B I O C H E M I S T R Y W O R K B O O K

for students of the Faculty of Medicine
and the Faculty of Health Sciences

Medical University of Białystok

edited by

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Białystok 2013

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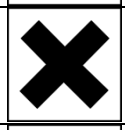
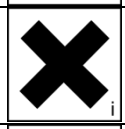
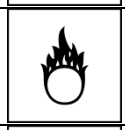
Warning signs and symbols used on chemical reagents

Sign	Symbol	Description
	**T+**	Highly toxic
	**T**	Toxic
	**Xn**	Harmful
	**C**	Corrosive
	**Xi**	Irritant
	**N**	Nature polluting
	**E**	Explosive
	**O**	Oxidizing
	**F+**	Extremely flammable
	**F**	Highly flammable

Laboratory regulations

1. Students are allowed in the laboratory only in the presence of a tutor.

2. Before entering the laboratory, you need to wear a laboratory coat and soft shoes.

3. In the laboratory, you need to work carefully, avoid unnecessary conversations and keep your workplace clean.

4. Please read the warning signs and symbols placed on the reagents prepared for exercises.

5. Preparations and reagents must not be examined by taste.

6. Eating and drinking are not allowed in the laboratory.

7. Use distilled water, electricity and gas efficiently.

8. Take particular caution when handling concentrated acids, bases, poisons and flammable liquids. Concentrated acids, bases and poisons should only be collected by dipping the pipette. Used acids and bases should be poured out into the sink in such a manner as to avoid burns caused by drops of liquid deflected from the wall of the sink (while pouring, hold the mouth of the vessel as close as possible to the drain, gently rinse with water).

   Flammable liquids should be used on premises without ignited burners or other sources of open flames. They should be stored in tightly sealed vessels.

9. The gas installation should be used with caution. When igniting a gas burner, first you must close the air supply, then draw a lit match closer to the outlet of the burner chimney and slowly open the gas valve. Adjust the air supply (the flame should not roar nor smoke). Unnecessary burners should be immediately turned off.

10. In the case of burns to the skin, mouth or eyes, immediately wash the corrosive liquid with plenty of tap water and notify the tutor. Then, neutralize the acids with 5% sodium bicarbonate, and the bases with 1% acetic acid. Compounds used for neutralization can be found in each laboratory.

    Prior to starting an exercise, make sure that the above-mentioned reagents are in the laboratory.

11. In the event of ignition of the reaction mixture, the table or the student's laboratory coat, one must immediately extinguish the fire using a fibreglass fire blanket (hangs on the wall in the lab) or a fire extinguisher (located in the room) and notify the assistant.

12. Before leaving the lab, the workplace, reagents and equipment should be put in order. Wash the lab glass. Close the gas valves. Turn off the taps.

Attention!

It is forbidden to write the results and take notes in the workbook. Comments on the exercises, experiment protocols, and result interpretations are to be entered into the notebook intended for biochemistry exercises.

Amino acids and proteins

Aim of the exercise: ***to learn about some of the properties of amino acids and proteins***

Amino acids

Amino acids are among the best-known components of living organisms. They are derived from organic acids, in which a hydrogen atom most often located near the α-carbon is substituted by the amino group. Some amino acids have two amino groups located at different carbon atoms, a few contain two or even three carboxyl groups. Two amino acids, proline and its hydroxylated derivative - hydroxyproline, have no amino group but an imino group, which is why they are called imino acids.

There are more than 300 different amino acids described. The vast majority of them occur in free form or in non-protein combinations, **and only 20** commonly occur in almost all proteins. The presence and location of amino acids in the structure of protein molecules is genetically determined. Some amino acids such as hydroxyproline and hydroxylysine appear in proteins by modifying the amino acid residues previously built into the protein chain.

A fragment of the amino acid molecule, composed of the **α**-carbon, the α-amino group and the α-carboxyl group is a common structural element of all protein amino acids (except imino acids). At physiological pH (about 7.4), most of the carboxyl groups are dissociated, create anion **$-COO^-$**, and most of the amino groups bind **H^+** creating cation **$-NH_3^+$.** Under these conditions, the dominant form of the amino acid is therefore a zwitterion, which has two opposite electric charges. Therefore, for didactic purposes, the structural formula notation of the amino acids with the amino group in the cationic form **$-NH_3^+$** and the carboxyl group in the anionic form **$-COO^-$** was assumed as the rule.

The chemical properties common to all amino acids are due to the presence of the α-carboxyl group and the α-amino group in their molecules. All amino acids, containing a free α-amino group, in a **reaction with ninhydrine** form products of a violet-blue colour, while proline and hydroxyproline, containing the imino group, create yellow-coloured products.

During a ninhydrine reaction, the amino acid decarboxylates and deaminates, and the released ammonia is fixed with ninhydrine to form a violet-blue-coloured product.

Other fragments of molecules of amino acids, fixed with the α-carbon, are called side chains or side substituents. They are marked with an **R** symbol. They are the ones that give the amino acids their individual characteristics. The side chain structure determines the role of the amino acid in protein. However, side chains differ in the elemental composition, the spatial structure, size, the electric charge, the ability to generate hydrogen bonds and chemical reactivity. In these substituents, the following may occur: an additional amino group, an amide group, an additional carboxyl group, the -SH group, the -S-CH_3 group, the -OH group, the guanidine group and ring substituents: phenyl, hydroxyphenyl, indole or imidazole. The presence of these groups makes it possible to detect individual amino acids in biological material using simple methods, possible to be used in a student laboratory. This applies to both free amino acids, as well as those forming the protein molecules.

The aromatic rings of **phenylalanine**, **tyrosine** and **tryptophan** under the effect of nitric acid form yellow-coloured nitro derivatives. This process is called the **xanthoproteic reaction.**

Tyrosine, like other phenols, reacts with Millon's reagent, which is a solution of mercury nitrates (III) and (V) in nitric acid. Nitrophenols, formed from tyrosine by the action of nitric acid (V), form red-coloured complexes with mercury. Heating a mixture containing free or peptide fixed tyrosine as well as Millon's reagent causes the formation of red sediment.

Sulphur-containing amino acids: **cysteine** and **methionine**, in a strongly alkaline environment degrade releasing sulphide ions, which react with lead acetate (II). The brown-black lead sulphide (II) is formed.

The **tryptophan** indole ring reacts with glyoxylic acid in the presence of sulphuric acid (VI) to form a product of a red-violet colour. Glyoxylic acid occurs (as a polluting component) in the commercial preparation of concentrated acetic acid.

Peptides and proteins

Proteins are constructed out of L-α-amino acids fixed with **peptide bonds**. Two amino acids bind to each other by a reaction of the α-carboxyl group of one with the other's amino group. A water molecule detaches and the peptide bond forms. The reaction product of two amino acids is a dipeptide retaining a free amino group of one of the amino acids and a free carboxyl group of the other one. The dipeptide carboxyl group can react with the amino group of the third amino acid to form the next peptide bond. This way the dipeptide transforms into a tripeptide, etc. Peptides constructed of several - more than a dozen amino acids are oligopeptides, longer ones are called polypeptides. A polypeptide containing over 100 amino acid residues is called a protein.

Protein amino acid composition is very diverse. Some, such as **albumin, egg protein**, contain all the protein-building amino acids, others such as **gelatine** (denatured collagen) do not contain cysteine and tryptophan, or contain only very small undetectable in our conditions amounts of phenylalanine and tyrosine.

The **peptide bond** has the characteristics of a double bond of the *trans* configuration. Oxygen of the C=O group and hydrogen of the N-H group are directed in opposite directions. The C and O atoms of the C=O group and the N and H atoms of the N-H group, together with the neighbouring C-α atoms, lie in one plain. The structure of peptide bonds resembles the binding occurring in a simple compound called a **biuret**. From it comes the name of the **biuret reaction**, characteristic for both: peptides and proteins.

The **biuret reaction** is a commonly used colour reaction, used for the detection and quantification of peptides and proteins. It is characteristic of structures that have at least two peptide bonds. In the presence of peptide or protein, the biuret reagent, which is a solution of $CuSO_4$, NaOH and sodium-potassium tartrate changes colour from blue to purple. In an alkaline environment, forms a complex of Cu^{2+} with a peptide or protein and with tartrate. The last increases the solubility of the complex. The colour intensity is proportional to the concentration of proteins in the solution.

The protein structure can be examined on four "levels". These are primary, secondary, tertiary and quaternary structures. The last three are known collectively as **protein conformation**.

The **denaturation of protein** involves the destruction of its spatial structures while retaining the primary structure. The continuity of the polypeptide chain remains intact. The essence of denaturation is the disintegration of low-energy bonds, which stabilize the spatial structure of the protein. The denaturising factors are primarily: elevated temperature (usually above 58-60°C), organic solvents, acids, alkalis, heavy metal ions (such as Hg^{2+}, Pb^{2+}), concentrated solutions of urea or guanidine hydrochloride. Denatured protein loses its biological activity, e.g. an enzyme loses its catalytic properties, an antibody - its antigen binding ability, collagen the ability to create fibres, and haemoglobin the ability to bind oxygen. The denaturation of protein generally changes its solubility. Soluble protein loses solubility, insoluble protein becomes soluble.

Soluble proteins form colloidal or real solutions. The stability of protein solutions mainly depends on the electric charge of the particles, their degree of hydration and temperature. Protein, which as a result of the denaturation agent action lost its colloidal character, usually precipitates from the solution.

E X E R C I S E

1. Ninhydrin reaction - common to all amino acids

To 1 ml of diluted neutral amino acid solution, add a few drops of ninhydrin solution, and then heat in a boiling water bath for several dozen seconds. Observe the change in colour.

2. Reactions specific to individual amino acids

a. the detection of aromatic amino acids - the xanthoprotein test

To 2 ml of **albumin** solution and 2 ml of **gelatine** solution, add 0.5 ml of concentrated nitric acid (V) and heat in a boiling water bath for about 30 seconds. During heating, a yellow colour starts to show. It intensifies after adding a few drops of 20% NaOH solution. Compare the results.

b. the detection of sulphuric amino acids - the cysteine test

To 0.5 ml of **albumin** solution and 0.5 ml of **gelatine** solution, add 0.5 ml of 20% NaOH solution and heat in a boiling water bath for one minute. Then, to the two test tubes, add 1-2 drops of solution of lead acetate (II). Only the albumin solution turns brown or black as a result of the formation of a lead sulphide (II) suspension.

c. the detection of tryptophan

To 1.0 ml of **albumin** solution and 1.0 ml of **gelatine** solution, add 1.0 ml of concentrated acetic acid (with glyoxylic acid), and then carefully add, pouring on the test tube's side wall, about 0.5 ml of concentrated sulphuric acid. Only in the test tube containing albumin, a reddish-purple ring will appear at the connection line of the layers, which indicates the presence of tryptophan.

3. Detection of the peptide bond - the biuret test

To 1.0 ml albumin solution, add 0.5 ml of 2M NaOH, and then add drops of copper sulphate (II) solution. The fluid changes colour from blue to purple.

4. Thermal denaturation of proteins

Heat 3 ml of albumin in a boiling water bath. The fluid turns opalescent, but precipitate does not form. Cool the contents of the test tube and gradually add drops of 1% acetic acid. At first, precipitate forms, which then dissolves in excess of acetic acid.

5. Ethanol precipitation of protein

Cool 1 ml of blood serum and 5 ml of 96% ethanol by immersion in a mixture of water with ice in separate test tubes. Then mix the two liquids.

Observe the precipitation of protein from the solution. Filter off the precipitated protein and dissolve it (through a filter) in distilled water.

Do a second test without cooling and let the mixture of serum and ethanol stand at room temperature for one hour and then filter. The resulting sediment should not dissolve in water.

6. The action of concentrated nitric acid on protein

Pour into a test tube 1 ml of concentrated nitric acid (V), and then carefully pouring on the test tube's side wall add a similar volume of albumin solution (avoid mixing the two liquids). On the border of the two liquids, a white-yellow layer of denaturised protein forms.

ASSIGNMENT

Determine whether the tested solution contains protein. Is it albumin or gelatine?

For this purpose make:

- test for the presence of protein
- xanthoproteic test for the presence of aromatic amino acids
- test for the presence of tyrosine
- cysteine test for the presence of sulphur amino acids
- test for the presence of tryptophan.

Properties of proteins in solutions

Aim of the exercise: ***to learn about some of the properties of proteins in solutions, the measurement of protein concentration***

The **solubility** of proteins is highly variable. Some are completely insoluble (such as keratin, elastin) or show negligible solubility (e.g. collagen). Others dissolve very well (e.g. haemoglobin, albumin). The following are solvents for proteins: water or aqueous solutions of salt, acids and alkalis, urea or detergents. Solubility depends on the presence of polar amino acids in the protein molecule. Proteins with a high content of polar amino acid dissolve in a water environment. Polar groups of amino acid side chains produce hydrogen bonds with water molecules. The protein molecule is surrounded by a water jacket. Proteins, which are dominated by non-polar amino acids, have a limited ability to bind water and are therefore insoluble.

Protein solutions are generally **real solutions** with a monomolecular degree of dispersion. Sometimes, however, protein molecules associate to form aggregates composed of two or several particles. The protein solution then takes on the characteristics of the **colloidal solution**.

Proteins exhibit **amphoteric properties**. In the solution (depending on pH), they act like acids or bases. This characteristic is mainly conditioned by the presence of polar groups with the electric charge in the side chains of certain amino acids. The **$-NH_2$** groups bind the **H^+** ions present in the solution preventing acidification, and protons separated during the dissociation of the **-COOH** groups neutralize the **$-OH^-$** ions preventing alkalization.

This characteristic of amino acids, peptides and proteins has been important in maintaining the **acid-alkaline balance** of tissues and body fluids. The acidic characteristics are given to protein primarily by the **β**-carboxyl groups of aspartic acid residues and the **γ**-carboxyl groups of the glutamic acid residues, which dissociate releasing (H^+) protons and create the negatively charged $-COO^-$ group. An alkaline environment is conducive to the dissociation of the carboxyl groups and the transformation of proteins into the anionic form. The ε-amino group of lysine residues, the guanidine groups of the arginine residues and the imidazole rings of histidine residues give protein

alkaline characteristics. They can bind protons (H^+), giving the protein molecule a positive charge. An acidic environment is conducive to the binding of protons by the before mentioned groups and the transformation of protein into cationic form.

Single α-amino and α-carboxyl groups found in N-terminal and C-terminal amino acids have little influence on the net electric charge of the protein molecules.

Each protein has its own characteristic pH value, where the number of positive and negative charges on the surface of a particle offset each other. This pH value is called the **isoelectric point** - **pI.** In the isoelectric point, the net electric charge of a protein molecule equals zero. In these conditions, protein does not move in the electric field, and its solubility is the lowest.

In an environment with a pH above the isoelectric point, proteins show a **negative** net charge and can form compounds with cations of heavy metals. The acidic action impedes the course of these reactions, because the dissociation of the carboxyl groups reverses. Protein loses anionic properties and does not react with cations.

In an environment with a pH lower than the isoelectric point, the resultant protein charge becomes **positive**. Protons from the dissociation of acids present in the solution bind with the amino groups creating positively charged $-NH_3^+$ groups. Protein, in cationic form can be precipitated from a solution with alkaloid reagents, which are carriers of a negative charge. These include, among others, acids: sulphosalicylic and picric, and hexacyanoferrate. Alkaloid reagents owe their name to the ability to precipitate alkaloids from solutions. Alkaloids are nitrogenous organic compounds with the nature of weak bases. Some of them show pharmacological activity (e.g. morphine).

Proteins can be separated from the low-molecule compounds in a process called **dialysis**. The dialysis bags used for this purpose are made from a semi-permeable membrane (made for example from cellophane). Low-molecule compounds found in the solution inside the bag penetrate into the water surrounding the bag in order to level-out the concentration on both sides of the membrane. However, macromolecular compounds, such as proteins, do not pass through the semi-permeable membranes. Through multiple changes of dialysis fluid (e.g. water) and with a sufficiently long

period of dialysis (2-3 days), the protein solution can be almost completely released from the salts contained in it. In the same way, you can introduce salt inside the dialysis bag or replace a salt solution with another without changing the protein concentration in the dialysed solution.

Neutral salts such as ammonium sulphate and sodium chloride have a substantial effect on the solubility of proteins. Low concentrations of these salts increase the solubility of many proteins. A progressive increase in salt concentrations in the solution causes protein dehydration and as a result lowers their solubility. At sufficiently high concentrations of salt, protein can be completely precipitated from the solution. This phenomenon is called **salting out**. This is one of the methods of protein fractionation. Serum proteins can be separated by ammonium sulphate ($(NH_4)_2SO_4$) salting out. Globulins precipitate from the solution at a 50% saturation of ammonium sulphate and albumin in a completely saturated salt solution.

EXERCISE

1. Protein precipitation with concentrated salt solutions - salting out

To 5 ml of undiluted blood serum, add 5 ml of saturated ammonium sulphate solution and mix. Filter through a dry filter into a dry test tube. The precipitated globulins will remain on the filter and the albumins will remain in the solution. Transfer the filter to another test tube and dissolve globulin precipitate in a small amount of distilled water (the amount of salt needed to dissolve them is on the filter) - globulins will transfer to the globulin solution. Perform the biuret test for the presence of protein in this solution.

Divide the albumin-containing filtrate into two parts. Perform the biuret test with one of the parts. Transfer the second to a porcelain bowl and add ammonium sulphate (in the substance), which should be ground (using the submerged end of the test tube) until complete saturation of the solution. Filter and subject the obtained filtrate to the biuret test. Compare the results.

2. The amphoteric properties of proteins

To the two test tubes, add 2 ml of water and 3 drops of thymol blue (a pH indicator). To one of them, add enough diluted NaOH so the fluid turns blue (alkaline environment), and to the second add diluted HCl so that a clear red colour appears (acidic environment). Avoid excess of NaOH and HCl. To both test tubes, add as much protein solution (blood serum) dropwise as to neutralize both the acid and the base, which can be determined by the change of the colour of the thymol blue (pH indicator) to the original.

3. Precipitation of anionic protein with salts of heavy metals

a. test with Fe^{3+} ion

To 3 ml of egg white solution, add drops of diluted $FeCl_3$.solution Precipitate forms, which dissolves after further addition of the reagent.

b. test with Pb^{2+} ion

To 3 ml of egg white solution, add drops of solution of lead acetate (II). Precipitate forms.

4. Precipitation of the cationic protein with alkaloid reagents

a. test with hexacyanoferrate

To 2 ml of protein solution, acidified with a few drops of concentrated acetic acid, add a few drops of potassium hexacyanoferrate (III) solution. A white precipitate forms.

b. Essbach's test

To 2 ml of protein solution, add an equal volume of Essbach's reagent consisting of picric acid and citric acid. A yellow precipitate forms.

c. test with sulphosalicylic acid

To 2 ml protein solution, acidified with a few drops of concentrated acetic acid, add a few drops of 10% sulphosalicylic acid. A white precipitate forms.

5. Dialysis

Into a test tube, add 5 ml of blood serum, 5 ml of 0.9% NaCl and 5 ml of 10% glucose solution. Mix the content of the test tube and pour into a dialysis bag. The bag should be tied and attached to a glass rod. Arrange the glass

rod horizontally on the edge of a 200 ml beaker and add distilled water so that its level equals the level of fluid in the dialysis bag. Mix the contents of the beaker every 10-15 minutes by gently shaking or moving the bag. After 2 hours, perform tests for the presence of chloride, glucose and protein in the dialyzed solution (taken from inside of the dialysis bag) and in the dialysis fluid.

a. test for the presence of chloride

Prepare two test tubes. Transfer 1 ml of dialyzed solution to the first test tube, and 1 ml of dialysis fluid to the second. Add a few drops of 0.1 M $AgNO_3$ to both test tubes and mix. Chloride ions react with silver ions to form insoluble AgCl.

b. test for the presence of glucose

Prepare two test tubes. Transfer 0.5 ml of dialyzed solution to the first one, and 0.5 ml of dialysis fluid to the second. To each test tube, add 2 ml of Benedict's reagent (blue), containing Cu^{2+} ions, and mix. Heat both test tubes for 5 minutes in a boiling water bath, and then cool. Glucose reduces Cu^{2+} to Cu^{+}. Observe the formation of orange precipitate of copper oxide (Cu_2O).

c. test for the presence of protein - biuret test

Prepare two test tubes. Transfer 1 ml of dialyzed solution to the first test tube, and 1 ml of dialysis fluid to the second one. To both test tubes, add 0.5 ml of 2M NaOH, and next 0.5 ml of copper sulphate (II) solution. Compare the colour.

6. Quantitative determination of protein using the biuret method

To test tubes 1 trough 4, add successively 1 ml of standard protein solutions with concentrations of: 10, 20, 40 and 60 mg/ml. To the 5^{th} test tube, add 1 ml of the tested sample, and to the 6^{th} test tube 1 ml of H_2O (control test). Next, add 4 ml of biuret reagent to each of these test tubes and mix thoroughly. Protein-containing solutions turn purple, its intensity increasing over time reaching a maximum after 20 minutes. After that time, read off the absorbance in the individual tests (test tubes 1-5) at a wavelength of 550 nm compared to the control test (test tube 6).

Prepare a calibration graph, taking into account the dependence of absorbance on the concentration of protein (on the Y-axis indicate the

absorbance, on the X-axis - the concentration of the protein) and then read off the concentration of protein in the tested sample.

A S S I G N M E N T

Determine the protein concentration using the biuret method. Present the measurement results in mg/ml.

Blood proteins

Aim of the exercise: ***to learn about some of the properties of blood proteins***

Blood is the only fluid tissue in constant motion. It primarily fulfils transport functions in the body. Almost all disease conditions are reflected in its composition. In the course of various pathological processes, components, which do not occur in the blood of the healthy, appear in the circulating blood. The content of many of the blood components increases or decreases in the course of a disease. The ease of blood collection (without harm to the patient) allows for its extensive use as a diagnostic material. If blood is collected into a vessel containing a substance inhibiting coagulation (*anticoagulant*) such as heparin or a substance binding Ca^{2+} ions, such as sodium versenate (*EDTA*), oxalate or citrate, it does not coagulate. The blood cells settling on the bottom of the tube can be separated by centrifugation from the liquid **blood plasma**. Plasma contains all the extracellular blood protein components, including fibrinogen and other protein coagulation factors. If blood is collected into a container without anticoagulant, it coagulates within a few minutes. The clot shrinks (*retracts*), releasing a fluid called serum. A shrunk clot containing mainly fibrin (converted fibrinogen) and cells "trapped" in its interior can be removed by centrifugation. **Blood serum** gathers above the precipitate.

The blood serum does not contain fibrinogen and several other factors of protein used during coagulation. Besides that, serum and plasma composition is very similar.

Blood is a suspension of cells (red blood cells, white blood cells and platelets) in liquid plasma. Blood plasma is composed of 90% water and 10% solids. Blood solids can be divided into:

1. **Organic substances:**

   nitrogenous:

   - **proteins:** albumin, globulin, fibrinogen, enzymes, protein hormones
   - **non-protein components**: urea, uric acid, creatinine, bilirubin, free amino acids

   non-nitrogenous: glucose, lipids

2. Inorganic substances - cations and anions: Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, HCO_3^-, $H_2PO_4^-$, HPO_4^{2-}

The main protein of red blood cells is **haemoglobin**. Its biological role is to bind oxygen during blood flow through the capillaries of the lungs and O_2 transfer to extra-pulmonary tissues. Haemoglobin is a haemoprotein made up of four pairs of identical α and β subunits. The composition of the haemoglobin molecule can be noted using the symbol: $\alpha_2\beta_2$. Each of the subunits contains a haem molecule with the iron ion Fe^{2+}, able to bind a single molecule of oxygen. Binding oxygen (haemoglobin oxygenation) does not change the level of iron oxidation.

