Laboratory Exercises in Medical Chemistry and Biochemistry I

1st year, General Medicine

SUMMER SEMESTER

Department of Medical Chemistry and Biochemistry

Faculty of Medicine in Pilsen
Charles University

A lab coat is required to be worn over your clothes, when working in a lab!

After the work is done, clean your working place so that it is the same as it was in the beginning!

Before you leave the lab, ask laboratory assistant for check of your working place!

Rules of occupational safety

1. Only practising students, specified by the timetable, are right of entry at the practical classes. No admittance of any visitors. Authorized personnel only.
2. Students are required to familiarize with their task. Laboratory coats and working instructions are obligatory. Long hair must be adapted for working with a burner without any risk of injury. Overgarments and bags must be put on the given place.
3. Any leaving is allowed just with a lecturer´s permission.
4. Only prescribed activities are allowed in laboratories. No eating, no drinking, no smoking and no storing food in laboratories. Laboratory equipment is not allowed to use for any other purposes.
5. If there is a leakage of harmful chemicals possible, the extraction must be ensured. Working with fuming substances, substances irritating to the respiratory, toxic gases and vapours, as well as annealing and combustion is allowed to do just in a fume chamber.
6. Students must be careful during the manipulation with a safety bulb pipette filler. Pieces of broken glass must be put in a specific container, label "GLASS".
7. It is possible to pour out only the solvents perfectly miscible with water into the sink. They must be sufficiently diluted (at least 1:10), maximum of 0.5 litre. Aqueous solutions of acids and alkalis must be diluted at least 1:30. Solvents immiscible with water, poisons, acids and alkalis over the given concentration and substances loosing toxic gases and gases irritating to the respiratory must be disposed into the special waste container.
8. An acid is pouring into the water during the dilution of acids, never vice versa.
9. It is forbidden to suck in solution into a pipette per mouth. A safety bulb pipette filler must be used.
10. Spilt acids must be washed by water immediately, if need be neutralized by sodium carbonate. Spilt alkalis must be just washed by water.
11. All burners and electrical current must be switched off due to spilling of flammable liquids and it is necessary to clear the air. Pouring liquids must be absorbed by suitable porous material and it is liquidate in the appropriate way.
12. During the heating of a liquid in a boiling flask superheating must be prevented by using a boiling chip.
13. It is necessary to check all devices before the start of working. Possible faults and defects must be reported to a lecturer or a laboratory technician.
14. Intentional handling with electrical device and substances is forbidden. To switch on a device and to light a burner is allowed by the approval of a lecturer or a laboratory technician.
15. All centrifugation procedures must be controlled by a lecturer or a laboratory technician. Vessels for the centrifugation must be well balanced and the top of the centrifuge must be closed safely during the operation.
16. The gas intake and electrical current must be switch off and clear the air if there is a leakage of gaseous fuels.
17. A lighted burner without supervision is not permited. If there are any problems with a turner, it is necessary to switch off the gas intake and the burner must be regulated.
18. Students are obliged to inform a lecturer of any accident, injury, or in case of ingestion chemicals.
19. Serious breach of rules because of a lack of discipline or ignorance is the reason of leaving the practical classes as an unexcused absence.
20. Students must be informed about classification of toxic, carcinogenic, mutagenic and damaging fertility substances. Safety sheets of particular substances are available in laboratories.
21. Students must be informed about rules of occupational safety with highly toxic substances (label T+) using in laboratories (e.g. mercury, potassium cyanide, ethidium bromide, mercury (II) nitrate).

Program

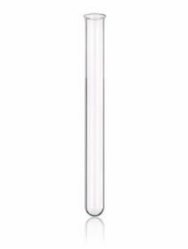
Name: *Date:*
Group:
Co-worker(s):

Lab 1: Essential laboratory skills

a) Laboratory glassware and equipment

Task: Get to know the names of laboratory aids

Different laboratory glassware and other aids are put on the laboratory table. Assign the cards with the appropriate name. After completing the task, have it checked by an assistant or laboratory technician, collect the cards and shuffle them for the next working group.

















Volumetric glass


b) Training of volume measurement (pipetting) and weighing

Task 1: Pipetting of distilled water with checking of the precision by weighting

a) Pipetting of large volumes, pipettes with fixed volume

Place *a small empty beaker* on a balance pan. The mass of the empty vessel is called the tare. Press the TARE button to get a reading of 0.000 g.

Take the beaker from the balance, place it on the table and pipette into it distilled water of the following volumes:

3 × 2000 μL
2 × 500 μL

For pipetting of 2 mL (=2000 μL), use the pipette with the fixed volume. Select the proper tip for the pipette (big white). To attach the tip, firmly press the shaft of the pipette into the large open end of the tip with light force to ensure a good seal. Similarly, for pipetting of 0.5 mL (=500 μL), use the pipette with the fixed volume and proper tip.

After you finish the pipetting, place the beaker on a balance pan to read the weight of distilled water inside. Compare the result with a theoretical expected value obtained as a sum of pipetted volumes recalculated to mass using the density. For doing this, density of distilled water at the temparature in the laboratory is $\rho = 1.000$ g/cm^3.

Expected volume	*Expected mass*	*Measured mass*

b) Pipetting of small volumes, pipettes with adjustable volume

Place an empty small plastic test tube of a volume 1.5 mL, so called *Eppendorf tube*, on a balance pan, and press the TARE button.

Take the open *Eppendorf tube* in your hand and pipette into it distilled water of the following volumes:

375 μL
25 μL

For pipetting of 375 μL, use the pipette with adjustable volume in the range 100-1000 μL and proper tip.
For pipetting of 25 μL, use the pipette with adjustable volume in the range 20-200 μL and proper tip (yellow).

After you finish the pipetting, close the *Eppendorf tube* and place it on a balance pan to read the weight of distilled water inside. Compare the result with a theoretical expected value obtained as a sum of pipetted volumes recalculated to mass using the density. For doing this, density of distilled water at the temparature in the laboratory is $\rho = 1.000$ g/cm^3.

Expected volume	*Expected mass*	*Measured mass*

Conclusion:

Task 2: Determination of the density of an unknown solution

Place *an Eppendorf tube* on a balance pan and press the TARE button.
Into *the Eppendorf tube*, pipette exactly 1.000 mL (=1000 μL) of a solution, density of which you want to determine.

For pipetting of 1000 μL, use the pipette with adjustable volume in the range 100-1000 μL and proper tip.

Close the *Eppendorf tube* and place it on a balance pan to read the weight of a solution inside. From a known volume and mass measured, calculate the density.

Volume	***Mass***	***Density***

Conclusion:

Task 3: Preparation of a solution and its aliquoting into individual tubes

Pipette into an *Eppendorf tube*:	distilled water	93 μL
	dye solution	7 μL

For pipetting of 93 μL, use the pipette with adjustable volume in the range 20-200 μL and proper tip (yellow). For pipetting of 7 μL, use the pipette with adjustable volume in the range 0.5-10 μL and proper tip (very small white). At this step, you are adding a very small volume. The best way how to do it: The orifice of the tip must be dipped below the level of the solution which is already in the *Eppendorf tube.* By adding these 7 μL, you will simultaneously mix the solution, read further how to do this!

Mix thoroughly the content of the *Eppendorf tube*. It can be done by so called "*pipetting up and down*" several times, *i. e.* repeatedly pressing and releasing the button of the pipette causing movement of the solution in the tip "up and down". In this exercise, the solution is coloured, so you can see what is happening and check if mixed sufficiently. Taking the tip out of the *Eppendorf tube,* be careful to make the tip empty!

Prepare 5 microtubes (0.2 mL) in a rack and pipette into each exactly 20 μL of the solution you have prepared.

Conclusion (evaluate the precision and accuracy of your pipetting):

Task 4: Pipetting of large volumes, pipettes with fixed volume

Pipette into a *titration flask:*	distilled water	10.0 mL
add:	0.1 M HCl	5.0 mL

Use a glass pipette with rubber suction bulb to pipette 10.0 mL of distilled water. Add 5.0 mL of 0.1 M HCl from the dispenser. The dispenser is designed for measuring aggressive reagents. It is a vessel with a piston on which the needed volume can be set. The volume of 5 mL is already set for your task, only the titration flask is added to the spout. The piston is pulled up all the way and then slowly pushed back down. In the last step, add 5 drops of indicator (methyl red).

Conclusion:

Name: *Date:*
Group:
Co-worker(s):

Lab 2: Preparation of solutions, reactions of inorganic compounds

One of the following quantities can be used to describe the composition of the solution:

Quantity	***Fill in definition:***	***Unit***
Molar /Substance concentration		
Mass concentration		
Mass fraction		

a) Preparation of a solution of known concentration

Task: Prepare 100 mL of 100 mmol/L calcium chloride solution

Estimate g of $CaCl_2$. 2 H_2O (M = 147.03 g/mol) that is needed for the preparation of 100 mL solution in concentration 100 mmol/L.

	Result
	g

Weight needed amount exactly:

Use balance, named pre-balance, easy and fast to work and enough accurate for common needs. The balance can be tared, it means to adjuste zero when a vessel for weighting is put on.

Put a small beaker used for weighting on the balance and press a button TARE. Add solid calcium chloride untill having exactly appropriate amount, use a little spoon.

A weighted amount of calcium chloride dissolve in the beaker in a „little“ volume of distilled water.

Content of the beaker pour into a 100 mL volumetric flask.

Add another little volume of distilled water (approx. 20 mL) into the beaker in which you have dissolved calcium chloride and pour into the volumetric flask too.

Use a wash bottle to fill in the volumetric flask to the mark exactly (*i. e.* to the volume 100 mL).

Close the volumetric flask by a cork and mix properly.

Estimate the molar concentration of dissolved ions in the prepared solution.

Ca^{2+}	mmol/L
Cl^-	mmol/L

Estimate the mass concentration of Ca^{2+} ions in the prepared solution. $A_r(Ca) = 40.08$

	Result
	g/L
	mg/L

Attention! Do not pour out the solution - you will need it in the next part b).

b) Filtration, centrifugation

Explain what filtration and centrifugation are.

Task: Precipitate part of the prepared calcium chloride solution with oxalic acid, separate the formed precipitates by filtration and centrifugation

1. Precipitation

Ca^{2+} ions can be removed from the solution with oxalic acid.

Pipette 3.0 mL of the calcium chloride solution prepared according to the instructions in section a) into each of the two prepared centrifuge tubes (conical bottom).

Calculate how many ml of oxalic acid solution (c = 0.1 mol/L) are needed to completely precipitate Ca^{2+} ions in a measured 3.0 mL solution.

	Result
	mL

Pipette the calculated volume of oxalic acid solution into the centrifuge tubes with calcium chloride solution.

Describe the changes observed:

Separate the precipitate in the first tube by filtration, in the second by centrifugation:

2. Filtration

Fold the filter paper in half and then in a quarter and open it to form a cone. Insert the filter prepared in this way into the funnel. Moisten it with distilled water so that it adheres to the walls.

Insert the filter funnel into the filter ring fixed on the stand. Place the beaker under the funnel so that the cut stem of the funnel touches the longer end of the inner wall of the beaker.

Pour the precipitated solution onto the filter along the glass rod to the wall of the funnel. The stream must not be directed into the orifice of the funnel to prevent the filter from rupturing.

3. Centrifugation

The tubes inside the centrifuge must be balanced, we need an "anti-tube". Add approximately the same volume of water to the second identical centrifuge tube.

Ask your assistant to perform centrifugation.

Evaluate results:

Conclusion:

c) Selected reactions of inorganic compounds

Tasks: Try selected reactions of inorganic compounds.

For each of the following exercises, you need ONE clean test tube. It does not matter on the exact measurement of volumes. You can pour the solutions into test tubes directly from bottles in appropriate small volume (about 1 mL, *i.e.* approx. 1 cm of the column height of liquid in a test tube). ***Do not pipette the solutions!***

1. Reaction of Ag^+ ions with diluted solution of HCl

Pour into a test tube about 1 mL of solution with Ag^+ ions.

CAUTION: contamination of the skin with Ag^+ ions causes unremovable black spots
(! not only pouring the solution on your skin is risky, touching on dirty glass as well !)

Add about 1 mL of dilute solution of HCl into the test tube and watch the reaction.

Describe the changes observed:

Chemical equation in ionic form	*Colour of the precipitate*

2. Reaction of Fe^{3+} ions with a solution of potassium ferrocyanide

Potassium ferrocyanide – formula:

Pour into a test tube about 1 mL of solution with Fe^{3+} ions.

Colour of the solution containing Fe^{3+} ions:

Add several drops of potassium ferrocyanide solution into the test tube and watch the reaction.

Describe the changes observed:

Chemical equation in ionic form	*Colour of the solution*

What is the traditional name for a dye produced by reaction of Fe^{3+} ions with ferrocyanides?

3. Reaction of Fe^{3+} ions with SCN^- ions

Pour into a test tube about 1 mL of solution with Fe^{3+} ions. Add several drops of a solution with SCN^-.

Describe the changes observed:

4. Reaction of Cu^{2+} ions with ammonia

Pour into a test tube about 1 mL of solution with Cu^{2+} ions.

Colour of the solution containing Cu^{2+} ions:

Add about 1 mL of dilute solution of ammonia into the test tube and watch the reaction.

Describe the changes observed:

Chemical equation in ionic form	*Colour of the solution*

5. Reaction of Ca^{2+} ions with oxalic acid

Free Ca^{2+} ions play many roles in body fluids. Ca^{2+} ions are very important in the process of blood clotting (hemocoagulation). Removal of Ca^{2+} can prevent blood clotting *in vitro*. This can be used in clinical medicine. Ca^{2+} ions can be removed from the solution (bound) by the use of organic acids with more carboxylic groups, *e.g.* oxalic acid or citric acid.

oxalic acid – structural formula	*citric acid – structural formula*

Let's try the reaction of Ca^{2+} ions with oxalic acid. Pour into a test tube about 1 mL of Ca^{2+} solution. Add about 1 mL of oxalic acid solution into the test tube and watch the reaction.

