

ARVYDAS KAMINSKAS | ASTA MAŽEIKIENĖ | DOVILĖ KARČIAUSKAITĖ

# MEDICAL BIOCHEMISTRY

LABORATORY MANUAL





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## LABORATORY MANUAL

VILNIAUS UNIVERSITETO LEIDYKLA

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# Rules and Regulations in a Biochemistry Laboratory

There are two major concerns to consider when working in a biochemistry laboratory. The first is safety: this can never be overemphasized. The general guidelines for safety are discussed below. The second major concern is efficiency in laboratory operations. Although the latter very much depends on the individuals doing the experiments, there are general rules that students are advised to follow:

1. Keep the benches and shelves clean and well-organized;
2. Avoid contaminating the chemicals; use only clean glassware and spatulas; label glassware in use;
3. Plan your experiments before starting to carry them out;
4. Mind other colleagues in the laboratory.

## **LABORATORY SAFETY**

Students working in a biochemistry laboratory must always be aware that the chemicals used are potentially toxic, irritating and flammable. Such chemicals are hazardous but only when mishandled. Students who show up to a laboratory session must have a complete understanding of laboratory procedures to be carried out and be familiar with both the physical and chemical properties of the chemicals and reagents to be used. Since the carelessness on the part of one student can often cause injury to others, one must have special concern for the safety of one's classmates. Students must be familiar with the general safety practices, facilities and emergency actions.

### **General Safety Rules**

1. Do not work alone in the laboratory;
2. Unauthorized experiments are not allowed;



3. Eating, drinking and smoking in the laboratory are all strictly prohibited;
4. Become familiar with the location and use of standard safety features in the laboratory. The laboratory is equipped with fire extinguishers, eyewashes, safety showers, fume hoods and first-aid kits. Any questions regarding the use of these facilities should be addressed to your instructor;
5. Special care for eye protection is required. Safety glasses must be used when certain procedures are being carried out. The instructor will call the students' attention to those procedures. The use of contact lenses is not recommended, since they reduce the rate of the self-cleansing of the eye.

### **Special Safety Rules**

1. While heating a solution, one should make sure not to overheat it; therefore, a vigorous mixing of the solution by shaking or stirring is required. The mouth of the glassware containing the solution to be heated should never be pointed toward anyone.
2. The handling of strong acids and bases requires special attention. When diluting concentrated acids, the acid should be poured into water and never the opposite;
3. The pipettes should never be filled with solutions of toxic substances, biological fluids, strong acids and bases by mouth suction. Use either automatic pipettes or pipette pumps;
4. Volatile liquids and solids that are toxic or irritating should be handled under fume hoods;
5. While handling flammable liquids such as ether, alcohols or benzene, a naked flame (burners, matches) must not be in use. The abovementioned liquids must not be stored near radiating heat sources, such as laboratory ovens;
6. Before using electrical appliances, make sure they are grounded;
7. Flasks with flat bottoms or thin walls should not be desiccated;
8. Before leaving the laboratory, electrical equipment should be turned off and gas burners extinguished. No tap water should be left running.

### **In Case of Accidents and Injuries**

*Chemical splatters into the eyes.* First, the eyelid should be opened by using the thumb and the pointing finger. Then, by using the eye wash kit, the eye should be rinsed with large amounts of water. When an acid or alkaline solution gets into the eye, the eye should be rinsed with 1%  $\text{NaHCO}_3$  or 1% boric acid, respectively. The victim should be taken to the doctor as soon as possible.

*Burning.* The burned spot on the skin should not be treated with water; rather, a special bandage should be used. See a doctor if necessary.

*Poisoning.* Prompt medical treatment should be obtained.

All injuries and accidents must be reported to the instructor.

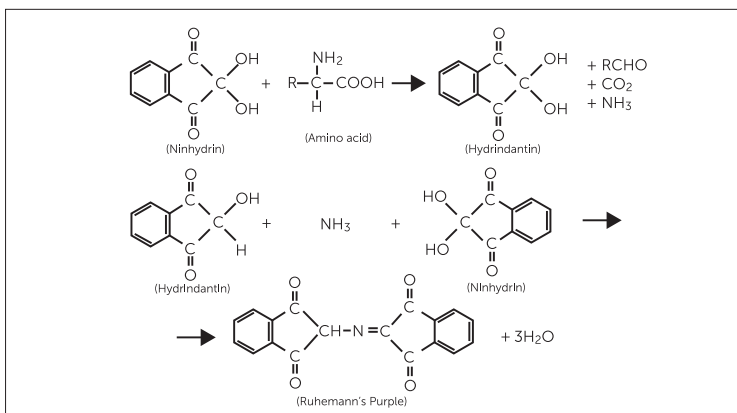
## Colour Reactions of Amino Acids

**AIM:** to qualitatively detect the presence of amino acids by using the Ninhydrin Test, Xantoproteic Test, Milons Test, Lead Acetate Test and Sakaguchi's Test.

### THE NINHYDRIN REACTION

In the pH range of 4-8, all  $\alpha$ -amino acids react with ninhydrin, a powerful oxidizing agent, to give a purple-colored product. This reaction is the most important method of detecting proteins, peptides and  $\alpha$ -amino acids and indentifying their content of a free  $\alpha$ -NH<sub>2</sub> group.

When amino acids with a free  $\alpha$ -NH<sub>2</sub> group are treated with a ninhydrin solution,  $\alpha$ -amino acid is oxidized and decomposed into aldehyde, CO<sub>2</sub> and NH<sub>3</sub>, while ninhydrin is reduced. Then, the reduced ninhydrin condenses with ammonia and a non-reduced ninhydrin molecule, which leads to the formation of the violet-blue condensation product.



## 1. Colour Reactions of Amino Acids

Only proline – imino acid containing a secondary amino group – produces the yellow condensation product.

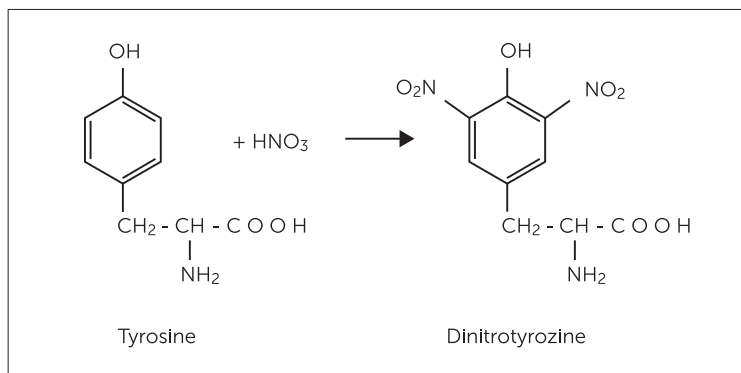
The color intensity is proportional to the concentration of ammonia from the amino groups. That is why the reaction with ninhydrin can be used for a quantitative analysis of amino acids and free amino groups in proteins.

Ammonium salts, amino sugars and ammonia also give positive ninhydrin test results. In order to obtain the reliable quantitative results, the test solution should not contain the compounds mentioned above.

**Procedure.** Add 5 drops of diluted egg protein or serum to one test tube and 5 drops of 1% gelatin to another test tube. Into each test tube, add 3 drops of 0.5% ninhydrin and heat the tubes in a boiling water bath. After 2–3 minutes observe a change in color.

### THE XANTHOPROTEIC (YELLOW PROTEIN) REACTION

Aromatic amino acids, such as tyrosine, tryptophan and phenylalanine, respond to this test. In the presence of a concentrated nitric acid, the aromatic phenyl ring gets nitrated to give yellow-colored nitro derivatives. In the presence of an alkali, these derivatives form orange-colored salts.

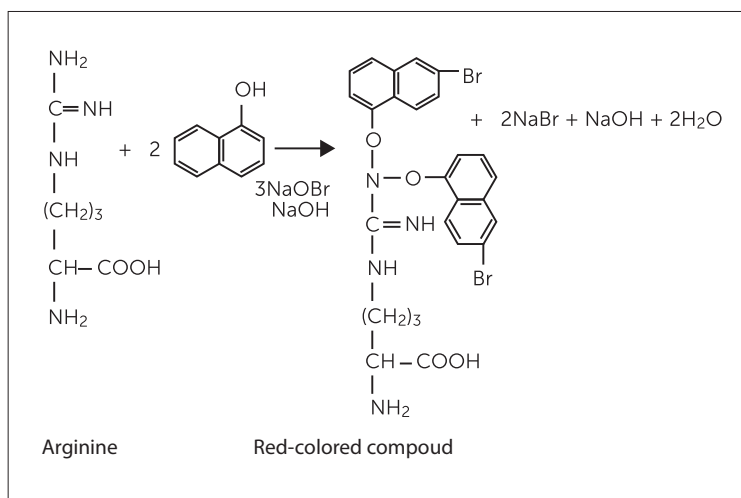


**Procedure.** Add diluted serum or egg protein to a test tube, then add 3 drops of concentrated nitric acid and heat the substance (be careful). Observe a color change.

After cooling, add 10 drops of concentrated ammonia and 30% NaOH into the test tube. Observe a color change.

## THE SAKAGUCHI REACTION

This test is specific for arginine, as it is the only amino acid containing a guanidine group. This moiety reacts with  $\alpha$ -naphthol and an oxidizing agent, such as hypobromite, to give off a red color.

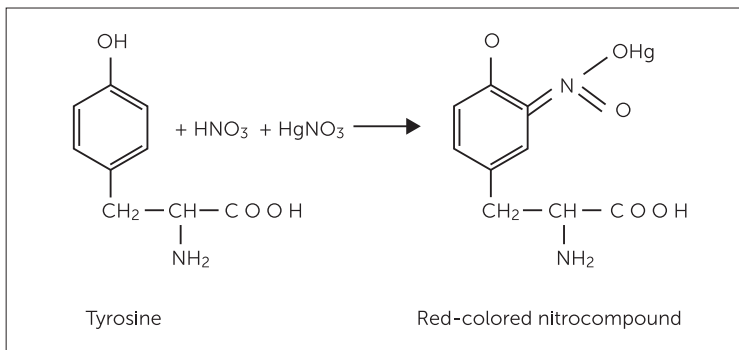


**Procedure.** Add 5 drops of diluted blood serum or egg protein into a test tube and 5 drops of 1% gelatin (partly hydrolyzed collagen) into another test tube. Into each test tube, add 5 drops of a 10% NaOH solution, 3 drops of 0.1%  $\alpha$ -naphthol in the ethanol solution and 1 to 5 drops of 2% sodium hypobromite. Observe a color change.

## THE MILLON REACTION

Phenolic amino acids, such as tyrosine and its derivatives, respond to this test. Compounds with a hydroxybenzene radical react with Millon's reagent (mercury in nitric acid solution) to form a reddish-brown precipitate.

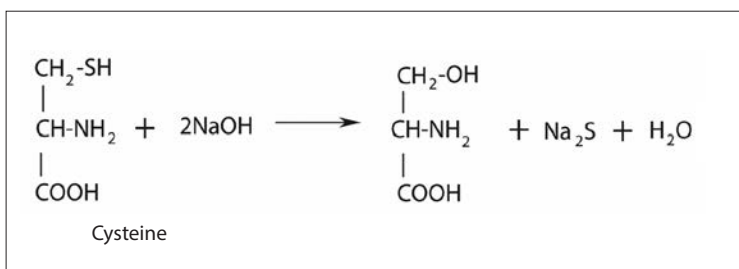
## 1. Colour Reactions of Amino Acids



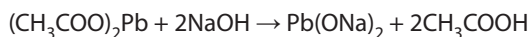
**Procedure.** Add 10 drops of diluted egg protein or blood serum into one test tube and 10 drops of 1% gelatin solution into another test tube. Into both tubes, add 1 to 2 drops of Millon's reagent. Heat the tubes (be careful). Observe a color change.

### LEAD ACETATE TEST

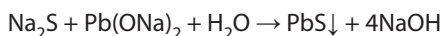
This is a specific test for sulfur-containing amino acids, such as methionine and cysteine. On heating under alkaline conditions, the sulfur of the amino acids combines with the lead acetate to form black lead sulfide.



Lead acetate and sodium hydroxide produce  $\text{Pb}(\text{ONa})_2$ :



Sodium sulfide reacts with  $\text{Pb}(\text{ONa})_2$  to form dark lead sulfide sediments:



**Procedure.** Add 5 drops of diluted egg protein or blood serum into one test tube and 5 drops of 1% gelatin into another test tube. Into both test tubes, add 5 drops of 30% sodium hydroxide and 1 drop of 5% lead acetate. Observe a color change.