Haemoglobin absorbs some of the components of the visible light spectrum. Oxygenated haemoglobin (oxyhaemoglobin) and de-oxygenated haemoglobin (deoxyhaemoglobin) differ in the absorption spectrum of visible light. Oxyhaemoglobin solution when viewed through a spectroscope shows two absorption bands in the yellow and green parts of the spectrum (578 nm and 540 nm, respectively). pH-lowering substances, such as sodium hydrosulphate (IV) ($NaHSO_3$), cause the transition of oxyhaemoglobin into deoxyhaemoglobin, resulting in a change in the absorption spectrum of visible light. Deoxyhaemoglobin solution turns red-purple and shows a single broad absorption band at 565 nm on the border of the yellow and green parts of the spectrum.

Blood plasma proteins

The concentration of proteins in blood plasma is 6-8% (w/v). These proteins differ in both structure and function. Depending on the method of allocation used, different protein fractions have been received and described. The simplest method for the separation of proteins is their fractionation by adding various salts (e.g. ammonium sulphate, sodium chloride) in increasing concentrations (salting out). This allows the division of plasma proteins into several major fractions, in the simplest case into **albumins, globulins** and **fibrinogen**.

Albumins represent more than half of all blood plasma proteins. They readily solubilise in water, maintain proper osmotic pressure, and fulfil transportation functions. **Globulins** poorly solubilise in water, and dissolve well in salt solutions. They fulfil the role of enzymatic, transportation and immunological proteins.

Fibrinogen is a plasma protein enabling blood coagulation. The essence of this process is the transformation of soluble fibrinogen into insoluble fibrin. This protects the body from excessive blood loss in the event of interruption of the continuity of the vascular wall. One of the factors determining coagulation is calcium ions (Ca^{2+}). Citrate, oxalate or versenate anions bind Ca^{2+} ions forming very poorly dissociating or insoluble salts. Because of this, soluble and well-dissociating citrates, oxalates, or versenates (sodium, potassium, ammonium) are used as substances that prevent blood coagulation. They allow to keep the extravasated blood (or plasma) in the liquid state. The addition of excess calcium ions (such as a well-dissociable $CaCl_2$) binds all the aforementioned anions, and the remaining Ca^{2+} ions, which did not react with oxalate or citrate, restore the blood's (or plasma's) ability to coagulate. The addition of active *thrombin* (an enzyme catalyzing the conversion of fibrinogen to fibrin monomer) causes immediate coagulation of blood (or plasma) regardless of the presence of Ca^{2+}. *Thrombin* acts directly on fibrinogen, which is a specific substrate for this enzyme.

In order to determine the **concentration of fibrinogen** "oxalate" or "citrate" plasma is subjected to the action of $CaCl_2$. A clot forms. Soluble fibrinogen transits into insoluble fibrin. After washing out other proteins, the clot is dissolved in NaOH solution and the tyrosine in it can be determined - an amino acid, the content of which in fibrinogen is a known value. Based on the content of tyrosine in the clot, you can calculate the concentration of fibrinogen in plasma, which has undergone coagulation. The content of tyrosine is determined by a colorimetric technique - the Lowry method. This method is based on the reduction by tyrosine of the phosphomolybdic and phosphotungstic acids contained in the Folin-Ciocalteu's reagent. The solution becomes a blue colour, whose intensity is a measure of tyrosine content.

Blood plasma protein electrophoresis

One way of plasma protein separation is gel electrophoresis. Fractions obtained in this way form the basis of the generally accepted division of protein into **albumin, α1-globulin, α2-globulin, β-globulin, γ-globulin** and **fibrinogen**. The mutual quantitative relations between the individual fractions of healthy human plasma proteins are fairly constant. Blood plasma (or serum) protein placed in an electric field migrates to the anode or cathode depending on the pH of the environment.

In paper electrophoresis, the strip of paper saturated with the buffer is the carrier. At pH 8.6, these proteins acquire a negative charge and therefore migrate to the anode. The migration rate of individual proteins in the electric field depends on the load and the size and shape of the molecules. After the conducted separation, the strips are dried at 105°C, and individual fractions are stained with the appropriate dye, such as bromophenol blue. The amount of dye bound with the protein is roughly proportional to the amount of protein. Individual fractions can be separated by cutting out the appropriate strips from the electrophoregram. The dye can be rinsed and its concentration determined colorimetrically. This way the percentage share of each fraction in the total amount of protein can be determined.

Other methods of blood protein separation (electrophoresis on a gel carrier, immunoelectrophoresis, column chromatography, isoelectric focusing) allow the separation of blood plasma proteins into several dozen fractions.

A variety of changes can be observed in the pathological processes, not only changes of the total amount of protein in the plasma (**hypo-** and **hyperproteinemia**), but also changes in the quantitative relationship between the individual fractions (**dysproteinemia**). Electrophoresis is one of the laboratory techniques allowing the identification of dysproteinemia.

E X E R C I S E

1. Spectroscopic study of light absorption of the visible spectrum for oxyhaemoglobin and deoxyhaemoglobin

a. oxyhaemoglobin study

Into a test tube, add about 5 ml of haemoglobin and shake intensely for 2-3 minutes. Contact with air causes the haemoglobin saturation with oxygen and become oxyhaemoglobin, which is characterized by the red colour. Observe through a spectroscope the two absorption bands in the yellow and green parts of the spectrum (540 nm and 578 nm).

b. deoxyhaemoglobin study

To 5 ml of oxyhaemoglobin (obtained in the previous experiment), add a few crystals of sodium hydrosulphate (IV) and mix vigorously.

Oxyhaemoglobin loses oxygen and transfers into deoxyhaemoglobin. The light-red solution turns red-purple. Observe the broad absorption band at 565 nm at the border of the yellow and green parts of the spectrum.

2. Determination of plasma fibrinogen

To 0.5 ml of plasma, add 5 ml of 0.9% NaCl and 4.5 ml of 0.025M $CaCl_2$. Mix very thoroughly. Insert a glass rod into the test tube and leave at room temperature for at least 60 minutes. The plasma clots, and the formed clot shrinks around the rod. After this time, move the clot to the filter and rinse three times with a small amount of 0.9% NaCl and three times with a little distilled water to remove soluble plasma proteins. Then, transfer the clot (preferably with a needle tip) to a volumetric test tube, add 2 ml of 5% NaOH and heat in a boiling water bath (for about 10 minutes) until the complete dissolution of the clot. Next, fill the test tube with distilled water up to 10 ml and mix well. Transfer 1 ml of liquid to another test tube containing 3 ml of 10% Na_2CO_3, and then add 0.5 ml of Folin's reagent and mix well. After 20 minutes, measure the absorbance at 670 nm compared to the control sample containing 1 ml of 1% NaOH, 3 ml of 10% Na_2CO_3 and 0.5 ml of Folin's reagent. The solution's colour intensity is a measure of the tyrosine content in the sample.

In order to determine the content of the tyrosine, a calibration curve should be drawn. To 1 ml solution containing different amounts of tyrosine: 10, 20, 40, 50, 100, and 160 µg, add 3 ml of sodium carbonate and 0.5 ml of Folin's reagent. After 20 minutes, measure the absorbance of each sample and draw the calibration curve. Indicate the absorbance on the Y-axis, on the X-axis - the amount of tyrosine.

The tyrosine content in the sample should be read from the calibration curve. Fibrinogen concentration in plasma is calculated from the following equation:

$$\mathbf{X} = \frac{T \times 10{,}8 \times 10}{0{,}9} = \mathbf{T} \times \mathbf{120}$$

X - fibrinogen concentration in mg per ml of plasma
T - the amount of tyrosine released in mg
10.8 - factor allowing to convert the quantity of fibrinogen into the amount of tyrosine
10 - dilution of the sample
0.9 - initial volume of plasma (1 ml sample contains 0.9 ml plasma + 0.1 ml of oxalate or citrate)

3. Paper electrophoresis of blood serum proteins

Please refer to the instructions for electrophoresis apparatus. Cut two strips from a sheet of *Whatman 1* filter paper (dimensions given in the instructions) and make a pencil mark at the place of serum application. Place these strips in the apparatus chamber and leave for the period necessary for the absorption with barbital buffer (approximately 30 minutes). Then, apply 5-10 ml of serum with a capillary tube onto each strip. It should be kept in mind that the applied protein should form the thinnest line possible, at least 0.5 cm away from the edges of the strip. Apply voltage according to the instructions. The separation takes a few hours.

Dry the electrophoregrams at 105°C and stain with bromophenol blue solution in ethanol. To do this, roll them into a tube, place in a beaker, pour the bromophenol blue solution and leave for 30 minutes. The liquid should be stirred several times by shaking the beaker. Pour the dye back into the bottle (it is suitable for reuse). Wash out the dye not bound with protein by adding 0.5% acetic acid. Complete removal of the unbound dye is obtained by shaking the electrophoregrams in a beaker for 1 hour and changing the acetic acid several times. After destaining, rinse the strips several times with distilled water and dry on a glass at room temperature. Separated proteins turn to a blue-green colour.

The dye bound to the protein can be rinsed (eluted) and determined colorimetrically. This experiment should be performed on two strips. To do this, prepare 12 test tubes marked with the numbers 1 through 12. On each of the strips, indicate with a pencil the boundaries of individual fractions. Select the appropriate sized part of uncoloured filter paper (6, 12 - control tests). Cut the marked fractions out of the strips and put them into the prepared test tubes according to Table I. For each test tube, measure 5 ml of 0.1 M NaOH and elute the dye from the scraps for 45 minutes, shaking the test tubes every few minutes. Determine the absorbance of the tested samples in relation to the control test at a wavelength of 560 nm.

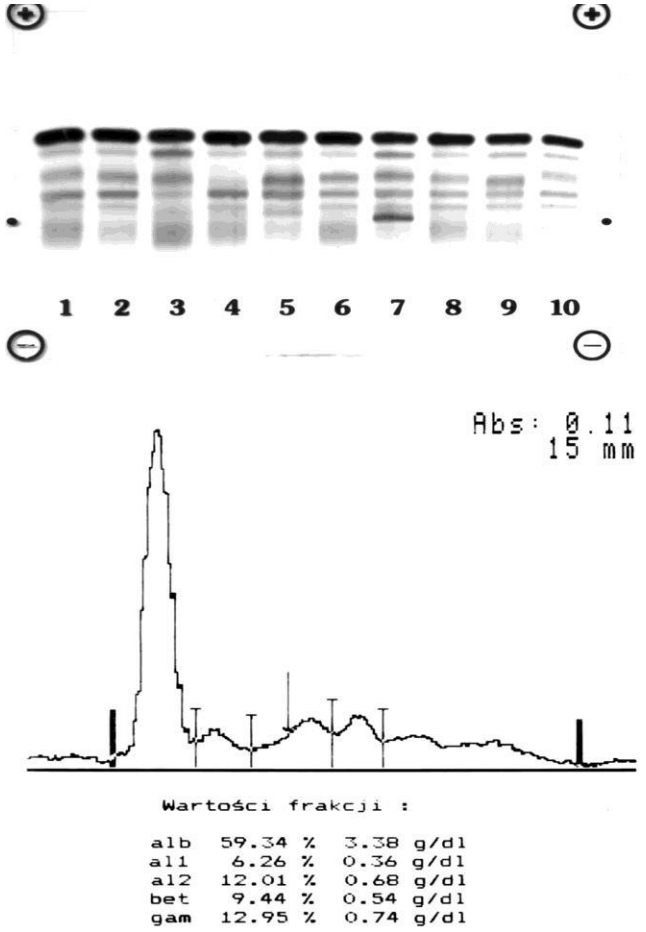
Calculations are based on the assumption that the total absorbance of all protein fractions corresponds to 100% of protein applied onto the strip. Absorbances of individual fractions, expressed as a percentage of this sum, give the percentage composition of the protein serum fractions - a proteinogram. Present the calculated values in Table I.

Table I

	Strip I			Strip II		
	Test No.	A_{560}	% fraction	Test no.	A_{560}	% fraction
albumin	1			7		
α1-globulin	2			8		
α2-globulin	3			9		
β-globulin	4			10		
γ-globulin	5			11		
control	6			12		

4. The analysis of electrophoretic separations of serum proteins on cellulose acetate and a densitometric evaluation of selected electrophoregrams

Look at the completed electrophoregrams. Notice the quantitative variation of the serum protein fractions. The attached figure presents a sample electrophoregram.



The figure shows electrophoregrams of serum proteins with the densitometric evaluation enabling a quantitative measurement of individual fractions.

ASSIGNMENT

Determine the concentration of fibrinogen.

Nucleic acids

Aim of the exercise: ***to study the composition and some properties of nucleic acids***

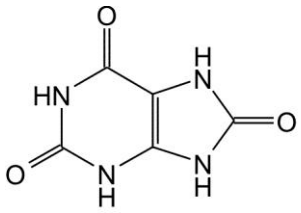
Nucleic acids are made up of **nucleotides**. A component of each nucleotide is the purine base (adenine or guanine) or pyrimidine base (cytosine, uracil or thymine), pentacarbon sugar: ribose or deoxyribose and orthophosphoric acid residue. The base binds the sugar by the β-N-glycosidic bond, orthophosphoric acid residue binds the sugar component by an ester bond through the -OH group at carbon 3' or 5' of ribose or deoxyribose. Individual nucleotides are bound with phosphodiester bonds between carbons 3' and 5'.

The acidic character of the nucleic acids is caused by orthophosphate residues, each of which contains H^+ capable of dissociation. Due to this, nucleic acids are **polyanions** - carriers of many negative charges, and this makes them capable of interacting with **polycations**, particularly with alkaline proteins - which are carriers of positive charges. They also bind with micromolecular compounds of an alkaline nature, such as methylene blue. In the natural environment, DNA mainly binds with alkaline proteins - **histones**, whereas RNA mainly binds with neutral proteins, which are part of the ribosome. Complexes of nucleic acids with proteins are called nucleoproteins. In the student laboratory conditions, artificial nucleoproteins can be obtained by mixing a solution of nucleic acid with blood serum. Nucleoproteins dissociate into its constituents in concentrated salt solutions. In an alkaline environment, nucleic acids form salts - **nucleates**, which are soluble in water. They can be precipitated from solution with ethanol.

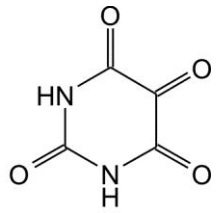
Sugar constituents of nucleic acids: ribose and deoxyribose can be detected directly in the solutions of these acids or their salts without prior hydrolysis. **Ribose** contained in the RNA, purine nucleosides and nucleotides, heated with concentrated HCl dehydrates to furfural, which with orcin in the presence of Fe^{3+} ions forms a complex of a stable green colour. **Deoxyribose**, contained in the DNA, when heated with concentrated sulphuric acid is converted into hydroxylevulinyl aldehyde. This compound forms a blue colour complex in reaction with diphenylamine. Purine bases can only be detected in the hydrolysis products of nucleic acids. The sample of

nucleic acid, intended for the detection of purines, should be hydrolysed in sulphuric acid at 100°C. Nucleic acids are hydrolysed, initially to mononucleotides. Purine mononucleotides are further hydrolysed to bases, pentoses and orthophosphoric acid. The action of this acid leads to the hydrolytic breakdown of the β-N-glycosidic bonds between the purine and the ribose or deoxyribose. The following purine bases are released: adenine and guanine. **Purine bases** precipitate easily as insoluble complexes with the ions of copper or silver. In the same conditions, pyrimidine nucleotides are stable and do not decompose.

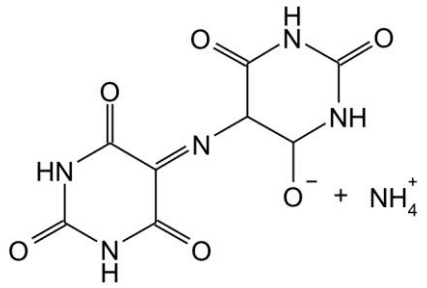
The final product of purine bases' transformations in the human organism is poorly soluble **uric acid**. Sodium salts of this acid (sodium urate) are quite soluble, while ammonium, silver and copper urates are sparingly soluble in water. The reducing uric acid properties make it an important biological antioxidant. It inactivates reactive forms of oxygen and prevents them from forming.



Uric acid



Alloxan



Murexide

Uric acid is oxidized by the action of nitric acid (V) to alloxan and urea. Condensation of two molecules of alloxan with ammonia induces the formation of murexidic acid. Its salts are called murexidates. In reaction with

the NH_4^+ ion a purple-coloured murexide is formed, and in reaction with the Na^+ ion, a blue-coloured sodium murexidate is formed.

EXERCISE

1. Preparation of nucleic acids from yeast

Suspend about 25 g of baker's yeast in 30 ml of 10% NaCl. Grind with the submerged end of a test tube, add 30 drops of alcohol solution of phenol red, and then neutralize the suspension, first with 10% NaOH, then 2% NaOH to a red-orange colour. Heat the suspension in a boiling water bath for 30 minutes, stirring periodically. Nucleic acids transfer to the solution. After cooling, centrifuge or filter the mixture. Nucleic acids remain in the filtrate (in the case of filtration) or in the supernatant (in the case of centrifugation). Insoluble components (precipitate) should be disposed. To the thus obtained solution of nucleic acids, add three times the volume of ethanol and allow to stand for 15 minutes. Nucleic acids then precipitate from the solution in the form of sediment, which must be removed by filtration or centrifugation and then dissolved in 15 ml of 0.1 M NaOH.
The resulting preparation of nucleic acids - sodium nucleate - use for further experiments.

2. Solubility of nucleic acids

a.

To 1 ml of sodium nucleate, add drops of 2 M HCl. Nucleic acid sediment precipitates - the liquid looks opalescent. Add drops of 2M NaOH to the same test tube. The precipitate dissolves.

b.

To 1 ml of sodium nucleate, add 2 ml of ethanol. Precipitate forms.

3. Precipitation of nucleic acids with protein solutions

To one test tube, add 1 ml of sodium nucleate, and to another add 1 ml of water. To both test tubes, add 2-3 drops of diluted (1:20) acetic acid and 1 ml

of diluted (1:10) serum. Observe the precipitation in the test tube containing the nucleic acid. Next, add 2 ml of saturated NaCl solution to both test tubes. Observe the dissolution of the precipitate in the test tube with the artificial nucleoprotein (resulting from the addition of serum), while in the test tube containing a serum-protein only, sediment starts to precipitate.

4. Precipitation of nucleic acids with methylene blue

To 1 ml of sodium nucleate add 2-3 drops of diluted (1:20) acetic acid, and then add a few drops of 1% methylene blue. The colour salt of the alkaline dye and nucleic acid precipitate.

5. Detection of sugar components of the nucleic acids

a. general reaction to sugars - test with α-naphthol

To 1 ml of sodium nucleate, add one drop of 10% α-naphthol alcohol solution, and then carefully add 0.5 ml of concentrated H_2SO_4 pouring down the test tube's sidewall in order to "sublayer" the fluid in it. At the border of structural constituents, a red-violet-coloured layer is formed, spreading to the entire solution after gentle mixing.

b. detection of ribose - test with orcin

To 1 ml of sodium nucleate, add 1 ml of orcin reagent (orcin with $FeCl_3$ in concentrated HCl), mix and place into a boiling water bath for 20 minutes. A green colour emerges.

c. detection of deoxyribose - test with diphenylamine

To 1 ml of sodium nucleate, add 2 ml of a diphenylamine-containing reagent (in a mixture of acetic and concentrated sulphuric acids). Mix and put in a boiling water bath for 10 minutes. A blue colour emerges.

6. Detection of phosphate

Into a test tube, add 3 drops of sodium nucleate, 2 ml of distilled water, 1.5 ml of 10% TCA, 0.5 ml molybdenum reagent, and 0.5 ml of reagent with eiconogen (in this order), and mix thoroughly. Observe the emergence of a blue colour characteristic of the presence of phosphate anions.

7. Detection of purine bases in nucleic acid hydrolysates

a. hydrolysis of nucleic acids

Collect 4 ml of sodium nucleate into a test tube, add 1 ml of 2.5 M H_2SO_4 solution and mix. Place in a boiling water bath for 1 hour. Cool the hydrolysate and carefully neutralize (checking on the litmus paper) by adding drops of 2M NaOH solution to the point of a weak acid reaction.

b. detection of purines - through precipitation with Cu^+ ions

Collect into a test tube 1 ml of hydrolysate and bring to a boil. Add a few drops of $CuSO_4$ (blue) solution, and then add drops of saturated solution of $NaHSO_3$ (reducing the Cu^{2+} ion to a Cu^+ ion). A yellow precipitate of an insoluble purine-copper complex forms. Repeat the exercise using water instead of nucleic acid hydrolysate. Observe the differences.

c. detection of purines - through precipitation with Ag^+ ions

Collect into a test tube 1 ml of hydrolysate and add a few drops of $AgNO_3$ ammonia solution and 1 ml of NH_4OH solution. A precipitate, which is a purine complex with Ag^+ ions, forms. Repeat the exercise using water instead of nucleic acid hydrolysate. Precipitate will not form.

8. Some properties of uric acid

a. detection of uric acid - murexide test

Add 1 ml of disodium urate and a few drops of nitric acid (V) into a small evaporator. Evaporate until dry. A red precipitate of murexydic acid forms. On one side of the precipitate, apply a drop of NH_4OH solution- a purple-coloured ammonium murexide forms. On the other side of the precipitate, apply a drop of NaOH solution - a blue-coloured sodium murexidate forms.

b. solubility of uric acid salts

To four test tubes, add 1 ml of disodium urate each. Then, to the first test tube add 1 ml 2M HCl solution, to the second – 1 ml NH_4Cl, to the third test tube – 1 ml $CuSO_4$ and to the last 1 ml $AgNO_3$. Soluble disodium urate transfers into insoluble or poorly soluble products, which form a precipitate (uric acid and its ammonium, copper and silver salts, respectively).

c. demonstration of the reducing properties of uric acid

To 1 ml of disodium urate, add 0.5 ml of Folin's reagent containing sodium tungstate in phosphoric acid. Alkalize the sample by adding 0.5 ml of 2M NaOH. In the presence of uric acid, blue-coloured tungsten oxides form ($WO_2 \times 3WO_3$).

ASSIGNMENT

Examine whether the solution contains ribose, deoxyribose or uric acid.

Carbohydrates

Aim of the exercise: ***to learn about some of the properties of simple and complex sugars***

Carbohydrates (sugars, saccharides) are compounds of aldehyde or ketone derivatives of higher polyhydric alcohol. **Monosaccharides**, which are simple sugars, can be classified according to various criteria, such as the number of carbon atoms in the molecule, the nature of the reactive groups or the construction of the ring. In the human body, sugars containing from three to seven carbon atoms primarily occur. Hexoses, and among them **glucose** which is the main monosaccharide consumed and processed in the human body, are most abundant. The joining of two hexoses by the glycosidic bond causes the formation of disaccharide. Longer chains composed of 3-10 monosaccharide units are called oligosaccharides. **Polysaccharides** usually contain hundreds or thousands of monosaccharide units. They are divided into **homopolysaccharides** (*homoglycans*), composed of equal units of sugar (starch, glycogen, cellulose) and **heteropolysaccharides** (*heteroglycans*), composed of various sugar and non-sugar units *(glycosaminoglycans*).

Simple sugars, mainly hexoses, are subject to various modifications in the tissues, resulting in such derivatives as: glycosides, aminohexoses, uronic acids and sialic acids.

The presence of **aldehyde** or **ketone** groups and **hydroxyl** groups causes that the sugars have typical reactions for aldehydes/ketones and alcohols.

The **oxidoreductive properties** of sugars deserve special attention. They can easily oxidize to appropriate aldonic acids at the expense of the reduction of the oxidizing agent. The aldehyde group oxidizes to the carboxyl group. Sugar is converted into the appropriate aldonic acid, e.g. glucose into gluconic acid, galactose into galactonic acid. The oxidation of the $-CH_2OH$ group at the opposite end of the molecule leads to the transformation of sugar in the appropriate uronic acid. Glucose is converted into glucuronic acid, and galactose into galacturonic acid.