Describe the changes observed:

Chemical equation in ionic form	*Colour of the precipitate*

6. Reaction of carbonates with diluted solution of HCl

Pour into a test tube about 1 mL of sodium carbonate solution. Add about 1 mL of dilute solution of HCl into the test tube and watch the reaction.

Describe the changes observed:

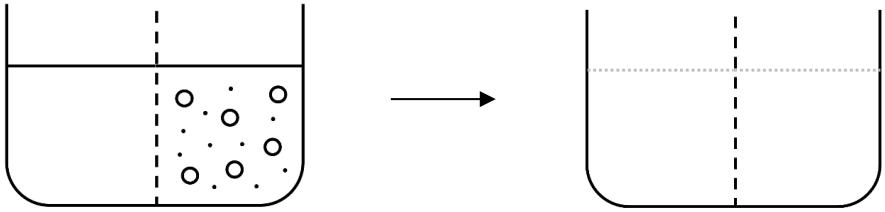
Chemical equation	*Gas, that is released*

Name: *Date:*
Group:
Co-worker(s):

Lab 3: Osmosis, osmotic pressure, osmolality

We consider the vessel divided into halves by a membrane (in the picture there is a dashed line). At the beginning, there is a cleare water on the left, and there is a mixture of large and small molecules (ions, or another particles) on the right. There is the same level of water on both sides. We are thinking of three different membrane behavior. Draw on the right side of the picture the situation on both sides after reaching equilibrium (amount of large and small molecules and water level). Answer the questions at the picture.

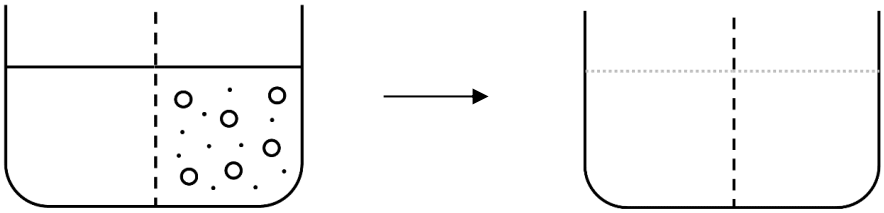
1) the membrane is permeable to water, large and small molecules



Explain what happened:

Name the process:

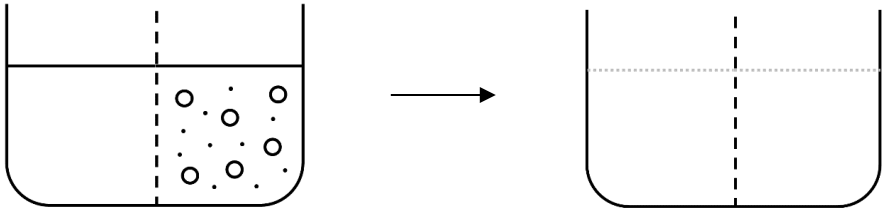
2) the membrane is only permeable to water and small particles



Explain what happened:

This principle is used for separation method named:

3) the membrane is only permeable to water but dissolved particles do not pass.



Explain what happened:

Name the process:

To be complete, there could be another possibility, the membrane is not permeable at all but it is not interesting. ☺

The membrane described in the case 2 and 3 is permeable just to something, we name such membrane as **semipermeable**. Next, we only deal with the phenomenon described in the case 3, the membrane is permeable just to solvent (in our case to water). The described phenomenon is due to the presence of osmotically active particles in the solution. No matter the particles size, charge or the shape. There is important only number of "pieces" of these particles. The presence of osmotically active particles does not cause just the phenomenon describe above, but it changes a set of properties of the solvent (in our case the solvent is always water).

It causes: decreasing of melting point (= cryoscopic effect)
increasing of boiling point (= ebulioscopic effect)

One of the following quantities can be used to describe the properties of the solution causing the described phenomena:

Quantity	***Fill in definition:***	***Unit***
Osmotic pressure		Pa
Osmolarity		mol/L
Osmolality		mol/kg of solvent

It is important to realize of the only thing that matters is the concentration of all potential particals in the solution (ions, moleculs etc.), regardless of type, size, charge or shape of particle.

Estimate osmolarity of the glucose solution in concentration 100 mmol/L.	Result
	mmol/L

Estimate osmolarity of the NaCl solution in concentration 100 mmol/L.	Result
	mmol/L

Estimate osmolarity of the $CaCl_2$ solution in concentration 100 mmol/L.	Result
	mmol/L

Estimate osmolarity of the solution consisting of: NaCl 140 mmol/L glucose 10 mmol/L urea 10 mmol/L	Result
	mmol/L

In biochemistry there is prefered osmolality (it is not the same as osmolarity, but it is not significantly different).

The osmolality of the internal environment (= extracellular fluid, blood plasma):

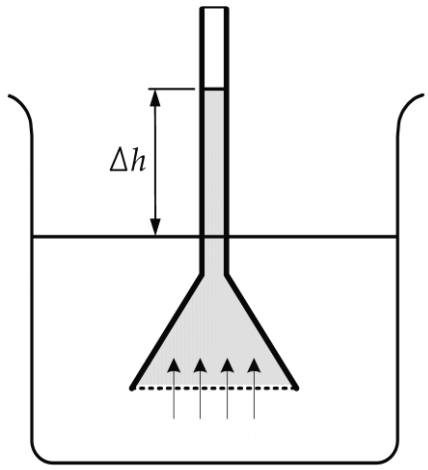
The osmolality of the internal environment is maintained constant off the urine osmolality. Urine osmolality can be in a wide range of values (50 – 1200 mmol/kg of water), depending mainly on liquid intake but even on another effects (e.g. sweating).

Comparing the osmolality (osmolarity, osmotic pressure) of two solutions, there are used terms:

Fill in the explanation of the following terms:

hypoosmotic	
isoosmotic	
hyperosmotic	

a) Demonstration of osmosis



We will try to carry out a classical experiment on demonstration of osmosis. The principle is shown in the figure. Water moves from the solution of lower osmolality, across the semipermeable membrane, into the solution of higher osmolality, and its level rises. In theory, the process should stop in time of balancing the osmotic pressure by hydrostatic pressure of the column of the solution inside a tube.

$$\pi = \Delta h \, \rho \, g$$

h ... difference in solution levels
ρ ... density of the solution
g ... gravitational acceleration

Experiment

We have a commercially available equipment for demonstrating capillary phenomena and osmosis.

Osmometer DM555-1A

The tube has a very thin diameter (capillary tube), and capillary phenomena due to surface tension, which greatly facilitate the upward movement of the liquid against the direction of gravity, are also very important. Surface tension causes the surface of the liquids to behave like an elastic layer, trying to achieve the least energy possible.

Sucrose solution is poured in the osmometer flask (stained with blue food colour for better visibility).

On a wider portion of the tube, fasten the cellophane, which will serve as a semi-permeable membrane.

The osmometer is placed in a beaker of distilled water.

Start timing on the stopwatch. Record the time it takes for the surface to rise to the top of the capillary tube and turn into an enlarged flask.

The beginning: *"What time is it?"*	The end: *"What time is it?"*	Time period: min
Difference in solution levels (mm):		

Why do we use sucrose?

The cellophane we use as a membrane certainly does not behave as an ideal theoretical semipermeable membrane, it permeates not only solvent (water), but unfortunately also other small particles the size of Na^+ and Cl^- ions, so we can not use common salts solution (such as NaCl or $CaCl_2$, which are very soluble) for this demonstration. Sucrose ($C_{12}H_{22}O_{11}$) already has a large enough molecule to prevent the pores in our cellophane from passing through. It is a sugar with extreme solubility in water, which also allows to prepare a fairly "concentrated" solution.

What is the substance concentration of saturated sucrose solution (M = 342.3 g/mol)?
Solubility at 25°C: 67.9 g of sucrose in 100 g of solution (density: 1.338 g/cm^3)

	Result
	mol/L
	mmol/L

What is the **osmolarity** of this solution?

	Result
	mol/L
	mmol/L

What is the **osmolality** of this solution?

	Result
	mol/ $kg_{\text{of water}}$
	mmol/ $kg_{\text{of water}}$

Conclusion:

b) Preparation of isotonic infusion solutions

Task 1: Preparing of 200 mL of physiological saline solution

The physiological saline solution is 0.9% solution of NaCl. It is isotonic solution, it means it is isoosmotic to blood plasma.

Calculate, how many g of NaCl is necessary for preparing of 200 mL 0.9% solution of NaCl (density is 1.00 g /cm^3).

	Result
	g

Weight exactly amount of NaCl.

Transfer the NaCl to the beaker and disolve it in a small amount of distilled water (approx. 50 mL).

All content pour to 200 mL volumetric flask.

Add another little volume of distilled water (approx. 50 mL) into the beaker to disolve NaCl and pour into the volumetric flask again.

Use a wash bottle to fill in the volumetric flask exactly to the mark (to the volume **200 mL**).

Close the volumetric flask by a cork and mix properly.
Estimate the molar concentration of the prepared solution. M(NaCl) = 58.5 g/mol

	Result
	mol/L
	mmol/L

Estimate the mass concentration of the prepared solution.

	Result
	g/L

Estimate the **osmolarity** of the solution.

	Result
	mol/L
	mmol/L

Estimate the **osmolality** of the solution.

	Result
	mol/ $kg_{\text{of water}}$
	mmol/ $kg_{\text{of water}}$

Task 2: Preparing of 250 mL of Ringer's solution

Physiological solution in form of 0.9% NaCl contains only Na^+ and Cl^- ions. However, there are also other ions in the blood plasma. In clinical practice, there are more types of infusion solutions in use, some of them more similar in ionic composition to blood plasma. Ringer's solution is one of them.

Weigh stepwise using a plastic weighing boat and transfer weighed quantities into an Erlenmayer flask (of volume of 250 mL):

sodium chloride (NaCl)	2.150 g
potassium chloride (KCl)	0.075 g
calcium chloride ($CaCl_2$)	0.083 g

Into the same Erlenmayer flask, flush also unobservable remnants from the plastic weighing boat using a wash bottle with distilled water.

Into the same Erlenmayer flask, add distilled water to a volume about 100 – 150 mL and by swirl mixing thoroughly dissolve the contents.

Using a funnel pour the content of Erlenmayer flask into a 250 mL volumetric flask. Rinse the Erlenmeyer flask at least 2× with a little of distilled water, pour everything into a volumetric flask.

Fill the volumetric flask exactly to the mark with distilled water (i.e. a volume of 250 mL).

Close the volumetric flask by a stopper and thoroughly mix the contents.

What is the concentration of the individual ions in the solution prepared?

M(NaCl) = 58.45 g/mol
M(KCl) = 74.56 g/mol
M($CaCl_2$. $2H_2O$) = 146.99 g/mol

	Result	
	Na^+	mmol/L
	K^+	mmol/L
	Ca^{2+}	mmol/L
	Cl^-	mmol/L

Estimate the **osmolarity** of the solution.

	Result
	mol/L
	mmol/L

Estimate the **osmolality** of the solution.

	Result
	mol/ $kg_{\text{of water}}$
	mmol/ $kg_{\text{of water}}$

Conclusion:

c) Determination of osmolality using cryoscopy

We will determine the osmolality based on the cryoscopic effect. The higher the osmolality of the solution, the lower the freezing (melting) point. The difference between the freezing (melting) point of the solution and the freezing (melting) point of the pure solvent (ΔT, freezing point depression) is directly proportional to the osmolality. The proportionality constant is the so-called cryoscopic constant.

$\Delta T = K_f \times$ osmolality cryoscopic constant of water: $K_f = 1.86\ °C \times kg \times mol^{-1}$

An ebulioscopic effect could also be used to measure osmolality. The higher the osmolality of the solution, the higher the boiling point. The difference between the boiling point of a solution and the boiling point of pure solvent (ΔT, boiling point elevation) is directly proportional to osmolality. The proportionality constant in this case is the ebulioscopic constant.K_b.

ebulioscopic constant of water: $K_b = 0.513\ °C \times kg \times mol^{-1}$

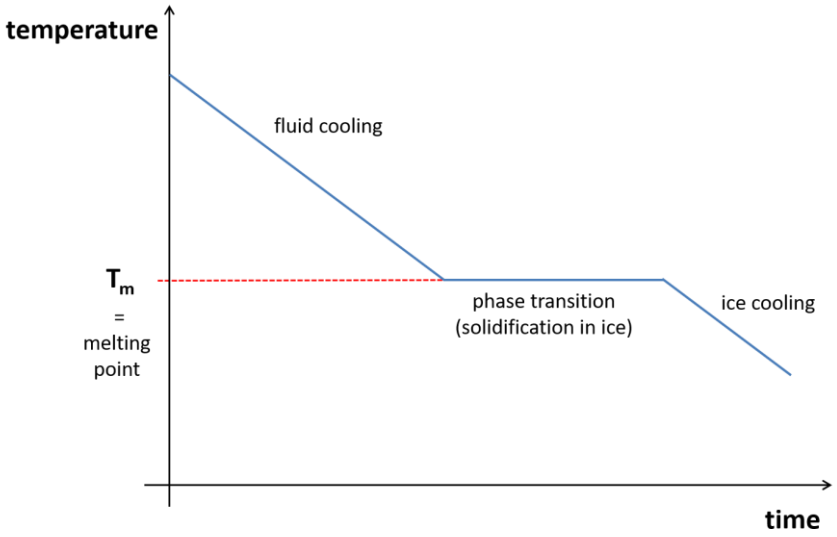
What is the freezing (melting) point of an aqueous solution with osmolality 3.5 mol/kg_{water} ?	Result
	°C

This knowledge will allow us to translate the task "How to measure osmolality?" to the task "How to measure freezing (melting) point?".

The freezing (melting) point can be easily deduced from the time course of the temperature drop during cooling of the analyzed sample.

What happens to the fluid if we cool it?

The temperature gradually decreases. When freezing (melting) point is reached, the temperature decrease stops until all the liquid has solidified into ice. Only then can the temperature drop continue and stop at the ambient temperature where the sample is located.