### REVIEW QUESTIONS

1. The major biological functions of proteins.
2. The chemical composition of proteins. The nitrogen content of proteins.
3. Amino acids: their properties and structural formulas.
4. A peptide bond and its properties.
5. The physical and chemical properties of amino acids.
6. The color reaction of amino acids.

## Paper Chromatography of Amino Acids

**AIM:** *Separation of amino acids using paper chromatography.*

### PRINCIPLE:

The chromatographic separation of components of mixture depends on their distribution between two different phases: the mobile phase (solvent or developer) and the stationary phase (sorbent). The term “paper chromatography” used in this experiment identifies the composition of the stationary phase.

The filter paper is composed mostly of cellulose and is very hydrophilic. Next, a hydrophobic organic solvent is drawn up the paper by capillary action. As the solvent moves over the location of the biomolecule, the biomolecule begins to move up the paper. The rate at which the biomolecule moves up the paper is related to its relative affinity for the paper (which is hydrophilic) and the solvent (which is hydrophobic). Hydrophobic molecules will move faster, because they are more attracted to the hydrophobic solvent than the hydrophilic paper. On the other hand, hydrophilic molecules will move slower, because they are attracted more to the paper than the hydrophobic solvent.

Paper chromatography is especially useful in characterizing amino acids. The different amino acids move at differing rates on the paper because of the differences in their R groups.

The rate of movement of a biomolecule during paper chromatography is reported as its relative mobility ( $R_f$ ).  $R_f$  is simply the distance that the biomolecule has moved through the filter paper divided by the distance the solvent moved through the paper.



**Procedure.** At the centre of the filter paper, draw a small circle with the radius of 1 cm – the starting line, where the amino acids have to be applied. Cut 1 cm wide tape from the edge of the paper up to the small circle at the center, which will be the wick. Place the paper disc on a petri dish and immerse the wick into the petri dish filled up with the solvent mixture (ethanol and 1M ammonium acetate 7:3). Leave the chromatogram to develop for 1–1.5 hours or until the solvent reaches the edge of the paper. Then, dry the paper in a drying oven at the temperature of 50 °C. The detection of amino acids on the chromatography paper is performed by dyeing it with a 0.1% ninhydrin solution, following a drying in oven for 5–10 minutes at 50 °C to reveal purple spots.

Measure the distance travelled by amino acids (a) and the distance travelled by the solvent (b) and calculate the partition coefficient  $R_f$  for each amino acid by the formula:

$$R_f = a : b$$

The partition coefficient is a constant characteristic of each amino acid and depending on test conditions.

## REVIEW QUESTIONS

1. Steps of protein purification from a mixture.
2. The determination of protein concentration in eluates.
3. The description of chromatographic methods.
4. Ion-exchange chromatography.
5. Molecular filtration using sephadex.
6. Affinity chromatography.
7. The criteria for purity of protein.
8. The determination of an amino acid composition and the sequence of proteins.
9. Paper chromatography.

## Determination of Protein Concentration in Urine

**AIM:** *to estimate protein amount in a urine sample by precipitation with sulfosalicylic acid.*

### BACKGROUND:

Small quantities of albumin are normally filtered at the glomerulus and reabsorbed by the proximal tubule. The amount of the protein that is not reabsorbed does not exceed 150 mg/24 hr, so that the proteinuria in excess of this amount is regarded as pathologic.

Protein is precipitated in urine by the addition of sulfosalicylic acid (SSA). The urine becomes turbid as a result of the addition of SSA. The level of turbidity is roughly proportional to the amount of protein present in the sample.

**Procedure.** Add 1.25 ml of filtered urine and 3.75 ml of a 3% sulfosalicylic acid solution into a test tube and mix the contents. To prepare a control solution, add 1.25 ml of urine and 3.75 ml of a 0.9% NaCl solution into another test tube. Wait for 5 minutes and measure the extinction with a colorimeter-nephelometer using a red filter and a 5 mm cell. Estimate protein concentration using the standard curve.

In order to determine the actual concentration of protein in the unknown samples, it is necessary to graph the standard curve. In the calibration curve, the different standard protein levels are plotted on the x-axis, and their extinction values, after the reaction with sulfosalicylic acid, on the y-axis.

Below is a table for making the standard curve (including the standard sample preparation from a stock albumin solution – 10 mg/ml).

Serial No.	Standard solution (ml)	0.9% NaCl (ml)	Protein concentration g/l	Extinction
1	0.05	9.95	0.05	
2	0.1	9.9	0.1	
3	0.2	9.8	0.2	
4	0.5	9.5	0.5	
5	1.0	9.0	1.0	

## REVIEW QUESTIONS

1. The definition and classification of proteinuria.
2. The mechanism of proteinuria. Why does a plasma albumin appear more easily in urine in comparison to globulins?
3. Origin of Bence-Jones protein in urine.
4. The method for detecting protein in urine. Could sulfosalicylic acid be replaced with sulfuric acid?
5. The importance of electrophoresis for the detection of Bence-Jones protein and the diagnostics of renal proteinuria.

# 4.

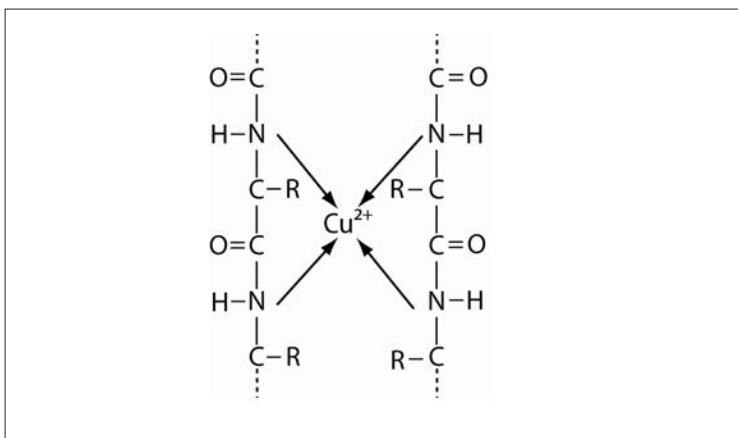
## Determination of Protein Concentration in Serum

**AIM:** to estimate the protein amount in a blood serum sample with the Biuret Method.

### BACKGROUND:

Proteins are the polymers of amino acids, which are linked serially by peptide bonds. The Biuret test is the general method for detecting peptide bonds.

Proteins containing two or more peptide bonds react with alkaline copper sulfates to produce a purple-colored product, which is developed due to the formation of a cupric coordination complex.



The intensity of the color produced is proportional to the number of peptide bonds present in the sample.

## Procedure.

REAGENT	<b>R1: biuret reagent</b>			
	Potassium iodide	30 mmol/l		
	Potassium sodium tartrate	100 mmol/l		
	CuSO <sub>4</sub>	30 mmol/l		
	NaOH	3.8 mmol/l		
	<b>R2: standard</b>			
	Solution of albumin	60 g/l		
PROCEDURE	1. Into the first test tube, add 1 ml of the R1 reagent; this tube is called “blank.”			
	2. Into the second test tube, add 1 ml of the R1 reagent, 10 µl of the standard R2 (the known concentration of a protein solution); this tube is called “standard.”			
	3. Into the third test tube, add 1 ml of the R1 reagent and 10 µl of the sample (blood serum); this tube is called “sample.”			
		“Blank”	“Standard”	“Sample”
	R1 reagent	1 ml	1 ml	1 ml
	R2 standard	–	10 µl	–
	Blood serum	–	–	10 µl
	4. Mix and incubate for 5 min. at +37 °C.			
	5. The analyzer measures the light absorption rates and shows the protein concentration in g/l.			
	CALCULATION	$\frac{\text{ABS sample}}{\text{ABS standard}} \times \frac{\text{standard concentration (g/l)}}{\text{protein concentration (ABS – absorbance)}}$		
REACTION CONDITIONS	Wavelength	546 nm (520–570 nm)		
	Temperature	+37 °C		
	Cell	1 cm		
REFERENCE VALUE	Serum: Adult subjects	65–85 g/l		

### REVIEW QUESTIONS

1. Blood plasma proteins and their concentration.
2. What is the difference between a blood serum and plasma?
3. Hypo-, hyper- and disproteinemia, their causes. Relative and absolute hyperproteinemia.
4. Paraproteins, their causes. The diagnostic possibilities of paraproteinemias.
5. The principle of the Biuret Method. What is a biuret?

## Electrophoresis of Serum Proteins

**AIM:** *to separate serum proteins and evaluate electrophoretic fractions.*

### BACKGROUND:

Electrophoresis separates proteins on the basis of their electric charge densities. Protein, when placed in an electric field, will move according to its charge density, which is determined by the pH of the surrounding buffer. At a pH greater than the pI, the protein is negatively charged ( $\text{AA-COO}^-$ ) and vice versa ( $\text{AA-NH}_3^+$ ). The direction of movement depends on whether the charge is positive or negative; cations (the positive net charge) migrate to the cathode (the negative electrode), whereas anions (the negative net charge) migrate to the anode (the positive electrode).

The speed of the migration can be estimated from the difference between the pI of the protein and the pH of the buffer. The more the pH of the buffer differs from the pI, the greater is the magnitude of the net charge of that protein and the faster it will move in the electric field. In addition to the charge density, the velocity of the movement also depends on the electric field strength, size and shape of the molecule; influencing factors are also temperature as well as the characteristics of the buffer, such as pH, qualitative composition and ionic strength.

With the standard method for serum protein electrophoresis (SPE), serum samples are applied close to the cathode end of a support medium that is saturated with an alkaline buffer (pH 8.6). All major serum proteins carry a net negative charge at pH 8.6 and mi-

grate toward the anode. Using standard SPE methods, serum proteins are separated into five fractions: albumin travels farthest to the anode, followed by an  $\alpha$ 1-globulin fraction, then by  $\alpha$ 2,  $\beta$  and  $\gamma$  – globulins, in that order. The width of the band of proteins in a fraction depends on the number of proteins present in that fraction. Homogeneous proteins give a narrow band.

After separation, the protein fractions are fixed by immersing the support medium in an acid solution (e.g., an acetic acid) to denature the proteins and immobilize them on the support medium. Next, the proteins are stained. The proteins appear as bands on the support medium. A visual inspection of the membrane can be done, but usually, the cleared transparent medium is placed on a scanning densitometer for reading. The pattern on the membrane moves past a slit through which light is transmitted to a phototube to record the absorbance of the dye, which is bound to each protein fraction. This absorbance is normally recorded on a strip-chart recorder to obtain a pattern of the fractions.

### Procedure.

1. *Preparing for electrophoresis.* Add 300–400 ml of buffer (the level must be equal in both chambers) into the chambers when the power is switched off.
2. *Preparing the plate(s).* Before electrophoresis on cellulose acetate plate(s), place a plate into the buffer for 2–3 minutes, then dry it (to remove the excess buffer) and mount it on the flexible part of the stretched bridge. Place the ends immersed in the buffer solution.
3. *Applying the sample to the plate.* Take 10–13  $\mu$ l of blood serum with a micropipette and move the sample into a cellulose acetate plate with a dry applicator.
4. *Electrophoresis.* Place the plate containing a specimen into the electrophoresis chamber so that the test samples will be near the cathode (-) field. The buffer is used with a pH higher than the protein isoelectric point (pH 8.6). Proteins contain the negative charge and move toward the positive electrode. Close the electrophoresis chamber with a cover; the electric power must be switched on.



Switch the power off after the migration; carefully remove the cover and take the plate out.

5. *Visualizing the protein bands.* Place the plate in the dye solution and keep it there for about 10 minutes.
6. *Fade.* Transfer the plate into a destaining solution; then, place it on a glass plate. If the solution forms any excess air bubbles, removed them.
7. *Drying.* Place the plate into a drying oven at 120–180°C for 5–10 minutes until it becomes dry and transparent.
8. *Evaluating the protein bands.* Scan the plates in a densitometer using a 525 nm filter.

The integrated lens of the densitometer provides a light flux that passes through the slot and the protein fractions. The photodetector light energy is converted into electric signals resulting in a curve that shows 5 to 6 protein fractions: albumin,  $\alpha_1$ ,  $\alpha_2$ ,  $\beta$  ( $\beta_1$ ,  $\beta_2$ ) and  $\gamma$  globulins. Also, the densitometer calculates the relative proportions of the fractions. When albumin makes about 60% of the total serum protein, globulin represents about 40% of it,  $\alpha_1$ -globulin fraction about 4%,  $\alpha_2$  – 8%,  $\beta$  – 12%,  $\gamma$  – 16%.

