Practice 1.

SELECTED METHODS OF SEPARATION AND DETECTION OF BIOMOLECULES DERIVED FROM BIOLOGICAL MATERIALS

Exercise 1. Gel-filtration chromatography – separation of albumin from $(NH_4)_2SO_4$

Principle

Molecules can be separated according to their size by column chromatography using so-called molecular sieves, i.e. cross-linked polymer gels with defined pore size. Smaller molecules require larger volume of the mobile phase for their elution because they diffuse into the pores of the polymer gel and therefore move through the column more slowly than larger molecules. Large molecules elute more quickly due to the fact that they enter less or do not enter at all into the pores of the polymer gel. Both molecular weight and three-dimensional shape contribute to the degree of retention. Gel-filtration chromatography may be used for analysis of molecular size, for separations of components in a mixture, or for salt removal or buffer exchange from a preparation of macromolecules.

Reagents and accessories
Chromatography gel (stationary phase): Sephadex G-25 (presoaked); Sephadex is commercially prepared by cross-linking dextran with epichlorohydrin;
Solution for elution (mobile phase): distilled water;
Sample: colloid solution of proteins in $(NH_4)_2SO_4$;
Reagent for determination of sulphates: $BaCl_2$ solution;
Chromatography column, cotton wool, stand and clamp, test tubes, spectrophotometer, quartz cuvette.

Procedure

The chromatography column is already prepared in a following way: wet cotton wool is placed at the column bottom; previously well mixed gel suspension is carefully poured into the column, avoiding formation of air bubbles; gel settles within the column and forms homogenous stack; gel is washed out with approximately 2 mL of water, the clamp is fastened.

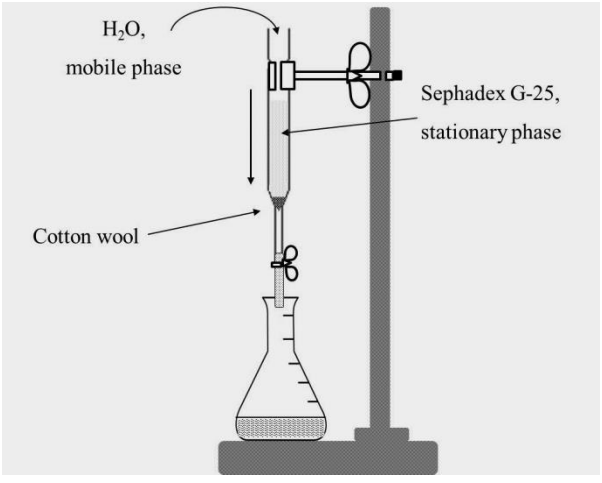


Figure 1. Gel-filtration chromatography

- Change the test tube under the column and carefully load the 100 μL sample mixture onto the gel, taking care not to disturb the gel.

- Subsequent to the sample loading onto the column, slowly loosen the clamp and start the elution, by draining each 2 mL of eluent into test tubes. Fractions obtained in such way should be analyzed for the presence of proteins and sulphates. Pour the eluate from every single test tube into quartz cuvette and measure the absorbance by spectrophotometer at 280 nm, using distilled water as a blank probe.

- Carry out the test for sulphates using a few drops of the $BaCl_2$ solution in each single fraction.

- Write down the precipitation reaction of barium sulphate (write balanced molecular and net ionic equations).

_____

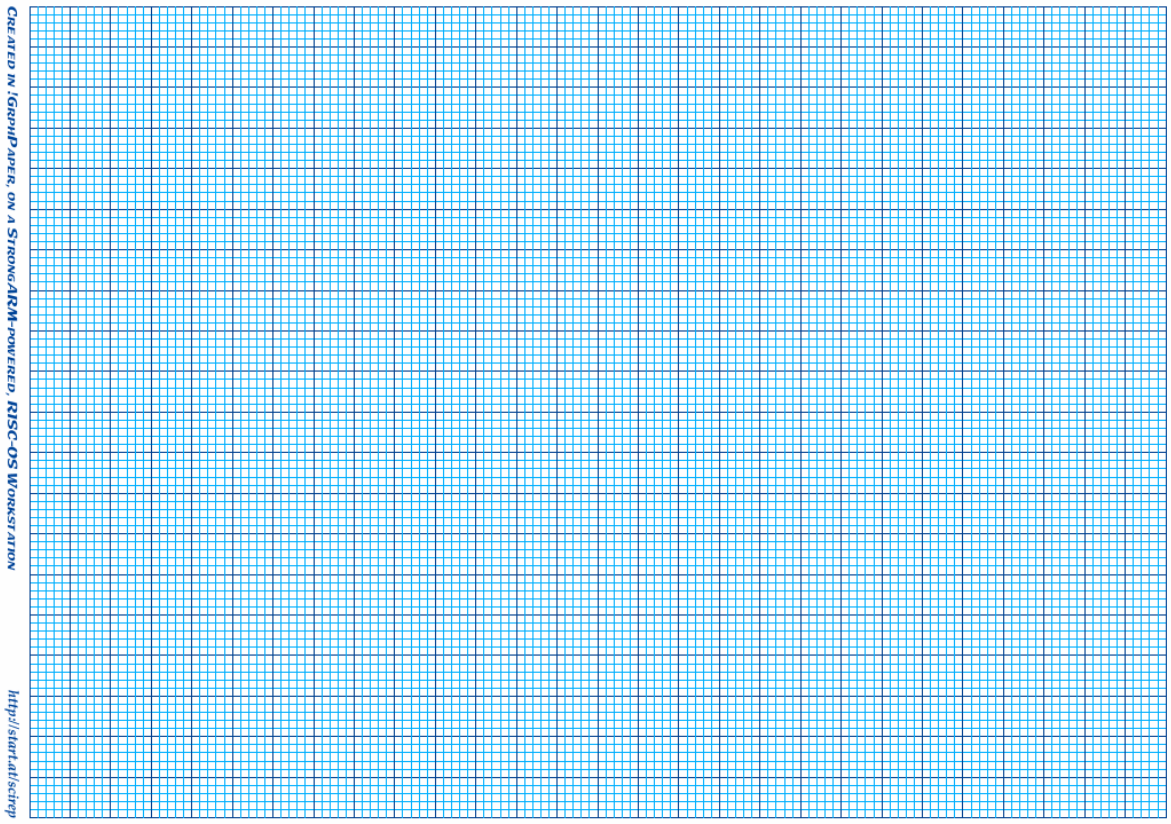
_____

Note

Protein solutions absorb light of 215 nm (peptide bonds) and of 280 nm (aromatic tyrosine rings). The method is very sensitive, and the absorbance is mainly measured at 280 nm. In this way, it is possible to easily determine total proteins concentration.

Results and graphical presentation

Fraction No:	1	2	3	4	5	6	7
Proteins A_{280}							
SO_4^{2-} Positive (+) Negative (-)							



Exercise 2. Determine the composition of sugar mixture using thin-layer chromatography

Reagents and accessories:
Plate for thin-layer chromatography (silica gel);
Mobile phase: ethyl acetate, acetic acid, methanol and water in volumetric ratio 60:15:15:10;
Reagent for color developing: 6% ethanol solution of orcinol + 1% solution of $FeCl_3$ in 10% solution of H_2SO_4 in volumetric ratio 1:10.
Standard solution of sugar;
Sample: sugar mixture;

Procedure:
On the chromatography plate, mark lightly the starting line (start) 1 cm above the bottom edge of the plate using graphite pencil, and the front line 0.5 cm below the upper edge of the plate. Apply the standard 0.5 cm from the left edge and the sample 0.5 cm from the right edge so that distance between them in not less than 1 cm. For applying the standard and the sample in the horizontal thin layer use capillary and leave the plate to dry out. Fill the chromatographic bathtub with the freshly prepared mobile phase, vertically plunge the chromatography plate and cover it. Because of the capillary force, solvent mixture moves across the plate and drags sugars with it and they will stop at different distances (heights).
After approximately 30 minutes or when solvent front reaches upper edge of the chromatography plate take it out and dry the plate. Spray the dry plate with the developing color solution and leave 3 minutes in a dryer on 100 ºC. Based on the color and R_f value, determine which sugars are present in the sample.

Colored reactions on sugars

Sugar	Anisaldehyde-sulphate acid	Orcinol-sulphate acid
Ribose	blue	gray
Xylose	gray	light blue
Arabinose	yellow-green	blue-gray
Fructose	violet	dark red
Mannose	green	light blue
Glucose	light blue	gray-blue
Galactose	gray-green	gray-blue
Sucrose	violet	red-brown
Lactose	greenish	red-violet

Standard	R_f	Sample	R_f
1. Xylose			
2. Glucose			
3. Sucrose			
4. Lactose			

Sugars in the above table are listed according to the chromatographic mobility, from the most mobile (1) to the least mobile structure (4).

Which sugars have you detected in the sample?

_____

_____

Schematically represent your thin-layer chromatography result!

Exercise 3. Detection of human chorionic gonadotropin (hCG) in urine, using immunochemical method – pregnancy test

Human chorionic gonadotropin (hCG) is a peptide hormone produced by the placenta during pregnancy. Simple and rapid pregnancy test is based on immunochemical reaction in which specific antibody (Ab) recognizes its specific antigen – hCG.

Principle

Direct ELISA (enzyme-linked-immunosorbent assay) is the method used for detection of human chorionic gonadotropin (hCG). Specific primary anti-hCG antibody is attached to a microtiter plate surface. In the case of presence of the antigen (hCG) in a urine sample, specific binding of antibody and antigen occurs. Visualisation of the antigen-antibody reaction is performed using secondary antibody conjugated to enzyme alkaline phosphatase and its reaction substrates (BCIP, 5-bromo-4-chloro-3'-indolyl-phosphate; NBT, nitrotetrazolium blue). Due to catalytic activity of alkaline phosphatase a blue coloration, resulting from formation of the insoluble compound NBT-diformazane, appears in a reaction mixture. Positive reaction indicates a presence of increased amount of hCG in urine and thus confirms the pregnancy.

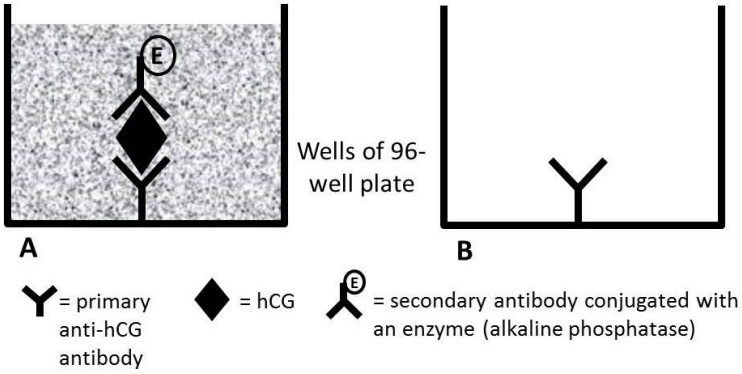


Figure 4. The principle of direct ELISA (explained in the text above). A) hCG is present in the sample. B) hCG is not present in the sample.

Reagents and accessories
Urine samples
Coloration buffer: 0.1 M Tris-HCl, 100 mM NaCl, 4 mM $MgCl_2$, pH=9,5
Anti-hCG antibody conjugated to alkaline phosphatase
Substrate BCIP (5-bromo-4-chloro-3-indolyl phosphate), 50 mg/mL (stock-solution)
Substrate NBT (nitrotetrazolium blue chloride, nitroblue), 10 mg/mL (stock-solution)
Microtiter plate, pipettes, pipette tips

Procedure

- Apply the samples into prepared microtiter plate wells, as follows:
  - positive control
  - negative control
  - sample
  (Note: For pipetting, use new, clean tips for each sample!)
- Add 84 µL of coloration buffer into each microtiter well containing a sample.
  (Note: For pipetting a buffer, you may use the same pipette tip but should not dip the tip into the solution!)
- Add 8 µL of BCIP and 8 µL NBT into each microtiter well.
  (Note: For pipetting, use clean tips for each substrate!)

	V sample (µL)	*V* buffer (µL)	*V* BCIP (µL)	*V* NBT (µL)
positive control	5	84	8	8
negative control	5	84	8	8
sample	5	84	8	8

"Pregnancy test" result: POSITIVE/NEGATIVE

Date: _____ Signature: _____

Practice 2.

AMINO ACIDS AND PROTEINS

1. What characterizes majority of naturally occurring amino acids?

_____

_____

_____

2. What characterizes the isoelectric point of amino acids?

_____

_____

_____

3. Present all ionic forms and calculate the isoelectric point of the following amino acid _____, using the data from table 5.1.

Table 5.1. Properties of amino acids.

Amino acid	Abbreviation/ symbol	M_r	pK_a values		
			pK_1 (—COOH)	pK_2 ($—NH_3^+$)	pK_R (R group)
Nonpolar, aliphatic R groups					
Glycine	Gly G	75	2.34	9.60	
Alanine	Ala A	89	2.34	9.69	
Proline	Pro P	115	1.99	10.96	
Valine	Val V	117	2.32	9.62	
Leucine	Leu L	131	2.36	9.60	
Isoleucine	Ile I	131	2.36	9.68	
Methionine	Met M	149	2.28	9.21	
Aromatic R groups					
Phenylalanine	Phe F	165	1.83	9.13	
Tyrosine	Tyr Y	181	2.20	9.11	10.07
Tryptophan	Trp W	204	2.38	9.39	
Polar, uncharged R groups					
Serine	Ser S	105	2.21	9.15	
Threonine	Thr T	119	2.11	9.62	
Cysteine	Cys C	121	1.96	10.28	8.18
Asparagine	Asn N	132	2.02	8.80	
Glutamine	Gln Q	146	2.17	9.13	
Positively charged R groups					
Lysine	Lys K	146	2.18	8.95	10.53
Histidine	His H	155	1.82	9.17	6.00
Arginine	Arg R	174	2.17	9.04	12.48
Negatively charged R groups					
Aspartate	Asp D	133	1.88	9.60	3.65
Glutamate	Glu E	147	2.19	9.67	4.25

Calculation:

pI = _____

Proteins

Beside water, proteins represent quantitatively the most important component of all cells and plasma. Variety and significance of their functions are obvious from the fact that proteins are present in all biological processes, whether they take part in these processes themselves or have a regulatory role.

Exercise 1. Determination of protein concentration using the Lowry method

Principle

Method is based on the formation of colored complex by reaction of Cu^{2+} ions in basic medium and nitrogen atoms from polypeptide or protein chain, and also by reduction of phosphomolybdate and phosphowolphramate in Folin-Ciocalteu reagent by the effect of phenol compound, e.g. tyrosine. Generated complex is blue, obtains the absorption maximum at 740 nm and is stable up to 1 hour. Sensitivity of the method is 10 – 100 μg of protein.

Reagents and accessories
A: 2% solution of Na_2CO_3 in 0.1 M NaOH (non-water Na_2CO_3);
B: 0.5% solution of $CuSO_4$ x 5 H_2O;
C: 2% solution of K, Na-tartarate;
Lowry reagent: 100 mL of solution A + 1 mL of solution B + 1 mL of solution C;
Folin-Ciocalteu reagent: 1 mL of Folin-Ciocalteu solution + 2 mL of distilled water;
Standard solution: bovine serum albumin in water, 1 mg/mL;
Sample: albumin solution of unknown concentration;
Spectrophotometer, vibration blender (Vortex), test tubes 12x100 mm, automatic pipette.