Task: Measure the osmolality of prepared infusion solutions and other samples

There is a modern freezing point osmometer **Osmomat 3000** in the laboratory. The total osmolality of aqueous solutions is determined by comparative measurements of the freezing points of pure water and of solutions. The instrument requires very small sample volumes (50 μL), test time is short (60 s).

Preparing samples

1. Distilled water – it is available in the laboratory
2. Sample of your urine
3. Physiological saline solution
4. Ringer's solution – you have already prepared (previous exercise)
5. Ringer's solution + ethanol

Using a graduated cylinder, measure 50 mL of Ringer's solution, pour it into a small beaker and add by pipetting **0.25 mL** of 40% solution of ethanol. M(ethanol) = 46 g/mol

Density of pure ethanol: 0.789 g/cm^3

Calculate what rise of osmolality (osmolarity) should the addition of ethanol result in:

6. Ringer's solution + glucose

Using a graduated cylinder, measure 50 mL of Ringer's solution and pour it into a small beaker. Weigh using a plastic weighing boat **270 mg** of glucose and dissolve it in the solution in the beaker. M(glucose) = 180 g/mol

Calculate what rise of osmolality (osmolarity) should the addition of glucose result in:

Pipette 50 µl of each sample into cryoscopic tube. *Without bubbles!*
Measure osmolality.

Sample	***Measured osmolality***
Distilled water	mmol/kg
Sample of urine	mmol/kg
Physiological saline solution	mmol/kg
Ringer's solution	mmol/kg
Ringer's solution + ethanol	mmol/kg
Ringer's solution + glucose	mmol/kg

Conclusion:

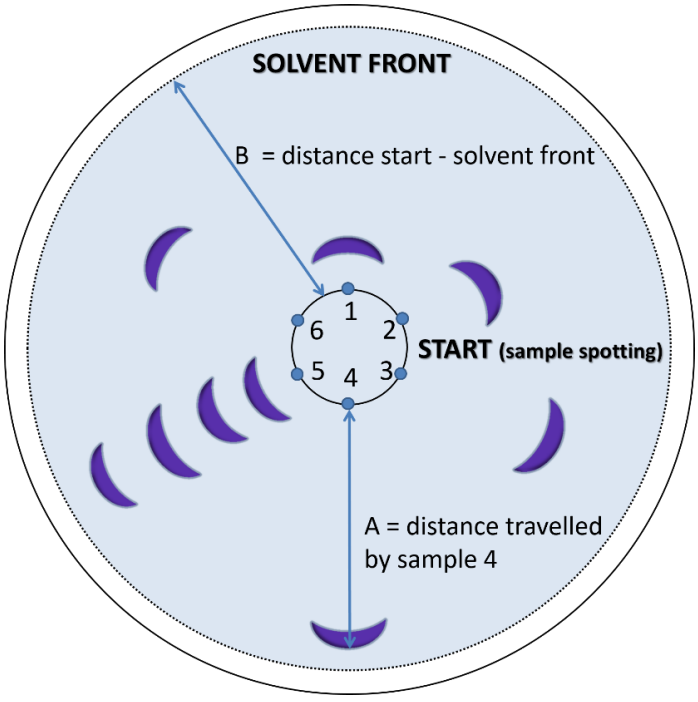
Name: *Date:*
Group:
Co-worker(s):

Lab 4: Chromatography

Define in general what chromatography is:

a) Paper chromatography of amino acids

The separation takes place on chromatographic paper, which is a carrier of the stationary phase, i.e. water, which is permanently bound to the cellulose in an amount of about 5 %. The mobile phases are various organic solvents or mixtures thereof with a certain proportion of water in order to avoid elution of the stationary phase from the carrier.
The samples are applied to the start with a capillary micropipette in a volume of 2 to 10 µl. During application, the solvent is evaporated to concentrate the sample into a small spot.



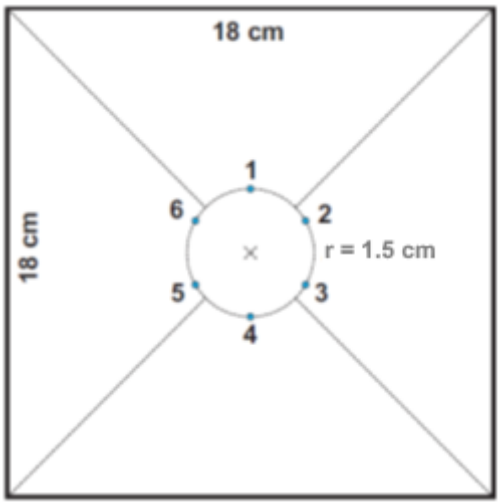
Development is performed in glass closed chambers. When the solvent front reaches the end of the paper, the chromatogram is removed, dried and developed as needed. (Spray with a suitable reagent that gives a strong colour reaction to all samples). Sometimes UV-detection is used. Many substances (often of natural origin) show colour fluorescence in UV light. The value of the retardation factor R_f is used for evaluation and a comparison is made with the standards applied in parallel.

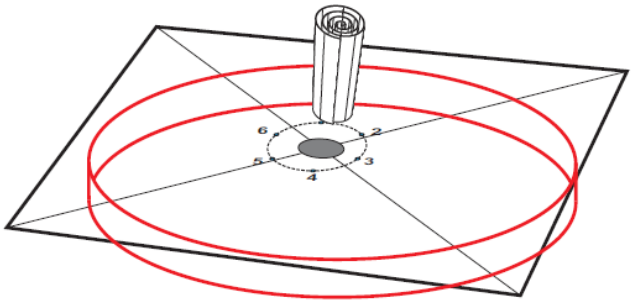
$$R_f = \frac{A}{B}$$

Task: Identify an amino acid in an unknown sample

Take a sheet of chromatographic paper (18 × 18 cm). Find out a centre of the sheet. Draw a circle around the centre about 3 cm in diameter. You can use prepared template. This is the "base-line", the start position. On the base-line make 6 marks evenly spaced and number them 1-6.

Using labelled capillaries make small spots (less than 0.5 cm in diameter) of about 5 µl of the standards and your unknown sample. The application should be repeated 2 times. Before the further application, the wet spots must be dried under the infra-red lamp or a ventilator. Use your pencil to perforate the paper exactly in the centre to draw through the hole a paper "wick".





Place the paper into a Petri dish partly filled
with a solvent mixture (n-butanol, acetic acid, water – 4:1:1) and cover the paper with a cap.

Do not take the Petri dish out from the fume chamber! All following procedures must be done in a fume chamber!

The chromatograms are left to develop until the front of a solvent overlaps the Petri dish cap. The paper is removed, dried, and sprayed with ninhydrine reagent (use protective gloves). Heat the paper at 80°C in order to develop the formation of blue complexes.

Insert a photo, redraw or paste a chromatogram here

Calculate the individual R_f according to the equation:

$$R_f = \frac{\text{distance between baseline and the centre of the spot}}{\text{distance between baseline and the front of the solvent}}$$

Site	***Amino acid***	***R_f***
1	Lysine	
2	Glycine	
3	Alanine	
4	Isoleucine	
5	The mixture of 1-4	- - -
6	Unknown sample	

Write structural formulas of amino acids:

alanine	*glycine*
isoleucine	*lysine*

Which of the following amino acids is the least polar?
Which of the following amino acids is the most polar?

Sample identification and conclusion:

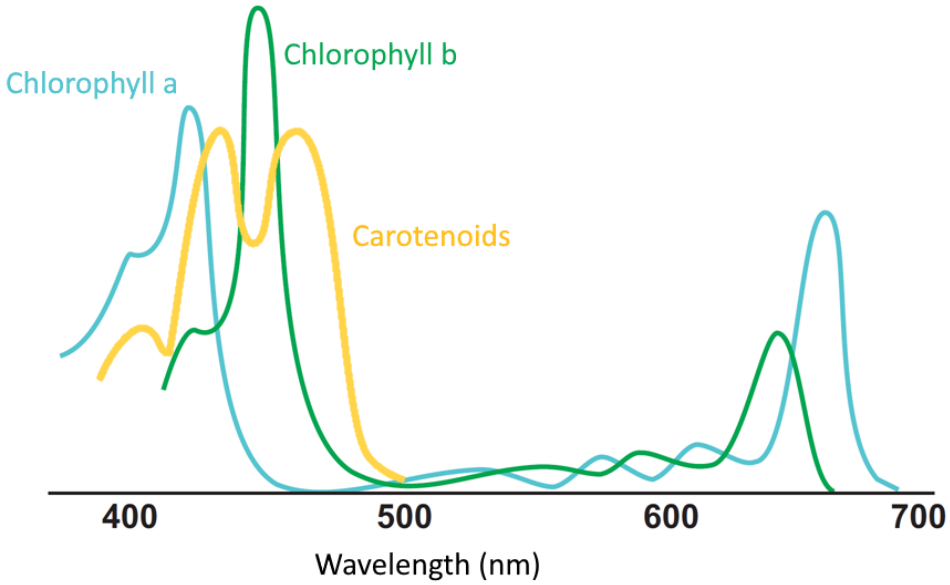
b) Separation of plant pigments by thin-layer chromatography

There are a few lipophilic pigments in green parts of plants. They have different chemical and functional properties. The primary function of pigments in plants is photosynthesis which uses the green pigment chlorophyll along with several red and yellow pigments - carotenoids. Chlorophylls are cyclic tetrapyrroles with a central magnesium atom, carotenoids include xanthophylls and carotenes.

List another cyclic tetrapyrrole, its central atom and degree of oxidation.

Indicate how xanthophylls (e.g. lutein, zeaxanthin) and carotenes (e.g. β-carotene, lycopene) differ (chemically).

These plant pigments can be separated using Thin Layer Chromatography (TLC). The principle of TLC is separation of the individual components of the mixture on the basis of their different interaction with the sorbent, which is applied in a thin layer on a solid substrate. The mobile phase is an organic solvent of suitable polarity. The choice of the most suitable sorbent, detection reagent and solvent depends on the type of mixture being separated.
The actual chromatography is usually carried out in ascending order so that the applied sample is not immersed in the liquid, but the solvent reaches the baseline level only by capping. The chromatographic chamber must be tightly closed throughout the solvent rise to prevent the escape of vapours and thus undesirable marginal phenomena which adversely affect the separation process. The thin-film plate is removed from the chromatographic chamber when the solvent front reaches a few millimeters from its upper edge. The chromatogram is then evaluated, which is the easiest for the coloured substances, because the position of the trace obtained is directly visible there. In the case of colorless substances, the traces obtained must be made visible by a detection reagent which, on reaction with the substance to be determined, produces a colour trace.



If we monitor which wavelengths a certain dye absorbs, we obtain the so-called ***absorption spectrum***. This spectrum shows maxima (so-called peaks), which are typical for a given substance. It is ideal to monitor the absorption spectrum in the visible region, which is sufficient to identify several dyes contained in the leaves. Decomposition products of chlorophyll are pheophytins, carotenoids pass through epoxides to colorless compounds.

Rapid separation of leaf pigments can be achieved by thin layer chromatography, where the adsorbent is silica fixed with a starch binder to an aluminum plate (Silufol). The components of the mixture are separated on the basis of the different adsorption affinity of the stationary phase to the solute molecules. After separation of the extract, the individual components can be eluted from the Silufol with organic solvents and determined spectrophotometrically.

Task: Separate plant pigments by TLC

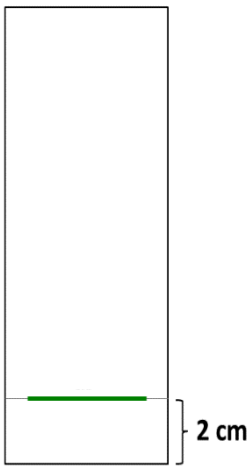
There are prepared samples of green leaves or needles on the working place. Cut it in pieces in a mortar. Then add a small spoon of calcium carbonate, a little bit of glass shards and crush it with a pestle in the presence of 1-2 mL of acetone.

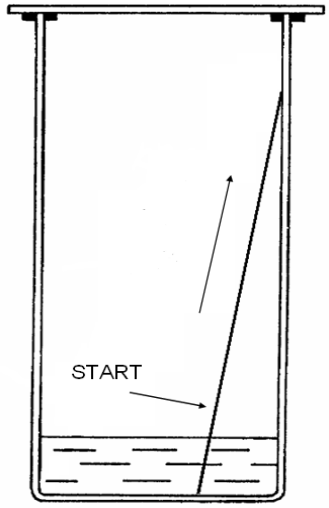
Protect your eyes using safety goggles!

Write structural formulas of:

calcium carbonate	*acetone*	*isopropanol*

Take TLC plate. After the separation, one is for you to be attached to the protocol, the second will be used further and destroyed by procedure of extraction of one of the pigments.





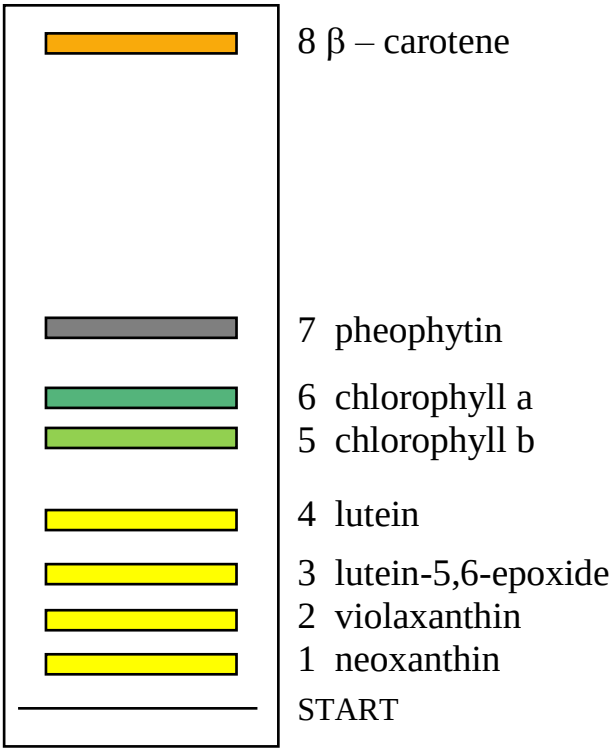
Draw the base-line about 2 cm from the shorter edge of the plate. Use the thin pencil. Do not press on the pencil to tear of the sorbent layer!

