ÚSTAV LÉKAŘSKÉ BIOCHEMIE A LABORATORNÍ DIAGNOSTIKY 1. LF UK A VFN

Enzymes

Practical lesson on medical biochemistry

General Medicine

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1. Proof of enzyme specificity

Principle:

The action of enzymes is highly specific with respect to the **substrate** as well as to the **type of catalyzed reaction**. As to **substrate specificity**, some enzymes exhibit very broad substrate specificity, e.g. esterases splitting both natural and artificial esters; some have a very narrow specificity, e.g. urease splitting only urea. In our experiment, the specificity will be demonstrated on enzyme **amylase** from saliva and yeast **sucrase**. Amylase cleaves α-1,4-glycosidic bonds in starch giving a mixture of oligosaccharides. Sucrase hydrolyses sucrose to glucose and fructose. Since reducing saccharides are the resulting products in both cases, Fehling´s test can be employed to show whether the cleavage of substrate occurred.

More information on the employed enzymes and their relatives:

Glycosidases belong to the class of hydrolases (subsubclass EC 3.2.1 of the international nomenclature of enzymes). They are specific towards the **type of glycosidic bond (**α-glycosidases or β-glycosidases).

Amylases, both of plant and animal origin, split **1,4-α-glycosidic bonds** of starch, glycogen and polysaccharides. They cannot split 1,4-β-glycosidic bonds, such as in cellulose.

α-Amylase (E.C. 3.2.1.1.) is an enzyme present in saliva and pancreatic juice of animals. It splits by **endohydrolysis 1,4-α-glycosidic bonds** in starch, glycogen and polysaccharides containing 3 or more glucose units bound as 1,4-α-glycosides. Thus, **amylose** is split hydrolytically to a **mixture of maltose and glucose** with hemiacetal hydroxyls released as α-anomers. **Amylopectin** and **glycogen** are split randomly at 1,4-α-glycosidic bonds, whereas **1,6-α-bonds remain intact,** so that the resulting products are **branched** and **unbranched oligosaccharides.** Enzymatic hydrolysis of **starch** passes through various stages, which can be demonstrated by the reaction with iodine. Starch gives with iodine a dark-blue color, whereas the polysaccharide cleavage products – dextrins are stained violet (amylodextrin), purple or red (erythrodextrin), or not at all if too short (achrodextrin). At the same time the amount of reducing sugars in the hydrolytic mixture increases.

β-Amylase (E.C. 3.2.1.2.) is an enzyme of **plant origin**, contained e.g. in malt, that splits 1,4-α-glycosidic bonds from the non-reducing end of polysaccharide chain. Thus amylose gives practically quantitatively maltose**,** released as β-anomer, thus leading to inversion in optical activity. Amylopectin (and glycogen) are split from the non-reducing ends as well, up to the 1,6-α- branching, the 1,6-α-bonds remaining intact. Dextrin originated from enzymatic hydrolysis of amylopectin by β-amylase is called limit dextrin. In summary, **neither animal α-amylase nor plant β-amylase can split 1,6-α- or 1,4-β-glycosidic bonds.**

γ-Amylase (glucoamylase, E.C. 3.2.1.20.) is a membrane bound enzyme in **brush border on the surface of enterocytes** of the small intestinal mucosa. It is relatively resistant to heat (thermostable maltase), splits **polysaccharides,** and yet better **maltose** from the non- reducing end at the **1,4-α-bonds,** releasing **β-glucose.**

Yeast sucrase is a **β-fructofuranosidase** that splits **β-glycosidic bond in sucrose**. In this respect it has a specificity different from the mammalian **small intestinal sucrase**, which is specific towards the **α-glycosidic** bond, so that in addition to **sucrose** it also cleaves **maltose**.

The **1,6-α-bonds** that remain in the oligosaccharides produced by α-amylase from starch and glycogen are eventually split by **isomaltase** subunit of **sucrase-isomaltase complex** of the intestinal brush-border, (oligo-1,6-glucosidase, EC 3.2.1.10).

The sucrase-isomaltase complex of the brush border is originally synthesized as a single polypeptide chain; the subunits originate secondarily, by proteolytic action of other proteases secreted into the intestinal lumen, and have a high degree of homology in their primary structures. The gene of the precursor pro-sucrase-isomaltase apparently evolved by duplication of a single ancestral gene. Both subunits are able to split **maltose** (representing about 80% of maltase activity of intestinal mucosa). Furthermore, as mentioned above, the **sucrase** subunit can splits **sucrose,** and the **isomaltase** activity can deal with **1,6-α- bonds** in isomaltose and dextrins.