If the oxygen of the aldehyde group of sugar is not bound with any other structure, it shows **reducing** properties. It, for example, can reduce metal cations such as Cu^{2+} into Cu^{+}, Ag^{+} into Ag^{0}. Reducing tests, although not specific for carbohydrates, are of great practical importance. They allow to conclude whether the aldehyde group responsible for this reaction is free or bound. They were and continue to be (although to a lesser extent) used both to detect sugars and to measure their concentrations in biological fluids. Currently reducing tests are replaced by more specific methods, including enzymatic, which allow identify individual sugars and a more accurate measurement of their contents.

In the Fehling's and Benedict's tests, copper hydroxide - $Cu(OH)_2$, containing blue-colour Cu (II) is reduced to orange copper oxide - Cu_2O containing Cu (I). Emerging compounds of Cu (I) copper easily precipitate as insoluble sediments in the reaction environment. Their re-solubilisation is possible by adding compounds containing the hydroxyl groups, such as: sodium potassium tartrate (in Fehling's reagent), or sodium citrate (in Benedict's reagent).

Most disaccharides (such as maltose, isomaltose, lactose) retain their reducing properties. **Sucrose** is a non-reducing sugar because the reducing groups of both constituent sugars are involved in the formation of the glycosidic bond. A similar observation applies to all polysaccharides (with the exception of their reducing end). Polysaccharide hydrolysis releases the reducing groups, and therefore hydrolysis products display such properties. Although ketones, in contrast to aldehydes, show no reductive ability, in an alkaline environment the ketoses undergo isomerisation to aldoses - e.g. fructose (*ketose*) isomerise to glucose (*aldose*) - therefore the ketoses are also reducing sugars.

The action of concentrated acids causes the **dehydration** of sugars. Pentoses transfer into furfural, and hexoses into hydroxymethylfurfural. These compounds form colourful connections with α-naphthol or thymol.

In the presence of concentrated sulphuric acid or hydrochloric acid, hexose resorcinols create a product of a salmon or red colour. In the same conditions, pentoses with orcin form a green-coloured product. Other sugars give a yellow or red coloration. Based on these tests, hexoses can be distinguished from pentoses.

The presence of **fructose** can be detected by using Seliwanow's reagent (resorcinol solution in hydrochloric acid). This test is specific to fructose only if it is positive during the heating period lasting no longer than 30 seconds. A salmon or red colour occurs. With a longer heating time, glucose has a similar reaction.

Starch is a plant polysaccharide. It is one of the main ingredients of human food. It consists of two fractions: **amylose** and **amylopectin**. Amylose is a linear unbranched polymer of glucose residues combined with α-1,4-glycosidic bonds. Amylopectin contains additional branches, where there are α-1,6 bonds.

Glycogen - animal polysaccharide - is built similarly to amylopectin, but has a higher degree of branching.

Both polysaccharides form coloured products in reaction with iodine. In this reaction, amylose, gives a blue-coloured product, amylopectin - purple-coloured, and glycogen - a brownish-red colour. The colour blue is characteristic of long spiral-twisted chains without side branches. As they reduce in length, the colour red intensifies. The degradation products of starch (dextrins) with long chains (amylodextrins) turn a bluish-violet colour. Products of medium chain length (erytrodextrins) dye red, and short chains (achromo-dextrins) take on the colour of iodine.

Starch molecules contained in the starch gruel are surrounded by a water jacket. The addition of water-binding substances (e.g. ammonium sulphate) causes the precipitation of starch from the solution.

Baker's yeast readily **ferments** glucose, fructose, maltose and sucrose, however it does not ferment lactose. The end products of this process are ethanol and carbon dioxide. The latter, as a gas product is released from the reacting system and is collected in the fermentation tube.

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$$

E X E R C I S E

1. Overall reaction to sugars - test with α-naphthol

To 1 ml of glucose solution, add 2-3 drops of 10% alcohol solution of α-naphthol, and then carefully add 0.5 ml of concentrated sulphuric acid (VI) pouring down the test tube's sidewall in order to sublayer the liquid in it. At the border of structural constituents, a red-violet-coloured layer is formed, spreading to the entire solution after careful shaking.

Repeat the test using a solution of sucrose, starch gruel, and a suspension of starch. Compare the results of the experiments.

2. Reducing tests

a. Fehling's test

Mix equal volumes (1 ml) of Fehling's reagents I and II. Then add 0.5 ml of glucose solution. Heat 5 minutes in a boiling water bath. An insoluble orange oxide copper (I) forms, which is maintained in suspension by creating a complex with tartrate present in Fehling's reagent (II). Repeat the test with the sucrose solution.

b. Benedict's test

To 2 ml of Benedict reagent, add 10 drops of glucose solution. Heat for about 5 minutes in a boiling water bath, and then cool. An orange insoluble copper oxide (I) forms, which is maintained in suspension by creating a complex with citrate, present in the Benedict's reagent. Repeat the test with the sucrose solution.

3. Fermentation of glucose

Grind a piece of baker's yeast the size of a large bean with 20 ml of 10% glucose solution. A suspension forms. Pour the liquid into the fermentation tube and leave at room temperature for two hours. Observe the process of fermentation. A gas appears (CO_2) under the closed dome of the tube and its volume increases through the course of the reaction.

4. Test for fructose

To 2 ml of Seliwanow's reagent, add a few drops of sucrose solution and heat the liquid in a boiling water bath for 30 seconds. A salmon or red colour forms.

5. Test for pentoses

To 1 ml of orcin reagent, add 3-4 drops of pentose solution and heat in a boiling water bath for 15 minutes. A characteristic green colour forms.

6. Properties of starch

a. preparation of the starch gruel

Mix approximately 0.3 g (1/3 of a flat teaspoon) of starch with 5 ml of cold distilled water, and then transfer the suspension to 25 ml of boiling water stirring constantly. Cook the resulting starch gruel for 30 seconds, and then cool.

b. test with iodine

To 1 ml of starch gruel, add one drop of iodine solution in potassium iodide (Lugol's solution). A blue colour forms, disappearing after a short heating and appears again after cooling.

c. reducing tests

Perform the Fehling's and Benedict's tests with the starch gruel in the manner described in sections 2a and 2b.

d. hydrolysis of starch

Take 2 ml of starch gruel, add 2 ml of 2M HCl, and heat in a boiling water bath for several minutes. Perform tests with Fehling's and Benedict's reagents and compare the results of the tests performed with the starch gruel - unhydrolysed.

ASSIGNMENT

1. Does the solution contain carbohydrates, and if so, are they reducing sugars?
2. Does the solution contain: glucose, fructose, pentose or starch?

Phospholipids, steroids and fat-soluble vitamins

Aim of the exercise: ***to isolate phospholipids and study of their composition; to detect certain steroids and vitamins***

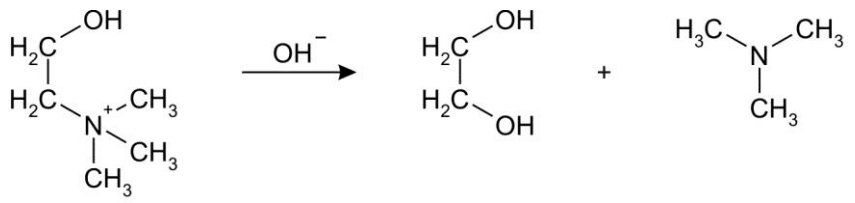
Phospholipids

Phospholipids occur in all biological membranes. They are amphipathic substances. Each phospholipid molecule contains a polar head and a non-polar tail. The molecular skeleton is made up of the residue of **glycerol** or a more complex alcohol - **sphingosine**. A recurring structural element of all phospholipids containing glycerol - called glycerophospholipids - is **phosphatidic acid**.

One of the glycerophospholipids is **phosphatidylcholine** (lecithin), whose molecule, beside glycerol, contains two chains of fatty acids, phosphate and choline. Lecithin is the most abundant phospholipid in eukaryotic cells, it is also found in egg yolk. It is easy to extract with a mixture of chloroform with methanol in a proportion of 2:1. Lecithin is difficult to dissolve in anhydrous acetone.

Lecithin is not soluble in water, but thanks to the presence of the hydrophilic phosphocholine group, it creates a persistent cloudy suspension in water. It is readily soluble in chloroform. During heating of lecithin, the dehydration of the glycerol molecule takes place. Unsaturated aldehyde forms - **acrolein** - a substance with an unpleasant, pungent odour. It can be shown with one of the reducing tests, for example with chromate (VI). Acrolein reduces potassium chromate (VI) (orange colour) to potassium chromate (IV) (green colour).

In a highly alkaline reaction, the hydrolysis of the ester bond takes place between choline and the phosphate residue of lecithin. The released **choline** decomposes to **ethylene glycol** and **trimethylamine**, which has a characteristic odour (figure below).



Choline Ethylene glycol Trimethylamine

Alkaline hydrolysis releases fatty acids in the form of potassium salts (soaps), which reduce the surface tension of water. Phosphate contained in lecithin is released during alkaline hydrolysis. In reaction with ammonium molybdate (VI), it creates ammonium phosphomolybdate, which colours the solution yellow.

Steroids

Steroids are derivatives of **cyclopentaneperhydrophenanthrene.** The most common representatives of steroids are alcohols known as sterols, which have a hydroxyl group next to the C-3 carbon, among which **cholesterol** is dominant. It is a substrate from which the majority of other steroid compounds are formed. These primarily include bile acids and steroid hormones. Vitamin D3 forms from 7-dehydrocholesterol - the precursor of cholesterol.

Cholesterol is a representative of animal sterols. It is synthesized by almost all organs; however, it forms most abundantly in the liver and intestinal wall. It dominates in cell membranes and blood plasma lipoproteins. Due to the presence of a double bond, cholesterol creates colourful products in the presence of strong acids. Under the influence of concentrated H_2SO_4 (Salkowski's reaction), the dissociation of water molecules takes place. A red disulphonic acid of bicholestadien forms, which in the presence of acetate anhydride (Lieberman-Burchard's test) forms a green-coloured monosulphonic acid of bicholestadien. Traces of water make this reaction impossible.

The most abundantly occurring **bile acids** are cholic and deoxycholoic acids, in smaller quantities - lithocholic and chenodeoxycholic acids. The presence of the polar carboxyl and hydroxyl groups gives the bile acids, as the only lipid group, solubility in a water environment. Bile acids are of an

amphipathic character. Their hydroxyl groups are directed to one side of the ring plane and the methyl groups to the other. Therefore, bile acid molecules have a non-polar side facing toward the lipid structural constituent and a polar side facing toward the aqueous structural constituent. Thanks to this characteristic, bile acids act as emulsifiers to the water-insoluble triacylglycerols and other lipids. They increase the degree of fat dispersion in the intestinal contents, thereby increasing the availability of enzymes for the lipid substrate.

Some bile acids form bonds with glycine or taurine - their salts are more effective emulsifiers than the free bile acids. In addition, bile acids bind with cholesterol enabling its solubility in bile and excretion from the liver through the bile.

Hydroxymethylfurfural, formed from hexose by the action of concentrated sulphuric acid (VI), reacts with the bile acids. A red ring forms on the border between the two layers.

The **surface tension** lowered by the bile acids present in the aqueous solution can be determined by the settling at the bottom of the test tube of colloidal sulphur (*sulphur flower*) and the formation of a permanent suspension (emulsion) of oil in water. Bile acids as cyclic compounds having several -OH groups act similarly to resorcin or α-naphthol. They condense with hydroxymethylfurfural, which is produced by the action of concentrated sulphuric acid on sucrose. A red-coloured condensation product appears, which indicates the presence of bile acids.

The **main forms of vitamin D** are **ergocalciferol** (vitamin D_2) and **cholecalciferol** (vitamin D_3). Provitamins of vitamin D are unsaturated sterols, which change into active vitamin D. Vitamin D_2 is created in the skin under the action of UV-light, which is a component of sunlight. Vitamin D_3 is present in cod-liver oil, and in the human body it is synthesized in the liver from 7-dehydrocholesterol.

The **A vitamins** have an **isoprene** structure. They are found exclusively in animal tissues in two forms: **retinol 1** (vitamin A_1) and **retinol 2** (vitamin A_2). Both are 20-carbon alcohols containing a six-member ring of beta-ionone with three methyl groups and a side chain containing two isoprene units. The vitamin A alcohol forms (retinols) are oxidized in the human body to the appropriate aldehydes (retinals) and these oxidize to the

appropriate retinoic acids. Plants contain a group of substances called carotenes, which are 40-carbon polyunsaturated derivatives of isoprene containing ionone rings. Carotenes: α, **β** and γ act as provitamin A in animal organisms. Due to the high number of conjugated double bonds, carotenes are coloured compounds.

The presence of conjugated double bonds, both in the structures of vitamin A and D_3, renders these substances to exhibit a characteristic colour reaction with antimony chloride ($SbCl_3$). With vitamin A_1, it forms a blue-coloured product, and with vitamin D_3 a violet-red-coloured product.

E X E R C I S E

1. Extraction of lecithin from egg yolk

Grind approximately 2-3 g of dried chicken egg yolk (flat teaspoon) in a mortar for 2-3 minutes with 10 ml of solvent consisting of chloroform and methanol in a 2:1 ratio. Filter the extract into a dry test tube through a filter paper moistened beforehand with the solvent. Evaporate the filtrate in a boiling water bath. On the walls of the test tube remains a brown oily substance, which after cooling becomes the consistency of Vaseline. It is lecithin, contaminated with other yolk constituents.

2. The solubility of lecithin

Transfer with a glass rod a pinch of lecithin into two test tubes. Add 1-2 ml of H_2O to one of them and heat for a few seconds in a boiling water bath. Observe a turbid suspension. Add 1 ml of chloroform to the second test-tube and similarly heat in a water bath. The lecithin dissolves in the chloroform. Add 2 ml of acetone - lecithin precipitate forms.

3. The chemical composition of lecithin

a. detection of glycerol - acrolein test

Transfer 2-3 drops of lecithin into a dry test tube, add 1.5 ml potassium chromate (VI) solution ($K_2Cr_2O_7$) and 3-4 drops of concentrated sulphuric

acid. Heat the prepared test tube in a boiling water bath for a few minutes. Observe the colour change of the solution.

b. detection of fatty acids – saponification reaction

Add 3 ml of 10% KOH alcohol solution to a pinch of lecithin in a test tube, and heat the mixture for several minutes in a boiling water bath. Then add 5 ml of distilled water and shake the test tube. The solution foams.

c. detection of choline

Add 2 ml of 20% NaOH to a test tube with a pinch of lecithin and heat in a boiling water bath for five minutes - observe the characteristic odour of the reaction product.

d. detection of phosphorus

Add 0.5 ml of 20% NaOH to a test tube with a pinch of lecithin and heat for 2 minutes in a boiling water bath. Then add 2 ml of ammonium molybdate (VI). Observe the colour of the solution.

4. Detection of cholesterol

a. Salkowski's reaction

Add 0.5 ml of chloroform solution of cholesterol into a dry test tube, and then carefully pouring on the test tube's sidewall add (sublayer) 0.5 ml of concentrated H_2SO_4. The chloroform-containing layer becomes red, and the sulphuric acid layer fluoresces green.

b. The Lieberman-Burchard's reaction

Into a dry test tube, add 1 ml of chloroform solution of cholesterol, 3 drops of acetic anhydride and carefully add 2 drops of concentrated H_2SO_4. The liquid turns red, and then changes colour to blue and finally green.

5. Detection of bile acids

In 1 ml of bile solution, dissolve a few crystals of sucrose. Then sublayer with the 1 ml of concentrated H_2SO_4 (by adding sulphuric acid pouring on the test tube's sidewall). Observe the formation of a red ring on the border of the layers.

6. Lowering the surface tension by bile acids

Add 3 ml of water to 2 test tubes. To one of them, add 2–3 drops of bile, and then to both test tubes pour a small amount of colloidal sulphur. Compare the sedimentation rate of sulphur.

7. Detection of fat-soluble vitamins

Into a dry test tube, add 1 ml of a saturated chloroform solution of antimony chloride ($SbCl_3$) and 2-3 drops of a pharmacological preparation of vitamins A and D_3. After mixing, a blue colour starts to form proving the presence of vitamin A, which rapidly changes into purplish-red associated with the presence of vitamin D_3.

ASSIGNMENT

Check whether the solution contains cholesterol or bile acids.

Enzymes

Aim of the exercise: ***to learn about some of the properties of enzymes***

Almost all biochemical reactions require the participation of biological catalysts (biocatalysts), called **enzymes**. The substance converted by an enzyme is called a **substrate**, and a substance resulting from the conversion of the substrate is called the **product**. The enzyme is not consumed during the reaction catalyzed by it, so one enzyme molecule is involved in the conversion of many substrate molecules.

Enzyme influence on reaction velocity

Enzymes accelerate the course of chemical reactions in the organism at least a million times. In the absence of enzymes, most chemical reactions occur so slowly that they are virtually unnoticeable. This can be demonstrated in an experiment with ***thrombin***. It is a proteolytic enzyme, which converts soluble fibrinogen of blood plasma into fibrin monomer, which spontaneously polymerises to form insoluble fibrin. This reaction is of fundamental importance in the process of blood coagulation. In a test tube without *thrombin* fibrinogen does not polymerise during the observation period, and in a test tube containing *thrombin* it coagulates after a few seconds.

Thermal inactivation of enzymes

Enzymes lose their ability to catalyze a reaction after undergoing thermal denaturation. This can be demonstrated on the example of ***thrombin*** and the enzymes catalyzing the process of alcohol fermentation.

The thermal inactivation of *thrombin* prevents the creation of a blood clot – the change of soluble fibrinogen into insoluble fibrin.

Yeasts contain a set of enzymes, which catalyze the process of alcohol fermentation of sugars. During fermentation, ethanol and CO_2 are formed. The last one is released as a gas product from the reacting system and is collected in the fermentation tube. Thermal inactivation of yeast prevents fermentation.

Effect of pH on enzyme activity

Changing the concentration of hydrogen ions in the environment changes the electric charge of the enzymatic protein. This causes changes in the chemical and physical properties of the enzyme. For this reason, each enzyme exhibits maximum activity at a strictly specified pH range (optimum pH).

Pepsin causes hydrolytic breakdown of peptide bonds in proteins. Its action can be easily demonstrated by the use of an insoluble artificially-coloured protein substrate - fibrin. As a result of its hydrolysis, short peptides form, which dissolve together with the bonded dye causing colorization of the solution.

Starch is dyed with iodine (Lugol's solution) and does not reveal reducing properties. ***Amylase*** causes hydrolytic breakdown of glycosidic bonds in starch. The action of this enzyme causes starch to break down to maltose and short oligosaccharides. The progress of starch hydrolysis can be easily demonstrated by the use of the reaction with iodine and reduction tests. As a result of starch hydrolysis, products appear that do not react with iodine, but they demonstrate reducing properties. The last feature is an effect of the release of aldehyde groups as a result of glycoside bond disintegration.

Effect of activators on the activity of enzymes

The effect of activators on the activity of enzymes can be demonstrated on the example of **plasma thromboplastin** (a complex of blood plasma coagulation factors - V, X, and blood platelet phospholipids). Calcium ion (Ca^{2+}) is an activator of this enzyme. Plasma *thromboplastin* induces the transition of inactive *prothrombin* to active *thrombin*, which in turn transits soluble fibrinogen into insoluble fibrin. In the presence of sodium citrate, Ca^{2+} cations are bound by citrate anions forming poorly dissociating calcium citrate. Lack of Ca^{2+} ions prevents the formation of active plasma *thromboplastin* and, consequently, inhibits blood coagulation. This phenomenon is used in medicine and in laboratory diagnostics to store blood in liquid form.

Effect of inhibitors on enzyme activity

The effect of inhibitors on the enzymatic reaction can be demonstrated on the example of the action of cyanide on ***catalase***. *Catalase* is an enzyme from the *oxidoreductases* class, which catalyze hydrogen peroxide hydrolysis.

$$H_2O_2 \rightarrow H_2O + ½O_2$$

Potassium cyanide binds with enzyme irreversibly and deprives it of its catalytic activity.

The prevalence of certain enzymes

Certain enzymes are widely distributed in the plant world. These include ***peroxidase*** - the second of the enzymes, which decompose hydrogen peroxide. It decomposes H_2O_2 only in the presence of an oxygen acceptor such as pyrogallol, which oxidizes changing its colour to brown. In human blood, there are also enzymatic proteins of peroxidative properties.

The use of enzymes for analytical purposes

Enzymes are characterized by great specificity, due to this, some of them are used in the laboratory setting for detecting and quantitative determination of substances contained in mixtures.

Urease, present in the seeds of certain plants, is used for the detection and quantitative determination of urea in biological material. It catalyzes the breakdown of urea to CO_2 and NH_3. Ammonia can be detected and quantitatively determined with Nessler's reagent (an alkaline solution of potassium iodomercurate (II)). This reagent in a neutral and alkaline environment reacts with ammonium ion (NH_4^+) to form a product with a yellow-brown colour.

E X E R C I S E

1. Demonstration of enzyme influence on reaction velocity

Prepare two test tubes containing 0.5 ml of fibrinogen solution each. Add 0.2 ml of physiological saline solution to one of them, and 0.2 ml of *thrombin* to the second. Observe the differences in the speed of clot formation.

2. Thermal inactivation of enzymes

a. inactivation of alcohol fermentation enzymes

Grind a piece of baker's yeast (the size of a large bean) in 10 ml of distilled water. Divide the obtained suspension into two equal parts. Heat one of them in a boiling water bath for 5 minutes in order to inactivate the enzymes with heat. Then, add to both suspensions approximately 20 ml of 10% sucrose solution and transfer to fermentation tubes. Allow the two tubes to sit at room temperature for about 45 minutes. Compare the course of fermentation.

b. inactivation of thrombin

Add 0.2 ml of *thrombin* solution to 2 test tubes. Heat the contents of one of them in a boiling water bath for 5 minutes. Thermal denaturation of enzymatic protein takes place. To both test tubes, add1 ml of blood plasma and put in a water bath at a temperature of 37°C for 5 minutes. Observe the differences in clot formation.

3. Effect of pH on enzyme activity

Prepare two rows of test tubes (4 test tubes in each row) and mark them in each row from 1 to 4. To each of the test tubes, add successively 2 ml of the following solutions of different pH:

test tubes No. 1 - 0.1 M hydrochloric acid	- pH 1.0
test tubes No. 2 - 0.1% lactic acid	- pH 5.0
test tubes No. 3 - distilled water	- pH 7.0
test tubes No. 4 - 1% sodium carbonate	- pH 9.0

Add 2-3 drops of *pepsin* solution and 3-4 "flocks" of Congo Red dyed fibrin to the test tubes in the first row. To the each of the test tubes in the second row, add 1 ml of starch gruel and 2 ml of *amylase* solution.

Put all test tubes in a water bath at 37°C for 20 minutes. Observe which of the solutions containing fibrin and *pepsin* became coloured.

Place a piece of universal litmus paper into each test tube (of the second row) containing starch and *amylase*. Stirring constantly, add drops of diluted NaOH (test tube 1 and 2) or HCl (test tube 4) - bring the liquids to a neutral reaction using the colour of the litmus paper as a guide. Next, divide the content of each test tube into two parts (**A** and **B**).

Test solution **A** for the presence of starch by adding 2-3 drops of Lugol's solution. Perform a reduction test (Benedict's test) with the **B** solutions as described in the **Carbohydrates** exercise (page No. 36, point 2b).

Observe the relation of the tested enzyme activity to the pH of the solution. Determine the approximate optimum pH for both of the tested enzymes. Summarize the results in the Table indicating the effects of the reaction (depending on their intensity) with an appropriate number of pluses.