Dip the glass capillary into the acetone extract and touch the tip of the capillary to the plate at the position you've marked. Gently and quickly make spot according to the picture. Try to make as small spots as possible and avoid scratching of the thin layer. For higher concentration repeat the process of application for several times.

Prepared separation chamber should be kept close. There is approximately 1cm thick layer of liquid solvent mixture on the bottom and the rest volume is filled with its vapours.

Place the thin layer plate carefully in the chamber (chromatography tank), containing a solvent at the bottom. Cover the vessel with a glass plate and allow to separate about 15 min. The base – line must be situated above the level of a solvent.

When the front of a solvent almost overlaps the top of the TLC plate, remove the plate. Evaluate the dry plate for the presence of pigments:



Insert a photo, redraw or paste a chromatogram here

Explain the following terms:

Stacionary phase
Mobile phase
Conclusion:

c) Separation of dye mixture by gel chromatography

Task: Separate dextran blue and potassium chromate

Dextran blue	***Colour***	***Molar mass***
What are dextrans?		$\sim 2 \times 10^6$ g/mol

Potassium chromate	***Colour***	***Molar mass***
formula: In what form is it in solution?		g/mol

There is a chromatographic column filled with Sephadex gel on the working place. Sephadex is polysaccharide of dextran type.

Uncap the chromatographic column, open the tap and allow carefully run out the excess of fluid until the surface of the gel bed is reached. Pipette on the gel slowly 0.5 mL of the coloured mixture (potassium chromate and dextran blue). *Mix the mixture before pipetting!* Use the pipette with fixed volume (=500 µL) and proper tip (blue). *Pipette carefully!* Not to whirl the surface of the gel!

Colour of the mixture:

By turning the tap let the sample pass into the column.

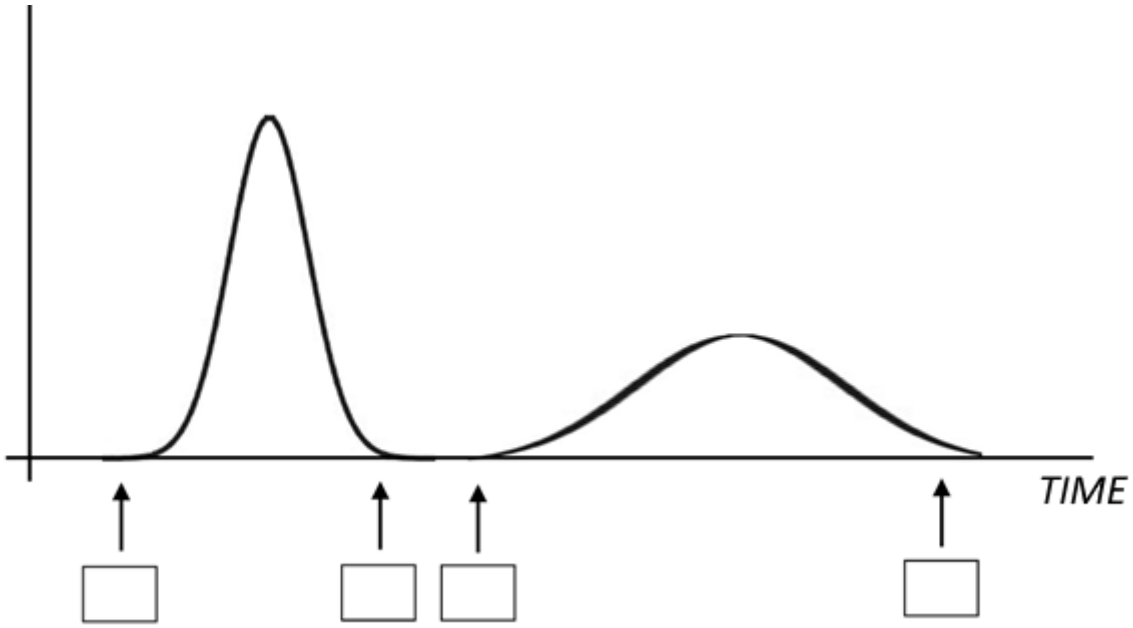
Pipette (using glass pipette and rubber suction bulb) about 3 mL of elution solution (physiologic solution; 0.9% solution of NaCl) and let it penetrate the gel in the same way.

What is happening in the column? Describe the changes:

Elute the column with sufficient amount of elution solution until the first fraction leaves the outflow. Recorde the first time in **s**. Collect this fraction in calibration test tube. *Use a thinner tube – volume 10 mL*. If the first fraction flows out recorde the second time.

At the moment the second color fraction appears recorde the time. Collect this fraction in calibration test tube. *Use a wider tube – volume 20 mL*. Record the end time of the second fraction.

Elution curve



Fraction order	***Colour***	***Substance***	***Fraction volume***
1			
2			

Explain the order of separated dyes (principle of separation by gel chromatography):

End of the experiment, cleaning the workplace

Finally, wash the column with a sufficient quantity of the elution solution (no traces of dyes must remain). Leave a layer of solution about 5 cm above the gel to prevent the gel from drying out. Close the column with a stopper.

Conclusion:

Name: *Date:*
Group:
Co-worker(s):

Lab 5: pH, buffers

Define what the pH is:
What is an acid-base indicator?
Explain the principle of pH measurement (using indicators and pH meter):
What is a buffer?
Write the Henderson-Haselbalch equation (including a description of the individual quantities): What does this equation describe?

a) Measurement of pH

Task: Determine the pH of the sample by different methods and compare these methods

On the working place is a bottle marked "pH measurement - sample". The task is to measure the pH of this solution by four methods. To measure using indicator papers and a pH meter, pour an appropriate amount of sample into a clean small beaker. Pipette 10.0 ml into a test tube for comparison with a range of buffers.

1) universal indicator strip

The universal pH indicator strips usually cover the full 0 – 14 pH range, but have low sensitivity of 1 pH unit. Intermediate range strips provide a sensitivity of 0.3 – 0.5 pH units and narrow range strips provide a sensitivity of 0.2 – 0.3 pH units. Intermediate and narrow range strips are often manufactured for special clearly defined use, *e.g.* urine pH measurement. Urine pH is somewhere between 4.5 and 7.5 therefore indicator strip for such purpose may be limited in range by these values.

wide pH range: 0 – 12
limited accuracy
(accurate to ± 0.5 pH unit)

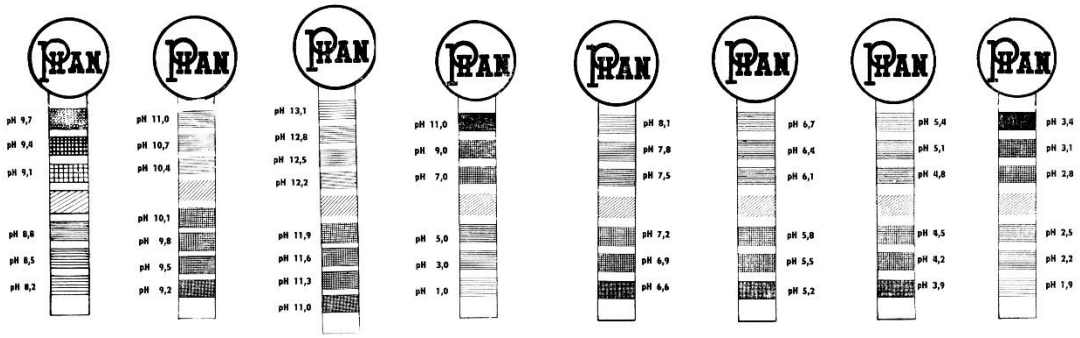
To use *universal indicator strip* test paper is by far the simplest way to measure the pH.

Take a piece universal indicator strip with tweezers and immerse it briefly (for approx. 1 second) directly into the test tube with your unknown sample to be tested.

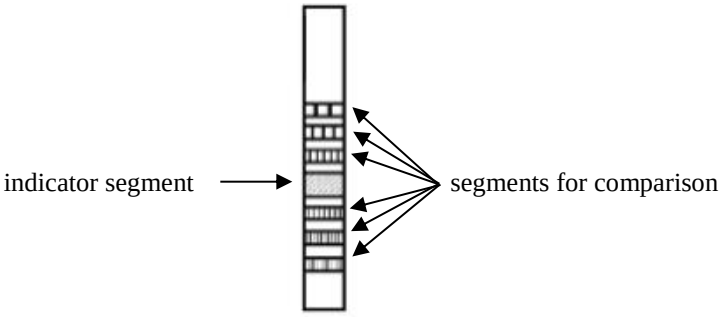
Be careful to prevent fall of indicator strip deep into the test tube. If this happens, remove the strip as soon as possible! Paper strip is impregnated with indicators that may bleed into the solution and change its colour. This could impair further experiments with the sample.

Immediately compare the colour of the wet indicator paper with the pH colour chart printed on the box and read the pH value.

2) "MULTIPHAN" indicator strip



pH range:	8.2-9.7	9.2-11.0	11.0-13.1	"universal"	6.6-8.1	5.2-6.7	3.9-5.4	1.9-3.4



narrow pH range of individual strip

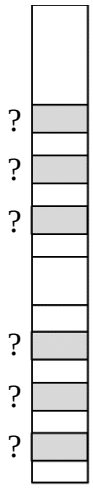
better accuracy

(accurate to ± 0.2 - 0.3 pH unit)

The kit consists of several indicator strips, each of them for different narrow pH range. First you have to select an appropriate strip so that pH you expect to measure is within the range. You already know approximate pH of your sample (pH determined by universal indicator strip).

pH range of selected indicator strip:

Take this indicator strip with a tweezer and immerse it briefly (for approx. 1 second) directly into the test tube with your unknown sample to be tested so that all the fields come into contact with the solution. Compare the colour of the indicator zone in the middle with the coloured segments placed on both sides. Look for the best match. Read the pH value from the enclosed schematic table.



From the schematic table enclosed in the kit,
copy the corresponding pH values into the fields.

MIDDLE indicator zone

Mark the zone which colour is the most similar to the colour of the middle indicator zone

3) by comparison of the colour of acid base indicator in set of pH standards (buffers)

Prepare the set of 8 buffers according to the table:

Standard number	***CH_3COOH (100 mmol/L)***	***CH_3COONa (100 mmol/L)***	***pH***
	mL	***mL***	
1	9.0	1.0	
2	8.0	2.0	
3	7.0	3.0	
4	6.0	4.0	
5	5.0	5.0	
6	4.0	6.0	
7	3.0	7.0	
8	2.0	8.0	

Use Henderson-Hasselbalch equation to calculate pH in individual test tubes.

pK_a (CH_3COOH) = 4.75

Add exactly 20 drops of the **bromocresol green** (acid base indicator) into all eight test tubes.

bromocresol green: pH interval of colour change: (acidic) **yellow** **3.8 – 5.4** **blue** (alkaline)

Add exactly 20 drops of the **bromocresol green** into the test tube with 10.0 mL unknown sample. Mix the content of all the test tubes thoroughly.

Close the test tube with rubber stopper and slowly turn the test tube upside down.

Compare the colour of your unknown sample with the scale of pH standards (8 test tubes prepared). Find the best match. You may use the"comparator" (cube with holes for test tubes) to help you.

Colour in the sample test tube is the most similar to standard number:

4) by using pH meter

Determination of pH can be done by simple colorimetric methods using acid-base indicators (pH test strips). Nevertheless, the precision of such methods is mostly insufficient. For exact pH measurement, laboratories are equipped with pH meters with usual resolution of 0.01 pH units, high-end instruments with resolution of 0.001 pH units.

There is a pH meter with combined electrode (consisting of both the glass and the reference electrode) at your working place.

Electrode must be kept moist using the storage solution. Remove the protective plastic cover with storage solution before using the electrode. Keep the storage solution inside the plastic cover, do not pour it out. At the end of your work, you have to place the electrode back into the cover with storage solution.

Never touch the glass membrane of the electrode with your fingers!

Electrodes should be rinsed between samples with distilled water. After rinsing, gently blot the electrode with cotton paper piece to remove excess water.

Dip the electrode of the pH meter into the solution (which pH you want to determine) in the beaker, gently shake the content by slowly moving the beaker to make the membrane of the electrod be in contact with the solution. (Electrode is "dirty" with distilled water used for rinsing between samples.)

After stabilization of the value, read the pH on a display.

After measuring the sample, rinse the electrode thoroughly with distilled water, wipe and put back in the protective cover with the storage solution.

Method	***Determined pH***
universal indicator strip	
"MULTIPHAN" indicator strip	
comparison with buffer solutions	
pH meter	

Conclusion:

b) Demonstration of buffer functioning

Task 1:

For the demonstration, we will use "phosphate buffer" composed of **sodium hydrogen phosphate** and **sodium dihydrogen phosphate**.

Use Henderson-Hasselbalch equation to calculate volumes of components needed to prepare **10 mL** of the phosphate buffer with **pH = 7.0**. **pK_a = 7.21**

What you have: solution of sodium hydrogen phosphate c = 100 mmol/L
solution of sodium dihydrogen phosphate c = 100 mmol/L

Calculation:

Round the calculated volumes of buffer components to one decimal place.

Component	*Formula*	*Volume needed (mL)*
sodium hydrogen phosphate		
sodium dihydrogen phosphate		

There are two titration flasks on your working place. Pipette (using glass pipette and rubber suction bulb) exactly **10.0 mL** of **WATER** into one of the titration flasks**.**

Into the second titration flask, pipette (using glass pipette and rubber suction bulb) exactly **the calculated volumes of both components of phosphate buffer**.

Add 2-3 drops of indicator (**methyl red**) into both titration flasks. Notice the colour of the solution.

