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DE MEDICINĂ ȘI FARMACIE
„VICTOR BABEȘ" DIN TIMIȘOARA

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CLINICAL BIOCHEMISTRY

1st year, 2nd semester

Laboratory notes

GHIDURI ȘI ÎNDRUMĂTOARE DE LABORATOR

Editura "Victor Babeș"
Timișoara, 2026

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ISBN 978-606-786-584-4

ACKNOWLEDGEMENTS

We would like to express our sincere gratitude to the laboratory staff for their invaluable support, dedication, and professionalism in the preparation of the practical sessions.

Mirela Antonescu
Paula Ciordaș
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Introduction. Digestion and absorption of carbohydrates

In a typical adult diet, carbohydrates represent the primary source of metabolic fuel, with an average daily intake of about 250-350 g, accounting for roughly 45-65% of total energy intake as recommended by nutritional guidelines. The dietary carbohydrates are distributed as follows: 50% starch, 40% sucrose, 5-10% lactose and small number of other saccharides Regardless of their complexity, only monosaccharides (*glucose, fructose, and galactose*) can be absorbed across the intestinal epithelium. Thus, digestion of oligo- and polysaccharides is required to convert them into these absorbable units. The hydrolysis of complex saccharides is facilitated by amylases, a group of hydrolases that cleave glycosidic bonds within polysaccharide chains. There are two types of amylases: *salivary* (secreted by salivary glands) and *pancreatic* (secreted by the exocrine part of the pancreas).

Carbohydrate digestion begins in the oral cavity, where **salivary α-amylase** (also known as ptyalin), secreted by the salivary glands, initiates the breakdown of α-1,4 glycosidic bonds in starch. Due to the limited duration of food exposure in the mouth, this phase contributes modestly to overall carbohydrate digestion, yielding *maltose, maltotriose, and α-limit dextrins* as intermediate products. Once swallowed, the acidic pH of the stomach rapidly inactivates salivary amylase, halting further polysaccharide digestion in the gastric phase.

Upon entering the duodenum, pancreatic secretions containing **pancreatic α-amylase** continue the digestion of starch and glycogen. Operating optimally at neutral to slightly alkaline pH, pancreatic amylase hydrolyzes internal α-1,4 glycosidic bonds, generating a mixture of disaccharides and oligosaccharides that require further enzymatic processing.

Final digestion of carbohydrates occurs at the brush border of enterocytes in the jejunum, where a variety of disaccharidases are expressed on the apical surface of intestinal epithelial cells. Here, **maltase** cleaves *maltose* and maltotriose into glucose, while **isomaltase (α-1,6-glucosidase)** specifically targets the α-1,6 bonds present in *α-limit dextrins*, completing the breakdown of branched polysaccharides. **Sucrase (invertase)** hydrolyzes *sucrose* into glucose and fructose, and **lactase (β-galactosidase)** breaks down *lactose* into glucose and galactose. A low beta-galactosidase activity can lead to milk intolerance. The products of this terminal digestion, glucose, galactose, and fructose, are then absorbed by enterocytes through specific transport mechanisms.

Absorption and transport

Due to their hydrophilic nature and relatively large molecular size, monosaccharides cannot diffuse freely across the hydrophobic lipid bilayer of cell membranes. Instead, monosaccharides transport requires the assistance of specialized membrane proteins, which mediate their movement into and out of cells based on physiological needs and tissue-specific demands. The transport of glucose occurs across various epithelial and cellular barriers in distinct physiological contexts:

- From the intestinal lumen into enterocytes (nutrient absorption in the small intestine)
- From enterocytes into the bloodstream (systemic distribution via the portal circulation)
- From the bloodstream into insulin-dependent and -independent tissues
- From the renal tubular lumen into proximal tubular cells (reabsorption of filtered glucose)

Two major families of transport proteins mediate glucose movement across membranes: the SGLT (Sodium-Glucose Linked Transporters) and the GLUT (Glucose Transporters). These differ in their mechanism, directionality, and tissue distribution:

- **Sodium-glucose linked transporters** (SGLTs): Also called sodium-dependent glucose cotransporters, SGLTs transport glucose against its concentration gradient. SGLTs are secondary active transporters that use the energy stored in the electrochemical gradient of Na^+ ions. The sodium gradient that drives this transport is maintained by Na^+/K^+-ATPase on the basolateral membrane, which pumps Na^+ out of the cell. These transporters are important in epithelial tissues where glucose must be absorbed or reclaimed from low-concentration environments:
    - *Intestinal absorption*: In enterocytes of the small intestine, SGLT1 facilitates the active uptake of glucose and galactose from the intestinal lumen.
    - *Renal reabsorption*: In the proximal convoluted tubule of the nephron, SGLT2 and to a lesser extent SGLT1 reclaim filtered glucose from the primary urine.
- **Glucose specific transporters** (GLUTs): GLUT proteins mediate facilitated diffusion of glucose along its concentration gradient. They are energy-independent and vary in their tissue distribution, kinetics (K_M values), and regulation, particularly by insulin:
    - *GLUT1* (K_M = 1-2 mM, high affinity): Mainly expressed in RBCs, brain, fetal tissues, placenta. Operates efficiently at fasting glucose levels; ensures basal uptake in brain and RBCs.
    - *GLUT2* (K_M = 15-20 mM, low affinity): Mainly expressed in liver, pancreatic β-cells, kidney, intestine. Functions as a glucose sensor; active mainly after meals when blood glucose is high. In pancreatic β-cells, it contributes to the regulation of glucose-stimulated insulin secretion (GSIS).
    - *GLUT3* (K_M = 1 mM, very high affinity): Mainly expressed in brain and placenta. Provides constant glucose supply to neurons even under hypoglycemia.
    - *GLUT4* (K_M = 5 mM, intermediate affinity): Mainly expressed in skeletal muscle, cardiac muscle, and adipose tissue. Matches normal blood glucose; activity regulated by insulin, not substrate levels (insulin-responder).
    - *GLUT5* (K_M = 5-10 mM, moderate affinity): Mainly expressed in small intestine, testis, kidney, and adipose tissue. Is specific for fructose, not glucose; active after ingestion of fructose-rich foods.

These differences have important physiological consequences. As such, the normal glucose concentration in blood (5 mM/L) is equal to the K_M of GLUT4 and 4 times lower than the K_M of GLUT2. Therefore, the glucose transport will occur at maximum capacity in cells having GLUT4, being independent of blood glucose levels and only depending on the number of transporter molecules. On the contrary, in cells with GLUT2, the transport will be dependent on the blood glucose levels, occurring at higher rates under high glycemic conditions. In type 2 diabetes mellitus, impaired GLUT4 translocation leads to reduced glucose uptake by skeletal muscle and adipose tissue, contributing to insulin resistance and hyperglycemia.

Glucose determination in biological fluids

Accurate measurement of blood glucose is a cornerstone in the diagnosis and management of metabolic disorders, particularly diabetes mellitus. Several laboratory methods are available for quantifying glucose concentrations in biological fluids, with varying degrees of specificity, sensitivity, and practicality.

- **Chemical methods**: While relatively simple and inexpensive, these methods are less specific, as it may react with other reducing sugars such as galactose or fructose.
    - *Ortho-toluidine colorimetric method*: glucose reacts with ortho-toluidine in an acidic medium, forming a green-colored Schiff base.

- **Enzymatic methods**: These assays are currently the most specific and widely used techniques for glucose analysis. The methods rely on enzyme-substrate reactions that yield quantifiable products proportional to glucose concentration:
    - *Hexokinase method*: Considered the gold standard in clinical laboratories, this method involves the phosphorylation of glucose by hexokinase to form glucose-6-phosphate, which is subsequently oxidized by glucose-6-phosphate dehydrogenase, producing NADPH.
    - *Glucose oxidase method*: Glucose is oxidized by glucose oxidase to gluconolactone (and then gluconic acid) and hydrogen peroxide. The latter reacts with a chromogen in the presence of peroxidase, forming a colored compound measured by absorbance.
    - *Glucose dehydrogenase method*: Less commonly used, this involves the oxidation of glucose to gluconolactone by glucose dehydrogenase, with concurrent reduction of NAD^+ or a dye.

Preanalytical considerations:

Reliable glucose measurement requires meticulous sample collection, handling, and storage to prevent false decreases due to glycolysis. Post-collection, blood cells and bacterial contaminants can metabolize glucose, leading to a decline of approximately 10 mg/dL per hour at room temperature. To prevent this degradation:

- Immediate separation of serum or plasma from blood cells is essential. This can be achieved by centrifugation shortly after collection.
- If processing is delayed, the specimen should be stored at 4°C (refrigeration), where glucose remains stable for up to 8 hours.
- When neither immediate processing nor refrigeration is possible, blood should be collected in tubes containing a glycolysis inhibitor, such as sodium fluoride (NaF) at 1 mg/mL. NaF inhibits enolase, a key glycolytic enzyme, thereby preserving glucose levels.

EXPERIMENTAL PART

Determination of glucose using the glucose oxidase method

Principle

The glucose oxidase method is a highly specific and reliable enzymatic assay for the quantitative determination of glucose in serum or plasma. It is based on a two-step oxidative enzymatic reaction involving glucose oxidase and peroxidase. In the first reaction, glucose oxidase catalyzes the oxidation of D-glucose to D-gluconolactone, simultaneously reducing molecular oxygen to hydrogen peroxide (H_2O_2). In the second reaction, the peroxidase enzyme uses the generated hydrogen peroxide to oxidize 4-aminoantipyrine in the presence of phenol, forming a red-colored quinonimine dye. The intensity of the red color, which is proportional to the glucose concentration in the sample, is measured spectrophotometrically at 546 nm.

$$\alpha - D - glucose \xrightarrow{glucomutase} \beta - D - glucose$$

$$\beta - D - glucose + O_2 + H_2O \xrightarrow{glucose\ oxidase} D - gluconolactone + H_2O_2$$

$$2H_2O_2 + phenol + 4 - aminoantipyrine \xrightarrow{peroxidase} quinoneimine\ dye\ (red) + 4H_2O$$

Reagents

1. Working reagent:
   - phosphate buffer, 0.5 mol/l, pH 7.50
   - phenol, 7.5 mmol/l
   - glucose oxidase, 12000 U/l
   - peroxidase, 660 U/l
   - 4-aminoantipyrine, 0.4 mmol/l

   The reagent is stable 3 months at 2-8^{0}C or 3 weeks at 20-25^{0}C.
2. Glucose standard, 100 mg%

Procedure

Prepare 3 test tubes labeled: Sample, Standard, and Blank. Pipette the reagents as follows:

Reagents, µl	**Sample**	**Standard**	**Blank**
Working reagent	1000	1000	1000
Diluted standard	-	100	-
Diluted serum	100	-	-
Distilled water	-	-	100

Mix thoroughly and incubate at room temperature for 30 minutes. Measure the absorbance of both the sample and standard at λ = 546 nm, using the blank to zero the spectrophotometer. The red color is stable for up to 60 minutes, and the method demonstrates linearity for glucose concentrations up to 400 mg/dL.

Calculation

The glucose concentration in the serum sample is calculated as:

$$glucose(mg/dL) = \frac{A_{sample}}{A_{standard}} \cdot c_{standard} = \frac{A_{sample}}{A_{standard}} \cdot 100$$

Error sources

The accuracy of the glucose oxidase method can be compromised by interfering substances:

- Hemoglobin > 4 g/L, bilirubin > 200 mg/L, creatinine > 100 mg/L, and galactose > 1 g/L may produce positive or negative bias. EDTA (2 g/L) can inhibit enzymatic activity.
- Deproteinization with trichloroacetic acid or perchloric acid may lead to erythrocyte lysis and release of glutathione, which reacts with hydrogen peroxide and reduces color formation, yielding falsely low results. Preferred deproteinizing agent: Uranyl acetate, in isotonic solution, preserves RBC integrity and prevents glutathione release.
- Vitamin C (ascorbic acid) at concentrations > 5 mg% (usually from IV administration) may consume hydrogen peroxide, also resulting in falsely decreased readings. Oral vitamin C generally does not cause interference.

Normal values

- Serum, plasma: 75 - 115 mg/100 ml (4,16 - 6,38 mmol/l)

Clinical significance

- **Hyperglycemia:**
  - Physiological:
    - Post-prandial hyperglycemia: After ingestion of carbohydrate-rich meals, blood glucose levels may up to up to ~140 mg/dL.
    - Stress-induced hyperglycemia: Triggered by acute physical or emotional stress (e.g., surgery, trauma, pain, anxiety). Catecholamines and cortisol are released via activation of the sympathetic nervous system and hypothalamic-pituitary-adrenal axis, resulting in stimulation of gluconeogenesis and reduction in peripheral glucose uptake.
    - In puberty, the rise in growth hormone and sex steroids can transiently raise fasting glucose levels. In pregnancy, placental hormones such as human placental lactogen (hPL), estrogen, and cortisol exert anti-insulin effects, increasing insulin resistance and promoting mild postprandial hyperglycemia. This is physiological unless excessive (as in gestational diabetes).
  - Pathological:
    - Diabetes mellitus: insulin resistance / insulin deficiency.
    - Endocrine disorders: Cushing's syndrome (glucocorticoid excess), acromegaly (growth hormone excess), pheochromocytoma (catecholamine-secreting tumor).
    - Pancreatic disorders: chronic pancreatitis, pancreatectomy, pancreatic carcinoma.
    - Pharmacological causes: glucocorticoids, thiazide diuretics, β-agonists, antipsychotics, and protease inhibitors.
- **Hypoglycemia:** insulin overdose, insulinoma (insulin-secreting tumors), high alcohol intake, post-gastrectomy syndromes, or reactive hypoglycemia.

Urine glucose determination

Under normal physiological conditions, the kidneys efficiently reabsorb filtered glucose in the proximal tubules, such that only trace amounts (typically less than 350-500 mg/24 hours) appear in the urine. This minimal urinary glucose elimination is not detectable by routine dipstick methods and is considered within normal limits. The analytical methods employed for urinary glucose quantification are like those used in serum or plasma.

The renal threshold for glucose refers to the plasma glucose concentration at which the reabsorptive capacity of the renal tubules becomes saturated, and glucose begins to appear in the urine. In most individuals, this threshold lies around 180 mg/dL (10 mmol/L). When blood glucose levels exceed this value, the transporters responsible for reabsorption (primarily SGLT2 in the proximal tubule) become overwhelmed, resulting in glucosuria. However, individual variation in

renal threshold exists. Some healthy individuals may excrete glucose in the urine even at normal or near-normal glycemic levels, a condition referred to as benign renal glucosuria, due to a lower-than-average renal threshold.

- **Physiological glycosuria**: These occurrences are transient, clinically benign, and resolve without intervention
  - Postprandial glycosuria: Following the ingestion of a large quantity of carbohydrates, particularly simple sugars, transient mild hyperglycemia may result in glucose excretion.
  - Stress-induced glycosuria: During episodes of acute emotional or physical stress, catecholamine-induced hyperglycemia may transiently surpass the renal threshold, leading to temporary glucosuria.
- **Pathological glycosuria**: Persistent glucosuria is most associated with diabetes mellitus, particularly when blood glucose concentrations are chronically elevated. In such cases, urinary glucose serves as a qualitative or semi-quantitative reflection of poor glycemic control, although it is considered a less precise marker compared to blood glucose or HbA1c. Other causes of pathological glucosuria include:
  - Renal tubular defects (e.g., Fanconi syndrome)
  - Pregnancy (due to increased glomerular filtration and altered tubular handling)
  - Drug-induced effects (e.g., SGLT2 inhibitors)

Oral Glucose Tolerance Test (OGTT)

The oral glucose tolerance test (also called glucose tolerance test) is a diagnostic procedure used to evaluate glucose homeostasis and assess the body's ability to clear glucose from the bloodstream following an oral glucose load. It is primarily utilized in the diagnosis of diabetes mellitus, impaired glucose tolerance (IGT), insulin resistance, and occasionally in the evaluation of reactive hypoglycemia, acromegaly, and rare disorders of carbohydrate metabolism.

Indications:

- Individuals with fasting blood glucose values between **110-126 mg/dL**, suggesting impaired fasting glucose.
- Patients with risk factors such as obesity, positive family history of diabetes, metabolic syndrome, or gestational diabetes risk.
- Suspected hypoglycemia: a prolonged version of the test (up to 5 hours) may be used to investigate postprandial hypoglycemia.
- Evaluation of β-cell function and insulin sensitivity in certain endocrine conditions (e.g., acromegaly).