Test tube	**1**	**2**	**3**	**4**
pH	1.0	5.0	7.0	9.0
Pepsin				
Amylase				

4. Effect of calcium ions on plasma *thromboplastin* activity

Add to two test tubes 0.1 ml of 0.025 M $CaCl_2$. To one of them, add 0.1 ml of 0.2 M sodium citrate, and 0.1 ml of distilled water to the second. To both test tubes, add 0.5 ml of blood plasma and insert them into a water bath at 37°C for 10 minutes. Observe the differences in the formation of a blood clot.

5. Effect of cyanide on *catalase* activity

Add 5-6 drops of blood to 3 test tubes. Put one of them in a boiling water bath for several minutes, and then cool. To the second test tube, add 2-3 drops of potassium cyanide solution. Next, to all three tubes, add 1 ml of hydrogen peroxide. Compare the differences in the foaming of the solution.

6. The prevalence of peroxidative properties

a. demonstration of the presence of *peroxidase* in potato juice

To 2.5 ml of fresh potato juice, add 10 drops of pyrogallol solution and 10 drops of hydrogen peroxide solution. The appearance of brown coloration in the solution after 2-3 minutes indicates the presence of the enzyme.

b. demonstration of the peroxidative properties of blood

To 2.5 ml of blood, add 10 drops of pyrogallol solution and 10 drops of hydrogen peroxide solution. The brown coloration that appears after a few minutes indicates the peroxidative properties of blood.

7. The use of *urease* for analytical purposes

Add 0.5 ml solution of *urease* to 2 test tubes. Heat one of them in a boiling water bath for several minutes and then cool. Then, add 1 ml of urea solution to both test tubes, allow to stand for 15 minutes at room temperature, and next add 3-4 drops of Nessler's reagent to each of them. Compare the results of both test tubes.

A S S I G N M E N T

Demonstrate whether the tested fluid has the activity of *catalase/peroxidase* or whether it contains urea.

Digestive tract enzymes

Aim of the exercise: ***to learn about some properties of digestive juices***

Consumed nutrients are converted in the gastrointestinal tract by the action of enzymes contained in the digestive juices. The table gives the approximate volumes of digestive juices secreted during the day.

Digestive juice	**Volume** [l]
Saliva	1.5
Gastric acid	2.5
Pancreatic juice	0.5
Bile	0.5
Intestinal juice	3.0

The essence of digestion is the metabolism of complex nutrients (proteins, polysaccharides, fats - mainly acylglycerols) to simpler products (amino acids, monosaccharides, fatty acids), which can be absorbed into the bloodstream and consumed by the tissues. Digestion begins in the mouth and stomach and ends in the small intestine.

Saliva

Three pairs of major salivary glands and minor salivary glands secrete saliva, which as the first digestive juice comes in contact with the consumed foods. Saliva contains about 1% solids. These are mainly proteins, proteoglycans, glycoproteins, and electrolytes. The only enzyme of saliva, which has relevance in the process of digestion, is ***α-amylase***, hydrolyzing starch to maltose. The catalytic activity of the enzyme is conditioned by the presence of chloride ions (Cl^-).

Gastric juice

The main components of gastric juice are: **hydrochloric acid**, ***pepsin*** and **mucus** protecting the mucous membrane. Hydrochloric acid creates an acidic environment (pH about 1) optimal for the action of *pepsin*, and moreover denatures food proteins increasing their susceptibility to proteolysis. Active *pepsin* is created by proteolytic modification of an inactive precursor - pepsynogen. Its activation process involves HCl and activated *pepsin*. *Pepsin* breaks down peptide bonds between valine and leucine and those created with the participation of amino groups of aromatic and acidic amino acids.

The action of ***pepsin*** causes the insoluble protein substrate (in the laboratory - coloured fibrin) to decompose to low-molecules, soluble products, which together with the dye transit into the solution.

Gastric juice contains free hydrochloric acid at a concentration of about 0.1 M (*free acidity*), which corresponds to pH 1. In addition to free HCl, gastric juice contains a number of substances with the characteristics of weak acids. These are mainly acidic proteins and protein salts with hydrochloric acid (*related acidity*). The sum of free and related acidity gives a value called *total acidity*. During the titration of gastric contents of 0.1 M NaOH, first the free molecules of HCl and then weak acids have a neutralization reaction.

Free acidity is expressed by the number of millilitres of 0.1 M NaOH required to bind free HCl contained in 100 ml of gastric contents (adjusting the pH to 3.0). **Total acidity** is expressed by the number of millilitres of 0.1 M NaOH required to fully neutralize all the acids contained in 100 ml of gastric contents (adjusting the pH to 9.0). In healthy people (on an empty stomach), free acidity does not exceed 20 ml and total acidity 30 ml of 0.1 M NaOH.

If for any reason, the gastric contents do not contain free HCl, favourable conditions for **bacterial** growth are created. The effect of their action is the occurrence of bacterial metabolic products, mainly lactic acid, in the gastric contents. Lactic acid in pathological gastric juice in the presence of phenol solution and $FeCl_3$ transitions into canary yellow coloured iron (III) lactate.

Pancreatic and intestinal juice

In duodenum, the chyme is exposed to the secretions of two great digestive glands: the pancreas and the liver. The main producer of digestive enzymes

located in the duodenum is the **pancreas**. The pancreatic juice contains hydrolytic enzymes, which act on all the main food substrates. These are mainly: *α-amylase, lipase, phospholipase A, cholesterol esterases, deoxyribonuclease* and *ribonuclease* and proteolytic enzymes - *trypsin, chymotrypsin, elastase* and *carboxypeptidase*.

Pancreatic juice, besides enzymes, contains a number of electrolytes, mainly: Na^+, K^+, Ca^{2+} and HCO_3^-. Particularly important are the bicarbonate anions (HCO_3^-), which are involved in the neutralization of stomach juice and create optimum (alkaline) pH for the action of pancreatic enzymes.

The detection of **proteolytic enzymes** in pancreatic juice has a similar basis as the detection of *pepsin* in gastric juice. Insoluble coloured fibrin by the action of pancreatic proteases disintegrates into low-molecule, soluble products which transit along with the dye into the solution.

The presence of ***lipase*** can be demonstrated by using milk as a source of fat. Fresh milk has a slightly alkaline pH. It colours the litmus paper blue. Fatty acids released by *pancreatic lipase* alter the basic pH into an acid pH - they acidify milk changing the litmus colour from blue to red. ***Pancreatic amylase*** can be detected by means of starch, which under the action of this enzyme can be degraded to reducing sugars. In the sample containing starch and pancreatic juice, the hydrolysis of starch will occur, and its consequence is the loss of a reaction with iodine and the appearance of reducing properties, which are demonstrated by the starch degradation products.

Bile, produced by the liver, plays an important role in the digestion of acylglycerols and the absorption of lipolysis products into the bloodstream. The bile acids it contains emulsify fats. A fat suspension with a high degree of dispersion forms. This increases the contact surface of fat with *pancreatic lipase* (enzyme with substrate), which eases the process of lipolysis. The released fatty acids bind with the bile acids, and in this form are absorbed from the gastrointestinal tract. Proper digestion and absorption of fat determines the absorption of fat-soluble vitamins: A, D, E and K.

The bile acids reduce the surface tension, which can be determined by assessing the speed of descent of colloidal sulphur (*sulphur flower*) to the bottom of the test tube - in Hay's test. This can be confirmed by adding oil to the water - oil does not mix with water. The border of structural constituents

is visible between the water and the oil, and in a test tube containing bile acids a stable suspension (emulsion) forms.

Intestinal juice, produced by the glands of the small intestine, contains a number of hydrolytic enzymes finishing the digestive process of food to absorbable products. These are mainly: *aminopeptidase, carboxypeptidase, phospholipase C* and *D, maltase, isomaltase, lactase, saccharase* (*sucrase*), *5'-nucleotidase* and *nucleosidase.*

The main end-products of intestinal digestion are free amino acids, simple sugars and fatty acids, which are absorbed into the blood. Triacylglycerols are generally only partially degraded. They break off one or two fatty acid residues. The products of their degradation are involved in the re-synthesis of these lipids in the wall of the small intestine, and in this form they are absorbed into the lymph.

The large intestine is only the place where bacterial fermentations, water absorption and the excretion of certain salts take place.

E X E R C I S E

1. Detection of bile acids

In 1 ml of diluted bile solution, dissolve a few crystals of sucrose. Sublayer this solution with 1 ml of concentrated H_2SO_4 (adding sulphuric acid by carefully pouring it on the test tube's sidewall). On the border between the two layers, a red ring forms.

2. Properties of bile acids

a. Hay's test

Add 3 ml of water to 2 test tubes. To one of them, add 2–3 drops of bile, and then to both test tubes pour a small amount of colloidal sulphur. Compare the sedimentation rate of sulphur.

b. emulsifying action of bile acids

To 2 test tubes, add 3 ml of distilled water and a few drops of oil each. Add a drop of bile to one of them. Shake both test tubes vigorously. Observe the differences.

3. Detection of *pepsin*

To two test tubes, add 2 ml of 0.1 M HCl and 3-4 "flocks" of dyed fibrin. Then to one of the test tubes, add 2 ml of gastric juice, and 2 ml of water to the second. Insert both test tubes into a water bath at 37°C for 30 minutes. Shake after incubation. Compare the coloration of the solutions.

4. Detection of lactic acid in pathological gastric content

Add two drops of $FeCl_3$ solution to 2 ml of 1% phenol. The solution turns blue. Add 2 ml of gastric juice to this solution. Observe the change in colour.

5. Measurement of the acidity of gastric contents

Collect a sample of gastric juice (10 ml) and add 1 drop of Topfer's indicator. Titrate with 0.1 M NaOH until the appearance of a salmon colour (pH 3). Read and record the volume of the base used. Continue titrating until the appearance of a pink colour (pH 9).

Calculate the free, related and total acidity by multiplying by 10 the volumes of 0.1 M NaOH read from the burette.

6. Pancreatic enzymes

a. detection of the proteolytic enzymes

Into each of 2 test tubes, insert 3-4 "flocks" of dyed fibrin and add 2 ml of buffer with pH 8.0. Add 10 drops of pre-mixed pancreatic juice to one of the test tubes. Insert both test tubes into a water bath at 37°C for 45 minutes. Shake after incubation. Compare the differences in the coloration of the solutions.

b. detection of *lipase*

Add 2 ml of milk and a few drops of litmus to each of the two test tubes. The alkaline reaction of the milk causes the mixture to dye blue. To one of the test tubes, add 10 drops of pre-mixed pancreatic juice. Insert both test tubes into a water bath at 37°C for 30 minutes. Compare the colour.

c. detection of *amylase*

Add 2 ml of starch gruel to two test tubes. Add 10 drops of pancreatic juice to one of them. Insert both test tubes into a water bath at 37°C for 30 minutes. After incubation, divide the contents of each test tube into two parts. Perform a reduction test (Benedict's test) on one of the test tubes, and a test with Lugol's solution on the other (tests described in the **Carbohydrates** chapter, page No. 36, point 2b). Interpret the results of the experiment.

ASSIGNMENT

1. Examine whether the tested liquid contains *pepsin*, *amylase*, *lipase*, pancreatic proteases.

2. Measure the acidity of the gastric juice.

The enzymatic reaction maximum velocity and the Michaelis constant

Aim of the exercise: ***to measure the maximum velocity and determine the Michaelis constant of the reaction catalyzed by saccharase***

The velocity of the enzymatic reaction is measured by the amount of substrate transformed by the enzyme per single unit of time. The catalytic activity of the enzyme is based on substrate activation by the formation of a temporary complex, which then disintegrates into the reaction product(s) and a free enzyme:

$$\mathbf{E} + \mathbf{S} \rightarrow \mathbf{ES} \rightarrow \mathbf{E} + \mathbf{P}$$

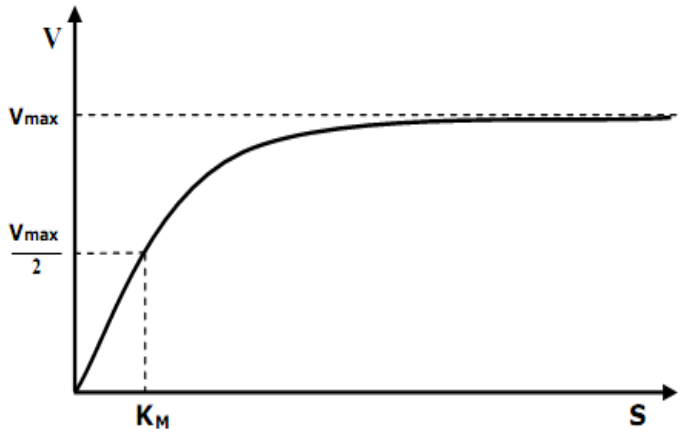
E - enzyme, **S** - substrate, **ES** - complex: enzyme-substrate, **P** - product.

The Enzyme-Substrate Complex is formed as a result of an "effective collision" of the enzyme molecule with the substrate molecule. It is such a collision after which the two molecules receive sufficient energy to enter into a chemical reaction. At a constant concentration of the enzyme, the number of such collisions increases with the increase of substrate concentration. With substrate deficiency in the reacting system, not all enzyme molecules are involved in the reaction. The increased substrate concentration causes that more enzyme molecules will be in contact with the substrate. For this reason, the velocity of the enzymatic reaction increases with the increase of the substrate concentration. After reaching a certain concentration value, all enzyme molecules come into contact with the substrate. Further increase in substrate concentration does not increase the velocity of enzymatic reaction. The reaction has reached maximum velocity (V_{max}). Some enzymatic reactions reach maximum velocity even at low concentrations of substrate. This demonstrates the high affinity of the enzyme to the substrate. In some cases, maximum velocity is achievable with high concentrations of substrate. This demonstrates the low affinity of the enzyme to the substrate.

A measure of enzyme affinity to the substrate is the **Michaelis constant**. This is such a concentration of substrate (expressed in moles per

litre) that the enzymatic reaction velocity equals half of the maximum velocity.

In order to determine the maximum velocity and the Michaelis constant, we incubate an enzyme of a fixed concentration with a substrate of a variable (increasing) concentration for the same amount of time. After stopping the incubation, we mark the reaction product in every test tube (a measure of the quantity of used substrate) and graph the dependence of the reaction velocity (on the Y-axis) on substrate concentration (on the X-axis). Observe the dependence shown in the Figure. It is called a Michaelis-Menten diagram. From this diagram you can read the value of the maximum velocity (V_{max}) and the Michaelis constant (K_M).



Saccharase (*sucrase*, *invertase*) is an enzyme that breaks down sucrose into glucose and fructose. During the reaction, one molecule of a non-reducing substrate (sucrose) splits into two molecules of reducing products (glucose and fructose). The progress of the reaction can be assessed by measuring the growth rate of the reducing sugars in the incubation liquid.

The reaction products, glucose and fructose, can be determined using the Folin-Wu method. This method uses the reducing properties of the formed sugars. When heated, hexoses reduce copper (II) ions contained in the alkaline solution of $CuSO_4$ to copper (I) ions. In the next stage, the copper (I) ions transfer electrons to the phosphomolybdic anion and form a coloured

complex known as molybdenum blue. The colour intensity is proportional to the amount of reducing sugars, which can be determined colorimetrically compared to the control sample, which eliminates, among others, the impact of copper (II) ions on the colour of the solution.

Two moles of generated products correspond to one mole of decomposed substrate.

EXERCISE

1. The course of the reaction

Prepare substrate solutions of different concentrations. To do this, add to four volumetric test tubes 1.5, 2.5, 3.5 and 5.0 ml of 0.4 M sucrose solution, respectively. Fill the contents of the test tubes 1-4 with distilled water up to 10 ml and mix thoroughly. The sucrose concentration in each test tube amounts to, respectively: 0.06M, 0.10 M, 0.14 M, and 0.20 M.

Next, prepare 6 new test tubes, numbered from **1** to **6**. To test tubes from **1** through **4** measure 1 ml of previously prepared sucrose solutions of the following concentrations:

test tube no. 1 - 0.06 M
test tube no. 2 - 0.10 M
test tube no. 3 - 0.14 M
test tube no. 4 - 0.20 M

To test tube no. **5** measure 1 ml of 0.40 M sucrose solution.

Place these test tubes into a water bath at 30°C for approximately 5 minutes in order to bring the samples to the reaction temperature.

To test tube **6** (control test) add 1 ml of yeast extract and heat for 5 minutes in a boiling water bath to inactivate the enzyme, then cool and add 1 ml of 0.40 M sucrose solution.

Measure 1 ml of yeast extract to test tubes **1** to **5** and keep incubating at 30°C for exactly 10 minutes. After that time, immediately insert the test tubes into a boiling water bath for 5 minutes to stop the reaction by thermal inactivation of the enzyme. Quantitatively transfer the content of each test tube (from **1** to **6**) to 100 ml volumetric flasks (with a stopper) (rinsing each test tube with distilled water 3 times). Next, fill the contents of the flasks with distilled water up to 100 ml and mix thoroughly.

2. Determination of reducing sugars in the post-incubation liquid

Make all determinations twice. Prepare new set of 12 tubes. To the numbered test tubes add:

test tubes 1-2	- 0.5 ml of fluid from flask no. 1
test tubes 3-4	- 0.5 ml of fluid from flask no. 2
test tubes 5-6	- 0.5 ml of fluid from flask no. 3
test tubes 7-8	- 0.5 ml of fluid from flask no. 4
test tubes 9-10	- 0.5 ml of fluid from flask no. 5
test tubes 11-12	- 0.5 ml of fluid from flask no. 6

Next, add 1 ml of alkaline solution of copper (II) sulphate to each test tube. Place all the test tubes in a boiling water bath for exactly 8 minutes, then immediately immerse them in cold water. After cooling, add 1 ml of phosphomolybdic acid to each test tube, mix, fill with distilled water to 5 ml and mix again. Determine the absorbance of light with a wavelength of 650 nm (test tubes **1-10**) compared to the control (test tubes **11-12**). Read the amount of reducing sugars from the calibration curve.

3. Preparation of calibration curve

Prepare five numbered test tubes (**1**-**5**). Add 1 ml of water to test tube No. **1** (control test). To the remaining test tubes (**2**-**5**) add successively: 0.1, 0.2, 0.5 and 1.0 ml of 0.0005 M glucose solution. Fill the contents of test tubes **2**-**4** up to 1 ml of distilled water. To each test tube, add 2 ml of alkaline solution of $CuSO_4$, heat in a boiling water bath for exactly 8 minutes and cool in cold water. Then, add 2 ml phosphomolybdic acid, fill up to 10 ml with distilled water and mix thoroughly. Determine the absorbance of the tested samples (**2**-**5**) at 650 nm compared to the control sample (test tube no. **1**). Calculate the glucose content in micromoles. Graph the relationship between

the absorbance and the amount of glucose. On the Y-axis indicate the absorbance and on the X-axis the quantity of glucose in micromoles.

4. Results presentation

The determined amount of reducing sugars in micromoles multiplied by a factor of 5 gives the reaction speed in the individual samples expressed in micromoles of sucrose decomposed within 1 minute. The factor was calculated taking into account the following data: the initial volume of the substrate (1 ml) was diluted to 100 ml. From one micromole of sucrose, 2 micromoles of hexose are formed. The duration of enzyme action is 10 minutes. Therefore, the measurement result should be multiplied by 100, divided by 2 and by 10.

Calculate the concentration of sucrose (substrate) in the reacting system remembering that the sucrose solutions were diluted twice with the yeast extract. Present the results in the Table.

Sample No.	**Sucrose concentration** [µmol/l]	**A_{650}**	**The amount of reducing sugars**	**Reaction velocity** [µmols of decomposed sucrose in 1 minute]
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				

Based on the data contained in the Table, graph the relationship between the reaction velocity [V] from the substrate concentration [S]. On the Y-axis indicate the reaction velocity and on the X-axis the concentration of the substrate. From the graph, read out the values of maximum velocity [V_{max}] and the Michaelis constant [K_M].

Enzymatic activity

Aim of the exercise: ***to determine enzyme activity using the example of yeast saccharase***

Enzyme activity is the velocity of enzymatic activity measured in specific conditions. The basic unit of enzymatic activity is a katal (kat). It is this enzyme activity that converts 1 mole of substrate in the product(s) during one second at a temperature of 30°C at the optimal pH, in zero-order reaction conditions (at full saturation of the enzyme by the substrate). Derivative units are milikatals (mkat = 1 x 10^{-3} kat), microkatals (μkat = 1×10^{-6} kat), nanokatals (nkat = 1×10^{-9} kat) and picokatals (pkat = 1×10^{-12} kat).

Enzyme activity can also be expressed using the **international enzyme unit (u)**. It is enzyme activity converting 1 micromole of substrate in 1 minute at 30°C, at the optimum pH, in zero-order reaction conditions. The international unit (u) corresponds to 16.67 nkat.

Saccharase (*sucrase*, *invertase*) is one of the disaccharidases associated with the surface of the ruffled border of the mucous membrane of the small intestine, which breaks down sucrose contained in foods into glucose and fructose. This enzyme is widespread in plant cells. The source of the *saccharase* used in the exercises is an extract from baker's yeast. Its preparation involves mechanical homogenization of yeast cells, lipid extraction with ether, saccharase extraction with distilled water and separation of the insoluble components of the homogenate by filtration on a *Büchner* funnel.

To determine the **activity** of this enzyme, an exactly defined quantity of yeast extract should be incubated with an equal volume of substrate (sucrose) at 30°C for different lengths of time - from 10 to 40 minutes. After the incubation, determine the quantity of the obtained products (reducing sugars) and calculate how many moles of sucrose had broken down during 1 second (activity in katals).

The reaction products, namely **glucose** and **fructose**, are determined using the Folin-Wu method. This method utilizes the reducing properties of the formed sugars. During heating, hexoses reduce copper (II) ions contained in the alkaline solution of $CuSO_4$ to copper (I) ions. In the next stage, the

copper (I) ions transfer electrons to the phosphomolybdic anion and form a coloured complex known as molybdenum blue. The colour intensity is proportional to the amount of reducing sugars, which can be determined colorimetrically against the control sample, which eliminates, among others, the impact of copper (II) ions on the colour of the solution.

From the quantities of the formed products (hexoses) calculate the amount of broken down substrate (sucrose), keeping in mind that from one mole of sucrose, 2 moles of hexoses (reducing sugars) form. For this purpose, a diagram should be drawn on graph paper showing the dependence of the quantities of broken down sucrose on the time of the reaction. On the Y-axis note the number of micromoles of decomposed substrate and on the X-axis the reaction time in minutes. Enzyme activity is calculated from the linear segment of the curve. It represents the directly proportional relationship between the amount of broken down substrate and the reaction time.

The calculation of enzyme activity involves the calculation of the number of moles of substrate, which had been broken down in one second by the *saccharase* contained in 1 ml of undiluted yeast extract. This is equivalent to the calculation of enzyme activity in katals or its derivatives.

E X E R C I S E

1. Preparation of *saccharase*

Students are given a ready extract of *saccharase*, which should be diluted.

2. Dilution of extract

Transfer 1 ml of *saccharase* extract to a 50 ml volumetric flask, fill with distilled water to the line and mix thoroughly. Then transfer 2.5 ml of this solution into another 50 ml flask, add 10 ml of 0.1 M acetate buffer with pH 5, fill with distilled water to the line and mix thoroughly. Calculate the dilution.

3. Measurement of enzyme activity

Prepare 10 test tubes and label them with the serial numbers from 1 to 10. Add 0.5 ml of the diluted enzyme extract to test tubes 1 and 2 (control samples) and heat for 5 minutes in a boiling water bath, then add 1 ml of alkaline solution of $CuSO_4$.

