LABORATORY MANUAL ON BIOLOGICAL CHEMISTRY

for students of stomatological faculty

PART I

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PREFACE

Biological chemistry is a fundamental medical discipline. A competence in biochemical processes, which take place on different levels of organization – cellular, organ, tissues and in a whole body – is necessary to medical students for understanding of metabolic processes and turnover of substances in tissues, energy production, anabolic and catabolic reactions, transfer of genetic information, processes providing elementary physiological functions as well for interpretation of biochemical data and their diagnostic and prognostic value.

In each practical lesson students would be learning topics, which are determined by a program, will perform investigations of different substances and compounds, and interpret their significance. In each sense modul the themes for independent studies are proposed.

A considerable attention is paid to clinical aspects of biological chemistry – inborn and acquired disorders of metabolism, enzymopathias, to employment of enzymes as medicinals, as well as to a pathochemical processes, which take place in development and in course of diabetes mellitus, atherosclerosis, rheumatism, myocardial infarction, diseases of digestive system, etc.

In order to improve the learning of educational material students are recommended to use modern textbooks and instruction materials, prepared by leading ukrainian specialists as well by coworkers of the Department of Biochemistry of Lviv national medical university.

The final aims in learning of the subject “Biological chemistry“ are determined with a thesis, that student in his future professional activity ought to be able:

- To analyze a correspondence of bioorganic compounds structure to physiological function, which fulfill these compounds in the body.
- To interpret peculiarities of physiological status of organism and development of pathological processes on basis of laboratory investigations data.
- To analyze reaction ability of carbohydrates, lipids and amino acids which provides their functional properties and metabolic transformations in the body.
- To interpret peculiarities of structure and transformations in tissues of bioorganic compounds as a background of their pharmacological activity as medicinals and drugs.
- To interpret biochemical mechanisms of pathogenesis of different disorders in human body and principles of their correction.
- To explain the main mechanisms of biochemical effect and directed action of different classes of pharmacological agents.
- To explain biochemical and molecular basis of physiological functioning of cells, organs and systems of human body.
- To analyze enzymatic reactions which occur in cell membranes and organelles for explanation an integration of metabolic processes
- To classify the results of biochemical investigation and changes of biochemical and enzymatic data, which are used in diagnostics of the most spread human diseases
- To interpret the significance of biochemical processes and their regulation in providement of normal function of organs, systems and whole body.
Avoid loose or synthetic clothing for lab work; remove loose jewelry; secure hair and clothing away from flames, equipment, and chemical contamination.

A laboratory is a workplace. The list of things not permitted in chemistry labs is long – begin with eating, drinking, cooking, applying makeup, smoking and anything else that might increase the chance of ingesting lab chemicals. Careful workers do not touch hands to their faces while working in lab. Professional and serious behavior is expected at all times; rowdy or boisterous play or pranks of any kind will be deemed cause for expulsion from lab.

Housekeeping. Store backpacks and other extra materials away from work areas and off floors to protect them and to keep walkways clear. Keep work areas clear; store extra glassware and materials as soon as you finish with them, keeping only essential materials on the workbench.

Use cotton towels to dry wet hands & clean surfaces. Use paper toweling for absorbing hazardous materials. Dispose of dirty waste paper in trash receptacles.

- Clean your work area:
- Check to be sure all reagents and waste containers are securely closed;
- Clean lab surfaces with sponges;
- Paper with absorbed hazardous chemicals should be placed in solid hazardous waste containers, not in the general trash.
- Broken glass contaminated with hazardous or smelly materials can be rinsed with appropriate solvent before placing shards in the broken glass container.

Handle hazardous materials with correct techniques.
Never touch hazardous chemicals with bare hands; use tools such as tongs and scoops.

Never remove chemicals from the laboratories. Do not attach samples to lab reports or notebook pages. In addition to causing disposal problems, taking samples from lab creates the potential for an accidental exposure.

Anhydrous materials (such as NaOH, CaCl₂, MgSO₄ or NaSO₄) may absorb water from the air and MUST be kept tightly closed between uses. Left in the air, NaOH or KOH pellets will absorb moisture and produce a puddle of concentrated corrosive liquid on the work bench – a serious skin exposure hazard.
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<td>Physico-chemical properties and structure of enzymes-proteins.Classification of enzymes</td>
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<td>3.</td>
<td>Study of mechanisms and kinetics of enzymatic reactions</td>
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<td>The role of cofactors, vitamins and their coenzyme forms in enzyme catalysis.</td>
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<td>Regulation of enzymatic reactions and mechanisms of enzymopathias appearance. Medical enzymology</td>
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<td>Studies on glycolysis – oxidation of carbohydrates under anaerobic conditions.</td>
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<td>Breakdown and biosynthesis of glycogen. Regulation of glycogen metabolism,</td>
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<td>Metabolism of complex lipids and ketone bodies</td>
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**THEMATIC PLANE OF LECTURES IN the 3rd SEMESTER**

<table>
<thead>
<tr>
<th>Theme of the lecture</th>
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<td>1 Enzymes. Regulation of enzymatic processes. Enzymology</td>
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<td>2 Molecular basis of bioenergetics: enzymes of biological oxidation; molecular organization of electron transport in mitochondria.</td>
<td>2</td>
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<tr>
<td>3 Metabolism of carbohydrates, its regulation and changes in pathology</td>
<td>2</td>
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<tr>
<td>4 Metabolism of lipids, its regulation and changes in pathology</td>
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Objective: Introduction to the assignments and methods of biochemical investigation; to learn specific methods of investigation of biologically active substances as well to make an acquaintance with instruments and devices used in biochemistry.

Actuality of the theme: Biochemistry is a science which investigates chemical composition of living organisms, chemical structure of constituents, their properties, localization, the pathways of their appearance and transformations, as well as chemical processes, which take place in living cell and provide turnover of matter and energy in the cell.

Biochemistry is on the way of solution of important problems and questions of natural history and medicine, e.g. problem of protein synthesis, life span prolongation, etc.

Modern physical, chemical and mathematical methods are used in biochemical investigations. Biochemical data are used in medical diagnostics, treatment and prevention of diseases

Specific aims:
- To know principal stages and regularities in origin and development of biochemistry as fundamental medical and biological science and educational discipline.
- To recognize principles of methods of investigation of functional status in human body in health and disease.
- To interpret data of biochemical investigations and evaluate the status of selected metabolic pathways
- To determine optical density of colored solutions at distinct light wavelength using a photocolorimeter, to interpret obtained results properly.

Theoretical questions:
1. The objectives and assignments of biochemistry and its principal trends and parts.
2. A short history of biochemistry and its main periods.
3. Contribution of scientists of the Dept. of Biochemistry of Lviv National Medical University to development of biochemistry.
4. The significance of biochemistry in the development of medical science and practical health care.
5. Chemical constitution of living organisms, the characteristic features of living systems.
6. The structural components of the prokaryotic and eukaryotic cells, their resolution with ultracentrifugation methods.
7. Biological material used in biochemical investigations.
9. Fractionation of material using differences in solubility (on the example of proteins precipitation).
11. Electrophoresis.
12. Chromatography.
13. Radioisotopic methods.
14. Enzyme immunoassays (ELISA).

Essay of the history of biochemistry unit

Unit of Biological Chemistry was founded in 1894 at the Medical department of Lviv University by Prof. V. Niemilovych (1863-1904) who had arrived from Vienna in 1891 upon invitation to work as an associate professor in Pharmacognosy and as a lecturer in chemistry.

From 1904 to 1919, the unit was headed by Prof. Bondzinski (1862-1929). He was the founder and the first president of the Academy of the Medical Sciences of Poland. Research of the unit's faculty under Prof. S. Bondzinski was the so-called oxyproteins - the products of protein turnover (metabolism) excreted in small amounts within urine.

From 1919 to 1922, the unit was supervised by Prof. W. Morachewsky, who also became rector of the Academy of Veterinary medicine in Lviv. W. Morachewsky was certified to teach medical chemistry at the University of Lviv in 1918 and he began his work as Professor at Lviv Academy of Veterinary medicine in 1921.

From 1922 until 1941 the unit was headed by the world-well known scientist Jakub Oscar Parnas. His most significant work at the Lviv schoole was connected with enzymatic products, their formation in association with muscular activity and alcohol fermentation. Some of his investigations have become classic works in biochemistry. They concern the rapid autolytic increase of ammonium content in extravasate blood and "postmortem" or traumatic ammoniogenesis in muscular tissue. Significantly important was his discovery of the higher phosphorylated derivatives of adenylic acid found in muscular fibers and erythrocytes, namely ATP and ADP. As alternatives to adenylic acid they are not subject to enzymatic deamination in cells. J. Parnas and his schoole studied biological and chemical properties of muscular adenylic acid and its derivatives and introduced analytical methods for its quantitative determination. J. Parnas achieved worldwide recognition in his research of anaerobic degradation of carbohydrates. His research is called in biochemical literature as the Embden-Meyerhof-Parnas pathway or "EMP scheme of glycolysis ". J. Parnas was the first who introduced isotopic methods into biochemical investigations.

From 1944 to 1973, the unit was headed by Prof. B.A. Sobchuk who began his scientific activity under J. Parnas conduction. B. A. Sobchuk investigated carbohydrate metabolism in the muscular tissue and yeast. He was awarded the degree of Doctor of Medicine (1937) for his research on the significance of pyruvate in glycogenolysis. For his thesis, "Synthesis of xanthopterin and its derivatives and their effect on experimental tumors in animals", B.A. Sobchuk was awarded the degree of Doctor of Biological Sciences in 1959. A year later he attained professorship of biochemistry. The co-workers of department studied the peculiarities of carbohydrate metabolism in tumor cells (the Krebtry's effect), as well as the synthesis and specific effect of xanthopterin and its derivatives iodopterin and porphyropterin on tumors. Another significant direction of the unit's work was the investigation of iodine metabolism, function of the thyroid gland and toxicology of carbon monoxide.
From 1974 to 1995, the unit was headed by prof. M.P. Shlemkevych. In close collaboration with the unit of oncology, the department of biochemistry studied the mechanisms of sensitivity and resistancy of tumor cells of stomach cancer to chemotherapeutic agents, namely 5-fluorouracil.

From 1995 to 1998, the head of biochemistry unit was prof. M.F. Tymochko. The main goal of his scientific research was the investigation of the fundamentals of adaptive-compensatory processes under various experimental conditions with emphasize on changes in oxygen-dependent reactions. He studied metabolic backgrounds of oxygen homeostasis in different functional conditions and dealt with solving of a wide range of important problems in medical practice. He also investigated the effect of harmful environmental factors on the functions of digestive system, the role of energy exchange in the pathogenesis of chronic diseases of the liver and cardiovascular system. He determined the degree of risk in surgery in abdominal cavity, endocrine and cardiovascular surgery and endogenous intoxication in oncological diseases.

Since 1998, the Biochemistry unit has been headed by O. Ja. Sklyarov, who started his scientific and educational activities at the unit of normal physiology. In 1993, for his thesis, "Mechanisms of combined action of mediators and hormones on the secretory function of gastric glands", he obtained the scientific degree doctor of medical sciences. In 1997 he was nominated as honorary associate professor by the fund of Soros and was noted by the fund "Revival". Prof. O.Ja.Sklyarov is concerned with the study of the mechanisms of regulation of gastric secretion under the influence of the combined action of neurohormonal substances. Alongside his research, prof. O.Ja.Sklyarov pays significant attention to educational work.

Currently, the unit's researchers are investigating the systemic influence of malignant tumors and endogenous intoxication in patients and in experimental animals. They are also studying the effects of stress on cytoprotective and ulcerogenic mechanisms of gastric mucosa, the ion-transport and metabolic processes in it and the action of exogenous factors on endoecological conditions.