Methyl red is a pH indicator with interval of colour change:

(acidic) **red** **4.4 – 6.2** **yellow** (alkaline)

Fill the burette with standard solution of **HCl (c = 0.100 mol/L)**. Adjust the level of standard solution in a burette to zero and add HCl (*i.e.* strong acid) into the solutions in titration flasks until colour change of the indicator (pink red colour). Read the volume of HCl used to reach this point.

How to titrate?

It is advisable to hold the titration flask in the right hand and to control the burette cock with the left hand. Slowly add the standard solution to the sample in the titration flask, stirring constantly. Always monitor the colour of the solution in the titration flask. You will end the titration at an equivalent point (you will recognize it by a sudden change in colour).

Be especially careful in the case of water!

	Volume in titration flask	***pH***	***Volume of HCl needed to change the pH***
water	**10 mL**	**7.0**	
phosphate buffer	**10 mL**	**7.0**	

Make conclusions:

Task 2:

Unbuffered solution - water

Using a graduated cylinder, measure 50 mL of deionized water, pour it into a clean beaker and determine the pH by pH meter. Record the value *(measurement 1)*. Pour out the water from the beaker. Using the same graduated cylinder, measure again 50 mL of deionized water, pour it into the same beaker and determine the pH by pH meter *(measurement 2)*. After the second measurement, do not remove the combined electrode from the beaker, let it dipped there. By pipetting add 100 µL of HCl solution (c = 0.1 mol/L) into the solution in the beaker and gently mix the content. After stabilization of the value record the pH.

pH of deionized water		***pH after addition of HCl***
measurement 1	*measurement 2*	
Describe how stable is the pH of an unbuffered solution:		

Buffer

Using a graduated cylinder, measure 50 mL of phosphate buffer, pour it into a clean beaker and determine the pH by pH meter. Record the value *(measurement 1)*. Pour out the buffer from the beaker. Using the same graduated cylinder, measure again 50 mL of phosphate buffer, pour it into the same beaker and determine the pH by pH meter *(measurement 2)*. After the second measurement, do not remove the combined electrode from the beaker, let it dipped there. By pipetting add 100 μL of HCl solution (c = 0.1 mol/L) into the solution in the beaker and gently mix the content. After stabilization of the value record the pH.

pH of the buffer		***pH after addition of HCl***
measurement 1	*measurement 2*	
Describe how stable is the pH of a buffer solution:		
Describe the differences between the behaviour of the buffer solution, and the pure water, upon the addition of a small amount of a strong acid:		

Conclusion:

Name: *Date:*
Group:
Co-worker(s):

Lab 6: Volumetric analysis

What do we use volumetric analysis for?
Explain the term equivalence point:

a) Alkalimetry

Task: Determine the concentration of acetic acid in a sample of vinegar

A. Standardization of NaOH solution

Pipette (using glass pipette and rubber suction bulb) exactly **10.0 mL** of primary standard solution of **oxalic acid (c = 0.050 mol/L)** into titration flask**.**

Add 2-3 drops of indicator (**phenolphtalein**). The solution remains colourless.

Use the funnel to fill the burette with standard solution of **NaOH (c ~ 0.1 mol/L)**. When it is done, take away the funnel from the burette, do not let it be there during the titration. Adjust the level of standard solution in a burette to zero (read the bottom of the meniscus, your line of sight must be in a direction perpendicular to the burette column).

How to titrate? It is the best to hold titration flask in your right hand (and mix gently the content all the time). Use your left hand to control the cock of the burette. Add slowly (drop by drop) standard solution from the burette to the sample in titration flask. Watch the colour of the solution in titration flask all the time. Stop the titration (addition of the solution from the burette) in the "equivalence point" (sudden change in colour) – the solution in titration flask turns **pink** colour.

Read the volume used to reach the endpoint (so called "consumption"). Repeat the titration one more time. If the difference in consumptions is higher than 0.5 mL, carry out the third titration. From all measured values, calculate the average.

If any measurement is incorrect "at the first site", do not use it, ignore this value.

Example: Consumptions you found are 1.5 mL and 7.4 mL. The third titration gives 7.2 mL. The value 1.5 mL is probably an error, do not use it. Calculate an average consumption from values 7.4 mL and 7.2 mL; average = 7.3 mL.

Calculate the exact concentration of standard solution.

chemical equation:

consumption 1 =	mL	consumption 2 =	mL	(consumption 3 =	mL)
average consumption =	mL				

c_{NaOH} = mol/L	c_{NaOH} = mmol/L

B. Estimation of concentration of CH_3COOH

The aim is to determine the concetration of acetic acid in a sample of vinegar for kitchen use.

Concentration of acetic acid in ordinary vinegar is too high for direct titration, it is necessary to dilute it before the procedure.

Pour appropriate volume of vinegar (approx. 20 mL) from the original bottle into a small beaker.

Why to do so? During your further work, you will avoid getting pollution inside orignal bottle.

From the beaker, pipette exactly **10.0 mL** (using glass pipette and rubber suction bulb) into a volumetric flask of volume 100 mL.
Fill the volumetric flask to a line etched on the neck with distilled water (from the stirrer or from the storage container for distilled water). The volume inside the flask is exactly **100 mL**.

Close the volumetric flask (rubber stopper) and mix the content thoroughly.

Pour the content of volumetric flask into Erlenmeyer flask.

Why? The neck of the volumetric flask is too narrow for the glass pipette.

From Erlenmeyer flask, pipette (glass pipette and rubber suction bulb) exactly **10.0 mL** of the sample into a titration flask.

Add 2-3 drops of indicator (**phenolphtalein**) and conduct the titration. In equivalence point (endpoint of titration), the colour of the solution turns to pink (sudden change). Repeat the titration one more time. If the difference in consumptions is higher than 0.5 mL, carry out the third titration.

Calculate the original concentration of acetic acid in a bottle of vinegar (molar and mass concentration, mass fraction and percentage). **CAUTION!** Do not forget that you have diluted the original solution before titration.

M(acetic acid) = 60.0 g/mol use the density 1.0 g/cm^3

VINEGAR				
manufacturer: declared concentration of acetic acid: %				
chemical equation: consumption 1 = mL consumption 2 = mL (consumption 3 = mL) average consumption $V_{standard\ solution}$ = mL molar concentration in titrated sample (diluted): $c_{titrated\ sample}$ = mol/L dilution:				
Results	Molar concentration	c = mol/L	Mass concentration	μ = g/L
	Mass fraction		Mass percentage	%

Compare the concentration you measured with the one declared by the manufacturer. By how much percent higher / lower concentration did you find?
Conclusion:

b) Chelatometry (complexometric titration)

Task 1: Determine the concentration of Mg^{2+} in mineral water

Chelatometry (complexometry) is a titration method that uses formation of **non-dissociated, but soluble** complexes of metal cations with complexing agents that are called **chelatons**.

Chelaton 3 = disodium salt of ethylenediaminetetraacetic acid

EDTA (complexon II)- structural formula:
Chelaton 3 (complexon III) - structural formula:

In general, chelatons react very similarly with all polyvalent metal cations (Ca^{2+}, Mg^{2+}, Ni^{2+}, Pb^{2+}, Bi^{3+}...). In mineral water you work with, assume that there are no ions other from Mg^{2+} reacting with chelatons.

Pour appropriate volume of water (approx. 50 mL) from the original bottle into a small beaker.

Why to do so? During your further work, you will avoid getting pollution inside orignal bottle of mineral water.

From the beaker, pipette exactly **10.0 mL** (using glass pipette and rubber suction bulb) into a titration flask.

Adjust the pH by addition of 1 mL of ammonia buffer (ammonia buffer is inside fume chamber).

Colour of the indicator strongly depends on pH, addition of the buffer prevents changes in pH during titration.
Add powdered indicator (eriochrome black T, Erio T), very small amount is sufficient. Solution gets light red wine colour. **Caution:** Deep coloured solutions (too much of indicator) are difficult to titrate, moreover, it is not accurate.

After addition of indicator (Erio T) to the sample, complex [indicator - Mg] is formed, that has different colour from the one of free indicator. In alkaline pH caused by the presence of ammonia buffer, free indicator is blue, complex [indicator - Mg] has the colour of red wine.

Use the funnel to fill the burette with standard solution of **Chelaton 3 (c = 0.010 mol/L)**. When it is done, take away the funnel from the burette, do not let it be there during the titration. Adjust the level of standard solution in a burette to zero (read the bottom of the meniscus, your line of sight must be in a direction perpendicular to the burette column).

How to titrate? It is the best to hold titration flask in your right hand (and mix gently the content all the time). Use your left hand to control the cock of the burette. Add slowly (drop by drop) standard solution from the burette to the sample in titration flask. Watch the colour of the solution in titration flask all the time. Stop the titration (addition of the solution from the burette) in the "equivalence point" (sudden change in colour) – the solution in titration flask turns **blue** colour.

Read the volume used to reach the endpoint (so called "consumption" of standard reagent).

Repeat the titration one more time. If the difference in consumptions is higher than 0.5 mL, carry out the third titration. From all measured values, calculate the average.

Use the average consumption to calculate molar and mass concentration of Mg^{2+}.

M(Mg) = 24.3 g/mol

Write down in structural formulas reaction between Chelaton 3 and Mg^{2+}:
Stoichiometric factor:

Mineral water:		declared concentration of Mg^{2+}	mg/L
consumption 1 = mL	consumption 2 = mL	(consumption 3 =	mL)
average consumption $V_{standard\ solution}$ = mL			

Mg^{2+}	Molar concentration c = mmol/L	Mass concentration μ = mg/L

Calculate, how much of Mg (mg) is inside 1.5 L bottle of mineral water.

Compare the concentration you measured with the one declared by the manufacturer.
By how much percent higher / lower concentration did you find?

Conclusion:

Task 2: Determine the Ni content (%) in a solid powder sample

Weight exactly about 0.15 g of analyzed sample.

Transfer the weighted powder into a titration flask and dissolve in 10 mL of distilled water. For measurement of H_2O volume, use graduated cylinder. Mix thoroughly so that the solid powder dissolves.

Adjust the pH by adding 2.5 mL of ammonia solution (caustic!). Ammonia solution is in the fume chamber.

Add powdered indicator (murexide), very small amount is sufficient. Solution gets light yellow colour.

Use the funnel to fill the burette with standard solution of Chelaton 3 (c = 0.010 mol/L). When it is done, take away the funnel from the burette, do not let it be there during the titration. Adjust the level of standard solution in a burette to zero (read the bottom of the meniscus, your line of sight must be in a direction perpendicular to the burette column).

Conduct the titration. In equivalence point (endpoint of titration), the colour of the solution in the titration flask will be purple (murexide released from Ni^{2+} complex).

Calculate the content of Ni (%) in analyzed sample. M(Ni) = 58.7 g/mol

Repeat the procedure one more time (i.e. steps 2-7).

	First experiment	*Second experiment*
Weighing (mg)		
Consumption (mL)		
Amount of Ni^{2+} in titration flask (= in weighing)		
Mass of Ni in titration flask (= weighing)		
Ni content (%) in analyzed sample		

From both results, calculate the average:

Conclusion:

Name: *Date:*
Group:
Co-worker(s):

Lab 7: Optical methods

Explain the principle on which spectrophotometry is based:

Define the transmittance: What units does it have?
Define the absorbance: What units does it have?
25 % of the radiation passed through the sample. What is the absorbance?
Beer–Lambert law:

a) Identification of acid-base indicator by absorption spectra

Absorption spectrum is a graph of absorbance as a function of wavelength of light (electromagnetic radiation). In UV-VIS range of radiation, absorption spectrum looks like a continuous curve with one or only few maxima, whose position is characteristic for particular substance. Absorption spectra can be used for identifying substances or for checking their purity.

Acid-base indicators are mostly organic dyes, which can be protonated (change ionization) according to pH of a solution into which they are introduced. A change in ionization is coupled with a change in structure and coloration. Acidic form and basic form of an indicator are different in colour. For identifying of an unknown acid-base indicator, we will measure absorption spectra of both acidic and basic form.

Task: Determine the acid-base indicator in an unknown sample

You will get a test tube with an unknown acid-base indicator to identify. It is an aqueous solution at pH more-less neutral (pH~7).

What is the colour of the indicator at neutral pH?

Take 2 clean test tubes. Mark one "A" (acidic) and the other one "B" (basic). Measure 1 mL of the indicator solution into both test tubes and then add 1 mL of sulfuric acid (0.05 mol/L) to "A" and 1 mL of sodium tetraborate (0.05 mol/L) to "B".

What is the colour of the acidic form?
What is the colour of the basic form?

On a spectrophotometer, measure the absorption spectra within the range 300 - 700 nm using the interval of 10 nm of both acidic and basic forms of the indicator against distilled water as a blank.

Do not overfill the plastic cuvettes, fill them only approx. 1 cm below the edge! Do not touch the walls of the cuvettes through which the light in the photometer will pass! If these walls are dirty, wipe them with dry lab wipe. Pay attention to the correct insertion direction into the cuvette hole in the photometer!

Wavelength [nm]	*Absorbance (acidic form)*	*Absorbance (basic form)*
300		
310		
320		
330		
340		
350		
360		
370		
380		
390		
400		
410		
420		
430		
440		
450		
460		
470		
480		
490		
500		

Wavelength [nm]	*Absorbance (acidic form)*	*Absorbance (basic form)*
510		
520		
530		
540		
550		
560		
570		
580		
590		
600		
610		
620		
630		
640		
650		
660		
670		
680		
690		
700		

There is a computer in the students' laboratory with a MS Excel file prepared to put the collected data and plot the graphs.

Compare the spectra with standards (from a catalogue available in the students' laboratory) and identify the indicator.

