pyruvate than lactate. It has been shown to be due to the formation of an abortive ternary complex or adduct of LD-NAD⁺-pyruvate with inhibits strongly the P → L reaction resulting in a non-linear reaction rate. Recently, Rivedal and Sanner have found that an increase in ionic strength can modify the stability of the ternary abortive complex and decreases inhibition. Thus, Vassault et al have shown that the addition of Na⁺ and Cl⁻ ions to the P → L reaction mixture modifies the stability of the inhibitory ternary complex. The proposed method includes the addition of 200 mmol per l NaCl to the Tris buffer reaction mixture (table II). The method is stated to show the following advantages: minimizing of differences for optimal concentration of pyruvate between LD 1 and LD 5, improvement in the linearity of the kinetics, and an increase in the upper limits of linearity.

Many P → L procedures initiate the reaction with pyruvate (table II), in order to provide a preincubation period to remove endogenous substrates reacting with NADH.

Lactate-to-Pyruvate Methodologies

The conditions for the primary methods for the continuously monitored rate (kinetic) measurement of LD in the lactate-to-pyruvate (L → P) direction are summarized in table III. Wacker,
Ulmen, and Vallee in 1956 were the first group to measure LD kinetically in the \( L \rightarrow P \) direction for clinical laboratory purposes. Subsequently, Amador, Dorfman, and Wacker found phosphate to be an inadequate buffer at a pH of 8.8, substituted pyrophosphate, and increased the lactate and NAD\(^+\) concentrations. These changes resulted in a markedly improved assay. King introduced glycine with added sodium chloride at pH 10.0 as a buffer system. While widely used, especially in Great Britain, the high pH is incompatible with the use of NAD\(^+\), which decomposes at very alkaline pH's. In addition, Fendley, Jacobs, Dunn, and Frings found the King assay at pH 10 to underestimate grossly LD 5 and have modified the pH to 8.8, which alleviates both problems.

While Morgenstern, Flor, Kessler, and Klein in 1965 developed an \( L \rightarrow P \) method for the Auto Analyzer utilizing AMP buffer with both colorimetric and kinetic (340 nm) versions, the 340 nm photometric measuring system was not made available as part of Technicon equipment until the 1970's. A similar manual kinetic method utilizing AMP buffer has been recommended by Demetriou, Drewes, and Gin in a well recognized textbook (table III).

An early very complete investigation of LD assay conditions was carried out by Gay, McComb, and Bowers. It utilized [crudely] extracted preparations of human heart and liver LD for investigation of the optimal conditions for the \( P \rightarrow L \) reaction in addition to the \( L \rightarrow P \) assay. However, this carefully executed study did not include the evaluation of a large number of buffers. The finalized optimal conditions for the \( L \rightarrow P \) reaction by Gay, McComb, and Bowers are minor modifications of the Amador et al pyrophosphate method (see table III). While they used pH 8.75 in their study, 8.55 was found optimal for a mixed isoenzyme sample.

<table>
<thead>
<tr>
<th>Method*</th>
<th>( T ) (°C)</th>
<th>Lactate (mmol/l)</th>
<th>NAD(^+) (mmol/l)</th>
<th>Buffer/conc. (mmol/l)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wacker, Ulmen, and Vallee (1956)</td>
<td>25</td>
<td>53 (DL)</td>
<td>5</td>
<td>Pif</td>
<td>8.8</td>
</tr>
<tr>
<td>Amador, Dorfman, and Wacker (1963)</td>
<td>25</td>
<td>77 (DL)</td>
<td>5.25</td>
<td>Ppi(^+)</td>
<td>8.6</td>
</tr>
<tr>
<td>King (1965)</td>
<td>25</td>
<td>175 (DL)</td>
<td>1</td>
<td>Glycine</td>
<td>10.0</td>
</tr>
<tr>
<td>Morgenstern, Flor, Kessler, and Klein (1965)</td>
<td>37.5</td>
<td>82 (DL)</td>
<td>7.5</td>
<td>AMP(^+)</td>
<td>188</td>
</tr>
<tr>
<td>Gay, McComb, and Bowers (1968)</td>
<td>30</td>
<td>77.5 (DL)</td>
<td>5.25</td>
<td>Ppi(^+)</td>
<td>50</td>
</tr>
<tr>
<td>Demetriou, Drewes, and Gin (1974)</td>
<td>30</td>
<td>90 (DL)</td>
<td>5.5</td>
<td>AMP(^+)</td>
<td>600</td>
</tr>
</tbody>
</table>