### REVIEW QUESTIONS:

1. The isoelectric point of a protein. The dissociation of protein depending on pH.
2. The principle of electrophoresis.
3. The electrophoretic fractions of serum proteins and their composition.
4. Disproteinemias.

## Qualitative Reactions of Oxidoreductases.

### Determination of Catalase Activity in Serum

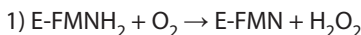
**AIM:** to determine the activity of catalase in serum.

#### BACKGROUND:

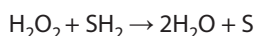
The first class of enzymes – **oxidoreductases** – catalyze oxidation-reduction reactions in the body. There are four groups of them:

- a) Anaerobic dehydrogenases – transmit protons ( $H^+$ ) and electrons ( $e^-$ ) from one substrate to another but not to oxygen. They have coenzymes: nicotinamide adenine dinucleotide and its phosphate ( $NAD^+$  and  $NADP^+$ ), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD);
- b) Aerobic dehydrogenases (oxidases); they oxidize substrates, are electron-transferring enzymes and catalyze the removal of one or two hydrogen atoms from the substrate by directly using  $O_2$  as a hydrogen acceptor. They have coenzymes FAD and FMN;
- c) Microsomal oxygenases that incorporate  $O_2$  into their substrates but are not related to the energy production. They have two subclasses: dioxygenases, which catalyze the incorporation of both the atoms of  $O_2$  into the substrate (e.g., tryptophan-2,3-dioxygenases), and monooxygenases or hydroxylases, which incorporate one oxygen atom into the substrate to form a hydroxyl group on it (e.g., mitochondrial  $cytP_{450}$  monooxygenase). These enzymes transfer reducing equivalents from NADPH or NADH;
- d) Oxidoreductases containing the heme or similar compounds. These enzymes belong to a group of cytochromes, catalase (mainly in red blood cells) and peroxidase (found in plants).

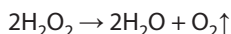
**Hydrogen Peroxide Formation and Detoxification.** Hydrogen peroxide is formed in cells (1) by oxidation – reduction reactions catalyzed by oxidase, where their flavin coenzymes take from the substrate hydrogen and transfer it directly to oxygen, or (2) by superoxide dismutase (SOD) catalysis:



Catalase acts in two ways: if the intracellular  $\text{H}_2\text{O}_2$  is limited, it acquires the properties of peroxidase and uses  $\text{H}_2\text{O}_2$  for the oxidation of the substrate:



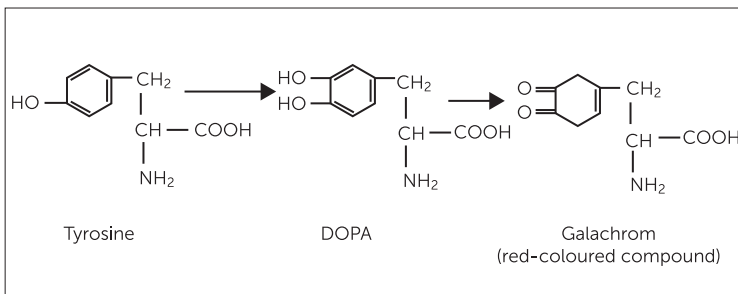
If  $\text{H}_2\text{O}_2$  is abundant, the catalase cleaves it for protecting the cells from the toxic action:



Peroxidase is catalyzing substrate oxidation reactions with hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). Glutathionperoxidase is very important for human red blood cells (it requires selenium). This enzyme-catalyzing reaction is important for the inactivation of hydrogen peroxide.

**The Determination of Tyrosinase Activity.** Tyrosinase is an oxidase containing 0.2 to 0.23% of copper. It is found in mushrooms, potatoes and animal melanocytes. This enzyme catalyzes the oxidation of tyrosine and some phenols into the corresponding quinones – the precursors of melanin.

## 6. Qualitative Reactions of Oxidoreductases. Determination of Catalase Activity in Serum



**Procedure.** Peel a raw potato and cut the upper layers into the blender. Add 10 ml of distilled water, crush some potato chips in and filtrate a mashed potato. To a clean test tube, add 2–3 drops of the tyrosine solution, 1–2 ml of the filtrate of the mashed potato and put it into the thermostat at 37 °C. The solution turns pink and then brown.

The plant oxidase oxidizes Guaiacum resin acid to ozonide – it turns blue in color.

**Procedure.** Add a few drops of the alcoholic solution of Guaiacum resin onto the upper layers of a peeled potato pellet. The edges of potatoes turn blue. Repeat the same reaction with a boiled potato; in this case, the same color remains due to the inactivation of the enzyme by cooking.

### The Determination of Catalase Activity in Blood Plasma:

a) A qualitative study.

**Procedure.** Into a test tube, add 1–2 ml of a 1%  $\text{H}_2\text{O}_2$  solution and a drop of blood. There is an intense release of oxygen.

b) A quantitative study. The enzyme activity is determined from the decrease of hydrogen peroxide content in the test sample. The rest of the  $\text{H}_2\text{O}_2$  in it, as well as all the added  $\text{H}_2\text{O}_2$  in the control sample, are titrated with  $\text{KMnO}_4$ .

**Procedure.** Into a 100 ml flask, add a small amount of distilled water and 0.1 ml of fresh blood. The solution is diluted to 100 ml (blood diluted 1000 times). Into two Erlenmeyer (flat) flasks, add 7 ml of distilled water and 0.1 ml of the diluted blood solution. Gently boil the contents of one flask for 2 minutes (for catalase inactivation); this will be the control sample. After cooling, add 2 ml of 1%  $\text{H}_2\text{O}_2$  to each flask and leave for 30 minutes at room temperature. Then, add 3 ml of a 10% sulfuric acid solution to both flasks. It stops the catalase functioning in the acidic medium. This is required for  $\text{H}_2\text{O}_2$  titration. Titrate it with a 0.02 M  $\text{KMnO}_4$  solution to develop a pale pink color. The difference of the potassium permanganate solution in ml consumed in the test (b) and the control (a) sample shows the amount of  $\text{H}_2\text{O}_2$  fragmented by catalase; 1 ml of 0.02 M  $\text{KMnO}_4$  is equivalent to 1 ml of 0.05 M (1.7 g/l)  $\text{H}_2\text{O}_2$ . The value of catalase activity, i.e., the content of hydrogen peroxide in mg, cleaved in 1 ml of blood within 30 minutes, is calculated by the following formula:

$$K = 1,7(a - b)1000 \text{ mg}$$

### REVIEW QUESTIONS:

1. Enzymes as biological catalysts.
2. The classification of enzymes, their nomenclature and code.
3. Environmental factors affecting enzyme activity.
4. The active centre of enzymes.
5. The inhibition of enzymes. Inhibitors and activators.
6. Major groups of oxidoreductases.
7. The structures and importance of  $\text{NAD}^+$ ,  $\text{NADP}^+$ , FMN, FAD.
8. The generation of hydrogen peroxide and other reactive oxygen compounds, detoxification. Antioxidants.
9. The method for measuring catalase activity in blood.

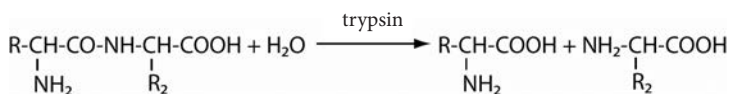
## 7.

## Analysis of Trypsin Enzyme Activity

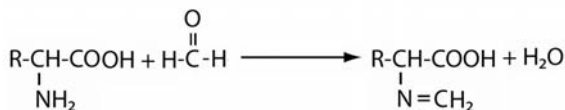
**AIM:** to evaluate trypsin activity.

### BACKGROUND:

Trypsin hydrolyzes the internal peptide bonds of protein while forming shorter peptides with free amino and carboxyl groups:



Amino groups react with formaldehyde in a slightly acidic medium (pH 6.8) (the methylene group is bound to the N atom, and the amino group loses its alkaline properties); free carboxyl groups must titrate with alkali.



**Procedure.**

1. Add 50 ml of a casein solution and 10 ml of trypsin into a flask. Mix.
2. Transfer 10 ml of this mixture into another flask and place the rest of it in a thermostat at 37–40°C.
3. Into the flask containing 10 ml of the mixture, add 2 ml of 0.4% HCl, 3 ml of formalin and a few drops of phenolphthalein. Titrate with NaOH up to a light pink color.
4. Repeat the previous step four times every 20 minutes.
5. Draw a graph based on the obtained results: put the time (min) on the abscissa axis and the amount (ml) of NaOH used for titration ml on the ordinate. Plot the curve.
6. Explain the results.

**REVIEW QUESTIONS**

1. Proteolytic enzymes. The importance of proteolysis processes in the body.
2. Proenzymes (zymogens).
3. Proteolytic hydrolases synthesized and secreted by the exocrine cells of the pancreas.
4. The conversion of trypsinogen (zymogen) to trypsin.
5. The specificity of trypsin and other proteases in the gastrointestinal tract.
6. The optimal pH of trypsin.
7. The method for determining trypsin activity.

## Measurement of Aspartate Aminotransferase and Alanine Aminotransferase Activity in Serum

**AIM:** to measure the activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum

### BACKGROUND:

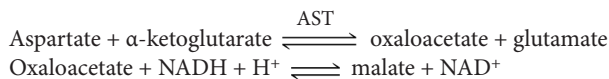
Measurements of the activity of enzymes in serum or plasma are valuable in the diagnosis and management of a wide variety of diseases. Most such enzymes are primarily intracellular, being released into the blood when cell membranes become damaged. Two aminotransferases are used in diagnosis and management: AST and ALT.

### ASPARTATE AMINOTRANSFERASE

AST is widely distributed in human tissues. The highest concentrations are found in cardiac tissue, liver and skeletal muscle, with smaller amounts found in the kidneys, pancreas and erythrocytes. The clinical use of AST is limited mainly to the evaluation of hepatocellular disorders and skeletal muscle involvement. AST levels are highest in acute hepatocellular disorders.

The assaying method for AST is based on the reaction catalyzed by AST and coupled with an enzymatic reaction using malate dehydrogenase (MD) as the indicator of the reaction; it also monitors the change in absorbance at 340 nm continuously as NADH is oxidized to NAD<sup>+</sup>.

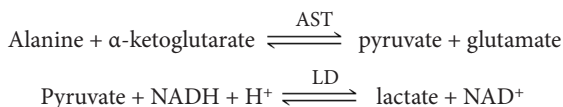




## ALANINE AMINOTRANSFERASE

ALT is distributed in many tissues, with comparatively high concentrations in the liver. It is considered the more liver-specific enzyme of the transferases. The clinical applications of ALT assays are confined mainly to the evaluation of hepatic disorders. Higher elevations are found in hepatocellular disorders than in extrahepatic or intrahepatic obstructive disorders.

The assaying procedure for ALT consists of a coupled enzymatic reaction using LDH as the indicator enzyme, which catalyzes the reduction of pyruvate to lactate with the simultaneous oxidation of NADH. The change in absorbance at 340 nm, measured continuously, is directly proportional to the ALT activity.



### Procedure.

1. Mix 1 ml of a reagent solution with 100  $\mu\text{l}$  of blood serum and incubate for 3 min at 37°C.
2. Measure the extinction at a wavelength of 340 nm.
3. Repeat the measuring after 2 or 3 minutes.
4. Calculate the change in extinction per minute ( $\Delta A_1$  and  $\Delta A_2$ ). The activity of AST and ALT is calculated in international units with the following formula:

#### 8. Measurement of Aspartate Aminotransferase and Alanin Aminotransferase Activity in Serum

$$\text{AST (U/L)} = 1746 \times \Delta A_1$$

$$\text{ALT (U/L)} = 1746 \times \Delta A_2$$

Normal AST and ALT activity rates at 37°C: up to 35 U/L for women and up to 40 U/L for men.

#### REVIEW QUESTIONS

1. The classification of enzymes.
2. Units of enzyme activity.
3. Transferases, their subclasses.
4. The mechanism of the aminotransferase reaction, the importance of the pyridoxal phosphate.
5. The relation between enzymes and vitamins.
6. The diagnostic value of AST and ALT activities.
7. The method for measuring AST and ALT activities.

## Lactate Dehydrogenase Activity in Serum

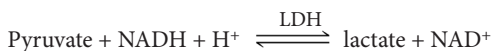
**AIM:** to measure the activity of lactate dehydrogenase (LDH) in serum.