To all the test tubes (1-10), add 0.5 ml of 0.1 M sucrose solution. Place the test tubes (3-10) and the prepared yeast extract for 5 minutes into a water bath at 30°C. Next, add 0.5 ml of diluted yeast extract to each of them (as soon as possible). From this moment, in test tubes 3-10 a reaction begins. In tubes 1-2, there is no reaction due to the thermal inactivation of the enzyme. Incubate each test tube for the period of time indicated in the Table. After the incubation time (different for each test tube), immediately stop the reaction, inactivating the enzyme by adding 1 ml of alkaline solution of $CuSO_4$. Place all test tubes (in rack) in a boiling water bath for exactly 8 minutes, then immediately immerse them in cold water. After cooling, add 1 ml of phosphomolybdic acid solution to each test tube, mix and fill up to 5 ml with distilled water. Measure the absorbance of samples 3-10 at 650 nm compared to the control samples (test tubes 1 and 2). Read the amount of reducing sugars from the calibration curve.

4. Preparation of calibration curve

Prepare a calibration curve as described in the exercise "The maximum velocity of enzymatic reaction and the Michaelis constant".

5. Results presentation

From the amount of micromoles of reducing sugars in the various tests, calculate the number of micromoles of sucrose decomposed by 1 ml of undiluted yeast extract. Take into account that one mole of sucrose creates two moles of reducing sugars. Summarize the results in the Table.

Based on the results contained in the Table, plot the relationship between the amount of decomposed sucrose (Y-axis) and the incubation time (X-axis). Calculate enzyme activity in international enzymatic units and microkatals per ml of undiluted extract.

Sample No.	Sample Content	Incubation time [min.]	A_{650}	Micromoles of reducing sugars	Micromoles of decomposed sucrose	
					Diluted enzyme	Undiluted enzyme
1 2	Denaturized enzyme + sucrose	0				
3 4	Active enzyme + sucrose	10				
5 6	"	20				
7 8	"	30				
9 10	"	40				

Competitive and non-competitive inhibition

Aim of the exercise: ***to learn about the difference between competitive and non-competitive inhibition using the example of succinate dehydrogenase inhibitors***

Inhibition is an occurrence of impeding the activity of enzymes. Substances that inhibit enzyme activity are inhibitors. They are divided into two groups: competitive and non-competitive inhibitors.

Competitive inhibition

A **competitive inhibitor** displays structural similarity to the substrate and competes with it for the enzyme active site. The enzyme "cannot" distinguish the substrate from the inhibitor and "accidentally" (instead of the substrate) binds the competitive inhibitor in its active site. The resulting Enzyme-Inhibitor complex cannot be transformed further. In the presence of the enzyme, substrate and inhibitor, two reactions can take place:

A. Enzyme + Substrate → Enzyme-Substrate → Enzyme + Product
B. Enzyme + Inhibitor → Enzyme-Inhibitor

The number of enzyme molecules (at a constant concentration) involved in the reaction depends on the relative concentrations of the substrate and the inhibitor. The higher the concentration of the substrate in relation to the inhibitor, the fewer molecules will bind to the enzyme inhibitor. A constant concentration of the enzyme and the inhibitor and increasing concentrations of the substrate cause a disconnection of a growing number of inhibitor molecules from the enzyme, and the inhibitor is replaced by the substrate. The Enzyme-Inhibitor complex is converted into the Enzyme-Substrate complex and the inhibitor is displaced from the enzyme active site. Inhibition of the reaction caused by a competitive inhibitor may therefore be reversed by increasing substrate concentration.

With appropriately high concentrations of substrate, the maximum reaction velocity (V_{max}) reaches the value observed in a system not containing an inhibitor, despite the presence of the inhibitor. In other words, to achieve

V_{max} in the presence of a competitive inhibitor, higher substrate concentrations are needed than in a system free of this inhibitor. Of course, a higher concentration of substrate is also needed to reach $V_{max}/2$, which means that the Michaelis constant (K_M) in the presence of a competitive inhibitor reaches a greater value.

Non-competitive inhibition

A non-competitive inhibitor shows no similarity to the substrate. It binds to the enzyme outside the active site (at a different place than the substrate). The active site of the enzyme binds the substrate, but "cannot" turn it into a product. Even a substantial increase in substrate concentration is no longer able to reverse inhibition. The inhibitor can bind with the free enzyme or the Enzyme-Substrate complex. The reactions may proceed according to the following diagrams:

A. Enzyme + Inhibitor → Enzyme-Inhibitor
B. Enzyme-Inhibitor + Substrate → Enzyme-Substrate-Inhibitor
C. Enzyme-Substrate + Inhibitor → Enzyme-Substrate-Inhibitor

Both the complex: Enzyme-Inhibitor and Enzyme-Substrate-Inhibitor are inactive and "cannot" transform the substrate into a product. In the presence of a non-competitive inhibitor V_{max} has a lower value regardless of substrate concentration, while the value of K_M does not change.

Succinate dehydrogenase oxidizes succinate to fumarate by disconnecting 2 hydrogen atoms. Their direct acceptor is **flavin adenine dinucleotide** (FAD), and then **coenzyme Q**, which transitions into a reduced form (QH_2). Starting with the latter, further transport of electrons occurs independently of the transport of protons. The electrons pass through **cytochrome b, cytochrome c_1, cytochrome c and cytochrome $a+a_3$** to oxygen. Oxide anion O^{2-} forms, which binds with two protons forming a water molecule.

In our experiment, there will be an artificial electron acceptor, yellow-green potassium hexacyanoferrate (III) solution - $K_3[Fe(CN)_6]$, which as a result of reduction transitions into the colourless potassium hexacyanoferrate (II) solution $K_4[Fe(CN)_6]$. Thus, a measure of the activity of *succinate dehydrogenase* is the degree of discoloration of the potassium hexacyanoferrate solution.

The **competitive** inhibitor of *succinate dehydrogenase* is malonate, a compound very similar to that of succinic acid, from which it differs by the absence of one group $-CH_2$. It competes for the enzyme active site. Malonate binds with dehydrogenase, and the obtained Enzyme-Inhibitor complex cannot be further transformed. As a result of this process, the reaction catalyzed by *succinate dehydrogenase* is inhibited. Increasing concentrations of the substrate (succinate) enables the reversal of this inhibition.

Non-competitive inhibitors of *succinate dehydrogenase* are heavy metal salts. The enzyme requires the presence of free -SH groups for its catalytic function. Heavy metal ions (e.g., Hg^{2+}) bind with the sulphur of the -SH groups inhibiting the activity of this enzyme. Increasing concentrations of the substrate (succinate) is not able to reverse this inhibition.

Table I. The main differences between a competitive and non-competitive inhibitor.

	Competitive inhibitor	**Non-competitive inhibitor**
Structure	similar to the substrate	not similar to the substrate
Binding site	enzyme active site	outside enzyme active site
Inhibition reversibility by higher substrate concentration	reversible	irreversible
$\mathbf{V_{max}}$	does not change, but is achieved with higher concentrations of substrate	decreases
$\mathbf{K_M}$	increases	does not change

This experiment consists in the incubation of rat liver homogenate containing *succinate dehydrogenase*, with succinate at different concentrations, in the presence or absence of inhibitors - malonate and $HgCl_2$, in a environment that contains an artificial electron acceptor - $K_3[Fe(CN)_6]$. After stopping the reaction with the help of trichloroacetic acid, in the clear filtrate we assess the dependence of the degree of discoloration of the solution on the presence and relationship of the substrate concentrations and the inhibitors at a constant enzyme concentration.

EXERCISE

1. Preparation of test tubes with incubation solutions

To the numbered test tubes (1-12), measure in any order reagents according to the attached Table, and additionally pour 2 ml of 10% trichloroacetic acid into test tube 2. The total volume of the reactive solution in each test tube will be the same and will amount to 4 ml.

Test tube no. 1 is a control sample without the substrate (with an equivalent volume of water). Test tube 2 is a control sample containing an inactive enzyme (denatured by trichloroacetic acid). Next, insert all test tubes (1-12) into a water bath with a temperature of 37°C for 5 minutes in order to bring the reacting fluids to this temperature.

Sample No.	**0.1M phosphate buffer** [ml]	**0.1M sodium succinate** [ml]	**0.05M sodium malonate** [ml]	**0.01M $HgCl_2$** [ml]	**0.5% $K_3[Fe(CN)_6]$** [ml]	**H_2O** [ml]
1	1	0	0	0	0.5	2.5
2	1	0.1	0	0	0.5	2.4
3	1	0.1	0	0	0.5	2.4
4	1	0.1	0	0	0.5	2.4
5	1	0.1	0.1	0	0.5	2.3
6	1	0.1	0.1	0	0.5	2.3
7	1	0.1	0	0.1	0.5	2.3
8	1	0.1	0	0.1	0.5	2.3
9	1	2.0	0.1	0	0.5	0.4
10	1	2.0	0.1	0	0.5	0.4
11	1	2.0	0	0.1	0.5	0.4
12	1	2.0	0	0.1	0.5	0.4

2. Incubation of reacting systems

After the initial incubation, add 1 ml of rat liver homogenate to each test tube. Mix and incubate for 30 minutes, and then to each test tube (except for test tube no. 2) add 2 ml of 10% trichloroacetic acid. Filter the samples through small filters to other test tubes bearing the same numbers.

3. Interpretation of the results

During the 30-minute incubation, prepare a Table according to the attached example. Calculate the substrate and the inhibitor concentrations in the individual test tubes.

Determine in which test tubes the discoloration of the potassium hexacyanoferrate (III) solution occurred (mark in the Table).

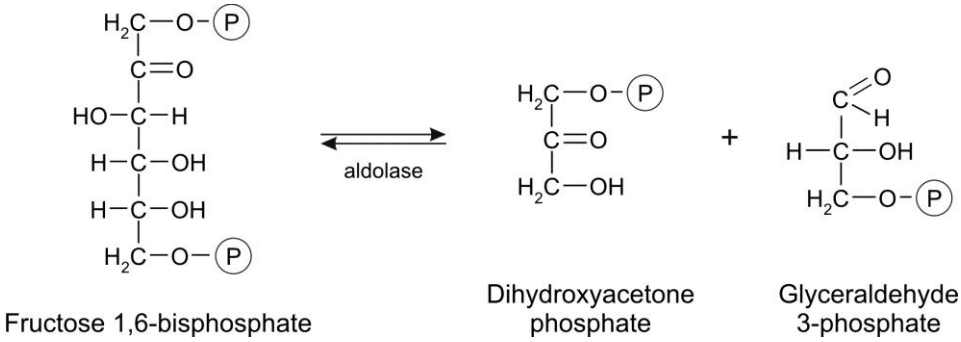
Sample No.	**Succinate concentration** [mM]	**Malonate concentration** [mM]	**$HgCl_2$ concentration** [mM]	**Discoloration of hexacyano-ferrate (+ or -)**

Based on the results compiled in the Table, determine in which test tubes the inhibition occurred. Analyze the relationship of the concentrations of the used substrate and the inhibitor. On this basis, indicate in which test tubes the reversal of inhibition took place. Analyze, in which case there was a change in the Michaelis constant.

Fructose 1,6-bisphosphate aldolase activity

Aim of the exercise: ***to follow the course of aldolase reaction***

Fructose 1,6-bisphosphate *aldolase* is an enzyme that belongs to the lyase class, catalyzing the reversible reaction of the degradation and synthesis of fructose 1,6-bisphosphate. As a result of this enzyme's action, two phosphotrioses are created or are used up dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. At equilibrium, the quantitative relationship between fructose 1,6-bisphosphate and phosphotrioses is set at a level of 89% to 11%. The *aldolase* reaction provides phosphotrioses in the glycolysis process, and vice versa consumes phosphotrioses in the gluconeogenesis process. The reaction that occurs with the involvement of this enzyme is the biochemical reaction form of reaction known in organic chemistry as aldol condensation. Hence the name of the enzyme - ***aldolase***.



The above figure shows a reaction catalyzed by *aldolase*.

Aldolase is a cytosol enzyme present in every cell. Several isoforms of this enzyme have been described. It is present in large quantities in skeletal muscle, follicular organs (liver, kidney) as well as in erythrocytes.

All isoenzymes are composed of four identical subunits with a molecular weight of about 40 kDa each. The tetrametric structure determines *aldolase* activity. Acidification causes the dissociation of the enzyme to

inactive subunits. After neutralization, the subunits spontaneously combine to form an active form of the enzyme.

The object of the exercise is to demonstrate the formation of phosphotrioses from fructose 1,6-bisphosphate through the action of *aldolase* contained in the extract of rabbit muscle homogenate and *aldolase* contained in blood serum. *Aldolase* activity in serum is small and it may increase in pathological conditions due to the penetration of the enzyme from damaged tissues into the blood.

For the determination of products of the aldolase reaction, the trioses colour reaction (aldehyde and ketone) with 2,4-dinitrophenylhydrazine are used. Free trioses go into a condensation reaction easier than their phosphate esters, therefore the formed phosphotrioses first undergo basic hydrolysis to disconnect the phosphate. As a result of triose condensation reaction with 2,4-dinitrophenylhydrazine, the appropriate 2,4-dinitrophenylhydrazones form.

In order to inhibit *glyceraldehyde 3-phosphate dehydrogenase* present in the tissues, which can oxidize the formed glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate, monoiodoacetic acid is applied. This is particularly important when examining the *aldolase* reaction in tissue homogenates, where (as opposed to blood serum) both of these enzymes (*aldolase* and *glyceraldehyde 3-phosphate dehydrogenase*) occur side by side. The presence of hydrazine during the incubation of the substrate with *aldolase* shifts the balance of the reversible enzymatic reaction in the direction of phosphotrioses creation.

EXERCISE

Prepare **6** test tubes: **3** test tubes for investigating the reaction catalyzed by muscle *aldolase* and **3** test tubes for testing the reaction catalyzed by blood serum *aldolase* (see Table). To all test tubes, measure 0.5 ml of fructose 1,6-bisphosphate solution in a hydrazine buffer (pH 8.2) containing monoiodoacetic acid. To the **second** and **third** test tubes add 0.1

ml of rabbit muscle extract, and to the **fifth** and **sixth** add 0.1 ml of blood serum each.

Place the test tubes (**1-6**) in a water bath at 37°C for 30 minutes. After the incubation, to all the test tubes, add 0.1 ml of 2M HCl to inactivate the enzyme. Then, to the first test tube add 0.1 ml of muscle extract, and to the fourth 0.1 ml of blood serum (test tubes **1** and **4** - control samples).

Further proceedings are the same for all the samples. Add 0.5 ml of 0.6 M NaOH to the test tubes and leave at room temperature for 30 minutes. Then, add 0.5 ml of 2,4-dinitrophenylhydrazine solution and put into a water bath at 37°C for 30 minutes. After this time, add 4.5 ml of 0.6 M NaOH and leave in a dark place for about 10 minutes.

Compare the intensity of the colour in the real tests with the corresponding control trials. Present the results in the Table, evaluating the intensity of coloration with three, two or one plus signs.

You can also determine the quantity of the formed trioses using the colorimetric method, by measuring the absorbance of light at wavelength 570 nm compared to the control. Dihydroxyacetone solution can be used as a standard.

<table>
<tr><th colspan="2">Muscle extract</th><th colspan="2">Blood serum</th></tr>
<tr><td>Control test
(test tube 1)</td><td>Real tests
(test tubes 2 & 3)</td><td>Control test
(test tube 4)</td><td>Real tests
(test tubes 5 & 6)</td></tr>
<tr><td>0.5 ml substrate</td><td>0.5 ml substrate
0.1 ml muscle extract</td><td>0.5 ml substrate</td><td>0.5 ml substrate
0.1 ml serum</td></tr>
<tr><td colspan="4">Incubation for 30 minutes at 37°C</td></tr>
<tr><td>0.1 ml 2M HCl
0.1 ml muscle extract</td><td>0.1 ml 2M HCl</td><td>0.1 ml 2M HCl
0.1 ml serum</td><td>0.1 ml 2M HCl</td></tr>
<tr><td colspan="4">0.5 ml 0.6M NaOH - 30 minutes at room temperature</td></tr>
<tr><td colspan="4">0.5 ml 2.4-dinitrophenylhydrazine - 30 minutes at 37°C</td></tr>
<tr><td colspan="4">4.5 ml 0.6M NaOH - 10 minutes in a dark place</td></tr>
</table>

RESULTS			
TEST TUBE NUMBER			
1	2 – 3	4	5 – 6

Oxidative decarboxylation of pyruvate

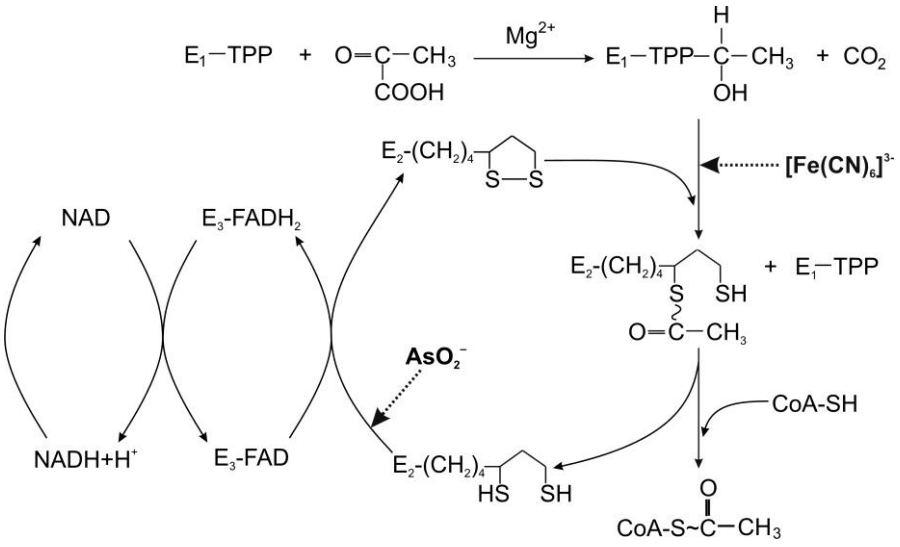
Aim of the exercise: ***to observe the course of the reaction of pyruvate oxidative decarboxylation***

Pyruvic acid is mainly the product of glycolysis and alanine deamination. In the physiological pH, it is in dissociated form as an anion called **pyruvate** and undergoes multi-transitions.

Most of the pyruvate is converted in the mitochondrial matrix to acetyl-CoA in the process of **oxidative decarboxylation**, catalyzed by the multienzymatic complex known as ***pyruvate dehydrogenase***. This complex is made up of *pyruvate decarboxylase* (E_1), *dihydrolipoyl transacetylase* (E_2) and *dihydrolipoyl dehydrogenase* (E_3). The coenzyme of *pyruvate decarboxylase* is thiamin pyrophosphate (TPP), the coenzyme of *dihydrolipoyl transacetylase* is lipoic acid, and the coenzyme of *dihydrolipoyl dehydrogenase* - oxidized flavin adenine dinucleotide (FAD), which transfers hydrogens taken from lipoate to the oxidized nicotinamide adenine dinucleotide (NAD^+).

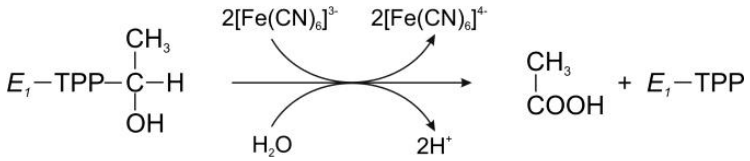
At the first stage, pyruvate joins thiamine pyrophosphate with concurrent decarboxylation (CO_2 disconnection). The obtained acetaldehyde, coupled with the tiasolic ring of thiamine pyrophosphate, is separated in the second stage with simultaneous oxidation (disconnecting the pair of hydrogens). The acetic acid residue joins by highly energetic binding (thioester) to the sulphur atom of lipoate. The hydrogen atoms from aldehyde are transferred to thiamine and the second sulphur atom of lipoate. In the third stage, the acetic acid residue is transferred to coenzyme A. Acetyl-CoA forms and lipoate is reduced. The latter is oxidized with the participation of *dihydrolipoyl dehydrogenase*, which transfers the hydrogen atoms extracted from lipoate to FAD. The reduced FAD ($FADH_2$) transmits the hydrogen atom and electron to NAD^+. The resulting $NADH + H^+$ is further oxidized in the respiratory chain.

The course of the oxidative decarboxylation of pyruvate is presented in a simplified manner in the following diagram:



Acetyl-CoA is oxidized in the citric acid cycle (Krebs cycle) to CO_2 and H_2O.

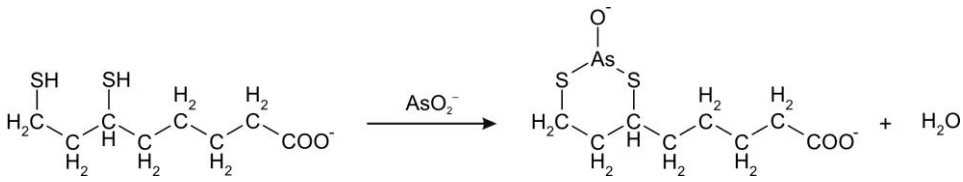
The object of the exercise is to demonstrate the consumption of pyruvate added to the homogenate of rat liver. It allows you to trace the redox reactions taking place and indicates the ability to inhibit this process by arsenate (III). The electron acceptor is oxygen or an artificial acceptor - potassium hexacyanoferrate (III) - $K_3[Fe(CN)_6]$. This complex directly oxidizes acetaldehyde (bound with the thiazole ring of thiamine pyrophosphate) into acetic acid.



The reaction is interrupted by adding trichloroacetic acid, and you evaluate the consumption of pyruvate by means of a colour reaction. In the test tubes containing potassium hexacyanoferrate (III), you also observe a colour change in the solution following the reduction of this compound (of a yellow colour) to colourless potassium hexacyanoferrate (II) $K_4[Fe(CN)_6]$.

Arsenate (III) (AsO_2^-) is a typical reaction inhibitor involving the -SH group. In the conditions of our experiment, it inhibits the reaction catalyzed by *dihydrolipoyl dehydrogenase.*

The mechanism of blocking the -SH groups by arsenate (III) is shown in the following equation:



The presence of potassium hexacyanoferrate (III), as an artificial electron acceptor, causes the stage inhibited by arsenate (III) to be bypassed. In these conditions, arsenate does not inhibit pyruvate consumption.

You asses the content of pyruvate in the reacting system by means of the reaction with 2,4-dinitrophenylhydrazine. All α-keto acids, and therefore pyruvate, react with 2,4-dinitrophenylhydrazine to form the corresponding phenylhydrazones. These compounds are soluble in organic solvents and can be extracted (e.g., using ethyl acetate). After adding ethyl acetate and vigorously shaking, the mixture separates into layers. In the aqueous phase (lower layer), water and salts contained in the tissue extracts remain. In the organic phase (upper layer), phenylhydrazones and excess of 2,4-dinitrophenylhydrazine accumulate. Phenylhydrazones and 2,4-dinitrophenylhydrazine can be easily separated. They differ in solubility in sodium carbonate solution. After removing the aqueous phase, we add the solution of Na_2CO_3 to the organic phase. While shaking, the phenylhydrazones transit into the aqueous phase, which contains sodium carbonate (bottom layer), and free 2,4-dinitrophenylhydrazine remains in the organic phase of ethyl acetate (upper layer). The pyruvate 2,4-dinitrophenylhydrazone after alkalization with NaOH solution changes colour from yellow to brown-red (like all aromatic nitro compounds). The colour intensity is proportional to the concentration of pyruvate in the reacting system.

E X E R C I S E

1. Incubation

To six numbered test tubes (**1-6**) add the appropriate amounts of reagents, according to Table I. Then, the control tubes (**1** and **4**) should be filled with 4 ml of 10% trichloroacetic acid (TCA). Add 2 ml of rat liver homogenate to every test tube and incubate in a water bath at 37°C for 45 minutes. Every 3-4 minutes, mix the contents of the test tubes by shaking.

After the incubation to the test tubes No.: **2, 3, 5** and **6** add 4 ml of TCA each. Filter the contents of all test tubes through small filters made of filter paper to the next series of short test tubes.