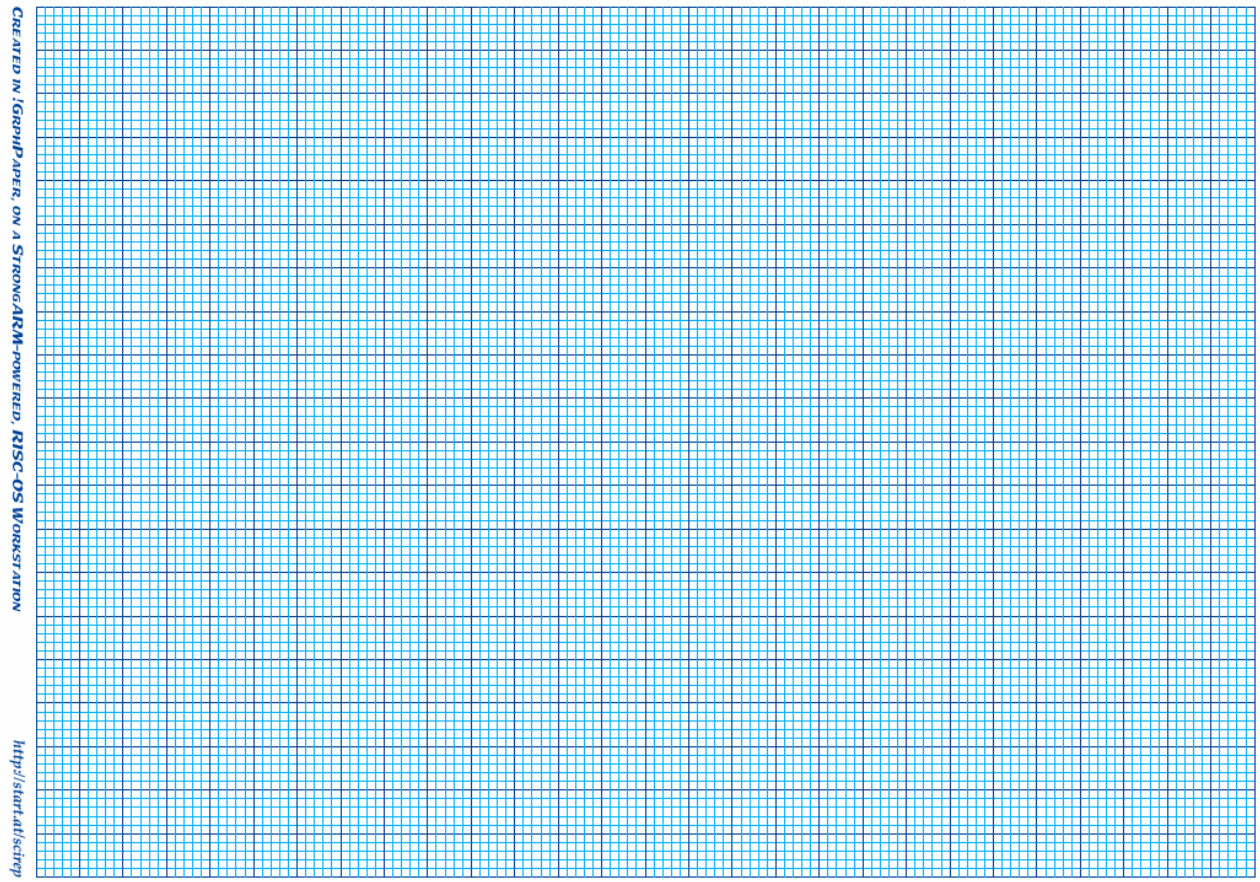
Procedure

Prepare the blank and the reaction mixture with the sample of unknown protein concentration (probe), and 5 reaction mixtures with standards (for the calibration line) according to the following table.

Test tube nr.	Blank	1	2	3	4	5	Probe
V (distilled water)/μL	200	190	180	170	160	150	-
V (standard solution)/μL	-	10	20	30	40	50	-
V (sample) /μL	-	-	-	-	-	-	200
V (Lowry reagent)/mL	2.0	2.0	2.0	2.0	2.0	2.0	2.0

Mix the test tubes content and leave at room temperature. After 12 minutes put 200 μL of Folin-Ciocalteu reagent into each test tube. Mix quickly and strongly and leave for 40 minutes at room temperature. Measure absorbance on spectrophotometer at 740 nm in relation to the blank. Draw calibration chart and determine protein concentration in the unknown sample.

	1	2	3	4	5	Probe
$m_{protein}$ /μg						
A						



γ(proteins) = _____ g/L .

Exercise 2. Separation and identification of the amino acids in mixture by thin-layer chromatography

Amino acids can be found in plasma and urine of healthy humans. Their content and composition are changed in many disorders, thus their determination is extremely important in diagnostics. A great number of inborn and acquired disorders of amino acids metabolism are known. Some of the disorders of the amino acids metabolism are lethal, while other disorders can be regulated by diet.

Reagents and accessories
Cellulose plates for thin-layer chromatography;
Mobile phase: n-butanol: acetone: cold acetic acid: water (in volume ratio 28:28:8:16);
Reagent for the color development: ninhydrin solution, c=0.4 mol/L;
Standard solution of amino acids (10 mg His, 15 mg Gly, 15 mg Ala, 20 mg Val and 30 mg Leu in 50 mL of 10% 2-propanol and one drop of 1 mol/L HCl);
Sample: amino acids mixture;
Beakers with cover, capillaries

Procedure

On cellulose plate, lightly mark the starting line (start) one centimeter above the lower edge of the plate using graphite pencil, and the regions to which sample and standards will be applied, having a width of 1 cm and distance between them of 0.5 cm. Mark the front line at 0.5 cm below the upper edge of the plate. Apply the solution of standards and sample to the plate with a capillary in the form of thin line. In the chromatography beaker, pour 80 mL of the mobile phase and 3 mL of the ninhydrin solution and leave the space above the solution for 10 minutes to become saturated with vapours. Subsequently, immerse the plate containing applied samples vertically into the beaker with the chromatography solution and cover the beaker. Due to capillary forces, solvents mixture migrates, taking amino acids with it, so that they fall behind at diverse heights. When the solvent front reaches the front line (migration time approximately 20 minutes), the plate should be taken out from the beaker and left for 3 minutes at 80 ^{o}C. The position of amino acids can be seen as a blue-purple coloring (or yellow in case of proline) of the spots on the chromatogram. Calculate the R_f values and compare them with the referent values. Standard solution consists of five amino acids, which are given in the table in descending order of their R_f values.

Standard	R_f **of standard**	**Sample**	R_f **of sample**
leucine			
valine			
alanine			
glycine			
histidine			

START

- Draw your thin-layer chromatogram.

- Conclusion

_____

_____

Exercise 3. Detection of phenylpyruvic acid in urine

Phenylalanine is an essential amino acid; its degradation pathway proceeds *via* tyrosine, hydroxylphenylpyruvic acid, homogentisic acid and other intermediates to fumaric and acetoacetic acid. Phenylketonuria is a disease caused by a defect in phenylalanine degradation due to deficiency of the enzyme phenylalanine hydroxylase, which converts phenylalanine into tyrosine. Under such conditions, phenylalanine is converted by transamination to phenylpyruvate which is secreted in urine of affected individuals. A disease is characterized by mental retardation. Early detection of the disease is crucial, like in all other metabolic disorders. If the disorder is detected on time, before irreversible pathological alterations occur, it is possible to prevent more severe symptoms by avoiding intake of food rich in phenylalanine.

Principle

Reaction between phenylpyruvic acid and Fe^{3+} ions produces green-colored chelate complex.

Reagents and accessories
0.37 M $FeCl_3$ solution;
Sample: urine
Test tube rack, funnel, filter paper.

Procedure

Into 2 mL of fresh urine add 1-2 drops of $FeCl_3$ solution, mix and observe the color change. Green to green-blue color, stable for 2-4 minutes, confirms the presence of the phenylpyruvic acid in urine.

The reaction in tested urine sample is positive/negative. *(underline)*

Draw the structures of phenylalanine, tyrosine and phenylpyruvate.

Date:_____ **Signature:**_____

Practice 3.

ENZYMES

1. What are enzymes and how are they classified?

2. What is an enzyme activity? Which units are used for its expression and how are they defined?

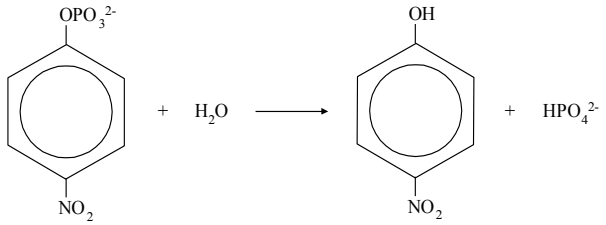
3. How does the substrate concentration influence the rate of enzymatic reaction? Explain v_{max} and K_m!

4. How does temperature influence the rate of enzymatic reaction?

Exercise 1. Influence of substrate concentration on the rate of an enzyme-catalyzed reaction: determination of K_m and v_{max}

Experimental determination of these values will be carried out on the example of serum alkaline phosphatase. Alkaline phosphatase is an enzyme belonging to the group of hydrolases; it catalyzes the reaction of phosphomonoesters hydrolysis yielding alcohol and phosphate. The enzyme was named alkaline due to its optimum pH in an alkaline range, unlike acid phosphatase, an enzyme with the pH optimum in acidic region that is also present in serum.

p-Nitrophenylphosphate (*p*NPP) is used as the substrate for determination of phosphatases activity. The hydrolysis of *p*NPP is presented by following equation:



***p*NPP**
colorless in acidic and alkaline medium

***p*-nitrophenol**
colorless in acidic, yellow in alkaline medium

Formed *p*-nitrophenol in an alkaline medium is converted into *p*-nitrophenolate ion, which is yellow-colored and its concentration is determined spectrophotometrically at 400-420 nm. The intensity of developed color is proportional to the concentration of formed product in the unit of time, and therefore also to the phosphatase activity.

Reagents and accessories
Substrate: p-nitrophenylphosphate, *c* (pNPP)=10 mmol/L;
Buffer: glycine /NaOH, pH=10.5;
NaOH solution, *c*(NaOH)=1 mol/L;
Sample: serum
Test tube rack, automatic pipette, burette, thermostated water bath, and spectrophotometer.

Procedure

Prepare six reaction mixtures containing different substrate concentrations. Add the enzyme solution (serum) shortly before the reaction starts. Measure out the volumes of given substrates and buffer solutions into six test tubes according to the following plan:

Test tube number:	1	2	3	4	5	6
V_{pNPP}/mL	0.10	0.20	0.30	0.50	0.75	1.00
$V_{buffer\ pH\ 10.5}$/mL	1.35	1.25	1.15	0.95	0.70	0.45
THERMOSTAT						
V_{serum}/mL	0.05	0.05	0.05	0.05	0.05	0.05

Place marked test tubes into thermostated water bath at 37 °C. After approximately 10 minutes, add specified volume of the enzyme solution (serum) into each test tube and write down the time when reaction started. After **exactly 10 minutes,** stop the reaction by adding 0.50 mL of NaOH solution. Take the test tubes out of the bath and measure the absorbance against water at 405 nm. For each substrate concentration prepare blank mixture according to the following plan:

Test tube number:	1	2	3	4	5	6
V_{pNPP}/mL	0.10	0.20	0.30	0.50	0.75	1.00
$V_{\text{buffer pH 10.5}}$/mL	1.35	1.25	1.15	0.95	0.70	0.45
$V_{\text{NaOH solution}}$/mL	0.50	0.50	0.50	0.50	0.50	0.50
V_{serum}/mL	0.05	0.05	0.05	0.05	0.05	0.05

Measure the absorbance of the blank solutions against the water. Subtract the measured blanks absorbances from the absorbance obtained for enzymatic reaction at a specified substrate concentration. Write the values into the table.

Test tube number:	1	2	3	4	5	6
$A_{\text{(reaction mixture)}}$						
$A_{\text{(blank mixture)}}$						
ΔA						

Calculate the value of the enzyme reaction rate for each substrate concentration as the change in product concentration per unit time:

$$v = \frac{\Delta c(p\text{-nitrophenol})}{\Delta t}$$

Calculate the exact concentrations of the formed product (*p*-nitrophenol) by using Lambert-Beer law and correct the absorbance values for the dilution of the reaction mixture:

$$v = \frac{\Delta c(p\text{-nitrophenol})}{\Delta t} = \frac{\Delta A \cdot 4/3}{\varepsilon(p\text{-nitrophenol}) \cdot l \cdot \Delta t}$$

v - rate of the enzyme reaction;

A- absorbance of the product (*p*-nitrophenol) in the reaction mixture (absorbance determined against blank must be corrected for the ratio of volumes of the final mixture after addition of NaOH solution and reaction mixture before addition of NaOH, i.e. 4/3

ε - molar absorption coefficient of *p*-nitrophenol, 18 200 L mol^{-1} cm^{-1};

l - pathlength of the spectrophotometer cell (cuvette), 1 cm;

t - time of the reaction (min).

Calculate the exact substrate concentration in each reaction mixture:

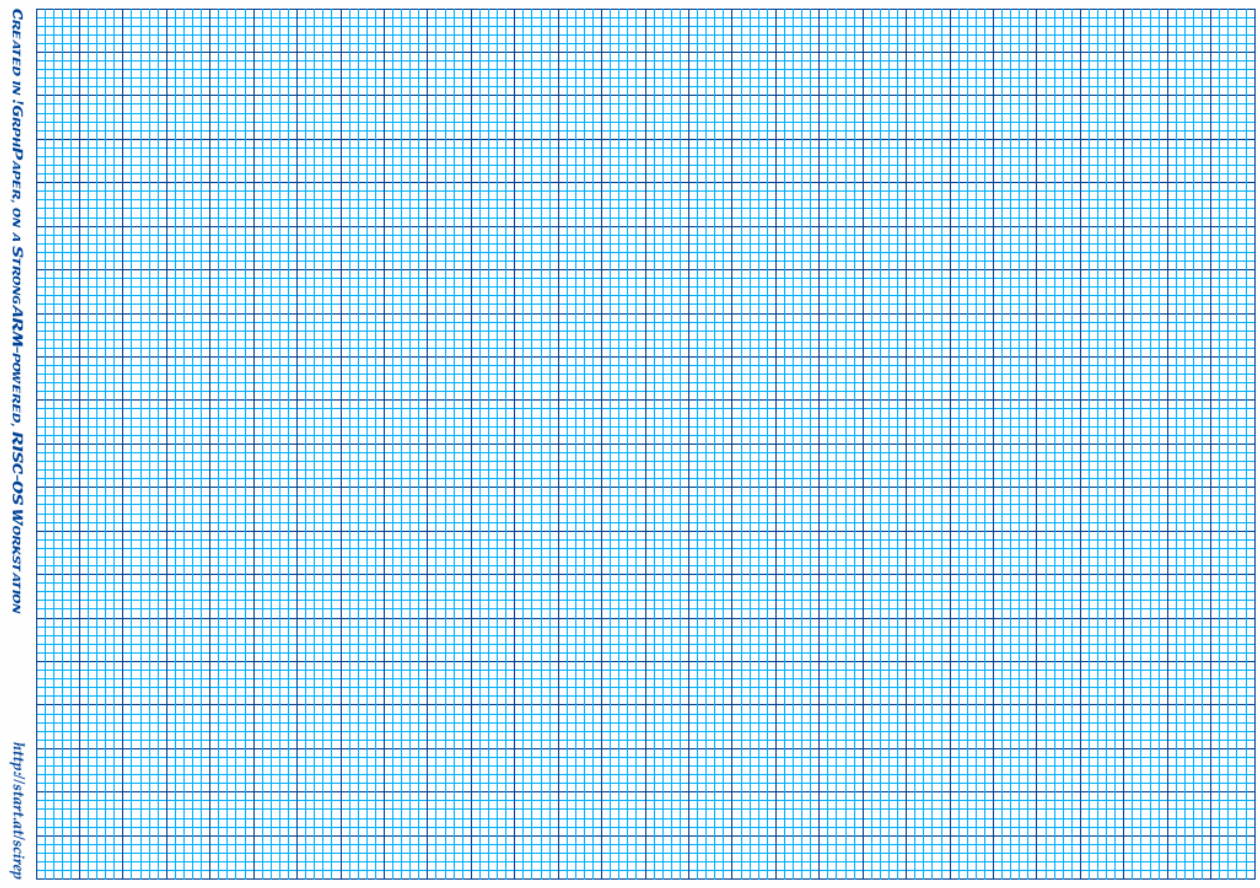
$c(\text{pNPP})_1$ = $c(\text{pNPP})_2$ =

$c(\text{pNPP})_3$ = $c(\text{pNPP})_4$ =

$c(\text{pNPP})_5$ = $c(\text{pNPP})_6$ =

On the basis of the obtained values, construct the Lineweaver-Burk diagram and graphically determine kinetic parameters K_m and v_{max}.

	c (s)	$1/c$ (s)	v	$1/v$
1				
2				
3				
4				
5				
6				



K_m = _____ v_{max} = _____

Exercise 2. Influence of pH on an enzyme-catalyzed reaction

Reagents and accessories
Buffers: Acetic, pH=4.5; Tris/HCl, pH=7.5; glycine/NaOH, pH=9.0; pH=10.5; KCl/NaOH, pH=12.5;
The rest is the same as in the previous exercise