Reagents:

1) Starch solution 10 g/l (1 g of starch is shaken or heated with water; pour the resulting suspension slowly to 100 ml of boiling water, keep boiling 2-3 minutes)
2) Sucrose 10 g/l (daily fresh)
3) Physiological solution (NaCl 9 g/l)
4) Yeast sucrase
5) Fehling′s solution I: copper sulfate 70 g/l 
6) Fehling′s solution II: sodium hydroxide 250 g/l 
   sodium potassium tartrate 350 g/l
7) Lugol solution: iodine 13 g/l and potassium iodide 25 g/l

Procedure:

a. Collect about 1 ml of your own saliva to a test tube, and shake with 9 ml of physiological solution. This will serve as a preparation of amylase.

b. Number four long glass tubes with a marker and pipette the following volumes into them:

Tube N^{o}	**1**	**2**	**3**	**4**
Starch	5 ml	-	5 ml	-
Sucrose	-	5 ml	-	5 ml
Amylase	1 ml	1 ml	-	-
Sucrase	-	-	1 ml	1 ml

c. Incubate the tubes for 30 minutes at 37 °C (thermo block).

d. Prepare Fehling′s reagent: in a beaker combine about 5 ml (use a plastic dropper!) Fehling I ($CuSO_4$) with an equal amount of Fehling II [sodium potassium tartrate (Seignett′s salt) with NaOH].

e. Use Fehling′s test to detect where the cleavage of substrate occurred: Take four <u>new</u> long test tubes, mark them 1-4 and put about 1 ml of Fehling′s reagent to each tube. Add several drops of the reaction mixtures from the corresponding original tubes, and place the tubes to a boiling water bath. Read the result after several minutes of heating. The original Fehling reagent is dark blue. Any color change to green, orange or red color due to formation of Cu_2O indicates the presence of reducing compounds, i.e., cleavage of substrate in this experiment.

f. Take the rest of incubation mixtures in the original tubes 1 and 3 (where starch was used) and perform a test for starch by adding Lugol solution (pre-diluted with water: 4 drops/10 ml of water). Presence of undigested starch manifests as a dark blue color.

Evaluation: Indicate to the table in your lab report where the cleavage of the substrate occurred using symbols + (substrate cleaved) or – (substrate not cleaved).

2. Effect of pH on the enzyme activity

Principle and theoretical background:

The effect of pH on enzymatic activity will be demonstrated using **pepsin**, an important digestive enzyme that cleaves proteins in the acidic environment of the stomach.

Various animal species produce slightly different forms of pepsin that have also been named differently. All of them belong to the subsubclass of **aspartate endopeptidases** (E.C. 3.4.23), which means they possess in their active sites two aspartate residues oriented opposite to each other, which participate in the acid base catalysis.

The pepsins work best in acidic environment. In humans the active pepsin is formed in the acidic medium of gastric lumen from the inactive precursor, pepsinogen, by cleavage of a part of the polypeptidic chain. This activation by limited proteolysis can either occur spontaneously at pH lower than 5 as an intramolecular reaction **(autoactivation)**, or by action of another, already activated molecule of pepsin **(autocatalysis).** The peptides cleaved in the process of pepsinogen activation remain bound to the pepsin and act as **inhibitors** of its activity. Thus, the last step of pepsin activation is release of these inhibitory peptides, which occurs at pH below 2.

Human **pepsin A** has 5 molecular forms. It hydrolyses certain peptidic bonds within the polypeptidic chain (endohydrolysis). Its specificity is directed to the presence of hydrophobic, preferentially aromatic (Phe), but also aliphatic (Leu), amino acid residues before or after the bond to be cleaved. **Pepsin B** is formed by a similar mechanism as pepsin A, but from pepsinogen B; it is produced mainly in pylorus. **Pepsin C** in humans is named as **gastricsin**; it splits by endohydrolysis preferentially peptide bonds after the amino acid residue Tyr, and is less specific than pepsin A.

For pepsin A the **pH optimum** depends on substrates: for egg-white albumin it is 1.5, for casein it is 1.8 and for hemoglobin the value is 2.3.

In the present exercise the proteolytic action of pepsin will be demonstrated on egg-white proteins as substrate.

Reagents:

1) Pepsin solution 10 g/l

2) Hydrochloric acid 0.1 mol/l

3) Egg-white suspension (boiled egg-white is homogenized in physiological solution – final protein concentration about 300 g/l)

Procedure:

Measure the following volumes (shake the egg white suspension before use!):

Tube N^o		**1**	**2**	**3**	**4**
Final pH		**1.2**	**1.5**	**2.5**	**control**
Pepsin	ml	2.0	2.0	2.0	-
HCl	ml	4.0	2.0	0.2	2.0
Distilled water	ml	-	2.0	3.8	4.0
Egg-white	ml	1.0	1.0	1.0	1.0

Place the tubes to thermo block set to 37 °C. Inspect the tubes every five minutes for up to 20 minutes for any sign of clarification. The egg-white suspension is cloudy and gradual digestion with pepsin makes the solution clearer.

