DETERMINATION OF ACTIVITY AND MICHAELIS CONSTANT OF LDH IN BIOLOGICAL MATERIAL

Attention: For a successful completion of this practical training is necessary to have 1 page of millimeter paper, ruler and calculator for each group (2 students)! Practical training follows the lecture “Enzymology” and seminar dedicated to the “Enzymology and enzyme kinetics”. The necessary part for your procedure in this practical training is on pages 3-6.

1. LACTATE DEHYDROGENASE: BIOCHEMISTRY AND CLINICAL MEDICINE

Lactate dehydrogenase (EC 1.1.1.27) is an enzyme that can be found in almost all tissues in human body. It catalyses the pyruvate - L-lactate change including the oxidation of NADH to NAD+. This enzyme consists of two types of polypeptide chains H and M, which form tetramer with molecular weight 140 kDa. There are 5 isoenzymes of LDH with the following composition M₄, M₃H, M₂H₂, MH₃, and H₄. In aerobic conditions pyruvate is converted to acetyl-CoA which enters Krebs cycle. In anaerobic conditions pyruvate is changed to lactate. The concentration of lactate in cytosol depends on NADH/NAD⁺ ratio. During tissue hypoxia the reoxidation of NADH and FADH₂ in respiratory chain in mitochondria is decreased. Decreased generation of ATP and energetic deficit can lead to a decreased function of the whole cell. The ratio NADH/NAD⁺ in cytosol is increased and it is related to the increase of lactate concentration as in anaerobic metabolism. Disorder of metabolism, hypoperfusion of tissues and deficiency of thiamine can indicate hyperlactemia or lactic acidosis. The use of different medicaments, together with combination of pathological changes of vascular system, liver or kidneys, leads in elderly people to increased risk of lactic acidosis with fatal prognosis.

Case report:
A 62 year old woman is admitted to hospital after she has been suffering from dyspnea and fuzziness for two hours. She denies chest pain. However, she feels a slight pressure near heart. She has been taking medications for diabetes type II, hypertension and hyperlipoproteinemia for many years. She takes 3 g of metformin in combination with 30 mg glitazide (diabetes therapy) every day. She takes 20 mg of atorvastatin every day (hyperlipoproteinemia therapy), as well as nitroglycerine and acetylsalicylic acid 100 mg for the treatment of stable angina pectoris. She is an ex-smoker and has not smoked for 5 years. She used to smoke 8 cigarettes per day. She denies alcohol abuse.

On admission, the patient had bradycardia (45/min) and deep and fast breathing (30/min). She was pale and sweating a lot. Her body temperature was 36.2 °C; blood pressure was 100/60 mmHg. Electrocardiogram showed the presence of the elevation of ST segment and the blockage of the left Tawara-branch block.

Basic biochemical and acid-base balance analyses were done. The results indicated the presence of lactic acidosis (Table 1).

Urine analysis: glucose +, other analytes were negative. The oxygen mask was immediately applied and the patient was treated with bicarbonate and the lost fluid was replenished. Renal function was satisfactory and the patient did not undergo hemodialysis. Cardiological intervention was undertaken by reperfusion. Further examination confirmed anteroseptal infarction as a result of damaged left coronary artery.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>135 mmol/L</td>
<td>136-146 mmol/L</td>
</tr>
<tr>
<td>K⁺</td>
<td>3.3 mmol/L</td>
<td>3.8-5.0 mmol/L</td>
</tr>
<tr>
<td>Glucose</td>
<td>10 mmol/L</td>
<td>3.9-5.6 mmol/L</td>
</tr>
<tr>
<td>pH</td>
<td>6.8</td>
<td>7.36-7.44</td>
</tr>
<tr>
<td>p₅CO₂</td>
<td>4.32 kPa</td>
<td>4.40-5.73 kPa</td>
</tr>
<tr>
<td>p₆O₂</td>
<td>9.07 kPa</td>
<td>10.4-14.3 kPa</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>22-26 mmol/L</td>
<td></td>
</tr>
<tr>
<td>Lactate</td>
<td>18 mmol/L</td>
<td>0.5-2.0 mmol/L</td>
</tr>
<tr>
<td>Creatinine</td>
<td>90 µmol/L</td>
<td>44-104 µmol/L</td>
</tr>
</tbody>
</table>