Patient Preparation: The reliability of OGTT is highly dependent on proper patient preparation. The following protocols must be followed:

- For at least 3 days prior to the test, the patient must consume a carbohydrate-rich diet **(≥150 g/day or 1.75 g/kg body weight**) to ensure adequate insulin reserve.
- Avoid smoking, physical exertion, and alcohol consumption for 24 hours.
- Discontinue/postpone medications affecting glucose metabolism (e.g., glucocorticoids, β-blockers, diuretics, biguanides, antihypertensives), unless clinically contraindicated.
- Fast for 8-14 hours before the test.

Preliminary Testing Before OGTT: Prior to administering the oral glucose load, the following parameters must be evaluated:

- **Urinary ketone bodies**: The presence of ketones suggests a state of carbohydrate deficiency (fasting, recent illness), invalidating test results. OGTT should be postponed.

- **Urinary glucose**: If positive, indicates pre-existing hyperglycemia or reduced renal threshold; OGTT is not performed.
- **Fasting blood glucose**: If fasting glucose exceeds 126 mg/dL, diabetes is already established, and the OGTT is unnecessary.

Test Procedure: In the most performed version of the test, a standard dose of glucose is ingested by mouth and blood levels are checked two hours later:

- Administer oral glucose at a dose of **1 g/kg body weight**, up to a maximum of 75 g, dissolved in approximately 400 mL of water. The solution should be ingested within 5 minutes. Because it is strongly hypertonic, nausea or vomiting may occur; combining glucose with maltose may improve tolerance. The patient should remain seated throughout the test, as gastric emptying is delayed in the supine position.
- Blood samples are collected at the following time points: Time 0 (fasting), 60 minutes after ingestion, 120 minutes after ingestion. Optional: If hypoglycemia is being investigated, additional samples are collected at 180, 240, and 300 minutes.
- A urine sample is collected at the end of the test to assess for glycosuria.

Many variations of the OGTT have been devised over the years for various purposes, with different standard doses of glucose, different routes of administration, different intervals and durations of sampling, and various substances measured in addition to blood glucose.

For adults <45 years of age with normal BMI and no major comorbidities, the results should be interpreted as follows:

	Time 0 (à jeun)	**After 60 minutes**	**After 120 minutes**
Normal glycemic curve	75-115 mg/100 ml	<140 mg/100 ml	75-115 mg/100 ml
Decreased glucose tolerance	115-126 mg/100ml	140-200 mg/100ml	126-200 mg/100ml
Diabetes mellitus	>126 mg/100 ml	>200 mg/100 ml	>200 mg/100 ml

Contraindications to OGTT: The test should not be performed in the following clinical conditions:

- Known diabetes mellitus (established diagnosis)
- Current weight-loss diets
- Gastrointestinal disorders impairing glucose absorption (celiac disease, gastric resection)
- Acute febrile illnesses
- Liver dysfunction (altered glucose metabolism)

Questions

1. Fill in the blanks: The digestion of carbohydrates begins in the _____ where _____ amylase (ptyalin) hydrolyzes internal α-1,4 glycosidic bonds in starch. Due to the short residence time and acidic environment, salivary amylase becomes inactive in the _____. In the small intestine, _____ amylase continues the hydrolysis of α-1,4 bonds, producing _____, maltotriose, and α-limit dextrins. _____ cleaves maltose into two glucose units, while _____ (α-1,6-glucosidase) breaks the α-1,6 bonds in branched oligosaccharides. Sucrose is hydrolyzed by _____ into glucose and _____, whereas lactose is hydrolyzed by _____ into glucose and _____.

2. Match each transporter to its location and function:

SGLT1	Transports fructose in the small intestine via facilitated diffusion
SGLT2	Active co-transport of glucose and sodium in the renal proximal tubule
GLUT2	Insulin-dependent glucose uptake in muscle and adipose tissue
GLUT4	Facilitates glucose and galactose absorption from intestinal lumen
GLUT5	Low-affinity glucose transporter in hepatocytes and pancreatic β-cell

3. A patient recently received IV vitamin C. You perform a glucose oxidase assay and get an unexpectedly low result. How can you explain this?

4. List the main physiological and pathological causes of hyperglycemia.

5. Why might glucose appear in urine even when blood glucose is below 180 mg/dL in certain individuals?

6. Sketch or describe the expected glycemic curve for a diabetic vs. non-diabetic patient after an oral glucose load (0, 60, 120 min).

7. Why should OGTT not be performed in a patient with current fever or gastrointestinal disease?

8. A 30-year-old patient with a family history of diabetes undergoes OGTT: Fasting = 120 mg/dL, 60 min = 168 mg/dL, 120 min = 182 mg/dL. How would you interpret the results? Justify based on reference ranges.

Lab Notes

Use this space for handwritten notes during the practical session.

L2. CARBOHYDRATE METABOLISM. GLYCOLYSIS. EMBDEN-MEYERHOF PATHWAY. PENTOSE PHOSPHATE PATHWAY. BREWER TEST

Glycolysis. Embden-Meyerhof pathway

Glycolysis is the central metabolic pathway by which glucose is converted into pyruvate, serving as a primary source of energy in human cells. It is a universal, cytoplasmic process that operates in all cell types and can proceed under both aerobic and anaerobic conditions. The classical sequence of reactions that define glycolysis is referred to as the **Embden-Meyerhof-Parnas (EMP) pathway**, named after the scientists who elucidated it. This pathway involves ten enzymatically catalyzed steps that collectively convert one molecule of glucose (six-carbon) into two molecules of pyruvate (three-carbon), while simultaneously generating a modest yield of energy: 2 molecules of ATP and 2 molecules of NADH per glucose molecule.

Under **aerobic conditions**, pyruvate, the end-product of glycolysis, enters the mitochondria where it is converted to acetyl-CoA by the pyruvate dehydrogenase complex. Acetyl-CoA then enters the citric acid cycle (Krebs cycle), leading to complete oxidation into CO_2 and H_2O, with high ATP yield through oxidative phosphorylation. The NADH generated during glycolysis is re-oxidized to NAD^+ in the mitochondrial electron transport chain, ensuring the continuation of glycolysis by maintaining the redox balance. Full aerobic oxidation of glucose via the TCA cycle yields ~30-32 ATP in total.

In **anaerobic conditions**, such as in exercising skeletal muscle or tissues with limited oxygen supply (e.g., ischemic tissues), oxidative phosphorylation is impeded. To sustain ATP production via glycolysis under these conditions, cells regenerate NAD^+ by reducing pyruvate to lactate, a reaction catalyzed by lactate dehydrogenase (LDH). This conversion allows glycolysis to continue producing ATP in the absence of oxygen, although at a much lower efficiency compared to aerobic metabolism. The net ATP yield per glucose molecule in anaerobic glycolysis is 2.

While anaerobic glycolysis supports energy production during acute energy demands, the accumulation of **lactate** can contribute to muscle fatigue and acidosis. But lactate is not merely a waste product. It can be exported from tissues into the bloodstream and taken up by the liver, where it enters the Cori cycle, being converted back into glucose via gluconeogenesis. This recycling of lactate helps maintain glucose homeostasis during intense exercise or fasting. In pathological states such as sepsis, shock, or hypoxia, excessive lactate production may lead to lactic acidosis, an important diagnostic and prognostic marker in clinical settings.

Pentose phosphate pathway

The pentose phosphate pathway (PPP), also referred to as the phosphogluconate pathway or the hexose monophosphate shunt, is a **non-energetic metabolic pathway**. This cytosolic metabolic route operates in parallel with glycolysis and represents an alternative oxidative pathway for glucose-6-phosphate catabolism. Although glycolysis and PPP share glucose-6-phosphate as a common intermediate, the two pathways diverge significantly in function, products, and regulation. Unlike glycolysis, which utilizes NAD^+ and yields ATP, the PPP employs $NADP^+$ as the electron acceptor and does not produce ATP. A hallmark feature of the PPP is the generation of CO_2, a product absent from the glycolytic pathway.

The primary functions of the PPP are:

- The generation of **NADPH**, a reducing equivalent for anabolic (biosynthetic) reactions, regeneration of glutathione and generation of superoxide anion.
- The production of **ribose-5-phosphate (R5P)**, essential for the synthesis of purine and pyrimidine-based nucleotides.

Tissue distribution

The PPP is ubiquitously present in all cells but exhibits elevated activity in tissues with significant requirements for NADPH or nucleotide precursors. NADPH produced via the PPP serves for:

- Reductive biosynthesis: In general, enzymes functioning in reductive biosynthesis, such as those involved in fatty acid and steroid hormone synthesis, require the $NADP^+$/NADPH coenzyme pair, in contrast to oxidative catabolic enzymes that rely on NAD^+/NADH. Consequently, tissues with high anabolic activity, including the liver, adipose tissue, adrenal cortex, testes, and lactating mammary glands, express elevated levels of PPP enzymes. Notably, up to 30% of hepatic glucose oxidation is channeled through this pathway.
- Detoxification of reactive oxygen species (ROS): by regenerating reduced glutathione via the NADPH-dependent enzyme glutathione reductase.
- Support of immune defense: particularly in neutrophils and macrophages, where NADPH fuels the NADPH oxidase complex, generating superoxide anion (O_2^-) in the respiratory burst that destroys engulfed pathogens.

Tissue	**Principal Function of PPP**
Liver	Fatty acid and cholesterol synthesis
Adrenal cortex	Steroid hormone synthesis
Testes and ovaries	Steroidogenesis
Mammary gland (lactating)	Fatty acid synthesis
Adipose tissue	Fatty acid synthesis
Red blood cells	Maintenance of reduced glutathione
Neutrophils and macrophages	NADPH-dependent respiratory burst

The PPP can also function as an anabolic pathway that utilizes the six carbons of glucose to generate five carbon sugars, particularly R5P that is required for purine and pyrimidine nucleotide biosynthesis. Rapidly dividing cells, including hematopoietic precursors and neoplastic cells, require substantial amounts of R5P and NADPH for DNA synthesis and redox balance, thus showing increased PPP activity.

PPP in erythrocytes

The principal metabolic pathways active in erythrocytes are anaerobic glycolysis, the pentose phosphate pathway (PPP), and the 2,3-bisphosphoglycerate (2,3-BPG) shunt, each fulfilling essential cellular functions. Glycolysis serves as the sole source of ATP in red blood cells, which lack mitochondria, thereby providing the energy required for maintaining membrane ion gradients via ATP-dependent pumps. In addition, glycolysis yields NADH, which is necessary for the enzymatic reduction of methemoglobin (oxidized hemoglobin, Fe^{3+}) back to its functional ferrous form (Fe^{2+}).

The PPP holds particular significance in erythrocytes, where it serves as the sole source of NADPH, essential for maintaining redox homeostasis through the regeneration of *glutathione*. Reduced glutathione detoxifies ROS, particularly hydrogen peroxide (H_2O_2), through the action of glutathione peroxidase, which converts H_2O_2 into water. Inadequate NADPH production compromises the regeneration of reduced glutathione, resulting in oxidative damage to membrane lipids (via lipid peroxidation) and to hemoglobin, whose sulfhydryl groups undergo oxidation leading to the formation of denatured, cross-linked aggregates (Heinz bodies). The ensuing membrane instability predisposes to hemolysis.

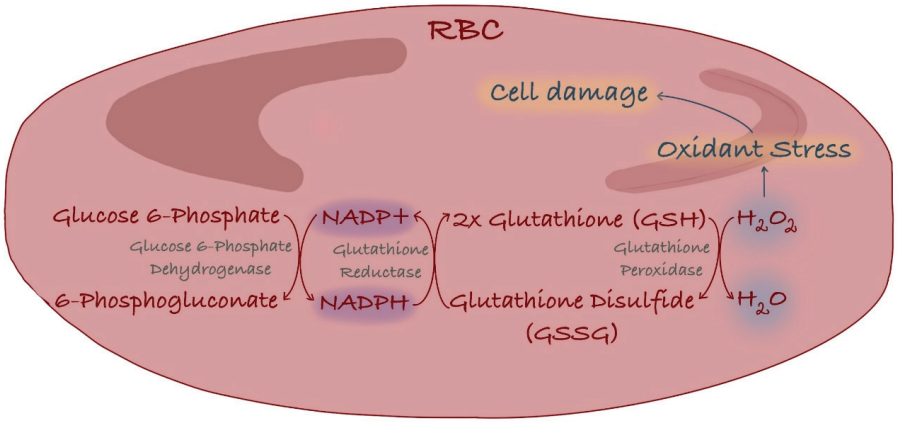


Figure 1. NADPH synthesis in erythrocytes

Given that the PPP is the exclusive source of NADPH in erythrocytes, any enzymatic deficiency, particularly of glucose-6-phosphate dehydrogenase (G6PD), significantly compromises cell viability under oxidative stress. G6PD deficiency, the most common enzymopathy worldwide, manifests clinically as *hemolytic anemia*, which may be precipitated by infections, ingestion of oxidant drugs (e.g., primaquine, sulfonamides), or fava beans (favism). Interestingly, G6PD deficiency also confers partial resistance to *Plasmodium falciparum*, the parasite responsible for the most severe form of malaria. The proposed mechanism is that the structurally compromised erythrocytes in G6PD-deficient individuals fail to provide a stable environment for parasite replication, thus impeding completion of the intraerythrocytic cycle.

PPP in leukocytes

Neutrophils and macrophages, the primary phagocytic cells of the innate immune system, exhibit the highest levels of PPP enzyme activity. Here, NADPH serves a critical role in host defense by fueling the NADPH oxidase complex, a membrane-bound enzyme system that catalyzes the one-electron reduction of molecular oxygen (O_2) to form superoxide anion ($O_2^{\bullet -}$).

The superoxide generated is further converted into a cascade of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and hypochlorous acid (HOCl), which exert potent microbicidal effects. This rapid production of ROS occurs during a heightened metabolic state known as the oxidative burst or respiratory burst, which is triggered upon phagocytosis of pathogens or exposure to inflammatory stimuli. The oxidative burst not only contributes to the destruction of engulfed microorganisms but also plays a role in signaling and inflammation.

The indispensable function of NADPH in this process underscores the PPP's immunological importance, particularly in innate immune surveillance and microbial clearance. Deficiencies in NADPH production, such as those resulting from G6PD deficiency or mutations in components of the NADPH oxidase complex (e.g., in chronic granulomatous disease), can severely impair ROS generation and increase susceptibility to recurrent infections.

The pentose phosphate pathway (PPP) is divided into two functionally distinct phases:

- In the **oxidative phase**, glucose-6-phosphate undergoes sequential dehydrogenation and decarboxylation reactions, catalyzed by enzymes such as *glucose-6-phosphate dehydrogenase* and *6-phosphogluconate dehydrogenase*, resulting in the production of ribulose-5-phosphate, CO_2, and two molecules of NADPH per glucose-6-phosphate molecule oxidized. This phase is irreversible and primarily functions to generate NADPH.
- The **non-oxidative phase** comprises a series of reversible sugar-phosphate interconversion reactions that rearrange the five-carbon sugar phosphates produced in the oxidative phase into ribose-5-phosphate and other glycolytic intermediates, such as glyceraldehyde-3-phosphate and fructose-6-phosphate. Ribose-5-phosphate serves as a precursor for the synthesis of nucleotides and nucleic acids, while erythrose-4-phosphate, another product of this phase, is essential for the biosynthesis of aromatic amino acids.