The experiment is done when you see clear differences among the tubes that indicate which pH is optimal for the pepsin action. Evaluate visually the appearance of solutions in the tubes and write the results to the table in your lab report. Use the symbols + for complete clarification, ± for partial clarification and − for persistent turbidity. The optimal pH is in the test tube with the clearest solution.

3. Oxidoreductases

Introduction: Significance of biological oxidations in organism

A major part of energy utilized in an animal organism originates from **oxidation-reduction (redox) processes**. Oxidized products of a redox reaction contain less potential energy than the original reacting substances, and the difference in energy content may appear as heat or can be transformed to other kinds of useful energy, e.g. energy of chemical bonds in the reacting system. Many redox reactions in the cell are coupled with the formation of "macroergic" phosphate esters of anhydride nature (ATP, ADP), which have a special importance in the **conservation** and **transformation of energy**. The source of the released energy in the cell is oxidation reactions. Oxidation-reduction reactions may proceed under **anaerobic** conditions, as in glycolysis, or as an **aerobic** process, e.g. during oxidation of the substrates of citric acid cycle (Krebs cycle) and β-oxidation of fatty acids in mitochondria.

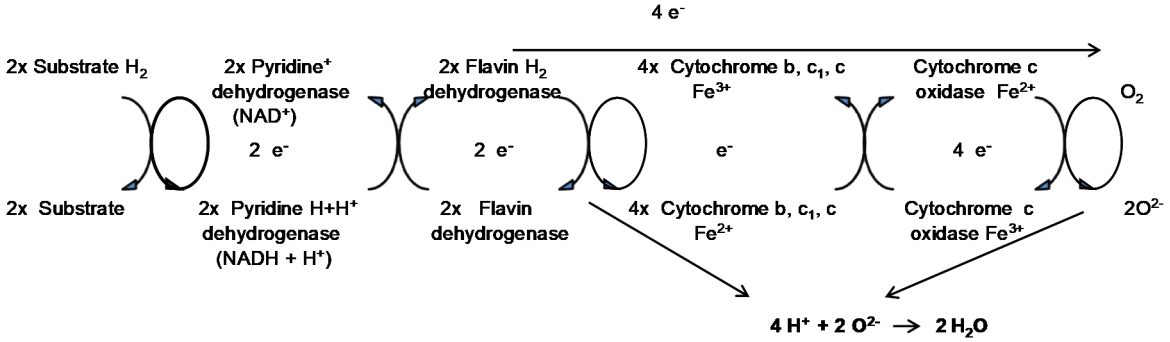
Saccharides, lipids and proteins represent the source of energy in the organism. However, their oxidation does not proceed directly with the molecular oxygen even in the aerobic organisms (with the exception of oxygenases).

During the oxidation-reduction processes in **mitochondria t**he electrons are transferred from the substrates to molecular oxygen via a **set of carriers**, which are organized as a system of ordered redox complexes. The chain of oxidation-reduction reactions (cell respiration) is represented by two phases:

1. Transfer of hydrogen atoms by the action of dehydrogenases, containing pyridine (nicotinamide) nucleotides and flavin nucleotides.

2. Transfer of electrons via cytochromes in mitochondria. The last step of electron transfer is represented by the cytochrome c oxidase, which reacts with molecular oxygen. The final product of oxygen reduction is the molecule of water.

The whole set of redox reactions in the inner mitochondrial membrane is called **respiratory chain**, which may be schematically described as a two-electron transfer, because the electrons are subtracted from the organic substrates during biological oxidations in pairs via coenzymes of dehydrogenases. At the level of cytochromes, however, the transfer becomes one-electronic and at the level of cytochrome c oxidase during the reduction of the molecule O_2 it is a four-electronic transfer.



During the two-electronic transfer from the substrates of citric acid cycle and β-oxidation to molecular oxygen in the inner mitochondrial membrane new molecules of ATP are synthesized – the process called **oxidative phosphorylation**.

Molecular oxygen is not the only possible acceptor of electrons transferred in redox processes. Some dehydrogenases transfer electrons to other acceptors, e.g. to pyruvate as in **lactate dehydrogenase** reaction of **glycolysis**, or in vitro to **artificial acceptors** like **methylene blue** (flavin dehydrogenases). Such processes belong to anaerobic oxidation-reduction reactions.