**Practical part**

**Determination of optical density of colored solutions according to their concentration**

The photoelectrocolorimetric method of analysis serves for determination of substances concentrations in coloured solutions, biological liquids or tissue extracts. It may be also used for determination of concentrations of colourless substances if they can be transformed into coloured state with the specific reagents. The method is one of the most widespread in biochemistry and clinical medicine.

**Experiment 1. Measurement of optical density (A) of solutions which contain different quantities of phosphorus.**

**Principle.** Phosphates in presence of sulfuric acid form phosphomolybdate complexes with molybdate, which are then reduced to a compound called molybden blue. The amount of phosphates is then measured colorimetrically according to a color intensity of molybden blue complex.

**Method.** Pipette the solutions into five labelled test tubes according to the table:
<table>
<thead>
<tr>
<th>№</th>
<th>Reagents</th>
<th>Tube number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Standard phosphate solution (10 mg/ml), ml</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>2</td>
<td>Distilled water, ml</td>
<td>5.0 4.5 4.0 3.0 2.0</td>
</tr>
<tr>
<td>3</td>
<td>Molybden reagent, ml</td>
<td>5.0 5.0 5.0 5.0 5.0</td>
</tr>
<tr>
<td>4</td>
<td>Phosphorus concentration, mg%</td>
<td>0 50 100 200 300</td>
</tr>
<tr>
<td>5</td>
<td>Results of optical density measurement</td>
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</tbody>
</table>

After mixing a color develops. After 5 min measure the optical density on a photoelectrocolorimeter with a red light filter. Then draw a plot (calibration curve) according to obtained results.

Examples of Krock-1 tests

1. The method consisting in removal of low-molecular impurities from colloidal systems and high-molecular compound solutions by semipermeable membrane diffusion is called:
   A. Dialysis
   B. Electrodialysis
   C. Ultrafiltration
   D. Decantation
   E. Compensatory dialysis

2. Choose from listed below methods ONE, which is used for fractionation of protein mixtures and isolation of individual proteins (enzyme, hormone, toxin etc):
   A. Affinity chromatography
   B. Precipitation with nitric acid
   C. Boiling of extracts
   D. Proteolysis
   E. Radioimmunoassay

3. Determination of C-reactive protein (CRP) in blood plasma is conducted with the use of antisera, containing specific antibodies against CRP. What type of analytical method is used in this case?
   A. Immunoprecipitation
   B. Spectrophotometry
   C. Electrophoresis
   D. Chromatography
   E. Polarography

4. Protein preparations from human blood plasma are frequently used in clinical medicine for treatment of many diseases. Fractionation of blood plasma and preparation of distinct protein fractions is achieved by the next method:
   A. Fractional precipitation with ammonium sulphate
   B. Fractional precipitation with ethanol by Cohn YI method
   C. Precipitation with salts of heavy metals
   D. Electrophoresis in agarose gel
   E. Ultracentrifugation
5. Determination of proportion between protein fractions in blood plasma or serum has an important clinical and diagnostic significance. The following routine method for obtaining results of this sort is most frequently used in clinical laboratories:

A. Salting out with neutral salts
B. Absorption chromatography
C. Precipitation with strong acids
D. Electrophoresis in agar gel or on acetyl-cellulose films
E. Immunoprecipitation

Individual independent students work

1. History of biochemistry and its main periods. The significance of biochemistry in the development of medical sciences and practical health care.
2. The fundamental discoveries in a branch of structural and functional significances in proteins and nucleic acids.

References:


Objective: Life in all its diverse manifestations is an extremely complex system of chemical reactions which are catalyzed by enzymes. The last play vital role in nearly all life processes. They are involved in living organisms in a vast multitude of interrelated chemical reactions such as synthesis, degradation and interconversion of a large number of chemical compounds. An understanding of their implications provides a deep insight into the sense and innermost enigmas of the fascinating phenomenon that we call life.

Specific aims:
• To interpret biochemical principles of structure and functioning of different classes of enzymes.
• On the basis of physical and chemical properties of enzymes as proteins to explain the dependence of enzymatic activity from pH of medium, temperature and other factors.
• To analyze values of the activity of enzymes in blood plasma in dependence from their localization in the cell, tissue or organ.  
• To analyze methods of determination of enzymatic activity for an optimal application in clinical biochemistry.

**Theoretical questions:**

1. Enzymes: definition, properties of enzymes as biological catalysts, difference between enzymes and inorganic catalysts.
5. Structure of enzymes: active centres and allosteric sites.

**Practical part**

**Salivary amylase**

Amylase – is an enzyme that catalyses the hydrolysis of starch into sugars. Amylase is present in the saliva of humans and some other mammals, where it begins the chemical process of carbohydrates digestion. Salivary gland makes amylase to hydrolyse α-1,4-glycosidic bonds of starch into disaccharides (maltose) which are converted by other enzymes to glucose to supply the body with energy. As diastase, amylase was the first enzyme to be discovered and isolated (by A. Payen in 1833).

![Section of a starch molecule](image) → ![Maltose molecule](image)

**Experiment 1. Investigation of starch hydrolysis by salivary amylase.**

**Principle.** The hydrolysis of starch is monitored by probes with iodine and the appearance of the of maltose, which is the final product of starch hydrolysis by salivary amylase. Starch with iodine gives a blue coloured complex. Dextrins (intermediate products of starch hydrolysis) in presence of iodine form red-brown colour or no change in colour at all. Maltose also does not form a coloured complex with iodine, but it can be detected with the Trommer’s reaction.

**Method:**

I. **Dilution of saliva.** To obtain diluted saliva (the source of amylase), rinse one’s mouth with distilled water during 1-2 min and collect that portion of fluid (saliva + water) into separate tube and use it for the analysis.

II. **Preparation of experimental mixtures and incubation**

1. Place 10 tubes in the tube holder and put into each tube 2 ml of water and 1 drop of Lugol solution.
2. Pour into a separate tube 5 ml of 0.5 % starch and 1 ml of diluted saliva.
3. Mix the contents of the tube and after each 1 min 0.5 ml of the fluid is taken and added to the tubes № 1, 2, 3 etc.
III. Color reactions for the detection of starch and maltose.

The hydrolysis of starch is considered to be complete when the probe will not change the colour of iodine in the test tube (starch with iodine gives a blue coloured complex).

*Trommer’s reaction.* Add 5 ml of 5% NaOH and several drops of CuSO₄ solution, mix the content of these tubes and heat it on a flame to boiling. The appearance of yellow-red sediment indicates the presence of reducing substances, namely, reducing disaccharide maltose.

Explain the results and draw a conclusion.

**Clinical and diagnostic significance.** Amylase (E.C. 3.2.1.1) hydrolyzes starch and dextrins. It is produced predominantly in salivary and pancreatic glands, although amylase activity can be detected in tissues of liver, kidneys, intestines and lungs. During normal conditions amylase activity in blood serum corresponds to 0.42-0.96 g of starch, hydrolyzed by 1 ml of enzyme in 1 minute.

An increase of amylase activity in serum is observed in pancreatitis, peritonitis, mesenteric vessels thrombosis, rupture of oviduct in case of extrauterine pregnancy and after the injection of some drugs, e.g. morphine, caffeine, ACTH and cortisol. The increase of the activity of amylase in saliva is observed in renal insufficiency, stomatitis, neuralgia. A decrease in amylase activity is noted in some cases of psychoses, accompanied by depression or excitation and in gastric secretion disorders (anaciditas).

**Examples of Krock-1 tests**

1. After the addition of an extract of pancreatic gland to the tube with starch solution a blue coloration of the sample with iodine have disappeared, which indicates on starch hydrolysis. What pancreatic enzyme is involved in this reaction?
   A. Amylase  
   B. Trypsin  
   C. Chymotrypsin  
   D. Lipase  
   E. Aldolase

2. In human saliva there is an enzyme able to hydrolyze the α[1→4] glucosidic bonds in the molecule of starch. Name this enzyme:
   A. α-Amylase  
   B. Phosphatase  
   C. Fructofuranosidase  
   D. β-Galactosidase  
   E. Lysozyme

3. The first position in classification of enzymes is occupied by:
   A. Oxidoreductases  
   B. Transferases  
   C. Isomerases  
   D. Hydrolases  
   E. Ligases

4. Ambulance delivered a patient to the hospital with a preliminary diagnosis “acute pancreatitis”. What enzyme activity must be estimated in blood and urine in order to support this diagnosis?
A. Alpha-amylase  
B. ALAT (GPT)  
C. AsAT (GOT)  
D. Gamma-amylase  
E. Lactate dehydrogenase

**Individual independent students work**
1. Multi-enzyme complexes and their advantages.
2. The employment of enzymes in biochemical investigations.

**References:**

**Topic № 3. Study of mechanisms and kinetics of enzymatic reaction.**

**Objective:** To show the catalytic role of enzymes on the basis of pancreatic proteinases and amylase activity measurements. To estimate clinical and diagnostic significance of determination of amylase activity in urine.

**Actuality of the theme:** Enzymes are biocatalysts with changeable activity, submitted to regulatory influences. The knowledge of kinetics of enzymatic reactions is important for the understanding of metabolic processes in cells, tissues and organs of human body.

**Specific aims:**
- To explain the mechanism of enzymatic action on basis of the affinity of enzyme to substrate and events which occur in enzyme-substrate complex.
- To interprete changes in activity of enzymes in biological fluids under conditions of different pathological processes.
- To explain the employment of inhibitors and activators of enzymes in cases of metabolic disorders or distinct pathology
- To analyse the mechanism of enzyme action using an example of chymotrypsin and acetylcholinesterase.

**Theoretical questions:**
1. Enzyme kinetics. Factors affecting enzymatic activity:
   - concentration of enzyme
   - concentration of substrate;
effect of temperature;
effect of pH
2. Michaelis-Menten constant and equation.
5. Units of enzymatic activity.
7. Isoenzymes, their role in enzymodiagnostics.

Practical part

Methods of enzymatic activity assays

Methods for the determination of enzymatic activity can be divided into: direct, indirect and coupled assays. The direct method is based on measurement of the substrate or product concentration as a function of time. For example, the enzyme cytochrome c oxidase catalyzes the oxidation of the heme-containing protein cytochrome c. In its reduced (ferrous iron) form, cytochrome c displays a strong absorption band at 550 nm, which is significantly diminished in intensity when the heme iron is oxidized (ferric form) by the oxidase. One can thus measure the change in light absorption at 550 nm for a solution of ferrous cytochrome c as a function of time after addition of cytochrome c oxidase; the diminution of absorption at 550 nm that is observed is a direct measure of the loss of substrate (ferrous cytochrome c) concentration.

In some cases the substrate and product of an enzymatic reaction do not provide a distinct signal for convenient measurement of their concentrations. Often, however, product generation can be coupled to another, nonenzymatic, reaction that does produce a convenient signal; such a strategy is referred to as an indirect assay. Dihydroorotate dehydrogenase (DHODase) provides an example of the use of an indirect assays. This enzyme catalyzes the conversion of dihydroorotate to orotic acid in the presence of the exogenous cofactor ubiquinone. During enzyme turnover, electrons generated by the conversion of dihydroorotate to orotic acid are transferred by the enzyme to a ubiquinone cofactor to form ubiquinol. It is difficult to measure this reaction directly, but the reduction of ubiquinone can be coupled to other nonenzymatic redox reactions.