### BACKGROUND:

LDH catalyzes the interconversion of lactic and pyruvic acids. It exists in body tissues as a tetramer. Two monomers, H and M, can combine in various proportions with the result of five isoenzymes of LDH that are known to us.

Increases in plasma LDH activity are seen in a wide variety of conditions, including acute damage to the liver, pancreas, skeletal muscle and kidneys, in megaloblastic and haemolytic anaemias.

The assaying procedure for LDH is based on its catalyzed reaction and the change in absorbance at 340 nm continuously as NADH is oxidized:



### Procedure.

1. Mix 1 ml of a reagent solution with 20 µl of sample.
2. Incubate for 30 seconds at 37°C and measure the extinction at the wavelength of 340 nm.
3. Repeat the measurements after 1, 2 and 3 minutes.

## 9. Lactate Dehydrogenase Activity in Serum

4. Calculate the change in extinction in  $\Delta A/\text{min}$ . LDH activity is calculated in international units (U) using the following formula:

$$\text{LDH (U/L)} = \Delta A \times 8095 (+37^\circ\text{C}).$$

The normal range for LDH activity is 230–460 U/L (+37°C).

### REVIEW QUESTIONS

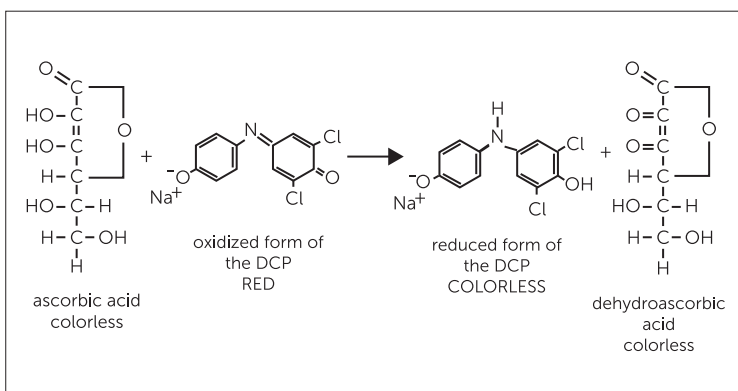
1. Anaerobic dehydrogenases, their coenzymes.
2. Isoenzymes. The isoenzymes of creatine kinase, AST and LDH.
3. The distribution of LDH isoenzymes in various tissues and its role.
4. The method for measuring LDH activity.

## Estimation of Vitamin C in Urine

**AIM:** to estimate ascorbic acid by titration using 2,6-dichlorophenolindophenol.

### BACKGROUND

The method for determining vitamin C is based on its reduction features. Ascorbic acid reduces to the blue 2,6-dichlorophenolindophenol (Tillman's reagent), making it appear colorless (in an acidic environment turn pink). Ascorbic acid is oxidized to dehydroascorbic acid during the reaction.



## 10. Estimation of Vitamin C in Urine

### Procedure.

1. Add 10 ml of urine, 100 ml of distilled water and 1 ml of concentrated acetic acid into a 200 ml flask.
2. Prepare a control solution by mixing the same amount of the sample solution with water and acetic acid.
3. Titrate the sample with Tillman's reagent until it appears pink.
4. Calculate the amount of vitamin C in urine, based on the fact that 1 ml of the Tillman' reagent reduces 0.0877 mg of vitamin C, and an average urine amount per day is 1500 ml.

## $\alpha$ -Amylase Activity in Serum

**AIM:** *to estimate  $\alpha$ -amylase activity in serum.*

### **BACKGROUND:**

$\alpha$ -Amylase is an enzyme of the hydrolase class that catalyzes the hydrolysis of  $\alpha$ -1,4-glucosidic bond in polysaccharides. Both straight-chain (linear) polysaccharides, such as amylose, and branched polysaccharides, such as amylopectin and glycogen, are hydrolyzed but at different rates. In the case of amylose, the enzyme splits the chains at  $\alpha$ -1,4-hemiacetal bonds. The enzyme does not cleave the  $\alpha$ -1,6-bonds at the branching points.

$\alpha$ -Amylase is present in a number of organs and tissues. The salivary glands secrete  $\alpha$ -amylase (S-type) to initiate a hydrolysis of starch while the food is still in the mouth and esophagus. The action of the salivary amylase is terminated by acidic environment in the stomach. In the pancreas, the enzyme (P-type) is synthesized by acinar cells and then is secreted into the intestinal tract. In the intestinal tract, the effective action of pancreatic and intestinal amylase is favored by mildly alkaline conditions in the duodenum.

Normally the enzyme present in blood and urine is predominantly of pancreatic and salivary gland origin. Blood  $\alpha$ -amylase activity is physiologically low, constant and greatly increases in acute pancreatitis and salivary gland inflammation.

The assays of choice for determining  $\alpha$ -amylase activity are ones with well-defined substrates with shorter glucosyl chains, e.g., small oligosaccharides and 4-nitrophenyl (4-NP)-glycoside substrates.

## 11. $\alpha$ -Amylase Activity in Serum

4-NP-glycoside substrates are prepared by bonding 4-NP to the reducing end of a defined oligosaccharide. If the oligosaccharide is maltoheptaose (G7), the substrate then is 4-NP-G7.

**The Principle.** The substrate p-nitrophenol maltoheptaoside (pNP-G7) is hydrolyzed by  $\alpha$ -amylase to shorter oligosaccharide chains with p-nitrophenol (p-NP-G). p-NP-G by the action of glucoamylase and  $\alpha$ -glucosidase is cleaved to glucose and pNP (yellow compound). Free pNP is detected by its absorbance at 450 nm.

<b>REACTIONS</b>	$\text{pNP-G7} \xrightarrow{\alpha\text{-amylase}} \text{pNP-G}$ $\text{pNP-G} \xrightarrow[\alpha\text{-glucosidase}]{\text{glucoamylase}} \text{p-nitrophenol (yellow)} + \text{glucose}$	
<b>REAGENTS</b>	<b>R1: buffer</b>	
	GOODS buffer (pH 7.2)	50 mmol/l
	Sodium chloride	50 mmol/l
	Calcium chloride	5 mmol/l
	<b>R2: substrate</b>	
	pNP-G7	>0.5 mmol/l
<b>PROCEDURE</b>	Prepare a working solution: gently mix (not shake) reagents R1 and R2.	
	Mix 1 ml of the working solution with 25 $\mu$ l of the sample and incubate for 1 minute at +37°C. Measure the change in extinction in 3 minutes ( $\Delta A/\text{min}$ ). Wavelength 450 nm.	
<b>CALCULATION</b>	$\Delta A/\text{min} \times 10480 = \text{U/l}$	
<b>REFERENCE VALUE</b>	Serum, plasma	Up to 180 U/l
	Urine	Up to 900 U/l



## REVIEW QUESTIONS

1. Dietary carbohydrates and their daily intake. The digestion of carbohydrates.
2. How  $\alpha$ -amylase is released into the blood and urine?
3. Enzyme activity in the serum and urine of healthy persons.
4. The diagnostic value of  $\alpha$ -amylase activity.
5. The method for measuring  $\alpha$ -amylase activity.

## Concentration of Seromucoids in Serum

**AIM:** *to estimate the concentration of seromucoids in serum.*

### BACKGROUND:

Serum seromucoids can be defined as that fraction of serum proteins which are not precipitated when perchloric acid is added to the serum. It has a high carbohydrate content, and this is probably important for protecting the fraction from denaturation and precipitation when perchloric acid is added to the serum. The seromucoid fraction is very heterogenous and the main components are  $\alpha_1$ -acid glycoprotein,  $\alpha_1$ -antitrypsin, haptoglobin and some other  $\alpha$  and  $\beta$  globulins.

**Procedure.** Add 0.5 ml of serum and 4.5 ml of a saline solution into a test tube. After mixing (thoroughly), add 2.5 ml of a 6% perchloric acid ( $\text{HClO}_4$ ), mix again, incubate at room temperature for 15 minutes and let the tube stand for 15 minutes at room temperature ( $16-25^\circ\text{C}$ ). During this time, other serum proteins precipitate, and seromucoids dissolve in perchloric acid. Use filtration paper to filter out the precipitated proteins. To measure the amount of proteins dissolved in perchloric acid, add 0.5 ml of a 5% phosphowolframic acid to 2.5 ml of the filtrate. The remainder of the filtrate is used as a control solution for nephelometry. After 10 minutes, perform the nephelometry of the sample and the control solution using a red light filter. The result is expressed in conditional units, which are obtained by multiplying the numeric value of the extinction coefficient by 1000. The reference value of seromucoides is about 100 conditional units.

## **REVIEW QUESTIONS**

1. The characteristics of glycoproteins.
2. Proteoglycans: their structure and functions.
3. Major glycosaminoglycans (GAGs) – hyaluronic acid, heparin, chondroitin sulphate, keratan sulphate, dermatan sulphate: structure, location, physiological role.
4. Mucopolysaccharidoses – diagnosis, causes and consequences.
5. The structure of plasma glycoproteins.
6. The structure of fucosis and sialic acid.

## Determination of Glucose Concentration in Serum

**AIM:** *to determine the concentration of glucose in serum based on glucose oxidase activity.*

### BACKGROUND:

Blood glucose concentration depends on the relative rates of the influx of glucose into the circulation and its utilization. Its concentration is normally subject to rigorous control, rarely falling below 2.5 mmol/L at any time or rising above 8.0 mmol/L in healthy subjects after a meal or above 5.0 mmol/L after an overnight fast. After a meal, glucose is stored as glycogen, which is mobilized during fasting. Blood glucose concentration usually falls to premeal concentrations within 4 hours of a meal, but then continues to fall somewhat as fasting continues and hepatic glycogen stores are used up until, after about 24 hours, adaptive changes lead to the attainment of a new steady state. After 72 hours, blood glucose concentration stabilizes and can then remain constant for many days. The principal source of glucose becomes gluconeogenesis, from amino acids and glycerol, whereas ketones, derived from fat, become the major energy substrate.

The integration of these various processes, and thus the control of blood glucose concentration, is achieved through the concerted action of various hormones: these are insulin (the actions of which tend to lower blood glucose concentration) and the “counterregulatory” hormones, namely glucagon, cortisol, catecholamines and the growth hormone, which have the opposite effect.

Measurements of glucose are critical to the diagnosis and management of diseases affecting carbohydrate metabolism. Glucose is

measured in whole blood, plasma, serum, cerebrospinal fluid, pleural fluid and urine for a variety of diagnostic and management purposes. Plasma glucose concentration tends to be 10% to 15% higher than that of whole blood, because a given volume of red cells contains less water than the same volume of plasma.

<b>METHOD</b>	Enzymatic-colorimetric	
<b>PRINCIPLE</b>	<p>In the presence of oxygen, glucose is oxidized by glucose oxidase (GOD) to gluconic acid and hydrogen peroxide (<math>\text{H}_2\text{O}_2</math>). The <math>\text{H}_2\text{O}_2</math> reacts with 4-chlorophenol and 4-aminoantipyrine in the presence of peroxidase (POD) to form quinoneimine dyes. The intensity of color formed is proportional to the glucose concentration and can be measured photometrically.</p> <p>1. <math>\text{Glucose} + \text{O}_2 \xrightarrow{\text{GOD}} \text{gluconic acid} + \text{H}_2\text{O}_2</math></p> <p>2. <math>2\text{H}_2\text{O}_2 + 4\text{-AA} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinoneimine (red)} + 4\text{H}_2\text{O}</math></p>	
<b>REAGENTS</b>	<b>R: reagent</b>	
	Phosphatate buffer (pH 7.4)	13.8 mmol/l
	Phenol	10 mmol/l
	4-aminoantipyrine (4-AA)	0.3 mmol/l
	Glucose oxidase (GOD)	$\geq 10000 \text{ U/l}$
	Peroxidase (POD)	$\geq 700 \text{ U/l}$
	<b>ST: standard</b>	
	Glucose	5.55 mmol/l 100 mg/dl
<b>SAMPLE</b>	Blood serum free of hemolysis; plasma collected on heparin or any glycolysis inhibitor.	
<b>REACTION CONDITIONS</b>	Wavelength	500 nm
	Temperature	+37°C
	Cuvette	1 cm light path