Procedure

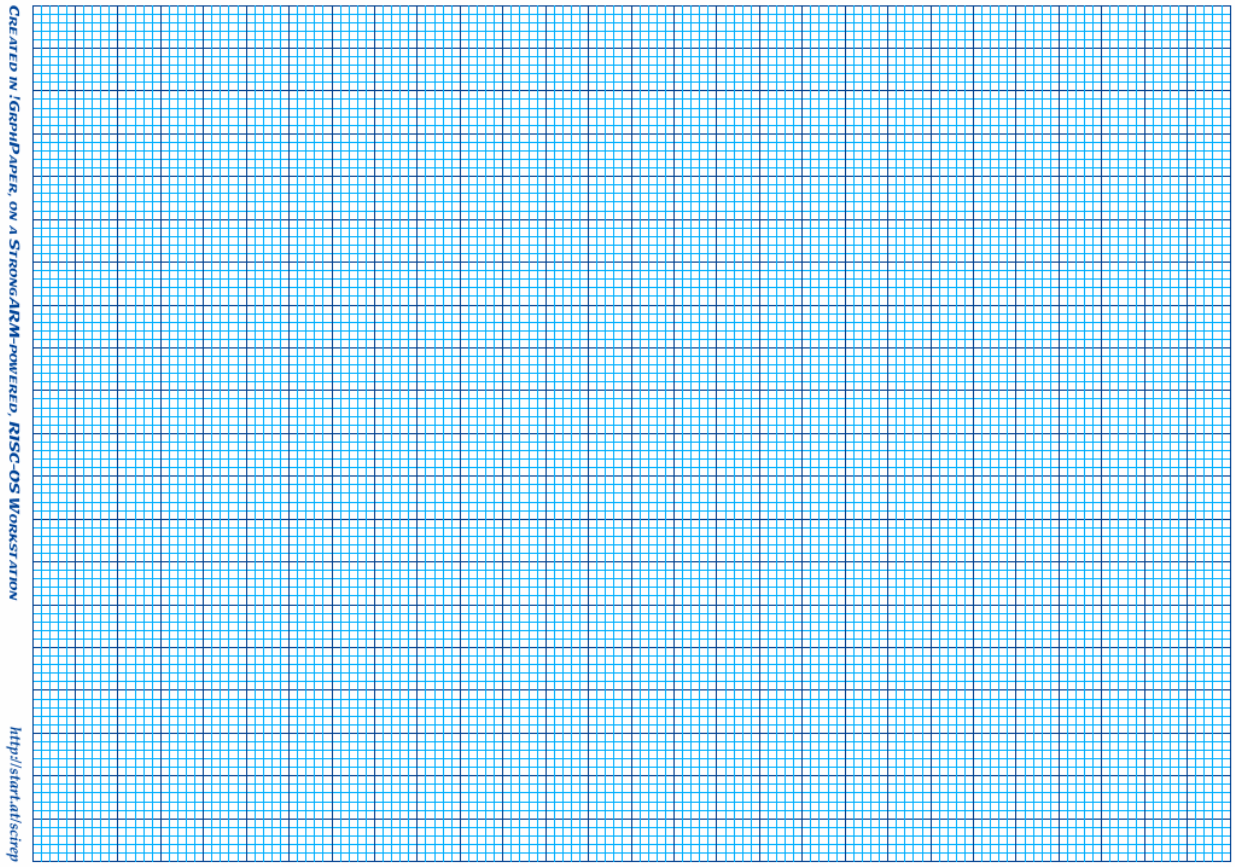
Prepare 5 test tubes and measure out substrate and buffer volumes according to the following plan:

Test tube number:	1	2	3	4	5
V substrate (pNPP) / mL	0.50	0.50	0.50	0.50	0.50
V buffer pH 4.5 / mL	1.00	-	-	-	-
V buffer pH 7.5 / mL	-	1.00	-	-	-
V buffer pH 9.0 / mL	-	-	1.00	-	-
V buffer pH 10.5 / mL	-	-	-	1.00	-
V buffer pH 12.5 / mL	-	-	-	-	1.00

Mix the test tubes content and put them into a water bath at 37 °C for about ten minutes. Subsequently, pour 0.025 mL of enzyme solution (serum) into each test tube, write down the time and return the test tubes into the bath. After **exactly 15 minutes**, add 0.50 mL of NaOH solution and measure absorbance against water at 405 nm.

	1	2	3	4	5
pH					
A					

Graphically present the dependence of the absorbance (reaction rate) on pH and explain the influence of pH of the reaction medium on the phosphatases activity in blood serum.



Explanation_____

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Date:_____ **Signature:**_____

Practice 4.

CARBOHYDRATES

Carbohydrates are the most abundant biomolecules in nature and have numerous biological functions: serve as energy sources (starch, glycogen, glucose); are structural components of complex compounds involved in cellular recognition and intercellular communication; act as synthesis precursors for other important biomolecules (amino acids, lipids, purines, pyrimidines). According to structural complexity, carbohydrates are classified as monosaccharides, disaccharides, oligosaccharides and polysaccharides.

For humans, the most important dietary source of carbohydrates are polysaccharides (starch) and disaccharides (lactose, sucrose). They are degraded to monosaccharide units by action of hydrolytic enzymes throughout the digestive system; monosaccharides are finally absorbed by intestinal cells and transported by circulation. Digestion of dietary carbohydrates begins in the mouth due to catalytic activity of salivary α-amylase. Salivary α-amylase is a hydrolytic enzyme, produced by salivary glands, required for degradation of starch. It catalyzes a hydrolytic cleavage of the internal α-1,4 glycosidic linkages in the starch structure, releasing branched and non-branched oligosaccharides containing approximately 6-7 glucose units. Dextrins, a mixture of low molecular weight polymers of D-glucose units, are the products of salivary α-amylase action. Further degradation of dextrins yielding oligosaccharides, maltotriose, maltose and isomaltose occurs in intestinal lumen, by the action of pancreatic amylase. Additional enzymes localized at surface of intestinal cells (so called brush-border enzymes) are involved in degradation of disaccharides.

Glucose has a central role in energy metabolism of animal cells and tissues and is quantitatively the most important monosaccharide produced by degradation of more complex carbohydrates. In addition to dietary sources, glucose may be released from glycogen stores in liver and skeletal muscles, or synthesized mainly in liver, in a metabolic pathway called gluconeogenesis. The concentration of glucose in blood is maintained in a narrow range (3.9 – 5.8 mmol/L) by regulatory mechanisms which involve antagonistic actions of pancreatic hormones, insulin and glucagon – hyperglycemia occurring after a carbohydrate-rich meal induces secretion of insulin, while hypoglycemia is leading to glucagon effects.

Hyperglycemia is one of the signs of diabetes mellitus, a disease in which regulation of glucose concentration in blood is disturbed due to complex pathogenetic mechanisms which include either lack of insulin production or insensitivity of target cells to insulin actions. Determination of blood glucose concentration is one of the most frequent routine tests in clinical laboratories. An additional test, such as determination of glycated hemoglobin (HbA_{1c}) is useful in interpretation of different hyperglycemic conditions. The amount of glycated Hb depends on the glucose concentration in blood; therefore elevated values are found in diabetic hyperglycemia. In general, the elevated values of 8–12% of glycated Hb are observed in cases of poorly controlled diabetes as well as in newly recognized diabetic patients. Due to the erythrocyte lifespan of 120 days, the values of glycated Hb are of relevance for evaluating metabolic control in period of up to 3 months. Therefore, after the normalization of blood glucose concentration is achieved, the HbA_{1c} value stays elevated for a certain period of time.

Chemical basis of modification of hemoglobin structure by glycosylation: Binding of glucose to the Hb is a slow, non-enzymatic process taking place in erythrocytes during their lifespan. The aldehyde (carbonyl) group of the glucose forms a Schiff base (an unstable aldimine) with an *N*-terminal valine from the α_2-chain of Hb. The aldimine is readily converted to a more stable ketoamine.

Exercise 1. Detection of salivary α-amylase

Principle

In reaction with elementary iodine, starch gives the intensively dark blue non-covalent product. Namely, the iodine molecules incorporate into cavities inside the coil of starch polysaccharide chain; due to high light absorption, the resulting so-called adsorptive compound shows characteristic dark blue color. As α-amylase degrades starch, the color of the adsorptive starch-I_2 compound disappears due to decay of its structure.

Reagents and accessories
2% starch solution;
Lugol solution: I_2 dissolved in aqueous KI solution; formation of a labile compound KI_3 occurs from which the I_2 is easily liberated;
Sample: saliva;
Test tubes, droppers, thermostated water bath, burner.

Procedure

To demonstrate the α-amylase activity in saliva the experiment should be prepared in two test tubes in parallel as follows:

Test tube 1: saliva (0.5-1 mL) + 2 mL of starch solution + 1 drop of Lugol solution;

- shake the reaction mixture;
- incubate reaction mixture for 30 minutes at 37 °C in a water bath.

For preparation of the test tube 2, boil the saliva sample for 5 minutes at 100 °C in a water bath, and then let it cool!

Test tube 2: boiled saliva + 2 mL of starch solution + 1 drop of Lugol solution;

- shake the reaction mixture;
- incubate reaction mixture for 30 minutes at 37 °C in a water bath.

- Comment your observation!

_____

_____

_____

- Examine the above prepared mixtures by performing the reaction according to Trommer!

Trommer's reaction (Fehling's test)

Principle

Trommers's reaction (Fehling's test) is used for determination of reducing properties of monosaccharides. The Fehling's reagent is very alkaline solution of complex copper(II) tartrate. This is the reason why in this reaction other reducing sugars or other reducing substances, besides glucose, are easily oxidized.

Reagents and accessories
Fehling's reagent:
Fehling I: $CuSO_4$ solution
Fehling II: alkaline solution of K,Na tartrate
Fehling I and Fehling II are mixed in 1:1 ratio.
Sample: glucose solution
Semi micro test-tube, dropper, burner, wooden peg

Procedure

In a test-tube prepare fresh Fehling's reagent by mixing approximately 2 mL of Fehling I and 2 mL of Fehling II. The reagent has dark blue color due to formation of complex salt of copper(II) tartrate. Take both samples from previous experiment. Mix the contents of each sample with reagent in volume ratio 1:1 (2 mL of the sample + 2mL of Fehling's reagent). Heat up both test tubes until boiling.

What do you observe?

Explain and conclude!_____

_____

_____

_____

Beside the salivary form, which is another form of α-amylase in humans? Compare them briefly!_____

_____

_____

_____

Exercise 2. Determination of glucose concentration in blood

2.1. *Enzyme (PAP) method*

Principle

Glucose-oxidase (GOD) catalyzes the oxidation of glucose to gluconic acid yielding the H_2O_2. In a peroxidase (POD) catalyzed reaction, the formed H_2O_2 oxidizes the colorless chromogen into a colored compound. The intensity of color is directly proportional to the glucose concentration in the sample.

$$\text{glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{gluconic acid} + H_2O_2$$

$$2\,H_2O_2 + \text{phenol} + \text{4-aminoantipyrine} \xrightarrow{\text{POD}} \text{quinoneimine} + 4\,H_2O$$

Reagents and accessories	
Working reagent:	solution of 4-aminoantipyrine (c = 0.25 mmol/L), glucose oxidase (GOD) > 15 kU/L, peroxidase (POD) > 1.5 kU/L; mutarotase > 2.0 kU/L; phosphate buffer, pH 7.5 (c = 100 mmol/L), solution of phenol (c = 0.75 mmol/L); sodium azide (0.095%)
Standard:	solution of glucose, c = 5.55 mmol/L;
Sample:	serum;
Test tubes, automatic pipette, spectrophotometer, cuvettes.	

Procedure

Prepare your sample for spectrophotometric determination according to the following table (blank and standard will already be prepared ahead of time):

	Blank	Standard	Sample
$V(H_2O)/\mu L$	10		
V(standard) /μL	-	10	-
V(serum)/μL	-	-	10
V(working reagent)/mL	1.0	1.0	1.0

Add the working reagent into the sample, mix and incubate for 10 minutes at room temperature. Read the absorbance of the standard and the sample at λ=500 nm against the blank. Calculate the concentration of glucose in the blood sample (serum).

A_{St}	
A_{Sm}	
c_{St} /mmol·L^{-1}	

c(glucose) = _____

Reference values: serum, plasma: 3.9 – 5.8 mmol/L

Conclusion:_____

_____

2.2. *Fast determination of blood glucose concentration using test-strips for GLUKOTREND® instrument*

Principle

A test-field on the strip contains a glucose-specific reagent. The instrument detects blood glucose concentrations in the range 0.6-33.3 mmol/L.

Procedure

Apply one drop of a blood onto the test-field on a strip. After 30 seconds read the concentration value determined by the GLUKOTREND® instrument. The instrument should be used according to the manufacturer's instructions.

The blood glucose concentration is _____ mmol/L.

Review questions:

1. What is the structural feature of a reducing sugar?

2. Which of the following carbohydrates are reducing and which nonreducing?

Starch	_____	Sucrose	_____
Cellulose	_____	Ribose	_____
Fructose	_____		

3. How does the structure of cellulose differ from starch and glycogen?

4. What would be the net result of the conversion of a molecule of sucrose to pyruvate?

5. Describe the fate of pyruvate under anaerobic and aerobic conditions.

Date: _____ **Signature: _____**

Practice 5.

LIPIDS

The lipids are a large group of structurally diverse organic compounds that are, in general, not soluble in water, but are soluble in nonpolar organic solvents (e.g. ether, chloroform, acetone, benzene). In humans and other mammals, triacylglycerols (simple esters) are storage and transport forms of fatty acids, serving as principal fuel molecules i.e. storage of energy. During metabolic oxidation of fatty acids derived from triacylglycerols by hydrolysis, their energy is either captured in ATP or released as heat. Triacylglycerols (fats and oils) are also major dietary lipids. Lipids with amphipatic properties, self-organizing in bilayers, are building components of biological membranes: cholesterol (simple lipid), glycerophospholipids, sphingomyelins and glycosphingolipids (complex lipids). Additionally, cholesterol is a precursor of many important cholesterol derivatives: bile acids as emulsifying agents in the digestive tract, steroid hormones, and cholesteryl esters as transport and storage forms of cholesterol. Also, during signalling, membrane complex lipids serve as precursors for enzyme-catalysed release of lipid second messengers, such as diacylglycerols, inositol-1,4,5-triphosphate, ceramides, sphingenine and other sphingoid bases, as well as arachidonate and its analogues as precursors for local hormones (eicosanoids). Certain isoprenoid lipids are lipid-soluble vitamins, electron carriers (coenzyme Q10) or sugar carriers (dolichol). Plasma lipoproteins, highly organized colloidal particles – spherical aggregates of lipids and (apolipo)proteins - serve as "transporting systems" of water insoluble lipids between tissues *via* blood. The major lipids packed in lipoproteins are triacylglycerols, cholesterol including its esterified form (cholesteryl esters), and phospholipids.

Blood triacylglycerols: In serum, triacylglycerols are associated with chylomicrons, which originate from small intestine and contain ingested (exogenous) lipids, and with low density lipoproteins (VLDL), which are produced in the liver and contain endogenously synthesized lipids. Since the composition of exogenous lipids depends mostly on dietary fats, it is of less importance for clinical diagnostics. Therefore, blood samples should be collected at least 12 hours after the last meal, the period in which chylomicrons are supposed to be cleared from the circulation in healthy persons. High concentration of triacylglycerols is present in disorders of lipid metabolism, or as secondary finding in diabetes, obstructive hepatitis, nephrosis and other disorders. Normal values for blood triacylglycerols are in the range from 0.70 to 1.90 mmol/L, and depend on age (lower in children), gender (lower in women), diet and a lifestyle.

Cholesterol in blood: Cholesterol is present in tissues and in plasma either as free cholesterol or in esterified form (cholesteryl esters). In plasma, both esterified and non-esterified cholesterol is transported by lipoproteins. Approximately half of the total amount of body cholesterol is derived from synthesis *de novo* (about 700 mg/day), while the remainder is provided by the average diet. Cholesterol is synthesized in many tissues from acetyl-CoA and is the main precursor of biologically important steroids such as corticosteroids, sex hormones, bile acids and vitamin D. The rate-limiting step of cholesterol synthesis is catalyzed by 3-hydroxy-3-methyl-glutaryl-CoA reductase, which is inhibited by both mevalonate and cholesterol. Blood cholesterol concentration is increased in primary hypercholesterolemia, but also in various disorders like obstructive icterus, diabetes, lipoid nephrosis etc. Hypercholesterolemia is found to be one of the most frequent risk factors for development of coronary heart disease; therefore determination of cholesterol concentration in blood serum is of particular importance. Serum cholesterol concentration reference values range from 3.2 to 5.2 mmol/L.

Exercise 1. Saponification of triacylglycerol

Principle

Saponification is the hydrolysis process of the fat onto glycerol and fatty acid by the action of the alkali, wherein the fatty acid form the corresponding salts. Salts of higher fatty acids are called soaps. Sodium and potassium soaps are insoluble in organic solvents, but soluble in water. Because of their amphiphilic character, they are used as detergents.

Reagents and accessories:
Ether;
2 mol/L NaOH;
Distilled water;
Samples: oil;
Test tubes and droppers, water-bath.

Procedure

Place approximately 0.1 mL (2 drops) of oil and 0.5 - 1 mL (10 drops) of ether into test tube and add 2 mL (15 drops) of NaOH solution. Leave the tube for 5 minutes, and then using a dropper, add 4-5 mL of distilled water and mix.

Describe the change!

_____

_____

Using structural formulas, write the reaction of saponification!

Exercise 2. Emulsifying and degradation of dietary lipids

Principle

During triacylglycerol digestion, free fatty acids and monoacylglycerols are released. Fat droplets are emulsified by bile acids, biological detergents synthesized by liver and secreted with the bile into the duodenum. Hydrolysis by pancreatic lipase and other lipolytic enzymes then occurs at the water-lipid surface.