Preserve the test tubes with filtrates until the end of class.

2. Demonstration of pyruvate loss

Prepare new set of six numbered test tubes. Measure 0.5 ml of corresponding filtrates to each and add 0.5 ml of 2,4-dinitrophenylhydrazine solution. Let them sit at room temperature for 15 minutes. Then, add 4 ml of ethyl acetate and mix vigorously for 1 minute using a suitable pipette (repeate aspirate–release cycles). Pipette carefully the lower aqueous layer using an appropriate pipette, to the rest measure 3 ml of 10% Na_2CO_3 solution, and mix again vigorously for one minute like before. From the bottom layer of water, collect by pipette 1.5 ml and transfer to a new series of identically numbered test tubes. To each, add 1.5 ml of 1.5M NaOH solution, mix. Based on colour differences, assess in which test tube pyruvate consumption took place.

Compare the consumption of potassium hexacyanoferrate (III) in test tubes **4, 5** and **6**.

Table I

Samples without potassium hexacyanoferrate						
Sample		**0.2M phosp. buffer pH 7.2**	**0.05M pyruvate**	**0.1M hexacyano-ferrate**	**0.02M arsenate**	**H_2O double distilled**
1	Control (denaturized enzymes)	0.2 ml	0.1 ml	**-**	**-**	0.2 ml
2	Real	0.2 ml	0.1 ml	**-**	**-**	0.2 ml
3	Real with an inhibitor	0.2 ml	0.1 ml	**-**	0.1 ml	0.1 ml

Samples with potassium hexacyanoferrate						
Sample		**0.2M phosp. buffer pH 7.2**	**0.05M pyruvate**	**0.1M hexacyano-ferrate**	**0.02M arsenate**	**H_2O double distilled**
4	Control (denaturized enzymes)	0.2 ml	0.1 ml	0.1 ml	**-**	0.1 ml
5	Real	0.2 ml	0.1 ml	0.1 ml	**-**	0.1 ml
6	Real with an inhibitor	0.2 ml	0.1 ml	0.1 ml	0.1 ml	-

3. Results presentation

Present the results in Table II and discuss them. In formulating observations, use the symbols: (+) - consumption, (-) - lack of consumption.

Table II Results.

	Samples <u>without</u> potassium hexacyanoferrate			**Samples <u>with</u> potassium hexacyanoferrate**		
Sample No.	**Control test** **1**	**Real test** **2**	**Real test with inhibitor** **3**	**Control test** **4**	**Real test** **5**	**Real test with inhibitor** **6**
Pyruvate						
Hexacyano-ferrate						

Glutaminase

Aim of the exercise: ***to compare glutaminase activity in the kidney and skeletal muscle***

Of more than twenty amino acids found in body fluids and animal tissues, **glutamine** plays a specific role in metabolic processes. Despite the large number of enzymatic reactions consuming glutamine, it is found in high concentrations (0.5 to 0.9 mM) in blood plasma and represents more than 50% of the intracellular pool of free amino acids. **Glutamine** is a neutral glucogenic amino acid, which can be synthesized in the body by a number of organs containing *glutamine synthetase*. Glutamine plays a key role in overall nitric metabolism. It is the donor of the amine groups for different biosyntheses and is the nitrogen conveyor between the different organs.

The major producers of glutamine are the liver and skeletal muscle. More than half of the total number of amino acids of the animal system is found in the skeletal muscle (mainly in proteins). Glutamine represents about 6% of the amino acids comprising the muscle protein and almost 25% of the amino acids released by the skeletal muscles. The hydrocarbon skeleton of glutamine can come from two sources. The first and probably most important is glutamate, derived from muscle protein breakdown. The second is α-ketoglutarate. The latter mainly forms as a metabolite of the citric acid cycle (Krebs cycle) or is produced during the transformation of certain amino acids. The formation of glutamine from α-ketoglutarate occurs in two stages. In the first stage, as a result of transamination, glutamate is formed. Binding ammonia by the γ-carboxyl glutamate group leads to glutamine formation.

Glutamine, released from skeletal muscle, may be transported through the blood and collected by other organs, in which it may be subjected to multi-directional transitions. The small intestine and lymphocytes play an important role in the metabolism of glutamine in physiological conditions, and in the lactating period the mammary gland as well.

In certain metabolic conditions, glutamine may be collected by the kidney in large numbers. This mainly occurs in the process of acidosis. Acidification of the kidney activates *glutaminase*, which breaks down glutamine into ammonia and glutamate. The released ammonia (**NH_3**) binds

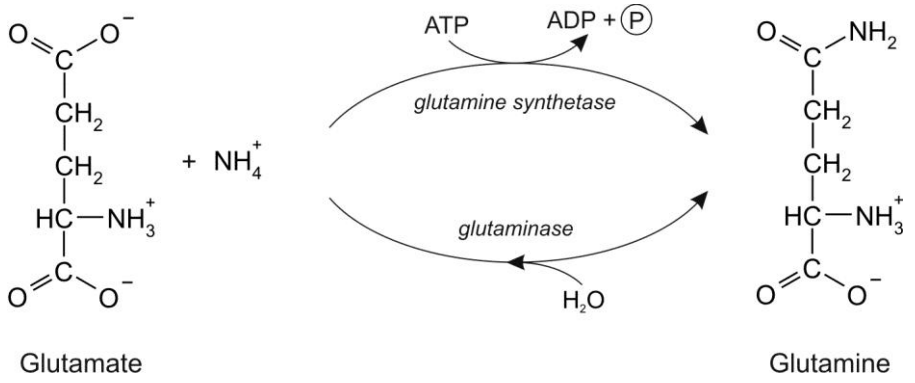
excess H^+ ions transitioning into ammonium ion (**NH_4^+**), which decreases the symptoms of acidosis.

In the metabolism of glutamine, the main role is played by two enzymes: *glutamine synthetase* and *glutaminase*.

Glutamine synthetase is widespread in animal tissues. It occurs in cytosol. The liver has the highest activity of this enzyme (expressed per gram of tissue), followed by the kidney, brain and stomach. Skeletal muscles have low *glutamine synthetase* activity, but given their large mass, the muscles are the main producers of glutamine.

Glutaminase is present in the mitochondria of many animal organs. It is activated by inorganic phosphate. In the absence of phosphate, enzyme activity is very low. There are two isoforms of *glutaminase*: liver and kidney. The first one only occurs in the liver, and the other one in the kidney and other organs.

The reactions catalyzed by *glutamine synthetase* and *glutaminase* are presented in the following figure.



We will compare the consumption of glutamine by the kidney and skeletal muscle. A glutamine consumption indicator is the amount of ammonia released as a result of its hydrolysis. The kidney is the organ that consumes glutamine. It contains *glutaminase*, therefore during the incubation of kidney cuttings with glutamine, ammonia is released. Skeletal muscle does not

contain *glutaminase*. During incubation of skeletal muscle cuttings with glutamine, ammonia is not released.

The determination of ammonia is based on its reaction with phenol in the presence of chlorate (I). A coloured reaction product forms, and the colour intensity of the solution is proportional to the concentration of ammonia.

E X E R C I S E

1. Tissues preparation

Cut out the skeletal muscles from a rat's hind limbs and the kidneys and place them in dry beakers. Next, weigh 0.5 g of each tissue. Without removing the weighed tissue from the beakers, cut them with clean scissors into small cuttings.

2. Glutamine solution preparation

Dissolve 200 mg of glutamine in 70 ml of 0.15 M phosphate buffer pH 8.2.

3. Incubation

Prepare 16 plastic test tubes for centrifuge marked with the symbols **I-5, I-10, I-15, I-20, II-5, II-10 …… IV-15, IV-20.** The first digit indicates the number of the reactive system (see Table), the second - the incubation time in minutes. To each of them, add 0.25 ml of 1M trichloroacetic acid.

To the beakers containing the cut tissue, add 10 ml of the appropriate incubation liquid, with glutamine or without glutamine (according to the Table), and then transfer the content to appropriately numbered beakers (**I-IV**) and incubate at 37°C for 20 minutes.

Reacting system	Organ	0.15M phosphate buffer pH 8.2 [ml]	Glutamine solution in 0.15M phosphate buffer [ml]
I	Skeletal muscle	10	**-**
II	Skeletal muscle	**-**	10
III	Kidney	10	**-**
IV	Kidney	**-**	10

After 5, 10, 15 and 20 minutes of incubation, collect from the beakers (I-IV) 0.5 ml of incubation fluid (without fragments of tissue) and transfer to the previously prepared and appropriately labelled plastic test tubes for centrifuge (I-5, I-10, I-15, I-20, II-5, II-10, II-15, II- 20, etc.) containing trichloroacetic acid (to stop the reaction by precipitation of protein). After five minutes after the end of incubation, add to each test tube 0.25 ml of 0.9 M KOH solution in order to neutralize the trichloroacetic acid. Mix the contents of all test tubes and centrifuge at a speed of 2000 revolutions per minute for 10 minutes. Determine the concentration of ammonia in the supernatants collected above the deposit of precipitated protein.

4. Colorimetric determination of ammonia

Prepare 18 test tubes with stoppers. To two test tubes (control samples), measure 1 ml of phenol reagent (phenol with sodium pentacyano-nitrosyl-ferrate (II)) and 0.1 ml of ammonia-free water.

To the properly labelled (same as the centrifuge test tubes) remaining sixteen test tubes, measure 1 ml of phenol reagent and transfer to them using an automatic pipette 0.1 ml of supernatants. Next, to each test tube and two control samples, add 1 ml chloride reagent (sodium chlorate (I) in 0.5% NaOH), close with the stoppers, mix, and place for 20 minutes in a water bath at 50°C. After that time, cool (do not open) and measure the absorbance compared to the control samples at a wavelength of 625 nm.

5. Results description

Based on the results obtained, present in one coordinate system diagrams of the absorbency dependence on the incubation time for all four incubation systems.

Pay attention to the differences in *glutaminase* activity in the skeletal muscle and the kidney.

Glucose consumption in the brain

Aim of the exercise: ***to assess glucose uptake by brain cytosol and to assess the impact of the cofactors and inhibitors of glycolysis on this process***

Glucose is one of the main energy substrates. The main way of sugar metabolism in animal cells is its conversion into **pyruvate**. This process occurs in the cytosol. In aerobic conditions, pyruvate transits into the mitochondria, where it undergoes oxidative decarboxylation to **acetyl-CoA**. The acetyl residues can oxidize in the citric acid cycle to **CO_2** and **H_2O**, providing the cell energy or posing as a substrate for biosynthesis, occurring both in the mitochondria and in the cytoplasm. In anaerobic conditions, oxidative decarboxylation of pyruvate is not possible, nor is the functioning of the citric acid cycle due to lack of oxygen as the final acceptor of the electrons and protons detached from the energy substrates.

In anaerobic conditions, during glucose conversion to pyruvate, in one of the stages, the oxidation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate takes place. The proton and electron acceptor is cytosol NAD^+, which transforms into a reduced form: $NADH+H^+$. In the cytosol, NAD^+ stocks are depleting, $NADH+H^+$ accumulates. The oxidation of consecutive molecules of glyceraldehyde 3-phosphate requires NAD^+ regeneration independent from the respiratory chain. This is possible by transferring protons and electrons from $NADH+H^+$ to pyruvate with the production of lactate and the regeneration of NAD^+. This reaction is catalyzed by *lactate dehydr*ogenase present in the cytosol.

The **brain** is an organ, which (in terms of health) only draws energy from the oxidation of glucose to CO_2 and H_2O. The object of this exercise is to assess the impact of glycolysis cofactors and inhibitors on glucose metabolism in the rat brain cytosol. The experiment is based on the incubation of rat brain cytosol with glucose in an environment of different composition, in the presence or absence of: ATP, NAD^+, iodoacetate, fluoride and citrate. For the experiment, we will use cytosol obtained from rat brains. The lack of mitochondria (they were centrifuged) prevents aerobic metabolism of glucose to CO_2 and H_2O. Glucose incubated with this extract may only be subject to anaerobic transformation into lactate. A necessary condition for this

transformation is the presence of ATP and NAD^+. Iodoacetate, fluoride and citrate inhibit this process.

Iodoacetate is a non-competitive inhibitor of *glyceraldehyde-3-phosphate dehydrogenase*. It binds covalently with sulphur of the -SH groups in the active site of this enzyme.

$$\text{Enzyme-}CH_2\text{-SH} + ICH_2COO^- \rightarrow \text{Enzyme-}CH_2\text{-S-}CH_2COO^- + HI$$

Fluoride ion (F^-) inhibits the activity of *enolase* by binding magnesium ion (Mg^{2+}) necessary for the functioning of this enzyme.

$$Mg^{2+} + 2F^- \rightarrow MgF_2$$

Citrate inhibits glycolysis, since it is an allosteric, negative effector of *phosphofructokinase.*

In the course of glycolysis, the glucose content is reduced. In the exercise, the amount of remaining glucose is determined using the ortho-toluidine method. It involves the reaction of glucose with ortho-toluidine during a brief heating in an acetic acid solution. A blue product forms. The intensity of the colour correlates with the glucose content.

EXERCISE

1. Preparation of cytosol from the rat brain

Dissect the brains of three rats and homogenize them carefully in 8 ml of cold, buffered KCl solution using a piston homogenizer. Transfer the homogenate into a centrifuge test tube. Centrifuge for 10 minutes at 4°C, at a speed of 15,000 rpm. Pour the supernatant containing the soluble part of the cytoplasm called **cytosol** into a test tube.

2. Preparation of the incubation mixtures

Dissolve 32 mg of glucose in 5 ml of 50 mM Tris-HCl buffer, pH 7.5 containing: 30 mM KH_2PO_4, 60 mM nicotinamide, and 10 mM $MgCl_2$. A glucose solution with a concentration of 35 mM forms.

In the centrifuge test tubes (**0-7**), prepare the incubation mixtures with the composition presented in Table I. Each component of the mixture should be placed on the bottom of the test tube.

Table I Composition of incubation mixtures.

Sample No.	Incubation mixture [ml]						
	35mM glucose	10mM ATP	10mM NAD^+	H_2O double distilled	50mM iodoactate	100mM NaF	50mM citrate
0	0.1	0.05	0.05	0.05	-	-	-
1	0.1	0.05	0.05	0.05	-	-	-
2	0.1	0.05	0.05	0.05	-	-	-
3	0.1	-	0.05	0.1	-	-	-
4	0.1	0.05	-	0.1	-	-	-
5	0.1	0.05	0.05	-	0.05	-	-
6	0.1	0.05	0.05	-	-	0.05	-
7	0.1	0.05	0.05	-	-	-	0.05

3. Incubation

To the test tube labelled **0** add 0.25 ml of 1M trichloroacetic acid solution. To all the test tubes (**0-7**) add 0.25 ml of cerebral cytosol and incubate in a water bath at 37°C for 40 minutes. Stop the reaction by adding 0.25 ml of 1M trichloroacetic acid solution to test tubes **1-7**.

During incubation, calculate the final concentrations of the components of the incubation mixture and mark them in Table II.

Table II.

Component of the incubation mixture	**Concentration** [mM]
Glucose	
Mg^{2+}	
ATP	
NAD^{+}	
H_2PO_{4-}	
Iodoacetate	
NaF	
Citrate	

4. Determination of glucose

5 minutes after finishing adding trichloroacetic acid, all samples should be carefully balanced, and then centrifuged for 10 minutes at 2000 rpm. After centrifugation, from each test tube collect 0.25 ml of supernatant using a pipette and transfer to the bottom of the test tubes with stoppers (appropriately labelled). Then, to each add 2 ml of ortho-toluidin reagent (ortho-toluidine in concentrated acetic acid). Place all test tubes sealed with stoppers in a boiling water bath for 8 minutes and then cool. Observe the formation of a colour reaction product and the diversity of colour intensity depending on the composition of the reacting mixture. Determine the absorbance of each sample in the colorimeter at a wavelength of 630 nm compared to water.

Calculate the percentage of glucose consumption (loss) in the individual samples. We assume that in the control sample **0**, the consumption of glucose is 0%. Glucose consumption is calculated according to the following formula:

$$\text{Glucose consumption} = \frac{(A_0 - A_n)}{A_0} \times 100\%$$

A_0 - absorbance of the "0" sample.
A_n - absorbance the "n" sample number (according to Table III)

Present the results in Table III.

Table III Glucose consumption in the individual samples.

Sample no. (n)	**A_{630}**	**$A_0 - A_n$**	**Glucose consumption** [%]
0		0	0
1			
2			
3			
4			
5			
6			
7			

Interpret the results of the conducted experiment, the dependency of glucose consumption on the presence of the cofactors and inhibitors of glycolysis must be assessed.

Assess:

- influence of ATP and NAD^+
- effects of citrate, iodoacetate and fluoride.

Glycogen synthesis and degradation

Aim of the exercise: ***to observe glycogen synthesis and breakdown***

Glycogen is the storage energy material, stored mainly in the liver and muscles. It is a branched homopolysaccharide composed of α-D-glucose residues joined by glycosidic bonds. Straight sections contain glucose residues joined by the α-1,4 bonds, and branches are formed by α-1,6- glycosidic bonds. For 8-10 glucose residues incorporated into the linear chain, there is one branching. These are multistage branchings. The high degree of glycogen chain branching helps fulfil its function. It improves the solubility of the polysaccharide and increases the number of terminal glucose residues, which (depending on need) may be released or may serve as acceptors for the next molecules of this monosaccharide. **Glycogen** acts as **reservoir of glucose**. The amount of glucose stored as glycogen is in dynamic equilibrium, rapidly changing depending on the state of the body and the organs synthesizing it: the muscles and the liver. High blood glucose (in states of satiety) causes that it is captured by liver cells and incorporated into glycogen.

The reduction in blood glucose (hypoglycaemia) increases the disintegration of hepatic glycogen to free glucose, which permeates into the blood, allowing to achieve the transitional state of normoglycaemia. Glucose from this source is transported with the blood and captured by the cells of various organs. Thus, the liver is a warehouse of this energy substrate for other tissues.

Muscle cells store glucose as glycogen for their own needs. Glycogen is broken down during a high demand for glucose, for example during exercise.

Glycogen synthesis proceeds in the same way in both tissues. It is a multistage process. For it to initiate, a fragment acting as a primer of this reaction is needed, in addition to the glucose. It is easily accessible in the cell, in which the supply of glycogen has not been fully used up. In the absence of a primer derived from the disintegration of a pre-existing glycogen molecule, it must be synthesized *de novo*. There is a specific protein - **glycogenin**, which serves as the acceptor of glucose residues. The transfer of the first glucose molecule from UDP-glucose to glycogenin is catalyzed by the *glycogen*

primer synthase. Glycogenin can bond subsequent glucose residues from UDP-glucose and a short oligosaccharidic chain (primer) forms, which becomes the acceptor of the subsequent glucose residues. Several enzymes convert glucose to glucose-6-phosphate, then to glucose-1-phosphate and UDP-glucose. During these changes the phosphate residues are released - their number can be a measure of the rate of glycogen synthesis. The principle of inorganic phosphate determination is based on the reaction of phosphate with ammonium molybdate (VI). The obtained phosphomolybdate transforms into "molybdenum blue" (a mixture of molybdenum oxides at a lower degree of oxidation) with the participation of the reducing agent, which is eikonogen (a solution of 1-amino-2-naphthol-4-sulfonic acid in 12% sodium pyrosulfite and 1.2% sodium sulphate (IV)).

In the final stages, ***glycogen synthase*** elongates the glycogen chains as a result of attaching glucose from UDP-glucose to the non-reducing end of the growing chain. The elongation product is a linear unbranched glycogen chain composed of glucose residues linked solely with α-1,4 bonds. The branchings of the glycogen chain are formed by the action of *1,4-1,6-transglucosidase*, also called the *branching enzyme*.

The **breakdown of glycogen** occurs through phosphorolysis involving ***glycogen phosphorylase*** and inorganic phosphate. *Glycogen phosphorylase* takes the form **"a" - active** (phosphorylated) or the form **"b" - inactive** (not phosphorylated). The product of phosphorolysis – glucose 1-phosphate is converted to glucose 6-phosphate, which is included in glycolysis in the muscle. However, in the liver cells there is an additional enzyme - *glucose-6-phosphatase*, which releases glucose from glucose 6-phosphate. As a result, the free glucose penetrates through the cell membrane into the blood and is delivered to other organs. A supporting mechanism of phosphorolysis is hydrolytic degradation.

Not only are the low levels of glucose concentration stimulators of glycogen degradation, but also the high content of 5'-AMP. In the muscle (in contrast to the liver), 5'-AMP activates *phosphorylase* regardless of enzyme phosphorylation.

The glycogen degradation process can be assessed by determining the amount of released glucose using the ortho-toluidin method described in the chapter: *Glucose consumption in the brain*.

E X E R C I S E

I. Preparation of rat skeletal muscle and liver homogenates

Cut out the skeletal muscles from a rat's hind limbs and the liver, and place them in beakers with cooled 0.9% NaCl solution. Weigh two portions of 1 g of each tissue to new, dry beakers and cut them with scissors. Next, homogenize in 5 ml of cooled 0.9% NaCl solution.

II. Assessment of glycogen synthesis

Prepare eight 1.5 ml test tubes for centrifuge (Eppendorf type) labelled as follows:

LA – 0	**LA – 20**	**MA – 0**	**MA – 20**
LB – 0	**LB – 20**	**MB – 0**	**MB – 20**

The letters indicate tissue types (L - liver, M - muscle) and the reacting systems (A, B), and the numbers indicate the incubation time in minutes.

To all tubes, add 0.5 ml of 7% chloric acid (VII) solution.

1. Preparation of samples for incubation

Prepare four incubation test tubes (plastic, with a capacity of 10 ml) marked:

LA and LB - for the liver homogenate,
MA and MB - for the skeletal muscle homogenate.

To the incubation test tubes, measure the reagents according to the table:

Reacting system	A	B
0.3M glucose 1-phosphate solution	50 µl	200 µl
H_2O distilled	150 µl	-

The **A** reacting system assesses basic glycogen biosynthesis, and the **B** system assesses the impact of the increase in substrate concentration on glycogen biosynthesis.

2. Incubation

Insert all incubation test tubes in a water bath at 37°C to heat the reactive solutions to this temperature. Then, to the incubation test tubes of the **L** system, add 1.5 ml of liver homogenate, mix and immediately collect 0.5 ml of the sample, transfer to previously prepared test tubes for centrifuge labelled with the **L0** symbol and mix thoroughly.

To the incubation test tubes of the **M** system, add 1.5 ml of muscle homogenate, mix and immediately collect 0.5 ml of the sample, transfer to previously prepared test tubes for centrifuge labelled with the **M0** symbol and mix thoroughly.

Then, incubate all the samples for 20 more minutes in a water bath at 37°C, stirring frequently. After that time, collect 0.5 ml of the sample from each test tube, transfer to previously prepared test tubes for centrifuge labelled with the appropriate symbols (**L20** and **M20**) and mix thoroughly.

3. Determination of inorganic phosphate

Centrifuge all the samples collected from the **A** and **B** systems for 5 minutes at 3000 rpm. After centrifugation, collect 50 microlitres of supernatant, transfer to clean, appropriately labelled test tubes, and then add (in the order listed): 2.5 ml of distilled water, 1.5 ml of 10% trichloroacetic acid - TCA (make sure to add the acid solution of the appropriate concentration), 0.5 ml of molybdic reagent and 0.2 ml of reagent with eikonogen. Mix the sample thoroughly. At the same time, prepare control sample consisting of: 2.5 ml of distilled water, 1.5 ml of 10% TCA, 0.5 ml of molybdic reagent, and 0.2 ml of eikonogen (mix the samples).

Let all samples sit at room temperature for 30 minutes. After that time, read the absorbance of each sample in the colorimeter at a wavelength of 690 nm compared to the control sample.