*Sample volume fraction 1/15.
†Phosphate buffer - Pi.
‡Pyrophosphate buffer - Ppi.
§AMP is 2-amino-2-methyl-1-propanol.
\(^\dagger\)Used pH 8.75 in their study but found 8.55 optimal for a mixed isoenzyme sample.
\(\Lambda\) - lactate.
Gay, McComb, and Bowers\textsuperscript{42} initiated the reaction with enzyme whereas King's method\textsuperscript{58} for the L → P assay specifies initiation with NAD\textsuperscript{+}. Buhl, Jackson, Lubinski, and Vanderlinde\textsuperscript{22} found human LD 1 and LD 5 activity, when assayed L → P, is adversely affected if the enzyme is preincubated with NAD\textsuperscript{+} and the reaction is initiated with lactate. The amount of inactivation depends on the buffer used; activities are decreased more in sodium pyrophosphate than in Tris. On the other hand, NAD\textsuperscript{+} has been reported to have a stabilizing effect on LD activity.\textsuperscript{8,33} Because this effect is more pronounced in phosphate buffers than in Tris buffer,\textsuperscript{63,106} it has been suggested that phosphate activates the NAD\textsuperscript{+} binding sites.\textsuperscript{63} These observations and the fact that the L → P reaction is an ordered sequential reaction, with NAD\textsuperscript{+} binding first,\textsuperscript{92} should lead to enhanced LD activity when the enzyme is preincubated with NAD\textsuperscript{+}. The contrary experimental findings may be the result of inhibitors, which have been found in NAD\textsuperscript{+} preparations from various sources.\textsuperscript{4} Also, a reduction of LD activity would be expected if the inhibitor-enzyme dissociation constant is less than the NAD\textsuperscript{+}-enzyme dissociation constant.\textsuperscript{22} Buhl interpreted the results to suggest also that lactate affects enzyme binding of NAD\textsuperscript{+}. Further, both LD 1 and 5 have higher L → P activity when assayed in Tris than when assayed in sodium pyrophosphate buffer.\textsuperscript{22} Although the latter system contains lower concentrations of NAD\textsuperscript{+} and lactate, this probably does not account for the lower activity observed because the NAD\textsuperscript{+} and lactate concentrations used are saturating.\textsuperscript{42} Buhl's results demonstrate also that buffer is an important factor in the LD reaction and that the buffer may interact with the enzyme and affect the binding of the substrates. The very complex issue of substrates, inhibitors and reaction mechanisms of the LD reaction has been ably reviewed by Everse and Kaplan.\textsuperscript{36} The lack of agreement in choice of buffer, the finding of inhibition by the pyrophosphate buffer, and the finding of discrepant results in assays of both purified isoenzymes and sera under various buffer conditions led Buhl et al\textsuperscript{21} to investigate seventeen buffers at a variety of pH's in the assay using highly purified human LD 1 and LD 5. The buffers selected included the four most commonly used (table III): pyrophosphate (PPi), Tris, 2-amino-2-methyl-1-propanol (AMP) and glycine and an additional 13 buffers with pK\textsubscript{a} values greater than 7.9.

Of the four most commonly used buffers, three proved unsatisfactory because of direct effects on LD. Glycine and PPi were found unsatisfactory because they inhibit LD activity with increasing concentration. With AMP the pH optimum for LD 1 is not compatible with the use of NAD\textsuperscript{+}, and the pH optima for LD 1 and LD 5 are vastly different. The concentration of AMP also greatly affects total LD activity; hence large amounts of AMP must be used to achieve maximal LD activity. Currently a concentration of 600 mmol/liter is used by Technicon\textsuperscript{99} and recommended by Demetriou et al.\textsuperscript{32} Additional disadvantages of AMP buffer are that LD activity is dependent on the method of preparation, the buffer has an unpleasant odor, and a gas is visibly released during pH adjustment. Although ethanolamine and dimethylyaminomethanol (DAE) were satisfactory buffers by some criteria, ethanolamine exerted a stimulating effect on LD activity and DAE showed potential undesirable chemical reactions as well as an obnoxious odor.

The three buffers Buhl et al\textsuperscript{21} found to have no detrimental effects on LD activity are Tris, diethanolamine (DEA), and 2-amino-2-methyl 1,3-propanediol (AMPdiol). He felt Tris may not be completely satisfactory because its pH opti-
mum for LD 1 is greater than 8.5, while its $P_{K_a}$ at 30°C is 8.0. Thus, Tris has limited buffering capacity at pH 8.5, the optimal reaction pH for mixtures of LD 1 and LD 5.