Reagents and accessories:
Pancreatic lipase;
Bile (diluted);
Lacmus-tincture
Na_2CO_3, $w = 0,02$
Samples: oil, milk;
Test tubes and droppers, water-bath.

2.1. *Bile acids as emulsifying agents*

Procedure

Place approximately 1 mL of bile solution and water into separate test tubes. Add 1 drop of oil into both tubes and mix.

Observe for a few minutes and describe changes.

_____

_____

_____

2.2. *Hydrolysis of dietary triacylglycerols by pancreatic lipase*

Procedure

Place 2 mL (take in droppers two times) of milk, add the same volume of pancreatic lipase solution, 4 drops of Lacmus-tincture and 8 drops of Na_2CO_3, until you reach mild alkaline conditions. Mix and place it in a water-bath at 37 ˚C for 30 minutes.

Observe and describe changes in the test tube!

_____

_____

_____

Exercise 3. Determination of serum triacylglycerols by colour-enzymatic PAP (*p*-aminoantipyrin) method

Principle

Reagent contains enzymes which first hydrolyse blood triacylglycerols to glycerol and fatty acids; in following steps the obtained glycerol is converted to a coloured product in the presence of chromogen. Measured absorbance is proportional to glycerol concentration and hence to the concentration of triacylglycerols.

$$\text{triacylglycerol} + H_2O \xrightarrow{\textit{lipoprotein lipase}} \text{glycerol} + \text{fatty acids}$$

$$\text{glycerol} + \text{ATP} \xrightarrow[Mg^{2+}]{\textit{glycerol kinase}} \text{glycerol 3-phosphate} + \text{ADP}$$

$$\text{glycerol 3-phosphate} + O_2 \xrightarrow{\textit{glycerol 3-phosphate oxidase}} H_2O_2 + \text{dihydroxyacetone 3-phosphate}$$

$$H_2O_2 + \text{4-aminoantipyrin} + \text{DHBS} \xrightarrow{\textit{peroxidase}} \text{kinonimine} + 2\,H_2O$$

Reagents and accessories

R1 reagent: Tris buffer pH 7.8 ± 0.1 (c= 100 mmol/L); ATP (c = 0.55 mmol/L); EDTA (c = 10 mmol/L); Mg^{2+} (c = 17 mmol/L); 4-aminoantipyrin (c = 0.40 mmol/L); DHBS (c = 1.60 mmol/L); glycerol kinase ≥ 16.7 μkat/L; glycerol 3-phosphate oxidase ≥ 66.7 μkat/L; peroxidase ≥ 2.5 μkat/L; lipoprotein lipase ≥ 16.7 μkat/L;

Standard: glycerol, c = 2.3 mmol/L

Sample: serum;

Test tubes, water-bath (37 °C), spectrophotometer.

EDTA, ethylenediaminetetraacetic acid; DHBS, 3,5-dichloro-2-hidroxybenzene sulfonate.

Procedure

Pipette the reagent into a test tube with the sample as follows:

	Sample
V (sample) /μL	50.0
V (reagent) /mL	1.0

Mix carefully and incubate in a water-bath at 37 °C for 10 minutes. Read absorbance at 510 nm against blank and calculate the concentration of triacylglycerols (TAG).

A_{Sm}	
A_{St}	
c_{St}	

c(TAG) = _____

Reference values: 0.70 - 1.90 mmol/L.

Exercise 4. Determination of serum cholesterol by color-enzymatic PAP method

Principle

Enzymes present in the reagent hydrolyze cholesteryl esters to cholesterol, and then convert free cholesterol into coloured products which can be measured by spectrophotometry.

$$\text{cholesteryl ester} \xrightarrow{\textit{cholesterol esterase}} \text{cholesterol + fatty acid}$$

$$\text{cholesterol + } O_2 \xrightarrow{\textit{cholesterol oxidase}} \text{cholest-4-en-3-on + } H_2O_2$$

$$2\ H_2O_2 + \text{4-aminoantipyrine + phenol} \xrightarrow{\textit{peroxidase}} \text{quinoneimine + 4 } H_2O$$

Reagents and accessories:

Reagent: cholesteryl esterase ≥ 150 KU/L; cholesterol oxidase ≥ 100 KU/L; peroxidase ≥ 5.0 kU/L; phenol (c = 5 mmol/L), 4-aminoantipyrine (c = 0.3 mmol/L); buffer pH 6.5 (c = 30 mmol/L); sodium azide (0.095%)

Standard: cholesterol , c = 5.17 mmol/L;

Sample: serum;

Test tubes, pipette, cuvette, spectrofotometer.

Procedure

	Sample
V (sample) / μL	10.0
V (reagent) / mL	1.0

Mix carefully and incubate in water-bath at 37 °C for 10 minutes. Read absorbance at 500 nm against blank and calculate the concentration of cholesterol (CHOL).

A_{Sm}	
A_{St}	
c_{St}	

c(CHOL)= _____

Reference values: 3.2 - 5.2 mmol/L

Comment the results obtained for triacylglycerol and cholesterol concentration in comparison with reference values.

_____

_____

_____

Exercise 5. Estimation of HDL-cholesterol in serum by color-enzymatic PAP method

Principle

Chylomicrons, VLDLs and LDLs can be precipitated by addition of polyanions and bivalent cations to serum or plasma. Precipitate is removed by centrifugation and HDL-cholesterol is estimated in clear supernatant by standard color-enzymatic (PAP) method.

Reagents and accessories:

Precipitating reagent: phosphowolfram acid (c = 0.55 mmol/L) and $MgCl_2$ solution (c = 25 mmol/L) diluted with water in the ratio 4:1 (v/v);

Reagent: same as in the Exercise no. 3.

Standard solution: cholesterol, c = 1.29 mmol/L;

Sample: serum;

Test tubes, pipette, centrifuge, cuvettes, spectrophotometer.

Procedure

Pipette 500 μL of precipitating reagent into centrifuge tubes containing 500 μL of serum. Mix well and after 10 minutes transfer the tubes into laboratory centrifuge and rotate for 15 min at 4 000 r/min. Use clear supernatant as a sample and pipette sample as follows (blank and standard will already be prepared ahead of time):

	Blank	Standard	Sample
V distilled water / μL	50	-	-
V standard solution/ μL	-	50	-
V sample / μL	-	-	100
V reagent R1 / mL	1.0	1.0	1.0

Mix carefully and incubate in water-bath at 37 ^{o}C for 5 minutes. Read absorbance at 500 nm against blank and calculate the concentration of HDL-cholesterol. For calculation, instead of c_{st}, use correction factor F=4.52 which includes c_{st} and dilution factor for given conditions.

A_{Sm}	
A_{St}	
c_{St}	

c(HDL-cholesterol) = _____

Notes:

- After precipitation, sample for HDL-cholesterol determination is stable 7 days at 20-25 °C, 3 weeks at 2-8 °C or 3 months at –20 °C.
- Results for HDL-cholesterol determination are affected by sample storage time, hypertriglyceridemia, precipitating reagent concentration, centrifugation, and the presence of ascorbic acid > 142 μmol/L, Hb > 1 g/L and bilirubin > 171 μmol/L.
- After centrifugation, HDL-containing supernatant should be clear. If TG concentrations are high (> 5.0 mmol/L), precipitation might be incomplete (supernatant opalescent). In that case, precipitation should be repeated with sample diluted in ratio 1:1 with saline and the obtained result should be multiplied by 2.

Clinical interpretation:

HDL-cholesterol		Expected values	Elevated risk	High risk
Men	mmol/L	> 1.4	1.4 – 0.9	< 0.9
Women	mmol/L	> 1.7	1.7 – 1.2	< 1.2

- Calculation of LDL cholesterol:

LDL cholesterol can be calculated according to the Friedwald's formula:

c(LDL-cholesterol)/ mmol/L =
c(total cholesterol) – [*c*(triacylglycerols)/2.2] – *c*(HDL-cholesterol)

c(LDL cholesterol) = _____

Clinical interpretation

LDL-cholesterol	Expected values	Increased risk	High risk
mmol/L	< 3.2	3.2 - 4.0	> 4.0

What can be concluded from the results for HDL- and LDL-cholesterol?

_____

_____

_____

Review questions:

1. Explain tissue localization and function of the following lipases:

Pancreatic lipase

Lipoprotein lipase

Hormone-sensitive lipase

2. Complete the table:

	Cellular/tissue site of synthesis	**Major function**
Chylomicrons		
VLDL		
LDL		
HDL		

3. Write the reaction of pancreatic lipase action on triacylglycerols**.**

4. Calculate the number of ATP produced by complete metabolic oxidation of 1 molecule of stearate.

Date: _____ **Signature: _____**

Practice 6.

NUCLEOTIDES AND NUCLEIC ACIDS

Nucleic acids are polymers of nucleotides. DNA analysis gives us the information about molecular structure of the genes or some genome parts. Therefore, we can detect the disease before any clinical signs appear using prenatal or postnatal molecular diagnostics.

DNA analysis begins with DNA extraction from tissue samples, blood samples, cells, etc.). Many DNA techniques include electrophoresis methods which enable:

- Analysis of the quality of DNA isolation
- Analysis of the quality of amplified DNA or RNA fragments
- Analysis of the polymorphisms and mutations in specific amplified oligonucleotide fragments derived from different samples.

Selected methods of nucleic acid research

Quality control of nucleic acids extracted from biological samples using Agilent bioanalyzer („Lab-on-a-chip")

In order to obtain optimal results in scientific experiments as well as in diagnostic purposes, the following parameters have to be determined:

1. concentration
2. purity
3. quality (state of degradation) of each sample.

Instead of tiresome procedures of checking each of these parameters separately (by using spectrophotometry and then gel electrophoresis), new methods are being used increasingly. The Agilent Bioanalyzer is a system which provides sizing, quantification and quality control of DNA, RNA and proteins. It is a microfluidics-based platform where only minimal sample consumptions are necessary (1 to 4 μL) and the analysis is very fast (up to 12 samples in 30 minutes) (as shown in Figure 1). The sample is prepared with special gel matrix and fluorescent dyes and then applied to a chip (different for DNA and RNA samples, Figure 1). The chip is placed in the bioanalyzer instrument which causes the sample to move through the microchannels from the sample well on a chip (1). The sample is then injected into the separation channel (2) where sample components are electrophoretically separated (3). Components are then detected by their fluorescence and translated into gel-like image (bands) and electropherograms (peaks) (4).

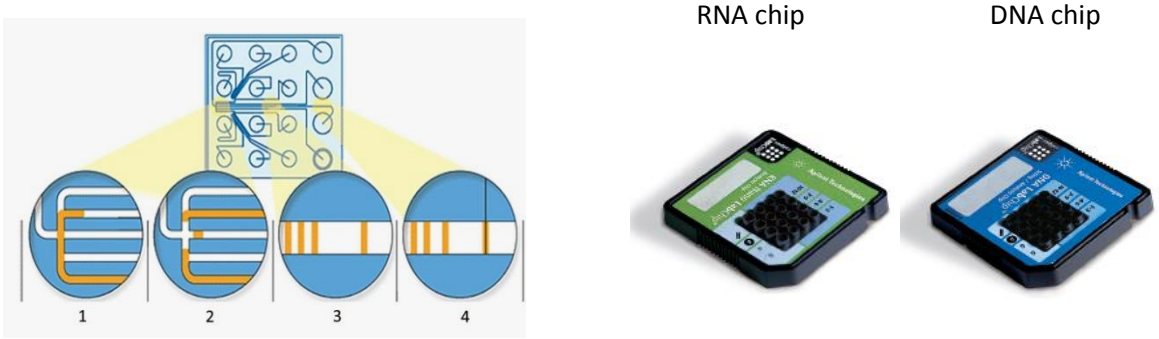
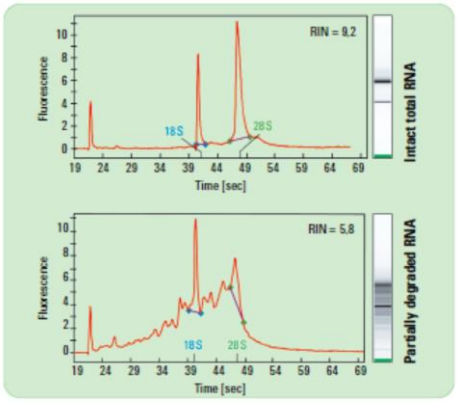


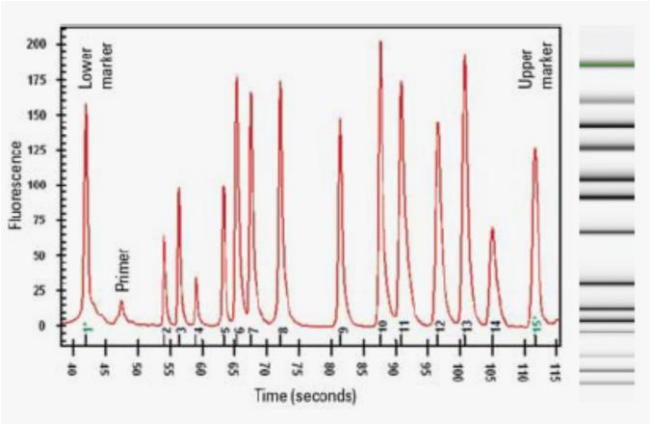
Figure 1. Qualitative analysis of nucleic acids using microfluidics-based platform (RNA/DNA chip)

A. RNA analysis



Apart from RNA concentration, parameters commonly used to estimate RNA quality are the ratio of 28S rRNA and 18S rRNA (which should ideally be 2:1), and "RNA integrity number" (RIN) which is a measure of degradation. The maximal RIN value is 10. Shown on the left is the example of electropherogram and gel-like image for intact and partially degraded RNA sample.

B. DNA analysis



Example of the Agilent Bioanalyzer electropherogram and gel-like image of 13 PCR products of different size range (99-995 base pairs).

Figure 2. Examples of qualitative and quantitative analysis of A. RNA and B. DNA using RNA and DNA chip

Restriction fragment length polymorphism (RFLP) is widely and routinely used method for determination of mutations and polymorphisms in genes of interest.

Restriction fragments are pieces of DNA produced by the action of restriction endonuclease.

Restriction endonucleases recognize specific base sequence in double-helical DNA and cleave both strands of the duplex at specific places. A striking characteristic of these cleavage sites is that they possess twofold rotational symmetry. The recognized sequence containing 4-8 base pair (bp) sequence is palindromic and cleavage sites are symmetrically positioned. Restriction enzymes are indispensable for analyzing chromosome structure, sequencing very long DNA molecules, isolating genes, and creating new DNA molecules that can be cloned. Restriction endonucleases are found in a wide variety of prokaryotes. Their biological role is to cleave foreign DNA molecules (i.e. restriction endonucleases from bacteria cleave viral DNA). The cell's own DNA is not degraded because the sites recognized by its own restriction enzymes are methylated. A fragment of DNA produced by the action of one restriction enzyme can be specifically cleaved into smaller fragments by another restriction enzyme.

Southern blotting is a hybridization technique, which includes: 1. Denaturation of DNA to form single-strands; 2. Separation of restriction fragments by agarose gel electrophoresis; 3.

Transferring of fragments to a nitrocellulose sheet; 4. Hybridization with labeled single-strand DNA-probes; 5. Autoradiography.

Polymerase chain reaction, PCR is a method for amplifying specific DNA sequence (i. e. some genes, parts of genes – exons, introns, or some restriction fragments), by using heat-stable Taq-polymerase. Millions of copies of the target sequence can readily be obtained by PCR.

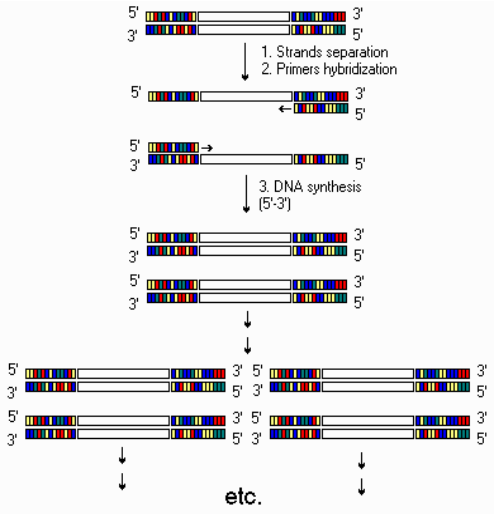


Figure 3. Polymerase chain reaction; only one double strand DNA-sequence can be amplified in 2^n copies (n =number of PCR-cycles).