### 13. Determination of Glucose Concentration in Serum

OPERATING PROCEDURE	1. Add 1 ml of the reagent (R) into the first cuvette (this cuvette is called a “blank”).																								
	2. Add 1 ml of the reagent (R) and 10 µl of the standard (ST) into the second cuvette (this cuvette is called a “standard”).																								
	3. Add 1 ml of the reagent (R) and 10 µl of the sample into the third cuvette (this cuvette is called a “sample”).																								
	<table><tr><td></td><td colspan="3">Cuvettes:</td></tr><tr><td></td><td>“Blank”</td><td>“Standard”</td><td>“Sample”</td></tr><tr><td>Reagent (R)</td><td>1 ml</td><td>1 ml</td><td>1 ml</td></tr><tr><td>Standard (ST)</td><td>–</td><td>10 µl</td><td>–</td></tr><tr><td>Sample</td><td>–</td><td>–</td><td>10 µl</td></tr></table>				Cuvettes:				“Blank”	“Standard”	“Sample”	Reagent (R)	1 ml	1 ml	1 ml	Standard (ST)	–	10 µl	–	Sample	–	–	10 µl		
		Cuvettes:																							
	“Blank”	“Standard”	“Sample”																						
Reagent (R)	1 ml	1 ml	1 ml																						
Standard (ST)	–	10 µl	–																						
Sample	–	–	10 µl																						
4. Mix and incubate the cuvettes at +37 °C for 15 min.																									
5. The analyzer reads light absorption rates (ABS) of the standard and the sample against the reagent blank and calculates glucose concentration.																									
CALCUATION	<table><tr><td>ABS sample</td><td rowspan="2">*      standard concentration</td><td rowspan="2">=</td><td>glucose</td></tr><tr><td>ABS standard</td><td>concentration</td></tr><tr><td colspan="2"></td><td></td><td>(mmol/l, mg/dl)</td></tr><tr><td colspan="2"></td><td></td><td>(mmol/l, mg/dl)</td></tr><tr><td colspan="2"><u>Conversion factor:</u></td><td colspan="2">mg/dl * 0.0555 = mmol/l</td></tr><tr><td colspan="2"></td><td colspan="2">mg/dl * 0.01 = g/l</td></tr></table>			ABS sample	*      standard concentration	=	glucose	ABS standard	concentration				(mmol/l, mg/dl)				(mmol/l, mg/dl)	<u>Conversion factor:</u>		mg/dl * 0.0555 = mmol/l				mg/dl * 0.01 = g/l	
ABS sample	*      standard concentration	=	glucose																						
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<u>Conversion factor:</u>		mg/dl * 0.0555 = mmol/l																							
		mg/dl * 0.01 = g/l																							
REFERENCE VALUE	Blood serum: 4.1–6.4 mmol/l Capillary blood: 3.33–5.55 mmol/l Cerebrospinal fluid: 2.78–3.89 mmol/l																								

## REVIEW QUESTIONS

1. Glucose concentration in capillary blood, plasma (serum) and cerebrospinal fluid. Hyper- and hypoglycemia.  
The importance of glucose for the brain.
2. Glucose-6-phosphate metabolism.
3. Glycolysis and its regulation.
4. Glucogenic compounds. Gluconeogenesis and its regulation.
5. The mechanism of hormone action  
(insulin, glucagon, adrenaline, and glucocorticoids) and their role in carbohydrate metabolism.
6. Diabetes. The glucose tolerance test.
7. The method for measuring glucose concentration in serum.

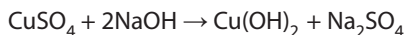
## Glucose Concentration in Urine

**AIM:** *to measure glucose concentration in urine.*

### BACKGROUND:

The presence of detectable amounts of glucose in urine is termed glycosuria; this condition occurs whenever the glucose level in the blood surpasses the renal tubule capacity for reabsorption. Glucose may appear in the urine at different blood glucose levels, and there is not always a concomitant hyperglycemia. Glomerular blood flow, tubular reabsorption rate and urine flow will also influence its appearance. When hyperglycemia is present, however, glycosuria usually occurs when the blood level is greater than 10 mmol/l.

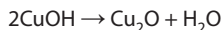
The Fehling's Test is based on reducing the property of mono-saccharides and disaccharides, which, in turn, depend on the presence of a free keto or an aldehyde group. This test is performed by adding a few drops of a sugar solution to a mixture of Fehling-I (i.e.,  $\text{CuSO}_4$ ) and Fehling-II (i.e.,  $\text{KOH}$  or  $\text{NaOH}$ ). When an alkaline solution of cupric hydroxide is heated in the presence of a reducing sugar, it gets reduced to insoluble, yellow- or red-colored, cuprous oxide.



Blue color



Yellow color



Red color



The Fehling's reagent consists of  $\text{CuSO}_4$  (Fehling-I), Segnet salt and a mixture of NaOH solutions (Fehling-II). 1 ml of a Fehling's reagent contains the amount of  $\text{CuSO}_4$  reduced to  $\text{Cu}_2\text{O}$  by 5 mg of glucose. The glucose concentration is calculated based on the volume of urine, used for titration.

**Procedure.** Into a flask, add 2.5 ml of a Fehling-I solution, 2.5 ml of a Fehling-II solution and 20 ml of distilled water. Heat the content of the flask and bring it to a boil; while heating, titrate it with a urine sample (pipette a few drops, wait a few seconds). The blue color of the solution turns green, then yellow. When the reddish color appears, the titration is completed.

Calculate the glucose concentration in urine based on the volume of the sample used for the titration.

## REVIEW QUESTIONS

1. Explain the difference between reducing and non-reducing sugars.
2. Explain the experiment procedure and the results.
3. Is the Fehling's Test specific for glucose? Explain.
4. Glycogen structure, its biological role and content in tissues.
5. Glycogenesis and glycogenolysis in the liver and muscles.  
The regulation of these processes. The activation of glycogen phosphorylase and glycogen synthase.
6. Glycogen hydrolysis in the digestive tract and tissues ( $\gamma$ -amylase).
7. Glycogenosis.
8. Renal threshold for glucose. Alimentary and renal glucosuria.

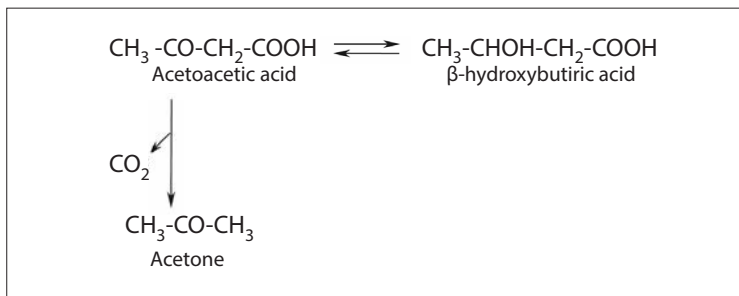
# 15.

## Qualitative Reactions for Ketone Bodies. Acetone Concentration in Urine

**AIM:** to test the given samples for the presence of ketone bodies.

### BACKGROUND:

In the human liver, three endogenous ketone bodies are formed: acetone, acetoacetic acid, and beta-hydroxybutyric acid.



Ketone bodies can be used as a source of energy. They are transported from the liver to peripheral tissues, where acetoacetate and beta-hydroxybutyrate can be reconverted to acetyl-CoA to produce energy via the citric acid cycle. Acetone is produced by spontaneous (produced without an enzyme) decarboxylation of acetoacetate in the liver in cases of the overproduction of acetoacetate, when it can't be used up by the peripheral tissues. A healthy human body is producing only a small amount of acetone. Acetone is harmful because it melts body lipids, i.e., the membrane may be vulnerable to it. Acetone

cannot be converted back to acetyl-CoA; hence, it is excreted in the urine and skin or exhaled through the lungs.

Under certain physiological conditions (when there's enough of carbohydrates, especially oxalacetate), the production of the ketone bodies is low, because acetyl-CoA is oxidized in the Krebs Cycle. Also, the excess acetyl-CoA can be moved from the cytoplasm to the mitochondria in the form of a citrate, and it can be used to synthesize fatty acids and cholesterol (Figure 2).

Synthesized in the liver, acetoacetic and beta-hydroxybutyric acids are transported with the blood to the mitochondria of the peripheral

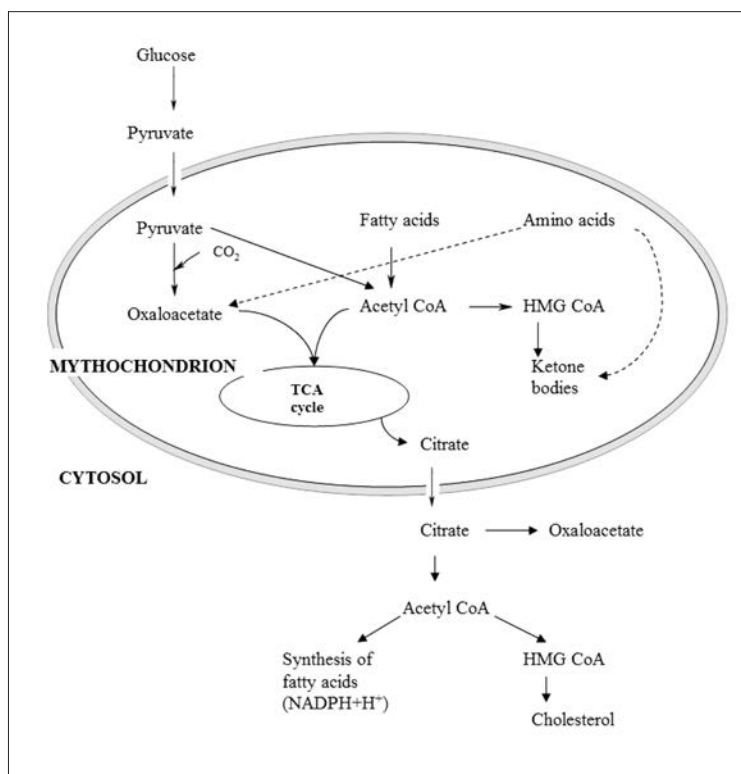


Figure 2. The formation and use of Acetyl – CoA.

**15. Qualitative Reactions for Ketone Bodies.**  
**Acetone Concentration in Urine**

tissue (brain, heart, kidney, skeletal muscle) cells, where the following acids can be reconverted to acetyl-CoA to produce energy via the citric acid cycle (ketolysis, Figure 3). The oxidation of  $\beta$  - hydroxybutyric

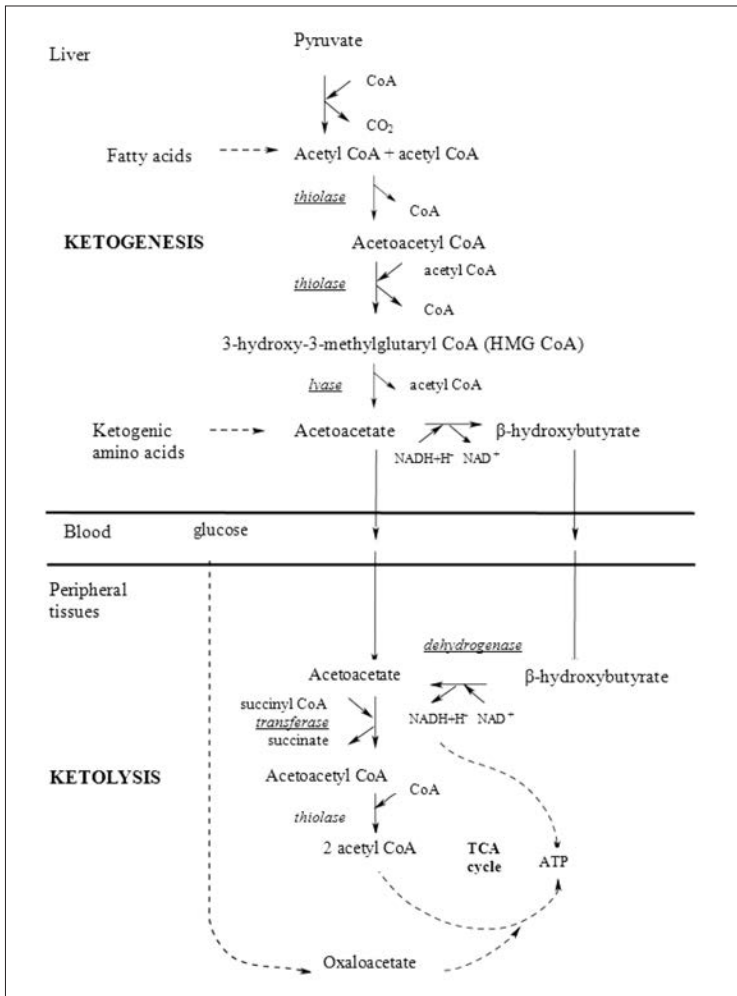


Figure 3. Ketogenesis and ketolysis

acid can produce 26 mol of ATP, and that of acetoacetic acid – 23 mol of ATP.