A third way of following the course of an enzyme-catalyzed reaction is referred to as the coupled assays method. Here the enzymatic reaction of interest is paired with a second enzymatic reaction, which can be conveniently measured. In a typical coupled assay, the product of the enzyme reaction of interest is the substrate for the enzyme reaction to which it is coupled for convenient measurement. An example of this strategy is the measurement of activity for hexokinase, the enzyme that catalyzes the formation of glucose 6-phosphate and ADP from glucose and ATP. None of these products or substrates provide a particularly convenient means of measuring enzymatic activity. However, the product glucose 6-phosphate is the substrate for the enzyme glucose 6-phosphate dehydrogenase, which, in the presence of NADP+, converts this molecule to 6-phosphogluconolactone. In the course of the second enzymatic reaction, NADP+ is reduced to NADPH, and this cofactor reduction can be monitored easily by light absorption at 340 nm.
Experiment 1. The investigation of the influence of pH of medium on amylase activity.

Principle. Each enzyme has an optimum pH at which the velocity is maximum. Below and above this pH, the enzyme activity is much lower and at extreme pH, the enzyme becomes totally inactive. Most of the enzymes of higher organisms show optimum activity around neutral pH (6-8).

Method.
I. Dilute saliva as described above (See topic 1, Experiment 1)
II. Preparation of experimental mixtures and incubation. Take 3 clean tubes and do the following procedures:
1. Add 2 ml of starch solution to each of the three tubes.
2. Add to the first tube 2 ml of phosphate buffer, pH 5.0.
3. Add to the second 2 ml of phosphate buffer, pH 7.0.
4. Add to the third 2 ml of phosphate buffer, pH 9.0.
5. Add into each tube 1 ml of diluted saliva.
6. Incubate tubes at 37 °C for 10 minutes.
5. Divide the content of each tube into two equal portions.

Stage 1 Preparation of mixtures and incubation

Stage 2. Color reactions for the detection of starch and maltose

III. Color reactions for the detection of starch and maltose.
8. Add 3 drops of iodine to the first portion of each test solution. Register changes in color.
9. Use the second portion of the probes for the performance of the Trommer’s reaction (see Topic 1Experiment 1).

Explain the results and draw a conclusion.
Examples of Krock-1 tests

1. Michaelis-Menten constants of two enzymes are $1.3 \times 10^{-5}$ M/l and $2.3 \times 10^{-3}$ M/l subsequently. Indicate true statement about the affinity of these enzymes to substrate.

A. The second enzyme has higher affinity to substrate
B. Enzymes possess equal affinity to substrate
C. The first enzyme has higher affinity to substrate
D. For decision an information on concentration of enzyme is needed
E. Data are incomplete and it is impossible to draw a conclusion

2. In an enzyme assay the substrate concentration was taken much higher than $K_m$. In this conditions the rate of the reaction will be as follows:

A. Shows zero-order kinetics
B. Approaches 50 % value of $V_{max}$
C. Is proportional to substrate concentration
D. Is independent of enzyme concentration
E. Is independent of temperature

3. Activity of many enzymes depends from the presence of free thiol groups in active center. What amino acid residue provides presence of these groups in enzyme molecule?

A. Cysteine
B. Lysine
C. Tryptophan
D. Methionine
E. Serine

4. Michaelis-Menten constant ($K_m$) reflects the next property of enzyme:

A. Affinity to substrate
B. Sensitivity to pH of medium
C. Thermolability
D. Affinity to a product of reaction
E. Sensitivity to competitive inhibitors

References:

Topic № 4. The role of cofactors, vitamins and their coenzyme forms in enzyme catalysis.

Objectives: To learn the structure, principles of classification and function of coenzymatic vitamins. To learn the methods of qualitative and quantitative determination of vitamins.

Actuality of the theme: Water soluble vitamin take part in metabolism as coenzymes and activators for many enzymatic reactions.

Deficiency in vitamin supply of the body or disorders of their metabolism which is caused by alteration of their absorption or transformation into coenzyme forms, substantially decrease the intensity of energetic and plastic metabolism. This is accompanied with functional disorders of brain, heart, liver and other organs, suppression of immune response to infection, loss of ability to accommodate effectively to unfavorable environmental conditions.

Specific aims:
- To interpret the role of vitamins and their biologically active derivatives in mechanism of catalysis by enzymes of different classes.
- To explain the application of antivitamins as inhibitors of enzymes in contagious diseases and in disorders of homeostasis.
- To explain the role of metals in mechanisms of enzymatic catalysis.
- To classify distinct groups of coenzymes according to their chemical nature and type of the reaction, which they catalyze.

Theoretical questions:
1. Classification of coenzymes due to their chemical nature and type of catalytic reaction.
2. Coenzymes as transporters of hydrogen atoms and electrons (examples of distinct reactions):
   - NAD⁺, NADP⁺ coenzymes – derivatives of vitamin PP;
   - FAD, FMN coenzymes – derivatives of vitamin B₂ – riboflavin;
   - Role of vitamin C in oxidative-reductive reactions
   - metalloporphyrins
3. Coenzymes as transporters of chemical groups (examples):
   - pyridoxal phosphate;
   - HS-CoA – coenzyme of acylation;
   - lipoic acid;
   - THF – derivatives of folic acid
4. Coenzymes of isomerisation, synthesis and cleavage of C-C bonds (examples):
   - thiamine pyrophosphate – coenzyme form of vitamin B₁;
   - biocytin – coenzyme form of vitamin H – biotin;
   - methylcobalamin and deoxyadenosylcobalamin – coenzyme forms of vitamin B₁₂

Practical part
In complex enzymes a protein component (apoenzyme) can be found out with the biuretic reaction. An unprotein component, containing a derivative of any vitamin, can be opened with the appropriate qualitative reactions for each vitamin.
Experiment 1. A method for the detection of nicotinic acid.

**Principle.** Heating of a mixture of nicotinic acid and copper acetate gives a blue sediment of copper nicotinate.

**Method.** Dissolve 10 mg of nicotinic acid in 10-20 drops of 10% acetic acid. Heat the tube to boiling and then add an equal volume of 5% solution of copper acetate. The liquid turns blue and after standing blue sediment appears.

Explain the results and draw a conclusion.

**Examples of Krock-1 tests**

1. In case of enterobiosis acrifine – the structural analogue of vitamin B₂ - is administered. The synthesis disorder of which enzymes does this medicine cause in microorganisms?
   A. FAD-dependent dehydrogenases  
   B. Cytochrome oxidases  
   C. Peptidases  
   D. NAD-dependent dehydrogenases  
   E. Aminotransferases

2. Hydroxylation of endogenous substrates and xenobiotics requires a donor of protons. Which of the following vitamins can play this role?
   A. Vitamin C  
   B. Vitamin P  
   C. Vitamin B₆
   D. Vitamin E  
   E. Vitamin A

3. A newborn child has convulsions that have been observed after prescription of vitamin B₆. This most probable cause of this effect is that vitamin B₆ is a component of the following enzyme:
   A. Glutamate decarboxylase  
   B. Pyruvate dehydrostase  
   C. Netoglubarate dehydromine  
   D. Aminolevulinate synthase  
   E. Glycogen phosphorylase

4. A disorder in the structure of collagen fibers associated with a deficiency of vitamin C leads to tooth loss. This can be explained, because vitamin C is a cofactor of:
   A. Lysine hydroxylase and proline hydroxylase  
   B. Glycosil transferase  
   C. Lysine hydroxylase and lysine oxidase peptidase  
   D. Procollagen peptidase

**References:**


**Topic № 5. Regulation of enzymatic activity and mechanisms of enzymopathias. Medical enzymology.**

**Objective:** To learn the main principles of regulation of metabolic pathways, the consequences of alteration of enzymatic activity in the cell and employment of enzymes in medicine.

**Actuality of the theme:** Enzymes are biocatalysts with changeable activity, submitted to regulatory influences. Estimation of enzymatic activity is routinely used in laboratory investigations with diagnostic purposes. Enzymes are also employed as medicines and drugs in practical medicine.

**Specific aims:**
- To analyze pathways and mechanisms of regulation of enzymatic reactions as a background of metabolism in health and disease.
- To explain the application of activators and inhibitors of enzymes as medicines and pharmaceuticals for correction of metabolic disorders in pathology.
- To explain changes in metabolic pathways and accumulation of distinct metabolic intermediates in the inborn (hereditary) and acquired disorders of metabolism – enzymopathias.
- To analyze changes in activity of indicatory enzymes in blood plasma in pathology of distinct organs and tissues.

**Theoretical questions:**
1. Enzyme inhibition (reversible, irreversible, competitive, non-competitive).
2. Regulation of enzyme activity in the living system:
   - allosteric regulation;
   - feedback regulation;
   - covalent modification of enzymes;
   - activation of latent enzymes by limited proteolysis;
   - cyclic nucleotides in regulation of enzymatic processes.
3. Control of enzymes synthesis (constitutive and adaptive enzymes).
4. Application of enzymes:
   - enzymes as therapeutic agents;
   - enzymes as analytic agents;
   - immobilized enzymes.
5. Diagnostical importance of enzymes (plasma specific and non-plasma specific enzymes).
6. Changes in enzymatic activity of blood plasma and serum as diagnostic indexes (markers) of pathological processes in distinct organs – myocardial infarction, acute pancreatitis, liver disease, pathology of muscle tissue.
7. Inborn (hereditary) and acquired metabolic defects, their clinical and laboratory diagnostics.
Practical part

Experiment 1. Study of the influence of activators and inhibitors on activity of salivary amylase.

**Principle.** Compounds, which enhance the activity of enzymes – called activators – are a number of metal ions, e.g. Na\(^+\), Mg\(^{2+}\), Mn\(^{2+}\), Co\(^{2+}\), as well as organic substances, especially metabolic intermediates. Amylase is activated by sodium chloride (NaCl), inhibited – by copper sulphate (CuSO\(_4\)). As indicator of the influence of mentioned compounds on the activity of amylase is a degree of starch cleavage under the action of amylase in the presence of NaCl or CuSO\(_4\).

**Method.**

I. **Dilution of saliva.** Dilute saliva two fold.

II. **Preparation of experimental mixtures and incubation.** Take 3 clean dry tubes and do the following procedures:

1. Add 1 ml of distilled water into the first tube.
2. Add 0,8 ml of distilled water and 0,2 ml of 1 % NaCl into the second tube.
3. Add 0,8 ml of distilled water and 0,2 ml of 1% solution of CuSO\(_4\) into the third tube.
4. Add 1 ml of diluted saliva into each tube
5. Add 2 ml of 1% starch solution into each tube. Mix them well and place tubes to thermostat at 37°C during 15 min.

III. **Color reaction.** Add 0,1% solution of iodine in 0,2% sodium iodide). Changes in color development are observed and registered in a table (see below).

<table>
<thead>
<tr>
<th>Tube content</th>
<th>№ of tube</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Water (ml)</td>
<td>1</td>
</tr>
<tr>
<td>NaCl, 1 %-solution (ml)</td>
<td>-</td>
</tr>
<tr>
<td>CuSO(_4), 5 %-solution (ml)</td>
<td>-</td>
</tr>
<tr>
<td>Saliva 2 fold diluted (ml)</td>
<td>1</td>
</tr>
<tr>
<td>Starch, 1 %-solution (ml)</td>
<td>2</td>
</tr>
<tr>
<td>Final color after addition of iodine</td>
<td></td>
</tr>
</tbody>
</table>

Explain the results and draw a conclusion.

**Clinical and diagnostic significance.** Inhibitors of enzymes are widely used in medicine as drugs and medicinals, e.g. acetylosalicylic acid (aspirin) is an inhibitor of cyclooxygenase (prostaglandin syntase) and employed as anti-inflammatory drug. Trasylol (inhibitor of trypsin) and contrical – inhibitor of different proteinases – (one of them are kallikreins) are used in treatment of pancreatitis, allopurinol –inhibitor of xanthine oxidase – is used for treatment of gout, etc.