PCR is carried out by adding several components to a solution containing the target sequence. These are: (1) a pair of primers – specific oligonucleotides, for hybridizing with the beginning of the forward and the reverse chain, (2) all four deoxyribonucleotides (dNTP), (3) a heat-stable DNA-polymerase, and (4) Mg^{2+}. A PCR procedure consists of three steps: (1) separation of DNA strands by heating to 94 °C, (2) annealing with the short synthetic DNA primers that flank the region to be amplified, at 50-60 °C, and (3) polymerization by extending the primers at 72°C. These three steps can be carried out repetitively in cycles. The target sequence of DNA, flanked by the primers, increases exponentially. We can analyze the quality of PCR-amplified fragments by electrophoresis in agarose gel.

Analysis of amplified DNA fragment: It can be analyzed by using the gel-electrophoresis, hybridization with nucleotide probes, and DNA sequence analysis.

DNA sequence analysis: Sanger dideoxy method: DNA-polymerase I is used to copy a particular sequence of a single-stranded DNA. There are four reaction mixtures each one containing polymerase, primer, four deoxyribonucleotides (radioactively or fluorescently labeled), and one 2',3'-dideoxynucleotide. The incorporation of this analog blocks further growth of the new chain because it lacks the 3'-hydroxyl terminus needed to form the next phosphodiester bond. Hence, fragments of various lengths are produced in which dideoxy analog is at the 3'-end. Four sets of chain-terminated fragments (one for each dideoxy analog) are then applied to electrophoresis, and the base sequence of the new DNA is read from the autoradiogram (Figure 4).

Each mixture contains:

a) DNA sample with sequence: 3'-CAGGACTGAATTGG-5'
b) DNA polymerase I
c) 5'-GTCCT primer
d) nucleotide mixture – dATP, dCTP, dGTP, dTTP
e) radioactively labelled dideoxy analog (for ex. ddATP)

Produced fragments:
3'- CAGGACTGAATTGG-5'
5'- GTCCTGACTTAA-3'
+
3'- CAGGACTGAATTGG-5'
5'- GTCCTGACTTA-3'
+
3'- CAGGACTGAATTGG-5'
5'- GTCCTGA-3'

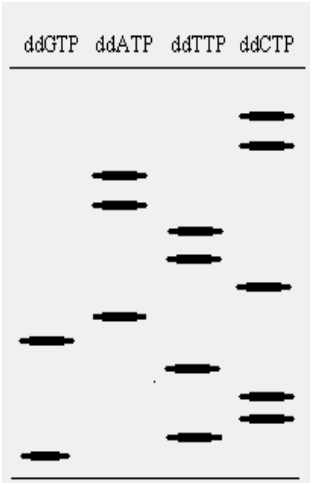


Figure 4. DNA fragments sequencing and detection by autoradiography:Fragments are produced by adding 2', 3'-dideoxy analog of dNTP to each of four polymerization mixtures. For example, the addition of the dideoxy analog of dATP results in fragments ending with A (as shown left). DNA fragments produced by sequencing are separated by electrophoresis. The sequence of original chain is complementary to the produced chain (as shown right).

An alternative to autoradiographic detection, and more widely used nowadays, is fluorescent detection - each of the four dideoxy analogs is labeled with different fluorescent dye. The fragments of DNA are then separated by electrophoresis and the sequence read using a laser beam and computer. The order in which the four fluorescent dyes are read gives the DNA sequence (Figure 3).

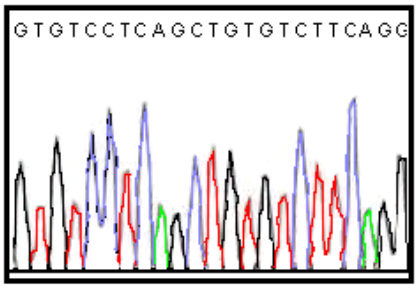


Figure 5. Sequencing of DNA fragments - fluorescent detection.

Exercise 1. Determination of DNA concentration

1.1. *UV-spectrophotometric determination of DNA concentration*

Principle

DNA concentration can be determined by UV-spectrophotometric measurement of the absorbance at 260 nm. Solution of DNA, which contains 50 μg DNA/mL, has the absorbance 1. DNA purity can be determined by the ratio of absorbances measured at 260 and 280 nm. Pure DNA sample has the A_{260}/A_{280} ratio ranging from 1.6 to 2.0.

Procedure

Dilute 1:10 the DNA solution (100 μL of DNA solution + 900 μL TE buffer). Measure the absorbance at the UV-spectrophotometer at 260 nm, and calculate the mass concentration of DNA in solution.

γ(DNA)= _____ μg/mL

Reagents and accessories

1) Tris-EDTA buffer (TE-buffer)

Tris (10mM)	1.21 g
Na_2EDTA (1 mM)	0.37 g
Distilled water, sterile	ad 1L

Adjust pH at 7.5 with HCl.
Sterilize and keep on room temperature.

2) DNA sample

3) UV-spectrophotometer, cuvettes.

1.2. *Determination of DNA concentration with indole-HCl*

Principle

This method is appropriate for determination of DNA in tissue homogenate. Determination of DNA in tissues can be useful to calculate the number of nuclei or the number of cells. The method is based on a reaction of indole with deoxyribose in acid medium; the reaction generates a yellow-orange complex.

Reagents and accessories

1) NaOH solution, c=2 mol/L
2) Indole/HCl reagent
   0,04%- indole solution
   Concentrated HCl solution

Mix before using in proportion 1:1.

3) Distilled chloroform
4) sample: tissue homogenate

Tube with cap, paraffin wax, automatic pipette, dispenser, centrifuge, spectrophotometer.

Procedure

- Add 300 µL of 2 M NaOH to the sample and mix it.
- Incubate 30 minutes at 50 °C.
- Add 300 µL of indole-HCl reagent, and mix it.
- Boil in WELL-CLOSED tubes, in the water bath at 100 °C, for 10 minutes.
- Cool at room temperature.
- Add 600 µL of water and 1000 µL of chloroform.
- Mix well and centrifuge shortly.
- Remove carefully the upper layer into cuvette and determine the absorbance at 490 nm. Prepare also the blank, using distilled water instead of the probe.

- Use the calibration chart and determine the mass of DNA in sample according to the absorbance. Calculate the quantity of DNA in tissue, in *mg per g* of fresh tissue. For calculation, keep in mind the following:

  - 10 µL aliquot of tissue homogenate was used for determination of DNA.
  - Tissue homogenate was prepared from 0.5 g of fresh tissue.
  - The total volume of homogenate was 3 mL.

  Total DNA content in analyzed tissue _____

  mg DNA/g fresh tissue _____

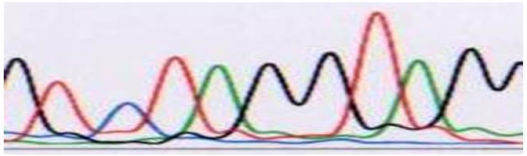
Exercise 2. DNA sequencing in practice

Note – Principle of DNA sequencing is explained in introductory part of Practice 6.

Problem: You sequenced a DNA sample from two patients. Partial sequences of isolated DNA are given below (5'→3').

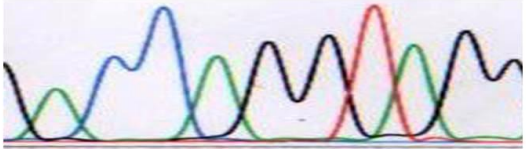
1)

G T C T A G G T A G G



2)

G A C C A G G T A G G



2nd base in codon

1st base in codon	U	C	A	G	3rd base in codon
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	STOP	STOP	A
	Leu	Ser	STOP	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

a) Assuming that the sense strand is sequenced, write the corresponding mRNA sequence for both patients.

1) _____

2) _____

b) Assuming that the first base is the start of the reading frame, write the corresponding amino acid sequences for both patients, using the RNA codon table.

1) _____

2) _____

c) Write down the structures of all (if any) amino acids that are different between the two patients. Explain what could be the effect of the changes in amino acid sequence on enzyme activity of the hypothetical enzyme coded by this gene.

Exercise 3. Restriction fragment length polymorphism analysis (RFLP)

3.1. *Determination of glutathione peroxidase (GPX1) gene polymorphism198Pro/Leu*

Glutathione peroxidase (GPX1) is the ubiquitous intracellular and key antioxidant enzyme within many cells, which converts hydrogen peroxide to water and lipid peroxides to their respective alcohols using reduced glutathione as an essential co-substrate. *GPX1*, the gene coding for glutathione peroxidase 1, is located on chromosome 3p21.3 and is composed of 2 exons. A genetic variant 198Pro/Leu, rs1050450 at codon 198 of *GPX1* gene, results with the substitution of proline (CCC) with leucine (CTC) due to nucleotide transition C-T. This polymorphism has been associated with a significantly decreased GPX1 enzymatic activity (as compared with the 198Pro allele) and therefore might be involved in pathogenesis of different disorders related to oxidative stress.

Principle

The method is based on amplification of oligonucleotide sequence of the exon 1 of *GPX1* gene and digestion of the amplified fragment by the restrictive endonuclease *Apa*I.

Reagents and accessories

1) Thermocycler device (used for DNA amplification)
2) DNA polymerase (5 U/L)
3) PCR buffer containing $MgCl_2$ (15 mmol/L)
4) Distilled sterile water
5) Deoxynucleotide mixture (dNTPs (dATP, dGTP, dTTP, dCTP) - 10 mmol/L of each
6) DNA primers (forward and reverse-20 μmol/L)
7) Restrictive endonuclease *Apa* I- 10 U/μL, and *Apa I* buffer
8) 0.5 %-agarose gel

Agarose	0.25 g
1x TAE buffer	50 mL

9) Ethidium-bromide, 10 mg/mL
10) TAE buffer
    50 x Tris-acetate-EDTA (TAE) stock buffer

Tris base	242 g
Acetic acid	57.1 mL
EDTA solution (0.5 M, pH 7.5)	100 mL
Distilled water	ad. 1 L

    1x TAE buffer: 20 mL of 50xTAE, distilled water, ad. 1L
11) Sample loading buffer

1%-solution of xylene-cyanole	1 mL
1%- solution bromine-phenol blue	1 mL
50 %- solution glycerol,	5 mL
50x TAE-buffer	190 μL
Distilled water	2.75 mL

12) DNA ladder (50 bp, 100 bp)
13) Incubator (37°C), mini submarine electrophoresis unit, UVT gel casting tray, UV gel-running tray, combs, Erlenmeyer flask (100 mL), automatic pipettes, power supply, gloves, vortex, safety googles, UV-transilluminator, digital camera.
14) Sample: extracted human DNA (500 ng of genomic DNA)

Procedure

The exon 1 of *GPX1* gene is amplified, using polymerase chain reaction method. Quality of the amplified fragment and its size (222 bp) is checked by gel electrophoresis. Next step is incubation of amplified fragment solution with restriction endonuclease *Apa*I,

following required optimal reaction conditions and using corresponding enzyme buffer. Finally, the restriction mixtures are analyzed by gel electrophoresis and individual genotypes are interpreted:

a. In the presence of 198Pro homozygous allele (C), the 222 bp PCR product will be cleaved into 2 fragments of 170 bp and 52 bp.
b. 3 fragments of 222 bp, 170 bp and 52 bp will be detected for the 198Pro/Leu (CT) heterozygote.
c. Uncleaved fragment, showing only the 222 bp PCR product, will be detected in the case of homozygous allele at 198Leu (T).

- Analyze your samples by performing gel electrophoresis:

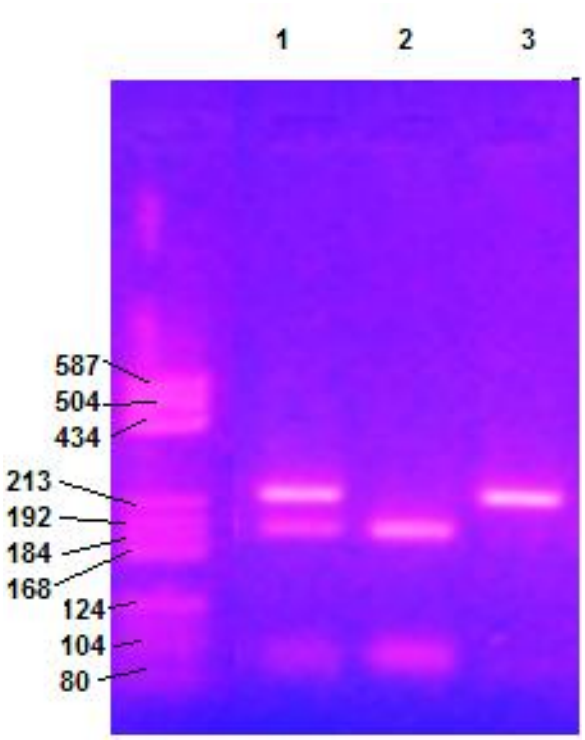
1. Install the running tray.
2. Seat the comb assembly on the rim of the casting tray.
3. Prepare 50 mL of 2% agarose in TAE-buffer. Boil it.
4. Add 1 μL of ethidium-bromide into prepared agarose solution. Pour the gel into the tray. Allow a minimum of 30 minutes for the gel to polymerize.
5. Fill buffer chambers in the submarine electrophoresis unit with 1x TAE-buffer.
6. Once the gel is set, transfer the running tray and gel to the submarine electrophoresis unit. Remove the comb carefully.
7. Load the samples to gel (9 μL of DNA in TE-buffer and 1 μL of sample loading buffer). Run the electrophoresis at 100 V, for 35 minutes.
8. Slide the gel onto transilluminator surface. View the sample under UV light. You can also photograph the gel. Wear UV safety goggles and protect skin while using UV lamp.

From the picture below, interprete the GPx genotypes for subjects 1-3:

1_____

2_____

3_____



Exercise 4. Colorimetric determination of uric acid in serum (PAP procedure)

Uric acid is degradation product of purine nucleotides in humans. Uric acid is excreted mostly with urine, and a small amount is excreted by feces. Meat nucleoproteins are the main dietary source of purine.

Genetic factors and life habits may influence on variation of uric acid concentration in serum and urine. High uric acid concentration can be caused by intensive synthesis of purine, by dietary purines, intensive purine metabolism, or low excretion of purines by kidney. Defect of control feedback mechanism in purine biosynthesis can cause primary hyperuricemia. Secondary hyperuricemia may be caused by intensive metabolism of purines or may accompany malignant diseases, especially leukemia. It can also occur in infections, psoriasis, and treatment with cytostatics - purine derivatives.

Inefficient excretion of uric acid by kidney can be caused by acute or chronic kidney failures. Gout is a disease in which uric acid, mostly in the form of sodium salts (urates) precipitates in joints, and is usually a consequence of primary hyperuricemia.

Inherited disorders of purine metabolism usually lead to high uric acid concentration, and complex symptomatology such as the one described in rare Lesch-Nyhan syndrome.

Low uric acid concentration is rare and it can be present in treatment of gout by alopurinol, which inhibits activity of enzyme xanthin-oxidase. Sometimes, low uric acid values can be present in different neoplasms and defects of kidney tubular function.

Principle

$$\text{urate} + 2\ H_2O + O_2 \xrightarrow{\textit{uricase}} \text{allantoin} + CO_2 + H_2O_2$$

$$2\ H_2O_2 + \text{4-aminopyrine} + \text{DHBS} \xrightarrow{\textit{peroxidase}} \text{quinoneimine} + 4\ H_2O$$

(DHBS=3,5-dichlore-2-hydroxybenzenesulfonate)

Reagents and accessories		
Reagent	borate buffer pH 7.0	50 mmol/L
	DHBS	4 mmol/L
	uricase	> 200 U/L
	peroxidase	> 1000 U/L
	4-aminoantipyrine	0.3 mmol/L
Standard	urate	c = 476 μmol/L
	sodium azide	0.095%
Sample:	serum	

Procedure

Prepare your sample according to the following table (reagent blank and standard will already be prepared ahead of time):

	Probe	Standard	Blank probe
V(sample)/μL	20	-	-
V(standard)/μL	-	20	-
V(distilled water)/μL	-	-	20
V(Reagent solution)/mL	1.0	1.0	1.0

Mix well and incubate for 10 minutes at room temperature. Read the absorbance of probe (A_{Pr}) and standard (A_{St}) at 520 nm. Color is stable for 30 minutes (plasma, serum).

A_{Pr}	
A_{St}	
c_{St}	

c(uric acid) =_____

Reference range:

Serum	Male	150 - 420 μmol/L
	Female	90 - 350 μmol/L
Urine	up to	4.43 mmol per 24 hours

Comment your result.

_____

_____

_____

Review questions:

1. Name and represent by structural formulas the pyrimidine nucleotides, components of nucleic acids:

2. Name and represent by structural formula the first precursor in biosynthesis *de novo* of pyrimidine nucleotides, which is also an intermediate in the urea cycle.

3. Name the precursors in biosynthesis *de novo* of purine nucleotides:

a) Three amino acids: _____, _____ and_____;

b) Donors of carbon units: _____ and _____;

c) Donor of ribose-5-phosphate: _____.

4. In *de novo* biosynthesis, the first intermediate with a complete purine ring is _____, which is then converted to _____ or _____.

Date: _____ **Signature: _____**

Practice 7.