Of the 17 buffers examined, DEA and AMPdiol at concentrations of 200 mmol per l and pH 8.7 were recommended as the buffers of choice for the measurement of LD activity in the $L \rightarrow P$ direction. However, DEA shows a significant cost advantage over AMPdiol. In a subsequent investigation Buhl, Jackson, Lubinski, and Vanderlinde established the optimal substrate conditions for the assay of the human LD isoenzymes 1 and 5 $L \rightarrow P$ in these two buffers at 25, 30, and 37°C (table III).

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. Krieg, Gorton, and Henry found crude human extracts of LD 1 and LD 5 to show more nearly the same velocity/substrate concentration profiles at 37°C than at 25°C as observed earlier by Vesell. However, the source, purity and type of lactate were not stated, and Buhl et al have demonstrated the purity and form of lactate to be extremely important. Hence, lithium-L(+)lactate of high purity was used by Buhl and his associates in their subsequent investigations. The lack of linearity in product formation (constant $\Delta A$ per min) has several components, which have been observed but not separated in previous studies. The initial rise ($<20$ s), Buhl suggested, is probably due to experimental design or deficiencies in instrument response while the decrease after 20 to 30 s has more than one component, including substrate depletion, and probably involves product inhibition. Substrate depletion can be overcome simply by adding additional substrate. However, attempts to remove the product formed with semicarbazide have met with mixed success, probably because of the complexity of the substrate inter-relationships and biochemicals of the LD reaction.

The apparent $K_m$'s for lactate and NAD$^+$ for nonhuman LD increase with temperature. Buhl's findings that increased amounts of lactate and NAD$^+$ are required for saturation of human LD 1 and LD 5 with increasing temperature is consistent with the earlier observations on animal isoenzymes.

Reaction conditions have been established which measure LD 1 and LD 5 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 normal and pathologic sera, as well as individual isoenzyme determinations from column procedures.

Buhl recommended that all $L \rightarrow P$ LD measurements be made within 60 s after the reaction is initiated. If this is not feasible because of delayed instrument response time, the measurement should be made as quickly as possible.

He also recommended that $L \rightarrow P$ assays be performed at 25°C or 30°C because at the lower temperatures the reaction response was linear for a longer time, less reagents were needed, and less interlaboratory and intralaboratory variation would be encountered on samples measured with a variety of instruments and on different isoenzymic mixtures having similar total LD activity.

Although it would seem more precise to have higher assay values, his results showed this not to be true. Because of an appreciably different decrease in $\Delta A$ per min for LD 1 and LD 5 at 37°C but not at 30°C or 25°C, it is more difficult to measure each isoenzyme at 37°C with equal efficiency, and the difference in efficiency was observed to become greater with time after the reaction is ini-
tiated. The consequence is that any assay of an isoenzymic mixture with elevated LD activity will have a large interinstrument variability. Also, more intrument variability would be encountered at 37°C than at 30°C or 25°C in samples with the same total LD activity but differing isoenzymic mixtures. The single-instrument variation will be greatest for instruments which measure LD activity one min or more after initiation.

Hence, Buhl\(^2\)\(^3\) recommended the measuring time interval be standardized and assays of LD measured \(L \rightarrow P\) be measured at 25°C or 30°C as they will show less variation attributable to instrument and isoenzyme content.

**Other Methodologies**

While clinical laboratories utilize widely continously monitored rate reactions at 340 nm involving the absorbancy change in \(NAD^+ - NADH\), with lactate or pyruvate as substrate, other methodological approaches for the measurement of LD have been advanced. Thus, Babson and Babson\(^5\) described in 1973 a kinetic colorimetric assay procedure in which the reduction of \(NAD^+\) is coupled to the reduction of a tetrazolium salt, 2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride (INT), with phenazine methosulfate serving as an intermediate electron carrier. The kinetic colorimetric method correlated well with 340 nm kinetic methods but did not require an ultraviolet spectrophotometer. A similar rapid single step kinetic colorimetric assay for LD in serum was developed by Allain, Henson, Nadel, and Knoblesdorff\(^2\) but used diaphorase as the intermediate electron carrier.