Our lesson includes a model oxidation of glucose with atmospheric oxygen catalyzed by methylene blue, which serves as a transient acceptor of electrons. The methylene blue is colorless in its reduced form, but when shaken on air it can react with oxygen and gets transformed back to the colored oxidized form.

As an example of dehydrogenases, in addition to lactate dehydrogenase, dehydrogenation by means of the enzyme **xanthine oxidoreductase (xanthine oxidase)** present in milk will be shown. In this reaction formaldehyde will serve as the substrate and methylene blue as the electron acceptor.

In addition to dehydrogenases and cytochromes further important representatives of oxidoreductases (among others) are **peroxidase** and **catalase**, both acting on H_2O_2. Peroxidase catalyzes oxidation of a suitable substrate by hydrogen peroxide releasing only water molecules as a by-product:

$$AH_2 + H_2O_2 \rightarrow 2\,H_2O + A$$

Whereas catalase releases molecular oxygen during the reaction:

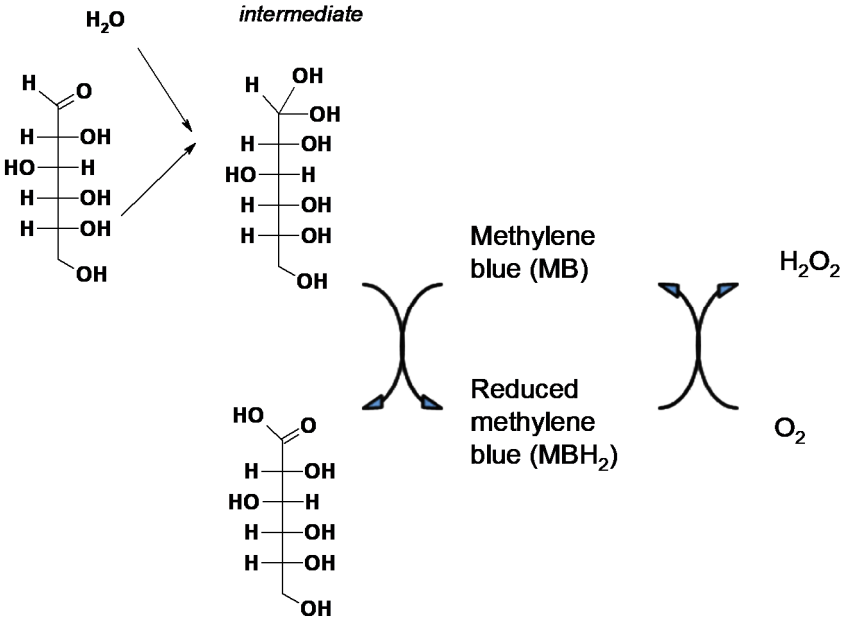
$$2\,H_2O_2 \rightarrow 2\,H_2O + O_2$$

Our experiment will demonstrate the activity of potato peroxidase using a benzidine (4,4'-diaminobiphenyl) derivative, which is oxidized in the presence of hydrogen peroxide as the substrate to a colored product (blue). Simultaneously we will test for pseudoperoxidase action of blood pigment and the blood catalase.

3.1. Oxidation of glucose with the air oxygen (model experiment)

Principle:

This experiment demonstrates a simple **non-enzymatic oxidation-reduction system**, where the hydrogen acceptor is a dye **methylene blue**; and the donor of reduction equivalents is **glucose**, which is oxidized. The original, oxidized methylene blue has a blue color; reduction by glucose changes it to the **colorless leucoform**. When shaken with air, the colorless reduced methylene blue is reoxidized by **molecular oxygen** and turns blue again.



Reagents:

1) Glucose 2.5 g/l
2) Methylene blue 0.5 g/l
3) Sodium hydroxide 80 g/l 

Procedure:

a. In a long glass test tube mix 1 ml of glucose solution with 50 µl of methylene blue, add 3 drops of sodium hydroxide solution, and heat in a boiling water bath. The blue solution of methylene blue (MB) is hydrogenated to its colorless leucoform (MBH_2).

b. As a negative control, do the same with another tube containing 1 ml of distilled water instead of glucose.

c. Cool both tubes and shake with air (oxidation).

d. Compare the course of the reaction in both test tubes and explain the difference.