**Examples of Krock-1 tests**

1. Marked increase of activity of MB-forms of CPK (creatinephosphokinase) and LDH-1 were revealed on the examination of the patient's blood. What is the most likely pathology?

A. Miocardial infarction  
B. Hepatitis
2. 12 hours after an acute attack of retrosternal pain a patient presented an increase of aspartate aminotransferase activity in blood serum. What pathology is this deviation typical for?
A. Myocardium infarction
B. Viral hepatitis
C. Collagenosis
D. Diabetes mellitus
E. Diabetes insipidus

3. Desulfiram is widely used in medical practice to prevent alcoholism. It inhibits aldehyde dehydrogenase. Increased level of what metabolite causes aversion to alcohol?
A. Acetaldehyde
B. Ethanol
C. Malonyl aldehyde
D. Propionic aldehyde
E. Methanol

4. In course of tuberculosis treatment a patient was administered isoniazide – a structural analogue of nicotinamide and pyridoxine. What type of inhibition by mechanism of action exhibits isoniazide?
A. Competitive
B. Irreversible
C. Uncompetitive
D. Allosteric
E. Noncompetitive

**Individual independent students work**
1. Regulators of enzymatic activity and their employment in clinical practice.
2. Enzymodiagnosics. The use of enzymes in enzymodiagnosics.

**References:**

Objectives: To learn the sequence of reactions in tricarboxylic acids (TCA) cycle and biological significance of TCA cycle as the final stage of catabolic pathway in the cell. To make an acquaintance with methods of TCA cycle investigation in mitochondria and to examine the effect of malonic acid upon this process.

Actuality of the theme: The peculiarities of TCA cycle functioning have an important significance in evaluation of its role for providement of the cell with energy as well as for understanding of its amphibolic significance. The analysis of TCA cycle function is necessary for estimation of its role in turnover of matter and energy in the cell.

Specific aims:
- To interprete biochemical principles of metabolic pathways: catabolic, anabolic, amphibolic pathways.
- To explain biochemical mechanisms of regulation of catabolic and anabolic reactions.
- To interprete biochemical principles of TCA cycle functioning and its anaplerotic reactions and their amphibolic sense.
- To explain biochemical regulatory mechanisms in TCA cycle and its principal position in turnover of matter and energy.

Theoretical questions:
2. Exergonic and endergonic biochemical reactions, role of ATP and other macroergic phosphate containing compounds in their coupling.
5. The most important metabolites of amphibolic pathways in turnover of proteins, carbohydrates, lipids, their significance for integration of metabolism in the cell.
6. Tricarboxylic acid (TCA) cycle:
   - Cellular location of TCA cycle enzymes;
   - Sequence of TCA cycle reactions;
   - Characterization of enzymes and coenzymes participating TCA cycle;
   - Reactions of substrate phosphorylation in TCA cycle;
   - The effect of allosteric modulators upon TCA cycle reactions;
   - Energetic effect of TCA cycle.
7. Anaplerotic and amphibolic reactions of TCA cycle.

Practical part

Experiment 1. Investigation of TCA cycle functioning in mitochondria and the effect of malonate upon this process.
**Principle.** The transformations of acetyl-CoA in presence of mitochondrial enzymes is accompanied with production of CO$_2$. As a source of acetyl-CoA, which is further incorporated into CTA cycle, is used pyruvate. The last under the action of multimeric pyruvate dehydrogenase complex is submitted to oxidative decarboxylation and acetyl-CoA and CO$_2$ are produced. If TCA cycle is inhibited with malonate bubbles of gaze does not occur. Malonate is a classic competitive inhibitor of succinate dehydrogenase – enzyme of TCA cycle. It binds with active center of this enzyme and hinder binding of true substrate – succinic acid (or succinate).

For binding of released CO$_2$ into incubation medium is added Ca(OH)$_2$. At the end of incubation a bound CO$_2$ is detected due to a production of gaze bubbles after addition of sulphuric acid solution into incubation medium.

**Method.** Fill three tubes – a control one, experimental 1 and 2 with reagents as indicated in the table:

<table>
<thead>
<tr>
<th>Content of tubes</th>
<th>Tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Tube 1</td>
</tr>
<tr>
<td>Phosphate buffer, pH 7.4, ml</td>
<td>2.0</td>
</tr>
<tr>
<td>Sodium pyruvate solution, ml</td>
<td>0.5</td>
</tr>
<tr>
<td>Malionic acid, ml</td>
<td></td>
</tr>
<tr>
<td>Saline, ml</td>
<td>0.5</td>
</tr>
<tr>
<td>Ca(OH)$_2$ solution, ml</td>
<td>0.5</td>
</tr>
<tr>
<td>Suspension of mitochondria</td>
<td>0.5</td>
</tr>
<tr>
<td>Boiled suspension of mitochondria</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Incubation in a thermostat for 15 min at 37ºC</strong></td>
<td></td>
</tr>
<tr>
<td>0.1 M solution of sulphuric acid</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Place tubes in a thermostat for 15 min at 37 ºC. Thereafter add 1.0 ml of 0.1 M solution of sulphuric acid into each tube and observe the appearance of CO$_2$ bubbles.

Explain the results and draw a conclusion.

**Examples of Krock-1 tests**

1. A patient was admitted into hospital with a diagnosis diabetes mellitus type I. In metabolic changes the decrease of oxaloacetate synthesis rate is detected. What metabolic passway is damaged as a result?
   A. Tricarboxylic acid cycle
   B. Glycolysis
   C. Cholesterol biosynthesis
   D. Glycogen mobilization
   E. Urea synthesis

2. Substrate phosphorylation is a process of phosphate residue transfer from macroergic donor substance to ADP or some other nucleoside diphosphate. What enzyme of tricarboxylic acid cycle participates in reaction of substrate phosphorylation?
   A. Succinyl thiokinase
   B. Citrate synthase
C. Succinate dehydrogenase  E. Alpha-ketoglutarate dehydrogenase complex
D. Fumarase

3. In a patient are manifested symptoms of intoxication with arsenic compounds. What metabolic process is damaged taking into account that arsen containing substances inactivate lipoic acid?
A. Oxidative decarboxylation of α-ketoglutarate
B. Fatty acids biosynthesis
C. Neutralization of superoxide anions
D. Coupling of oxidation and phosphorylation
E. Microsomal oxidation

4. Mitochondria are subcellular organelles and are present in a cytoplasm of every cell except mature red blood cells, bacteria, blue-green algae. What method is used principally for their isolation?
A. Differential centrifugation
B. Chromatography
C. Electrophoresis
D. Spectrophotometry
E. Gel-filtration

Individual independent students work
1. Anaplerotic and amphibolic role of tricarboxylic acid cycle.
2. Role of the most important metabolites (pyruvate, α-ketoglutarate, acetyl-CoA, succinyl-CoA) in the integration of metabolism.

References:


Objective: to learn general principles of enzymatic respiratory chain organization in mitochondria, distinct oxidoreductases and their functional significance in tissue respiration. To master the methods of investigation of the next oxido-reductases: phenol oxidase, aldehyde dehydrogenase and peroxidase.
**Actuality of the theme:** oxidoreductases catalyze reactions connected with transfer of electrons and protons and are in the background of macroergic compounds production. Investigation of their activity is necessary for detailed understanding of the mechanisms of tissue respiration and its changes in different functional status of the body.

**Specific aims:**
- To explain processes of biological oxidation of different substrates in the cell and reservation of released energy in a form of macroergic bonds of ATP.
- To analyze reactions of biological oxidation and their role in providement of fundamental biochemical processes in tissues.
- Malate-aspartate and glycerophosphate shuttle systems of transmembrane transfer of reduced NADH2 into mitochondria and their significance
- To explain the structural organization of electron transport chain and its macromolecular complexes.
- To interpret role of biological oxidation, tissue respiration and oxidative phosphorylation in generation of ATP in aerobic conditions.

**Theoretical questions:**
2. Pyridine dependent dehydrogenases, structure of NAD and NADP, their role in reactions of oxidation and reduction.
3. Flavine dependent dehydrogenases. Structure of FAD and FMN, their role in reactions of oxidation and reduction.
4. Cytochromes and their role in tissue respiration. Structure of their prosthetic group.

**Practical part**

*Experiment 1. Study of phenol oxidase activity.*

**Principle.** Polyphenol oxidase is an enzyme that catalyses the hydroxylation of monophenols to o-diphenols. They can also further catalyse the oxidation of o-diphenols to produce o-quinones. It is the rapid polymerisation of o-quinones to produce black, brown or red pigments (polyphenols) that is the cause of fruit browning.

\[
\begin{align*}
\text{OH} & \quad \text{OH} \\
\text{O} & \quad \text{O} \\
\text{Phenol} + \text{O}_2 & \xrightarrow{\text{Phenol oxidase}} \text{Quinone} + \text{H}_2\text{O}
\end{align*}
\]

Method is based on the phenomenon of pyrocatechol oxidation by oxygen in the presence of phenol oxidase.
Method.
I. The source of enzyme is fresh potato juice. Mince in a mortar 1 - 2 g of potato add 25 ml of distilled water, mix it and press the juice into tube. (Iron instruments must not be used because ions of iron induce the darkening of juice).
II. Add 0.5 ml of potato juice into 4 tubes. Add 0.5 ml of pyrocatechol solution to the first tube; 1-2 drops of Na$_2$S (an inhibitor of phenol oxidase) and 0.5 ml of pyrocatechol – to the second; boiled juice and 0.5 ml of pyrocatechol - to the third and 0.5 ml of water to fourth tube. Place tubes into the thermostat at 37° C for 30 min. Periodically shake the tube’s content for better aeration.

![Diagram showing the setup of tubes]

**Result:**
1. brown  2. colorless  3. colorless  4. colorless

### III. Result
In the first tube brown colour appears due to oxidation of pyrocatechol. The colours of other tubes don’t change: due to the presence of inhibitor (2 tube), denaturation of enzyme (3 tube ) and absence of substrate (4 tube).

Explain the results and draw a conclusion.

### Examples of Krock-1 tests
1. Enzymes of respiratory chain perform oxidation of substrates and transfer of reductive equivalents to oxygen with production of water molecules. Where they are located?
   A. On inner mitochondrial membrane.  
   B. On cytoplasmic membrane  
   C. In cytoplasm  
   D. On outer mitochondrial membrane  
   E. In nucleus

2. Cytochromes are components of respiratory chain in mitochondrias, which transfer electrons from ubiquinon to molecular oxygen. What part of cytochrome molecule take part in oxydative-reductive reactions?
   A. Iron atom  
   B. Vinyl residue  
   C. Pyrrole cycle  
   D. Proteinous part  
   E. Methen bridge

3. The next process occurs in suspension of mitochondria with ruptured inner membrane and provided with malate and oxygen:
   A. Transport of electrons along enzymes of respiratory chain  
   B. Phosphorylation of ADP  
   C. Decrease of pH in the external
D. Increase of pH in mitochondrial matrix

E. Oxydative phosphorylation will take place

4. The correct sequence of cytochrome carriers in respiratory chain is:
   A. Cyt b→cyt c₁→cyt c→cyt aa₃
   B. Cyt aa₃→ cyt b→cyt c→cyt c₁
   C. Cyt b→cyt c→cyt c₁→cyt aa₃
   D. Cyt b→cyt aa₃→cyt c→ cyt c
   E. Cyt aa₃→ cyt c₁→cyt c→ cyt b

**Individual independent students work**

1. Development of knowledge on biological oxidation, works of A. Bakh, V. Palladin, O.Warburg in this field.
2. Modern data about the organization and functioning of respiratory chain as a single polyenzyme complex.