Insert the obtained absorption spectrum here

Wavelength of the absorption maximum of the acidic form:
Wavelength of the absorption maximum of the basic form:

Name of unknown acid-base indicator:
Try to find somewhere the structural formula:

Conclusion:

b) Spectrophotometric estimation of Cu^{2+} concentration (calibration curve)

The hydrated copper complex ion $[Cu(H_2O)_4]^{2+}$ is light blue in colour. However, for photometric estimation, this coloration is of low intensity and it must be highlighted with a suitable reagent like solution of ammonia that results in formation of dark blue tetraammine copper(II) complex.

chemical equation:

For determination of the Cu^{2+} concentration, we will use a **calibration curve** method. By diluting of a stock solution (a standard solution of known high concentration) you will get a set of calibration solutions. Measure absorbances of them at optimal wavelength (absorption spectrum maximum). The calibration curve is a graph of absorbance as a function of concentration, A = f(c). According to the Beer-Lambert law, it should be a straight line passing through the origin, *i.e.* the absorbance (A) is directly proportional to the concentration (c). Once having a calibration curve, concentration of unknown samples can be easily read from it.

Task: Create a calibration curve to determine the concentration of Cu^{2+} in unknown samples

Into a test tube rack, place clean and dry test tubes and mark them with numbers:

1 to 10	calibration solutions
0	blank solution
S1, S2	unknown samples

According to the table, prepare the calibration solutions by diluting of the **stock solution of Cu^{2+} (c = 25 mmol/L)** with distilled water. Be careful with pipetting accuracy!

Number	***Stock solution***	***H_2O***	***$c(Cu^{2+})$***	***Absorbance***
-	***mL***	***mL***	***mmol/L***	-
0	-	5.0		0.000
1	0.5	4.5		
2	1.0	4.0		
3	1.5	3.5		
4	2.0	3.0		
5	2.5	2.5		
6	3.0	2.0		
7	3.5	1.5		
8	4.0	1.0		
9	4.5	0.5		
10	5.0	-		

Transfer by pipetting 5.0 mL of each unknown sample solution into the test tubes marked S1-S2.

Add 5 mL of ammonia solution into each test tube (blank, calibration solutions, unknown samples). For doing this, use the automatic dispenser.

Mix the content of all test tubes by turning upside down and back. For closing the tubes, use the rubber stopper not your thumb!

On a spectrophotometer, measure the absorbance of the calibration solution with the mean concentration (number 5) against the blank at wavelengths specified in the table below. Pour appropriate volume of calibration solution 5 into a clean cuvette, similarly fill the other cuvette with the blank solution. Be careful inserting the cuvettes into the spectrophotometer not to spill the content!

Wavelength [nm]	**450**	**500**	**550**	**600**	**650**	**700**
Absorbance						

Insert the obtained absorption spectrum here

Select the optimal wavelength for making the further measurements. Optimal wavelength is the wavelength with the highest absorbance.

Optimal wavelength [nm]	

Before plotting the calibration curve, you have to calculate the concentration of individual calibration solutions from known dilution of the stock solution.

On a spectrophotometer, measure the absorbances of all the calibration solutions against the blank at the optimal wavelength found in previous step.

Insert the calibration curve here

According to the Beer-Lambert law, the absorbance is directly proportional to the concentration. Therefore the calibration curve should be a straight line passing through the origin, *i.e.* the point (0.0).

There is a computer in the students' laboratory with a MS Excel file prepared to fit a linear regression line to the collected data. Before putting data into this MS Excel form, please finish the measurement of unknown samples (next step).

On a spectrophotometer, measure the absorbances of all unknown sample solutions against the blank at the optimal wavelength.

Sample	***Absorbance***	***Concentration***
	-	***mmol/L***
1		
2		

The regression equation for calibration curve generated in the previous step will be automatically used to calculate concentrations of unknown samples.

Conclusion:

c) Spectrophotometric estimation of Cl^- concentration (single standard)

Reagent for estimation of Cl^- concentration contains $Hg(SCN)_2$ and $Fe(NO_3)_3$. Cl^- reacts with $Hg(SCN)_2$ when the colorless complex $[HgCl_2]$ is formed. Free ions SCN^- yields with Fe^{3+} red complex suitable for the photometric determination.

Task: Determine the concentration of chlorides in the blood serum

You have prepared 3 small dry test tubes into a test tube rack. Mark them with **sa** (sample), **st** (standard) and **0** (blank). Pipette according to the table blood serum, standard and distilled water *(on the bottom of test tube, the solution mustn´t stay inside the tip!):*

Test tube 1 (sample)	20 µl blood serum (*in the eppendorf tube)*
Test tube 2 (standard)	20 µl standard Cl^- (c_{st} = 100 mmol/L)
Test tube 3 (blank)	20 µl distilled water

Pipette **2.0 mL** of reagent into all test tubes. Mix the content properly.

After **5 minutes** measure the absorbance of sample (A_{sa}) and standard (A_{st}) against blank at **450 nm.**

	Absorbance
Sample	
Standard	

The concentration of Cl^- calculate according to: $\mathbf{c_{sa}} = \frac{\mathbf{A\ sample}}{\mathbf{A\ standard}} \times \mathbf{c_{standard}}$

C_{sample} = mmol/L

Reference value of chlorides in the blood:

Conclusion:

Name: *Date:*
Group:
Co-worker(s):

Lab 8: Enzymology I

Enzymes are chemically proteins. For proper function, they require the correct conformation, which depends on relatively weak types of interactions (hydrogen bonds, ionic interactions, hydrophobic interactions, van der Waals forces). It is therefore not surprising that enzymes are sensitive to a number of external influences such as ambient temperature and pH.

Explain the terms:
primary structure of proteins

secondary structure of proteins

tertiary structure of proteins

quaternary structure of proteins

Other types of molecules than proteins may also exhibit catalytic activity
(Nobel Prize in Chemistry 1989).

Which?

Where do they apply?

Who won the Nobel Prize for this discovery?

With increasing temperature, the rate of chemical reactions generally increases (Arrhenius equation). However, this has its limits in enzyme-catalyzed reactions. The enzyme shows maximum activity at a temperature called the **temperature optimum** (for human enzymes it is around 37 °C). Higher temperatures cause conformational changes in the structure of the enzyme, i.e. partial to complete denaturation.

In nature, however, there are also enzymes that have a temperature optimum at more extreme temperatures, such as **Taq polymerase** from the bacterium *Thermus aquaticus*.

What is the temperature optimum of this enzyme?

Where is this enzyme used?

In addition to temperature, enzymes are also very sensitive to the pH. Enzymes are composed of amino acids which can contain functional groups whose ionization is pH dependent.

Which amino acids contain groups in the side chain that can carry a **negative charge**?

Write their structural formulas.

Which amino acids contain groups in the side chain that can carry a **positive charge**?

Write their structural formulas.

What form the ionizable functional group will be in depends on its dissociation constant (pK). Which of the above amino acids has a pK close to pH in the human body, and thus the pH changes that can occur in the human body have a relatively large effect on its ionization?

The pH dependence of enzyme activity usually has a Gaussian distribution. The enzyme shows maximum activity at a pH value called the **pH optimum**.

Most human enzymes have a pH optimum at a pH of around 7.

There are also human enzymes with a more extreme pH optimum.

Which human enzyme has a pH optimum at an extremely acidic pH (around 1.5)?

What is the function of this enzyme?

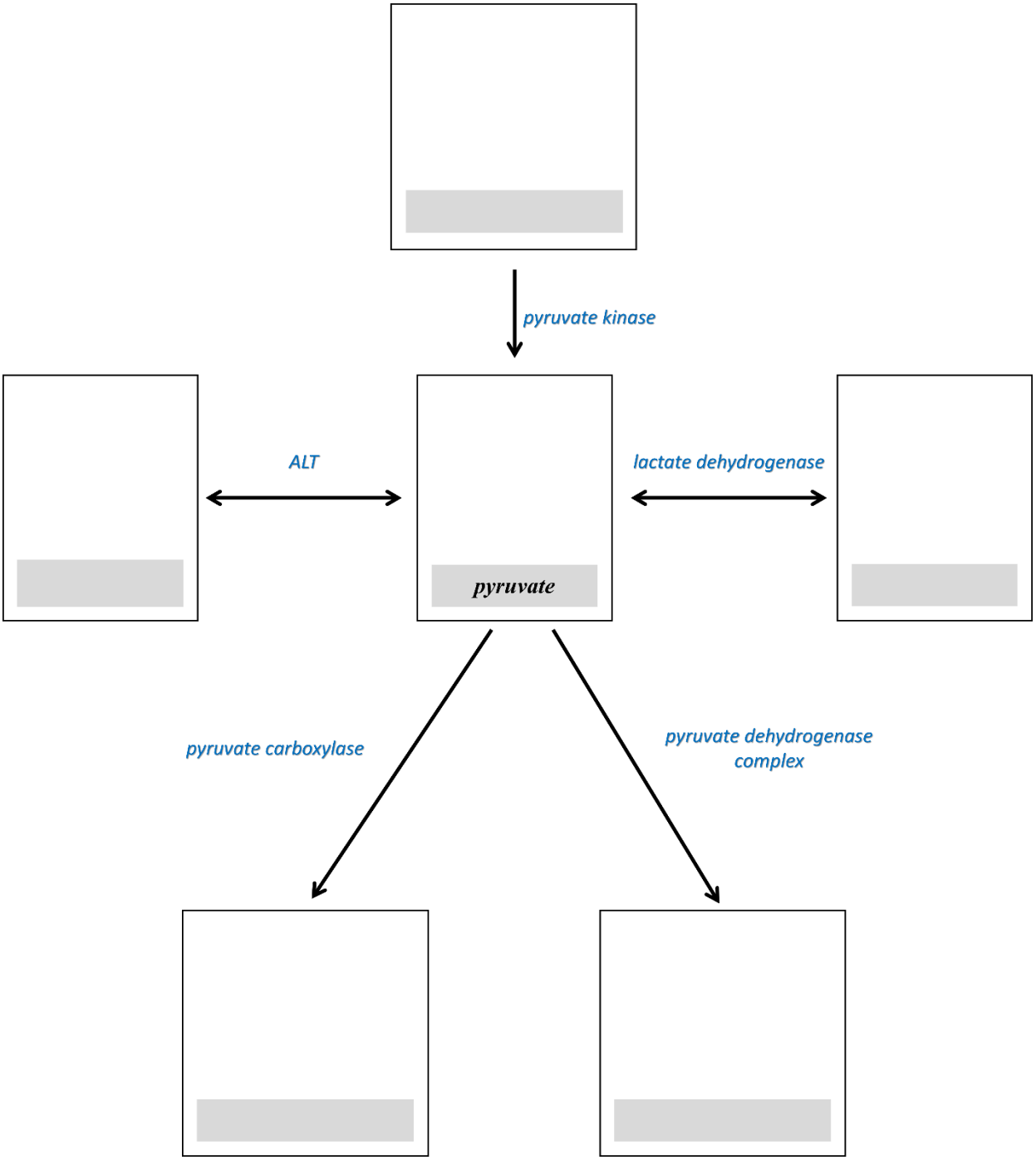
The main advantage of enzymes as "biocatalysts", in contrast to catalysts used in the chemical industry, is their specificity. Two types of specificity can be distinguished, functional specificity (reaction specificity) and substrate specificity.

Substrate specificity

Substrate specificity means that the enzyme acts on only a limited group of substrates and "ignores" others. In this sense, enzymes are variously specific, some show absolute specificity, catalyze the conversion of only one particular substrate, and "ignore" much similar molecules. Some enzymes can catalyze the reaction of several similar substrates, but often with different affinities (some substrates are preferred). An example is alcohol dehydrogenase, which can oxidize not only ethanol but also, for example, methanol.

Functional specificity

The enzyme catalyzes only one particular type of reaction. When it is a "carboxylase", it carboxylates, it will not hydroxylate or deaminate ☺. Let's see what all enzymes can work with pyruvic acid (pyruvate) as a substrate (product):



Fill in the boxes in the diagram with structural formulas and names of products (substrates), as well as the necessary coenzymes (cofactors). Note that some reactions are reversible (possible in both directions), some practically irreversible (the direction is indicated by an arrow).

a) Specificity of enzymes (sucrase, α-amylase)

In the task you will perform, you will have two enzymes, you will offer them two different substrates under different conditions and analyze what happened.

Enzymes: α-amylase annd sucrase Substrates: starch and sucrose

The first enzyme you will work with is **α-amylase**, the enzyme which can be easily and cheaply prepared.

Where in the human body is this enzyme formed?

What is a α-amylase substrate?

α-amylase cleaves bond.

What are the products of reaction catalyzed by this enzyme?

The second enzyme used, is **sucrase**.

What is a sucrase substrate? Draw the structure of this substrate.

Baker´s yeast *(Saccharomyces cerevisiae)* sucrase cleaves β-glycosidic bond of sucrose. This enzyme is different from sucrase in the intestinal juice of mammals which splits α-1 glycosidic bond.

What class of enzyme α-amylase and sucrase belong to?

Record the hydrolysis of starch by α-amylase and sucrose by yeast sucrase in the structural formulas. Mark the bonds that hydrolyzed these enzymes.

Hydrolysis of starch by α-amylase:

Hydrolysis of sucrose by yeast sucrase:

You will analyze the substrates and cleavage products by reacting with Fehling's reagent and iodine.

Which substances are generally detected by Fehling's reagent?

Explain the principle of the Fehling reaction:

What do we detect with iodine?

THEORY

	Fehling reaction	***Reaction with iodine***
starch		
cleavage products of starch		
sucrose		
cleavage products of sucrose		

Task: Monitor enzyme reactions with adequate and inadequate substrate

Preparation of enzymes:

α-amylase

Saliva collection is performed using the *Salivette*® kit. Uncap the tube, remove the swab from the top and place it in your mouth and chew for about 1 minute to suck the saliva into. Then put the swab back in the centrifuge tube and close the cap. Give the tube to the laboratory assistant.

To obtain a saliva sample, centrifuge for 2 minutes at 2,500 rpm.

Pipette 6 ml of distilled water into a clean tube, add 0.5 mL of saliva and mix by turning the tube. This will give you diluted saliva, which you will use as an **α-amylase solution** (also for the next task: "Dependence of enzyme activity on pH (α-amylase)").

Sucrase

Take approximately **0.5 g of yeast** and suspend **in 2 mL of distilled water**. This is the sucrase solution.