The feasibility of microcalorimetry for the determination of the kinetics of enzyme catalyzed reactions, specifically LD and uricase, was investigated by Rehak, Everse, Kaplan, and Berger\(^8\)\(^2\). The relationship between activity and concentration of both soluble and immobilized chicken LD 1 was determined using the rate of heat production in a batch-type calorimeter. Another novel approach to the measurement of LD was made by Nikolelis, Painton, and Mottola\(^7\)\(^6\) who found the air oxidation of NADH, catalyzed by peroxidase, to provide a useful "indicator reaction" for the determination of either serum LD or of NADH. They described application of this indicator reaction to repetitive determinations by sample injection into a continuously circulated reagent mixture through the monitoring of oxygen depletion with an amperometric sensor.

Curry, Pardue, Mieling, and Santini\(^3\)\(^0\) have designed a filter fluorometer that incorporates a photon-counting detector and evaluated its applicability in the fluorometric determination of serum LD. While reflectance spectroscopy has not been widely used in the clinical laboratory, Zipp, Watson, and Greyson\(^1\)\(^1\)\(^0\) have developed a solid state matrix system for the determination of LD in serum in which the quantitation is carried out by reflectance spectroscopy. They claim that this approach can be used to measure total LD in serum with the convenience and simplicity of a test strip without compromising the quantitative nature of present wet methods. In addition, the dry film based technology of the Eastman Kodak Company's new Ektachem 400 System utilizes the principle of reflectance spectroscopy,\(^9\)\(^8\) which is well established in the photographic field. However, while their amylase methodology is available, other enzymes including LD have just been released recently.

A vidicon spectrophotometric approach to the measurement simultaneously of two enzymes, exemplified by serum LD and alkaline phosphatase (EC 3.1.3.1), has been demonstrated by Milano and Pardue\(^7\)\(^2\) to give enzyme
activities equivalent to those in common use.

NADH and NAD+ Inhibitors

As early as 1961, the Kaplan group recognized the presence of an inhibitor in NADH which can strongly inhibit LD; some clinical chemists were aware of the problem in the middle sixties. In 1968 McComb and Gay published their findings on four commercial sources of NADH. Subsequently, Berry, Lott, and Grannis showed lot to lot variation in the amount of the inhibitor and suggested a procedure for assessing the quality of commercial NADH preparations. However, the problem was not unique to NADH and the pyruvate-to-lactate continuously monitored rate reaction as Dalziel in 1963 had demonstrated NAD+ to contain nucleotide impurities which interfered with the lactate-to-pyruvate reaction. Babson and Arndt confirmed Dalziel’s observation by showing that commercial preparations of NAD+ contained various amounts of one or more LD inhibitors but not the inhibitor described for NADH. Thus, in the early seventies, the quality, storage, and refreezing characteristics of NADH and/or NAD+, utilized in clinical laboratory kinetic measurements of LD in either direction, became of concern. This concern stimulated efforts in several additional fronts: namely, by the professional societies, by the National Bureau of Standards, and by the manufacturers, among others.

Since adenosine diphosphoribose (ADPR), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) are poor inhibitors for the lactate dehydrogenases, the inhibitors were presumably entirely different structures.

Gerhardt et al. under the auspices of the Scandinavian Society for Clinical Chemistry and Clinical Physiology evaluated practical methods for the detection of inhibitors in NADH and for monitoring its quality and described specifications for both reference and commercial preparations. The U.S. National Bureau of Standards group, under Margolis and Schaffer, has spent several years identifying impurities in NADH and developing specifications for reference quality NADH. The molar absorptivity of high purity NADH was evaluated in 1976 by McComb et al. in the United States and by Ziegenhorn et al. in Europe. McComb’s group as well as the NBS group studied the formation and properties of the LD inhibitors in NADH by column chromatography and/or high performance liquid chromatography (HPLC). Loshon et al. described at least two inhibitory components which can form in concentrated NADH solutions, only one of which can be separated on DEAE-cellulose or DEAE-Sephadex.

The second inhibitor, which separated on the trailing edge of the NADH peak in chromatography on DEAE-cellulose, was resolved from the NADH by Shaffer’s group by HPLC on u Bondapak C18. Because this new inhibitor had ultraviolet properties similar to those of NADH, a ratio of absorbance at 260/340 nm of less than 2.32 could not be used as an indication of an inhibitor free preparation and HPLC chromatography was deemed necessary to ensure its absence in preparations of NADH used for clinical assay. While Loshon et al. showed the presence of NAD+ to influence the rate of inhibitor production from unbuffered aqueous NADH solutions, no inhibitor was produced by NAD+ incubated alone under the same conditions. However, Gallati and Johnson postulated NAD to undergo ring opening of the nicotinamide ring in strongly alkaline solutions.