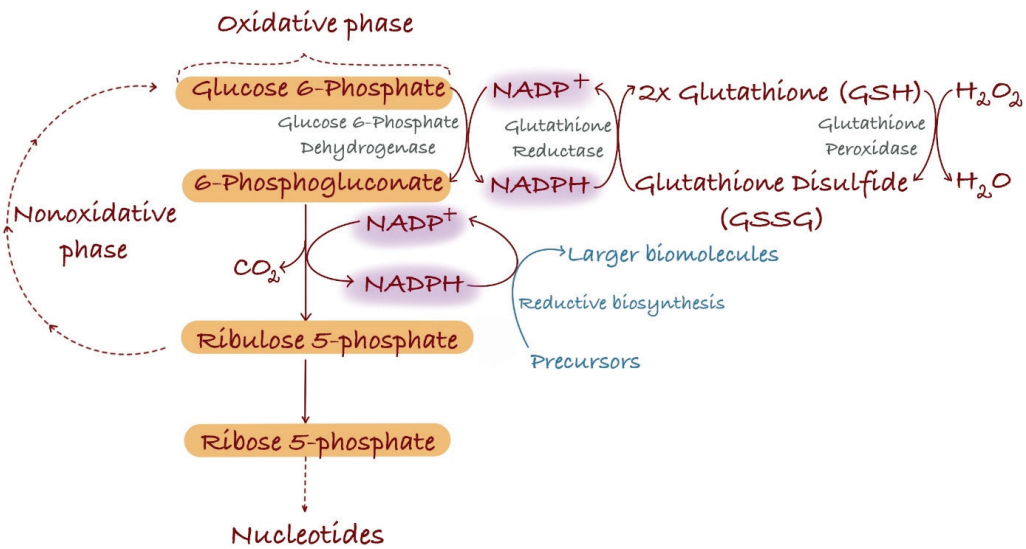


Figure 2. The oxidative and non-oxidative phases of PPP

Regulation

The principal regulatory enzyme of the PPP is glucose-6-phosphate dehydrogenase, which catalyzes the initial and rate-limiting step of the oxidative phase. Its activity is tightly controlled by the intracellular NADPH/$NADP^+$ ratio. $NADP^+$ acts as a positive allosteric effector, stimulating G6PD activity, whereas NADPH exerts feedback inhibition, thereby suppressing further NADPH production when cellular levels are sufficient. Under physiological conditions, particularly in hepatocytes, the NADPH:$NADP^+$ ratio is approximately 100:1, maintaining a highly reducing intracellular environment favorable for anabolic processes. Additionally, acetyl-CoA may serve as a metabolic signal to inhibit the pathway under certain conditions, reflecting crosstalk between carbohydrate and lipid metabolism.

EXPERIMENTAL PART

Brewer test

Principle

The Brewer test is a qualitative *in vitro* assay designed to evaluate the functional integrity of the PPP in erythrocytes by assessing the cells' capacity to reduce methemoglobin back to functional hemoglobin. This reduction process is dependent on the generation of NADPH via the PPP, primarily through the activity of glucose-6-phosphate dehydrogenase. In this test, hemoglobin within red blood cells is oxidized by sodium nitrite to methemoglobin, a form that cannot bind oxygen. The transformation is normally reversible in the presence of methylene blue, which acts as an artificial electron acceptor and is itself reduced by NADPH. Restoration of hemoglobin thus indicates a functioning PPP. Conversely, the persistence of methemoglobin suggests an enzymatic deficiency, most commonly a defect in G6PD, impairing NADPH production and, consequently, methemoglobin reduction.

Reagents

1. sodium nitrite 0.18m in 0.28M glucose
2. methylene blue 0.0004M in 0.9% NaCl
3. heparin 50 UI / 1 ml blood

Procedure

- Pipette reagents into three test tubes as follows:

Reagent	Tube 1 (control)	Tube 2 (control)	Tube 3 (Test sample)
Heparinized blood	2.00 ml	2.00 ml	2.00 ml
Sodium nitrite	-	0.100 ml	0.100 ml
Methylene blue	-	-	0.100 ml

Mix the solutions in each test tube and incubate the mixture for 3 hours at 37^0C.

Interpretation of results

- Tube 1 (bright red): Contains only blood; is a reference for normal hemoglobin coloration.
- Tube 2 (brown): Contains sodium nitrite; hemoglobin is oxidized to methemoglobin, resulting in a brown coloration.
- Tube 3: Contains sodium nitrite and methylene blue; the outcome is:
    - Red coloration (like T1): Indicates normal NADPH production via the pentose phosphate pathway-methemoglobin is reduced back to hemoglobin.
    - Brown coloration (like T2): Suggests a deficiency in PPP enzymatic activity (G6PD deficiency), leading to low NADPH production and persistent methemoglobin.

Clinical significance

This test is especially valuable in the screening of G6PD deficiency, a common enzymopathy with global prevalence, particularly in populations of Mediterranean, African, or Asian descent. Individuals with G6PD deficiency are at increased risk for oxidative hemolysis in response to specific drugs (e.g., primaquine, sulfonamides), infections, or ingestion of fava beans (favism). A positive Brewer test (failure to reduce methemoglobin) supports the diagnosis and guides patient management and preventive strategies.

Questions

1. Fill in the blanks: The oxidative phase of the PPP begins with the conversion of glucose-6-phosphate into _____, catalyzed by the enzyme _____, and produces one molecule of _____. _____ is then converted into _____ by the enzyme 6-phosphogluconate dehydrogenase, releasing one molecule of _____ and generating another molecule of _____.

2. Which of the following are major roles of the pentose phosphate pathway?
   A. Production of ATP
   B. Generation of NADPH
   C. Synthesis of ribose-5-phosphate
   D. Support of oxidative phosphorylation
   E. Provision of intermediates for nucleotide biosynthesis

3. Which of the following are direct consequences of glucose-6-phosphate dehydrogenase deficiency?
   A. Reduced NADPH generation
   B. Increased risk of hemolytic anemia
   C. Enhanced fatty acid synthesis
   D. Accumulation of hydrogen peroxide in erythrocytes
   E. Resistance to *Plasmodium falciparum* infection

4. What are the differences between aerobic and anaerobic glycolysis?

5. Name the tissues that have intense pentose phosphate pathway activity.

6. When is Brewer test indicated?

7. Explain importance of the pentose phosphate pathway for RBCs.

Lab Notes

Use this space for handwritten notes during the practical session.

L3. CARBOHYDRATE METABOLISM. REGULATION OF GLYCEMIA. DETERMINATION OF GLYCATED HEMOGLOBIN

Carbohydrates (glucids, saccharides) are the main source of metabolic energy for human cells. Among these, glucose play a central role, as the unique source of energy for the central nervous system and erythrocytes. The daily glucose requirement of the human body is approximately 250 grams of glucose per day. This requirement is met through three sources:

- **Dietary intake**: Glucose is absorbed in the small intestine following the enzymatic digestion of dietary carbohydrates polysaccharides (starch, glycogen) and disaccharides (sucrose, lactose, maltose).
- **Glycogenolysis**: The degradation of *hepatic* glycogen maintains plasma glucose concentrations during fasting states. *Skeletal muscle* glycogen, while abundant, serves local energy needs and does not contribute directly to circulating glucose due to the absence of glucose-6-phosphatase in myocytes.
- **Gluconeogenesis**: The *hepatic* (and to a lesser extent, *renal*) synthesis of glucose from non-carbohydrate precursors such as lactate, glycerol, and glucogenic amino acids ensures glucose availability during prolonged fasting or metabolic stress (insulin deficiency).

Glucose is a polar, hydrophilic molecule and therefore cannot diffuse passively across the lipid bilayer of the cell membrane. Instead, GLUT proteins ensure the passive transport (facilitated diffusion) of glucose down its concentration gradient, without the expenditure of ATP. The expression, tissue distribution, and regulation of GLUT isoforms determine the efficiency and control of glucose uptake in various organs. Certain GLUT transporters are regulated by insulin, while others function independently of insulin:

- **Insulin-Dependent Tissues**: skeletal muscle, adipose tissue, and myocardium express GLUT4, whose translocation to the plasma membrane is insulin-dependent. These tissues have limited glucose uptake in the absence of insulin.
- **Insulin-Independent Tissues**: liver (GLUT2), brain (GLUT1, GLUT3), erythrocytes, renal tubular cells, Langerhans islets, and intestinal mucosa take up glucose independently of insulin.

Regulation of glycemia

Maintaining glycemia within the physiological range is essential for metabolic homeostasis and the optimal function of glucose-dependent tissues such as the central nervous system and erythrocytes. Tight regulation is achieved through an integrated system involving a set of hormones with opposing actions:

- **Hypoglycemic hormones**: *insulin*. Secreted by pancreatic beta-cells, insulin lowers blood glucose by stimulating glucose uptake into insulin-dependent tissues (muscle, adipose), enhancing glycogenesis, lipogenesis, and protein synthesis and inhibiting gluconeogenesis, glycogenolysis, and lipolysis.
- **Hyperglycemic hormones**: *glucagon*, *adrenaline* (epinephrine), *glucocorticoids* (cortisol), *thyroxine* (T4)*, growth hormone* (somatotropin - STH). These hormones oppose the action of insulin, especially during fasting, stress, or high energy demand.

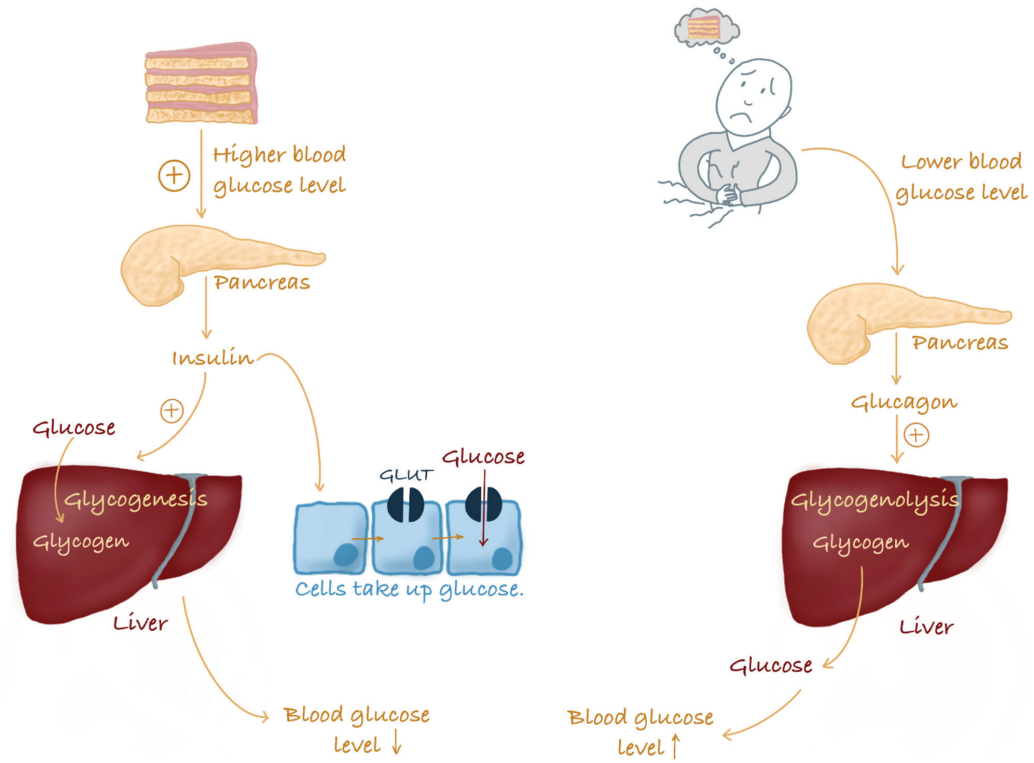


Figure 1. Early postprandial vs late postprandial regulation of glycemia

Glycemia is not static and varies based on feeding-fasting cycles. The regulatory mechanisms differ between the early postprandial and late postprandial (fasting) phases:

- **Early postprandial** (0-2 hours after eating): Glycemia is increased => insulin secretion is initiated to lower blood glucose:
  - *Glucose utilization as an energy source*: Insulin stimulates GLUT4 translocation in muscle and adipose tissue for glucose uptake.
  - *Glycogenesis/glycogen synthesis*: In both liver and muscle, insulin promotes the conversion of glucose to glycogen. Liver glycogen is a buffer for blood glucose; muscle glycogen is reserved for local use.
  - *Lipogenesis*: In adipose tissue and liver, excess glucose is diverted toward fatty acid synthesis. These are then esterified into triacylglycerols (TAGs) for storage.

- **Late postprandial** (> 4 hours after eating): Glycemia is decreased => insulin secretion is reduced, and hyperglycemic hormones contribute to elevation of blood glucose:
  - *Glycogenolysis*: Hepatic glycogen is broken down to glucose under glucagon stimulation. Muscle glycogen, although abundant, lacks glucose-6-phosphatase, and thus cannot export free glucose into circulation; instead, it serves as a local energy source.

- *Gluconeogenesis*: *De novo* synthesis of glucose in the liver (and kidneys during prolonged fasting) is stimulated using lactate (from pyruvate in Cori cycle), glycerol (from lipolysis), and glucogenic amino acids (from proteolysis).
- *Lipolysis and Ketogenesis*: Adipose tissue, under the influence of epinephrine and cortisol, breaks down triglycerides to free fatty acids and glycerol. The liver oxidizes fatty acids to generate ketone bodies (β-hydroxybutyrate, acetoacetate), serving as alternative fuels for peripheral tissues and for the brain during prolonged fasting or starvation.

Diabetes Mellitus

The most common and clinically significant disorder of carbohydrate metabolism is diabetes mellitus (DM), characterized by chronic hyperglycemia due to either absolute insulin deficiency (as in type 1 diabetes mellitus, T1DM), or relative insulin resistance and eventual secretory failure (as in type 2 diabetes mellitus, T2DM). Key metabolic features of diabetes mellitus include:

- **Hyperglycemia**: Fasting plasma glucose exceeds 126 mg/dL or random glucose exceeds 200 mg/dL.
- **Glycosuria**: When glycemia surpasses the renal threshold (~180 mg/dL), filtered glucose exceeds resorptive capacity of the proximal tubule. This results in glucose being excreted in the urine, often accompanied by polyuria, polydipsia, and electrolyte imbalance.
- **Enhanced catabolic pathways in insulin-dependent tissues**: Lipolysis is stimulated in adipose tissue, releasing free fatty acids into the circulation. Proteolysis is upregulated in muscle tissue to supply amino acids for gluconeogenesis. Gluconeogenesis increases, particularly in the liver, contributing further to hyperglycemia.
- **Ketoacidosis**: Increased lipolysis leads to elevated levels of free fatty acids, which are converted into acetyl-CoA in the liver. When acetyl-CoA exceeds the capacity of the TCA cycle (due to low oxaloacetate levels), it is diverted into the production of ketone bodies.

Several diagnostic tests are employed in clinical practice to assess glucose homeostasis, evaluate diabetes, and monitor glycemic control:

- the fasting and two-hour postprandial blood glucose
- the oral glucose tolerance test
- the glycated hemoglobin (HbA1c)

Glycated hemoglobin (HbA1c)

The determination of HbA1c plays a pivotal role in the assessment and long-term monitoring of glucose metabolism. Unlike single-point glucose measurements, HbA1c provides an integrated view of chronic glycemic status over a period of 2-3 months, the average lifespan of circulating erythrocytes.

HbA1c is formed by a **slow, non-enzymatic glycosylation (glycation) reaction** between glucose (which easily enters red blood cells) and N-terminal valine residue of the β-globin chains of adult hemoglobin (HbA).

The glycation process occurs in two phases:

1. **Formation of a labile Schiff base (aldimine)**. Glucose freely diffuses into erythrocytes and reacts with the free amino group on valine. The reaction is rapid and reversible, with a yield directly dependent on ambient blood glucose concentration. The aldimine is unstable and easily dissociated, thus not measured in most standardized HbA1c assays.

2. **Amadori rearrangement (ketoamine)**. Over several hours to days, the aldimine undergoes a spontaneous Amadori rearrangement. The resulting ketoamine is stable and represents glycated hemoglobin (HbA1c). Most methods for measuring HbA1c determine this glycated product without including the labile Schiff base, which is influenced by dietary variations.

The HbA1c level in blood correlates with both the **half-life of hemoglobin** and **the average blood glucose level** over the lifespan of red blood cells. This means that an increase in HbA1c level might reflect a high mean glycemia over the past 8-12 weeks. Therefore, HbA1c serves as a retrospective biomarker of chronic glucose exposure and metabolic control.