When fasting, only the brain can use these compounds for energy, because only they contain oxaloacetate (formed from blood glucose), which is required for acetyl-CoA oxidation. In the case of low blood glucose, most other tissues have additional energy sources besides ketone bodies (such as fatty acids), but the brain does not, because fatty acid  $\beta$ -oxidation does not occur in the brain.

In normal individuals, there is a constant production of ketone bodies by the liver and their utilization by extrahepatic tissues. The concentration of ketone bodies in blood is maintained around 0.01–0.02 mmol/L (0.15–2 mg%). When the rate of the synthesis of ketone bodies exceeds the rate of utilization, their concentration in blood increases; this is known as *ketonemia*. It is followed by ketonuria – the excretion of ketone bodies in urine. The overall picture of ketonemia and *ketouria* is commonly referred to as *ketosis*. The concentration of ketone bodies in blood in the case of ketosis can reach 20 mmol/l; it lowers the pH of the blood and leads to metabolic acidosis.

In healthy individuals, the excretion of ketone bodies in urine is very low (10–20 mg) and is undetectable by routine urine tests. In case of ketosis, the excretion of ketone bodies reaches 150 g, and it will cause a further removal of water and electrolytes from the blood. This can lead to exsiccosis – a water deficiency in the body.

## QUALITATIVE REACTIONS OF KETONE BODIES

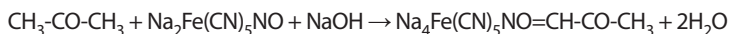
**Liben's Reaction.** The reaction of acetone with iodine in an alkaline medium gives the iodoform:



**Procedure.** Into a test tube containing 3 ml of the study sample, add 5 drops of an iodine solution and 5 drops of 10% NaOH, then mix. If the sample contains acetone, the characteristic odor of iodoform precipitate will transpire.

### 15. Qualitative Reactions for Ketone Bodies. Acetone Concentration in Urine

**Legal's Reaction.** The reaction of acetone and acetoacetic acid with sodium nitroprusside in an alkaline medium gives a red-orange-colored complex compound.



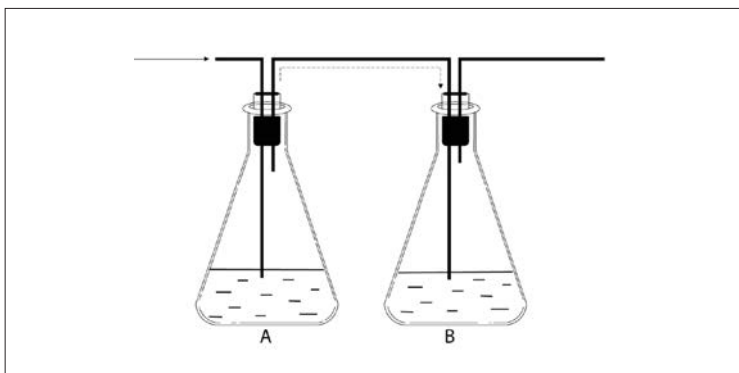
The addition of acetic acid changes the structure of the complex, and its red-orange color turns cherry-red.

**Procedure.** Into the test tube containing 3 ml of urine (the study sample), add 5 drops of 10%  $\text{Na}_2\text{Fe(CN)}_5\text{NO}$  and 1 ml of 10%  $\text{NaOH}$ ; after that, observe a change of color to orange. Upon adding 2 ml of concentrated acetic acid, it turns cherry red.

### THE DETERMINATION OF ACETONE CONCENTRATION IN URINE

The method is based on the detection of iodine, required to convert acetone into iodoform. Acetone is blown into an alkaline iodine solution. Non-reacted iodine is titrated with a sodium thiosulfate solution.

**Procedure.** Add 20 ml of the study sample, 0.2 g of oxalic acid and 10 g  $\text{NaCl}$  into Flask A. Into Flask B, add 20 ml of 0.1 N iodine solution, 15 ml of 25%  $\text{KOH}$  and 25 ml of water. Connect the stoppered



flasks to each other through an air stream for 30 minutes. Acetone will pass with a stream of air into Flask B, where acetone will react with iodine to form iodoform. After that, add concentrated HCl to Flask B until a bright, yellow color appears. Add a couple of drops of a starch solution into the flask. Titrate iodine that has not reacted with acetone with a 0.1 N  $\text{Na}_2\text{S}_2\text{O}_3$  solution until the blue color disappears.

*Calculation.* The quantity of acetone (mg) per 100 ml of study sample (mg/dl) can be calculated using the following formula:

$$\frac{(a - b) \cdot 0.967 \cdot 100}{c}$$

Where a = the volume (ml) of iodine solution, added to flask B;

b = the volume (ml) of 0.1 N  $\text{Na}_2\text{S}_2\text{O}_3$ , used to titrate the iodine that has not reacted with acetone;

c = the volume of the study sample (20 ml).

## REVIEW QUESTIONS

1. Write the structural formulas of ketone bodies; indicate the relationship between these compounds.
2. The synthesis of ketone bodies (ketogenesis). Ketogenic amino acids.
3. The oxidation of  $\beta$ -hydroxybutyric and acetoacetic acids in peripheral tissues (ketolysis). Write the reactions and note the energy value.
4. Why does hyperketonemia occur in the case of diabetes after a long fasting period or after receiving inadequate amounts of dietary carbohydrates? What is the concentration of ketone bodies in urine?
5. In the abovementioned cases, why does acetone form in the liver?
6. Ketosis and its consequences.
7. The qualitative reactions of ketone bodies.
8. The method for the quantitative determination of ketone bodies in urine.

## Determination of the Concentration of Triacylglycerols in Serum

**AIM:** *to estimate the concentration of serum triacylglycerols.*

### BACKGROUND:

Triglyceride is present in dietary fat and can be synthesized in the liver and adipose tissue to provide a source of stored energy; this can be mobilized when required – during starvation, for example. Although the majority of fatty acids in the body are saturated, certain unsaturated fatty acids are important as precursors of prostaglandins and in the esterification of cholesterol. Triglycerides containing both saturated and unsaturated fatty acids are important components of cell membranes.

Dietary fat is hydrolyzed in the small intestine, absorbed and re-synthesized by mucosal cells, and secreted into vessels as chylomicrons. Triglycerides in the chylomicrons are cleared from the blood by tissue lipoprotein lipase. Endogenous triglyceride production occurs in the liver, and then triglycerides are transported to the peripheral tissues by very-low-density lipoproteins (VLDL).

Elevated triglycerides ( $> 1,7$  mmol/L) are now considered an independent risk factor for coronary artery disease and a major risk factor for acute pancreatitis, particularly when the serum triglyceride levels are  $> 10$  mmol/L.



METHOD	Enzymatic-colorimetric Endpoint																			
PRINCIPLE	<p>This method is based on the enzymatic hydrolysis of serum or plasma triglyceride to glycerol and free fatty acids (FFA) by lipoprotein lipase (LPL). The glycerol is phosphorylated by adenosine triphosphate (ATP) in the presence of glycerolkinase (GK) to form glycerol-3-phosphate (G-3-P) and adenosine diphosphate (ADP). G3-P is then oxidized by glycerophosphate oxidase (GPO) to form dihydroxyacetone phosphate (DHAP) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). The H<sub>2</sub>O<sub>2</sub> reacts with 4-aminoantipyrine (4-AA) and phenol in the presence of peroxydase (PO) to produce red chromogen. The intensity of color formed is proportional to the concentration of triglycerides in the sample and can be measured photometrically.</p> <p>1. Triglycerides + 3H<sub>2</sub>O <math>\xrightarrow{\text{LPL}}</math> Glycerol + 3FFA</p> <p>2. Glycerol + ATP <math>\xrightarrow{\text{GK}}</math> Glycerol-3-P + ADP</p> <p>3. Glycerol-3-P + O<sub>2</sub> <math>\xrightarrow{\text{GPO}}</math> DHAP + H<sub>2</sub>O<sub>2</sub></p> <p>4. H<sub>2</sub>O<sub>2</sub> + 4-AA + 4Phenol <math>\xrightarrow{\text{POD}}</math> Quinoneimine (red) + H<sub>2</sub>O</p>																			
REAGENTS	<b>R: reagent</b> <table><tr><td>Buffer (pH 6.8)</td><td>50 mmol/l</td></tr><tr><td>Phenol</td><td>3 mmol/l</td></tr><tr><td>4-aminoantipyrine (4-AA)</td><td>0.5 mmol/l</td></tr><tr><td>ATP</td><td>2 mmol/l</td></tr><tr><td>Mg<sup>2+</sup></td><td>40 mmol/l</td></tr><tr><td>Lipoprotein lipase (LPL)</td><td>≥ 1200 U/l</td></tr><tr><td>Glycerolkinase (GK)</td><td>≥ 1000 U/l</td></tr><tr><td>Peroxidase (POD)</td><td>≥ 10000 U/l</td></tr></table> <b>ST: standard</b> <table><tr><td>Glycerol</td><td>2.26 mmol/l</td></tr></table>		Buffer (pH 6.8)	50 mmol/l	Phenol	3 mmol/l	4-aminoantipyrine (4-AA)	0.5 mmol/l	ATP	2 mmol/l	Mg <sup>2+</sup>	40 mmol/l	Lipoprotein lipase (LPL)	≥ 1200 U/l	Glycerolkinase (GK)	≥ 1000 U/l	Peroxidase (POD)	≥ 10000 U/l	Glycerol	2.26 mmol/l
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Peroxidase (POD)	≥ 10000 U/l																			
Glycerol	2.26 mmol/l																			

**16. Determination of the Concentration  
of Triacylglycerols in Serum**

SAMPLE	Blood serum obtained from the patient after an overnight fast.			
REACTION CONDITIONS	Wavelength	500 nm (±20 nm)		
	Temperature	+37°C		
	Cuvette	1 cm light path		
OPERATING PROCEDURE	1. Add 1 ml of the reagent (R) into the first cuvette (this cuvette is called a “blank”);			
	2. Add 1 ml of the reagent (R) and 10 µl of the standard (ST) into the second cuvette (this cuvette is called a “standard”);			
	3. Add 1 ml of the reagent (R) and 10 µl of the sample into the third cuvette (this cuvette is called a “sample”).			
		Cuvettes:		
		“Blank”	“Standard”	“Sample”
	Reagent (R)	1 ml	1 ml	1 ml
	Standard (ST)	–	10 µl	–
Sample	–	–	10 µl	
	4. Mix and incubate the cuvettes at +37°C for 5 minutes.			
	5. The analyzer reads the light absorption rates (ABS) of the standard and the sample against the reagent blank and calculates the triglyceride concentration (mmol/l).			
CALCULATION	$\frac{\text{ABS sample}}{\text{ABS standard}} \times \frac{\text{standard concentration (mmol/l)}}{\text{triglycerides concentration (mmol/l)}}$			
REFERENCE VALUE	≤ 1.7 mmol/l			

## REVIEW QUESTIONS

1. The characterization of lipids, their biological importance.
2. Lipid classification.
3. TAG classification and their properties.
4. Fatty acids and their properties. Essential fatty acids.
5. The synthesis of  $\omega$ -6 and  $\omega$ -3 polyunsaturated fatty acids.
6. The synthesis of eicosanoids and their biological role.
7. TAG hydrolysis in the body: in the digestive tract, adipose tissue and blood.
8. The emulsifying of lipids. Emulsifiers.
9. Soaps. What soaps can be produced in the human digestive tract?
10. Fat hydrogenation. Margarine. Advantages and disadvantages.
11. The diagnostic significance of the determination of serum TAG concentration.

## Determination of Total Cholesterol Concentration in Serum

**AIM:** *to estimate serum cholesterol concentration in the given sample.*

### **BACKGROUND:**

Cholesterol is an important compound in membrane structure and is the precursor of steroid hormones, bile acids and vitamin D. Cholesterol is present in dietary fat and can be synthesized in the liver by a mechanism that is under close metabolic regulation. Cholesterol can be excreted in the bile either *per se* or after metabolism to bile acids.

Cholesterol accounts for almost all of the sterol in plasma. It exists as a mixture of unesterified (30 to 40%) and esterified (60 to 70%) forms; the proportion of the two forms is fairly constant among normal individuals.

Elevated plasma cholesterol concentrations are a major risk factor for cardiovascular disease, because in the majority of people, most plasma cholesterol is present in the LDL fraction.