More recently, Guilbert and Johnson...
have demonstrated the pseudobase hydroxide ring addition adduct of NAD\(^+\), ψNAD-OH, to be reversibly formed and, subsequently, to undergo ring opening at the number six carbon of the nicotinamide ring to form open ring NAD designated as ONAD. ONAD has pK\(_a\) values at 1.9 and 11.2 and absorbs maximally at 350 nm in its acidic form, at 372 nm in its neutral form, and at 340 nm in its anionic form. The hydrolysis of ONAD leads to the formation of 2-carboxamideglutacondialdehyde and adenosine diphosphate ribosylamine with the former breaking down further to the base fluorescent compound, 2-hydroxynicotinaldehyde. Thus, NAD\(^+\) in alkaline solution can form at least 5 intermediate end products. More recently, a cooperative group formed between the Louis Pasteur University at Strasbourg and Boehringer Mannheim GmbH\(^{13}\) and a second group in Copenhagen under Godtfredsen\(^{46,47}\) have made great strides in the structural elucidation and interrelationships between the various LD inhibitors occurring in both NADH and NAD\(^+\). Biellmann et al\(^{13}\) found NADH on storage in a moist atmosphere to result in three inhibitory compounds, only two of which were inhibitors. The structure of one inhibitor was not elucidated owing to its instability; another inhibitor was demonstrated not to originate directly from NADH but from contaminating NAD\(^+\). It was shown to be phosphate-NAD\(^+\) adduct formed in small amounts by the addition of a phosphate ion to the 4-position of the nicotinamidinium ring of the contaminating NAD\(^+\). This 4-phosphoryloxy-1, 4-dihydro-NAD adduct was shown to be a potent competitive inhibitor of LD with an inhibitor constant (K\(_i\)) of 2 × 10\(^{-7}\)M and to be identical with the inhibitor prepared by Gallati\(^{40,41}\) from NAD\(^+\) plus phosphate in alkaline medium. The third inhibitor product isolated from NADH was identified by Biellmann et al\(^{13}\) as a dimer of NAD\(^+\). Subsequently, Godtfredsen and Ottesen\(^{46}\) showed NADH under humid conditions to form 1,6-dihydro-NAD, a potent inhibitor of LD with a K\(_i\) of 2.5 × 10\(^{-7}\)M. They report their 1,6-dihydro-NAD inhibitor to have spectroscopic properties at 340 nm similar to those of the dimer of NAD\(^+\) identified by Biellmann\(^{14}\) and present evidence to support Biellmann’s compound most likely being 1,6-dihydro-NAD.

Furthermore, Godtfredsen et al\(^{47}\) present evidence to show that this compound is probably the major inhibitor formed in moist NADH at pH 8.8 and have presented experimental data to support their hypothesis\(^{46}\) that the 1,6-dihydro-NAD is generated in NADH preparations by a bimolecular reaction between NADH and the small quantities of NAD\(^+\) which are usually present in samples of NADH. One major implication of this finding is that no or very little inhibitor formation should take place in NADH preparations completely free of NAD\(^+\). Thus it would seem fairly well established that the major two inhibitors in NADH are: (a) 1,6-dihydro-NAD\(^+\) which absorbs at 340 nm similar to NADH and cochromatographs on DEAE cellulose or Sephadex with NADH and (b) 4-phosphoryloxy-1,4-dihydro-NAD\(^+\), a phosphate adduct of NAD\(^+\).

Also, the pH of the preparation and the nature of the inorganic salts present, particularly phosphate, alter which inhibitors occur in various NADH and NAD\(^+\) preparations.

Wenz et al\(^{104}\) showed in 1976 various commercial preparations of NADH to yield up to 12 different compounds which can act as LD inhibitors. All manufacturers have been aware of the inhibitor problem with NAD\(^+\) and particularly NADH for many years. Consequently, many of the manufacturers offer good routine service quality NADH and NAD\(^+\) preparations as well as chromatopure or ultrapure preparations of both NADH and NAD\(^+\) at 50 to 100 percent increased cost. Buhl, Jackson, and
AMEASUREMENT OF TOTAL LACTATE DEHYDROGENASE ACTIVITY

GrafFunder obtained about 10 percent higher activities with the pure human LD 1 and LD 5 isoenzymes with a more pure commercial B-NADH preparation but found no difference in substrate requirements. However, equally important are the numerous factors such as the humidity and storage conditions, freezing and thawing of solutions, the nature of the contaminants, which salts are present, and pH of the preparation which effect the stability of NADH and NAD+ maintained within the user’s laboratory.