PORPHYRINS AND BILE PIGMENTS

Hemoglobin

A common name used for haemoglobin is blood pigment because of its presence in erythrocytes. It enables cell respiration by transporting oxygen to peripheral tissues and carbon dioxide from tissues to the lungs. Haemoglobin is one of haemoproteins, complex proteins whose prosthetic group is hem - the iron-containing pyrrole ring. The protein component of haemoglobin consists of four polypeptide chains, and each polypeptide chain contains one hem as a prosthetic group. Thus, one haemoglobin molecule has the capacity to combine with four oxygen molecules. Heme contains iron in reduced form (Fe^{2+}, ferrous ion). In this form, the iron can share electrons and bond with oxygen to form oxyhaemoglobin in the lungs. Dissociation of oxyhaemoglobin enables releasing of the oxygen to the tissue and a formation of deoxyhaemoglobin (reduced haemoglobin, the form in which the hem iron is still reduced - Fe^{2+}).

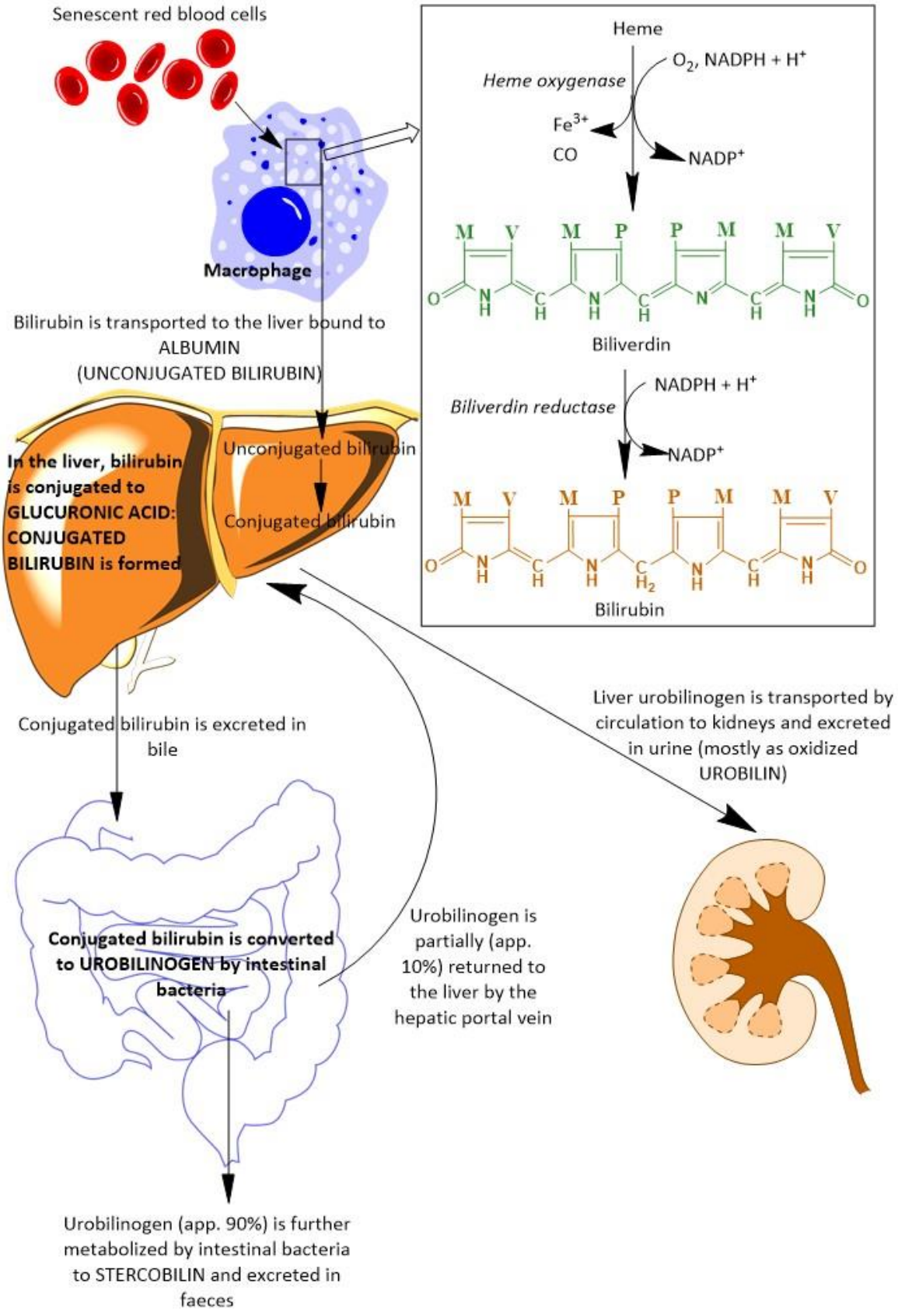
Hem represents non-protein component of various haemoprotein. Precursors for biosynthesis are succinyl-CoA and glycine**.** Metabolic disorders of hem biosynthesis, rare hereditary disease are due to lack of the biosynthetic enzymes. Accumulation of different porphirynogen precursors leads to oxidation into porphyrins. Related disorders could be anaemia or porphyries. One of the porphyries is so called erytropoetic congenital porphyria which is characterized with specific symptoms: photosensitivity, increased uroporphyrin in in blood, urine and faeces, and **erythrodontia of teethes**.

In adult human, several polypeptide chain species may be distinguished - HbA_o or HbA_1 (95%), HbA_2 and HbF (1.7%), while HbF (fetal haemoglobin) is predominant form of haemoglobin in a new-born (85%). Defects of genes that control the expression of the haemoglobin protein can produce abnormal haemoglobins and anaemia, and lead to conditions termed haemoglobinopathies. Haemoglobinopathies may be a consequence of structural changes in the haemoglobin molecule (HBC; HbS, sickle cell haemoglobin; HBM, methaemoglobin), or diminished production of one of the two subunits of the haemoglobin molecule (*i.e.* α-thalassemia or β-thalassemia).

Bile pigments

Bile pigments are degradation products of the haemoglobin metabolism. Bilirubin is formed from haemoglobin by a series of reactions in the reticuloendothelial system (RES). From the reticuloendothelial cells, bilirubin enters the circulation and binds to albumin (**unconjugated bilirubin**). By an active transport mechanism it enters the liver and in the parenchymal liver cells it conjugates with glucuronic acid giving bilirubin glucuronide (**conjugated bilirubin**). In this form it is concentrated through the Golgi apparatus to the surface of the bile ductile membranes and is excreted into the bile. From the bile it reaches the small intestine, where it is removed from the glucuronides (by specific glucuronidases) and subsequently reduced to urobilinogen by anaerobic intestinal flora. Urobilinogen is partly excreted in the faeces, but up to 20% of urobilinogen is reabsorbed from the intestine and enters the enterohepatic circulation. Most of the reabsorbed urobilinogen is taken up by the liver and re-secreted in the bile. Small fraction enters the general circulation and appears in urine (Figure 2).

Clinical and scientific studies have shown that bilirubin can be disposed in solid tooth tissue which results in **discoloration and / or hypoplasia** of tooth enamel.



Disorders of bilirubin metabolism result in jaundice.

Prehepatal jaundice - hemolitic jaundice: Several uncommon conditions give rise to overproduction of bilirubin. One of these conditions might include rapid destruction of red

blood cells. The bilirubin in the blood in these conditions usually is only mildly elevated. Urobilinogen in urine can also be mildly elevated.

Posthepatal jaundice - obstructive jaundice is caused by an interruption to the drainage of bile in the biliary system. The most common causes are gallstones in the common bile duct, and tumors. Inability of conjugated bilirubin excretion into bile results with increased bilirubin in the circulation and its appearance in urine, while urobilinogen might be absent.

Hepatal jaundice causes include acute hepatitis, hepatotoxicity, alcoholic liver disease, some genetic disorders of bilirubin metabolism, primary biliary cirrhosis, and neonatal jaundice (hepatic machinery for the conjugation and excretion of bilirubin does not fully mature until approximately two weeks of age). In hepatal jaundice both bilirubin fractions in blood are increased; in urine, conjugated bilirubin is present, and urobilinogen is increased.

In most cases the isolated elevation of bilirubin concentration (without other signs of hepatobiliary disorders) is due to an **inherited disorder of bilirubin metabolism**. **Gilbert syndrome** is the result of a mutation in the promoter region of a gene coding for the enzyme UDP-glucuronosyltransferase. **Crigler-Najjar** syndrome is elicited by a lack or deficiency of the enzyme uridine-diphosphate glycosyltransferase (UGT). Gilbert syndrome and Crigler-Najjar syndrome are examples of the unconjugated hyperbilirubinemias. Defective excretion of conjugated bilirubin, its reabsorption into the blood and excretion in the urine are symptoms of conjugated hyperbilirubinemia (**Dubin-Johnson syndrome** and **Rotor syndrome**).

Exercise 1. Determination of hemoglobin in blood

Principle

Hemoglobin is oxidized by potassium hexacyanoferrate(III), $K_3[Fe(CN)_6]$ (potassium hexacyanide) to methemoglobin, which forms a stable reddish coloured complex of the cyanmethemoglobin in reaction with potassium cyanide.

Reagents and materials

R1: Drabkin's reagent $K_3[Fe(CN)_6]$ 0.61 mmol/L,
KCN 1.03 mmol/L,
KH_2PO_4 0.77 mmol/L.

Standard solution of cyanmethemoglobin, γ = 0.6 g/L, corresponds to hemoglobin of γ = 150 g/L;
Working reagent: Dilute 20 mL of the reagent R1 with 980 mL of distilled water;
Sample: whole blood;
Test tubes, automatic pipette, spectrophotometer, cuvettes.

Procedure

Prepare your sample according to the following table (standard will already be prepared ahead of time):

	Standard	Sample
V (working reagent)*/mL	-	2.50
V (sample)/mL	-	0.02
V (standard)/mL	2.50	-

***CAUTION: CYANIDE IS A POISON**

Mix well. After a 5 minutes of incubation at room temperature (20-25 ^{o}C), transfer the content of the test tube into the spectrophotometric cuvette and measure the absorbance of the

sample, A_{sample}, against water at the wavelength of 546 nm. Calculate the mass concentration of hemoglobin in the sample.

A_{sample}	
A_{St}	
γ_{St}	

γ(Hb) = _____

Reference values: Women 115-155 g/L
Men 125-175 g/L

Exercise 2. Determination of total and conjugated bilirubin in serum

Principle

Bilirubin reacts with 2,4-dichloroaniline by forming the coloured azobilirubin with an absorption maximum at 546 nm. The intensity of the colour depends is proportional to the concentration of bilirubin.

Unconjugated bilirubin reacting in the presence of detergent is determined as total bilirubin, whereas only conjugated bilirubin reacts in the absence of detergent.

Reagents and materials
R1: 2,4-dichloroaniline 2.22 mmol/L,
HCl 53.33 mmol/L,
detergent;
R2: 2,4-dichloroaniline 2.22 mmol/L,
HCl 53.33 mmol/L;
R3: sodium nitrite 222.20 mmol/L;
Working reagent 1: mix R1 and R3 in the 100:1 ratio;
Working reagent 2: mix R2 and R3 in the 100:1 ratio;
Sample: serum;
Test tubes, automatic pipette, spectrophotometer and cuvettes.

2.1. *Determination of the total bilirubin concentration*

Procedure

Prepare your sample according to the following table (reagent blank will already be prepared ahead of time):

	Sample	Blank
V (sample)/mL	0.1	0.1
V (working reagent 1)/mL	1.0	-
V (solution R1)/mL	-	1.0

Mix and leave the sample at room temperature protected from the light. After 10 minutes, measure the absorbance of the sample and the blank against distilled water at 546 nm.

A_{sample}	
A_{blank}	

Calculation:

The concentration of bilirubin expressed in μmol/L is obtained by multiplication of the difference of the absorbance values of the sample and the blank by the constant 214**.** The constant value (214) is calculated from volumes of the reaction mixture and the serum, the conversion factor of mol/L to μmol/L and the molar absorption coefficient (ε) of azobilirubin.

c(total bilirubin) = _____

2.2. *Determination of the conjugated bilirubin concentration*

	Sample	Blank
V (sample)/mL	0.1	0.1
V (working reagent 2)/mL	1.0	-
V (solution R2)/mL	-	1.0

Mix and leave the sample at room temperature protected from light. After exactly 5 minutes measure the absorbance of the sample and the blank against distilled water at 546 nm.

A_{sample}	
A_{blank}	

The calculation is the same as in the Exercise 2.1.

c(conjugated bilirubin) = _____

Reference values:

Total bilirubin	
Adults	≤ 19 μmol/L
Newborns	≤ 225 μmol/L
Conjugated bilirubin	≤ 5 μmol/L

Remarks

1) The analyzed sample must be protected from direct light to avoid false low results, which may arise from the bilirubin breakdown.
2) Hemolytic samples aren't useful. Namely, during the preincubation with HCl, hemoglobin oxidizes to methemoglobin along with the formation of H_2O_2. Azobilirubin formed in the main reaction is degraded by H_2O_2. This is the cause of the false decreased bilirubin concentrations in hemolytic samples.
3) Bilirubin interferes with the determination of glucose and creatinine, causing the false decreased serum concentrations of these compounds.

Exercise 3. Determination of bile pigments in urine

Normally, a neglectable amount of bilirubin is excreted in the urine if any. If the liver function is impaired or when biliary drainage is blocked, some of the conjugated bilirubin leaks out of the hepatocytes and appears in the urine, turning it dark amber (hepatocellular and cholestatic jaundice). Urobilinogen is normally excreted in urine but in lower amounts

(approximately 0.5-5 μmol daily). Urobilinogen in urine can be absent in cholestasis and increased in hepatocellular damage. Estimation of a difference between increased urine bilirubin and increased urine urobilinogen helps to distinguish between various disorders of hepatobiliary system.

Reagents and materials
Iodine solution in alcohol, *c*=039.4 mmol/L
Ehrlich's reagent: para-dimethylaminobenzaldehyde, 0.134 mol/L
HCl, *c*= 5.4 mol/L
Sample: urine
Test tubes and droppers

3.1. *Qualitative analysis - Rosin's test for detection of bilirubin*

In the presence of iodine, bilirubin is oxidized to green biliverdin or blue bilicyanin.

Procedure

Pipette 2 ml of urine into a test tube and carefully pour out the sample with 0.5-1 mL of iodine solution to avoid mixing. Formation of the green-blue ring at contact surfaces indicates the bilirubin presence in urine.

3.2. *Qualitative analysis - Ehrlich's test for detection of urobilinogen*

The test is based on a reaction of para-dimethylaminobenzaldehyde and urinary urobilinogen in a strongly acidic medium. Urobilinogen reacts with Ehrlich's reagent to form a red-coloured compound.

Procedure

Pipette 2 ml of urine into a test tube and add 2-4 drops of Ehrlich's reagent. Light-orange coloured mixture indicates the presence of urobilinogen. Intensive orange or red colour of the mixture indicates the increased urobilinogen excretion in urine.

- On the basis of the obtained results for total concentration of bilirubin in serum, conjugated bilirubin in serum, and presence of bilirubin and urobilinogen in urine, assume a probable type of jaundice! Use the data from laboratory differential diagnosis of jaundice given in the following table.

	No jaundice	Hemolytic *	Cholestatic*	Hepatocellular*
Total bilirubin Bilirubin in urine Urobilinogen in urine Hemoglobin in blood	≤ 19 μmol/L Negative Normal Women 115-155 g/L Men 125-175 g/L	Up to 75 μmol/L Negative Increased (+) Decreased	Increased (+++) Positive (+++) Negative (-)	Increased *** Positive (+ to +++) Increased (++)

Remarks:
*Determination of the category of jaundice includes determination of enzyme activities (AST, ALT, LDH and GGT) and determination of total protein and albumin concentrations.
** Clinical sensitivity and specificity of conjugated bilirubin/total bilirubin ratio are limited.
***Depends on damage intensity.

Conclusion

Total bilirubin		Jaundice type:
Conjugated/total bilirubin ratio		
Bilirubin in urine		
Urobilinogen in urine		

Review questions:

1. a) In mammals, the porphyrin biosynthesis starts with biosynthesis of (A)_____ from two precursors:
   (B)_____ and (C)_____.
   The reaction is catalyzed by the action of the enzyme _____.

2. Which clinical disorders are associated with defects in biosynthesis of porphyrins? Name and describe the most common disorder of porphyrin synthesis.

3. The first reaction in degradation pathway of heme is catalysed by the enzyme_____.
The products of that reaction are:_____, _____ and _____.
Describe the fates of all three products of the reaction:
_____
_____
_____

4. What are solubility properties of bilirubin?
_____

Can this solubility be altered during the bile pigment formation and transport? Name the reaction responsible for that change.

_____

_____

Where does the mentioned reaction occur? Name the responsible enzyme.