Prepare a set of 8 test tubes, mark them and pipette according to the table. Perform the described experiment:

		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
ENZYME	*α-amylase (mL)*	0.5	0.5	0.5	-	-	-	-	-
	sucrase (mL)	-	-	-	0.5	0.5	0.5	-	-
	distilled water(mL)	-	-	-	-	-	-	0.5	0.5
BOILING		-	-	YES	-	-	YES		
SUBSTRATE	*starch (mL)*	2.0	-	2.0	-	2.0	-	2.0	-
	sucrose(mL)	-	2.0	-	2.0	-	2.0	-	2.0
Place into the water bath (***37 ºC***) for ***30 minutes***.									
Then divide the content of each tube into two parts. (Pour about half of the contents of the respective tube into a parallel row of 8 tubes.)									
The **first** portion will be examined with **Fehling reagent**: Mix 2 mL of Fehling I + 2 mL of Fehling II. Add 0.5 mL of this solution to each tube and heat it in the water bath (***75 ºC***) for ***5 minutes***. The **second** with **iodine:** 3-5 drops of iodine solution + 5 mL of water									
Results of Fehling reaction									
Coloration created with iodine									

Evaluate the result in each test tube and make conclusions.

Tube No.	*Explanation*
1	
2	
3	
4	
5	
6	
7	
8	

Conclusion:

b) Dependence of enzyme activity on pH (α-amylase)

Task: Determine the pH optimum of salivary α-amylase

Take diluted saliva *from previous experiment as amylase solution*.

Prepare a set of 7 test tubes and mark them. Pipette the buffers according to the table:

Test tube No	*Na_2HPO_4 (mL)*	*Citric acid (mL)*	*pH*
1	1.0	4.0	3.1
2	2.5	2.5	4.8
3	3.0	2.0	5.8
4	3.5	1.5	6.4
5	4.0	1.0	7.0
6	4.5	0.5	7.5
7	5.0	0	9.0

Add 1 mL of 1% **starch solution** *(not containing buffer!)* into each tube as substrate.

Add 0.5 mL of **diluted enzyme solution** *(diluted saliva)* to each tube and leave in water bath (37 °C). Start measuring time from the moment you put the set of test tubes in the water bath.

The activity in the prepared enzyme solution can vary greatly from sample to sample. There are students, whose even very dilute saliva can split all the starch offered under any conditions in a short time. On the contrary, there are also students who do not even have amylase in their saliva ☺. To evaluate the experiment, it will be necessary to take repeated samples from the tubes incubated in the water bath.

After 10 minutes of incubation pour approximately 1-2 mL of the reaction mixture into another set of tubes and the remaining part leave on the water bath (for another 5 - 10 minutes).

To each of the samples taken of the incubation add 5 drops of iodine solution *(Lugol)* and 5 mL of water. Register the colour that develops.

It may happen that the pH optimum after the first sampling cannot be determined yet. This situation occurs if unhydrolyzed starch is present in all tubes. In this case, it is necessary to continue the incubation and take new samples from the tubes incubated in the water bath for a longer time. Otherwise, the experiment can be terminated.

After the second incubation is completed take the samples of the bath, add again 5 drops of iodine and 5 mL of water to each tube. Register the colour again.

Insert a photo (or description) of the result here.

What colour does unhydrolyzed starch give with iodine?

What is the colour in the test tubes with starch hydrolysis products?

Partial hydrolysis:

Complete hydrolysis:

Enzymatic hydrolysis of starch goes through various stages, which are also manifested by reaction with iodine. Starch is coloured dark blue with iodine, polysaccharides formed by starch partial cleavage - dextrins purple (amylodextrin), purple to red (erythrodextrin), or does not stain with iodine at all (achrodextrin).

Explain how you know the pH optimum:

What pH is the most suitable for α-amylase?

Conclusion:

Name: *Date:*
Group:
Co-worker(s):

Lab 9: Enzymology II

a) Estimation of Michaelis‘ constant (K_M) of acid phosphatase

What is Michaelis´constant?

Phosphatases cleave esters bonds of phosphoric acid and a relevant alcohol are arised. A synthetic substrate – *p*-nitrophenylphosphate which is possible for a direct photometric estimation is used for observation the enzyme activity.

Fill in the reaction equation, which is the basis for the estimation in the structural formulas. Mark the reactant whose absorbance is measured.

The reaction will be done with different substrate concentration and the relation of the reaction velocity and substrate concentration will be observed. Graphic expression will be in system of reciprocal values.

Read the whole procedure before you start working!

To obtain good results it is necessary to pipette exactly and to keep the time of incubation!

Task: Determine the Michaelis constant of acid phosphatase

Prepare **10 test tubes** and mark them.

Pipette according to the table citrate buffer at first and then substrate (use the mechanical micropipettes, it is possible to use the same tip, after the finishing the work waste the tip).

Test tube No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Citrate buffer [μL]	450	400	350	300	250	200	150	100	50	–
Substrate [μL]	50	100	150	200	250	300	350	400	450	500

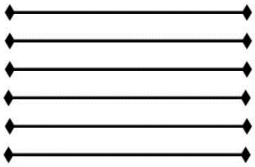
Place the set of test tubes into the water bath at **37 °C** for **5 minut**. Meanwhile, bring the enzyme from the refrigerator, prepare stop watch and the pipette of **50 μL** with a clean tip.

Now the reaction needs to run for exactly 10 minutes in each of the test tubes. The reaction will be started by the addition of the enzyme, terminated after exactly 10 minutes by the addition of an inhibitory solution, which denatures the enzyme by changing the pH.

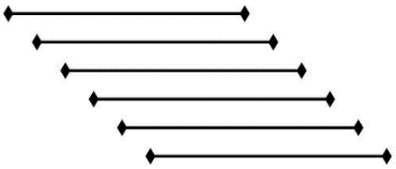
How to achieve this? What are the options?

a) Process each of the test tubes in turn. *It would take too long.* ☹

b) Process all test tubes at once. *Not technically feasible.*☹



c) Process particular test tubes with a time shift. ***Our choice.*** ☺



Enzyme solution must be pipetted in absolutely exact **30 seconds** period. Add **50 μL** of the enzyme to the test tube No. 1, press the stop watch and let it run during the whole experiment. Than pipette the same volume of the enzyme solution to other test tubes in **30 seconds** period. Just take out the test tube from the water bath and after adding the enzyme solution place it back immediately. Put the pipette tip approximately 0.5 cm above the level, it is not advisible to touch the substrate. It is necessary to drop the enzyme solution in the substrate not on the wall of the tube.

Prepare the second test tube and add the enzyme after **30 seconds** exactly. Continue in the same way, the period of adding into tubes must be the same**, 30 seconds**!!!

Prepare the inhibition solution which stops the enzyme reaction. The inhibitor must be added in **10 minutes** after adding the enzyme, it means the reaction runs for 10 minutes in each test tube.

Add **2 mL** of **inhibition solution** to each test tube and mix. Do not place back the tubes to the water bath than switch off the stop watch.

Measure the absorbance of all samples at **405 nm** against **water (blank)**. Start with tube No. 1 and continue in sequence to tube No. 10. Do not rinsen the cuvettes, only pour them and dry them carefully.

Test tube No.	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
concentration of substrate (mmol/L)	0										
absorbance	0										

Enter the obtained values into the table in the computer. The concentration of basic substrate solution is **2.5 mmol/l**. Measured absorbance is proportional to the enzymatic reaction velocity in each tube because reaction velocity means amount of converted substrate in time. In this experiment the result is amount of the arisen product in 10 minutes. The program creats Lineweaver-Burk`s graph, it is dependence of **1/v** on **1/[S]** and the equation for the line is given.

Calculation

Michaelis` constant is estimated from the equation or from the graph where the value of $\mathbf{-1/K_M}$ is found.

Insert the table here

Insert the obtained graphs here

Calculation of Michaelis´constant:

Determined K_M:

Conclusion:

b) Estimation of catalase activity

Catalase of erythrocytes is a part of a system reducing unadvisable reactive forms of oxygen, more precisely their products. Catalase released from erythrocytes decomposes hydrogen peroxide.

Complete the equation of the catalase-catalyzed reaction:

Explain what reactive oxygen species are.

Concentration of substrate, i.e. hydrogen peroxide, is determined by manganometric titration.

Manganometry belongs to redox titration methods. A solution of potassium permanganate is used as the standard solution for this method.

Potassium permanganate – formula:	oxidation state of manganese:

Colour of the solution of potassium permanganate:

Chemical equation in ionic form:

Task: Assess the kinetics of hydrogen peroxide decomposition using catalase

Prepare **8 titration flasks**, mark them numbers 0 - 7. Put **2.5 ml of sulfuric acid** into each flask (it will stop the enzymatic reaction and it is also necessary for the manganometric titration).

Preparation of enzyme solution

You will use hemolyzed capillary blood diluted with distilled water as the enzyme solution.

Pipette 1.5 ml of distilled water into the ependorf tube. Then collect the capillary blood from your fingertip.

Take one glass capillary before blood collection. Disinfect and dry your fingertip. Inject with the lancet.. Wipe the first drop and fill the capillary with blood from the second drop at least halfway. Insert the capillary with the sample into the water in the ependorf tube. Close the ependorf tube and mix by inverting repeatedly so that all the blood from the capillary enters the water.

Dispose of the infectious material in a prepared special container!

Setting up enzymatic reaction:

Measure **60 ml of substrate** into **100 ml Erlenmayer flask** (buffered hydrogen peroxide solution) and add a magnetic stirrin bar. Place the flask on a magnetic stirrer.

Prepare 5 ml pipete for a sampling and transfer **5 ml of substrate** into the titration flask number 0. Be careful, the pipete must not be contaminated with sulfuric acid or else it could not be used for another sampling.

Measure **1 ml of enzyme solution** and add it to the substrate and run the stop watch immediately and let it run.

Prepare 5 ml pipete for a sampling **in 1 minute intervals** and transfer samples into titration flasks immediately. The moment of sample releasing into the titration flask with sulfuric acid that stops the enzymatic reaction is decisive. It means, suck in the sample several seconds before the minute and empty the pipete in a specific moment exactly. All samples are titrated with the solution of permanganate and the concentration of substrate (hydrogen peroxide) in each sample is determined.

Calculation of substrate - hydrogen peroxide - concentration:

Values obtained from the titration put in the Excel table in the computer and the expression of changes of substrate concentration and changes of reaction velocity on time.

Assess the kinetics and try to estimate a reaction order.

Time	***V(t)***	***c(H_2O_2)***	***n(H_2O_2)***	***Δn / Δt***
min	***mL***	***mmol/L***	***μmol***	***μmol/min***
0				-
1				
2				
3				
4				
5				
6				
7				

Insert the obtained graphs here

Conclusion:

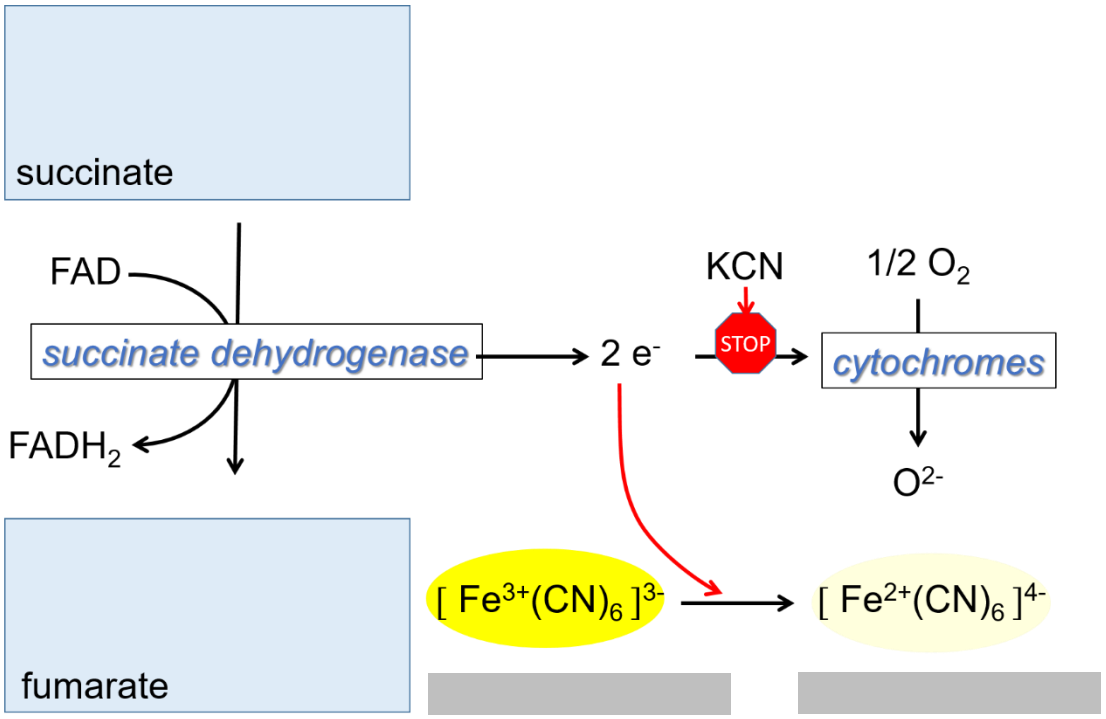
Name: Date:
Group:
Co-worker(s):

Lab 10: Enzymology III

a) Inhibition of succinate dehydrogenase with malonate

Succinate dehydrogenase is an enzyme of citrate cycle. It belongs to the riboflavine-linked dehydrogenases presented in the inner mitochondrial membrane. It converts succinate to fumarate under normal conditions. Malonate is known as an competitive inhibitor of this reaction. In the nature conditions this enzyme transfers electrons through the respiratory chain to the oxygen. In order to measure this reaction in our experiment we have to block the respiratory chain, particulary the function of cytochromes with KCN, and we supply an artificial electron acceptor – *potassium hexacyanoferrate (III), $K_3[Fe(CN)_6]$*, which is reduced to *potassium hexacyanoferrate (II), $K_4[Fe(CN)_6]$*. This reduction can be observed photometricaly, and it goes with coloure changing, fading of a yellow solution.