Furthermore, research and standardization involving dehydrogenases such as LD and alcohol dehydrogenase (EC 1.1.1.1) measurements which are especially sensitive to inhibitors may require highly purified NAD+ and NADH sources whereas the less expensive commercial preparations may suffice for routine clinical laboratory measurements.

Choice of Methodologies

In recent years, laboratories in the United States have increasingly shifted from colorimetric end-point enzyme measurements to continuously monitored rate (kinetic) reactions and to the measurement of LD by the L → P rather than the P → L reaction. For example, the clinical laboratories in New York State, outside of New York City, increased their utilization of kinetic rate methods for LD from 21.7 percent of all laboratories in 1971 to 61.0 percent in 1975. In addition, the percentage utilizing an L → P method instead of P → L jumped from 43.4 percent in 1971 to 81.0 percent in 1975. The major reason for this shift was the increased use of automated enzyme analyzers utilizing the L → P reaction at 340 nm, especially continuous flow 340 nm based kinetic analyzers. A secondary reason was the shift from end-point to kinetic assays by many smaller laboratories.

The College of American Pathologists (CAP) Enzyme Chemistry Program employs sets of linearly related enzyme specimens including LD which are sent to participants quarterly. The 1982 Enzyme Survey V-A Summary Report provides valuable participant information on the instruments/reagents and number of laboratories utilizing each methodology which are summarized in table IV. All methods utilized by ten or more laboratories involve the L → P reaction, and only a minor number of participants use P → L procedures.

Howell, McCune, and Shaffer in 1979 re-examined the L → P and P → L assays for LD and state that the P → L assay can now yield linearity equal to or better than that obtained by the L → P assay. In addition, they state there are significant advantages to the P → L reaction, namely: (a) a greater change in absorbance per unit of time, which

### Table IV

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Reagents</th>
<th>Number of Laboratories</th>
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</thead>
<tbody>
<tr>
<td>Abbott ABA-100</td>
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<tr>
<td>Abbott VP</td>
<td>Abbott</td>
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<td>American Monitor KDA</td>
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<td>DuPont ACA</td>
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<td>ENA Gemini</td>
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<td>Smith-Kline</td>
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<tr>
<td>Gilford-Various Systems</td>
<td>Worthington</td>
<td>33</td>
</tr>
<tr>
<td>Gilford-Various Systems</td>
<td>Gilford</td>
<td>15</td>
</tr>
<tr>
<td>Hycoel Super or Mark 17</td>
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<tr>
<td>Technicon SNA 12/60</td>
<td>Fisher Scientific</td>
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<tr>
<td>Technicon SNA 12/60</td>
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<td>Technicon SMAC</td>
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<td>Baker Diagnostics</td>
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<td>Chemetrics II</td>
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<td>11</td>
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</table>


†Instruments/reagents shown only for 10 or more participating laboratories. All involve L→P methods but utilize varying buffers (PPI, Tris, AMP), temperatures (25°C, 30°C, 37°C) and substrate concentrations.

* Technicon Instruments Co., Tarrytown, NY.
allows a more accurate spectrophotometric readout; (b) lower reactant concentrations which thereby reduce costs; (c) solid reagents are used to prepare the assay solution; and (d) the reagent solutions are more stable. They point out that inhibitors in commercial NADH preparations may substantially effect LD activities measured P → L and state that a Standard Reference Material for NADH is being developed for issuance by the NBS. However, for a number of reasons this project has been postponed.80

On the other hand, Buhl and Jackson,19 who have compared serum LD catalysis under optimal conditions developed for the pure human isoenzymes in both the L → P and P → L reactions at 25°C, 30°C, and 37°C, found the results from the L → P reaction to be more reliable for interinstrument and interlaboratory comparisons. Interconversions of results between the bidirection reactions were not practicable. Measurements of LD in either direction at 25°C, 30°C, or 37°C were stated to be equally reliable if the volume fraction and the resulting ΔA per min is small. However, they recommended that the P → L reaction be measured as soon as possible after start of the reaction, the measuring interval be reasonably short (<10 s) and that the L → P reaction be measured within the first 40 s. Furthermore, they suggested that any future reference method or selected method include a recommendation for the time and duration of the measuring interval after the reaction is initiated. While they found neither the P → L nor the L → P response to be linear, they report that at equal ΔA per min the activity of the L → P reaction does not decrease as rapidly and is therefore more reliable. Buhl and Jackson19 have reported, “Some available preparations and improperly stored β-NADH contain inhibitors of LD, whereas many β-NAD⁺ preparations are free of LD inhibitors.” A current review of representative biochemical reagent catalogs showed many of the manufacturers to offer good routine service laboratory quality NADH and NAD⁺ preparations as well as chromatopure or ultrapure preparations of NADH and NAD⁺ at 50 to 100 percent increased cost (See section on NADH and NAD⁺ inhibitors).