**References:**


**Topic № 8. Oxidative phopshorylation, its regulation. Microsomal oxidation**

**Objective:** To learn the mechanisms of tissue respiration and oxidative phosphorylation, role of oxidoreductases, determination of their activity, evaluation and the diagnostic significance of catalase activity determination in blood.

**Actuality of the theme.** Chemiosmotic theory of oxidative phosphorylation explains molecular mechanisms of ATP production in course of biological oxidation. In some pathological processes, which are accompanied by hypoxia, it may happen incomplete reduction of oxygen molecule in a respiraqtory chain and accumulation of hydrogen peroxide, the last is neutralized by catalase. In that sense the determination of catalase activity is an important index for evaluation of antioxidative potential of human body.

**Specific aims:**

- To explain mechanisms of coupling of biological oxidation and oxidative phosphorylation – biosynthesis of ATP.
To analyze structural and functional peculiarities of respiratory chain functioning, which provides effective synthesis of ATP.

To explain the main principles of chemiosmotic theory of oxidative phosphorylation.

To analyse the action of inhibitors and uncouplers of oxidative phosphorylation of natural and artificial origin and their physiological significance.

**Theoretical questions:**

3. Inhibitors of electron transport in a respiratory chain of mitochondria.
4. Uncouplers of electron transport and oxidative phosphorylation in a respiratory chain of mitochondria.
5. Microsomal oxidation

**Practical part**

*Experiment 1. A study of peroxidase activity.*

**Principle.** Peroxidases are a large family of enzymes that typically catalyze a reaction of the form:

ROOR' + electron donor (2 e⁻) + 2H⁺ → ROH + R'O

For many of these enzymes the optimal substrate is hydrogen peroxide, but others are more active with organic hydroperoxides such as lipid peroxides.

**Method.** Add the following reagents into 3 tubes:

1. 3-4 drops of benzidine solution in acetic acid, 3-4 drops of 3 % solution of H₂O₂, 3-4 drops of horseradish extract.
2. 3-4 drops of benzidine solution, 3 - 4 drops of H₂O₂ solution, 3 - 4 drops of a boiled horseradish extract.
3. 3-4 drops of benzidine solution, peroxidase inhibitor, 3 - 4 drops of horse radish peroxidase and 3-4 drops of H₂O₂ solution.

**Place**

**Result.** In the first tube a blue color is developed due to oxidation of benzidine by H₂O₂ under catalysis of peroxidase. In the 2 and 3 tubes color is not developed.

![Result](image)

**Result:**

1. blue  2. colorless  3. colorless
Clinical and diagnostical importance. Horseradish peroxidase can be conjugated to a labeled molecule. It produces a coloured, fluorimetric, or luminescent derivative of the labeled molecule when incubated with a proper substrate, allowing it to be detected and quantified. Horseradish peroxidase is often used in conjugates (molecules that have been joined genetically or chemically) to determine the presence of a molecular target. For example, an antibody conjugated to horseradish peroxidase may be used to detect a small amount of a specific protein in a western blot. Here, the antibody provides the specificity to locate the protein of interest, and the horseradish peroxidase enzyme, in the presence of a substrate, produces a detectable signal. Horseradish peroxidase is also commonly used in techniques such as ELISA and Immunohistochemistry due to its monomeric nature and the ease with which it produces coloured products. Peroxidase, a heme-containing oxidoreductase, is a commercially important enzyme which catalyses the reductive cleavage of hydrogen peroxide by an electron donor.

Examples of Krock-1 tests

1. CO is extremely dangerous poison as it irreversibly blocks respiratory chain of enzymes. At which point is arrested electron transport in presence of CO?
   A. Cytochrome oxidase
   B. Succinate dehydrogenase
   C. Ubiquinon-cytochrome c reductase
   D. Respiratory complex III
   E. NADH₂-ubiquinon reductase

2. Some hormones are acting as uncouplers of oxidative phosphorylation. Chose from listed below hormones one which is considered as the best uncoupler;
   A. Thyroxine
   B. Norepinephrine
   C. Testosterone
   D. Insulin
   E. Cortisol

3. The production of thyroid hormones is stimulated under thyrotoxicosis. It leads to body weigh loss, tachycardia, and rise of psychic irritability. Choose the biochemical mechanism by which thyroid hormones affect the tissue bioenergetics from the listed below:
   A. Uncoupling of oxidation and oxidative phosphorylation
   B. Blockage of mitochondrial respiratory chain
   C. Activation of substrate level phosphorylation
   D. Blockage of substrate level phosphorylation
   E. Activation of oxidation and oxidative phosphorylation

4. Cyanides are extremely dangerous poisons as they irreversibly block respiratory chain of enzymes. At which point is arrested electron transport in presence of cyanides?
   A. Cytochrome oxidase
   B. Succinate dehydrogenase
   C. Ubiquinon-cytochrome c reductase
   D. Respiratory complex III enzymes
   E. NADH₂-ubiquinon reductase
Individual independent students work
1. Uncouplers of oxidative phosphorylation and regulation of thermogenesis
2. Universal significance of chemiosmotic hypothesis for living systems.

References:

The aim of the lesson: To learn fundamental principles of intracellular oxidation of glucose in anaerobic and aerobic conditions and pathways of its regulation. To interpret the role of coenzymes and enzymes in glycolytic pathway. To explain reactions of oxidative phosphorylation and ATP synthesis.

Actuality of the theme: The occurrence of uninterrupted glycolysis is very essential in skeletal muscle during strenuous exercise where oxygen supply is very limited. Glycolysis in the erythrocytes leads to lactate production, since mitochondria - the centres for aerobic oxidation - are absent. Brain, retina, skin, renal medulla and gastrointestinal tract derive most of their energy from glycolysis. Concentration of lactate in blood increases after hard muscle exercises and in some diseases. Under anaerobic conditions, 2 ATP are synthesized while, under aerobic conditions, 8 or 6 ATP are synthesized-depending on the shuttle pathway that operates.

Specific aims:
- To interpret biochemical pathways of intracellular oxidation of glucose in anaerobic conditions
- To analyze peculiarities of glycolytic reactions, which occur with involvement of ATP
- To analyze peculiarities of substrate phosphorylation and production of ATP in this way
- To interpret role of coenzymes and enzymes in glycolytic reactions
- To analyze regulatory mechanisms of glucose oxidation in anaerobic conditions
Theoretical questions:
1. Glucose as an important metabolite in carbohydrate metabolism: general scheme of sources and turnover of glucose in the organism
4. Alcohol fermentation, common and different reactions in glycolysis and fermentation.

Practical part

Experiment 1. Quantitative determination of lactic acid in blood serum after Buchner method.

The occurrence of uninterrupted glycolysis is very essential in skeletal muscle during strenuous exercise where oxygen supply is very limited. Glycolysis in the erythrocytes leads to lactate production, since mitochondria-the centres for aerobic oxidation-are absent. Brain, retina, skin, renal medulla and gastrointestinal tract derive most of their energy from glycolysis.

Principle. By heating with concentrated sulfuric acid lactic acid is converted to acetic aldehyde, which, when followed by a reaction with hydroquinone, forms a red-brown substance.

Preparation of a filtrate.
1. Pour into 2 clean tubes 6 ml of water.
2. Add to the first tube 1 ml of standard solution of lactate (test tube) and to the second one add 1 ml of blood serum (control tube).
3. For protein precipitation add to each tube 1 ml of metaphosphoric acid and after several minutes filter the solutions.
4. Add to each filtrate 1 ml of copper sulfate (10%) and 0.5g of calcium hydroxide. Mix the probes with a glass rod and filter after 5 min.

Method.
1. Add 1 ml of each filtrate into 2 clean, dry tubes,
2. Add 0.1 ml of copper sulfate, mix the solutions
3. Add 4 ml of concentrated sulfuric acid to each tube and place them into a boiling water bath for 1.5 min.
4. After cooling add 0.1 ml of alcohol solution of hydroquinone and incubate the tubes in boiling water bath for 15 min.
5. Cool the tubes and measure optical density at blue light filter.

Calculations. Lactic acid concentration is calculated according to a formula:

$$C_{lac} = \frac{C_{stand} \times A_{probe}}{A_{stand}}$$
Where, C is concentration and A is optical density. Explain the results and draw a conclusion.

Clinical diagnostic significance. Venous blood of healthy person contains 0.5 - 2.2 mmol/l of lactic acid. An increase of lactic acid content is associated with strenuous muscular effort during short time term intervals when there is a deficiency of oxygen. In this case the oxidative decarboxylation of pyruvate to acetyl-CoA does not occur and the production of the lactate takes place. Lactate can be utilized during a restoration period with a sufficient supply of oxygen. The increase production of lactic acid is also observed during epilepsy, tetanies, convulsions and hypoxia, and it is associated with cardiac and pulmonary failure, malignant tumors, liver diseases and other pathologies.

Examples of Krock-1 tests
1. An untrained person who has not been practicing physical exercises for a long time complains of a muscle pain as a result of intensive manual work. What is the probable reason of the pain syndrome?
   A. Accumulation of lactate in muscles
   B. Decreasing of lipids level in muscles
   C. Increased disintegration of muscle proteins
   D. Accumulation of creatinine in muscles
   E. Increase of ATP level in muscles

2. The high speed sprint causes a feeling of pain in skeletal muscles of untrained people that occurs due to lactate accumulation. The activation of what biochemical process is it resulting from?
   A. Glycolysis
   B. Gluconeogenesis
C. Pentose phosphate pathway  E. Glycogenesis  
D. Lipogenesis

3. A 7-year-old girl manifests obvious signs of anemia. Laboratory tests showed the deficiency of pyruvate kinase activity in erythrocytes. The disorder of what biochemical process is a major factor in the development of anemia?
A. Anaerobic glycolysis  D. Oxidative phosphorylation  
B. Deamination of amino acids  E. Breaking up of peroxides  
C. Tissue respiration

4. During consumption of cakes or sweets in mixed saliva a transient increase in lactate level takes place. Activation of what biochemical process causes this effect?
A. Anaerobic glycolysis  D. Gluconeogenesis  
B. Tissue respiration  E. Microsomal oxidation  
C. Aerobic glycolysis

Individual independent students work
1. Disorders of carbohydrate metabolism and its pharmacological correction.  

References:

Topic № 10. Aerobic oxidation of glucose. Biosynthesis of glucose – gluconeogenesis

Objective: To learn the role of pyruvate dehydrogenase multienzyme complex in aerobic metabolism of glucose. To interpret reactions of gluconeogenesis, their peculiarities and principles of their regulation.

Actuality of the theme: Metabolism of carbohydrates includes all complex process of carbohydrates transformation starting from digestion, absorption, transport
and utilization in cells up to formation of end products – CO₂ and H₂O. In aerobic conditions pyruvate, as a product of glycolysis, releases CO₂ and is transformed to acetyl-CoA, which is further oxidized in tricarboxylic acid cycle (Crebs cycle) to CO₂ and H₂O. The rate of reactions in TCA cycle depends from requirements of the cell in ATP. Regulatory reactions in TCA cycle are synthesis of citrate and oxidative decarboxylation of alpha-oxoglutarate, which are regulated by amount of ADP, succinyl-CoA and NADH₂.

**Specific aims:**

- To interpret mechanisms of monosaccharides transformation to final metabolic products and energetic effect in aerobic conditions
- To analyze structural and functional peculiarities of pyruvate dehydrogenase complex
- To analyze specific features of gluconeogenesis reactions and substrates of this process.
- To explain and interpret regulatory mechanisms of gluconeogenesis.

**Theoretical questions:**

1. Stages of aerobic oxidation of glucose.
2. Oxidative decarboxylation of pyruvic acid: structure of multienzyme pyruvate dehydrogenase complex; peculiarities of function of pyruvate dehydrogenase complex; mechanism of oxidative decarboxylation of pyruvate; role of vitamins and coenzymes in transformation of pyruvate to acetyl-CoA.
4. Metabolic pathways and substrates of gluconeogenesis, mechanisms of regulation, compartmentalization of enzymes, biological significance of the process.

