

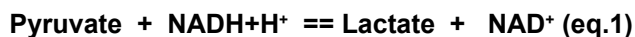
THE ISOENZYME DISTRIBUTION OF LACTATE DEHYDROGENASE

Determining the serum level of lactate dehydrogenase (LDH) activity combined with the results of other clinically important enzyme assays (GOT, SGOT, CRK) provides an excellent tool for the physician to make decisions in cases accompanied by tissue damage or with a change in membrane permeability. In a myocardial infarct within 12 hours the LDH level of serum begins to rise, reaching its maximal value after 48-72 hours. The enhanced enzyme level is proportional to the damage of the heart muscle tissue and in serious cases its elevation could even be threefold. The elevation of serum LDH activity might represent other diseases (e.g. anemias, tumors, liver diseases) as well, so it is important to know from what tissue the LDH was released into the bloodstream. The characterization of the LDH isoenzyme profile helps to solve this problem.

The LDH molecule itself is a tetramer, built from two types of subunits coded by different genes. From the linear combination of the H(heart) and M(muscle) type of subunits, five combinations can be obtained: H₄(LDH-1), H₃M₁(LDH-2), H₂M₂(LDH-3), H₃M₃(LDH-4) and M₄(LDH-5).

While LDH-1 and LDH-2 can be found mainly in the heart muscle and in erythrocytes, the liver and smooth muscle tissue contain mostly the LDH-5 isoenzyme.

All isoenzymes catalyze the following reversible reaction:



The different isoenzymes show different enzymekinetical behaviour due to their different amino acid compositions, which also provides the possibility to separate the isoenzymes by electrophoretic methods.

I. Enzymekinetical methods to characterize the LDH isoenzymes.

A. Heat stability

The serum samples obtained are heat treated for 30 minutes both at 57°C and 65°C in the presence of NADH. The LDH activities of the cooled samples are determined. A control sample held at room temperature presents the total amount of LDH activity. While the difference in the enzymic activities between the control and the 57°C treated sample gives the heat labile LDH activity, the enzyme activity measured from the 65°C treated samples provides the heat stable LDH activity, which is elevated in myocardial infarction.

B. Substrate concentration dependence

This method is based on the fact that while the LDH-5 isoenzyme is maximally active in the presence of 250 mM lactate, the optimal lactate concentration for the LDH-1 isoenzyme is 10 mM, and there is a 50 % inhibition of LDH-1 activity when it is assayed using 250 mM lactate as a substrate. By measuring LDH activity using both 10 and 250 mM lactate concentrations, the calculated ratio of the two activities helps to judge whether the sample

contains LDH-1 or LDH-5 isoenzyme.

C. Substrate specificity

The LDH isoenzymes also show different substrate specificities towards pyruvate and 2-oxobutyrate as substrates. While LDH-1 shows a higher specific activity when 2-oxobutyrate is used, the LDH-5 isoenzyme is more active with pyruvate. The ratio of LDH activities measured using the two substrates (HBDH/LDH) varies between 0.63-0.81 in normal cases. Ratios higher than 0.83 are characteristic for a myocardial infarction. Liver diseases are diagnosed when the ratio of enzyme activities is lower than 0.61.

II. Electrophoretic method for the separation of LDH isoenzymes:

Due to their different amino acid compositions the LDH isoenzymes can be separated by electrophoretic methods (agarose, cellulose-acetate, polyacrylamide can be used as solid carrier). In an electric field at pH 8.8 the LDH-1 migrates fastest towards the anode while the LDH-5 is the slowest isoenzyme. After electrophoresis the LDH isoenzymes can be visualized by an activity staining process where the product of the enzymic reaction is a water insoluble stain precipitating in the gel where the LDH proteins are located.

Practical part

A. Spectrophotometric assay:

Assaying LDH activity is based on the fact that NADH has an absorbance maximum around 340 nm which can not be found in the spectra of NAD. So when the enzyme assay is carried out using oxo-substrate and NADH according to eq.1., the time dependent decrease in the optical activities of the samples measured at 340 nm is proportional to the LDH activity ($\epsilon_{340}=6.2 \times 10^6 \text{ M}^{-1}\text{cm}^2$).

Solutions used:

1. 50 mM phosphate buffer, pH7.5
2. 8 mM NADH dissolved in phosphate buffer
3. 10 mM pyruvate dissolved in phosphate buffer
4. 100 mM 2-oxobutyrate dissolved in phosphate buffer
5. LDH-1 enzyme solution
6. LDH-5 enzyme solution

Pipet directly into photometric cuvettes

	1	2	3	4	5
Phosphate buffer μl	1000	910	910	910	910
NADH μl	-	30	30	30	30
LDH1 μl	-	30	30	-	-
LDH5 μl	-	-	-	30	30
pyruvate μl	-	30	-	30	-
2-oxobutyrate μl	-	-	30	-	30

The cuvettes are placed into the photometer and the reactions are started by the addition of the oxo-substrate. Previously the optical activities of sample 2, 3,4 and 5 are determined against sample 1 (blank). The decrease of absorbance measured at 340 nm is followed for 3 minutes. The enzyme activities are calculated from the linear part of the curves obtained by plotting A vs. time. The HBDH/LDH ratios are calculated for both LDH-1 and LDH-5 enzymes.

$$\text{Calculation: Activity (U/L)} = \frac{E * V * 1000}{\text{min} * 6.22 * v}$$

B. PAGE separation of LDH isoenzymes

Materials:

1. premade 6.5% polyacrylamide gel slabs
2. tank buffer (2.4 g Tris base, 11.6 g glycine/liter)
3. samples made from liver, heart muscle, and kidney in sample loading buffer
4. 1 M Tris- HCl, pH 8.0
5. NAD, 10 mM
6. tetrazolium-blue, 1 mg/ml
7. phenazine-methosulphate, 1.6 mg/ml
8. 1 M Na-lactate

In our experiments premade 6.5% polyacrylamide slab gels will be used. The sample slots are rinsed out with the tank buffer and the gels with the glass plates together are clamped to the buffer tank using bulldog clips. Load 40 μl from each sample into different slots of the gel. Carefully cover the samples with tank buffer. Pour tank buffer into the reservoirs and connect the electric cables. The positive pole is at the bottom. Turn the power source on and set the voltage setting to the value given by your instructor. Proceed for two hours. Turn off electricity, separate the two glass plates by prying them apart with a spatula and place the gel into the developing chamber

which already contains the developer solution (H₂O 18.4 ml, 1 M Tris 4 ml, tetrazolium-blue 12 ml, phenazine-methosulphate 4 ml, Na-lactate 4 ml and NAD 1.3 ml). Incubate at 45 °C to develop color reaction for 20 minutes. In the color reaction NAD and lactate serve as substrates, phenazine-methosulphate is the electron acceptor and tetrazolium-blue is the final electron acceptor. Wash the gel with water and dry the gel between paper sheets.

Answer the following questions:

1. How many bands can be observed in the different samples?
2. Is there any difference between the intensities of the different bands?
3. How can you explain the symmetrical distribution of the isoenzymes?
4. What is the explanation for the different distances of migration?
5. How does affinity staining work?
6. What properties of the LDH isoenzymes is the spectrophotometric method based on?