HbA1c measurement is a key test for ***long-term evaluation*** and monitoring of glycemic control in patients with diabetes mellitus. It also has a predictive role in assessing the risk of diabetes complications, such as ketoacidosis, nephropathy, and retinopathy. To enhance clinical interpretability, HbA1c can be converted into an estimated average glucose (eAG) value using the following formula:

eAG = estimated average glucose (mg/dL) = 28.7 x HbA1c (%) – 46.7

HbA1c	eAG	
%	mg/dL	mmol/L
6	126	7
6.5	140	7.8
7	154	8.6
7.5	169	9.4
8	183	10.1
8.5	197	10.9
9	212	11.8
9.5	226	12.6
10	240	13.4
11	269	14.9
12	298	16.5

The frequency of HbA1c testing depends on the type of DM and the stability of glycemic control:

- Every 3-4 months for Type 1 diabetes patients on conventional treatment
- Every 1-2 months for Type 1 diabetes patients on intensive therapy
- Every 6 months for Type 2 diabetes patients with stable glycemic control
- Every 1-2 months for pregnant women with diabetes

Advantages of HbA1c Compared to Blood Glucose Measurement

- Fasting is not required.
- Greater pre-analytical stability.
- Lower individual biological variability compared to blood glucose.
- Less affected by stress and acute illnesses.

Disadvantages of HbA1c Compared to Blood Glucose Measurement

- Higher cost per test.
- Limited availability in some laboratories.

- Lack of correlation with average blood glucose in some individuals (e.g., obesity, hypothyroidism, hemolytic anemia, malignancies).
- Not reliable in patients with abnormal red blood cell turnover (e.g., hemolytic or iron-deficiency anemias); in such cases, diabetes diagnosis must rely solely on glycemia.

Interpretation of results

- Increased HbA1c indicates hyperglycemia over the past 2-3 months.
- High levels are found in patients with poorly controlled or newly diagnosed diabetes.
- Diabetes is considered well-controlled when HbA1c is below 7%.
- In cases of poor glycemic control, HbA1c can rise to 20%.
- HbA1c decreases gradually over several months, as older red blood cells are replaced by new ones with normal glycated hemoglobin levels.

Methods for Determining HbA1c Concentration

- Chromatography: Ion-exchange column chromatography, Affinity chromatography
- High-performance liquid chromatography (HPLC)
- Electrophoretic methods
- Photometric methods (based on color reaction with thio barbituric acid)
- Immunological methods
- Enzymatic methods

EXPERIMENTAL PART

Determination of HbA1c - Enzymatic method

Principle

Hemolyzed blood is prepared by adding the Hemolysis reagent (R3) to the blood sample. The proteases present in R1 reagent hydrolyze the proteins in the lysed blood sample and, therefore, release amino acids such glycated valine from the beta globin chains. The enzyme fructosyl-valine oxidase (R2) acts on the N-terminal ends of glycated valines and generates hydrogen peroxide. This will react with a corresponding chromogenic substance in the presence of horseradish peroxidase and further, it will generate a colored compound.

Reagents

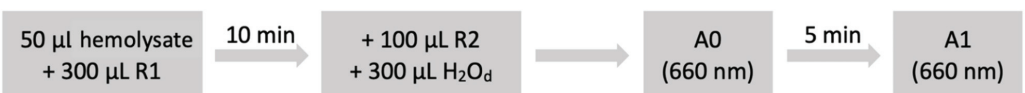
- R1 (Buffer) = pH =7 buffer solution; proteases; Triton X-100 (detergent); redox agents.
- R2 (Enzyme/Chromogen) = Fructosyl-valine oxidase (FVO); horseradish peroxidase (POD); chromogenic substance.
- R3 (Hemolysis reagent) = Cyclohexane-2-aminoethane sulphonic acid; Triton X-100; Sodium dodecyl sulphate (SDS); Redox agents
- R4 (Calibrators) = C1 (N), %HbA1c = 4.6; C2 (H), %HbA1c = 10.87
- Distilled water
- Blood collected on EDTA

Materials and consumables

- Hemolyzed blood - 50 µL in a 0.2 mL Eppendorf tube
- Reagents required - 300 µL R1 and 100 µL R2 in 0.5 mL Eppendorf tubes
- Distilled water
- Spectrophotometric cuvettes
- Automatic pipettes and pipette tips

Procedure

1. Preparation of hemolyzed blood (Already prepared!)
- add 20 µL blood and 500 µL R3 to an Eppendorf tube and mix thoroughly by pipetting "up and down"
- incubate for 5 minutes at laboratory temperature
2. Preparation of the test sample
- work directly in the spectrophotometric cuvette
- add 300 µL R1 and 50 µL hemolysate and mix well by pipetting up and down
- incubate at room temperature for 10 minutes
- add 100 µL R2 and 300 µL distilled water and mix well by pipetting up and down
3. Reading of absorbances
- the spectrophotometer is set to wavelength λ = 660 nm
- set the absorbance to 0 using the blanck solution
- the initial absorbance of the sample (A0) is read immediately
- after 5 minutes read the final absorbance (A1)

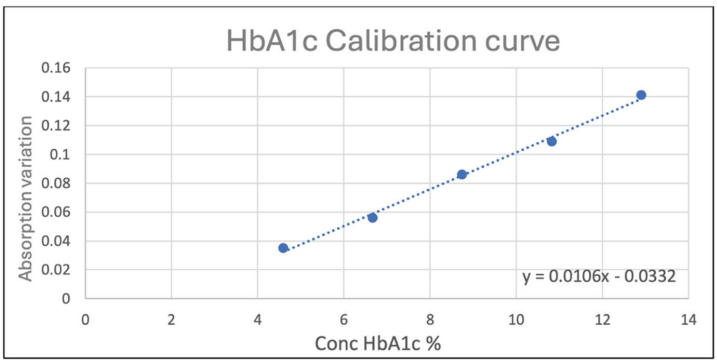


Reagents (µL)	Sample	Blank
Hemolyzed blood	50	-
Distilled water	-	50
R1	300	300
Mix well and incubate at room temperature for 10 minutes		
R2	100	100
Distilled water	300	300

Calculation

- calculate the absorbance variation, ΔA = A1 - A0
- determine the HbA1c concentration (%) by extrapolation, knowing the equation of the calibration curve drawn beforehand

Conc HbA1c (%) = 94.34 x ΔA + 3.13



Results obtained with the present method can be compared with those obtained by a certified standardized method (NGSP) or compared with the standardized method of the 'International Federation of Clinical Chemistry' (IFCC), using the formulas below:
%HbA1c-NGSP = (0.9148 x %HbA1c-IFCC) + 2.152
%HbA1c-NGSP = (0.09148 x HbA1c-IFCC (mmol HbA1c/mol Hb)) + 2.152

Clinical significance

	NGSP % (National Glycohemoglobin Standardization Program)	IFCC % (International Federation of Clinical Chemistry)
Normal (healthy, non-diabetes mellitus)	4 - 6	2 – 4.2
Impaired glucose tolerance	6 – 6.5	4.2 – 4.8
Diabetes mellitus patients with effective treatment	6.5 – 8	4.8 – 6.4
Diabetes mellitus patients with ineffective treatment	> 8	> 6.4

Questions

1. Fill in the blanks: In the regulation of glycemia, _____ plays the central role in lowering blood glucose levels after a meal by promoting glucose uptake in insulin-dependent tissues such as _____ and _____. This hormone stimulates _____ in the liver and muscle and promotes _____ in adipose tissue. In contrast, during fasting or stress, hormones like glucagon and cortisol stimulate processes such as _____ and _____ to restore blood glucose levels.

2. Explain why glycogen from skeletal muscle does not contribute directly to maintaining blood glucose concentration.

3. List the major hormones involved in glycemia regulation and classify them as hypoglycemic or hyperglycemic.

4. What hormonal and metabolic adaptations take place in the late postprandial (fasting) state?

5. What is the pathophysiological basis of ketoacidosis in uncontrolled diabetes mellitus?

6. Explain what glycated hemoglobin is and how is it formed.

7. Provide two advantages and two limitations of using HbA1c to monitor diabetes control.

8. A patient has an HbA1c of 9.5%. Estimate their average blood glucose (eAG) over the last 2–3 months.

9. What is the role of fructosyl-valine oxidase (FVO) in the enzymatic HbA1c assay?

10. Which of the following statements regarding HbA1c are TRUE?
    A. It reflects blood glucose levels over the last 2–3 days.
    B. It is unreliable in patients with hemolytic anemia.
    C. It requires the patient to be in a fasting state.
    D. It is formed through an enzymatic glycosylation of hemoglobin.
    E. It has a high analytical stability

Lab Notes

Use this space for handwritten notes during the practical session.

L4. LIPID METABOLISM. DETERMINATION OF TRIGLYCERIDES. IDENTIFICATION OF KETONE BODIES IN URINE

Lipids are a heterogeneous group of biomolecules that play essential roles in energy storage, structural organization of biological membranes, and cellular signaling. Due to their high energy density, lipids represent the most efficient form of energy storage in the human body, providing approximately 9 kcal/g upon oxidation. The principal classes of biologically relevant lipids include triglycerides (triacylglycerols), phospholipids, cholesterol, and fatty acids.

In a typical diet, lipids account for approximately 20-35% of total daily energy intake, with triglycerides representing the predominant form. Because lipids are hydrophobic and insoluble in aqueous environments, their digestion and absorption require specialized mechanisms involving emulsification and enzymatic hydrolysis. These processes ensure the conversion of complex lipid molecules into absorbable units that can be transported across the intestinal epithelium.

Digestion and absorption

Due to their hydrophobic nature, dietary lipids require emulsification and enzymatic hydrolysis prior to absorption. Lipid digestion begins in the stomach, where gastric lipase initiates the hydrolysis of triglycerides, although this contribution is relatively limited. The major phase of lipid digestion occurs in the small intestine, where bile salts, synthesized in the liver and stored in the gallbladder, emulsify large lipid droplets into smaller particles, thus increasing the surface area accessible to digestive enzymes.

Pancreatic lipase, together with its cofactor colipase, plays a central role in triglyceride digestion by hydrolyzing ester bonds at positions 1 and 3, resulting in the formation of free fatty acids and 2-monoacylglycerols. Additional enzymes, including phospholipase A_2 and cholesterol esterase, contribute to the digestion of phospholipids and cholesterol esters, respectively.

The products of lipid digestion are incorporated into mixed micelles formed by bile salts, which facilitate their transport through the aqueous environment of the intestinal lumen to the brush border of enterocytes. Following absorption, fatty acids and monoacylglycerols are re-esterified within enterocytes to form triglycerides. These are subsequently packaged together with cholesterol, phospholipids, and apolipoproteins into chylomicrons, which are secreted into the lymphatic circulation and eventually enter the bloodstream, ensuring the transport of dietary lipids to peripheral tissues.

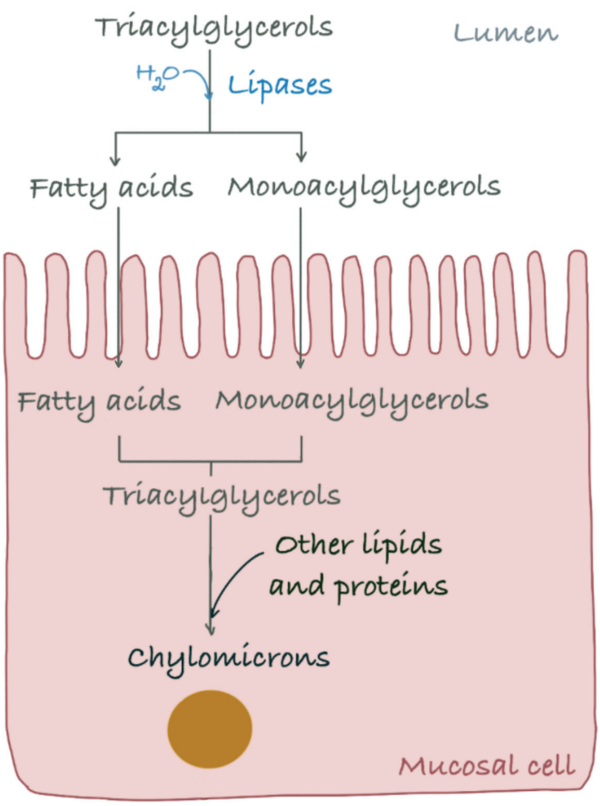


Figure 1. Digestion and absorption of triglycerides

Once in circulation, triglycerides contained in chylomicrons are hydrolyzed by lipoprotein lipase located on the endothelial surface of capillaries, particularly in adipose tissue and muscle. This process releases free fatty acids, which are taken up by cells and either oxidized to produce energy or re-esterified and stored as triglycerides. The chylomicron remnants are subsequently taken up by the liver.

Lipid metabolism is characterized by a dynamic balance between lipid storage and mobilization, primarily represented by lipogenesis and lipolysis, which are regulated according to the nutritional and hormonal status of the organism.

Lipogenesis represents the anabolic pathway by which excess energy, mainly derived from carbohydrates, is converted into fatty acids and subsequently into triglycerides. This process occurs predominantly in the liver and adipose tissue and is stimulated by insulin in the postprandial state. The synthesized fatty acids are esterified with glycerol to form triglycerides, which are stored in adipose tissue as an energy reserve.

In contrast, lipolysis is the catabolic process through which stored triglycerides are hydrolyzed into free fatty acids and glycerol. This process is activated during fasting, physical activity, or stress and is stimulated by hormones such as glucagon and catecholamines, while being inhibited by insulin. Free fatty acids released into the circulation are transported bound to albumin and utilized by peripheral tissues through β-oxidation, whereas glycerol is transported to the liver, where it participates in gluconeogenesis or glycolysis.

The balance between lipogenesis and lipolysis ensures the maintenance of energy homeostasis, allowing the organism to adapt efficiently to variations in energy intake and demand.

The dynamic interplay between lipid absorption, transport, storage, and mobilization is reflected in the concentration of circulating lipids, particularly triglycerides. Under physiological conditions, plasma triglyceride levels vary according to nutritional status, hormonal regulation, and metabolic demands. Alterations in these processes may lead to changes in triglyceride concentrations and the production of metabolic intermediates such as ketone bodies.

The evaluation of these parameters provides important information regarding lipid metabolism and its disorders. Therefore, the determination of plasma triglycerides and the identification of ketone bodies in biological fluids represent essential laboratory methods for the assessment of both normal metabolic function and pathological conditions.

DETERMINATION OF SERUM TRIACYLGLYCEROLS

The determination of serum or plasma triacylglycerol concentration is used to evaluate and differentially diagnose the primary or secondary hyperlipidemias, and for the evaluation of the risk factors of acute pancreatitis. The methods used nowadays determine the glycerol from the triacylglycerol molecule, determination that can be done using chemical or enzymatic methods.

The technique with pyruvate kinase, having a final measurement in ultraviolet, at 340 nm, has the following sequence of reactions:

$$Triacylglycerols \xrightarrow{lipase} glycerol + fatty\ acids$$

$$Glycerol + ATP \xrightarrow{glycerolkinase} glycerol - 3 - phosphate + ADP$$

$$ADP + phosphoenolpyruvate \xrightarrow{pyruvatekinase} ATP + pyruvate$$

$$pyruvate + NADH + H^{+} \xrightarrow{lactate\ dehydrogenase} lactate + NAD^{+}$$

The technique with glycerol phosphate oxidase is used and explained in the experimental part, presented below.

EXPERIMENTAL PART

Serum triacylglycerol determination with the enzymatic method

Principle

Triglycerides are enzymatically hydrolyzed to glycerol and free fatty acids in the presence of specific lipases. Glycerol reacts further according to the following reaction scheme:

$$Glycerol + ATP \xrightarrow{glycerolkinase} glycerol - 3 - phosphate + ADP$$

$$Glycerol - 3 - phosphate + O_2 \xrightarrow{glycerol-3-P\ oxidase} Dihydroxyacetonephosphate + H_2O_2$$

$$H_2O_2 + 2 - chlorophenol + 4 - aminoantipyrine \xrightarrow{peroxidase} 2H_2O + HCl + 4 - (o - benzoquinone - monoimino) - phenazone$$

Procedure

Reagents (µl)	Sample	Standard	Blank
Serum	100	-	-
Standard (200 mg/dl)	-	100	
Distilled water	-	-	100
Reactive mixture	1000	1000	1000

Mix and incubate 10 minutes at room temperature. Read the sample and standard absorbance at 546 nm zeroing the spectrophotometer against the blank.

Calculation

To calculate the serum triglyceride concentration, use the formula:

$$Triacylglycerol\ (mg/dl) = \frac{A_{sample}}{A_{standard}} \cdot c_{standard} = \frac{A_{sample}}{A_{standard}} \cdot 200$$

Extinction-concentration linearity is observed for concentrations up to 800 mg triglycerides/dl. For higher concentrations, the serum is properly diluted with distilled water.