It is not possible to assess at this time new findings such as the Société Française de Biologie Clinique recommendation101 of a new more linear L → P assay (see L → P section).

Sources of variation

Preferred Blood Sample

Serum is the preferred sample for all enzyme activity measurements including LD. Haymond and Knight50 found the LD activities of capillary serum and capillary plasma were significantly greater than in simultaneously assayed venous serum, the greatest difference being between capillary and venous serum. Although some difference is attributable to tissue fluid, most of the difference was attributed to platelets. They recommend that when capillary blood is to be used for enzyme assay, it should be processed as plasma. Subsequently, Tothwell, Jendrzejczak, Becker, and Doumas88 investigated the LD activity of venous serum versus venous plasma and demonstrated platelets to be the source of up to a four fold increase in plasma LD. More recently, Bais, Prior, and Edwards7 have shown platelets to increase LD as measured by the SMAC continuous flow analyzer* and the detergent in the detection buffer to further enhance the effect. Hence, if plasma must be used as the source of specimen, the sample must be centrifuged such as to provide a platelet-free plasma.

* Technicon Instruments Corp., Tarrytown, NY.
Various anticoagulants such as oxalate and fluoride are inhibitory to LD while heparin was reported by Batsakis and Henry to be erratic. More recently, Robertson, Chesler, and Elin found serum collected with the antiglycolytic agent, lithium iodoacetate to be unsuitable for the measurement of LD.

**Hemolysis**

The high intra-erythrocytic concentration of LD 1 isoenzyme requires rapid separation of serum from the clot. The calculated error per gram of hemoglobin for LD activity is 463 percent, while the observed error per gram of hemoglobin was 550 percent. Hence, hemolysis in sera for LD measurements must stringently be avoided.

**Stability of Serum**

Schmidt and Schmidt report the average loss of serum LD activity on storage at 4°C to be 0 percent at 24 hours, four percent at 48 hours, eight percent at three days, nine percent at five days and 12 percent for seven days, whereas at 25°C the corresponding losses are 0, 1, 2, 10, and 15 percent, respectively. However, because LD 1 is relatively stable whereas LD 5 is thermolabile, stability may vary with the isoenzyme composition of individual sera. In general, freezing is to be avoided.

**Activators, Inhibitors and Interferences**

The “nothing-dehydrogenase” reaction while mainly associated as an artifact in serum isoenzyme analysis based on tetrazolium methods may occur also in the serum where its origin is unknown. Sometimes it is said to represent LD activity but since a correction with serum proteins and particularly their sulphhydryl groups can be made, Somer believes that protein-bound sulphhydryl groups are responsible. Recently, Bails and Edwards have shown that alcohol dehydrogenase can be present in pathological sera, can be identified as a sixth LD band, and shows considerable activity at pH 9 in buffers used to measure LD in the L → P direction including DEA, AMPdiol, and AMP.

Howell has shown that Zn⁺⁺ can be an inhibitor for the LD catalyzed reduction of pyruvate-to-lactate at even the upper normal limit of serum Zn⁺⁺, but inhibition is greatly reduced by pyruvate concentrations in excess of 1 mmol per 1 which is usual in the P → L assay.

The diuretic, triamterene (2,4,7-triamino-6-phenylpteridine), has been found to induce spurious elevations in serum LD as measured by an automated fluorometric assay but not spectrophotometrically.

A serum inactivator to the M subunit of LD isoenzymes which migrated electrophoretically between the beta and gamma globulins was reported by Nagamine. Also, Acheampong-Mensah has reported a persistent increase in serum LD activity to be related to an enzyme-IgG-Lambda immunoglobin complex. Several LD-immunoglobulin complexes in human serum have been described previously by Biewanga and Feltkamp.

**Sources of Error**

The sources of error in measuring LD are failure to maintain the reaction at constant temperature, use of poor quality reagents, e.g., NADH or NAD⁺, hemolyzed samples, and deviation from the prescribed procedure. Increasing or decreasing the temperature will change the LD result approximately seven percent per degree. Various sources of NADH and NAD⁺ contain inhibitors of the LD reaction and different commercial sources of lactic acid also affect the
amount of LD measured. Erythrocytes contain large amounts of LD, and any hemolysis will give abnormally high results. Changes in the procedure—such as substitution of buffer, change of pH, or change in the order of addition of reagents to the reaction mixture—will also alter the amount of LD measured.