Add structural formulas and names of reactants to the reaction scheme!



In this experiment there is possible to observe the kinetic of enzyme reaction easily even effects of inhibitors.

structural formula of malonate

Explain what is competitive inhibition:
Explain what is non-competitive inhibition:

Task: Monitor the effect of a competitive inhibitor (malonate) on the rate of the enzyme reaction (catalyzed by succinate dehydrogenase)

Prepare an ice bath. Put several ice cubes and distilled water into **250 mL beaker.**

Take a calibrated tube and 5× diluted phosphoric buffer, **2 mL of phosphate buffer + 8 mL of distilled water**. Mix it and place into an ice bath.

Preparation of the enzyme solution

Take a small piece of pork heart, put it into a mortar and cut it with a scalpel into small pieces. Wash the pieces with approximately 3 ml of diluted phosphate buffer and homogenize the tissue with a pestle. Transfer the crude homogenate into the centrifugation tube and spin it down for **5 minutes** at **2,000 rpm**. Meanwhile, clean the mortar and pestle.

During the preparation of the enzyme solution, it is desirable to prepare a „basic solution".

After centrifugation pour the supernatant carefuly in the waste. Put the sediment that must be colourless, into the mortar and resuspend it. (Any colour would interfere with photometric measurements.) Add approximately 3 ml of diluted phosphate buffer and glass pieces for better homogenisation (use protective glasses!). Transfer the mixture into the centrifugation tube and spin it down for **5 minutes** at **2 000 rpm**. After that collect the supernatant because it is our enzyme solution. Store it in the ice bath because mitochondria is very sensitive compages.

Preparing of a „basic solution"

Mix **3 mL** of **potassium cyanide, KCN**, **3 mL** of **potassium hexacyanoferrate (III), $K_3[Fe(CN)_6]$**, and **10 mL** of **phosphate buffer** (undiluted).

Pipette the solutions into test tubes according to the table:

Test tube No.	**1**	**2**	**3**	**blank**
„basic solution" (mL)	2.0	2.0	2.0	-
sodium succinate (mL)	1.0	1.0	-	-
sodium malonate (mL)	-	0.5	0.5	-
distiled water (mL)	0.5	-	1.0	3.5

Switch on the photometer, adjust the wavelength **415 nm**, and prepare 4 photometric cuvettes, stop watch, and 0.5 mL pipette.

Add **0.5 mL** of **enzyme solution** to the **blank**, mix and pour it into the cuvette and adjust A = 0.000.

Add **0.5 mL of enzyme solution** into the test tube **No. 1**, mix and pour it into the cuvette immediately and measure absorbance at time 0 and start to measure the time. Note the value of absorbance.

Pipette **0.5 mL of enzyme solution** into the test tube **No. 2** very quickly, mix and pour it into another cuvette. Place the cuvette into the photometer and measure the absorbance. The time of

measurement must be **30 seconds** after the measurement of the test tube No. 1 accurately.

Change the cuvettes in a period of **30 seconds**. Each cuvette is measured in a period of **1 minute**. Make **20 measurements** for each cuvette overall.

Process the test tube No. 3 which is chacking in the same way. It contains just an inhibitor, no substrate. Add **0.5 mL of enzyme solution** and measure the absorbance in a period of **1 minute**. There can be observed just slight decrease of the absorbance what is a systhematical error of the method.

Time	***Succinate***	***Succinate + Malonate***	***Malonate***
min	***A***	***A***	***A***
0			
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			

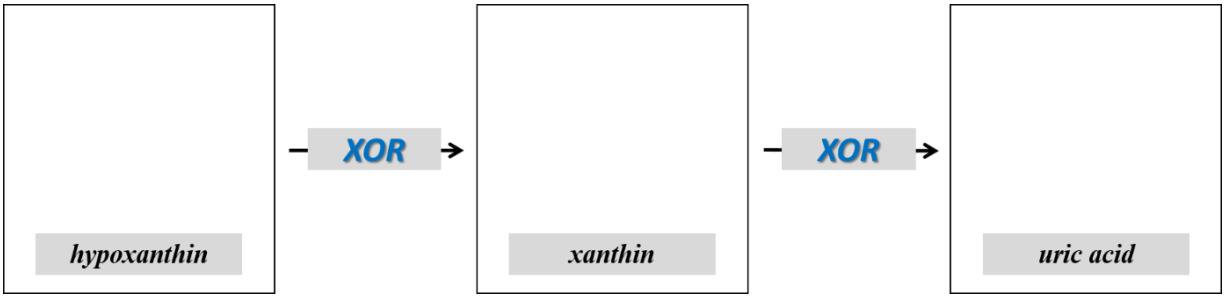
Write down all absorbance values in the table into the computer and a graphic processing will be done.

Insert the obtained graph here

Evaluate the results and make a conclusion:

b) Monitoring of milk xanthine oxidase activity

*Xanhtine oxidase (XOR) i*s a complex molybdenum containing flavoproteine. It is able to convert hypoxanthine to xanthine, and xanthine to uric acid.

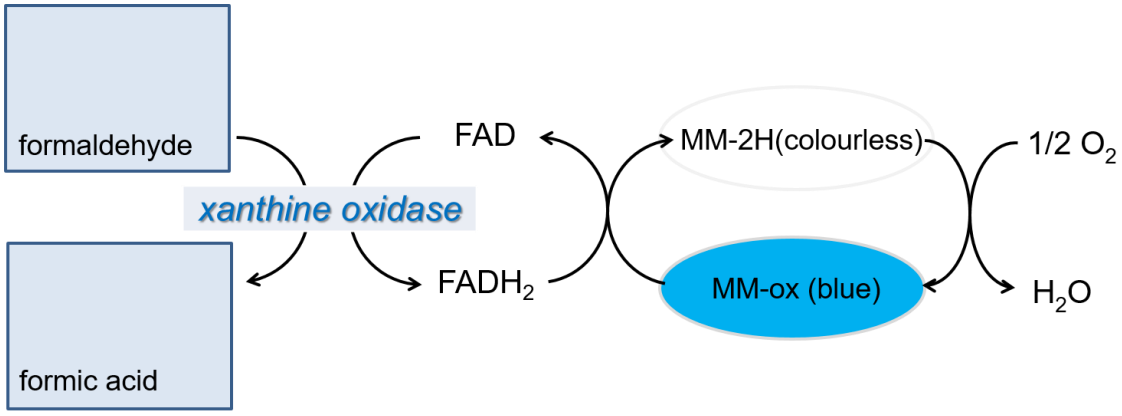


Fill in the structural formulas.

An inhibitor of this enzyme has found application in clinical practice. Compare its structure with hypoxanthine: What is its therapeutic use?	*name* *structural formula*

Xanhtine oxidase is present in many organs and also in milk. Its substrate specificity is not absolute and therfore even non-specific substrates can be oxidated by this enzyme, if a suitable electron acceptor is present. This acceptor is usually the molecular oxygen which subsequently forms water. In this reaction also the one-electron reduction product is often formed, i. e. the superoxide anion O_2^- which has to be disproportionated by superoxide dismutase and catalase.
In our experiment formaldehyde as a substrate for the xanthine oxidase and the methylen blue as the first electron acceptor are used. The final electron acceptor is the atmospheric oxygen.

Add the structural formulas of the reactants to the reaction scheme!



This experiment can be used as a simple test for milk pasteurisation. Higher temperature causes enzyme denaturation and adding of formaldehyde cannot raise discolouration of methylen blue.

Methylene blue is a redox dye, ie the colour depends on oxidized or reduced form. What is the colour of the oxidized form? What is the colour of the reduced form?

Task: Monitor milk xanthine oxidase activity using methylene blue under different conditions

Pipette solutions into **3 test tubes** according to the table:

Test tube No.	**1**	**2**	**3**
Fresh milk	2 mL	2 mL	2 mL
Heat test tube No. 3 in the water bath (***75 ºC***) for ***5 minutes***. Next continue according to the table:			
Methylen blue	3 drops	3 drops	3 drops
Formaldehyde	3 drops	-	3 drops

Shake well the tubes and place them into the water bath **at 37 ° C**. Look where discolouration can be observed and explain the reason.

Take out the test tube where the blue colour disappeared and shake vigorously until the blue colour is restored. Then continue the reaction in the water bath.

You can repeat this cycle several times until all formaldehyde is exhausted. Then methylen blue will not loose the colour any more. In such a case few drops of additional formaldehyde will restore this ability again.

Tube No.	*Explanation*
1	
2	
3	

Conclusion:

Table of Reference Values for Clinical Testing *update 26. 1. 2024*

A result of a laboratory investigation is a measured value that may be physiological, increased or decreased. For classification of lab results as physiologic or pathologic we need to compare measured values with a physiological (reference) range of values. The reference range is obtained by measuring the reference (means healthy) population. Obtained values are arranged in ascending order and then a definite percentage (usually 2.5%) of the extreme minimal and the extreme maximal values are discarded. Then the lowest and the highest remaining values signify the lower and upper reference limits. In this case 95% of healthy people belong to the reference range. But on the other hand, there are about 5% of healthy people, whose values do not belong to the reference range. However, significant deviations of values are almost always associated with pathology.

For some substances any significant deviations from the physiological range are associated with pathologies (*e.g.* glucose). There is a danger only if the level of value of some substances exceeds a certain limit or if the value declines under the limit. For such substances is used only the upper or lower reference value (*e.g.* lowering of cholesterol does not signify a clinical danger).

Reference population, laboratory reagents or procedure of measurement, could be different among particular laboratories in some detail. That is the reason of different reference values used in different labs. The students should be familiarized with the most important reference values used in University Hospital Pilsen in present in the enclosing list. Some reference ranges even include for example a patient's age or a gender. In such cases for study purposes we tried to simplify these ranges. But it should still provide at least a general idea of the average values for healthy adults.

The same metabolites may be found in various body fluids in a different concentration. Therefore, it is necessary to mark an analyzed metabolite with an abbreviation of an investigated material. In common the special term is used for a concentration of certain substances in the specific body fluids. For example, glycaemia refers to blood glucose, the term glycorrhachia relates to cerebrospinal fluid and glycosuria means glucose in urine. If the blood glucose is under the physiological limit we talk about hypoglycaemia. High level of blood glucose is called hyperglycemia.

E.g.:

B_glucose - means glucose concentration in full blood

S_glucose - means glucose concentration in serum

P_glucose - means glucose concentration in blood plasma

Csf_glucose - means glucose concentration in cerebrospinal fluid

U_glucose - means glucose concentration in urine

Common Lab Values to Remember

Lab Investigation	Normal Range
Blod Gas Analysis (Arterial Blood Gas)	
pH	7.36 - 7.44
pCO_2	4.8 - 5.9 kPa
pO_2	9.6 kPa and higher
HCO_3^-	22 - 26 mmol/L
Base Excess (BE)	± 2.5 mmol/L
Anion Gap (AG)	14 - 18 mmol/L
Blood Tests	
Plasma Osmolality	275 - 295 mmol/kg H_2O
Sodium	136 - 144 mmol/L
Potassium	3.8 - 5.2 mmol/L
Total Calcium	2,2 - 2,6 mmol/L
Ionized Calcium	1.15 - 1.30 mmol/L
Magnesium	0.7 - 0.9 mmol/L
Chloride	98 - 109 mmol/L
Phosphorus	0.7 - 1.7 mmol/L
Serum Iron	6 - 35 µmol/L
Lactic Acid	less then 2.2 mmol/L
B/S Glucose	3.6 - 5.6 mmol/L
Glycated Hemoglobin (HbA1c)	20 - 42 mmol/mol
Results of Glucose Tolerance Test	
Normal Glucose Tolerance	less than 7.8 mmol/L
Impaired Glucose Tolerance	7.8 - 11.0 mmol/L
Diabetes Mellitus	11.1 mmol/L and higher

Total Bilirubin	less than 25 µmol/L
Bilirubin - Conjugated ("direct")	less than 8 µmol/L
Total Cholesterol	less than 5 mmol/L
Triglycerides	less than 1.7 mmol/L
LDL Cholesterol	less than 3 mmol/L
HDL Cholesterol	♂ 1 mmol/L and higher
	♀ 1.2 mmol/L and higher
Urea	3 - 8 mmol/L
Uric Acid	♂ 210 - 450 µmol/L
	♀ 140 - 360 µmol/L
Creatinine	♂ 60 -100 µmol/L
	♀ 50 - 90 µmol/L
Ammonia	less than 50 µmol/L
Alpha-amylase (AMS)	less than 2.0 µkat/L
Aspartate Transaminase (AST)	♂ less than 0.8 µkat/L
	♀ less than 0.6 µkat/L
Alanine Transaminase (ALT)	♂ less than 1.2 µkat/L
	♀ less than 0.8 µkat/L
Alkaline Phosphatase (ALP)	0.7 - 2.2 µkat/L
Lactate Dehydrogenase (LD)	less than 4.0 µkat/L
Gamma-glutamyltransferase (GGT)	♂ less than 1.2 µkat/L
	♀ less than 0.7 µkat/L
Lipase (LPS)	less than 1.0 µkat/L
C-reactive Protein (CRP)	less than 5 mg/L
Serum Total Protein	63 - 80 g/L
Albumin	37 - 52 g/L
Serum Protein Electrophoresis Fractions	
Albumin	0.530 (53%) - 0.650 (65%)
Alpha-1 Zone	0.020 (2%) - 0.040 (4%)
Alpha-2 Zone	0.080 (8%) - 0.130 (13%)
Beta Zone	0.090 (9%) - 0.160 (16%)
Gamma Zone	0.115 (11.5%) - 0.19 (19%)

Cerebrospinal Fluid Tests	
Protein	less than 0.5 g/L
Glucose	2.7 - 4.5 mmol/L
Lactic Acid	1.2 - 2.2 mmol/L
Urine Tests	
Density	1010 - 1030 g/L
pH	5 - 6
Glucose	0
Protein	0
Alpha-amylase (AMS)	less than 8.0 µkat/L
Creatinine Clearance Rate	1.6 - 2.6 mL/s