**Practical part**

*Experiment 1. Quantitative determination of pyruvic acid in urine by colorimetric method.*

*Pyruvate* is converted to acetyl CoA by oxidative decarboxylation. This is an irreversible reaction, catalysed by a multi-enzyme complex, known as **pyruvate dehydrogenase complex (PDH)**, which is found only in the mitochondria. High activities of PDH are found in cardiac muscle and kidney. The enzyme PDH requires five cofactors (coenzymes), namely-TPP, lipoamide, FAD, coenzyme A and NAD+. (lipoamide contains lipoic acid linked to amino group of lysine).

**Principle.** Pyruvic acid and 2,4-dinitrophenyl hydrazine form in alkaline medium a 2,4-dinitrophenylhydrazone pyruvate (brown-red color compound). The intensity of the color is proportional to the pyruvic acid concentration and is evaluated calorimetrically.
Materials and reagents. Sample of urine, standard solution of pyruvic acid (625 mg in 100 ml of water), 0.1% solution of 2,4-dinitrophenylhydrazine (in 2 N hydrochloric acid), 12% solution of sodium hydroxide, distilled water, tubes, pipettes, colorimeter.

Method. To one tube is added 0.1 ml of tested urine, to another tube – 0.1 ml of standard pyruvate solution. To each tube is added 0.9 ml of distilled water. Thereafter is added 0.5 ml of 2,4-dinitrophenylhydrazine and tubes are leaved for 20 min in a dark place. Then to each tube is added 1 ml of 12% solution of sodium hydroxide and after 10 min the intensity of color is measured in colorimeter with a blue light filter.

Calculation:
Pyruvic acid concentration ($C_{exp}$) is calculated according to the formula:

$$C_{exp} = \frac{C_{stand} \times A_{exp} \times V}{A_{stand} \times a}$$
where:

$C_{\text{stand.}}$ – concentration of standard pyruvate
$C_{\text{exper.}}$ – concentration of pyruvate in a sample of urine
$A_{\text{exper.}}$ – optical density of tested urine
$A_{\text{stand.}}$ – optical density of standard pyruvate
$V$ – daily volume of excreted urine (1500 ml)
a – 0.1 ml of urine, taken for analysis.

Compare the obtained result with normal value. Draw the conclusion.

**Examples of Krock-1 tests**

1. **In a patient are manifested symptoms of intoxication with arsenic compounds.** What metabolic process is damaged taking into account that arsenic containing substances inactivate lipoic acid?
   - A. Oxidative decarboxylation of pyruvate
   - B. Fatty acids biosynthesis
   - C. Neutralization of superoxide anions
   - D. Coupling of oxidation and phosphorylation
   - E. Microsomal oxidation

2. **What biochemical process is stimulated in the liver and kidneys of a patient exhausted by starvation?**
   - A. Gluconeogenesis.
   - B. Synthesis of urea.
   - C. Synthesis of bilirubin.
   - D. Formation of hippuric acid.
   - E. Synthesis of uric acid.

3. **In a patient a painfulness along a great nerve trunks is observed as well as increase of pyruvate in blood. Insufficiency of what vitamin may cause these symptoms?**
   - A. Vitamin B$_1$
   - B. Vitamin C
   - C. Vitamin B$_6$
   - D. Vitamin K
   - E. Vitamin PP

4. **Lipoic acid is a cofactor of the next enzyme complex:**
   - A. Pyruvate dehydrogenase
   - B. Lactate dehydrogenase
   - C. Succinate dehydrogenase
   - D. Cytochrome oxidase
   - E. Transketolase

**References:**

Topic № 11. Breakdown and biosynthesis of glycogen. Regulation of glycogen metabolism

Objectives: To learn reactions of synthesis and breakdown of glycogen and mechanisms of humoral regulation of glycogen metabolism in liver and muscles.

Actuality of the theme: Glycogen is the storage form of glucose in animals, as is starch in plants. It is stored mostly in liver (6-8 %) and muscle (1-2 %). Due to more muscle mass, the quantity of glycogen in muscle (250 g) is about three times higher than that in the liver (75 g). Glycogen is stored as granules in the cytosol, where most of the enzymes of glycogen synthesis and breakdown are present.

Specific aims:
- To explain characteristic features of glycogen breakdown and biosynthesis.
- To analyze mechanisms of humoral regulation of glycogen metabolism in liver and muscles.
- To explain hereditary disorders of glycogen metabolism.

Theoretical questions:
1. Mechanism and peculiarities of enzymatic reactions of glycogenesis.
2. Glycogenolysis, common and different reactions with glycolysis.
3. Cascade mechanisms of ATP-dependent regulation of glycogen phosphorylase and glycogen synthase activities.
4. Peculiarities of hormonal regulation of glycogen metabolism in liver and muscles.

Practical part

Experiment 1. Iodine Test for Starch.

Principle. Starch is a polysaccharide consisting of glucose units joined together by glycosidic bonds. The chains formed during the condensation reaction are either linear or highly branched molecules.

Linear - both straight and helical - molecules of starch are referred to as Amylose.

Whereas branched molecules of starch are called Amylopectin.
The Iodine Test for Starch is used to determine the presence of starch in biological materials. The chains formed during the condensation reaction are either linear or highly branched molecules. Iodine on its own (small non-polar molecule) is insoluble in water. Therefore Potassium triiodide solution - Iodine dissolved in potassium iodide solution - is used as a reagent in the test. To be more specific, potassium iodide dissociates, and then the Iodide ion reacts reversibly with the Iodine to yield the triiodide ion. A further reaction between a triiodide ion and an iodine molecule yields the pentaiodide ion.

\[
(C_6H_{10}O_5)_n + I_2 \xrightarrow{\text{heating}} (C_6H_{10}O_5)_n I_2
\]

starch complex of starch with Iodine

Since molecular iodine is always present in solution, the bench iodine solution appears brown; the iodide and triiodide pentaiodide ions are colourless.

The triiodide and pentaiodide ions formed are linear and slip inside the helix of the amylose (form of starch).

**Reagents:** 1% solution of starch, Lugol solution (iodine dissolved in potassium iodide), tubes.

**Method.**
1. Add 1ml of the starch solution to a clean, dry test tube.
2. Add about 5 drops of Lugol solution to the test tube.
3. Note any colour changes. Write a conclusion.

**Experiment 2. Detection of glycogen in the liver.**

**Principle.** A positive test for glycogen is a brown-blue color. A negative test is the brown-yellow color of the test reagent. Glycogen, as well as starch, forms a colored compound with iodine (starch forms blue, glycogen - a red-brown compound). It is thought that starch and glycogen form helical coils. Iodine atoms can then fit into the helices to form a starch-iodine or glycogen-iodine complex. Starch in the form of amylose and amylopectin has less branches than glycogen. This means that the helices of starch are longer than glycogen, therefore binding more iodine atoms. The result is that the color produced by a starch-iodine complex is more intense than that obtained with a glycogen-iodine complex.

**Reagents.** Fresh or frozen liver tissue, Lugol solution (iodine dissolved in potassium iodide), 1% solution of acetic acid, porcelain mortar, water bath, paper filters.
Generation of filtrate
1. Put 0.5 g of liver tissue into a tube, add 4 ml of distilled water and boil it (2-3 min in order to inactivate enzymes).
2. Transfer liver into the mortar and grind it.
3. Transfer the obtained homogenate into the tube, add 1 ml of distilled water and boil the solution on a water bath (20 min). Add 5-10 droplets of acetic acid solution for protein precipitation.

Method.
1. Take a clean, dry tube. Put 1 ml of filtrate.
2. Add 2-3 droplets of Lugol solution.
3. Note any colour changes. (In presence of glycogen a red-violet color is observed). Compare the color with a color obtained in the previous experiment. Explain the results.

Clinical diagnostic significance. Glycogen is a polysaccharide, which serves as a main reserve of carbohydrates in the body. It is stored mainly in liver and muscles. Normal blood level – 16.2 – 38.7 mg/l. The prime function of liver glycogen is to maintain the blood glucose levels, particularly between meals. Liver glycogen stores increase in a well-fed state which are depleted during fasting. Muscle glycogen serves as a fuel reserve for the supply of ATP during muscle contraction.

The metabolic defects concerned with the glycogen synthesis and degradation are collectively referred to as glycogen storage diseases. These disorders are due to defects in the enzymes which may be either generalized (affecting all tissues) or tissue-specific. The inherited disorders are characterized by deposition of normal or abnormal type of glycogen in one or more tissues. Increase in blood glycogen concentration is observed in some infection diseases, which are accompanied with leukocytosis, diabetes mellitus, and malignancies.

Examples of Krock-1 tests
1. In a weak apathic infant an enlarged liver was detected, which in investigation of biopsy pieces showed an excess of glycogen. Blood glucose concentration is under the normal value. What may be the cause of this disease?
   A. Lowered activity of glycogen phosphorylase in a liver
   B. Lowered activity of glycogen synthase
   C. Lowered activity of glucose 6-phosphate isomerase
   D. Lowered activity of glucokinase
   E. Deficiency of gene responsible for synthesis of glucose 1-phosphate uridyl transferase

2. During biochemical investigation of blood in a patient was detected hypoglycemia in fasting condition. Investigation of liver biopptates revealed the failure of glycogen synthesis. What enzyme deficiency may cause such status?
   A. Glycogen synthase
   B. Phosphorylase
   C. Aldolase
   D. Fructose bis-phosphatase
   E. Pyruvate carboxylase
3. In an infant with point mutations in genes the absence of glucose 6-phosphatase, hypoglycemia and hepatomegaly were revealed. What disease is characterized by these symptoms?
A. Gierke disease  D. Cori disease
B. Adison disease  E. Mac Ardle disease
C. Parkinson disease

4. In a patient a lowering in ability to physical load was revealed, while in skeletal muscles the glycogen content was increased. The decrease in activity of what enzyme may cause this condition?
A. Glycogen phosphorylase  D. Glycogen synthase
B. Phosphofructokinase  E. Glucose 6-phosphatase
C. Glucose 6-phosphate dehydrogenase

**Individual independent students work**

1. Principles of regulation of glycogen biosynthesis and breakdown.
2. Hereditary disorders of synthesis and breakdown of glycogen and glycoconjugates.

**References:**

**Topic № 12. Studies of mechanisms of metabolic and hormonal regulation of carbohydrate metabolism. Diabetes mellitus.**

**Objectives:** To interpret the role of hormones in regulation and maintenance of constant blood glucose level. To learn the peculiarities of changes in metabolism of carbohydrates, lipids and proteins in diabetes mellitus. To interpret metabolic pathways of fructose and galactose in human body. To learn the sequence of enzymatic reactions in Pentose Phosphate Pathway (PPP).
**Actuality of the theme:** Diabetes mellitus is a clinical condition characterized by increased blood glucose level (hyperglycemia) due to insufficient or inefficient insulin. In other words, insulin is either not produced in sufficient quantity or inefficient in its action on the target tissues. As a consequence, the blood glucose level is elevated which spills over into urine in diabetes mellitus. Determination of blood glucose level in clinical laboratory investigations is of great importance in diagnostics of diabetes mellitus and many other diseases and disorders.

**Specific aims:**
- To explain the sequence of reactions in PPP and significance of this process
- To analyze metabolic pathways of fructose and galactose transformations in human body.
- To analyze the principal sources and metabolic pathways of utilization of blood glucose
- To explain the role of hormones in maintenance of constant glucose level in blood
- To explain disorders in metabolism of carbohydrates in diabetes mellitus.