_____

5. Newborn infants may sometimes develop jaundice. How is this condition explained at a biochemical/metabolic level? Which procedure is used as an efficient treatment for this condition?

6. Which regulatory mechanism of iron metabolism explains high values of TIBC and UIBC, common findings in sideropenic anemia?

Date: _____ **Signature: _____**

Practice 8.

URINE ANALYSIS

Urine is a transparent, aqueous solution in which the excess water, mineral salts and various products of metabolism, especially nitrogen compounds, are excreted from organism. Analysis of urine is important for the evaluation of normal kidney physiology and function. Various pathological conditions may cause abnormalities of urine composition, thus the urine analysis is useful in diagnosis of different disorders. Also, urine analysis is frequently used for determination of intake and metabolizing of drugs and toxic substances.

The amount of daily urine is between 1000 and 1500 mL, urine density is from 1.015 to 1.025 g/mL and pH in the range of 5 to 7 (4.6 to 8). Urine contains 96% of water, 1.5% of inorganic and 2.5% of organic substances.

Normal urine components

a) Inorganic substances

The most abundant cations of urine are sodium and potassium ions, and there are twice as many sodium ions as potassium ions. Ammonium ions are also present in the urine, while calcium and magnesium ions are usually found in small quantities. Traces of iron, copper, zinc and manganese ions may also be present.

Chlorides are the most prevalent anions in urine; phosphates, sulfates and small quantities of hydrogen carbonate ions are also present in normal urine.

b) Organic substances

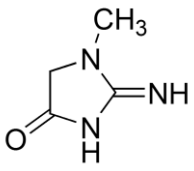
Organic substances of urine can be classified as nitrogen-containing and non-nitrogen-containing compounds. Most of the nitrogen excreted from the body in the urine (approximately 95%) is in the form of urea, uric acid, creatinine and ammonium salts. Non-nitrogen-containing organic substances are found in neglectable quantities in normal urine. Traces of certain enzymes, vitamins and hormones may be also detected in urine.

Pathological urine components

Main dietary compounds include carbohydrates, proteins and fats. Their degradative products are normal components of the urine. Their presence in urine in a non-metabolized (non-degraded) form indicates a disorder either of the kidney function or a metabolic disorder. Pathological components of the urine include: proteins, carbohydrates, ketone bodies, hemoglobin, bile pigments, red blood cells, white blood cells, epithelial cells and casts.

Creatinine clearance

Clearance methods are used for determination and quantification of kidney function. Glomerular filtration rate is determined as a clearance of the substances which are filtered in the glomerules and are not reabsorbed or secreted in the tubules. It is most accurately determined using intravenous administration of inulin, a fructose polymer. However, simpler creatinine clearance is routinely used. Creatinine (structure shown in figure) is a product of skeletal muscle metabolism and its concentration in serum is a relatively constant value. As creatinine is filtered and reabsorbed by kidney in neglectable quantities, its concentration in urine reflects the function of glomerular filtration. Creatinine clearance is a very good test for kidney function evaluation and is a more sensitive indicator of kidney insufficiency than serum creatinine levels alone. Normal creatinine clearance ranges from 94 to 156 mL/min (1.57-2.60 mL/s).



Decreased creatinine clearance is found in disorders affecting the filtration:

a) decreased renal blood flow;
b) decreased number of functional glomerules (kidney parenchyma lesions, glomerulonephritis, glomerulosclerosis);
c) decreased glomerular filtration rate due to low blood pressure, higher osmotic pressure as a consequence of hemoconcentration or dehydration, diarrhea, hemorrhage or increased intracapsular pressure in the Bowman's capsule.

Exercise 1. Determination of creatinine in urine

1.1. *Determination of creatinine concentration in urine*

Principle

Creatinine reacts with alkaline picrate to form an orange-red compound and the intensity of color is determined colorimetrically or photometrically.

Reagents and accessories
Picric acid solution, c =35 mmol/L;
NaOH solution, c = 1.6 mol/L;
Distilled water;
Creatinine standard solution, c = 8.84 mmol/L;
Sample: urine;
Test tubes, automatic pipette, dispenser, spectrophotometer, and cuvettes

Procedure

Prepare your sample according to the following table (reagent blank and standard will already be prepared ahead of time):

	Reagent blank	Standard	Sample
V(distilled water)/ mL	2.0	-	-
V(standard solution)/ mL	-	2.0	-
V(sample)/ mL	-	-	2.0
V(picric acid solution)/mL	0.5	0.5	0.5
V(NaOH solution)/mL	0.5	0.5	0.5

Mix well, incubate for 25 minutes at room temperature, then measure the absorbance of the sample and standard against the reagent blank using the spectrophotometer at 490 nm.

A_{Samp}	
A_{St}	
c_{St}	

Calculation

c (urine creatinine) = _____mmol/L

1.2. *Determination of creatinine clearance*

The glomerular filtration rate (GFR) may be estimated by calculating creatinine clearance (C), using a simple expression:

$$C = \frac{c_U}{c_S} \cdot \frac{V}{t}$$

C - creatinine clearance
c_U - creatinine concentration in urine (mmol/L)
c_S - creatinine concentration in serum (μmol/L)
V - daily urine volume (mL)
t - 24 hours in which daily urine is collected (min).

Calculation:

Calculate creatinine clearance using the above expression and following data:

- Creatinine concentration in urine (as determined in 1.1.) =_____mmol/L
- Given creatinine concentration in serum =_____μmol/L
- Given volume of daily urine =_____mL

Creatinine clearance = _____mL/min.

- Compare your results with reference values. For calculation of total quantity of creatinine excreted during 24 hours, take into account given values on daily urine volume and concentration of creatinine in urine.

	Reference creatinine range
Urine	Males: 8.8 - 17.7 mmol/day Females: 7.1 - 15.9 mmol/day
Serum	62 - 125 μmol/L
Clearance	94 - 156 mL/min

Comment briefly your result:

_____

_____

_____

Exercise 2. Analysis of urine composition using test-strips

Principle

Test-strip contains 10 test-fields with specific reagents for determination of: urine density, pH-value, glucose, bilirubin, ketone bodies, blood, proteins, urobilinogen, nitrites and white blood cells.

Procedure

Immerse the test-strip (Multistix® 10 SG) into the urine sample for 1 to 2 minutes, then compare the colors of the test-fields with the scales.

Interpretation of the results

	Normal values	Obtained values
Glucose	-	
Bilirubin	-	
Ketone bodies	-	
Density	1.015–1.025 g/cm^3	
Blood	-	
pH	4.6-8	
Proteins	<0.15 g/dm^3	
Urobilinogen	<16 μmol/dm^{3*}	
Nitrites	-	
White blood cells	-	

* 3.2 μmol/dm^3 = 0.2 mg/dL = 0.2 EU/dL; according to the manufacturer of Multistix® 10 SG

_____

_____

Exercise 3. Determination of the pathological urine components

In the given urine sample, determine the presence of **proteins**, **glucose**, **ketone bodies** and **blood pigments**, using described methods.

Reagents and accessories
20% sulfosalicylic acid solution;
Nylander's reagent – the solution containing: bismuth(III) oxidenitrate ($BiONO_3$), *c* = 95.6 mmol/L, potassium sodium tartarate, c = 142 mmol/L, NaOH, *w* = 0.5;
Trommer's reagent;
1% sodium nitroprusside solution, $Na_2[Fe(NO)(CN)_5]$;
50% CH_3COOH solution;
10% NaOH solution;
Concentrated CH_3COOH solution;
Concentrated NH_3 solution;
10% aminopyrine solution in ethanol;
3% H_2O_2 solution;
Sample: urine;
Test tubes, droppers

3.1. *Detection of proteins with sulfosalicylic acid*

Principle

Presence of proteins in urine is determined by precipitation reactions based on colloid properties of proteins. Color-developing reactions are not suitable because of the color of urine itself. If the addition of sulfosalicylic acid solution causes opacity of urine or white precipitate formation, this indicates the presence of proteins. The test is very sensitive, and as little as protein concentration of 0.01 g/L causes slight opalescence (trace of proteins); 0.1 g/L causes opacity; 0.5 g/L causes muddiness; more than 1 g/L forms precipitate.

Procedure

Pour approximately 2 mL of clear urine into a test tube and add a few drops of sulfosalicylic acid, drop by drop.

What have you noticed?

_____

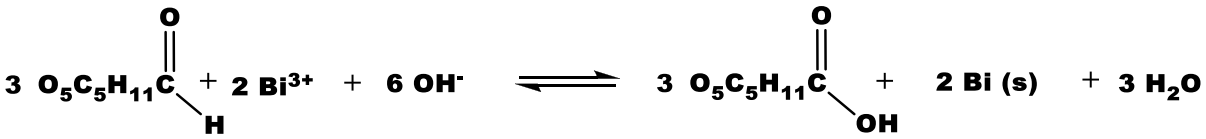
_____

3.2. *Detection of glucose*

3.2.1. *Test according to Nylander*

Principle

Reductive sugars, when heated, reduce Bi^{3+} ions in alkaline medium (Nylander's reagent) to the elementary bismuth. If sugar is present, the urin quickly darkens and the black precipitate of elementary bismuth is formed. Glucose, for instance, reacts following the equation below, and is oxidized to gluconic acid:



Procedure

Add 0.2 mL of Nylander's reagent into a test tube containing 2 mL of urine and boil.

What have you noticed?

_____

_____

Note:

Proteins, present in urine in certain pathological conditions, form black precipitate of bismuth(III) sulfide with the reagent, because they are made of amino acids and some of them contain sulfur (cysteine, methionine). Because of that, the proteins, if detected, should be removed prior to this test. The reagent is sometimes reduced by creatinine, homogentisate, uric acid and certain drugs (salicylates, tetracyclines), but their interference is usually irrelevant when the test is done with a sample of urine diluted with distilled water in 1:1 ratio.

3.2.2. *Fehling's test (Trommer's reaction)*

Note: Principle and procedure of Fehling's test is explained in **Practice 1**, **Exercise 1**.

Using Fehling's test, what have you noticed in your urine sample?

_____

_____

3.3. *Determination of ketone bodies*

3.3.1. *Legal reaction*

Principle

Acetone, acetoacetate and β-hydroxybutirate are ketone bodies. Most of the tests for determination of ketone bodies in urine are based on the reaction of the sodium nitrosylpentacyanoferrate(II) (sodium nitroprusside), which forms a red complex with acetone and acetoacetate in alkaline medium:

$$CH_3\text{-}CO\text{-}CH_3 + OH^- + [Fe(NO)(CN)_5]^{2-} \rightarrow [Fe(CN)_5NOCH_2\text{-}CO\text{-}CH_3]^{3-} + H_2O$$

More intensive purple-red color following the addition of concentrated acetic acid indicates the presence of ketone bodies in urine. If the color disappears after acidification, the ketone bodies are not present; the coloration which later disappears is in this case the result of the reaction with creatinine (normal urinary component).

Procedure

Add a few drops of freshly prepared sodium nitroprusside solution, $Na_2[Fe(NO)(CN)_5]$, into a test tube containing 2 mL of urine. Mix well and add a few drops of NaOH solution.

What have you noticed?

_____

_____

Add a few drops of concentrated CH_3COOH solution!
What have you noticed? Conclusion!

_____

_____

3.3.2. *Modification of Legal reaction*

When testing for small quantities of acetone, which we might fail to observe it, a modified Legal's reaction is used.

Procedure

Add a few drops of acetic acid and sodium nitroprusside solution to the sample of urine; carefully add a few drops of concentrated NH_3 solution to a test tube. If the reaction is positive for acetone, a purple ring forms at the contact surface between two layers.

What have you noticed? Conclusion!

_____

3.4. *Detection of hemoglobin using aminopyrine test*

Principle

Hemoglobin shows pseudoperoxidase activity, and in presence of peroxides oxidizes various chromogenic polyphenols and aromatic amines. There are a number of tests for hemoglobin in urine based on that principle. Aminopyrine, for instance, is oxidized in the presence of hemoglobin and forms a purple compound.

Procedure

Add 2 mL of urine into a test tube containing an equal volume of aminopyrine solution in ethanol, a few drops of 50% acetic acid and hydrogen peroxide, and mix well.

In case of positive reaction, the solution turns purple.

What have you noticed?

_____

Write down and explain the results for the analyzed urine sample!

Compound	Method	Result
Proteins	Precipitation by sulfosalicylic acid	
Carbohydrates	Nylander Trommer	
Ketone bodies	Legal Modification of Legal	
Hemoglobin	Aminopyrine test	

_____

_____

Review questions:

1. a) Represent ketone bodies using structural formulas.

b) Which metabolic condition and/or metabolic disorder lead to excessive production of ketone bodies and ketonuria?

2. Mammals excrete most nitrogen atoms as urea. Describe how increasing concentration of ammonia stimulates urea cycle.

3. Describe metabolic basis and symptoms of maple syrup urine disease.

Date: _____ **Signature: _____**

Practice 9.

ACID-BASE AND MINERAL STATUS OF HUMAN ORGANISM

Sodium

Approximately two-thirds of the total content of Na^+ ions in a human body is in the body fluids; the rest is bound in minerals building bones. Na^+ ions are quantitatively the most abundant cations in all extracellular body fluids, and are primarily accompanied by Cl^- ions. The average Na^+ concentration in a blood plasma is 143 mmol/L. It is crucial for the maintenance and regulation of osmotic pressure of blood plasma and other extracellular fluids as well as for balancing distribution of water in a body. Sodium hydrogen carbonate and sodium hydrogen phosphate are soluble salts forming important inorganic buffers of blood plasma and other extracellular fluids. Na^+ has an important role in depolarization of neuronal and muscular cell membranes, i.e. it takes part in generating of an action potential. Sodium is consumed by food; the daily needs for NaCl are 5 to 15 g depending on a physical activity i.e. on a loss by sweating. It is absorbed in the small intestine and enters across the membrane into the intestinal cell by the protein transport system known as glucose/Na^+ co-transport. It is excreted from the body by urine and sweat. Hormone aldosterone stimulates its re-absorption in the distal renal tubules reducing its loss.

Potassium

Potassium ion is quantitatively the most abundant cation of the intracellular fluid (140 mmol/L), while its concentration in the extracellular fluid is low (approx. 4 mmol/L); the concentration in a blood plasma is 5 mmol/L. In a human body, K^+ has several important roles. It influences activity of muscles, especially myocardium. The potassium hydrogen and the potassium dihydrogen phosphate are components of the major intracellular inorganic buffer. K^+ maintains and regulates the osmotic pressure of cellular plasma and participates in maintaining of cell membrane potential in a resting state as well as in membrane depolarization. A high intracellular concentration of K^+ has a positive influence on ribosomal synthesis of proteins. It acts as an activator of many enzymes such as for example a glycolitic enzyme pyruvate kinase. Potassium is taken in by food and our body's daily need for it is about 4 g. Like Na^+, K^+ is also absorbed in the small intestine. It is excreted through kidney and in part through intestine by feces. Healthy kidneys are very efficient in elimination of this cation, so the possibility of hyperkalemia in healthy body is minimal. During K^+ elimination by the intestine, a part of it is reabsorbed. Digestion disorders accompanied by diarrhea could cause a significant decrease of K^+ concentration in blood; that is manifested as general body weakness even collapse, and particularly as weakness of myocardial activity. Like Na^+, the metabolism of K^+ is regulated by aldosterone causing the elimination of K^+ from blood plasma, acting at the level of distal renal tubules. Thus, aldosterone regulates the correct ratio of Na^+/K^+ concentrations.

Chlorides

Chloride ion is quantitatively the most abundant anion of extracellular fluid and usually accompanies the Na^+. The average concentration of Cl^- in blood plasma is 103 mmol/L. It takes part in maintaining and regulation of osmotic pressure. In blood, it regulates acid-base equilibrium as it enters from plasma to erythrocytes instead of HCO_3^- ion, which diffuses from erythrocytes to plasma. Cl^- anion is also a component of gastric juice (as HCl) and regulates the circulation of water in the body.

Cl^- is consumed in a form of NaCl (salt). It is absorbed in small intestine, and eliminated through kidneys and skin. The metabolism of chloride ions is regulated in relation to the regulation of Na^+ metabolism by mineralocorticoids.

Hydrogen carbonates

In a human body, hydrogen carbonate ions (HCO_3^-, bicarbonate) are continuously generated from CO_2. CO_2 is a final catabolic product of majority of organic molecules. CO_2 reacts with water generating carbonic acid; the reaction is catalyzed by the enzyme carboanhydrase. The carbonic acid dissociates liberating HCO_3^-. Erythrocytes contain carboanhydrase, so they are able to synthesize HCO_3^-.

HCO_3^- is eliminated from the body through renal secretion. Its metabolism is regulated by mineralocorticoids inducing secretion of HCO_3^- at the level of distal renal tubules, but saving Cl^- ions.