Reference Ranges

Laboratories using different reagents and/or different procedures will have different normal ranges and should use values established preferably by their own laboratory or as documented by the manufacturer of their kit or reagent instrumental system. However, it is not widely recognized that pediatric LD values decrease with maturity and older individuals show significantly higher LD activities.

LD Standardization Efforts

Most clinical chemists accept the need for national and international standardization of the conditions under which enzymes are measured.

The German Society for Clinical Chemistry was the first national society to put forth a recommendation for the standardization of a method for the estimation of serum lactate dehydrogenase. Their “standard method” recommended in 1972 is a modification of the Wroblewski and LaDue method carried out at 25°C. They also included a “standard method” for α-hydroxybutyrate dehydrogenase.

The Committee on Enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology published Recommended Methods for the Determination of Four Enzymes in Blood which included LD in the P → L direction. However, the Scandinavian Society opted for Tris instead of phosphate buffer, measurement at 37°C, and a pyruvate substrate concentration two-fold that which was recommended by the German Society for 25°C.

While the British Association of Clinical Biochemistry-Working Party has made method recommendations for three enzymes, LD was not included. Very recently, the Société Française de Biologie Clinique (S.F.B.C.) has described a P → L method for total LD at 30°C with the goal of measuring each isoenzyme with optimal conditions. They have found that the addition of Na⁺ and Cl⁻ ions to the reaction mixture modifies the stability of the ternary complex between enzyme, coenzyme, and substrate which usually inhibits the P → L reaction. They report the method to show the following advantages: minimization of the differences in the optimal concentration of pyruvate required for LD 1 and LD 5, improved linearity, and an increase in the upper limits of linearity.

has been requested or issued for lactate dehydrogenase.

The National Bureau of Standards in February of 1982 issued Standard Reference Material (SRM) 909, Human Serum (Enzymes Values Determined).\textsuperscript{79} SRM 909, a Human Serum Standard Reference Material, has been available for use in assessing the accuracy of clinical methods for six analytes: namely, glucose, calcium, chloride, lithium, potassium, and uric acid, for calibrating instrumentation used for these analytes, and for validating in-house or commercially produced quality control materials. The revised certificate now provides uncertified values, for information only, of the catalytic concentrations of seven enzymes including lactate dehydrogenase. The enzymes were determined cooperatively by teams of experts using the “best available” methodology. Where possible, methods of the IFCC or the American Association for Clinical Chemistry were used. The certificate provides outlines of the methods and the final assay reaction conditions, and gives literature references. The Bowers\textsuperscript{15} procedure for $P \rightarrow L$ was utilized except for temperature where a reaction temperature of 29.77°C was used, verified by a Gallium Melting Point Cell, SRM 1968. Sodium pyruvate is available also from the National Bureau of Standards as SRM 910.

A hierarchy of definitive method, reference method, and Standard Reference Material (SRM) can be developed to provide an accuracy base for many of the inorganic, and biochemical analytes in human serum measured routinely by clinical laboratories.\textsuperscript{78,81} In the area of clinical enzymology, standardization efforts have centered primarily on the development of reference and selected methods rather than on enzyme materials. However, the National Committee on Clinical Laboratory Standards has recently published a report entitled "C7-T-Tentative Guidelines to Kinetic Analysis of Enzyme Reactions."\textsuperscript{75} While several scientists and organizations have suggested requirements for enzyme reference materials,\textsuperscript{27,84} to date only a material for aspartate aminotransferase (E.C. 2.6.1.1) has been prepared, characterized and validated\textsuperscript{25,83,85,80} as a proposed biological validation and transfer material (BVTM).\textsuperscript{90} The availability of a BVTM for aspartate aminotransferase in conjunction with the IFCC proposed reference method for this enzyme\textsuperscript{11} provides a model system for the standardization of other enzymes of clinical laboratory interest including LD. Some of the developmental work for an analogous material for lactate dehydrogenase has been accomplished in that a highly purified LD 1 has been used as a reference material in the New York State Proficiency Testing Program.\textsuperscript{15} Also, the inter-converting of results for serum samples and for highly purified LD isoenzymes 1 and 5 assayed $L \rightarrow P$ in pyrophosphate, Tris and AMP has been investigated.\textsuperscript{24}

Acknowledgments

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