New Instrument for Rapid Determination of Activities of Lactate Dehydrogenase Isoenzymes

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Lactate dehydrogenase isoenzymes can be distinguished kinetically by the fact that isoenzyme H is strongly inhibited a few seconds after the reaction is started if high concentrations of pyruvate are present, in contrast to the M isoenzyme. A new instrument that exploits this fact can measure both the total activity and the proportion of H isoenzyme in serum or plasma in 8 to 10 s. The instrument consists of a simplified stopped-flow apparatus in which the plasma is assayed for lactate dehydrogenase activity, and an electronic device that measures the rate of the reaction at two pre-set time intervals. The first rate is taken between 0.2 and 0.4 s after the reaction is started, a time at which both isoenzymes are fully active, and at which the rate obtained thus reflects total lactate dehydrogenase activity in the plasma sample. The second rate is measured 4 to 6 s after the start of the reaction, at which time the H isoenzyme has become inhibited and the observed rate compared to the initial rate is therefore proportional to the percentage of H isoenzyme activity in the serum. These two rates are electronically displayed on two three-digit voltmeters, the first display being the total activity, the second a number proportional to the inhibited slope. The percentage of M isoenzyme can then be calculated from the initial and final rate. A total of five to six repeat assays may be done within a minute on 1 ml of plasma or serum. This instrument may be of significant value in following the progress of myocardial infarctions and other diseases.

Additional Keyphrases: heart disease • following enzyme activity with time • analytical systems

A myocardial infarction generally results in an increase in the activities of various enzymes in the serum, including creatine kinase (EC 2.7.3.2), lactate dehydrogenase (EC 1.1.1.27; LDH), aspartate aminotransferase (EC 2.6.1.1), and several other enzymes normally present in cardiac muscle cells. These increases are the result of release of the enzymes into the blood stream from the damaged heart tissue.

Lactate dehydrogenase consists of four polypeptide chains (subunits), which are strongly, but noncovalently bound to each other. Two different subunits have been identified (I-3); they are known as the M-type LDH or LDH-A and the H-type or LDH-B. Both subunits may be present in a single LDH molecule, which allows for a total of five isoenzymes: H4, H3M, H2M2, HM3, and M4, also known as LDH-1, LDH-2, LDH-3, LDH-4, and LDH-5.

The LDH in certain tissues such as heart, kidney cortex, and erythrocytes contains a high percentage of the H isoenzymes, whereas other tissues such as liver and skeletal muscles have predominantly the M isoenzyme of LDH. Release of LDH from the heart tissue into the serum therefore results in an increased proportion of the H type in the serum LDH (4), whereas liver damage results in an increased proportion of the M isoenzyme (5). In both cases one observes an increase in total LDH activity of the serum.

The determination of the relative activities of the LDH isoenzymes in serum has thus far been laborious, time-consuming, and expensive. They usually are separated by electrophoresis, followed by a staining that is based on enzymatic activity, and densitometry of the resulting colored spots or bands (7). Other techniques depend on the fact that the M isoenzyme is more susceptible to heat (8) or urea (9) denaturation than is the H isoenzyme, or make use of other physical or physico-chemical differences between the two isoenzymes. All these methods are too involved for convenient routine use in the clinical laboratory.

The relative isoenzyme activities in a given specimen may also be determined kinetically (4, 10). Kinetic methods are generally fast, inexpensive, and easily adapted for automated instrumentation. The kinetic properties of the H and M LDH differ in several ways, one of which is exploited in developing an instrument that can be used to make such measurements in seconds.

Kinetics of LDH at High Substrate Concentrations

LDH activity is inhibited in the presence of high concentrations of pyruvate (2, 11-14), as illustrated
in Figure 1, which shows that the activity of the H₄
isoenzyme of human origin is particularly sensitive to
high pyruvate concentrations: at a pyruvate concen-
tration of 10 mmol/liter only 40% of the activity re-
 mains, whereas the M₄ isoenzyme still has about 70%
of its activity.