Exercise 1. Determination of hydrogen carbonates and chlorides according to Scribner

1.1. *Volumetric determination of hydrogen carbonates in serum*

Principle

If an excess of HNO_3 is added, the HCO_3^- ions quantitatively convert to the equal quantity of CO_2(g), liberating from a sample as gas. The unreacted excess of HNO_3 is determined by titration with standard solution of NaOH using diphenylcarbazone as an indicator.

Reagents and accessories
Standard solution of HNO_3 (c = 0.1000 mol/L);
Standard solution of NaOH (c = 0.1000 mol/L);
Solution of indicator: 0.4% solution of diphenylcarbazone in ethanol;
Sample: human serum;
Hagedorn test tube, burette, micro-burette.

Procedure

Mix 1.00 mL of serum and 1.00 mL of standard solution of HNO_3 in wide test-tube and shake circularly at least half a minute until the produced CO_2(g) is liberated from the mixture. Add 3-5 drops of the indicator. Perform retitration of the excess of HNO_3 by standard NaOH solution until the first drop in excess induces appearance of red color of the indicator. Red color must be visible for at least one minute.

Calculation

Chemical equations of reactions:

$HCO_3^- + HNO_3 \rightarrow$

$HNO_3 + NaOH \rightarrow$

V(standard NaOH solution) =

From the sequence of reactions and stoichiometric relations follows the expression:

$$n(HCO_3^-) = n(HNO_3) - n(NaOH)$$

By inserting the known values it becomes:

$$c(HCO_3^-) = \frac{c(HNO_3) \cdot V(HNO_3) - c(NaOH) \cdot V(NaOH)}{V(HCO_3^-)} =$$

=

Result: $c(HCO_3^-)$ = _____mol/L = _____mmol/L

Reference values: 24-28 mmol/L

Comment the result in comparison with reference values!

_____

_____

_____

1.2. *Volumetric determination of chlorides in serum*

Principles

The concentration of the Cl^- ions in serum could be quantitatively determined by titration with the standard solution of $Hg(NO_3)_2$ (mercury(II) nitrate). Cl^- ions are quantitatively bound into the mercury(II) chloride. Ions of mercury(II) from the first excess drop react with the indicator diphenylcarbazone, forming the violet complex. The acidic medium is necessary for the reaction (pH 3-4.5) to prevent the hydrolysis of the Hg^{2+} ions as well as the binding of that ion to the SH-group of proteins and other potentially present halogenide ions; therefore the solution of HNO_3 is added.

Reagents and accessories
Solution of HNO_3 (c = 0.10 mol/L);
Standard solution of $Hg(NO_3)_2$ (c = 0.0500 mol/L):
Solution of indicator: 0.4% solution of diphenylcarbazone in ethanol;
Sample: human serum i.e. the reaction mixture remaining after the determination of hydrogen carbonates;
Hagedorn-tube, burette, micro-burette.

Procedure

To the reaction mixture remaining after determination of hydrogen carbonates (Exercise 1.1.), containing the original sample, add 2 mL of HNO_3 solution and perform titration with the standard solution of $Hg(NO_3)_2$ until the color of the titrated mixture is changed to violet from the first excess droplet of standard solution which reacts with indicator resulting in formation of the violet complex of the indicator with the Hg^{2+} ions.

Calculation

Chemical reaction equations:

$Cl^- + Hg(NO_3)_2 \rightarrow$

V(standard $Hg(NO_3)_2$ solution) =

$n(Cl^-)$ =

$c(Cl^-)$ =

Result: $c(Cl^-)$ =_____mol/L = _____mmol/L

Reference values: 95-106 mmol/L Cl^-

Discuss the obtained results in relation to the reference values! _____

_____

_____

_____

Calcium

The average content of calcium in a human body is 1180 g; it is always present in the oxidation state +2. Even 99% of the total content is bound in slightly soluble salts (minerals) building bones and teeth. The rest of 1% of calcium is primarily present in extracellular fluids. The smaller part is present in cells, either bound or free as Ca^{2+}(aq) cation.

Calcium in blood, either as a free ion or bound in compounds, is primarily localized in blood plasma, while small quantities are present in erythrocytes. Therefore the serum is used for diagnostic determination of calcium; the concentration in healthy condition is 2.25–2.75 mmol/L. The calcium concentration in fetal serum is 2.75–3.0 mmol/L.

In blood serum, calcium is present in two forms: diffusible and non-diffusible (Table 8.1). The non-diffusible calcium is bound to serum proteins. Diffusible calcium accounts for 50-60% of the total calcium in serum; it could be ionized (Ca^{2+}(aq), a free ion form) and/or bound in complexes with citrate, hydrogen carbonate, sulfate and hydrogen phosphate. The solubility of the calcium phosphate is higher in the blood then in the water; it depends on pH, partial pressure of CO_2, ionic strength, and on concentration of proteins, magnesium and inorganic phosphate. At the constant pH of blood (7.4), the solubility is reciprocally proportional to the concentration of HCO_3^- and HPO_4^{2-}, and directly proportional to the concentration of magnesium and albumins. The majority of diffusible calcium is ionized and only ionized calcium is a physiologically active form. Its concentration in serum changes reciprocally proportional to the pH, but the concentration of total calcium is not changed. This means that if a concentration of H^+-ions decreases, the concentration of ionized calcium also decreases and vice versa. Also, a concentration of Ca^{2+}(aq) decreases with an increase of either HCO_3^- or HPO_4^{2-} concentration.

Table 8. 1.

Total serum calcium: 2.25 – 2.75 mmol/L		
Non-diffusible: 1.12 – 1.25 mmol/L	Diffusible: 1.12 – 1.50 mmol/L	
	Ionized: 1.05 – 1.40 mmol/L	Bound in complexes: 0.05 – 0.125 mmol/L

Exercise 2. Determination of the total calcium in serum by spectrophotometric method

Principle

o-Cresolphthalein (1-hydroxy-2-methylbenzene-phthalein) is a complexone reacting with calcium ions in an alkaline medium forming a violet complex. The intensity of the violet color of the complex in the mixture is measured spectrophotometrically at λ = 570 nm.

Reagents and accessories
Buffer: lysine buffer (pH 11.1) c = 0.2 mol/L
sodium azide (0.095%);
Color-reagent:
o-cresolphthalein (c = 0.10 mmol/L)
8-hydroxykinolyn (c = 14 mmol/L)
hydrochloric acid (40 mmol/L)
sodium azide (0.095%);
Working reagent: mix the buffer and the color reagent in ratio 1:1 (according to the number of samples);
Standard: solution of calcium salt (c = 2 mmol/L)
sodium azide (0.095%);
Sample: human serum;
Test tubes, pipette.

Procedure

Prepare your sample according to the following table (reagent blank and standard will already be prepared ahead of time):

	Blank	Standard	Probe
V(sample)/μL	-	-	20
V(distilled water)/μL	20	-	-
V(standard)/μL	-	20	-
V(working reagent)/mL	1.0	1.0	1.0

Mix and leave to stand for 5 minutes at a room temperature. Measure the absorbances of the standard (A_{St}) and the probe (A_{Pr}) against the blank at 575 nm.

A_{St}	
A_{Pr}	
c_{St}	

Calculation

Result: $c(Ca^{2+})$ =_____mmol/L

Reference values: Serum 2.25 – 2.65 mmol/L;
Urine 2.50 – 7.75 mmol/*V* 24 h
Comment the obtained result in relation to the reference values! _____

_____

_____

Magnesium

Human body contains about 25 grams of magnesium, always in the oxidation state +2. It is either bound in compounds or present as a free ion Mg^{2+}(aq). More than 50% of magnesium (Mg^{2+}) is a component of bones in the form of water-insoluble salts. The rest of it is mainly within cells, and less in extracellular fluids, predominately in the ion form.

Erythrocytes contain most of the magnesium in the blood, about 2.25 to 3 mmol/L. That is three times more than the concentration in blood serum, which is 0.60-1.10 mmol/L. About 70-85% of serum magnesia is diffusible and rest is bound to proteins, mainly albumin. Most of the diffusible serum magnesium is ionized and the rest is in the form of phosphate, citrate and other complexes.

Exercise 3. Spectrophotometric determination of magnesium in the blood serum

Principle

Mg^{2+} and xylidyl blue form colored complex in alkaline medium. The color intensity is measured spectrophotometrically at 520 nm.

Reagents and accessories:
Reagent: CAPS (*N*-cyclohexyl-3-amino-1-propanesulfonic acid) (*c* = 50 mmol/L)
GEDTA (glycoletherdiamine-*N*,*N*,*N'*,*N'*-tetraacetic acid) (*c* = 13 mmol/L)
Xylidyl Blue (*c* = 0.09 mmol/L)
sodium azide (0.095%);
Standard: magnesium salt solution (*c* = 1.03 mmol/L);
Sample: blood, serum;
Test tubes, automatic pipette, spectrophotometer and cuvettes.

Procedure

Prepare your sample according to the following table (reagent blank and standard will already be prepared ahead of time):

	Blank	Standard	Probe
V(distilled water) / μL	10	-	-
V(standard) / μL	-	10	-
V(blood serum) / μL	-	-	10
V(working solution) / mL	1.0	1.0	1.0

Mix and leave for 10 minutes at a room temperature. Measure the absorbances of the standard (A_{St}) and the probe (A_{Pr}) against the blank, at 520 nm.

Notes:
GEDTA eliminate interference with calcium and other metal ions.

Hyperhemoglobinemia, hyperlipemia and hyperbilirubinemia do not affect the accuracy of determination.

A_{St}	
A_{Pr}	
c_{St}	

Calculation**:**

Result: $c(Mg^{2+})$ =_____mmol/L

Reference values

Serum: 0.60 – 1.10 mmol/L

Comment the obtained result!

_____

_____

Exercise 4. Qualitative analysis of selected ions

4.1. *Precipitation*

Principle

Detection of an ion in a sample solution includes addition of a reagent solution, which may cause the precipitation of the insoluble salt - product of the sample cation and the anion from the reagent or vice versa. Identification of an ion in the sample solution is then made on the basis of the physical and chemical characteristic of the formed precipitate (color, solubility, etc.).

Reagents and accessories:

Solution of sodium hexanitrocobaltate(III), $Na_3[Co(NO_2)_6]$ (c = 0.1 mol/L);
$(NH_4)_2CO_3$ solution (c = 2.0 mol/L);
$Na_2C_2O_4$ solution (c = 0.25 mol/L);
NaOH solution (c = 2.0 mol/L);
NH_4OH solution (c = 2.0 mol/L);
NH_4Cl solution (c = 2.0 mol/L);
Na_2HPO_4 solution (c = 0.33 mol/L);
$AgNO_3$ solution (c = 0.1 mol/L);
$BaCl_2$ solution (c = 0.25 mol/L);
$MgCl_2$ solution (c = 0.4 mol/L);
HCl solution (c = 1.0 mol/L);
HNO_3 solution (c = 1.0 mol/L);
CH_3COOH solution (c = 1.0 mol/L);
Samples: solutions of unknown salts;
Semimicro-test tubes, droppers.

NaCl solution (c = 0.1 mol/L);
K_3PO_4 solution (c = 0.1 mol/L);
$MgCl_2$ solution (c = 0.1 mol/L);
$CaCl_2$ solution (c = 0.1 mol/L);

Procedure

Pipette 3-4 drops of the sample solution in a clean semimicro-test tube and add the equal amount of reagent solution and mix. Repeat the procedure with various reagents according the following table.

Note: procedures of the reactions labeled in the table with numbers 1 and 2 are described under the table!

Ion	Reagent	Positive reaction for identification of an ion
K^+	$Na_3[Co(NO_2)_6]$	Yellow crystalline precipitate _____.
Ca^{2+}	$(NH_4)_2CO_3$	White crystalline precipitate _____, soluble in diluted HNO_3 or diluted HCl.
	$Na_2C_2O_4$	White crystalline precipitate _____, soluble in mineral acids, and insoluble in diluted CH_3COOH.
	NaOH	White precipitate_____.
	NH_4OH	White precipitate_____.
Mg^{2+}	NaOH	White amorphous precipitate _____, insoluble in the excess of the reagent solution.
	NH_4OH	White amorphous precipitate _____. Dissolves by addition of ammonium salt solution, e.g. NH_4Cl solution.
	NH_4OH/ NH_4Cl/ Na_2HPO_4 [1]	White crystalline precipitate _____. (magnesium ammonium phosphate)
Cl^-	$AgNO_3$	White precipitate _____, insoluble in diluted HNO_3.
PO_4^{3-}		Yellow precipitate _____, soluble in diluted HNO_3.
	$BaCl_2$	White precipitate _____, soluble in diluted HNO_3 or HCl.
	Magnesium-mixture [2] $MgCl_2$/ NH_4OH/ NH_4Cl	White crystalline precipitate _____. (magnesium ammonium phosphate)

[1] Add NH_4OH solution drop-by-drop into the sample solution till the appearance of the white precipitate of $Mg(OH)_2$. Then add drop-by-drop NH_4Cl solution, and mix, until the precipitate is dissolved. Add Na_2HPO_4 solution to the mixture.

[2] Preparation of reagent solution: add drop-by-drop NH_4OH solution to $MgCl_2$ solution till the appearance of the white precipitate of $Mg(OH)_2$. Then add drop-by-drop NH_4Cl solution until the precipitate is dissolved and mixture is clear. Put 1-2 drops of sample into the reagent solution.

- **Write the chemical equations of all positive reactions used for determination of the particular ion! Use the ionic equations (examined ion + ion from a reagent) and describe the visible changes!**

Example of the chemical equations of the positive reactions for the identification of examined ion, and description of the visible changes:

$Mg^{2+} + NH_4^+ + HPO_4^{2-} \rightarrow MgNH_4PO_4$ (s) + H^+ (white precipitate)

($MgCl_2 + NH_4OH + Na_2HPO_4 \rightarrow MgNH_4PO_4$ (s) + 2 NaCl + H_2O (white precipitate)

_____

_____

_____

_____

_____

_____

4.2. *"Flame coloration"*

Principle

The evaluation of flame colorations is used for the qualitative analysis of elements. Volatile salts of some ions color flame (see table below). In a flame, following processes occur rapidly: an aqueous solution of e.g. sodium chloride is sprayed into flame and the solvent is vaporized while NaCl partially dissociates into atoms; part of the produced atoms (Na) in the gaseous state are excited by thermal energy. As a result of the return of the electrons to the ground state, light is emitted (for sodium at the main wavelength of 589 nm, yellow light).

Na^+	Yellow
K^+	Purple
Ca^{2+}	Red

Procedure

Check the color of the flame by putting the filter paper soaked with the sample solution into flame.

4.3. *Qualitative analysis of the unknown salt*

Identify and determine the cation and anion present in the obtained sample solution. Use all previously described reactions.

Empiric formula of the determined salt:

_____

Write the chemical equations of the positive reactions, and description of the flame coloration method:

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

Appendix

Characteristic diagnostic parameters used for interpretation of the acid-base balance state in body

The corresponding reference values are given in brackets!

1. **Blood pH** (7.36-7.44) - The negative logarithm of the hydrogen ion concentration in the blood.
2. **Partial pressure of CO_2 in blood, pCO_2** (4.80-5.87 kPa; 36-44 mmHg) - The pCO_2 is directly related to the blood CO_2 concentration, which is in balance with the blood H_2CO_3 concentration.
3. **Partial pressure of O_2 in blood, pO_2** (arterial blood 10.67-13.83 kPa or 80-104 mmHg; venous blood 2.67-6.53 kPa or 20-49 mmHg)
4. **Saturation of blood (i.e. hemoglobin) with oxygen, sO_2** (95-98%) - The percentage of the maximal amount of oxygen, which could be bound to hemoglobin in blood.
5. **Base excess** (-2.5 to +2.5 mmol/L) - Denotes an excess or a deficit of bases in blood, i.e. the corresponding deficit or the excess of nonvolatile acids. It is usually expressed as the amount (**mmol**) of the acid or the base that would be spent for the titration of the completely oxygenized blood until the normal pH (pH 7.4) is achieved, at the physiologic pCO_2 (5.33 kPa ili 40 mmHg), and the physiologic temperature (38 °C).
6. **Standard HCO_3^-** (22-26 mmol/L) - The concentration of HCO_3^- in completely oxygenized blood at pCO_2 5.33 kPa and temperature of 38 °C.
7. **Actual HCO_3^-** (22-26 mmol/L) **-** The concentration of HCO_3^- in plasma of the blood taken at anaerobic conditions.
8. **Total CO_2** (23-27 mmol/L) - Total amount of CO_2 in 1.00 L of plasma of the blood taken at anaerobic conditions. Includes dissolved CO_2, and CO_2 bound to hemoglobin, H_2CO_3 and HCO_3^-.
9. **Buffer bases** (45.5-50.5 mmol/L) - Concentration of buffer anions, primarily HCO_3^-, and protein anions in the oxygenized blood at pCO_2 5.33 kPa and temperature of 38 °C.

Date: _____ **Signature:** _____