

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 (subclass 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 split **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°	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 new 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 subclass 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°	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
Purified 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.

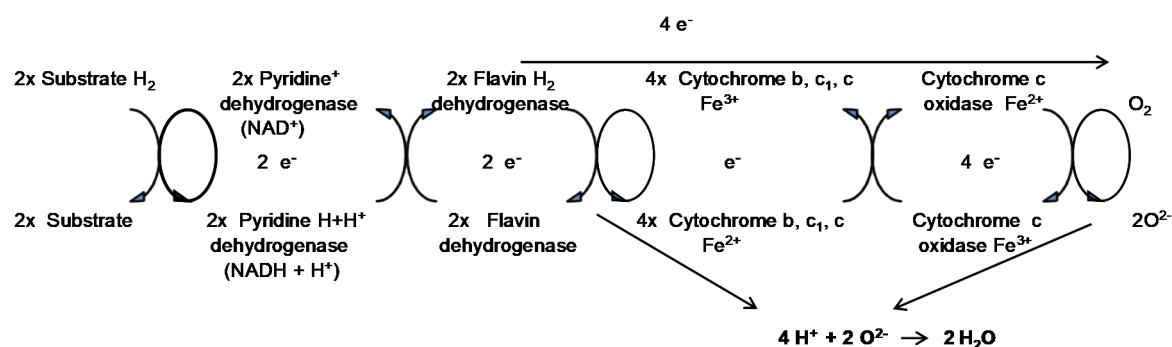
Saccharides, lipids and proteins represent the source of energy in the organism. However, their oxidation does not proceed directly with the molecular oxygen even in the aerobic organisms (with the exception of oxygenases).

During the oxidation-reduction processes in **mitochondria** the 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.



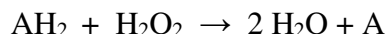
During the two-electronic transfer from the substrates of citric acid cycle and β -oxidation to molecular oxygen in the inner mitochondrial membrane new molecules of ATP are synthesized – the process called **oxidative phosphorylation**.

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.

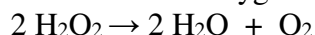
Our lesson includes a model oxidation of glucose with atmospheric oxygen catalyzed by methylene blue, which serves as a transient acceptor of electrons. The methylene blue is colorless in its reduced form, but when shaken on air it can react with oxygen and gets transformed back to the colored oxidized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



Whereas catalase releases molecular oxygen during the reaction:

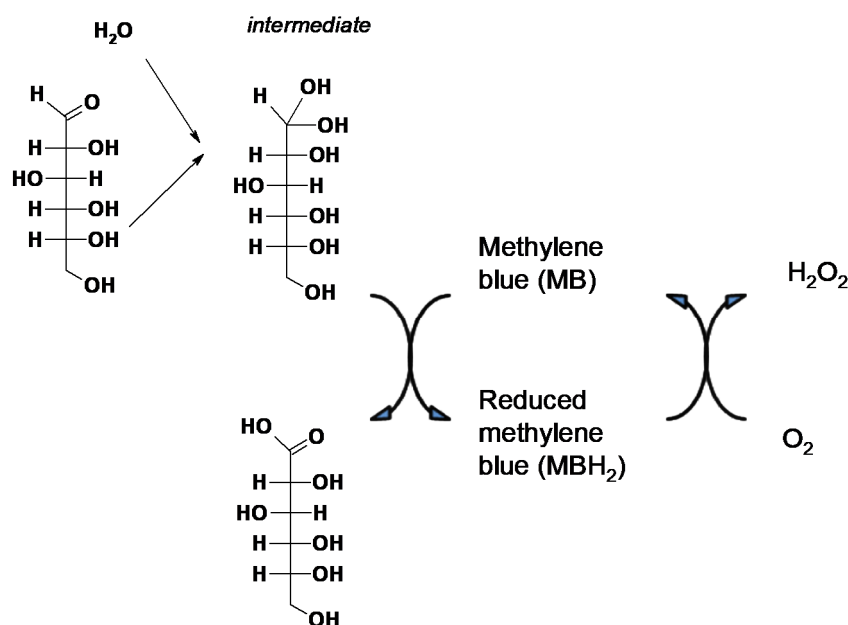


Our experiment will demonstrate the activity of potato peroxidase using a benzidine (4,4'-diaminobiphenyl) derivative, which is oxidized in the presence of hydrogen peroxide as the substrate to a colored product (blue). Simultaneously we will test for pseudoperoxidase action of blood pigment and the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