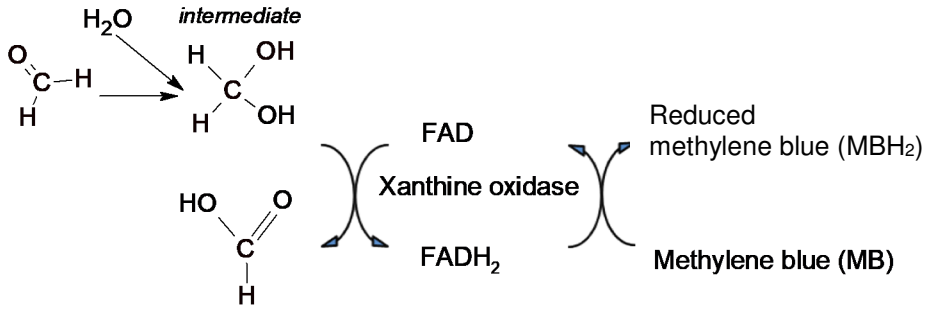
3.2. Dehydrogenation with xanthine oxidoreductase (xanthine oxidase)

Principle:

Xanthine oxidase (E.C. 1.17.3.2) is a relatively non-specific dehydrogenase contained e.g. in milk (Schardinger's enzyme), mammalian liver and intestine. It converts **purines** (hypoxanthine and xanthine) to **uric acid**. In addition, xanthine oxidase is able to oxidize also some other substrates, including **simple aldehydes** (i.e., it possesses also an aldehyde oxidase activity, EC 1.2.3.1). It is a flavoprotein, whose coenzyme is **flavin adenine dinucleotide (FAD)**, but its active center contains also atoms of metals (Mo, Fe). The substrate dehydrogenation produces $FADH_2$, which can be reoxidized with **molecular oxygen** (autoxidation) forming H_2O_2, or with an **artificial acceptor** (methylene blue). The enzyme is not inhibited by cyanide, but is destroyed by boiling.

In the mammalian liver and other tissues under physiological condition *in vivo* the xanthine oxidoreductase exists mostly in the **dehydrogenase form** (D-form, E.C. 1.17.1.4) with NAD^+ as a coenzyme. In pathological states and also under denaturing condition *in vitro* the enzyme is converted to the **oxidase form** (O-form, EC 1.17.3.2), unable to bind NAD^+.

In our experiment we will use a solution of formaldehyde as a non-specific substrate, methylene blue as an electron acceptor for $FADH_2$ reoxidation, and non-pasteurized cow milk as a source of the enzyme.



Reagents:

1) Fresh milk (unpasteurized)
2) Boiled milk
3) Methylene blue 0.5 g/l
4) Formaldehyde solution 4 g/l 
5) KCN 10 g/l 

Procedure:

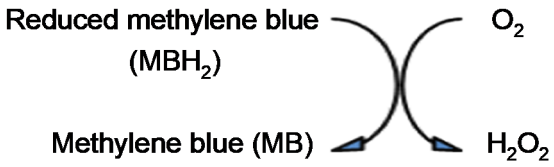
a. Take 3 test tubes and prepare the reaction mixtures according to this scheme:

	1	**2**	**3**
Fresh milk	2 ml	-	2 ml
Boiled milk	-	2 ml	-
Methylene blue	100 µl	100 µl	100 µl
KCN (poison!)	-	-	200 µl
Deionized H_2O	200 µl	200 µl	-
Formaldehyde	250 µl	250 µl	250 µl
Mix well the tube contents			
Paraffin oil* (overlay immediately after mixing)	1 ml	1 ml	1 ml

N.B. ** Paraffin oil is added in order to prevent autoxidation (to provide anaerobic condition for the reaction)*

b. Place the tubes to thermo block, incubate at 37 °C and keep checking the reaction course every 5 minutes until the tube No. 1 becomes completely destained.

c. Shake the Tube No. 1 with air. The milk is expected to turn blue again because of the reaction:



d. Place the tube back to thermo block – the methylene blue will lose its color again.

Evaluation:

Describe the observations in all three tubes; compare the obtained results in tubes No. 2 and 3 with the one in tube No. 1. Explain the results.

3.3. Peroxidase and catalase

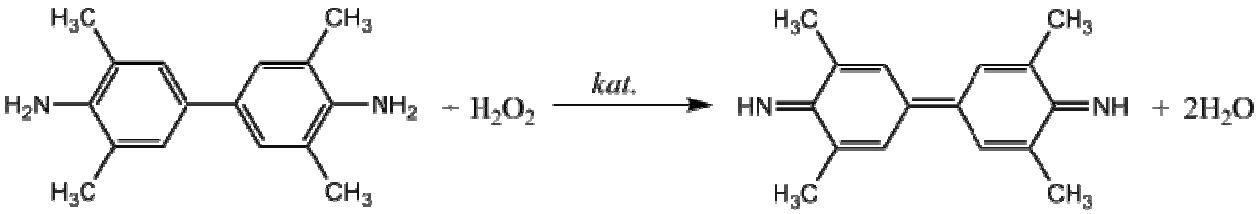
Principle:

Hydrogen peroxide H_2O_2 is a by-product of the oxidative processes in the cell and is subsequently removed by specialized enzymes called **peroxidases** and **catalases.** They belong to the group of hemin enzymes (hemoproteins). The reaction mechanisms of the two groups of enzymes are different.