Normal values

Women: 40-140 mg%
Men: 60-165 mg%

Pathological values

- **Increased** triglyceride levels are found in primary hyperlipoproteinemias (except type IIa), type II diabetes mellitus (T2DM), obesity, hypothyroidism, liver diseases, obstructive jaundice, nephrotic syndrome, acute pancreatitis, pregnancy, and during treatment with corticosteroids or estrogens.
- **Decreased** values are found in malnutrition, malabsorption syndromes, hyperthyroidism, chronic obstructive liver disease, severe anemia, and chronic wasting diseases. Low values may also occur in patients on low-fat diets or undergoing treatment with lipid-lowering drugs (such as fibrates).

KETONE BODIES

Ketogenesis is a metabolic process that occurs in the liver, where fatty acids and certain ketogenic amino acids are broken down to produce ketone bodies. This process provides an alternative source of energy, especially during fasting, prolonged exercise, starvation, or insulin deficiency, when glucose availability is low.

The three main ketone bodies are:

- **acetoacetic acid (acetoacetate) -** the primary ketone body produced in the liver
- **β-hydroxybutyric acid (β-hydroxybutyrate) -** formed by the reduction of acetoacetate
- **acetone** - a volatile byproduct that is exhaled through the lungs. Its presence can give the breath a sweet, fruity odor, often observed in diabetic ketoacidosis

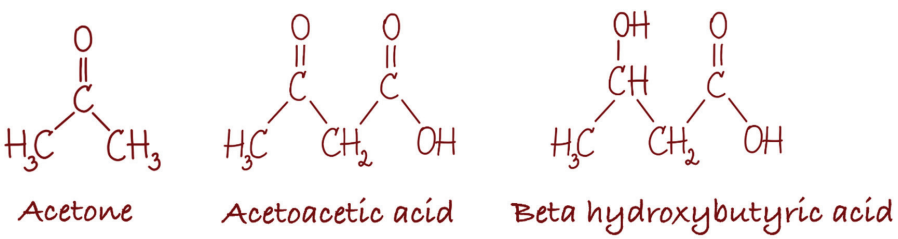


Figure 2. The ketone bodies: acetoacetate, β-hydroxybutyrate, and acetone

Production site

Ketogenesis occurs exclusively in the liver, within the mitochondria of hepatocytes, with acetoacetic acid being the primary product. For the liver, acetoacetate is an inert metabolite that cannot be catabolized, as the liver lacks the necessary enzymatic machinery to activate acetoacetate (specifically, succinyl-CoA-acetoacetate-CoA transferase).

In contrast, extrahepatic tissues, such as skeletal muscle, the heart, the kidneys, and, under prolonged fasting, the brain, can convert acetoacetate and β-hydroxybutyrate into energy.

Physiological Role

Under normal conditions, the production of ketone bodies is low, and their urinary excretion is minimal (the urine ketone test is negative). When glucose availability decreases, ketone body synthesis increases as an adaptive response to provide fuel for tissues and to spare glucose for those that are strictly dependent on it (such as red blood cells and parts of the brain).

Pathological Ketogenesis

Pathological ketogenesis occurs when the production of ketone bodies in the liver exceeds the capacity of peripheral tissues to use them for energy. This leads to an accumulation of ketone bodies in the blood (ketonemia) and their appearance in the urine (ketonuria). The condition represents a metabolic imbalance often associated with insulin deficiency or severely impaired glucose utilization.

Under normal conditions, insulin suppresses lipolysis (the breakdown of fat/stored triglycerides) in adipose tissue. However, when insulin levels are low, as in uncontrolled diabetes mellitus type 1, or, to a lesser degree, during starvation or prolonged fasting, this inhibition is lost. Therefore, the following processes will occur:

1. ***Increased lipolysis*** - Triglycerides in adipose tissue are broken down into free fatty acids (FFAs) and glycerol.
2. ***Transport to the liver*** - FFAs enter the liver and are converted into acetyl-CoA via β-oxidation in the mitochondria.
3. ***Accumulation of acetyl-CoA*** - When carbohydrate metabolism is impaired (low insulin → low glucose uptake → low oxaloacetate levels), the Krebs cycle slows down and cannot handle all the acetyl-CoA, leading to its accumulation.
4. ***Shift toward ketogenesis*** - The excess acetyl-CoA is diverted into the ketogenic pathway, resulting in the formation of ketone bodies (acetoacetate, β-hydroxybutyrate, and acetone).

Consequently, ketone bodies accumulate in the blood because their production exceeds their rate of utilization by extrahepatic tissues **(ketonemia).** Once blood concentrations rise above the renal threshold, ketone bodies are excreted in urine **(ketonuria).** Since ketone bodies are acids, their accumulation lowers blood pH, leading to **metabolic (keto)acidosis**. The body attempts to compensate via hyperventilation (Kussmaul breathing) to expel CO_2 and restore pH balance. Urinary excretion of ketone salts depletes sodium, potassium, and bicarbonate (alkaline reserve), worsening dehydration and acidosis.

Pathological ketogenesis is most seen in:

- Uncontrolled diabetes mellitus (especially type 1) → diabetic ketoacidosis (DKA)
- Prolonged starvation or fasting
- Low-carbohydrate ("ketogenic") diets (usually mild and physiological, but may mimic early ketosis)
- Alcoholic ketoacidosis (due to ethanol metabolism and poor nutrition)

Patients with pathological ketogenesis, particularly in diabetic ketoacidosis, may present:

- Nausea, vomiting
- Dehydration and excessive thirst
- Rapid, deep breathing (Kussmaul respiration)
- Fruity (acetone) odor on the breath
- Altered consciousness (in severe cases)

EXPERIMENTAL PART

Ketone bodies identification in urine using Rothera's Test

Principle

The sodium nitroprusside reaction is used to detect **acetone and acetoacetate** in biological samples, but it does **not react with β-hydroxybutyrate**. The assay is generally free from interference by most drugs or metabolic products.

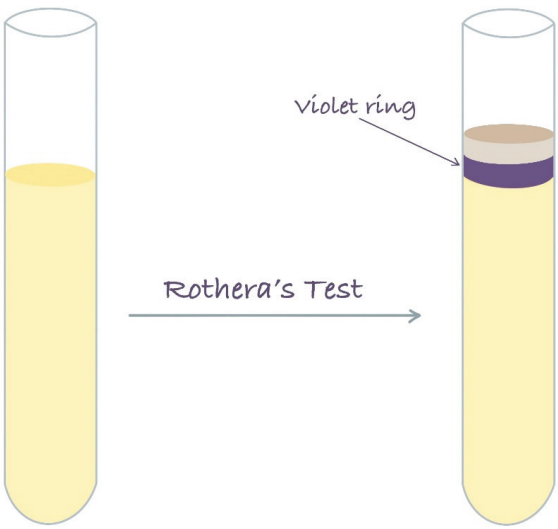
In the Rothera test (named after its originator), 1 g of ammonium sulfate is added to 5 ml of urine and shaken until completely dissolved. Then, approximately three drops of a freshly prepared sodium nitroprusside solution are added and mixed thoroughly. Next, 1-2 ml of a saturated ammonium hydroxide solution is carefully layered, along the side of the test tube, onto the sample. After 2 minutes, the appearance of a purple ring at the interface indicates the presence of ketone bodies; the color intensity is graded from 1+ to 4+.

Procedure

Reagents	
Urine	5.0 ml
Ammonium sulphate	1 g
Sodium nitroprusside, drops	3-4 drops
Ammonium hydroxide	1-2 ml

The nitroprusside reaction can detect 1-2 mg/dL of acetoacetate or 10 mg/dL of acetone.

Normal urine will not form the violet ring.



Questions

1. Explain what happens to monoacylglycerols and free fatty acids after absorption by intestinal epithelial cells.

2. Explain the role of bile salts in the digestion of triglycerides.

3. What is the principle of the pyruvate kinase method for measuring triglycerides, and how is the result detected spectrophotometrically?

4. Name the three ketone bodies. Where are they formed and from what precursor?

5. When does ketogenesis occur?

6. What is the rate-limiting enzyme of ketogenesis?

7. A 52-year-old man with the following symptoms: polyuria, polydipsia and nausea, comes to the emergency unit. On examination, he is dehydrated, tachycardic, and has deep, rapid breathing (Kussmaul respiration). His breath has a fruity odor.
   Laboratory tests reveal:
   Blood glucose: 450 mg/dL
   Arterial pH: 7.30(↓)
   Positive Legal-Imbert test for ketone bodies in urine.
   - What is the most likely diagnosis? Explain.

8. A 44-year-old woman has a high-fat diet and is physically inactive. Her fasting serum sample appears lipemic and triglyceride levels are 390 mg/dL. How do you interpret the elevated triglycerides, and what might explain this finding?

Lab Notes

Use this space for handwritten notes during the practical session.

L5. LIPID METABOLISM. PLASMA LIPOPROTEINS. DETERMINATION OF TOTAL AND HDL CHOLESTEROL FROM PLASMA LIPOPROTEINS

Cholesterol, a steroidal lipid ($C_{27}H_{46}O$), is a major constituent of the cell membranes of animal cells. It is characterized by a rigid sterol nucleus composed of four fused hydrocarbon rings, a hydrocarbon tail and a single free hydroxyl group.

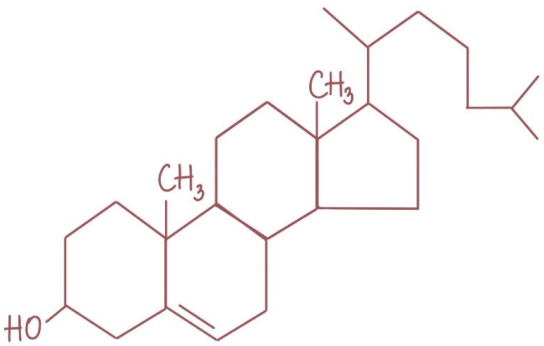


Figure 1. Cholesterol structure

The body requires approximately 1 gram of cholesterol per day, which is supplied through both **endogenous synthesis** and **dietary intake**. The relative contribution of these two sources varies depending on dietary habits and physiological needs. The exogenous intake is variable, close to 1 gram of cholesterol per day. During digestion, in the presence of bile salts, only half of it is absorbed in the intestine. Most of the absorbed cholesterol is converted to cholesteryl esters by microsomal enzyme acyl-CoA-cholesterol acyltransferase (ACAT) in the intestinal mucosa. Along triacylglycerols, some free cholesterol and other dietary lipids, the cholesteryl esters are packaged into chylomicrons and transported to the peripheral tissues.

Cholesterol biosynthesis

Cholesterol is synthesized in the body at a rate of approximately 0.5 to 1 gram per day, depending on dietary intake. Although all nucleated cells can produce cholesterol, the liver is the primary site of synthesis, contributing to about half of the total amount formed in the body. Cholesterol synthesis starts with acetyl-CoA, which is used to synthesize 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). All steps downstream of HMG-CoA occur in the smooth endoplasmic reticulum. *HMG-CoA reductase* reduces HMG-CoA to mevalonate; this enzyme is the major target of regulation in the entire pathway. Mevalonate is converted to various isoprene intermediates. Squalene is cyclized to the first sterol intermediate. This molecule, lanosterol, is then converted to cholesterol by several successive modifications. The last biosynthetic precursor of cholesterol, 7-dehydrocholesterol, is also the precursor of vitamin D3 (cholecalciferol).

Figure 2. Cholesterol synthesis

Cholesterol biosynthesis - regulation

Biosynthesis of cholesterol is directly regulated by the existing cholesterol levels, though the homeostatic mechanisms involved are only partly understood. The amount of cholesterol that is synthesized in the liver is tightly regulated by dietary cholesterol levels. When dietary intake of cholesterol is high, synthesis is decreased and when dietary intake is low, synthesis is increased. The synthesis of endogenous cholesterol is controlled by ***HMG-CoA reductase***.

Cholesterol - use and elimination

Cholesterol is the precursor for the synthesis of steroid hormones, primary bile acids and vitamin D. The steroid ring system of cholesterol cannot be degraded in the human body. Therefore, the excretion of cholesterol is through the biliary system, in the form of bile acids.

Lipoproteins

Plasma lipoproteins are macromolecular complexes responsible for the transport of lipids, (insoluble in water), through the bloodstream between different organs and tissues. These structures consist of:

- a lipid component: mainly triacylglycerols, cholesterol, and phospholipids
- a protein component known as apolipoproteins.

Their structure is adapted to the aqueous environment of blood: the polar groups of phospholipids and apolipoproteins are oriented toward the surface, forming a hydrophilic shell, while nonpolar lipids are located in the hydrophobic core. This organization ensures lipid solubility and efficient transport.

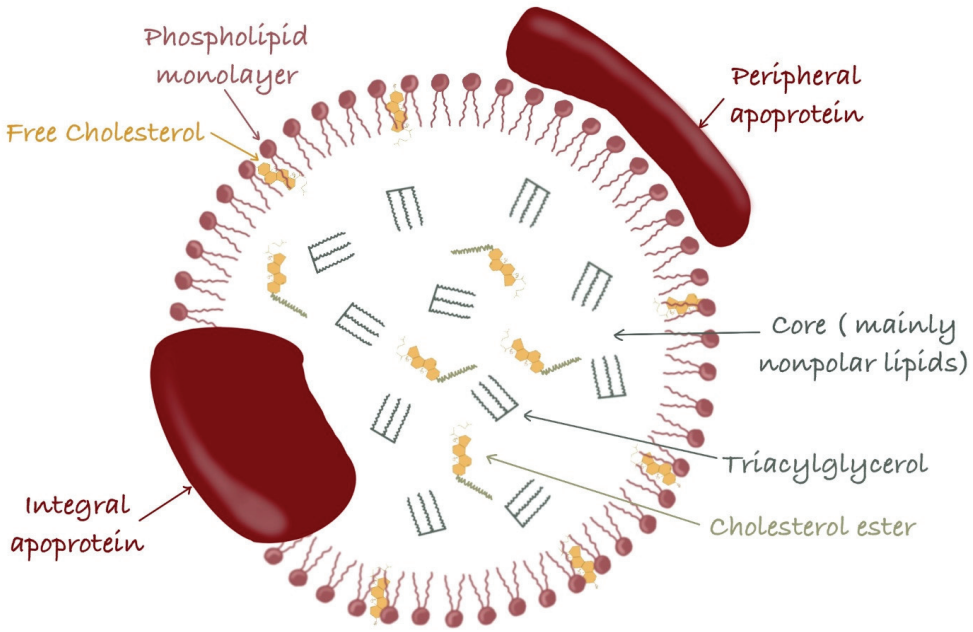


Figure 3. General structure of a lipoprotein

Lipoproteins are classified according to their density, which reflects the relative proportion of lipids and proteins. In order of increasing density, they are: chylomicrons, very-low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). A higher protein-to-lipid ratio corresponds to greater density.

Chylomicrons are the least dense lipoproteins and are responsible for the transport of exogenous (dietary) lipids from the intestine to peripheral tissues. They contain apolipoprotein B-48 and acquire apolipoproteins C and E from HDL in the circulation. Structurally, they consist of a phospholipid-apolipoprotein shell surrounding a core rich in triglycerides. After delivering triglycerides to tissues, the cholesterol-rich chylomicron remnants are taken up by the liver.

Very-low-density lipoproteins (VLDL) are synthesized in the liver and transport endogenous triglycerides to extrahepatic tissues. In the circulation, VLDL particles are progressively metabolized into intermediate-density lipoproteins (IDL) and subsequently into low-density lipoproteins (LDL).