<b>METHOD</b>	Enzymatic-colorimetric	
<b>PRINCIPLE</b>	<p>All cholesterol esters present in a specimen are hydrolyzed quantitatively into free cholesterol and free fatty acids (FFA) by cholesterol esterase (CHE). In the presence of oxygen, free cholesterol is then oxidized by cholesterol oxidase (CHO) to cholesten-4-ene-3-one and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). The H<sub>2</sub>O<sub>2</sub> reacts with 4-chlorophenol and 4-aminoantipyrine in the presence of peroxidase (POD) to form quinoneimine dyes. The intensity of color formed is proportional to the cholesterol concentration and can be measured photometrically between 480 and 520 nm.</p> <p>1. Cholesterol ester + H<sub>2</sub>O <math>\xrightarrow{\text{cholesterol esterase}}</math> Cholesterol + FFA</p> <p>2. Cholesterol + O<sub>2</sub> <math>\xrightarrow{\text{cholesterol oxidase}}</math> Cholesten-4-ene-3-one + H<sub>2</sub>O<sub>2</sub></p> <p>3. 2H<sub>2</sub>O<sub>2</sub> + 4-chlorophenol + 4-aminoantipyrine <math>\xrightarrow{\text{peroxidase}}</math> Quinoneimine (red) + 4H<sub>2</sub>O</p>	
<b>REAGENTS</b>	<b>R: reagent</b>	
	Buffer (pH 7.2)	50 mmol/l
	p-chlorophenol	2 mmol/l
	Sodium chlorate	8 mmol/l
	4-aminoantipyrine	0.6 mmol/l
	Cholesterol esterase (CHE)	≥ 400 U/l
	Cholesterol oxidase (CHO)	≥ 200 U/l
	Peroxidase (POD)	≥ 500 U/l
	<b>ST: standard</b>	
	Cholesterol	5.17 mmol/l
<b>SAMPLE</b>	Blood serum obtained from the patient after an overnight fast.	

**17. Determination of Total Cholesterol  
Concentration in Serum**

REACTION CONDITIONS	Wavelength	510 nm (480–520 nm)		
	Temperature	+37°C		
	Cuvette	1 cm light path		
OPERATING PROCEDURE	1. Add 1 ml of the reagent (R) into the first cuvette (this cuvette is called a “blank”).			
	2. Add 1 ml of the reagent (R) and 10 µl of the standard (ST) into the second cuvette (this cuvette is called a “standard”).			
	3. Add 1 ml of the reagent (R) and 10 µl of the sample into the third cuvette (this cuvette is called a “sample”).			
		Cuvettes		
		“Blank”	“Standard”	“Sample”
	Reagent (R)	1 ml	1 ml	1 ml
	Standard (ST)	–	10 µl	–
	Sample	–	–	10 µl
	4. Mix and incubate the cuvettes at +37°C for 5 minutes.			
	5. The analyzer reads the light absorption rates (ABS) of the standard and the sample against the reagent blank and calculates cholesterol concentration (mmol/l).			
CALCULATION	$\frac{\text{ABS sample}}{\text{ABS standard}} \times \text{standard concentration (mmol/l)} = \text{cholesterol concentration (mmol/l)}$			
REFERENCE VALUE	< 5.2 mmol/l			

## REVIEW QUESTIONS

1. The biological function of cholesterol.
2. The structural formula of cholesterol. The formation of cholesteryl esters in tissues.
3. Exogenous and endogenous cholesterol.
4. The synthesis of endogenous cholesterol.
5. Cholesterol metabolism in the gut and liver.
6. Cholesterol and lipoprotein interface. The role of lecithin cholesterol acyl transpherase.
7. Total, LDL and HDL cholesterol concentrations in serum. The antiatherogenicity of HDL cholesterol.

## Determination of Low Density Lipoprotein Concentration in Serum

**AIM:** *to measure low density lipoprotein (LDL) concentration in serum.*

### BACKGROUND:

LDL is the principal carrier of cholesterol, mainly in the form of cholesteryl esters. LDL is formed from VLDL via IDL. It can pass through the junctions between capillary endothelial cells and bind to LDL receptors on cell membranes that recognize apo B-100, both in the liver and in peripheral tissues. This is followed by internalization and lysosomal degradation with the release of free cholesterol. Cholesterol can also be synthesized in these tissues, but the rate-limiting enzyme, HMG-CoA reductase (hydroxymethylglutaryl coenzyme A reductase), is inhibited by cholesterol, with the result that, in healthy adults, cholesterol synthesis occurs only in the liver.

LDL receptors are saturable and subject to down-regulation by an increase in intracellular cholesterol. Macrophages derived from circulating monocytes can take up LDL via scavenger receptors. This process occurs at normal LDL concentrations but is enhanced when LDL concentrations are increased and by modification (e.g., oxidation) of LDL. The uptake of LDL by macrophages in the arterial wall is an important event in the pathogenesis of atherosclerosis. When macrophages become overloaded with cholesteryl esters, they are converted to foam cells, the classic components of atheromatous plaques.

All circulating lipoproteins share common lipid and apolipoprotein components, and the central problem in lipoprotein analysis is the separation of different lipoprotein classes from one an-



other. Some lipoproteins are precipitated with polyanions such as heparin sulfate, dextran sulfate, phosphotungstate and others in the presence of divalent cations such as  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ , and  $\text{Mn}^{++}$ . Conditions have been established in which the major classes of lipoproteins can be precipitated in stepwise fashion beginning with the lower-density, lipid-rich lipoproteins. The more dissimilar the lipoproteins are from one another, the better the separation. LDLs form a complex with heparin which, in the presence of  $\text{CaCl}_2$ , forms residues.

**Procedure.** Add 2 ml of 0.025 M  $\text{CaCl}_2$  and 0.2 ml of blood serum into each of two test tubes. Into one of them, add 0.04 ml of a 1% heparin solution. Mix and, after 4 minutes, perform a colorimetric measurement using a red light filter.

Calculate the LDL concentration using the following formula:

$$[\text{LDL}] = (E_x - E_k) * 1164 \text{ mg\%}$$

Where  $E_k$  – light absorption of the control solution,  $E_x$  – light absorption of the test solution.

## REVIEW QUESTIONS

1. The structure of lipoproteins.
2. Lipoprotein classes. Methods for their separation.
3. The metabolism of chylomicrons. Lipoprotein lipase.
4. The metabolism of very low density lipoproteins (VLDL).
5. The metabolism of low density lipoproteins (LDL).
6. The metabolism of high density lipoproteins (HDL).
7. Dyslipoproteinemia.
8. The principle for measuring LDL concentration in plasma.

## Qualitative Reactions for Bile Pigments and Acids

**AIM:** *to test samples for bile salts and bile pigments.*

### REACTIONS OF BILE PIGMENTS

The reaction for recognizing bile pigments is based on their oxidation, which leads to the composition of colored oxidation products: greenish biliverdin and other blue, pink, yellow derivatives.

**Procedure. Gmelin's Reaction.** Add 1 ml of bile in to the tube. Carefully pour 1 ml of  $\text{HNO}_3$  on the inner wall of the tube. In the liquid junction, residues of bile acids and protein and colored rings of bile pigments will form.

**Rozenbach's Reaction.** Filter the bile through the filter paper several times. The bile pigments will remain on the filter paper. Then, spread out the filter paper and add a drop of concentrated  $\text{HNO}_3$  in the middle. This will result in colored bile rings.

**Razin's Reaction.** Add a few drops of acetic acid into the tube with the bile. Carefully pour 1 ml of an iodine solution in the ethanol on the inner wall of the tube. A greenish ring will appear in the liquid junction.

### REACTIONS OF BILE ACIDS

**Hay's Test.** The presence of bile acids in a sample solution causes the reduction of its surface tension. When sulfur powder is sprinkled on such samples, they sink. This is in contrast to samples without bile acids, on which sulfur powder keeps floating.

**Procedure.** Add 2 ml of distilled water into two test tubes. Pour a few drops of bile into one of them and mix. Then, add sulfur powder to both tubes. In the tube containing the bile, the sulfur will sink. The reaction is based on the ability of bile acids to reduce the surface tension.

**Pettenkofer's Reaction.** Mix 1 ml of  $\text{H}_2\text{SO}_4$  and 1 ml of sucrose. Pour the bile into another test tube and add a mixture of sulfuric acid and sucrose. In the liquid junction, residues of bile acids and a purple ring will be formed. After cooling the tube and gently shaking its contents, the liquid will acquire a cherry color. It will depend on the formation of the colored compound during the reaction of cholic acid and oxymethylfurfural (derived from the reaction of sucrose by sulfuric acid).

## REVIEW QUESTIONS

1. The qualitative and quantitative composition of bile.
2. What is the difference between hepatic bile and gall bladder bile? The factors that stimulate secretion of bile.
3. What amount of bile acids is produced per day?
4. The synthesis of bile acids and their importance.
5. Fat digestion. Cholic acids.
6. The formation and removal of bile pigments.
7. Gallstones.
8. Mechanical jaundice and cholemia. Other types of jaundice.
9. Reactions for the detection of bile pigments and acids.

## Determination of Urea Concentration in Serum

**AIM:** *to measure the concentration of urea in serum.*

### **BACKGROUND:**

Urea is synthesized in the liver, primarily as a by-product of the deamination of amino acids. Its elimination in the urine represents a major route for nitrogen excretion. It is filtered from the blood by the glomeruli, but significant tubular reabsorption occurs through passive diffusion.

Urea production is increased by a high protein intake, in catabolic states and by the absorption of amino acids and peptides after gastrointestinal bleeding. Conversely, production is decreased in patients with a low protein intake and sometimes in patients with liver disease.

Changes in plasma urea concentration are a feature of renal impairment, but it is important to consider any possible extra-renal influences on urea concentrations.

<b>METHOD</b>	Enzymatic-colorimetric	
<b>PRINCIPLE</b>	<p>Urea (<math>\text{H}_2\text{N}-\text{CO}-\text{NH}_2</math>) is hydrolyzed by urease into ammonia (<math>\text{NH}_3</math>) and carbon dioxide (<math>\text{CO}_2</math>). The generated ammonia reacts with alkaline hypochlorite (<math>\text{NaClO}</math>) and sodium salicylate in the presence of sodium nitroprusside as a coupling agent to yield a blue chromophore called indophenol. The intensity of the color formed is proportional to the concentration of urea in the sample.</p> <p>1. <math>\text{H}_2\text{N}-\text{CO}-\text{NH}_2 + \text{H}_2\text{O} \xrightarrow{\text{urease}} 2\text{NH}_3 + \text{CO}_2</math></p> <p>2. <math>\text{NH}_4^+ + \text{salicylate} + \text{NaClO} + \text{nitroprusside} \xrightarrow{\text{OH}^-} \text{indophenol} + \text{NaCl (blue)}</math></p>	
<b>REAGENTS</b>	<b>R1a: enzyme reagent</b>	
	Urease	> 500 U/ml
	Stabilizers	
	<b>R1b: buffered chromogen</b>	
	Phosphate buffer (pH 6.9)	20 mmol/l
	EDTA	2 mmol/l
	Sodium salicylate	60 mmol/l
	Sodium nitroprusside	3.4 mmol/l
	<b>R2: Alkaline hypochlorite</b>	
	Sodium hypochlorite	10 mmol/l
	NaOH	3.4 mmol/l
	<b>ST: standard</b>	
	Urea	8.3 mmol/l (50 mg/dl)
<b>SAMPLE</b>	Serum or heparinized plasma free of hemolysis and urine	
<b>REACTION CONDITIONS</b>	Wavelength	600 nm ( $\pm 10$ nm)
	Temperature	+37°C
	Cuvette	1 cm light path

## 20. Determination of Urea Concentration in Serum

<b>OPERATING PROCEDURE</b>	1. Preparation of the working reagent No. 1: mix the enzyme reagent (R1a) with the buffered chromogen (R1b). 2. Preparation of the working reagent No. 2: dilute alkaline hypochlorite (R2) in 100 ml of distilled H <sub>2</sub> O. 3. Add 1 ml of the working reagent No. 1 into the first cuvette (this cuvette is called "blank"). 4. Add 1 ml of the working reagent No. 1 and 10 µl of the standard (ST) into the second cuvette (this cuvette is called a "standard"); 5. Add 1 ml of the working reagent No. 1 and 10 µl of the sample into the third cuvette (this cuvette is called "sample").		
		"Blank"	"Standard"
	Working reagent No. 1 (R1a+R1b)	1 ml	1 ml
	Standard (ST)	–	10 µl
	Sample	–	10 µl
	6. Mix and incubate the cuvettes at +37°C for 5 minutes.		
	7. Pipette 1 ml of the working reagent No. 2 into all cuvettes:		
	Working reagent No. 2	1 ml	1 ml
	8. Mix and incubate the cuvettes at +37°C for 5 minutes.		
	9. The analyzer reads the light absorption rates (ABS) of the standard and the sample against the reagent blank and calculates urea concentration (mmol/l).		
<b>CALCULATION</b>	$\frac{\text{ABS sample}}{\text{ABS standard}} \times \frac{\text{standard concentration (mmol/l)}}{\text{urea concentration (mmol/l)}}$		
<b>REFERENCE VALUES</b>	Serum or plasma:	1,7–8,3 mmol/l	
	Urine:	15–30 g/24h	

## REVIEW QUESTIONS

1. Ammonia formation in the body, its concentration in the blood.
2. Ammonia uptake and detoxification.
3. Ammonia detoxification in the brain. Glutamine synthetase.
4. Ammonia detoxification in the liver: Urea cycle. The origin of urea nitrogen atoms.
5. Urea concentration in plasma and daily amount in urine. Hyperuremia.
6. Renal glutaminase. The formation of ammonium salts. Hyperammoniurea and acidosis.
7. Nitrogen-containing compounds in the urine.
8. The method for measuring urea concentration in serums.