Peroxidases catalyze the oxidation of a substrate by hydrogen peroxide releasing water molecules as a by-product (see the introductory section). Peroxidases (EC 1.11.1.7) are found in plant as well as animal organisms.

Catalase (EC 1.11.1.6) decomposes hydrogen peroxide to water and molecular oxygen as described in the introduction; in addition it may act also as a peroxidase. It is present in greater or smaller amounts in all tissues (in structures called peroxisomes) and body fluids. Its major significance lies in decomposition of H_2O_2, harmful for the organism.

In the present experiment the peroxidase reaction will be demonstrated qualitatively by the oxidation of a derivative of benzidine (4,4´-diaminobiphenyl) to a blue product. In further experiments, pseudoperoxidase activity in urine containing blood pigment will be shown (non-specific effect of heme iron), and the action of catalase present in blood will be demonstrated as release of O_2 from H_2O_2.



Tetramethylbenzidine (colorless) — Benzidine blue

Tetrametylbenzidin. In *WikiSkripta* [online]. Praha : MEFANET, 2008- [cit. 2012-01-05]. Obtained from WWW: <http://www.wikiskripta.eu/index.php?title=Tetrametylbenzidin&oldid=109016>. ISSN 1804-6517.

Reagents:

1) Extract from potatoes (with peroxidase activity)
2) Urine with blood
3) Blood diluted with water 1 : 50
4) 3,3´-dimethylbenzidine (tolidine) 
5) Glacial acetic acid 
6) Hydrogen peroxide 30 g/l
7) KCN 10 g/l 

3.3.1. Proof of peroxidase by benzidine reaction

Principle:

Derivative of benzidine (3,3´-dimethylbenzidine - tolidine) is in the presence of peroxidase and hydrogen peroxide oxidized to a colored product.

Procedure:

a. Dissolve a few crystals of tolidine in cca 1 ml of concentrated acetic acid
b. Prepare the reaction mixtures to 4 tubes as follows:

N.B. ** In the tube 2 boil the potato extract for 5 min in boiling water bath and cool down.*

	1	**2**	**3**	**4**
Potato extract	1.0 ml	-	-	1.0 ml
Potato extract boiled	-	1.0 ml*	-	-
Deionized H_2O	-	-	1.0 ml	100 µl
H_2O_2	100 µl	100 µl	100 µl	100 µl
Tolidine in acetic acid	100 µl	100 µl	100 µl	-

Evaluation:

Compare the result obtained in tube 1 with the ones in tubes 2, 3, and 4. Explain the differences.

3.3.2. Pseudoperoxidase reaction

Principle:

Blood in urine catalyzes oxidation of benzidine derivative by hydrogen peroxide even after boiling. It indicates a non-specific effect of iron in heme, rather than an enzyme activity.

Procedure:

a. Separate the urine with blood to two aliquots.
b. Heat one aliquot in water bath to boiling.
c. Filter both aliquots of urine.
d. Examine both filter papers for the peroxidase activity by adding several drops of tolidine solution (from previous experiment) and then hydrogen peroxide.

Evaluation:

Compare the result on both filter papers with the experiment 3.3.1. What is the difference between the two experiments and what does it indicate?

3.3.3. Proof of catalase

Procedure:

Prepare the reaction mixtures as follows:

N.B. ** In the tube 3 boil the diluted blood for 5 min in boiling water bath and cool down.*

	1	**2**	**3**
Diluted blood 1 : 50	2 ml	2 ml	-
Boiled diluted blood 1 : 50	-	-	2 ml*
KCN 10 g/l (poison!)	-	0.5 ml	-
Deionized H_2O	0.5 ml	-	0.5 ml
H_2O_2	1 ml	1 ml	1 ml

Evaluation:

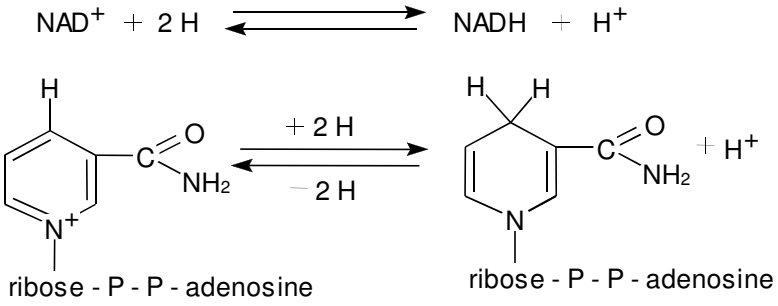
Compare the result in tube 1 with those obtained in tubes 2 and 3, try to analyze and explain your observations.