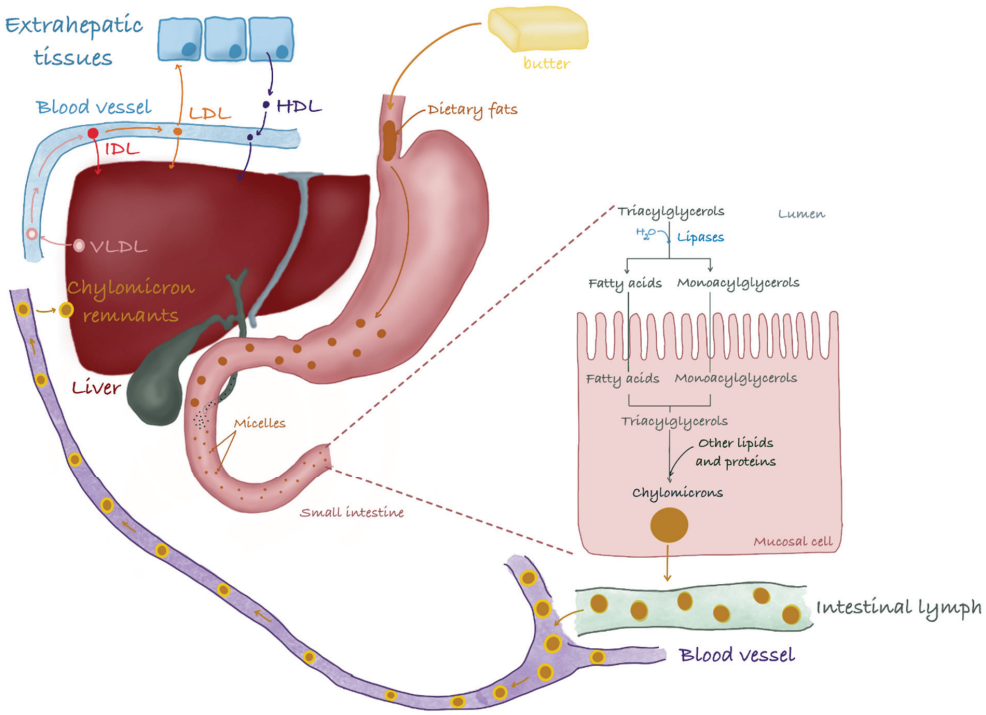


Figure 4. Plasma triacylglycerol and cholesterol transport in humans.

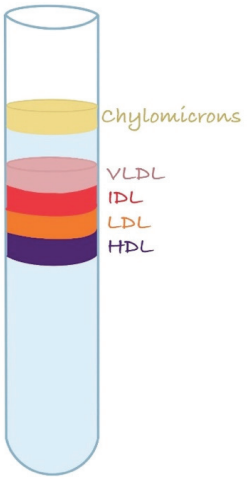
Low-density lipoproteins (LDL) are the principal carriers of cholesterol in the blood and are often referred to as "bad" cholesterol. They deliver cholesterol from the liver to peripheral tissues. LDL particles contain a single molecule of apolipoprotein B-100, which is recognized by specific LDL receptors on cell surfaces. The expression of these receptors is regulated by intracellular cholesterol levels through sterol regulatory element-binding proteins (SREBPs). High intracellular cholesterol suppresses receptor synthesis, whereas low cholesterol levels stimulate receptor expression.

High-density lipoproteins (HDL) are involved in reverse cholesterol transport, carrying excess cholesterol from peripheral tissues back to the liver for excretion or reutilization. HDL particles also serve as a reservoir of apolipoproteins for other lipoproteins. Higher HDL levels are associated with a reduced risk of atherosclerosis, whereas low levels are linked to increased cardiovascular risk; therefore, HDL is referred to as "good" cholesterol.

Methods for plasma lipoprotein fraction separation

Plasma lipoproteins can be separated using different methods:

1. Physicochemical methods:

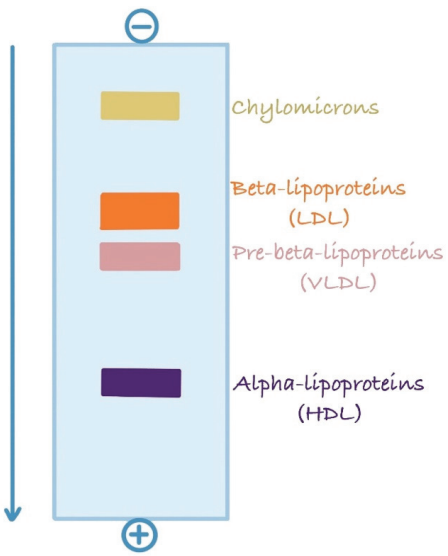


Ultracentrifugation is based on the density difference of plasma lipoproteins according to their lipids and proteins content. Using high gravity fields (approx. 100000g) and according to the medium density, lipoproteins will sediment (if their densities are higher than the medium one) or float (if their densities are lower). Using this method, in a buffer with a density of 1.063 g/cm^3, four fractions can be separated:

- chylomicrons d ≤ 0.96 g/cm^3
- VLDL (very low density lipoproteins) d = 0.96-1.006 g/cm^3
- LDL (low density lipoproteins) d = 1.006-1.063 g/cm^3
- HDL (high density lipoproteins) d = 1.063-1.21 g/cm^3

Electrophoresis - plasma lipoproteins migrate in an electric field because proteins and phospholipids bear electrical charges. The separation is done at a pH = 8.6 and the fractions are visualized with specific dyes. The separated fractions are:

- chylomicrons - practically do not migrate due to their low content in proteins and phospholipids
- β-lipoproteins - correspond to LDL
- pre- β-lipoproteins - correspond to VLDL
- α-lipoproteins - correspond to HDL



In a normal serum, collected after 8-10 fasting hours, chylomicrons do not appear, and VLDL (pre- β) appears only in traces.

Size-exclusion chromatography - separates particles based on their size as they pass through a porous gel resin; larger particles are excluded from pores and elute first, while smaller particles enter the pores and elute later. It separates lipoproteins, typically isolating large particles like chylomicrons (largest) and VLDL from smaller ones (LDL, HDL). While useful for separating very large particles, SEC alone often yields poor separation between smaller lipoproteins or from similarly sized extracellular vesicles (EVs).

2. Chemical methods

The separation of lipoprotein fractions can be realized using cations (2^+), polyanions or surface-active substances. The cholesterol in the separated fractions can be determined using the same method as for total cholesterol. HDL fraction is separated by precipitation with a phosphotungstic reagent, and the HDL + LDL fractions are precipitated with sodium dodecyl sulfate.

Lipid Metabolism and Cardiovascular Risk: Clinical and Paraclinical Indicators

Lipid metabolism dysfunction has a significant impact on the development of degenerative diseases such as dyslipidemias. In most cases, improper dietary habits exacerbate a preexisting genetic predisposition.

Metabolic disturbances can be identified relatively easily through the correlation of **anthropometric indices** (**body mass index - BMI, waist circumference and waist-to-hip ratio**) with **paraclinical indicators** assessing lipid metabolism (**triglycerides, total cholesterol, HDL-cholesterol, LDL-cholesterol**). These measurements help assess the severity of metabolic dysfunction and estimate an individual's risk of developing diabetes, dyslipidemia or a cardiovascular disease.

ANTHROPOMETRIC INDICATORS

A. Body Mass Index (BMI)

- Reflects overall body adiposity and is associated with cardiovascular risk and mortality.
- Can be calculated quickly.
- Defined as the ratio between body weight (kg) and height squared (m^2):

$$BMI = \frac{weight(kg)}{height^2(m^2)}$$

Interpretation:

- BMI < 18.5 kg/m² – underweight (possible malnutrition or eating disorders)
- BMI: 18.5–24.9 kg/m² – normal weight
- BMI ≥ 25 kg/m² – overweight
- BMI ≥ 30 kg/m² – obesity

B. Waist Circumference and Waist-to-Hip Ratio

- Indicators of abdominal obesity
- Waist circumference is measured halfway between the lower rib and the iliac crest
- Visceral fat is a key risk factor for cardiovascular disease, type 2 diabetes, hypertension, sleep apnea and certain cancers

Interpretation:

- Waist circumference > 94 cm in men and > 80 cm in women indicates abdominal obesity
- Values above 102 cm (men) or 88 cm (women) are associated with significantly increased cardiovascular risk
- Waist/hip ratio:

< 0.95 in men (normal)
< 0.80 in women (normal)
> 1.0 in men and > 0.85 in women associated with elevated cardiovascular risk

C. Broca Formula and BMR (Basal Metabolic Rate)

a. Broca Formula

- It is used to calculate the **ideal body weight**.
- Calculation: subtract 100 from the height in centimeters, then subtract 10% from the result.
- Example: For an individual with a height of 175 cm:
    - Normal weight = 175 – 100 = 75 kg
    - Ideal weight = 75 – 10% = 75 – 7.5 = 67.5 kg

b. BMR (Basal Metabolic Rate)

- It is used to calculate the number of **kilocalories needed per 24 hours**.
- Calculation:
  - For men:
    $BMR\ (kcal\ /\ 24h) = 66.47 + 13.7\ x\ weight(kg) + 5\ x\ height(cm) - 6.8\ x\ age(years)$
  - For women:
    $BMR\ (kcal\ /\ 24h) = 655.1 + 9.6\ x\ weight(kg) + 1.8\ x\ height(cm) - 4.7\ x\ age(years)$

PARACLINICAL INDICATORS

A. Determination of Total Cholesterol

Principle

Cholesterol and its esters are liberated from lipoproteins by the action of tensioactive agents. Cholesterolesterase hydrolyses the esters and cholesteroloxidase oxidizes the substrate resulting hydrogen peroxide.

$$cholesterol\ esters + H_2O \xrightarrow{cholesterolesterase} cholesterol + fatty\ acids$$

$$cholesterol + O_2 \xrightarrow{cholesteroloxidase} colesten - 3 - one + H_2O_2$$

$$2H_2O_2 + phenol + 4 - aminoantipirine \xrightarrow{peroxidase} quinoneimine\ dye + 4H_2O$$

Procedure

Pipette according to the table:

Reagents, µl	Sample	Standard	Blank
Standard	-	100	-
Serum	100	-	-
Distilled water	-	-	100
Working reagent	1000	1000	1000

Mix vigorously and then incubate 5 minutes at 37^0C. Measure the sample and standard extinctions at 546 nm, zeroing the spectrophotometer with the blank (the color is stable for 60 minutes).

Calculation

$$Cholesterol\ (mg/dl) = \frac{A_{sample}}{A_{standard}}\ x\ 200$$

Normal values: 140 - 220 mg%

Pathological values

Hypercholesterolemia occurs in familial hypercholesterolemia, diabetes mellitus, nephrotic syndrome, alcoholism, pregnancy.
Low values are found in hepatic cirrhosis, hyperthyroidism.

B. Determination of HDL Cholesterol

Direct enzymatic method
In the first step, chylomicrons, LDL and VLDL fractions are eliminated by a specific reaction. In the second step, the reactions are identical with those of total cholesterol determination.

Procedure

Pipette according to the table:

Reagents, µl	**Sample**	**Standard**	**Blank**
Standard	-	120	-
Serum	120	-	-
Distilled water	-	-	120
R1 reagent	600	600	600
Mix and incubate 5 min at 37^0C, then add:			
R2 reagent	200	200	200

Incubate at 37^0C for 5 minutes.
Read the extinction of sample and standard at 600 nm against the blank. Calculate:

Calculation

$$HDL\ Cholesterol\ (mg/dl) = \frac{A_{sample}}{A_{standard}} x\ 50.5$$

Normal values

Men: 35-55 mg HDL cholesterol/100 ml serum
Women: 45-65 mg HDL cholesterol/100 ml serum

Atherogenic risk	**Men**	**Women**
low	> 55 mg/100 ml	> 65 mg/100 ml
high	< 35 mg/100 ml	< 45 mg/100 ml

The **atherogenic risk** can be also appreciated by the calculation of the **ratio C_T/C_{HDL}**.

- C_T/ C_{HDL} = 3.5 - 5 normal ratio
- C_T/ C_{HDL} < 3.5 low atherogenic risk
- C_T/ C_{HDL} > 5 high atherogenic risk

C. Calculation of LDL cholesterol (Friedewald equation):

$$LDL\ cholesterol\ (mg/dl)\ serum = Total\ cholesterol - (HDL + VLDL)$$

$$LDL\ cholesterol\ (mg/dl)\ serum = Total\ cholesterol - (HDL + \frac{Triglycerides}{5})$$

Normal values

- < 150 mg LDL/100 ml: no medical treatment needed
- 150-190 mg LDL/100 ml: medium atherosclerosis risk
- > 190 mg LDL/100 ml: high atherosclerosis risk-treatment needed

Primary and Secondary Lipid Metabolic Disorders

1. Primary Dyslipidemias

Inherited or familial disorders caused by genetic defects in lipoprotein metabolism.

Type	Type I	Type IIa	Type IIb	Type III	Type IV	Type V
	Hyper-chylomicronemia	Isolated Hyper-cholesterolemia	Combined Hyper-cholesterolemia and Hyper-triglyceridemia	Dysbeta-lipoproteinemia ("Broad beta disease")	Hyper-prebeta-lipoproteinemia	Familial Mixed Hyper-lipidemia
Lipoprotein	↑ Chylomicrons	↑ LDL	↑ LDL + ↑ VLDL	↑ IDL	↑ VLDL	↑ VLDL + Chylomicrons
Total cholesterol	N or ↑	↑	↑	↑	N or ↑	N or ↑
TG	↑↑	N	↑	↑	↑	↑↑
LDL-cholesterol	N or ↓	↑	↑	N or ↓	N	N
HDL-cholesterol	N or ↓	N or ↓	N or ↓	N or ↓	N or ↓	N or ↓

2. Secondary Dyslipidemias

Acquired lipid disorders due to underlying diseases, medications or physiological states.

Increased Cholesterol	Increased Triglycerides
Diet	Obesity
Hypothyroidism	Severe acute hepatitis
Cholestatic liver diseases	Diabetes mellitus, pancreatitis
Nephrotic syndrome	Alcoholism
Pregnancy	Nephrotic syndrome
Corticosteroid therapy	Insulin resistance
Thiazide diuretics	Pregnancy
	Estrogens, corticosteroids, thiazide diuretics, beta blockers

Common Dyslipidemias

Atherosclerosis

Hypercholesterolemia, associated with certain genetic defects or a high-cholesterol diet, contributes to the development of atherosclerosis.

Atherosclerosis is a chronic disease that develops gradually and involves the buildup of low-density lipoprotein (LDL), commonly known as "bad cholesterol", in the walls of large arteries, especially the coronary arteries. The process appears to begin with the association of lipoproteins, particularly LDL, with proteoglycans in the vessel wall. This interaction leads to the retention of LDL particles in the arterial intima, where they become oxidized over time.

Oxidized LDL then activates the endothelial cells lining the blood vessels, prompting them to express adhesion molecules that attract monocytes. These monocytes migrate into the vessel wall and transform into macrophages, which engulf the oxidized LDL and become foam cells. The foam cells release inflammatory molecules that attract additional immune cells, resulting in the formation of a fatty plaque composed of cholesterol, cellular debris and smooth muscle cells. Over time, this plaque may harden, leading to stiffened arteries.

Although an extensive plaque can occlude the lumen of the artery (Figure 5), blood flow is usually not completely blocked unless the plaque ruptures. This plaque rupture triggers the formation of a blood clot that can prevent circulation to the heart (causing myocardial infarction) or the brain (causing stroke) and trigger other severe cardiovascular conditions.

High circulating LDL levels are strongly linked to the development of atherosclerosis. Risk increases with poor diet, smoking, infections, and genetic predisposition. Smoking, for instance, promotes oxidation of LDL, making it more likely to be taken up by macrophages.

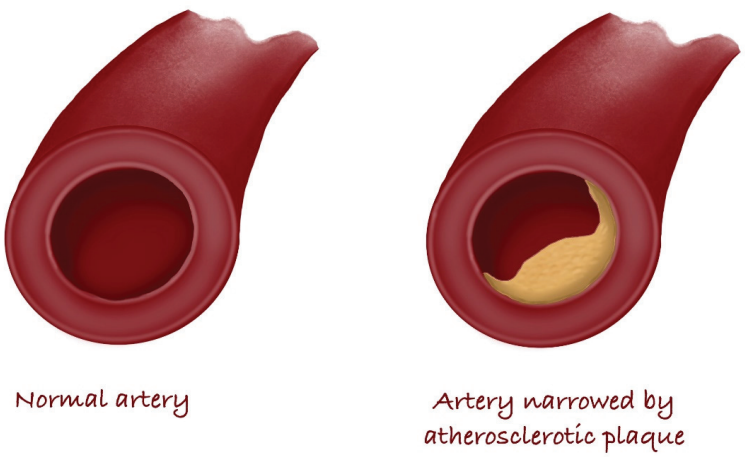


Figure 5. The effects of excess cholesterol.