**Theoretical questions:**
1. Pentose phosphate pathway (PPP) of glucose utilization:
   - scheme of reactions in oxidative and nonoxidative stages of PPP;
   - enzymes and coenzymes of PPP reactions;
   - biological significance of PPP;
   - disorders of PPP in red blood cells;
   - enzymopathias of glucose-6-phosphate dehydrogenase.
3. Biochemical processes which provides the constant blood glucose level. Role of different pathways of carbohydrate metabolism in regulation of blood glucose level.
4. Hormonal regulation of carbohydrate metabolism:
   - Insulin, its structure, mechanism of action, role in carbohydrate metabolism.
   - Adrenalin and glucagone, mechanism of their regulatory effects on carbohydrate metabolism.
   - Glucocorticoids, their effect on carbohydrate metabolism.
6. Insulin dependent and noninsulin dependent forms of diabetes mellitus.
7. Characterization of metabolic disorders in diabetes mellitus

**Practical part**

*Experiment 1. Estimation of blood glucose by o-toluidine method.*

**Principle.** In this method of glucose determination, a primary aromatic amine, o-toluidine, reacts in hot glacial acetic acid with the terminal aldehyde group of glucose to produce a blue-green color. The absorbance of this product is measured...
photometrically and glucose concentration can be calculated. The absorbance in 600 - 700 nm region is directly proportional to the glucose concentration.

Reagents and materials. Blood sample, 3% solution of trichloroacetic acid (TCA), o-toluidine reagent, glucose standard (4 mM/L, i.e. 720 mg of glucose dissolved in 1 L of water), distilled water, tubes, pipettes, micropipette 0.1 ml, centrifuge, centrifuge tubes, electrocolorimeter, water bath.

Preparation of supernatant. Add 0.9 ml of trichloroacetic acid into two centrifuge tubes. Into test tube add 0.1 ml of blood specimen, into control tube – 0.1 ml of standard glucose solution. Centrifuge the tubes at 3000 rpm for 10 min. Separate test and control supernatants for further investigations.

Method.
1. Take two clean, dry tubes. Into the first tube add 0.5 ml of control supernatant, into second – 0.5 ml of tested supernatant.
2. Add 4.5 ml of o-toluidine reagent to each tube.
3. Place the tubes on a boiling water bath for 8 min.
4. Cool tubes and measure optical density (A) in a colorimeter at wavelength 630 nm (red filter).

Calculation. The concentration of glucose is calculated using the formula:

\[ C_{\text{test}} = \frac{C_{\text{stand}} \times A_{\text{test}}}{A_{\text{stand}}} \]

where:
\( C_{\text{test}} \) – concentration of glucose in blood, mmoles/L;
\( C_{\text{stand}} \) – concentration of glucose in standard solution
\( A_{\text{test}} \) – optical density of test probe
\( A_{\text{test}} \) – optical density of standard glucose probe.

Compare the obtained result with normal value, draw the conclusion.

Clinical diagnostic significance. The fasting blood glucose level in normal individuals is \( 3.3-5.5 \text{ mmol/l} \) and it is very efficiently maintained at this level. When the blood glucose concentration falls, the symptoms of hypoglycemia appear. The
manifestations include headache, anxiety, confusion, sweating, slurred speech, seizures and coma, and, if not corrected, death. All these symptoms are directly and indirectly related to the deprivation of glucose supply to the central nervous system (particularly the brain) due to a fall in blood glucose level.

Elevation of blood glucose concentration is the hallmark of uncontrolled diabetes. **Hyperglycemia** is primarily due to reduced glucose uptake by tissues and its increased production via gluconeogenesis and glycogenolysis. When the blood glucose level goes beyond the renal threshold, glucose is excreted into urine (**glycosuria**).

Examples of Krock-1 tests

1. A 46-year-old woman complains of dryness in the oral cavity, thirst, frequent urination, general weakness. Biochemical research of the patient’s blood showed hyperglycemia and hyperketonemia. Sugar and ketone bodies are revealed in the urine. Diffuse changes in myocardium are marked on the electrocardiogram. Make an assumptive diagnosis of the illness.
   A. Diabetes insipidus.  
   B. Alimentary hyperglycemia.  
   C. Acute pancreatitis.  
   D. Diabetes mellitus.  
   E. Ischemic cardiomyopathy.

2. A patient was admitted to a hospital in comatous state. The accompanying mates explained that the patient loss his consciousness during the training on the last stage of marathon distance. What coma type can be recognized?
   A. Hypoglycemic  
   B. Hyperglycemic  
   C. Hypovolemic  
   D. Hypothyroid  
   E. Hepatic

3. A patient addressed to physician with complaints on permanent thirst. In laboratory investigation it was revealed hyperglycemia, polyuria and increased content of 17-ketosteroids in urine. What disease is the most probable?
   A. Steroid diabetes  
   B. Insulin dependent diabetes mellitus
C. Addison disease
D. Glycogenosis of the 1 type
E. Myxoedema

4. Essential fructosuria is a hereditary disease, connected with disorders of fructose metabolism. The symptoms of lesions of liver and kidneys are manifested. This disease is caused by insufficiency of enzyme, which catalyze transformation of fructose to the next compound:

A. Fructoso-1-phosphate
B. Fructoso-6-phosphate
C. Fructoso-1,6-bisphosphate
D. Glucoso-6-phosphate
E. Glyceraldehyde phosphate

References:


Objective: to learn the processes of biosynthesis of triacylglycerols and the main pathways of intracellular metabolism of lipids. To learn the methods of determination of phospholipids concentration and to interpret the obtained results.

Actuality of the theme: The knowledge of main pathways of intracellular metabolism of lipids under normal conditions and in pathology are necessary for medical students in further studies of general pathology, pharmacology and related clinical disciplines for correct interpretation of results of laboratory investigations and recognition of metabolic disorders in distinct cases.

Specific aims:
- To interpret biochemical function of simple and complex lipids in organism: their involvement in formation of structure and function of biological membranes, reserve and energetic significance, the role as precursors in biosynthesis of biologically active compounds of lipid nature.
- To explain the principal pathways of intracellular lipid metabolism.
- To explain enzymatic reactions of catabolism and biosynthesis of triacylglycerols.
➢ To analyze the main pathways of lipid metabolism in human body in normal conditions and in pathology.
➢ To explain hormonal regulation of lipid metabolism.

**Theoretical questions:**
2. Involvement of lipids in formation of structure and function of biological membranes. Liposomes. Application of liposomes in medical practice.
5. Biosynthesis of triacylglycerols, the significance of phosphatidic acid as a precursor.

**Practical part**

*Experiment 1. Quantitative determination of phospholipids in blood serum.*

**Principle:**
Phospholipids are precipitated with trichloroacetic acid together with plasma proteins. After mineralization of sediment the quantity of phosphorus is determined colorimetrically and content of phospholipids is calculated.

**Method.**
I. **Precipitation of phospholipids with trichloroacetic acid (Attention!!!! This part of experiment was previously done by technitions):**
1. Take a clean dry centrifuge tubes.
2. Add 0.2 ml of blood serum, 2 ml of distilled water and 3 ml of 10 % solution of trichloroacetic acid.
3. Mix the content of the tube and after 2-3 min. centrifuge it during 5 min at 3000 rpm.
4. Carefully remove the supernatant. Sediment contains lipoproteins. Add to the sediment 1 ml of 56 % HClO₄ put the tube into a bath with a boiling water for 30 min. The final mineralizeat must be colorless.

II. **Color reaction:**
1. Take 2 clean dry tubes.
2. To the 1ˢᵗ (experimental) tube add 5 ml of mineralizate, 1 ml of ammonium molybdate and 1 ml of 1% solution of ascorbic acid.
3. To the 2ⁿᵈ tube (standard) add 5 ml of standard phosphorus solution (0,05 g/l) 1 ml of ammonium molybdate and 1 ml of 1% solution of ascorbic acid.
4. Mix the content of both tubes and incubate 15-20 min at room temperature.

III. **Measurement of optical desity:**
After 5 min measure the optical density on a photoelectrocolorimeter with a red light filter.

**IV. Calculation:** Use the following formula:

\[
[T\text{otal phospholipids in serum}] = \frac{A_{\text{exp}} \times 0.05}{A_{\text{st}} \times 0.2} \times 25 \text{ g/l}
\]

where \(A_{\text{exp}}\) – optical density of the experimental probe;
\(A_{\text{st}}\) – optical density of the standard probe;
0.05 – concentration of phosphorus in standard (mg/ml)
0.2 – volume of analyzed serum
25 – coefficient for calculation of total phospholipids content.

**Clinical and diagnostic significance.** The determination of phospholipid content in blood has an important diagnostic significance. The concentration of total phospholipids in blood serum of healthy adult is 1.5 – 3.6 g/l. The increase of phospholipids level in blood serum (hyperphospholipidemia) is observed in heavy form of diabetes mellitus, nephrosis, obturative jaundice. Decrease of phospholipid level (hypophospholipidemia) may be observed in atherosclerosis, anemias, fever, alimentary distrophias, liver diseases.

**Examples of Krock-1 tests**

1. **In patients suffering from diabetes mellitus an increase in a content of non-esterified fatty acids (NEFA) in blood is observed. It may be caused by:**
   A. Increase in activity of triacylglycerol lipase
   B. Stimulation of ketone bodies utilization
   C. Activation of synthesis of apolipoproteins A\(_1\), A\(_2\), A\(_3\)
   D. Decrease in activity of phosphatidylcholine-cholesterol-acyltransferase in blood plasma
   E. Accumulation in cytosol of palmitoyl-CoA

2. **The essence of lipolysis, that is the mobilization of fatty acids from neutral fats depots, is an enzymatic process of hydrolysis of triacylglycerols to fatty acids and glycerol. Fatty acids that release during this process enter blood circulation and are transported as the components of:**
   A. Serum albumins
   B. Globulins
   C. HDL
   D. LDL
   E. Chylomicrons

3. **Which one of the following statements about the absorption of lipids from the intestine is correct?**
   A. Dietary triacylglycerol is partially hydrolyzed and absorbed as free fatty acids and monoacyl glycerol
   B. Release of fatty acids from triacylglycerol in the intestine is inhibited by bile salts
   C. Dietary triacylglycerol must be completely hydrolyzed to tree fatty acids and glycerol before absorption
   D. Fatty acids that contain ten carbons or less are absorbed and enter the circulation primarily via the lymphatic system
E. Formation of chylomicrons does not require protein synthesis in the intestinal mucosa.

4. After consumption of lipids in the body than begins their digestion and absorption in intestines. What products of lipid hydrolysis are absorbed in the intestine?

A. Monoacylglycerol, fatty acids  
B. Amino acids  
C. Polypeptides  
D. Monosacharides  
E. Lipoproteins

References:

**Topic № 14. Metabolism of complex lipids and ketone bodies**

**Objective:** to learn the processes of biosynthesis of phospholipids and sphingolipids. To know metabolic pathways of ketone bodies under normal conditions and in pathology and to determine their amount in urine.

**Actuality of the theme:** Determination of ketone bodies concentration in blood and in urine has important significance in diagnostics of several pathological processes, including diabetes mellitus and starvation.

**Specific aims:**
- To explain enzymatic reactions of catabolism and biosynthesis of phospholipids.
- To interpret enzymatic reactions of synthesis of sphingolipids.
- To analyze the main pathways of lipid metabolism in human body in normal conditions and in pathology.
- To analyze the metabolism of ketone bodies.
- To explain the mechanism of excessive accumulation of ketone bodies in diabetes mellitus and in starvation.