## Determination of Creatinine Concentration in Urine

**AIM:** to measure creatinine concentration in urine.

### BACKGROUND:

Creatine is synthesized in the kidneys, liver and pancreas by two enzymatically-mediated reactions. Creatine is then transported through blood to other organs, such as the muscles and the brain, where it is phosphorylated into phosphocreatine, a high-energy compound. The interconversion of phosphocreatine and creatine is a particular feature of the metabolic processes of muscle contraction. A proportion of free creatine in muscle (thought to be between 1 and 2%/d) spontaneously and irreversibly converts into its anhydride waste product, creatinine. Thus, the amount of creatinine produced each day is fairly constant and is related to the muscle mass. Creatinine is freely filtered at the glomerulus, and its concentration is inversely related to the glomerular filtration rate (GFR).

### QUALITATIVE REACTIONS

**Procedure. Weill Reaction.** Add 3–4 ml of urine and 3–5 drops of a 10% NaOH solution into a test tube. Add a few drops of sodium nitroprusside. The color of the solution will rapidly turn from pink to yellow.

**Procedure. Jaffe Reaction.** Add 3–4 ml of urine, 3–5 drops of 10% NaOH and several drops of picric acid into a test tube. Creatinine picrate will form. The color of the solution turns orange.



## THE QUANTITATIVE DETERMINATION OF CREATININE CONCENTRATION

Determination of creatinine which is a colorless compound is performed using the color Joffe reaction.

**Procedure.** Take two 50 ml volumetric flasks. Add 1 ml of a standard creatinine solution into one of them and 1 ml of urine into the other. Then, add 1 ml of a 10% NaOH solution and 1.5 ml of picric acid into both flasks. Gently mix the content of the flasks and leave for 5 minutes at room temperature. Then, fill up both flasks with distilled water up to their marks and perform a colorimetric measurement using a green light filter. The control cuvette is filled with distilled water.

Calculation:

$$C_1 = \frac{E_x \cdot C_2}{E_{st}} \text{ (g /dl)};$$

$$X = \frac{C_1 \cdot 1500}{100} \text{ g};$$

$$X \cdot 8.8 = \text{mmol/24h};$$

Where  $C_1$  – concentration of the sample solution (g/dl);

$C_2$  – concentration of the standard solution (0.1 g/dl);

$E_x$  – extinction coefficient of the sample solution;

$E_{st}$  – extinction coefficient of the standard solution;

X – creatinine quantity (g) per day in urine;

8.8 – conversion factor to mmol.

### **REVIEW QUESTIONS**

1. The synthesis of creatine phosphate. The biological role of creatine phosphate.
2. S-adenosylmethionine (SAM) formation and the importance of the methylation process.
3. Creatinine generation. How much creatinine is excreted in the urine per day? Creatinine ratio.
4. Creatine and creatinine levels in blood serums. Hypercreatininemia.
5. Hypercreatinemia, creatinuria.
6. The method for determining creatinine concentration in urine.

## Determination of Uric Acid Concentration in Serum

**AIM:** to measure concentration of uric acid in serum.

### BACKGROUND:

Uric acid is an end product of nucleoprotein metabolism and is excreted by the kidneys. An increase in serum uric acid concentration occurs with increased nucleoprotein synthesis or catabolism (blood dyscrasias, the chemotherapy of leukemia or a solid tumor) or a decreased renal uric acid excretion (e.g., thiazide diuretic therapy or renal failure).

A measurement of serum uric acid is predominantly used in the investigation of gout, either as a result of a primary hyperuricemia or caused by other conditions or treatments that give rise to secondary hyperuricemias.

**Principle.** The reduction of uric acid with phospho-wolframic acid (the part of Folin's reagent) forms colored compounds, which can be measured using photoelectrocolorimetry.

**Procedure.** Add 1.5 ml of serum, 1.5 ml of distilled water and 1.5 ml of 20% trichloroacetic acid ( $\text{CCl}_3\text{COOH}$ ) into the centrifuge tube. Mix the contents of the tube thoroughly. After 30 minutes, centrifuge the tube at 3000 rpm. Then, add the following reagents into two empty tubes:

Sample	Standard	Super-natant	$\text{CCl}_3\text{COOH}$	Distilled $\text{H}_2\text{O}$	$\text{Na}_2\text{CO}_3$	Folin's reagent
I (ST)	0.5 ml	–	0.5 ml	0.5 ml	0.7 ml	1 drop
II(S)	–	1.5 ml	–	–	0.7 ml	1 drop

## 22. Determination of Uric Acid Concentration in Serum

After 10 minutes, perform a calorimetric measurement using a green light filter. The uric acid concentration is calculated using the following formula:

$$C_S = \frac{C_{ST} \cdot E_S \cdot 100}{a \cdot E_{ST}} \text{ MG/100mL};$$

$$C_S (\text{mg/100ml}) \cdot 59 = C_s (\mu\text{mol/l}),$$

Where  $C_S$  – concentration of uric acid in the serum (mg/dl);

$C_{ST}$  – concentration of uric acid in the standard (I) solution (0.02 mg/ml);

$E_S$  – extinction of the sample (II);

$E_{ST}$  – extinction of the standard solution;

$a$  – volume of the supernatant;

59 – the conversion coefficient from mg/100ml to  $\mu\text{mol/l}$ .

### REVIEW QUESTIONS

1. The structural formulas of purine and pyrimidine bases. The properties of these bases.
2. The structure, importance and properties of mononucleotides.
3. The degradation of purine nucleotide.
4. The concentration of uric acid in blood serum and its amount in daily urine. Hyperuricemia. Xanthinuria.
5. Gout and its causes.
6. The synthesis of purine nucleotides. The sources of carbon and nitrogen atoms in the purine ring.
7. The method for the determination of uric acid concentration in blood serum.

## Determination of Total Bilirubin Concentration in Serum

**AIM:** *to measure the total bilirubin concentration in serum.*

### BACKGROUND:

Bilirubin is produced from the catabolism of protoporphyrin IX by a microsomal heme oxygenase. Daily bilirubin production from all sources averages from 250 to 300 mg. Approximately 85% of the total bilirubin produced is derived from the heme moiety of hemoglobin released from senescent erythrocytes, which are destroyed in the reticuloendothelial cells of the liver, spleen and bone marrow. In blood, bilirubin is bound to albumin and is transported to the liver. Inside the hepatocytes, bilirubin is rapidly conjugated with glucuronic acid to produce bilirubin monoglucuronide and diglucuronide, which are then excreted into bile.

Hyperbilirubinaemia can be caused by an excess of either conjugated or unconjugated bilirubin or both. A separate measurement of these entities is useful in the diagnosis.

Jaundice is a condition characterized by hyperbilirubinemia and the deposition of bile pigment in the skin, mucous membranes and sclera, with a resulting yellow appearance of the patient; it is also called *icterus*.

### Procedure.

1. Into three tubes, add the following reagents in the sequence shown below (mix after each addition):

### 23. Determination of Total Bilirubin Concentration in Serum

Reagents	Test tubes		
	First tube (total bilirubin)	Second tube (direct bilirubin)	Third tube (control)
Serum	0.5 ml	0.5 ml	0.5 ml
Caffeine reagent	1.75 ml	–	1.75 ml
NaCl 0.9%	–	1.75 ml	0.25 ml
Ehrlich's diazo reagent	0.25 ml	0.25 ml	–

2. Measure the extinction of samples using a green light filter and a cuvette with a 5 mm ply:  
After 10 minutes, transfer the contents of the second tube (direct bilirubin) to the cuvette and perform a calorimetric measurement.  
After 20 minutes, transfer the contents of the first (total bilirubin) and the third tubes (control) to the cuvettes and perform a calorimetric measurement.
3. Calculate the concentration of bilirubin.  
Subtract the extinction value of the control sample from the extinction values of the first and the second samples. Use the calibration curve to find the total and direct bilirubin concentrations. The indirect bilirubin is the difference between the total and the direct bilirubin.  
To convert values expressed in mg/dL to  $\mu\text{mol/L}$ , multiply by a conversion factor 17.104.

## REVIEW QUESTIONS

1. The formation of bilirubin in the body.
2. The concentration of total bilirubin and bilirubin fractions in blood serums.
3. The formation of bilirubin glucuronide in the liver. Bilirubin transformation in the gut.
4. Stercobilinogen excretion. Stercobilinogen content in stool and urine. Causes of the increased or decreased stercobilinogen levels in stool and urine.
5. Changes of total bilirubin and its fraction concentrations in blood serums in cases of obstructive, parenchymal (hepatic) and haemolytic jaundice.
6. Bilirubinuria.
7. Urobilinogen; the causes of its appearance in urine.

## Qualitative Reactions for the Detection of Blood and Its Pigments in Urine

**AIM:** *to test the presence of hemoglobin in urine samples.*

In the presence of hemoglobin, organic compounds are oxidized with hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and converted into colored compounds.

### **BENZIDINE REACTION.**

This is a very sensitive reaction and can be performed with **highly** diluted blood – up to 200 000 times.

Dissolve some benzidine crystals in 0.5 ml of concentrated acetic acid; add 1 ml of urine containing blood and a few drops of  $\text{H}_2\text{O}_2$ . The solution will turn green or blue.

Gaiac Resin Reaction.

Add some urine containing blood into the test tube; add 2 ml of Gaiac resin and a few drops of  $\text{H}_2\text{O}_2$ . The solution will turn blue.

### **HEMOCHROMOGEN CRYSTALS.**

During the forensic medical examination of blood stains, hemoglobin is converted to hemochromogen, which can be identified by its characteristic absorption spectrum or by obtaining its crystals.

Add a drop of blood on the slide and a few drops of the reagent (containing pyridine, sodium hydroxide and glucose) beside. Gently warm the slide at  $37^\circ\text{C}$ . The heme interacts with pyridine; the formed compound composes crystals. They can be observed with a microscope.



## TEICHMANN REACTION.

The reaction is based on the formation of hemin chloride crystals (hemin is a heme product).

Add a drop of blood on the slide and dry it out (the temperature cannot be higher than 30°C). Then, place several crystals of NaCl on top of dry blood and put a glass cover on; drip 1–2 drops of glacial acetic acid on the top of the cover glass. Heat up the slide. Small, needle-shaped hemin chloride crystals can be observed through the microscope.

## REVIEW QUESTIONS

1. The physiological role of hemoglobin and its concentration in blood serum.
2. Hemoglobin structure. Heme structure.
3. Compounds containing heme-like substances. The physiological role of these compounds.
4. Fetal and adult forms of hemoglobin.
5. The pathological forms of hemoglobin.  
Hemoglobinopathies: thalassemia, hemoglobin variants and their causes.
6. The structural properties and incidence of the hemoglobin variant HbS.
7. Iron metabolism. Transferrin and ferritin and their physiological role. Hemosiderin. Hemosiderosis (haemochromatosis).
8. Heme synthesis.
9. Porphyrrias, their classification, clinical and biochemical features. Anemia.
10. Hematuria, hemoglobinuria, myoglobinuria.
11. Qualitative reactions for detecting blood and its pigments in urine.

**Arvydas Kaminskas, Asta Mažeikienė, Dovilė Karčiauskaitė**

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Universiteto str. 1, LT-01513 Vilnius, Lithuania

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