Hypercholesterolemia is a major risk factor for atherosclerotic disease through the following mechanisms:

a. Elevated total cholesterol

The risk of developing atherosclerotic disease and the mortality rate from coronary artery disease increase when cholesterol levels exceed 160 mg/dL.

A 10% reduction in total cholesterol concentration is associated with a 50% reduction in risk.

b. Elevated LDL Cholesterol

Coronary risk begins to rise when LDL cholesterol exceeds 150 mg/dL.

The "2:1:1 rule" states that a 2% decrease in LDL cholesterol results in a 1% reduction in the risk of developing coronary artery disease and a 1% regression of existing lesions.

c. Reduced HDL Cholesterol

A low HDL cholesterol level is an independent and significant predictor of coronary risk, especially when associated with hypertriglyceridemia.

The atherogenic index (total cholesterol / HDL cholesterol) is predictive of cardiovascular risk when values exceed 5.

The refined atherogenic index (LDL cholesterol / HDL cholesterol) is predictive when values exceed 3.5 in men and 2.5 in women.

Global Cardiovascular Risk Assessment

The European Atherosclerosis Society recommends the use of the EURO Score Chart to quantify overall cardiovascular risk.

This chart incorporates the following primary and additional risk factors: sex, age, smoking status, total cholesterol, systolic blood pressure, diabetes mellitus, HDL cholesterol levels, family history, obesity, and hypertriglyceridemia.

Individual cardiovascular risk is determined by locating the appropriate cell in the chart based on the individual's profile, followed by adjustment according to the presence of additional risk factors.

Obesity

Obesity is a type of secondary dyslipidemia resulting from a long-term imbalance between energy intake and energy expenditure, where excess energy is stored in the form of triglycerides within adipose tissue. Even a small daily caloric surplus, such as 1%, can lead to significant weight gain over time, contributing to an increased body mass index (BMI).

According to the World Health Organization (WHO) 2024 guidelines, obesity is clinically diagnosed when a person's body mass index (BMI) is ≥ 30 kg/m^2. To further assess health risk, especially related to abdominal (visceral) fat, the following additional indicators are recommended: waist circumference and waist-to-hip ratio. While BMI is used for diagnosis, these additional measures help stratify the severity and risk of obesity-related complications, such as type 2 diabetes, dyslipidemia and cardiovascular disease.

Questions

1. How is the biosynthesis of cholesterol regulated in response to dietary intake, and what is the role of HMG-CoA reductase in this process?

2. Why cannot cholesterol be directly catabolized and what are its main metabolic fates?

3. Describe the differences between chylomicrons, VLDL, LDL, and HDL in terms of their density, size, content and role.

4. Describe the process by which LDL contributes to the formation of atherosclerotic plaques.

5. A 52-year-old woman comes in for a cardiovascular risk assessment. Her lipid profile shows the following values:
   Total cholesterol: 260 mg/dL
   HDL cholesterol: 50 mg/dL
   Triglycerides: 150 mg/dL
   - Calculate her LDL cholesterol using the Friedewald equation.
   - Calculate her atherogenic risk

6. A 23-year-old sedentary male with irregular eating habits weighs 90 kg and is 172 cm tall. To assess his metabolic status, calculate his BMI and interpret the result. Then, determine his BMR using the standard formula. What does the BMR suggest about his energy needs? Based on his profile, how would you assess his health risk, and what lifestyle recommendations would you make?

Lab Notes

Use this space for handwritten notes during the practical session.

L6. PROTEIN METABOLISM. DETERMINATION OF TRANSAMINASES

Introduction. Digestion and absorption of proteins

Dietary protein is a vital source of amino acids. Proteins ingested in the diet are digested into amino acids or small peptides that can be absorbed by the intestine and transported in the blood. Another source of amino acids is the degradation of defective or unneeded cellular proteins.

Protein digestion begins in the stomach, where the acidic environment favors protein denaturation. Denatured proteins are more accessible as substrates for proteolysis than are native proteins. The primary proteolytic enzyme of the stomach is ***pepsin***, a nonspecific protease that is synthesized as pepsinogen (proenzyme/zymogen). It is activated to pepsin at an acidic pH and is maximally active at pH = 2. This is the reason why pepsin can be active in the highly acidic environment of the stomach, even though other proteins undergo denaturation there.

Protein degradation continues in the lumen of the intestine with the activity of proteolytic enzymes secreted by the pancreas (***trypsin***, ***chymotrypsin***, ***elastase***, *and* ***the carboxypeptidases***). These enzymes are secreted as inactive zymogens and then converted into active enzymes. The enzymes display a wide array of specificity, and so the substrates are degraded into free amino acids as well as di- and tripeptides. Digestion is further enhanced by proteases, such as ***aminopeptidase N***, ***dipeptidase***, that are located in the plasma membrane of the intestinal cells. Aminopeptidases digest proteins from the amino-terminal end. Single amino acids, as well as di- and tripeptides, are transported into the intestinal cells from the lumen and subsequently in the portal vein, liver, and systemic circulation. From the extracellular medium, the amino acids pass into the cells using an active transport, against the concentration gradient.

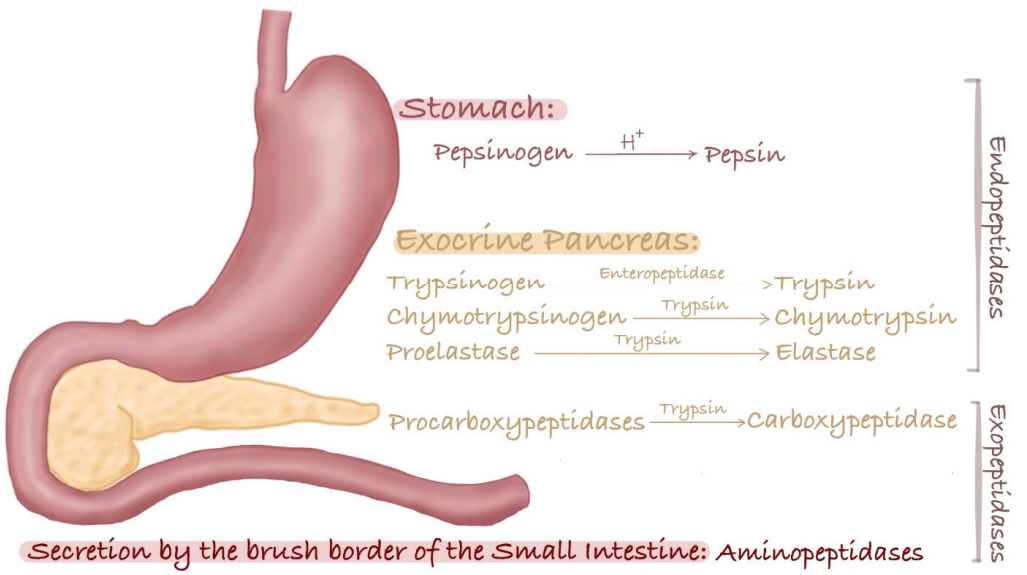


Figure 1. Digestion of proteins

Transamination Reactions

Transamination is the major process for removing nitrogen from amino acids. Most amino acids undergo transamination reactions, but a few, including lysine, threonine, proline, and hydroxyproline, do not. The enzymes catalyzing these reactions are known as **transaminases** or **aminotransferases**. These enzymes have pyridoxal phosphate as cofactor. In most instances, the nitrogen, in the form of an amino group, is transferred from the original amino acid to α-ketoglutarate, forming glutamate, whereas the original amino acid is converted into its corresponding α-keto acid.

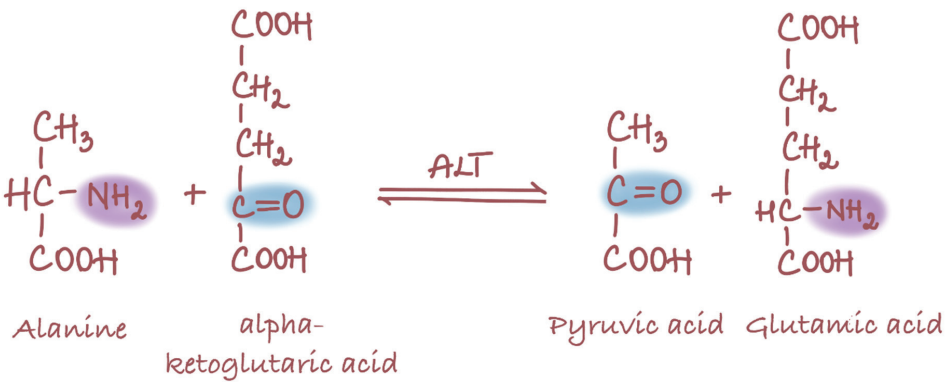
$$amino\ acid + \alpha - keto\ acid \xleftrightarrow{Transaminase} \alpha - keto\ acid + amino\ acid$$

Overall, in a transamination reaction, an amino group from one amino acid becomes the amino group of a second amino acid. Because these reactions are readily reversible, they can be used to remove nitrogen from amino acids or to transfer nitrogen to α-keto acids to form amino acids. Thus, they are involved both in amino acid degradation and in amino acid synthesis.

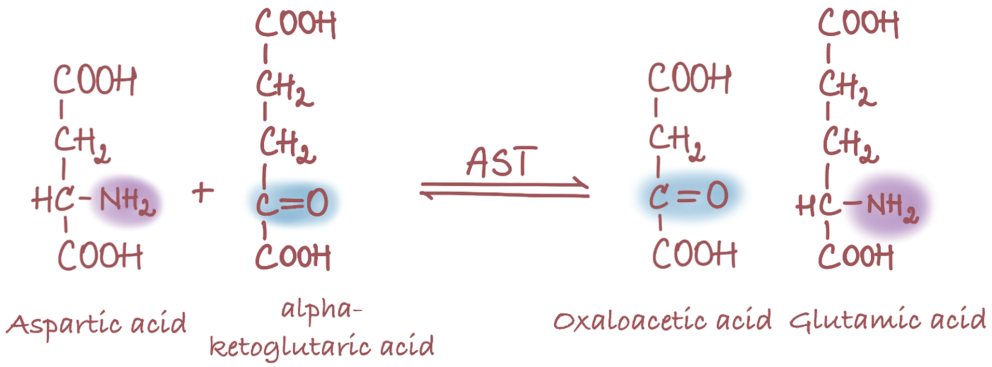
Two important transaminase enzymes are **alanine transaminase/alanine aminotransferase (ALT/ALAT)** or **serum glutamate-pyruvate transaminase (SGPT/GPT)** and **aspartate transaminase/aspartate aminotransferase (AST/ASAT)**, also known as **serum glutamate-oxaloacetate transaminase (SGOT/GOT)**.

Although measurement of their activities is commonly used as a ***test of hepatocellular damage***, these enzymes have a wide tissue distribution.

Alanine aminotransferase catalyzes the transfer of an amino group from L-alanine to α-ketoglutarate, the products of this reversible transamination reaction being pyruvate and L-glutamate. ALT is commonly measured clinically as part of *liver function tests*. When used in diagnostics, it is almost always measured in international units/liter (IU/L) or μkat.



Aspartate aminotransaminase catalyzes the interconversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate.



AST is similar to ALT in that both enzymes are associated with liver parenchymal cells. The difference is that ALT is found predominantly in the liver, with clinically negligible quantities found in the kidneys, heart, and skeletal muscle, while AST is found in the liver, heart (cardiac muscle), skeletal muscle, and also kidneys, brain, and red blood cells. As a result, ALT is a more specific indicator of liver inflammation than AST, as AST may be elevated also in diseases affecting other organs, such as myocardial infarction, musculoskeletal diseases, and acute pancreatitis, acute hemolytic anemia, acute renal disease.

AST is commonly measured clinically as a part of diagnostic *liver function tests* (together with ALT). However, it is important to keep in mind that the source of AST (and, to a lesser extent, ALT) in blood tests may reflect pathology in organs other than the liver.

Different transaminase isoenzymes are found in the cytoplasm and mitochondria of the cells. AST is bilocular, with localization in both the mitochondria and the cytoplasm, while ALT is present only in the cytoplasm. For example, in the conditions of a mild injury to the liver tissue, the isoenzyme that appears in the serum comes from the cellular cytoplasm, although a small amount of the mitochondrial isoenzyme is also present. Upon severe tissue damage and mitochondrial isoenzyme is released in significant amounts.

The determination of serum transaminase activity can be performed by several methods, including the color reaction method and the optical test, which is presented below.

EXPERIMENTAL PART

Optical test - Principle

It is not possible to monitor transamination reactions directly using the optical test, because their coenzyme pyridoxal phosphate, does not produce a detectable signal. However, the advantages of continuous-monitoring assays can be achieved by coupling the transamination reactions to specific dehydrogenase reactions. The keto acids produced in the transamination reaction are measured indirectly by their enzymatic reduction to the corresponding hydroxy acids, and the accompanying by the oxidation of NADH to NAD^+. This change in NADH concentration is monitored spectrophotometrically at 340 nm. The coupled reactions for the two enzymes are as follows:

For ALT (GPT):

Pyruvate, formed in the ALT reaction, is reduced to lactate in the presence of lactate dehydrogenase (LDH):

$$L-alanine+\alpha-ketoglutarate \overset{ALT}{\longleftrightarrow} pyruvate+glutamate$$

$$pyruvate+NADH+H^+ \overset{LDH}{\longleftrightarrow} lactate+NAD^+$$

For AST (GOT):

Oxaloacetate, formed in the AST reaction, is reduced to malate in the presence of malate dehydrogenase (MDH):

$$L-aspartate+\alpha-ketoglutarate \overset{AST}{\longleftrightarrow} oxaloacetate+glutamate$$

$$oxaloacetate+NADH+H^+ \overset{MDH}{\longleftrightarrow} malate+NAD^+$$

The substrate, NADH, and the auxiliary enzymes, LDH and MDH, must be present in sufficient quantity so that the reaction rate is limited solely by the concentrations of GOT and GPT, respectively. As the reactions proceed, NADH is oxidized to NAD^+. The decrease in NADH is monitored by measuring the decline in absorbance at 340 nm, either continuously or at frequent intervals over several minutes. The change in the absorbance per minute (ΔA/min) is directly proportional to the micromoles of NADH oxidized, and therefore to the micromoles of substrate transformed per minutes, expressed in International Units (IU).

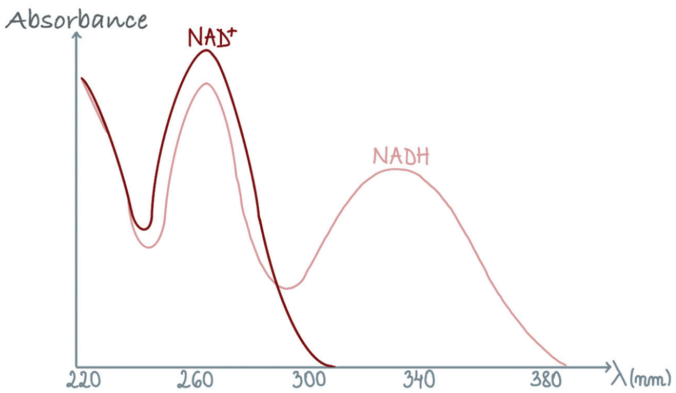


Figure 2. The absorption spectra of NAD^+ and NADH

Working procedure for ALT (GPT) determination:

In a cuvette, pipet 1000 µl of working reagent and 100 µl of serum. Homogenize by pipetting up and down, incubate 1 minute at room temperature, and then read the absorbance against air at la 340 nm, after 1, 2 and 3 minutes. Calculate ΔA/minute.

$$ALT\ (enzymatic\ activity) = \frac{\Delta A}{min}\ x\ 1746$$

Normal values: **- men: <50 U/L**
- women: <35 U/L

Working procedure for AST (GOT) determination:

In a cuvette, pipet 1000 µl of working reagent and 100 µl of serum. Homogenize by pipetting up and down, incubate 1 minute at room temperature, and then read the absorbance against air at la 340 nm, after 1, 2 and 3 minutes. Calculate ΔA/minute.

$$AST\ (enzymatic\ activity) = \frac{\Delta A}{min}\ x\ 1750$$

Normal values: **- men: <55 U/L**
- women: <35 U/L

Clinical significance

Transaminases are widely distributed in human tissues. Both AST and ALT are normally present in human plasma, bile, cerebrospinal fluid, and saliva, but none is found in urine unless there is kidney lesion. Below are the main pathologies associated to increased transaminases:

- **Liver-related pathologies**
  - In *viral hepatitis* and other liver diseases involving hepatic necrosis, serum ALT and AST are elevated even before the clinical signs and symptoms (such as jaundice) appear. Activities of both enzymes may reach values as 100 times the upper reference limit, although 20 – 50-fold elevation are most observed.
  - Moderately increased levels of AST and ALT activity may also occur in *extrahepatic cholestasis.*
  - In *cirrhosis* enzyme levels vary depending on the stage and severity of the disease. Typically, values range from upper-normal to four to five times the normal level, with AST activity higher than ALT.
  - In *primary or metastatic liver cancer*, five- to tenfold elevations of both enzymes may be seen, with AST usually exceeding ALT. However, enzyme levels may remain normal during early stages of malignant infiltration.