**Theoretical questions:**
1. Biosynthesis of phospholipids from phosphatidic acid.
3. Metabolism of ketone bodies.
• enzymatic reactions of ketone bodies biosynthesis (ketogenesis);
• reactions of ketone bodies utilization (ketolysis), energetic effect;
• metabolism of ketone bodies in pathology. Mechanism of excessive accumulation of ketone bodies in diabetes mellitus and in starvation.

**Practical part**

**Experiment 1. Qualitative reaction on acetone and acetoacetic acid (Lange reaction).**

**Principle.** It is based on a property of acetone and acetoacetic acid to produce compounds with sodium nitroprussid of red color in the basic pH of medium.

\[
CH_3 – CO – CH_3 + Na_2 [Fe (CN)_5 NO] + 2 NaOH \rightarrow \\
\rightarrow Na_4 [Fe (CN)_5 NO = CHCOCH_3] + 2 H_2O.
\]

Under the action of acetic acid it turns to a violet product:

\[
Na_4 [Fe (CN)_5 NO = CHCOCH_3] + CH_3COOH \rightarrow \\
Na_3 [Fe (CN)_5 NOCH_3COCH_3] + CH_3COONa.
\]

compound after an overlay of conc. ammonia solution over the mixture of test sample, containing sodium nitroprussid and acetic acid. The rate of color ring formation between two layers of liquids depends from acetone concentration. It is assumed, that appearance of the ring after 3-4 min corresponds to the acetone concentration 0.0085 g/l (8,5 mg/l).

**Method.**

1. Take two clean dry tubes.
2. Add 0.5 ml of urine of a healthy person to the first tube (blank).
3. Add 0.5 ml of urine of a patient with diabetes mellitus to the second one (test sample).
4. Add 0.5 ml of 10 % NaOH and 5-7 drops of fresh 10% sodium nitroprussid solution into both tubes. Color in the 2\textsuperscript{nd} tube turns red.
5. Add 5-7 drops of acetic acid into both tubes. Color in the 2\textsuperscript{nd} tube turns violet.

Explain the results, draw a conclusion.

**Clinical and diagnostic significance.** Ketone bodies include the following substances – acetoacetic acid, β-hydroxybutyric acid, acetone. Biosynthesis of ketone bodies (ketogenesis) takes place in liver from intermediates of fatty acid oxidation, namely, from acetyl-CoA.

Acetoacetic acid, produced in liver, is transported to body tissues (brain, muscles, kidneys, heart etc.), where it serves as an energetic material. In healthy person the content of ketone bodies in blood is in ranges of 13-185 µmoles/l (1.5-20 mg/l). With urine is excreted 20-40 mg of ketone bodies daily, which are preferentially acetoacetic and β-hydroxybutyric acids. Acetone appears under pathological conditions.

The increase of ketone bodies concentration in blood (ketonemia) and in urine (ketonuria) is observed in diabetes mellitus, deficiency of sugar in nutrition, overproduction of hormones, antagonistic to insulin (corticosteroids, thyroxine, hormones of adenohypophysis). The decrease of ketone bodies content has no clinical value. In early
childhood prolong disorders of digestion (toxicosis, dysenteria) may lead to ketonemia due to the permanent starvation and exaggeration.

**Examples of Krock-1 tests**

1. **In a patient suffering from diabetes mellitus in blood was detected acetone. Note the process of its production in the body.**
   - A. By condensation of two molecules of acetyl-CoA
   - B. In course of α-oxidation of fatty acids
   - C. In course of β-oxidation of fatty acids
   - D. In course of γ-oxidation of fatty acids
   - E. In tricarboxylic acid cycle.

2. **Fabry’s disease (one of sphingolipidoses) is an autosomal recessive disease. Major symptoms of this disease: skin rash, kidney failure, pain in lower extremities. It is caused by a deficiency of:**
   - A. α-Galactosidase A
   - B. Hexosaminidase A and B
   - C. Gm1 Gangliosidase
   - D. Galactocerebrosidase
   - E. Ceraminase

3. **In diabetes mellitus and starvation there is an increase of ketone bodies content in blood, which are utilized as energetic material by tissues. Note the substance which is used in ketone bodies synthesis.**
   - A. Acetyl-CoA
   - B. Citrate
   - C. Succinyl-CoA
   - D. α–Ketoglutarate
   - E. Malate

4. **Patients who suffer from severe diabetes and don't receive insulin have metabolic acidosis. This is caused by increased concentration of the following metabolites:**
   - A. Ketone bodies
   - B. Fatty acids
   - C. Unsaturated fatty acids
   - D. Triacylglycerols
   - E. Cholesterol

**Individual independent students work**


**References:**


**Topic № 15. β –Oxidation and biosynthesis of fatty acids. Studies on metabolism of fatty acids and ketone bodies.**

**The aim of the lesson:** To learn reactions of biosynthesis and oxidation of fatty acids.

**Actuality of the theme:** Oxidation of lipids, respectively fatty acids, as well as ketone bodies metabolism are important constituents of energetic metabolism in sense of providing tissues and cells with ATP.

**Specific aims:**
- To study reactions β -oxidation of long chain fatty acids.
- To interpret biosynthesis of long chain fatty acids and regulation of biosynthetic process on the level of acetyl-CoA-carboxylase and fatty acid synthetase.

**Theoretical questions:**

1. β –Oxidation of long chain fatty acids:
   - localization of the process of β-oxidation of fatty acids;
   - activation of fatty acids, the role of carnitine in transport of fatty acids into mitochondria;
   - the sequence of enzymatic reactions in β –oxidation of fatty acids;
   - energetic balance of β –oxidation of fatty acids;

2. Mechanism of glycerol oxidation, bioenergetics of this process.

3. Biosynthesis of long chain fatty acids:
   - localization of biosynthesis of long chain fatty acids;
   - metabolic sources for biosynthesis of fatty acids;
   - stages in synthesis of saturated fatty acids;
   - characteristic of the synthetase of long chain fatty acids, the significance of acyl transporting protein and biotin;
   - sources of NADPH₂ for biosynthesis of long chain fatty acids;
   - the sequence of enzymatic reactions in biosynthesis of long chain fatty acids
   - regulation of biosynthetic process on level of acetyl-CoA-carboxylase and fatty acid synthetase;
   - elongation of carbon chain of fatty acids;

**Practical part - Clinical cases and situational tasks**

**Examples of Krock-1 tests**

1. Aerobic oxidation of substrates is typical of a cardiac muscle. Which of the following is the major oxidation substrate of a cardiac muscle?
   A. Fatty acids.  
   B. Triacylglycerols.  
   C. Glycerol.  
   D. Glucose.  
   E. Amino acids.
2. Carnitine is recommended to a sportsman as a preparation that increases physical activity and improves achievements. What biochemical process is mostly activated under the action of carnitine?

A. Transport of fatty acids into mitochondria.
B. Ketone bodies synthesis.
C. Lipids synthesis.
D. Tissue respiration.
E. Steroid hormones synthesis.

3. A 1-year-old child was brought to a clinic with signs of muscle weakness. Through the inspection, the deficiency of carnitine in the muscles was determined. The biochemical mechanism of the development of this pathology consists in the disorder of the process of:

A. Transport of fatty acids into mitochondria.
B. Regulation of the level of Ca^{2+} in mitochondria.
C. Substrate level of phosphorylation.
D. Utilization of lactate.
E. Synthesis of actin and myosin.

4. Lipids are obvious energetic material for the body. What is the main pathway of fatty acids metabolism in mitochondria?

A. β-Oxidation
B. Decarboxylation
C. α-Oxidation
D. Reduction
E. γ-Oxidation

References:


Objective: To learn the pathways of cholesterol biosynthesis and biotransformation. To study main disorders of lipid metabolism such as atherosclerosis and obesity.

Actuality of the theme: Disorders in cholesterol biotransformation processes cause several diseases, such as atherosclerosis, obesity et al. In this connection the investigation of lipid metabolism indexes is obvious for diagnostics and treatment of different diseases.
Specific aims:
- To interpret stages of cholesterol biosynthesis.
- To explain regulation of cholesterol production in human body.
- To analyze pathways of cholesterol biotransformation: esterification, synthesis of bile acids, steroid hormones, vitamin D₃, excretion of cholesterol from the body.
- To interpret pathology of lipid metabolism: atherosclerosis, diabetes mellitus, obesity, steatorrhea.

Theoretical questions:
1. Biosynthesis of cholesterol in human body.
   - localization of the process and its significance;
   - stages of cholesterol biosynthesis;
   - enzymatic reactions of biosynthesis of mevalonic acid;
   - regulation of cholesterol synthesis.
3. Pathways of cholesterol biotransformation (esterification, production of bile acids and steroid hormones, synthesis of vitamin D₃, excretion from the body).
5. Lipoproteins: structure, classification, characteristics of apolipoproteins.
7. Disorders of plasma lipoproteins (classification of hyperlipoproteinemias, characteristics of hypolipoproteinemias.
8. Fatty liver (steatosis), lipotropic factors.
9. Pathological processes which leads to the development of obesity.

Practical part

Experiment 1. Qualitative reaction on bile acids (Petenkoffer reaction)

Principle. The reaction is based on formation of colored products after condensation of bile acids with hydroxymethylfurfural. The last is produced under the action of sulfuric acid upon fructose, which in turn is formed from sucrose due to its hydrolysis with sulfuric acid.

Method.
1. Take a clean dry tube.
3. Add ~5 droplets of a fresh 20% sucrose solution and mix it.
4. Carefully overlay 1 ml of conc. sulfuric acid without mixing of two fluids.
5. Observe the precipitation of bile acids in the borderline between two fluids (appearance of violet color ring).
6. Mix it well and observe the appearance of red-violet color.

Clinical and diagnostic significance. The bile acids and their salts are detergents that emulsify fats in the gut during digestion. They are synthesized from cholesterol in the liver by a series of reactions. The bile acids are produced in liver, about 10-15 g daily. Bile acids include cholic, deoxycholic, chenodeoxycholic, lithocholic et al., which are excreted
in bile in free state or conjugated with glycine or taurine. Bile acids emulsify lipids, activate lipase, are involved in absorption of fatty acids, form cholefinic complexes, stabilize cholesterol. Deficiency of bile acids in intestines may be caused with liver diseases (occlusive jaundice, hepatitis, cirrhosis), disorders in gallbladder or bile ducts (cholelithiasis, tumors of bile ducts). In coprologic investigations decrease or complete absence of bile pigments in feces are observed, as well as high content of soaps, especially calcium soaps.

**Examples of Krock-1 tests**

1. **A patient suffers from arterial hypertension due to atherosclerotic injury of blood vessels. The consumption of what dietary lipid needs to be limited?**
   A. Cholesterol.  
   B. Oleic acid.  
   C. Lecithine.  
   D. Monooleateglycerol.  
   E. Phosphatidylserine.

2. **Fats of phospholipids is disordered due to fat infiltration of the liver. Indicate which of the presented substances can enhance the process of methylation during phospholipids synthesis?**
   A. Methionine  
   B. Ascorbic acid  
   C. Glucose  
   D. Glycerin  
   E. Citrate

3. **In a patient after investigation it was detected an increased content of low density lipoproteins in blood serum. What disease can be expected in this patient?**
   A. Atherosclerosis  
   B. Pneumonia  
   C. Gastritis  
   D. Acute pancreatitis  
   E. Kidney disease

4. **A child 5 years old suffers from transient abdominal pains. Blood serum is turbid in fasting conditions. Cholesterol content – 4,3 mmoles/l, total lipids – 18 g/l. For precisement of diagnosis electrophoresis of blood lipoproteins is administered. What classes of lipoproteins are expected to be increased?**
   A. Chylomicrons  
   B. HDL  
   C. IDL  
   D. LDL  
   E. VLDL

**Individual independent students work**

1. Lipid peroxidation, its role under normal conditions and in pathology.

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