4. Estimation of catalytic activity of lactate dehydrogenase in serum by means of the Warburg optical test

Principle:

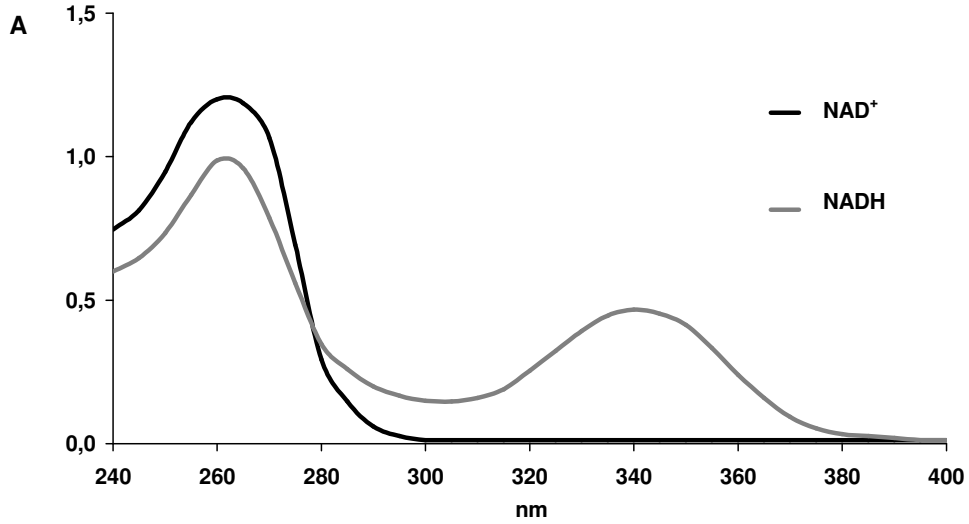
Nicotinamide adenine dinucleotide, NAD in short, is a coenzyme consisting of nicotinamide, adenine, two ribose molecules and two phosphates, connected together in the same way as nucleotides are (adenosine diphosphate, then ribose and then nicotinamide attached).

In the cell NAD participates **in redox reactions**, i.e. it is a coenzyme of **oxidoreductases**. The coenzyme occurs in two forms: **NAD^+** is the oxidized form that accepts electrons from other molecules and is itself reduced. In this way **$NADH + H^+$** originates which provides electrons and itself gets oxidized. This electron transfer is the major function of NAD^+.



Reversible hydrogenation of nicotinamide adenine dinucleotide, which occurs on the pyridine ring in nicotinamide, leads to the reduced form and is associated with a distinct change in the **absorption spectrum**. The oxidized form (NAD^+) has an absorption maximum at wavelength 260 nm. Reduction cancels the aromatic character of the pyridine ring and its transition to a quinoid form ($NADH + H^+$) is associated with emergence of **another absorption maximum at 340 nm**. This maximum is utilized for estimation of concentration of the coenzyme in a reaction mixture.

Absorption spectrum of NAD^+ and NADH



The used **molar absorption coefficients** are:

at 340 nm $6.22 \cdot 10^3$ $l \cdot mol^{-1} \cdot cm^{-1}$
at 365 nm $3.41 \cdot 10^3$ $l \cdot mol^{-1} \cdot cm^{-1}$

We can prepare a reaction mixture from the estimated enzyme, e.g. **lactate dehydrogenase** (LD, EC 1.1.1.27.), its coenzyme (NADH) and corresponding substrate (pyruvate) and under optimal condition follow the reaction rate by measuring changes in absorbance of NADH at the wavelength 340 nm or 365 nm.

$$CH_3\text{-}CHOH\text{-}COOH + NAD^+ \longleftrightarrow CH_3\text{-}CO\text{-}COOH + NADH + H^+$$

lactate — pyruvate

If the reaction mixture containing the enzyme, pyruvate and NADH is placed into a cuvette of a spectrophotometer equipped with UV-light source and ability of continuous recording, the enzyme activity will manifest as a gradual and steady decrease in absorbance at 340 nm, appearing as declining straight line (zero-order kinetics).

Then, if the molar absorption coefficient of NADH at this wavelength is known, dilution of the sample is taken into account and time interval 1 sec. is chosen, it is possible to express the activity of LD in sample (serum) in µkat/l. If continuous recording is not available, it is possible to resort to a discontinuous measurement of 340 nm absorbance in 1 minute intervals ($\Delta A_{340\,nm}$), and convert to the interval 1 sec.