Table 1: Selected results of biochemical examination.
Biochemical aspects of cardiac ischemia:
Metformin is a *per oral* antidiabetic drug and it belongs to the class of biguanides. It is the medicament that is prescribed to obese patients with diabetes type II and with normal renal function. Metformin decreases both basal and postprandial concentration of plasma glucose. It decreases gluconeogenesis and glycogenolysis in liver and enhances recapturing of glucose at periphery, as well.

Lactic acidosis is a rare but very serious complication of metformin therapy. Metformin is contraindicated in patients who suffer from renal insufficiency, serious infection or a condition associated with tissue hypoxia. Patients with cardiac disorder (stable angina pectoris) can suffer from insufficient blood supply in myocardium if they do physical exercises. Decreased supply of oxygen can cause ischemia and a subsequent pain. Heart muscle has a high oxygen demand. Temporary ischemia and decreased oxygen supply result in the decrease of ATP production in mitochondria, inhibition of Krebs cycle, and increase of NADH/NAD+ ratio with conversion to anaerobic metabolism. Increasing concentration of lactate in cells and decreasing pH result in the inhibition of glycolytic enzymes. Production of ATP via anaerobic metabolism is weakened and the resulting accumulation of toxic products leads to myocardial necrosis. Development of massive lactic acidosis in the acute myocardial infarction and metformin therapy is observed. Ionic and acid-base homeostasis is impaired and this affects the vascular system; therefore, the risk of patient’s death is increased. Lactate dehydrogenase is an enzyme used in diagnosis of heart ischemia (mainly LDH1 and LDH2). LDH1/LDH2 ratio higher than one suggests increased probability of myocardial infarction. Other significant markers of myocardial infarction are, among others, myoglobin, troponin I or T, and CK-MBmass. LDH is also a tumour marker.

2. **GENERAL PRINCIPLES OF PROTEIN ISOLATION**

For isolation of the required protein, the choice of right material plays a crucial role. The material should be easily available and should contain the required protein in sufficient quantity. Strategy of isolation depends on the localisation of the protein in cell. The main aim of the first step is to dissolve the protein and discard all insoluble parts. For isolation of intracellular proteins it is important to homogenise the cells and transfer the cell material to appropriate buffer.

2.1 **Homogenisation**
The type of homogenisation is dependent on the character of used material. For animal tissues it is possible to use the meat grinder. Homogenisation can be carried out by mixer or by homogeniser, e.g. Potter and Elvehjem.

2.2 **Extraction**
Proteins from homogenised animal tissue are then extracted into 2 – 5 times volume of buffer. This buffer should have sufficient buffer capacity. Extraction should be carried out in the solution with stable pH. It is recommended to work at lower temperatures, since at these temperatures proteins are more stable (4 °C – the denaturation process is slow).

2.3 **Precipitation**
Proteins are then precipitated by the addition of neutral salt to the water solution. Protein molecules without solvation cover form aggregates when the salt is added. Eventually, these protein aggregates do precipitate. By applying the correct concentration of the salt in solution we can precipitate particular type of proteins due to the different water solubility of proteins. Salting effect is higher if pH equals pI (pH = pI), and the resulting charge of protein is therefore minimal. This means that the solubility decreases at
this pH. The most commonly used salt for this process is ammonium sulphate. It is very important to add salt to solution gradually by low amounts with continuous mixing.

### 2.4 Centrifugation

Centrifugation is often faster and more suitable process than filtration. Sometimes it can lead to better separation of solid and liquid phase even in one step.