4. Description of the results

Calculate the amount of released phosphate based on the following formula:

Amount of phosphate = 177 × (A_{20} – A_0) [micromoles / g of tissue]

177 - numerical coefficient, taking into account the molar calibration factor and the method of preparing the homogenate and the samples.

III. Assessment of glycogen degradation

Prepare eight 1.5 ml test tubes for centrifuge (Eppendorf type) labelled as follows:

LC – 0	**LC – 20**	**MC – 0**	**MC – 20**
LD – 0	**LD – 20**	**MD – 0**	**MD – 20**

The letters indicate tissue types (L - liver, M - muscle) and the reacting systems (C, D), and the numbers indicate the incubation time in minutes.

To all test tubes for centrifuge, add 0.5 ml of 20% trichloroacetic acid solution - TCA (make sure to add the acid solution of the appropriate concentration).

1. Preparation of samples for incubation

Prepare 4 incubation test tubes (plastic, 10 ml volume) inscribed as follows:

LC and LD - for the liver homogenate,
MC and MD - for the skeletal muscle homogenate.

To the incubation test tubes, measure the reagents according to the table:

Reacting system	C	D
10% glycogen solution	100 µl	100 µl
40 mM AMP solution	-	100 µl
distilled H_2O	100 µl	-

The **C** reacting system assesses the basic degradation of glycogen, and the **D** system assesses the impact of AMP on glycogen degradation.

2. Incubation

Insert all incubation test tubes in a water bath at 37°C to heat the samples. Then, to all the incubation test tubes of the **L** reacting system, add 1.5 ml of liver homogenate, mix and immediately collect 0.5 ml of the sample to previously prepared test tubes for centrifuge labelled with the **L0** symbol and mix thoroughly.

To all incubation test tubes of the **M** system, add 1.5 ml of muscle homogenate, mix and immediately collect 0.5 ml of the sample to previously prepared test tubes for centrifuge labelled with the **M0** symbol and mix thoroughly.

Then, incubate all the samples for 20 more minutes in a water bath at 37°C, stirring frequently. After that time, collect 0.5 ml of the sample to the appropriate test tubes for centrifuge labelled **L20** and **M20**, and mix thoroughly.

3. Determination of the glucose amounts

Leave all the centrifuge test tubes of the **C** and **D** systems for 10 minutes at room temperature. Then, centrifuge them for 5 minutes at 3000 rpm. After centrifugation, collect 0.5 ml of supernatant and transfer to clean, appropriately labelled test tubes with a stopper. Add 2 ml of ortho-toluidin reagent, close, mix and place into a boiling water bath for exactly 8 minutes. Next, cool the samples (without opening), and determine the absorbance in

the colorimeter at a wavelength of 630 nm compared to distilled water (at too high absorbances dilute the samples by adding 2.5 ml of distilled water).

4. Description of the results

Calculate the amount of released glucose based on the following formula:

$$\text{Amount of glucose} = 74 \times (A_{20} - A_0) \text{ [micromoles / g of tissue]}$$

74 - numerical coefficient, taking into account the molar calibration factor and the method of preparing the homogenate and the samples.

IV. Analysis of the results

Based on the obtained results, discuss the process of synthesis and degradation of glycogen in both tested organs. Assess the impact of substrate concentrations and AMP on the course of these reactions.

Synthesis and degradation of starch

Aim of the exercise: ***to observe the course of synthesis and degradation of starch***

Starch is a polysaccharide made up of many glucose residues bound by α-glycosidic bonds. In the plant world, it serves as a storage energy substrate. It consists of two fractions: **amylose** and **amylopectin**. Amylose creates straight chains. Only α-1,4-glycosidic bonds occur between glucose residues Amylopectin is a branched polysaccharide. In addition to α-1,4-glucosidic bonds, it also has α-1,6-glycosidic bonds forming polysaccharide chain branching. The molecular weight of starch chains is highly variable and ranges from 2 to 20 kDa.

A characteristic property of starch is the **reaction with iodine**. The reagent Lugol's solution, which contains I_2 in KI solution, is used. Particles of starch concentrate free iodine on their surface. Amylose is coloured blue by iodine, and amylopectin purple.

Phosphorolytic degradation of starch in plant tissues is catalyzed by *starch phosphorylase*. The action of this enzyme with the participation of inorganic phosphate causes the sequential disconnection of isolated glucose residues from the non-reducing end of the starch chain. The -OH group at the anomeric carbon (C-1) of glucose, formed as a result of disintegration of the glycosidic bond, binds with phosphate. Glucose 1-phosphate forms. The degradation of the ester bond in the alkaline environment and the accompanying change of the glucose ring form into chain form causes that in position C^1, the aldehyde group with reducing properties occurs.

Reducing sugars can be detected using the test with Benedict's reagent. Copper (II) hydroxide - $Cu(OH)_2$ is reduced to an orange copper (I) oxide - Cu_2O. The sample must be heated in a boiling water bath. If reducing sugars are present, an orange precipitate of copper (I) oxide forms. The intensity of the colour depends on the amount of reducing sugars in the tested sample.

The degradation of starch also occurs with the participation of **hydrolytic enzymes** - *amylases*. Due to their activity, we distinguish *α-*

amylase and *β-amylase*. The first one (*α-amylase*) hydrolyses α-1,4-glycosidic bonds within the starch chain, creating as a result a mixture of maltose (disaccharide) and oligosaccharides (dextrins). The second one (*β-amylase*) detaches maltose molecules from starch from the non-reducing end. A measure of the progress of starch hydrolysis catalyzed by both *amylases* is the emergence and increasing quantities of reducing sugars and the gradual disappearance of the ability of the disintegrating starch to interact with iodine.

The **synthesis of starch** in plant tissues can occur by reversal of the phosphorolysis reaction. During this process, the glucose residue is collected from glucose 1-phosphate and transferred to the acceptor, called a primer. The role of the primer is played by the already existing fragment of oligo- or polysaccharide, whose non-reducing end is elongated by the attachment of subsequent glucose residues obtained from glucose 1-phosphate. The synthesis product can be detected through a reaction with Lugol's solution. The characteristic for starch violet-blue colour (which appears shortly after the addition of Lugol's solution) becomes more vibrant in the course of the synthesis reaction of this polysaccharide.

Starch synthesis by reversal of the phosphorolysis reaction is of secondary importance *in vivo*. The main pathway of polysaccharide synthesis takes place with the participation of UDP-glucose, just as in the synthesis of glycogen in the liver and muscles.

In laboratory conditions, we can perform starch hydrolysis in diluted sulphuric acid. As the time of hydrolysis passes, the amount of released glucose increases. There is a progressive loss of reaction with Lugol's solution (substrate consumption) and increase in the intensity of reducing tests with Benedict's reagent (increase in hydrolysis products).

E X E R C I S E

1. Enzymatic synthesis of starch

a. preparation of *phosphorylase*

Peel and grate two clean potatoes. Transfer the potato pulp to a mortar and add 50 ml of distilled water. After mixing thoroughly, insert the mortar with the pulp into the freezer for 10-15 minutes, and then squeeze the pulp in a linen bag. Centrifuge the filtrate for 10 minutes at 3000 rpm. The liquid above the precipitate contains *phosphorylase*.

b. preparation of 2% glucose-1-phosphate solution

Dissolve 100 mg glucose 1-phosphate in 5 ml of distilled water.

c. observation of the course of starch synthesis

To 1 ml of *phosphorylase* solution, add 1 ml of 2% glucose 1-phosphate solution and no more than 2 drops of 0.4% starch gruel (reaction primer) and mix. Carry out the incubation at room temperature. At the appropriate intervals: after 5 min., 15 min., 30 min., 45 min., 1 hr. and 1.5 hours collect onto a watch glass a sample of incubated mixture (2-3 drops) and add 1-2 drops of Lugol's solution.

2. Phosphorolysis of starch

Collect 10 ml of 1.9% starch gruel containing phosphate buffer, pH 6.8 into the test tube. Add 2.5 ml of freshly prepared solution of *phosphorylase* (potato extract). Mix the contents. Collect 0.5 ml of this mixture and perform a test for the presence of reducing sugars.

Divide the remaining liquid into two parts. To one part add 2 drops of toluene and leave at room temperature for 24 hours. Heat the second part in a boiling water bath for 10 minutes to inactivate the *phosphorylase*, and then cool. Add 2 drops of toluene and leave at room temperature for 24 hours. Both samples should be appropriately labelled.

Asses the course of phosphorolysis in the next exercise by performing tests for the presence of reducing sugars as described in the chapter *Carbohydrates* (page No. 36, point 2b).

3. Starch acid hydrolysis

Transfer to a beaker 30 ml of 1.7% starch gruel. Add to it 0.5 ml concentrated H_2SO_4 and mix. Heat the gruel with acid in a boiling water bath. Every 5 minutes, collect two samples of 0.5 ml each. Examine one for the presence of starch - perform a test with Lugol's solution, and alkalize the second with 2M NaOH solution using the universal litmus paper as a guide, and test for the presence of reducing sugars (Benedict's test). Continue incubation until the end of hydrolysis.

4. Enzymatic hydrolysis of starch

a. preparation of *β-amylase*

Grind about 10 g of dry barley malt in a mortar, transfer to a conical flask and pour 60 ml of distilled water. Mix thoroughly and leave for 1 hour at 37°C, then filter. The resulting extract contains *β-amylase*.

b. the course of the hydrolysis

To a high-beaker, collect 25 ml of 0.8% starch gruel in phosphate buffer, pH 6.8. Add 0.25 ml of 30% NaCl solution. Heat the gruel to a temperature of 37°C and add 5 ml of *β-amylase* extract. Incubate the mixture in a water bath at 37°C. Observe the course of the reaction by collecting every 5 minutes two 1 ml samples and performing parallel tests for the presence of starch (with Lugol's solution) and reducing sugars (Benedict's reagent).

Catalase

Aim of the exercise: ***to learn about one of the methods of enzyme isolation from biological material and measuring its activity***

Catalase is an enzyme that belongs to a class of *oxidoreductases*. It is a haemoprotein with four iron atoms. This enzyme catalyzes the degradation of hydrogen peroxide into water and oxygen, according to the equation:

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

The biochemical function of *catalase* is the degradation of toxic hydrogen peroxide, which is formed primarily during the oxidation of amino acids with the participation of *amino acid oxidases*, interacting with flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD). *Catalase* is one of the most active enzymes. It is present in every cell. A particularly large amount of this enzyme occurs in the liver and the red blood cells.

Catalase is very sensitive to inhibition. Even the substrate (H_2O_2), especially in higher concentrations, results in the enzyme inactivation. A number of compounds that react with Fe^{3+} also have an inhibitory effect, for example: cyanides, azides, fluorides. Sulphuric acid immediately inactivates *catalase* and stops the reaction of H_2O_2 degradation.

Enzyme activity is measured in terms of providing an excess of the substrate (zero-order reaction), temperature of 30°C, the presence of cofactors, and optimum pH for its action. *Catalase* activity is expressed in the amount of H_2O_2 distributed in a time unit. At the beginning of the reaction (time 0), in the reacting system there is an excess of substrate. During the reaction, gradual consumption of the substrate occurs with a simultaneous slow inactivation of the enzyme, which is why over time the reaction velocity decreases.

We denote the content of H_2O_2 in the reacting system at the start of the reaction - at time 0 and after its cessation by sulphuric acid - at time *t*. We determine the remaining quantity of H_2O_2 in the obtained samples

iodometrically. To do this, KI solution is added. In an acidic environment, KI transforms into HI, and H_2O_2 oxidizes HI to free iodine. The role of the reaction catalyst is played by molybdic (VI) acid.

$$H^+ + KI \rightarrow HI + K^+$$

$$H_2O_2 + 2HI \rightarrow I_2 + 2H_2O$$

One mole of H_2O_2 releases 1 mole of molecular iodine. Free iodine is titrated with sodium thiosulphate in the presence of starch gruel, which is coloured blue by iodine. The reduction of I_2 to $2I^-$ (at the expense of oxidation of the sulphur contained in the thiosulphate) causes a discoloration of the solution.

$$2Na_2S_2O_3 + I \rightarrow 2NaI + Na_2S_4O_6$$

The volume of the sodium thiosulphate solution used allows to calculate the amount of H_2O_2 remaining in the individual samples. The difference in the volume of used thiosulphate in the titration sample "0" and "t" is the amount of H_2O_2 distributed in time *t*.

We express ***catalase*** **activity** in katals (the number of moles of substrate converted in 1 second) or in International Units (the number of micromoles of substrate converted in 1 minute). When calculating enzyme activity per gram of tissue, you should take into account the dilution of *catalase* during its preparation.

E X E R C I S E

1. Catalase preparation

Cut five grams of fresh bovine liver into small pieces and homogenize in 10 ml of H_2O using a piston homogenizer. Transfer the homogenate into a conical flask, add 5 ml of alcohol and chloroform (1:1) mixture, seal it tightly and shake vigorously for about 1 minute. Most of the proteins contained in the homogenate will precipitate. Centrifuge the resulting suspension at a speed of 5000 rpm for 5 minutes. *Catalase* will remain in the supernatant (basal liquid). Collect the supernatant, measure its volume (note down) and

gradually add portions of ammonium sulphate (3 g per every 10 ml of the supernatant). Stir the preparation constantly until the added salts completely dissolves. *Catalase*, under the action of ammonium sulphate, precipitates out of the solution and transits into sediment, which must be separated by centrifugation at 5000 rpm for 15 minutes. Discard the supernatant, and dissolve the precipitate (containing *catalase*) in 0.15 M phosphate buffer (pH 7.2) of the volume equal to the amount of supernatant obtained during the first centrifugation. The resulting solution contains *catalase*.

Dilute the enzyme in two stages using two 50 ml volumetric flasks. Prepare the **first** dilution by combining 1 ml of *catalase* solution with 19 ml of 0.15 M phosphate buffer (pH7.2), and mix thoroughly (20-fold dilution). Prepare the **second**, final dilution by mixing 1 ml of first *catalase* dilution (diluted 20-fold) with 19 ml of the same buffer. In this way, a 400-fold dilution of the enzyme is obtained. This diluted enzyme should be used for activity measurement.

2. Measurement of *catalase* activity

Prepare three 100 ml conical flasks (or beakers) and mark them: **0, 2, 4**. To each, add 5 ml of 1M H_2SO_4. Prepare a beaker (tall, narrow) containing 24 ml of 10mM H_2O_2 in phosphate buffer and place it in a water bath at 30°C for a few minutes to heat the solution to the temperature of enzymatic reaction. Then, add to this beaker 1 ml of the 400-fold diluted preparation of *catalase*. Mix immediately after adding, collect 5 ml of the mixture and transfer to the flask marked **0**. Collect the next samples (5 ml) **2** and **4** minutes after collecting sample **0**, and transfer to the flasks marked **2** and **4**, respectively.

Next, add 1 ml of 5% KI solution and 0.5 ml of a saturated solution of molybdic acid (VI) to each flask, mix and set aside for 3 minutes. Titrate the separated I_2 with 2.5 mM sodium thiosulphate in two stages. Titrate to a pale yellow colour. Add 3 drops of 0.1% starch gruel. A blue colour appears. Continue titrating the 2.5 mM sodium thiosulphate solution until the complete discoloration of the solution. Note the volume of the sodium thiosulphate solution used (from the beginning of titration until complete sample discoloration).

Express enzyme activity in microkatals and international units per 1 gram of tissue.

3. Results presentation

The following <u>example</u> shows how to calculate *catalase* activity.

For the titration of H_2O_2 contained in sample **0**, 9.2 ml of 2.5 mM thiosulphate was used. For the sample incubated for **2** minutes, 5.4 ml of thiosulphate was used. Within 2 minutes of incubation, such a quantity of hydrogen peroxide reacted which corresponds to 3.8 ml (9.2 - 5.4 = 3.8) of sodium thiosulphate solution.

It is known that one millilitre of 2.5 mM thiosulphate solution is equivalent to one millilitre of 1.25 mM hydrogen peroxide solution. It should be taken into consideration that 1 ml of 1.25 mM solution contains 1.25 micromoles of H_2O_2.

From the following, you can calculate the number of micromoles of degraded substrate:

1.0 ml of 2.5mM $Na_2S_2O_3$	-	1.25 micromole of H_2O_2
3.8 ml of 2.5mM $Na_2S_2O_3$	-	X micromoles of H_2O_2

$$X = 4.75 \text{ micromole of } H_2O_2$$

To express enzyme activity in international units, divide the number of micromoles of degraded hydrogen peroxide by the incubation time in minutes. In this case, the activity will be equal to 2.375 (4.75 ÷ 2) international units.

To calculate the activity of this enzyme in microkatals, divide the number of micromoles of degraded substrate by the incubation time in seconds (4.75 micromoles, 2 minutes = 120 seconds). The calculated activity is approximately 0.04 microkatals per 1 ml of diluted *catalase* solution.

The initial catalase preparation was diluted 400-fold. The initial activity in 1 ml of undiluted *catalase* solution is calculated by multiplying the result obtained by 400 (the dilution of the enzyme). From 5 g of liver tissue, 10 ml of *catalase* was obtained, for example. As a result, catalase activity in 1 g of tissue is calculated by multiplying the initial activity by 10 (initial volume of *catalase* preparation in ml) and dividing by 5 (the weight of tissue in grams).

Gel filtration

Aim of the exercise: ***to use gel filtration (molecular filtration) for protein separation, measurement of their molecular weight and desalination of protein solutions***

Gel filtration is also known as molecular filtration or molecular chromatography on molecular sieves. It is a method of separation of substances contained in solutions based on differences in molecular weight. The separation occurs during the flow of the mixture of substances separated by a column filled with porous particles of especially prepared dextran, known under the trade name **Sephadex.**

Sephadexes are insoluble in water, salt solutions and diluted solutions of acids and bases. They do not have absorption properties, and their hydrophilic nature and ability of gel formation is due to the presence of huge numbers of hydroxyl groups. The chemical modification of dextran involves partial hydrolysis and the establishment of numerous cross-links between individual dextran molecules. As a result, cross-linked three-dimensional structures form. Depending on the reaction conditions, preparations with varying degrees of cross-linking are obtained, only allowing penetration into the porous grains by particles of a certain size. The less cross-linked the Sephadex, the more water it absorbs and the larger the molecules that can penetrate into the interior of the tubules in its grains.

Table I Some properties of different types of Sephadexes

Sephadex type	**Water binding** [ml/g]	**Molecular weight fractionation range** [Da]
G – 10	1.0	to 700
G – 15	1.5	to 1 500
G – 25	2.5	1 000 – 5 000
G – 50	5.0	1 500 – 30 000
G – 75	7.5	3 000 – 70 000
G – 100	10.0	4 000 – 150 000
G – 150	15.0	5 000 – 400 000
G – 200	20.0	5 000 – 800 000

In addition to the Sephadex G, other molecular sieves can be used. Sephadex LH-20 (hydroxypropyl derivative of Sephadex G-25) is used to separate soluble substances in organic solvents (e.g., lipids). Sepharose is the product of agarose cross-linking and is used to separate substances of very big molecular weights. Sepharose 4B separates substances with a molecular weight from 3×10^5 up to 3×10^6, and Sepharose 2B separates substances with a weight from 2×10^6 to 25×10^6. Fractionation based on molecular filtration can also be carried out on Bio-Gel type molecular sieves both natural - agarose (type A Bio-gels) and synthetic ones (type P Bio-gels). The latter are copolymers of acrylamide and N,N'-methylenbisacrylamide. They are more resistant to bacterial enzymes than Sephadexes.

If you fill the chromatography column with Sephadex (or another gel) and apply on top a mixture of substances with different molecular weights, then the compounds with a molecular weight exceeding the upper limit of the resolving range (listed in Table 1) pass through the column without stopping and appear the earliest in the fluid leaking from the column. They are washed out with the smallest volume of fluid. The molecules of these compounds are larger than the diameter of the tubules in the Sephadex's grains, they do not penetrate and leave the column via the shortest route. In the case of proteins with lower molecular weights, a "retardation" of their passage through the column and elution with larger amounts of solvent is observed. Smaller size molecules penetrate into the interior of the tubules in the grains; the smaller their dimensions are in comparison with the diameter of the Sephadex tubules, the deeper they penetrate. Releasing these molecules from inside of the Sephadex spatial lattice is more difficult. **Greater** volumes of fluid are required for their washing out.

Sephadexes and other molecular sieves are used to separate mixtures of substances with different molecular weights, to separate macromolecular compounds from micromolecular compounds (for example protein from inorganic salt – desalting), and to determine molecular weights - mainly of peptides and proteins.

When **determining the molecular weight** using the molecular filtration method, the linear dependence between the elution volume and the common logarithm of molecular weight is utilized. Not all proteins are subject to this rule. It applies to globular proteins (of spherical shape). Fibrillar proteins (of fibrillar shape) do not fully correspond to this dependence.

To measure molecular weights, proteins (or other substances) with known molecular weights should be used and their elution volume measured. This term means the volume of liquid needed to wash out the added protein from the column. Elution volume increases with the decrease of protein molecular weight. The order of protein appearance in the liquid emerging from the column (eluate) is observed using analytic methods, which serve for the detection and quantitative determination of proteins. Most often, a measurement of light absorption at wavelength 280 nm is performed.

The elution volume of proteins with a known molecular weight is measured and a diagram of the dependence between the common logarithm of molecular weight and the elution volume is drawn (calibration curve). On the Y-axis, the common logarithms of molecular weights are marked and on the x-axis the elution volume in millilitres. Then, a protein's (of unknown molecular weight) elution volume is measured and its value is marked on the x-axis. From the calibration curve, a common logarithm of molecular weight, corresponding to the elution volume of the tested protein is read off, and then its molecular weight is calculated.

During the **separation of compounds mixtures** differing in molecular weight, the proper Sephadex (Sepharose or Bio-Gel) should be selected. Its resolving capacity should include the differences in molecular components of the separated mixture. If the purpose of filtration is to separate the proteins from the micromolecular compounds, low numbered gels should be used. The macromolecular proteins then pass through the column without going into the tubules of the Sephadex grains, and the micromolecular salts "fall" into the tubules and wash out with a delay. In other words, the proteins are washed out with a small volume of liquid and the salts with a large volume.

Desalting of protein solution through Sephadex filtration is much faster than other methods used to separate the micromolecular salts from macromolecular proteins. Desalting is carried out on columns filled with Sephadex G-25. In the case of desalting of water-soluble proteins, distilled water is used as the solvent.

EXERCISE

1. Measurement of the molecular weight of cytochrome C

At the top of a column sized 2×40 cm, filled with Sephadex G-100 and rinsed with 0.05 M ammonium bicarbonate solution, apply the entire mixture containing:

0.2 ml of haemoglobin solution (m.w. 68 000 Da)
0.2 ml cytochrome C solution
0.1 ml of methylene blue solution (m. w. 250 Da)

After applying the mixture to the top of the gel, its components should be rinsed. Initially, apply small volumes of elution liquid on top of the gel (2-3 times of about 1 ml), then connect the column to the dish with the liquid (0.05 M NH_4HCO_3). **Air should not be allowed into the column - the gel should constantly be covered with liquid**. Collect the eluate leaking from the column in a graduated cylinder until the haemoglobin emerges (red-orange colour). Next, collect 2 ml fractions until the methylene blue leaves the column. Assess visually in which test tube there is the maximum concentration of haemoglobin, cytochrome C (pink colour) and methylene blue (blue colour). Calculate the elution volume of the individual substances. Present the results in Table II.

Table II.

Separated substances	**Molecular mass** [Da]	**Logarithm of the molecular mass**	**Elution volume** [ml]
Haemoglobin	68 000	4.833	
Methylene blue	250	2.398	
Cytochrome C			

Prepare a calibration graph of the dependence of the elution volumes on the common logarithm of their molecular weight. Read the logarithm of the molecular weight of cytochrome C (of the tested protein) and calculate its molecular weight.