The mechanism of the inhibition by high con-
centrations of pyruvate is now known (6, 12, 15, 16).
LDH can form several abortive ternary complexes⁴
that can cause an inhibition of the enzymatic activ-
ity. The complex responsible for the inhibition of
the enzyme in the presence of high concentrations of
pyruvate consists of LDH, NAD⁺, and pyruvate. The
LDH reaction and the formation of the abortive com-
plex are illustrated in the following equations:

\[
\text{NADH + pyruvate + H}^+ \rightleftharpoons \text{NAD}^+ + \text{lactate (1)}
\]

\[
\text{NAD}^+ + \text{pyruvate + LDH} \rightarrow \text{ternary complex (2)}
\text{(enzymatically inactive)}
\]

The complex forms to a greater extent with the H-
isoenzyme than with the M-isoenzyme. In both cases,
however, the complex is formed relatively slowly (6).

Generally LDH is assayed by use of reaction 1
(pyruvate to lactate direction). Hence, at the start of
the assay the NAD⁺ concentration is zero, and no
substrate inhibition should occur, even in the pres-
ence of high pyruvate concentrations, because the
abortive complex cannot be formed until a certain
amount of NAD⁺ has been generated. After some
NAD⁺ has been formed, it reacts relatively slowly
with the enzyme and the pyruvate, and during this
time the inhibition will be increasing until a steady
state is reached. Thus, when one assays for the H iso-
enzyme—with high concentrations of pyruvate pre-
sent—the reaction is initially at its maximum rate,
and only after some time has elapsed does it become
partly inhibited. The initial time period is too short
to be observed with an ordinary spectrophotometer,
but may be recorded with a stopped-flow apparatus,
as shown in Figure 2. As illustrated, little or no inhi-
bition occurs with the M isoenzyme under the con-
tions used, whereas the H isoenzyme is considerably
inhibited after the first 0.5 s. Mixtures of the two ho-
mologous isoenzymes as well as the hybrid forms H₃M,
H₂M₂, and HM₃, yield curves that are interme-
tiate to these two curves, in proportion to their iso-
enzyme content (4, 10).

These data suggest that if we measure the rate of
the reaction within a few tenths of a second after the
reaction has started, we will obtain the total activity
of the LDH present, whether or not high concentra-
tions of pyruvate are present, because during this
time the reaction is not inhibited. On the other hand,
if we measure the rate of a few seconds after the reac-
tion has started the rate is a function of the amount
of H isoenzyme present in the specimen. Therefore, if
the rate of the reaction is measured a few tenths of a
second after its initiation, and again a few seconds
later, one can within a few seconds obtain both the
total lactate dehydrogenase activity in the sample and
its H isoenzyme activity.

The LDH Isoenzyme Analyzer

The data shown in Figure 2 were obtained with an
Aminco-Morrow stopped-flow apparatus (American
Instrument Co., Silver Spring, Md. 20910). Such a
commercial stopped-flow apparatus, complete with
data-retrieval system, etc., may be too large an in-
vestment for the average clinical laboratory. We have	herefore designed a new instrument, which yields
the same information but is within the budgets of
most clinical laboratories.

Figure 3 shows our instrument, used to obtain the
data presented below. The heart of the instrument
(Figure 4) consists of a powerful light source, an
interchangeable filter, a small observation-cell with a
light path of 1 cm, and a phototube—i.e., the basic
components of a simple spectrophotometer. The ob-
servation chamber is designed as a simplified stopped-flow mixing chamber, such that the reagents and the sample of plasma are completely mixed within 50 to 100 ms. The two entrances to the cell are made to fit disposable 1-ml syringes, which makes sample change quick and simple. The exit is connected to a piece of Tygon tubing via a one-way valve, to prevent any back-flow. The tubing leads to a receptacle to collect discarded liquids. The solutions are driven into the chamber by two pushrods, that operate under air pressure and travel independently (Figure 5). The volume of reagents used may be adjusted by a micrometer, which limits the travel distance of push rods; no stopping syringe is needed. In this manner, 10–12 observations may be made with 1 ml of serum. The reagents, pyruvate and NADH, will eventually be available in kit-form somewhat analogous to the well-known “Stat-packs.”