### 3. ISOLATION OF LACTATE DEHYDROGENASE FROM HEART MUSCLE

Enzyme is extracted into phosphate buffer and purified by ammonium sulphate. Catalytic activity of LDH is proportional to the produced pyruvate per time unit under standard conditions. Generated pyruvate reacts with 2,4-dinitrophenylhydrazine, forming coloured hydrazone, which can be measured photometrically.

![Reaction where the coloured hydrazone is generated](image)

Procedure:

1. Cut 30 – 40 g of chicken heart into small pieces and homogenise in mixer with 100 mL of 0.2 M phosphate buffer with 1 mM EDTA. Extract homogenate for 15 min and stir occasionally. Centrifuge at 4000 RPM at 4 °C for 10 min. Supernatant is called rough extract.
2. Add solid ammonium sulphate (until 35% saturation is achieved) into the rough extract with continuous mixing. Incubate suspension for 15 min and then centrifuge according to previously described conditions. Add ammonium sulphate up to 60% saturation into the supernatant and then centrifuge again.
3. Re-suspend sediment in small amount of 0.1 M TRIS buffer (maximal 5 mL).
4. DETERMINATION OF CATALYTIC ACTIVITY AND MICHAELIS CONSTANT OF LDH WITH LACTATE AS A SUBSTRATE

After the lecturer’s permission, pipette .......... µL of prepared fraction of LDH in tube and add .......... µL of 0.1 M TRIS buffer (final dilution: ............). Diluted sample keep on the ice.

4.1 Preparation of sample for activity determination and estimation of Michaelis constant for LDH with lactate as a substrate

Pipette solutions to 6 tubes as follows:

<table>
<thead>
<tr>
<th>Tube</th>
<th>0.9 M Lactate [mL]</th>
<th>3 mM NAD⁺ [mL]</th>
<th>Dist. water [mL]</th>
<th>0.1 M TRIS buffer [mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Blank)</td>
<td>0.10</td>
<td>0.15</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>2</td>
<td>0.01</td>
<td>0.15</td>
<td>0.09</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>0.02</td>
<td>0.15</td>
<td>0.08</td>
<td>0.1</td>
</tr>
<tr>
<td>4</td>
<td>0.04</td>
<td>0.15</td>
<td>0.06</td>
<td>0.1</td>
</tr>
<tr>
<td>5</td>
<td>0.05</td>
<td>0.15</td>
<td>0.05</td>
<td>0.1</td>
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<tr>
<td>6</td>
<td>0.10</td>
<td>0.15</td>
<td>-</td>
<td>0.1</td>
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</table>

Table 2

1. Put the tubes with all solutions into the water bath and incubate for 5 min at 37 °C.
2. Add 0.1 mL of isolated enzyme into tubes 2-6 exactly in 10 s interval and incubate in the water bath for another 5 min at 37 °C.
3. After incubation, pipette again exactly in 10 s interval to all tubes 0.25 mL of 2,4-dinitrophenylhydrazine. Let them stay for 10 min at laboratory temperature.
4. Finally, add to all tubes 2.5 mL of NaOH and after 10 min measure absorbance against blank at 505 nm.¹

5. EXPERIMENTAL DATA PROCESSING

5.1 Determination of LDH activity

For calculation of LDH activity, use absorbance value measured in tube 6 against blank. Activity will be expressed as the concentration of formed pyruvate per minute in one milliliter of the sample.

\[
\text{LDH activity} = \frac{\Delta A}{t} \times 378,6 \times F^2
\]

LDH activity: ........................................... µmol.min⁻¹.mL⁻¹

Calculation:

¹ In laboratory practise, reduction of produced pyruvate to lactate with NADH as a cofactor is used. NADH is oxidized to NAD⁺ in redox reaction. This oxidation induces change in kinetic mode caused by decrease of absorbance at 340 nm.
² F is a dilution factor. It determines how many times you diluted the prepared enzyme fraction before the sample was analysed (see the part 4 of this procedure).
5.2 Estimation of Michaelis constant for lactate:

Use Lineweaver-Burk plot (double-reciprocal) to determine Michaelis constant for lactate. To axis $x$ plot $1/[\text{lactate}]$ and to axis $y$ plot $1/A$ (use millimeter paper):

<table>
<thead>
<tr>
<th>Tube</th>
<th>c(lactate)</th>
<th>1/[lactate]</th>
<th>A</th>
<th>1/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Blank)</td>
<td>-</td>
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*Table 3*

Calculate the value of Michaelis constant for lactate from the intersection point of the straight line and the axis $x$.

$$K_M(\text{lactate}) = \ldots \ldots$$

Calculation:

Discussion:

1. Interpret the pathological results from biochemical analysis that was done for this patient.
2. Try to think why lactic acidosis is dangerous of the organism? What are the other causes of lactic acidosis?
3. Explain the term Michaelis constant and its importance.
4. Try to explain why the increasing order of lactate concentration and the constant $\text{NAD}^+$ concentration were used for determination of $K_M$ for lactate? What would you do if you were to determine $K_M$ for $\text{NAD}^+$?
5. Why is it good to add EDTA into the buffer used for homogenisation?

Conclusion:
APPENDIX 1: DOUBLE-RECIPROCAL PLOT

Enzymes are protein catalysts that accelerate chemical reaction in an organism. Enzymes accelerate the rate of reaching equilibrium between default substances (substrates) and products only by decrease of activation energy. Enzymes divide the reaction into several steps. Final composition of balance mixture is not affected by enzymes. Specific feature of enzymatic reaction is saturation by substrate. Michaelis and Menten described the simplest mechanism of enzymatic reaction. Enzymatic reaction occurs by following scheme: enzyme (E) reacts at first with substrate (S) and generates enzyme-substrate complex (ES) which subsequently splits to enzyme and product (P).

\[
E + S \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} ES \overset{k_2}{\rightarrow} E + P
\]

For initial velocity (v) of the reaction, which is in steady state (concentration of ES complex is constant) and in which the concentration of substrate is much higher than the concentration of enzyme, it applies that:

\[
v = \frac{V_{max} \cdot [S]}{K_M + [S]}
\]

where \( V_{max} \) is maximal velocity of reaction and \( K_M \) is the Michaelis constant, which characterises affinity of enzyme to substrate. Michaelis and Menten equation is hyperbolic function. It suits very well for experimental finding for many enzymes. Through simple mathematic operations it is possible to linearize the hyperbola. This adjustment was suggested by Lineweaver and Burk:

\[
\frac{1}{v} = \frac{K_M}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}
\]

Graph of the equation is a linear dependence of reciprocal value of measured absorbance \( 1/A \) to reciprocal value of concentration of lactate \( 1/[S] \). Intersection with axis y is \( 1/V_{max} \) and intersection with axis x is \( -1/K_M \).
APPENDIX 2: MATERIAL SAFETY DATA SHEET

1. Sodium hydroxide

Risk phrases:
H314 Causes burns.

Safety phrases:
P280 Wear suitable protective clothing, gloves and eye/face protection.

2. 2,4-dinitrophenylhydrazine

Risk phrases:
H228 Flammable solid.
H302 Harmful if swallowed.
H315 Irritating to skin.
H319 Causes eye irritation.
EUH001 Explosive if dry.

Safety phrases:
Protect from heat/sparks/flames/hot surface. – Smoking is not allowed.
P280 Wear suitable protective clothing, gloves and eye/face protection.

3. TRIS - Tris(hydroxymethyl)aminomethan

Risk phrases:
H319 Causes eye irritation.
H335 Irritating to respiratory system.
H315 Irritating to skin.

Safety phrases:
P261 Prevent inhalation of dust.