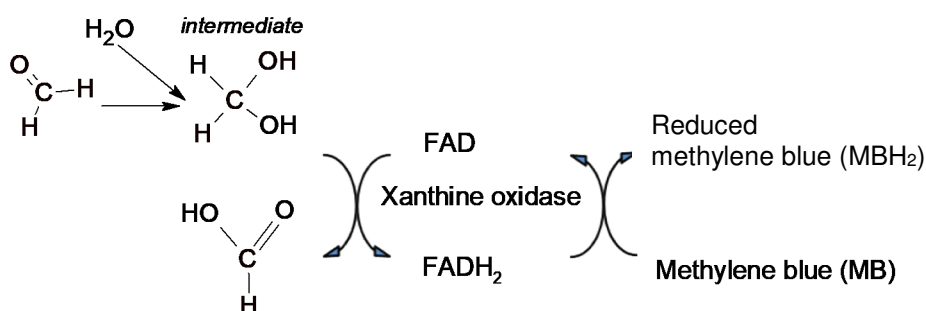
3.2. Dehydrogenation with xanthine oxidoreductase (xanthine oxidase)

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



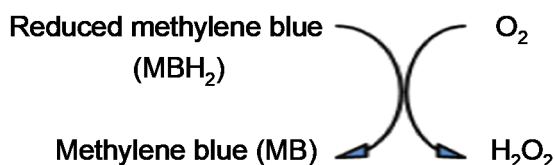
Procedure:

- 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 μ l	100 μ l	100 μ l
KCN (poison!)	-	-	200 μ l
Purified H₂O	200 μ l	200 μ l	-
Formaldehyde	250 μ l	250 μ l	250 μ l
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

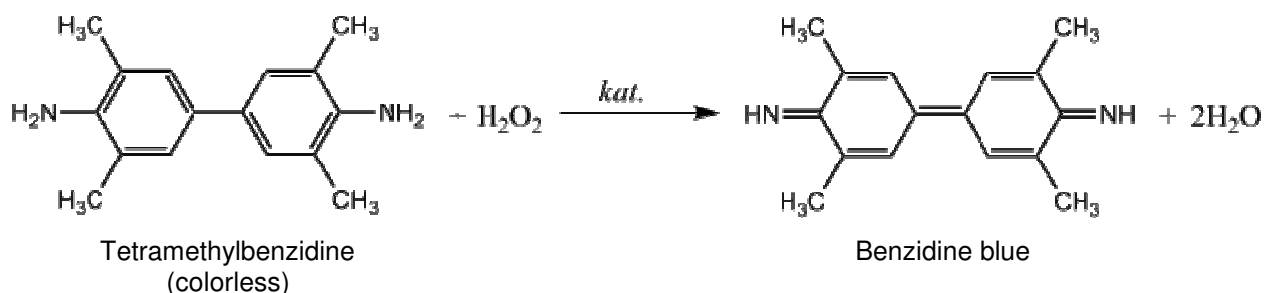
Principle:

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 heme 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 .



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 - toluidine) is in the presence of peroxidase and hydrogen peroxide oxidized to a colored product.

Procedure:

- a. Dissolve a few crystals of toluidine 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*	-	-
Purified H ₂ O	-	-	1.0 ml	100 µl
H ₂ O ₂	100 µl	100 µl	100 µl	100 µl
Toluidine 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. About 4 ml of urine with blood divide to 2 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 toluidine 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	1 ml	1 ml	-
Boiled diluted blood 1 : 50	-	-	1 ml*
KCN 10 g/l (poison!)	-	0.25 ml	-
Purified H₂O	0.25 ml	-	0.25 ml
H₂O₂	0.5 ml	0.5 ml	0.5 ml

Evaluation:

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