The data-retrieval system consists of two digital read-outs, displaying the rate during the early part of the reaction and the rate when the steady state is reached. The time-interval during which the rates are measured, as well as the initiation of the measurements, may be varied by selecting the time at which the two measurements begin and end. In our experience, best results were obtained by measuring the first rate at 0.2 to 0.4 s, and the second at 4.0 to 6.0 s after the reaction is begun. The reaction is started by simply pushing a button.

The instrument is calibrated with serum from which lactate dehydrogenase has been removed by affinity chromatography and replaced by a known activity of purified H or M isoenzyme. These calibration solutions of the purified isoenzymes are prepared in serum, in order to have comparable viscosities and absorbances with the unknown plasma samples. Both the first and the second read-outs are calibrated to display the activity of the samples in U/liter. (In the commercial unit to be available, the second read-out will display the percentage isoenzyme activity in the specimen and necessary calculations will be done electronically.)

Table 1 shows the results of repeated assays of three samples of pooled serum. After a new syringe with serum or reagents was put in the instrument, we routinely performed two determinations in order to drive any trapped air bubbles out of the system. The results of these two determinations were ignored. Subsequent determinations were then recorded, until the solution in one of the syringes was used up. The coefficient of variation we found for measurements of the first rate was about 5% (Table 1). An activity of
.100 U represents an absorbance change of about 5 milliunits in 0.2 s. The second measurement is made during a 10 times longer time span (2 s), and the coefficient of variation we obtained for those readings was about 3%. Obviously, at this sensitivity level, a small air bubble or solid particle in the observation cell will affect the read-out so drastically that the operator immediately realizes that something is wrong, and will repeat the assay.

Figure 6 shows results for known mixtures of purified H₄ and M₄ isoenzymes. The constitution of the LDH and its activity during the second time interval are linearly related; this correlation is the basis for the determination by our technique.

Some Results for Plasma Analyses after Myocardial Infarction

We have now made such measurements with our instrument for plasma samples obtained at regular time intervals from several patients who had suffered a myocardial infarction. The results for one of them are shown in Figure 7. The data show that serum lactate dehydrogenase activity increased soon after the initiation of the series, leveled off after about 24 h, and returned to normal values during the next 48 h. Concomitant with the increase in total activity, the percentage of H isoenzyme activity increased and then decreased to the original value after about 48 h.

Several of the samples were analyzed by conventional methods for total activity and for their isoenzymes. The results obtained were the same, within experimental error, as those obtained with the new analyzer (Table 2).

The LDH profile for patient B.R. (Figure 8) indicates that this patient may have additional problems. Although the H isoenzyme activity of the plasma showed the expected increase and decrease, the total activity was still high after three to four days, which indicates that M isoenzyme may have been released from some other tissue during this period.

We used the new instrument to monitor several other patients who had suffered a myocardial infarction. If the patient suffers a second attack, one observes two maxima in the values for H isoenzyme, indicating additional damage to the heart tissue. (Further clinical data will be forthcoming in a subsequent publication.) Frequently monitoring the isoenzymes in plasma may thus be of value, because the instrument can give the clinician an almost immediate picture of the patient's LDH pattern. Indeed, because of the speed and ease of such assays, it could be used in intensive-care units.
Other Uses

The usefulness of this instrument may not be limited to assays of lactate dehydrogenase in plasma; it could also be used to determine other enzymes in the same serum samples by merely exchanging the syringe containing the NADH and pyruvate for a syringe containing the substrates for another enzyme that one would like to determine.

The instrument may also prove quite useful in (e.g.) the enzymological research laboratory. Basically, it is a fast, highly precise, and sensitive spectrophotometer, for which relatively small amounts of enzyme are required. It could be used for routine enzyme assays—e.g., monitoring column eluents—as well as for studies on enzyme kinetics. Data may be obtained much more rapidly than with an ordinary spectrophotometer, because activities are determined in a few seconds rather than in minutes, and there is no need to rinse and refill cuvets.

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References


Table 2. Results Obtained for Six Sera with the Instrument Described, as Compared with Those Obtained by Electrophoretic Separation

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a Consecutive sera from a single patient who had suffered a myocardial infarction.
b Av. of five or more determinations.
c Results of two determinations.