2. Desalting albumin solution

Onto the column with Sephadex G-25 (1×40 cm), washed with distilled water, carefully apply with a pipette a solution containing:

0.5 ml albumin solution
0.3 ml potassium chromate (VI) solution

Put a test tube at the outlet of the column and open the bottom clamp. After the whole sample infiltrates into the gel, wash the column with a small amount of distilled water (2×1 ml) and then continually add larger portions of water. **Air should not be allowed into the column - the gel should constantly be covered with liquid.**

Collect fractions of approximately 2.5 ml until the complete washing out of potassium chromate (VI) (yellow colour). Determine the location of the coloured chromate, and then with each fraction do a test for the presence of protein by adding 0.5 ml of 20% trichloroacetic acid. In the test tubes containing the protein, there will be turbidity or sediment will appear. Observe the separation of protein from salt.

Protein nitrogen, amino acid transamination

Aim of the exercise: ***I - to learn the methods for measuring the nitrogen content in blood serum***
II - to demonstrate glutamate amino-transferase activity in the myocardium

In the tissues and body fluids, the proteins are the main nitrogen-containing macromolecular compounds. In other macromolecular compounds, nitrogen content is negligible. There is the concept of **"protein nitrogen"**, which means nitrogen contained in the amino and amide groups of amino acids and in the rings of: tryptophan, histidine, proline and hydroxyproline - incorporated in the protein chain. The nitrogen content in different proteins is fairly constant at around 16% of the weight of the protein. For this reason, the measurement of nitrogen content in material not containing other nitrogen compounds may be useful for the quantitative determination of protein. Multiplying the determined quantity of nitrogen by 6.25, we get the amount of protein in the tested biological material.

A separate group of compounds constitute the "**non-protein nitrogen**". The name applies as a general description of micromolecular components of plasma or serum containing nitrogen. It mainly includes urea, uric acid, creatine, creatinine and free amino acids and oligopeptides. In order to determine serum "protein nitrogen" and "non-protein nitrogen", you must first separate the protein from the above-mentioned micromolecule compounds. This can be achieved by molecular filtration, for example, or by precipitation of proteins with the appropriate salt concentrations.

The main carriers of nitrogen are the **amino groups** of amino acids. During the degradation of amino acids, amino groups may unlink in the form of ammonia (NH_3) or may be transferred onto keto acids. Ammonia is mostly included in the urea cycle and is excreted from the body in the form of urea. However, the amino groups transferred to keto acids form new amino acids, which may again be incorporated into proteins. The daily "turnover" of protein in the human body weighing 70 kg is about 400 g. Approximately 100 grams of this degrades and the quantity must be substituted with amino acids supplied in the diet. The others are used for the proteins resynthesis or serve as substrates for the synthesis of other biomolecules.

A measure of the metabolic turnover of proteins is the **nitrogen balance**. This is a comparison of the amount of nitrogen assimilated in a day to the quantities of excreted nitrogen. The nitrogen balance can be: **balanced** - when the amount of nitrogen assimilated and excreted is equal, **positive** - when the quantity of nitrogen assimilated is greater than the amount of nitrogen excreted, or **negative** - when the amount of nitrogen assimilated is less than the amount of nitrogen excreted.

I. Measurement of nitrogen content in the blood serum using the Kjeldahl method

During the heating of organic compounds in the presence of concentrated sulphuric acid (VI), their hydrocarbon skeletons oxidize to CO_2 and H_2O; whereas, nitrogen, released in the form of ammonia, is bound by concentrated sulphuric acid to form ammonium sulphate - $(NH_4)_2SO_4$. Under the action of concentrated NaOH, ammonium ion (NH^{4+}) is displaced by sodium ion (Na^+). In reaction with a base, the ammonium ion gives back a proton forming free ammonia (NH_3).

$$NH_4^+ + OH^- \rightarrow NH_3 + H_2O$$

The released ammonia can be distilled and bound with boric acid (III). Ammonium borate forms. The amount of bound ammonia can be determined by titration with titrated H_2SO_4 solution. Sulphate ions displace borate ions.

The Kjeldahl method of nitrogen determination is divided into 3 stages: mineralization, distillation and titration.

1. Mineralization

Mineralization is carried out in concentrated sulphuric acid (VI) in the presence of $CuSO_4$ as a catalyst. To increase the boiling temperature of the mixture potassium or sodium sulphate is added. During the mineralization, volatile substances are formed CO_2, H_2O, SO_2 as well as ammonium (NH_3), which immediately reacts with H_2SO_4 to form ammonium sulphate $(NH_4)_2SO_4$.

$$2NH_3 + H_2SO_4 \rightarrow (NH_4)_2SO_4$$

2. Distillation

Distillation is carried out in the Parnas-Wagner apparatus. The apparatus consists of a large flask used to produce steam, an intermediate part, a distillation flask with a funnel, a condenser and a receiver.

Under the action of concentrated NaOH, ammonium sulphate releases ammonia.

$$(NH_4)_2SO_4 + 2NaOH \rightarrow Na_2SO_4 + 2NH_3$$

The released ammonia passes with the water vapour into the receiver containing boric acid (III) with the addition of Tashiro's indicator solution (an alcohol solution of methyl red and methylene blue). In an acidic environment, this indicator turns the solution violet. The distilled ammonia bonds with the boric acid to form ammonium borate, which causes an increase in pH and the solution colour changes to green.

$$H_3BO_3 + 3NH_3 \rightarrow (NH_4)_3BO_3$$

3. Titration

After complete distillation of ammonia, the ammonium borate solution is titrated with a titrated solution of H_2SO_4 or HCl. The strong acid (H_2SO_4 or HCl) displaces the weak boric acid from its salts.

$$2(NH_4)_3BO_3 + 3\ H_2SO_4 \rightarrow 2\ H_3BO_3 + 3(NH_4)_2SO_4$$

The released boric acid once again acidifies the solution contained in the receiver. Tashiro's indicator once again turns violet.

Knowing the number of millilitres of H_2SO_4 consumed for titration, we calculate the amount of ammonia bonded with boric acid. The amount of secreted ammonia corresponds to the amount of nitrogen contained in the mineralized substance.

This method is applicable to the determination of total nitrogen content (protein nitrogen and non-protein nitrogen) in blood serum. The amount of non-protein nitrogen is determined in the serum after the

precipitation of proteins (e.g., with trichloroacetic acid). The content of protein nitrogen can be calculated from the difference between the total nitrogen content and the non-protein nitrogen quantity.

To obtain reliable results, it is recommended to use laboratory glassware and reagents free of contamination with ammonia.

EXERCISE

1. Mineralisation

Mineralization should be carried out in Kjeldahl flasks underneath the chemical hood.

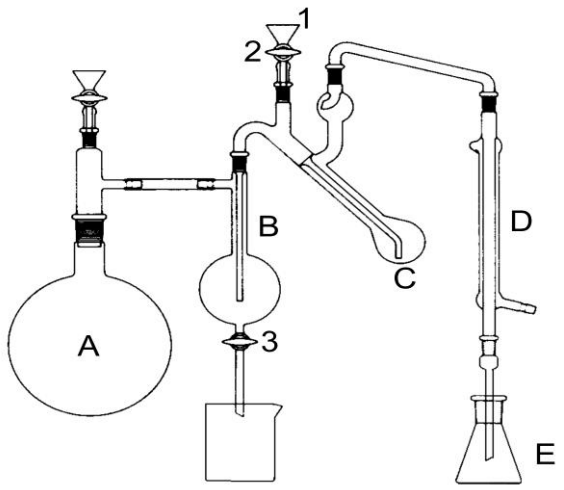
To a Kjeldahl flask, add 0.2 ml of blood serum, 0.2 ml of 40% $CuSO_4$, 1 ml of concentrated H_2SO_4 and about 1 g of K_2SO_4. The mixture will turn brown. Rinse the Kjeldahl flask walls with a little water. Place the flask at a slant (at a 45° angle) and heat over a gas burner, slowly at first to evaporate most of the water, then gradually increase the flame (the addition of glass beads enables self-mixing of the liquid during heating and prevents it from splashing outside). The flask fills with irritating smoke. You must then cover the outlet of the flask with loose glass stoppers (the flask neck acts as a reflux condenser). The released water vapour condenses in the neck, and the formed water returns to the heated fluid preventing excessive concentration of the mixture. During the mineralization, the brown colour fades. The solution brightens and turns blue from the $CuSO_4$. After complete liquid brightening, continue mineralization for 1 hour. After finishing, cool the flask to room temperature and add 3 ml of H_2O. The contents of the flask should be distilled in the Parnas-Wagner apparatus (Fig. 1).

2. Distillation

Prepare the receiver (**E**) - to a high 100 ml beaker measure exactly 10 ml of 4% H_3BO_3 and a few drops of Tashiro's indicator solution.

Put the receiver under the condenser so that its end is immersed in liquid. Move the mineralized substance from the Kjeldahl flask (10 ml) through the funnel (**1**) to the distilling flask (**C**) and add a few drops of methyl red. Alkalize the contents of the distilling flask by pouring through the funnel (**1**) 1 ml of 20% NaOH solution until the fluid colour changes from red to yellow. Rinse the funnel three times with a few ml of distilled water. Bring the water in flask **A** to a boil. Close valves **2** and **3**.

Continue the distillation for 30 seconds from the time of the colour change (from purple to green) of the solution in the receiver. Then set aside the receiver and stop heating. The contents of the distillation flask, as a result of a pressure difference, are passed into the intermediate flask (**B**). Remove the liquid from the intermediate flask by opening valves **2** and **3**. Rinse the distillation flask with distilled water several times.



A – steam production flask
B – intermediate part
C – distillation flask
D - condenser
E - receiver
1 - funnel
2, 3 - valves

Fig. 1. Diagram of the Parnas-Wagner apparatus

3. Titration

Titrate the contents of the receiver with 0.005 M H_2SO_4 until a violet colour appears. Calculate the nitrogen content.

One millimole of H_2SO_4 binds 2 millimoles of NH_3 (2 x 17 = 34 mg), which is equivalent to 28 mg of nitrogen. One millilitre of 0.005 M H_2SO_4

contains 0.005 millimole of acid, which binds 0.01 millimole of ammonia, which is equivalent to 0.14 mg of nitrogen. Multiplying the volume of acid used to titrate by 0.14, we get the number of mg of nitrogen in the tested sample.

$$x = V \times 0.14$$

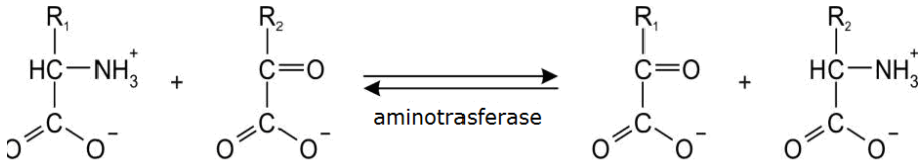
x - nitrogen content in the tested sample (mg)
V - volume of acid used (ml)
0.14 - conversion factor

II. Transamination

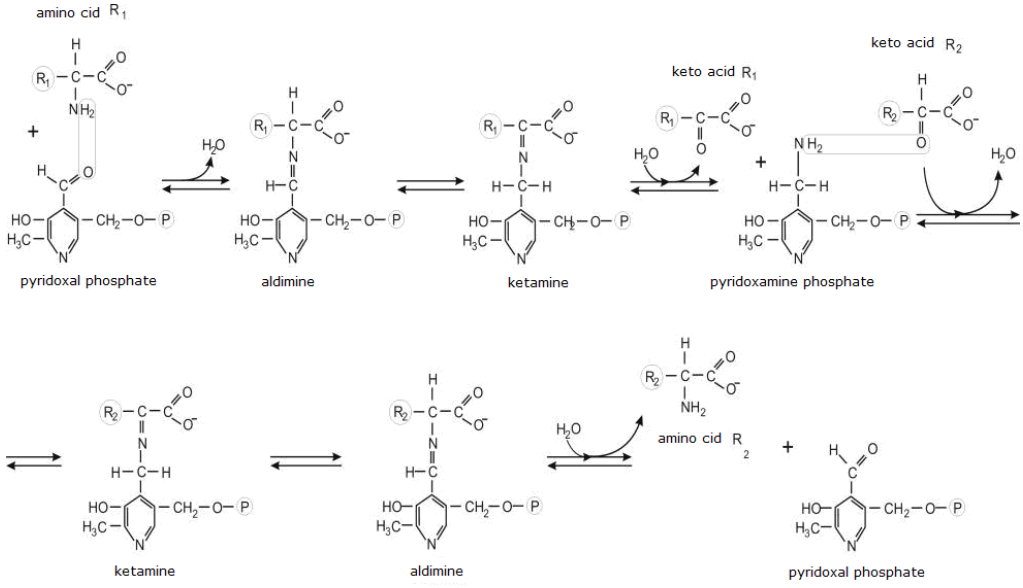
The first stage of amino acid degradation is generally **transamination**, which transfers the amino groups from different amino acids onto one of three α-keto acids: pyruvate, oxaloacetate or α-ketoglutarate. The amino group donors in transamination reactions are almost all amino acids, except lysine and threonine, as well as proline and hydroxyproline. An amino acid deprived of the amino group becomes a keto acid and the keto acid, which added an amino group becomes an amino acid. Therefore, as a result of transamination a new amino acid and a new keto acid are formed.

Transamination reactions are catalyzed by ***aminotrasferases***, whose coenzymes are pyridoxal phosphate and pyridoxamine phosphate. Pyridoxal phosphate forms a complex of a Schiff base with amino acids.

The course of transamination is shown in the following equation.



Pyridoxal phosphate participation in the transamination process is described in the following diagram:



The aim of the exercise is to demonstrate *glutamate aminotransferase* activity in the myocardium. Incubate aspartate solution and α-ketoglutarate solution with an extract of bovine heart muscle. As a result of *glutamate aminotransferase*, the present in the myocardium α-ketoglutarate is converted to glutamate with simultaneous transformation of aspartate to oxaloacetate. Analyze the result of transamination using paper chromatography.

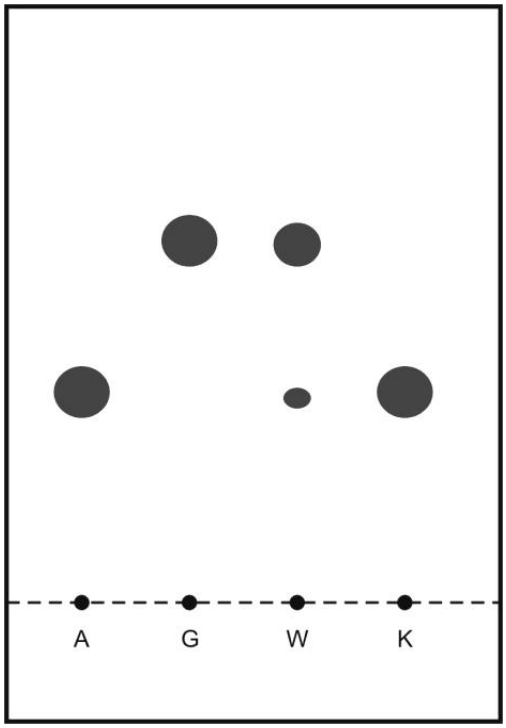
Paper chromatography is a form of liquid chromatography, in which the **solid phase** (resolution) is composed of a sheet of cellulose *Whatman* paper. Separated substances are applied to a spot approximately 3 cm from the bottom edge of the sheet (starting line) and then the paper is placed in the chromatography chamber. The paper is submerged in a few millimetres of a solvent mixture (**mobile phase**), so that the applied substances are above the surface of the solvent. Due to capillary action, the solvent moves gradually upward, dragging behind it at different speeds the ingredients present in the analyzed mixture. When the solvent front approaches the upper edge of the paper, the separation is complete. The individual components of the mixture follow the solvent front at different speeds. Due to the difference in migration velocities of the individual components of the mixture, at the end of the analysis they will be at different heights in relation to the starting line. Each of them corresponds to a single "spot". This phase is called the development of the chromatograph.

When the separated compounds are colourful, these spots can be directly seen on the paper. If the components are colourless, they must be stained with the appropriate reagents. In the case of amino acid chromatography, ninhydrin solution is used.

Each substance in the mixture subjected to paper chromatography is characterized by a specific factor – **Rf** (retention factor). It is expressed as a ratio of the distance travelled by the component of the mixture (S_1) to the distance travelled by the solvent front (S_2).

$$\mathbf{Rf} = \frac{S_1}{S_2}$$

S_1 - the migration distance of a component
S_2 - the migration distance of the solvent



The developed chromatogram shows the result of the process of transamination.

A - aspartate standard
G - glutamate standard
W – investigated sample
K - control sample

EXERCISE

To 2 test tubes measure:

- 0.2 ml of 0.5 M phosphate buffer, pH 8.3
- 0.1 ml of 0.2 M sodium aspartate
- 0.1 ml of 0.2 M α-ketoglutarate solution
- 1.5 ml suspension of myocardium

Mix the contents of one test tube and immediately place it in a boiling water bath for 10 minutes to inactivate the enzyme (control test). After mixing the contents, incubate the second tube at 37°C for 2 hours (real test). After the incubation, filter both mixtures to separate test tubes and perform chromatography.

Cut out a rectangle sized 12×24 cm from the *Whatman* paper. At a distance of 3 cm from the bottom edge of the paper, draw a starting line with a pencil and mark four points at equal distances from one another (A, G, W, K). On these points, apply 3 drops of the appropriate solutions using a micropipette (apply a drop at a time, each time drying the paper):

- on point A - 0.2 M sodium aspartate solution
- on point G - 0.2 M sodium glutamate solution
- on point W - real test filtrate
- on point K - control test filtrate

Suspend the paper in an ammonium chamber for a few minutes to neutralize the acid salts and free amino acids. Then, move it to a chamber containing a mixture of solvents – butanol: water (4:1). Chromatography should be continued until the solvent front is at a height of about 15 cm from the start line. This takes approximately 3-4 hours. Then remove the chromatogram, dry (first at room temperature, then at 90°C), spray with ninhydrin solution and then dry with a stream of hot air.

Calculate and compare the **Rf** for the individual spots. Pay attention to the appearance of spots on the chromatogram with a **Rf** value corresponding to glutamate.

Biochemical calculations

1. Calculate the length of the polypeptide chain containing 120 amino acid residues, if:
   - it occurs in the form of an α-helix;
   - it is fully extended.

2. What is the total length of all polypeptide chains in the bacterial cell if it contains 10^6 molecules of protein and each of these molecules has a molecular weight of 40 kDa and is in the form:
   - α-helix,
   - fully extended?

   Average molecular weight of one amino acid residue is approximately 100 Da.

3. Mammalian cells contain DNA in the amount corresponding to 3.9×10^9 of nucleotide pairs. What is the total length of DNA molecules contained in one cell?

4. Calculate (in katals and international units) the activity of lactate dehydrogenase, which at a temperature of 30°C, in optimal conditions for the action of this enzyme, converted 60 millimoles of lactate to pyruvate, in 5 minutes.

5. Calculate (in katals and international units) urease activity, which has turned a certain amount of urea into gaseous products with a total volume of 134.4 ml (measured in standard conditions) during one minute.

6. How many millimoles of NAD^+ are needed for the conversion of 3.6 g of glucose to acetyl-CoA?

7. How many millimoles of NAD^+ are needed for the conversion of 0.18 g of glucose to CO_2 and H_2O?

8. How many ml of CO_2 will be created as a result of the transformation of 90 mg of glucose into acetyl-CoA?

9. How many moles of ATP will form as a result of the transformation of 0.2 mol of fructose 1,6-bisphosphate into phosphoenolpyruvate?

10. How many moles of ATP will form as a result of the oxidation of 0.9 g of lactate to acetyl-CoA?

11. How many moles of ATP will form as a result of the transformation of 0.5 mol of phosphoenolpyruvate into acetyl-CoA?

12. How many moles of ATP will form as a result of the change of 8.8 g of pyruvate into CO_2 and H_2O?

13. How many moles of ATP will form as a result of the oxidation of 45 mg of glucose to CO_2 and H_2O?

14. How many moles of $NADH+H^+$ will be created as a result of the change of 3.6 g of lactate into CO_2 and H_2O?

15. How many mg of glucose has been oxidized to CO_2 and H_2O, if at the same time 19 mmol of ATP formed?

16. How many micromoles of ATP will be formed by substrate phosphorylation during the change of 5 micromoles of phosphoenolpyruvate into CO_2 and H_2O?

17. How many micromoles of ATP will be created by oxidative phosphorylation during the transformation of 4 micromoles of isocitrate into malate?

18. How many millimoles of ATP will be formed by substrate phosphorylation as a result of 0.9 g of glucose oxidation to CO_2 and H_2O?

19. How many micromoles of ATP will be formed by oxidative phosphorylation during the transformation of 5 micromoles of dihydroxyacetone phosphate to acetyl-CoA?

20. How many ATP moles will be formed as a result of complete oxidation (to CO_2 and H_2O) of the following substrates:
    - 0.2 moles of acetyl-CoA,
    - moles of phosphoenolpyruvate,
    - moles of glyceraldehyde 3-phosphate,
    - moles of 3-phosphoglycerate?

21. How many ml of CO_2 will be released as a result of oxidative decarboxylation of 4.4 mg of pyruvate?

22. How many moles of ATP are necessary for the conversion of 4.4 millimoles of pyruvate to oxaloacetate in the course of gluconeogenesis?

23. How can lactate be used as a substrate in the process of gluconeogenesis? Calculate the energy balance of the transformation of 4.5 g of lactate to oxaloacetate.

24. How many millimoles of $NADPH+H^+$ will be formed as a result of the transformation of 1.8 g of glucose to ribulose 5-phosphate?

25. How many millimoles of $NADPH+H^+$ are required for the biosynthesis of 5 micromoles of arachidic acid (20 C) from active acetate?

26. How many moles of ATP will be formed as a result of β-oxidation of 0.3 moles of lauryl-CoA (12 C)?

27. How many moles of ATP will be formed as a result of complete oxidation (to CO_2 and H_2O) of 0.4 moles of myristyl-CoA (12 C)?

28. How many moles of ATP will be formed as a result of complete oxidation (to CO_2 and H_2O) of 2 moles of arachidic acid (20 C)?

29. How many moles of ATP will be formed as a result of complete oxidation (to CO_2 and H_2O) of 3 moles of glycerol 3-phosphate?

30. How many millimoles of ATP are required for the biosynthesis of 3 millimoles of lecithin from glycerol, free fatty acids and choline?

31. How many moles of ATP are required for the biosynthesis of 0.2 moles of lecithin from the active forms of glycerol, fatty acids and choline?

32. How many millimoles of $NADPH+H^+$ are required for the formation of 0.04 moles of lauric acid (12 C) from acetyl-CoA?

33. Is the amount of $NADPH+H^+$, which has been formed as a result of the transformation of 4.5 g of glucose in pentose phosphate pathway, enough for the biosynthesis of 0.2 moles of lauric acid (12 C)?

34. How many micrograms of alanine got inside cells during the Meister cycle, if 12 micromoles of ATP had been utilized in this process?

35. How can glutamate be used as a substrate in the gluconeogenesis process? Calculate the energy balance of the transformation of 2.94 g of glutamate to oxaloacetate.

36. How can alanine be utilized as a substrate in the gluconeogenesis process? Calculate the energy balance of the conversion of 1.78 mg of alanine to oxaloacetate.

37. Is the amount of ATP, which has been formed as a result of the transformation of 45 mg of fructose to acetyl-CoA, enough for the synthesis of 30 mg of urea?

38. How many ml of gaseous products will be formed as a result of degradation (by urease) of urea, which was formed with the participation of 20 millimoles of aspartate?

39. How many moles of urea will be formed as a result of the complete degradation of 8 grams of protein (containing 16% nitrogen)?

40. How many mg of glucose must be oxidized to CO_2 and H_2O to provide the energy required for the synthesis of 15 mg of urea?

41. How many micromoles of ATP are required for the resyntesis of 20 micromoles of glutathione in the course of the Meister cycle (transport of amino acids)?