Reagents:

1) Reagent 1: Phosphate buffer 62.5 mmol/l, pH 7.5
2) Reagent 2: Phosphate buffer 62.5 mmol/l, pH 7.5
   Pyruvate 0.75 mmol/l
3) Reagent 3: Phosphate buffer 62.5 mmol/l, pH 7.5
   NADH 1.25 mmol/l
4) Serum

Procedure:

a. Cuvettes and solutions are pre-warmed to 37 °C.

b. Prepare the blank sample (directly to a cuvette):

800 µl reagent 2 (phosphate buffer and pyruvate)
200 µl reagent 1 (phosphate buffer)
20 µl serum.

c. Mix.

d. According to the instructions for use of the spectrophotometer Lightwave II+ set the measurement of absorbance at the wavelength 340 nm (**Lightwave II+ - short instructions**).

e. Insert the blank sample to the instrument and set it to zero.

f. Into another cuvette measure:

800 µl reagent 2 (phosphate buffer and pyruvate)
200 µl reagent 3 (NADH in phosphate buffer)
20 µl serum.

g. Mix well and after exactly 1 minute read A for the wavelength 340 nm, and write the value to the table.

h. Repeat the measurement 5-times more in **exactly one minute intervals**; write down the absorbances for the given wavelengths to the table.

i. From the measured absorbances A_{340} calculate the difference in absorbance per one minute (ΔA_{340}). Calculate the arithmetic mean of these differences. Use the average value of ΔA_{340} for calculation of the catalytic activity of LD related to 1 liter of undiluted serum and time interval 1 sec., utilizing the molar absorption coefficient of NADH for 340 nm:

$$\mathbf{LD\ (\mu kat/l) = \Delta A_{340} \times 136.7}$$

Explanation of calculation:

The calculation of enzyme activity is based on the **Lambert-Beer law:**

$$A = \varepsilon \cdot d \cdot c \qquad (1)$$

$$c = \frac{A}{\varepsilon \cdot d} \qquad \Delta c = \frac{\Delta A}{\varepsilon \cdot d} \qquad (2)$$

The estimation of enzymatic activity assumes the reaction follows the zero order kinetics, i.e. the course of the reaction is linear (consumption of substrate or accumulation of the product per unit of time is constant).

In the kinetic estimation of the enzymatic activity the change of substrate or NADH concentration (mol · l^{-1}) is related to time interval 1 sec. and volume of undiluted serum 1 liter.

$$\text{Catalytic activity/1 liter of serum} = \frac{\Delta A}{\varepsilon \times d} \cdot \frac{1}{60} \cdot \frac{V}{v} \cdot 10^6 \quad \mu mol \cdot l^{-1} \cdot s^{-1} \qquad (3)$$

The change of substrate concentration is expressed in terms of the equation (2), 1/60 is conversion from minutes to seconds, and V/v stands for dilution of the serum. The unit of enzyme activity is katal (kat) = mol · s^{-1}. In clinical chemistry the derived unit µkatal is preferred (1 kat = 10^6 µkat).

When measuring a specific enzyme, lactate dehydrogenase in our case, specific values are entered to the equation 3 (4):

$$LD = \frac{\Delta A_{340}}{\varepsilon \times d} \cdot \frac{1}{60} \cdot \frac{V}{v} \cdot 10^6 \quad \mu mol \cdot l^{-1} \cdot s^{-1} \qquad (4)$$

$$LD = \frac{\Delta A_{340} \times 1.02 \times 10^{-3} \times 10^6}{6.22 \times 10^3 \times 60 \times 0.02 \times 10^{-3}} = \Delta A_{340} \times 136.7 \ \mu kat/l$$

Symbol		**Unit**	Specific value for LD and used method
V	Volume of reaction mixture in cuvette	l	**$1.02 \cdot 10^{-3}$**
v	Volume of added serum	l	**$0.02 \cdot 10^{-3}$**
ε	Molar absorption coefficient (for NADH at 340 nm)	$l \cdot mol^{-1} \cdot cm^{-1}$	**$6.22 \cdot 10^3$**
d	Lightpath (cuvette thickness)	cm	1
10^6	Conversion from mol to µmol		10^6
60	Conversion from min. to sec.		60

Note: the first three values (in bold) depend on the estimated enzyme and method, whereas the following three values (lightpath, conversion from mol to µmol, conversion from min. to sec.) are usually the same for any kinetic measurement of enzyme activity.

EXAMPLE:

The experimental data:

Minute	A_{340}	ΔA_{340}
2.	0.882	
3.	0.854	0.028
4.	0.827	0.027
5.	0.800	0.027
6.	0.772	0.028
7.	0.745	0.027
Mean:		0.0274

Calculation: LD (µkat/l) = 136.7 × 0.0274 = **<u>3.74 µkat/l</u>**