  While both enzymes rise in liver cell injury, ALT is more liver specific. Elevated ALT levels are rarely seen outside of parenchymal liver disease and tend to persist longer than AST elevations.

- **Alcohol intake**
  - Mild to moderate increases in AST and ALT activities may occur following *alcohol consumption*.

- **Drug administration**
  - Enzyme elevations may be seen after *administration of certain drugs*, including opiates, salicylates, ampicillin, paracetamol, and most neuroleptics.
  - *Paracetamol overdosage:* paracetamol becomes a significant hepatic toxin when more than 10 grams are ingested, due to the formation of a reactive intermediate, N-acetyl-p-benzoquinone imine (NAPQI), which accumulates when the normal metabolic pathways are overwhelmed.

Of note:
- **ALT > AST** - Suggests viral hepatitis or non-alcoholic fatty liver disease (NAFLD)
- **AST > ALT (usually >2:1 ratio)** - Suggests alcoholic liver disease
- **Mild elevations (1–3× normal)** - Often seen in chronic conditions or drug-induced injury
- **Severe elevations (>10×)** - Typically indicates acute liver injury

The AST/ALT ratio (de Ritis ratio) is clinically useful:
- AST/ALT > 5 - extrahepatic cause
- AST/ALT > 2 - Strongly suggests alcoholic hepatitis
- AST/ALT < 1 - Common in late-stage viral hepatitis
- AST/ALT ~1 - Can be seen in cirrhosis or advanced liver disease

- **Heart-related pathologies**
  - In *myocardial infarction*, AST activity increases significantly in the serum. ALT levels typically remain within normal limits or are only slightly elevated, as heart muscle contains relatively low levels of ALT compared to AST.
  - AST levels may also rise in liver damage secondary to heart failure.

- **Muscle-related pathologies**
  - Elevated AST activity is observed in progressive *muscular dystrophy* and *dermatomyositis*, sometimes reaching up to 8 times the normal level.
  - In contrast, *neurogenic muscle diseases* generally do not show elevated transaminase levels.

- **Other pathologies**
  - *Pulmonary embolism* can cause AST levels to rise 2-3 times above normal
  - Mild to moderate elevations (2-5 times normal) may also occur in conditions as:
    - *acute pancreatitis*
    - *crushed muscle injuries*
    - *gangrene*
    - *hemolytic disease.*

Questions

1. Pepsin, trypsin, chymotrypsin, elastase, carboxypeptidase, aminopeptidase, dipeptidase.
   For each of the following categories, list the corresponding enzymes:
   a. Endopeptidase:
   b. Exopeptidase:
   c. Gastric (stomach) enzyme:
   d. Pancreatic enzyme:
   e. Intestinal enzyme:

2. Explain the general transamination reaction.

3. Briefly present the tissue and intracellular localization of transaminases.

4. Which method is used to determine serum transaminases in the laboratory? Explain the principle of GPT and GOT determination.

5. Explain why the absorbance level is determined at 340 nm and not 260 nm.

6. What is the clinical utility of determining serum transaminases?

7. What could a value of 200 IU/L of GOT indicate? Explain.

8. What could a value of 2000 IU/L of GOT and GPT indicate? What other markers do you think will be modified?

9. Which drugs can influence the serum values of transaminases?

Lab Notes

Use this space for handwritten notes during the practical session.

L7. PROTEIN METABOLISM. AMINO ACID METABOLISM. IDENTIFICATION OF AMINO ACID METABOLISM DISORDERS

In the human healthy organism, the main source of amino acids, for the endogenous proteins, is the dietary proteins. These proteins are enzymatically hydrolyzed in the digestive tube and the resulted amino acids pass into the blood and then extracted by the liver and other organs. They are used specially to synthesize the endogenous proteins.

Amino acid catabolism takes place especially at the liver and kidney level by the transamination reaction, coupled with the deamination reaction. The resulting compounds are ammonia and α-ketoacids. Ammonia is converted to urea and is eliminated mainly by urine. α-ketoacids are degraded in the Krebs cycle or by specific reactions.

The plasma concentration of amino acids varies during the day with approximately 30%, being higher after the meal. Amino acids from the blood are filtered out through the glomerular membrane but than are reabsorbed in the renal tubules by an active transport. The enhanced elimination of amino acids in urine is called ***aminoaciduria***. There are two types of aminoaciduria:

Primary aminoaciduria

It is due to the hereditary mutations at the DNA level which codes enzymes involved in the amino acid metabolism or in their renal reabsorption system. These mutations produce defective enzymes which block the normal metabolic pathways of their own substrates, so an accumulation of these substrates (amino acids) or of metabolic intermediaries appears. In some cases, this block of the normal metabolism sends the amino acids to other metabolic pathways, which produce pathologic degradative products. The diagnosis can be done at three levels:

a) the evidence of the DNA modifications
b) the evidence of the defective enzymes
c) the evidence of the produced metabolic disturbances

Table 1. The main errors in the metabolism of amino acids

Amino aciduria	**Incidence**	**Defective Enzyme**	**Excess in Blood**	**Excess in Urine**	**Clinical Symptoms**	**Treatment**
Hyperphenyl-alaninemia, Phenylketonuria	1:10,000	Phe hydroxylase	Phe	Phe, phenylpyruvic, phenylacetic acids, etc	Mental retardation, eczema	Phe restrictive diet
Tyrosinosis	1:100,000	Phenylaceto-acetate hydroxylase	Tyr, Met	Tyr and metabolites	Hepatic cirrhosis, renal disturbances	Restrictive diet in Phe, Tyr, Met
Alkaptonuria	1:250,000	Homo-gentisate oxidase	Homo-gentisic acid	Homogentisic acid	Degenerative arthritis, cartilage pigmentation	
Homo-cystinuria	1:200,000	Cystationine β-synthase	h-Cys, Met	h-Cystine, Met	Eye, vascular, bone disturbances	Pyridoxine, Low Met diet, supple-mentary Cys
Branched chain ketoaciduria (maple syrup urine disease)	1:250,000	Branched ketoacid decarboxylase	Leu, Ile, Val	Leu, Ile, Val and the corresponding ketoacids	Acidosis, vomiting, respiratory block, lethal evolution	Leu, Ile, Val restrictive diet

The clinical diagnosis is based on the evidence of the metabolic disturbances. Primary aminoaciduria symptoms and prognosis may vary from benign forms as ***alkaptonuria*** to lethal forms as ***maple syrup urine disease***. The accumulations of ketoacids (in the maple syrup urine disease, or of phenylalanine, in ***phenylketonuria*****)** are examples of substrate or intermediary accumulation with toxic effect. The main primary aminoacidurias are summarized in Table 1.

Secondary aminoaciduria

It affects simultaneously more amino acids and is provoked by the affections of some active organs in the amino acid metabolism, such as liver and kidney, also by advanced famine. There are used for identification and treatment of secondary aminoaciduria:

- the diagnosis of the ill infant or child
- routine tests at the new-born infants
- prenatal diagnosis

Precocious diagnosis of these diseases is a mandatory condition for the efficiency of the treatment. These affections are suspected when the child often vomits has neurological or hepatic disturbances and these disturbances are accentuated as the protein intake enhances.

Investigation methods that are used are clinical or microbiological, qualitative or semi-qualitative ones. These methods put in evidence individual amino acids or specific intermediary metabolites. Quantitative methods are used for the exact determination of the amino acid or the unknown metabolite in specialized medical centers. These methods use modern techniques of mass spectrometry, IR spectrophotometry, high pressure liquid chromatography, etc.

Further, the metabolism of Tyrosine and Phenylalanine, Tryptophan and Branched chain amino acids (Valine, Leucine, Isoleucine) are presented.

Metabolism of Phenylalanine and Tyrosine

Phenylalanine is an essential amino acid of mixed nature (both glucogenic and ketogenic). Tyrosine, on the other hand, is classified as non-essential since it can be synthesized endogenously, but its formation depends entirely on phenylalanine. For this reason, in states of phenylalanine deficiency, tyrosine itself becomes essential. Beyond their role as protein constituents, phenylalanine and tyrosine are central to the synthesis of several biologically important compounds, and disturbances in their metabolism account for some of the most clinically significant amino acid disorders.

Formation of Tyrosine

Tyrosine arises either from dietary protein intake or from the hydroxylation of phenylalanine. This reaction is catalyzed by phenylalanine hydroxylase, which requires tetrahydrobiopterin (BH_4), molecular oxygen, and NADPH as cofactors. Defects in this step underlie the most frequent inherited disorder of amino acid metabolism: phenylketonuria (PKU).

Catabolism of Tyrosine

The degradation of tyrosine follows a defined pathway that ultimately yields fumarate (a glucogenic intermediate) and acetoacetate (a ketogenic intermediate). The process begins with transamination to p-hydroxyphenylpyruvate, which is then converted to homogentisate. Homogentisate is oxidized to maleylacetoacetate, which is subsequently isomerized to fumarylacetoacetate. The final cleavage of fumarylacetoacetate produces fumarate and acetoacetate. Each of these steps is catalyzed by specific enzymes, and genetic defects at various levels give rise to distinct metabolic diseases.

Biosynthetic Roles of Tyrosine

Beyond its catabolic fate, tyrosine serves as a precursor for several essential biomolecules:

- **Catecholamines:** Tyrosine is hydroxylated to form Levodopa or L-3,4-dihydroxyphenylalanine (L-DOPA), which undergoes sequential decarboxylation and hydroxylation to produce dopamine, norepinephrine, and finally epinephrine. These catecholamines play major roles in neurotransmission, cardiovascular regulation, and the stress response.
- **Melanin:** Through the action of tyrosinase, tyrosine is converted into dopaquinone and ultimately melanin pigments, which are responsible for skin, hair, and eye coloration.
- **Thyroid hormones:** Within the thyroid gland, tyrosine residues in thyroglobulin undergo iodination to form triiodothyronine (T_3) and thyroxine (T_4).

Clinical significance: Pathology of Phenylalanine and Tyrosine Metabolism

The disorders of phenylalanine and tyrosine metabolism are among the most important amino acid metabolism pathologies, due to both their relatively high incidence and the severe neurological and systemic manifestations that may result.

- **Phenylketonuria:** is the most important congenital amino acid disorder, usually caused by deficiency of phenylalanine hydroxylase. Affected patients accumulate high levels of phenylalanine and its abnormal metabolites such as phenylpyruvic acid. The excess phenylalanine disrupts myelin synthesis and serotonin production, leading to profound intellectual disability historically termed "phenylpyruvic idiocy." Hypopigmentation results from reduced tyrosine availability for melanin synthesis. Clinically, urine develops a greenish color upon addition of ferric chloride due to phenylpyruvic acid. Early diagnosis and dietary restriction of phenylalanine (with tyrosine supplementation) can prevent neurological damage.
- **Alkaptonuria:** This rare inherited disorder results from deficiency of homogentisate oxidase. Homogentisic acid accumulates and is excreted in urine, which darkens upon contact with air, due to oxidation. Over time, ochronosis develops, with pigment deposition in cartilage and joints leading to arthritis.
- **Albinism:** A heterogeneous group of syndromes caused by deficiency of tyrosinase, leading to absent melanin synthesis. Affected individuals exhibit hypopigmentation of the skin, hair, and eyes, with clinical consequences including photophobia and increased risk of skin cancer.
- **Tyrosinemias:** These are rare disorders caused by different enzyme defects within the tyrosine catabolic pathway (fumarylacetoacetase, tyrosine aminotransferase, homogentisate oxygenase) leading to neurological manifestations or accumulation of toxic intermediates with hepatotoxic, nephrotoxic and carcinogenic effects.

Metabolism of Branched-Chain Amino Acids (Valine, Leucine, Isoleucine)

Valine, leucine, and isoleucine are the three branched-chain amino acids (BCAAs). They are essential amino acids, obtained exclusively from the diet. From the standpoint of their metabolic end products, valine is glucogenic, leucine is ketogenic, and isoleucine is both glucogenic and ketogenic. Unlike most amino acids, BCAA metabolism does not take place primarily in the liver. Instead, it occurs in extrahepatic tissues such as skeletal muscle, kidney, and brain, since branched-chain aminotransferase has a lower activity in the liver. This makes BCAAs important substrates for energy production, particularly in muscle during fasting and exercise.

BCAA Degradation

Initial Common Pathway

The first steps of catabolism are shared by all three BCAAs:

1. Transamination: Each amino acid undergoes transamination, catalyzed by branched-chain aminotransferase (BCAT), with pyridoxal phosphate (vitamin B_6) as a cofactor. This produces the corresponding branched-chain α-keto acids (BCKAs).

2. Oxidative Decarboxylation: The BCKAs are then decarboxylated by the branched-chain α-keto acid dehydrogenase complex (BCKD) that produces are the respective acyl-CoA derivatives. A genetic deficiency of BCKD causes maple syrup urine disease.
3. Dehydrogenation and Further Reactions: From this point, the pathways diverge, each BCAA yielding different metabolic intermediates.

Divergent Catabolic Pathways

- **Valine (Glucogenic):** Valine is ultimately degraded to succinyl-CoA, an intermediate of the TCA cycle. The pathway proceeds through intermediates such as α-ketoisovalerate, isobutyryl-CoA, and propionyl-CoA before yielding succinyl-CoA. Because the end product is a gluconeogenic substrate, valine is classified as glucogenic.
- **Leucine (Ketogenic):** Leucine is degraded to acetyl-CoA and acetoacetate, via intermediates including α-ketoisocaproate, isovaleryl-CoA, and HMG-CoA. This makes leucine exclusively ketogenic, contributing to ketone body and cholesterol synthesis, but not to gluconeogenesis.
- **Isoleucine (Mixed):** Isoleucine produces both acetyl-CoA and succinyl-CoA. As a result, isoleucine is considered both glucogenic and ketogenic, contributing to both glucose and ketone body formation.

Clinical significance: Pathology of BCAA Metabolism

The most important inherited disorder of branched-chain amino acid metabolism is **maple syrup urine disease (MSUD)**, also known as branched-chain ketoaciduria. This condition is caused by a deficiency of the branched-chain α-keto acid dehydrogenase (BCKD) complex, leading to the accumulation of BCAAs and their corresponding keto acids in blood and urine. Clinically, affected infants present with poor feeding, vomiting, developmental delay, neurological dysfunction, and, if untreated, death within the first year of life. A hallmark feature is the characteristic sweet, maple syrup-like odor of urine, due to excreted keto acids. Early recognition and dietary management (restriction of BCAAs) are critical for survival and neurodevelopmental outcomes.

Another relevant clinical link is **methylmalonic acidemia**, which can affect BCAA metabolism at the stage where propionyl-CoA is converted to succinyl-CoA. This reaction requires vitamin B_{12}; therefore, B_{12} deficiency can also impair valine and isoleucine degradation, leading to accumulation of methylmalonic acid with metabolic acidosis and neurological manifestations.

Metabolism of Tryptophan

Tryptophan is an essential amino acid that must be obtained through the diet. From a metabolic perspective, it is classified as a mixed amino acid, since it gives rise to both glucogenic products (alanine → pyruvate) and ketogenic products (acetyl-CoA and acetoacetate). Beyond its role in protein synthesis, tryptophan is a precursor for several biologically important compounds, including serotonin, melatonin, tryptamine, and NAD^+. Because of its importance in niacin and NAD^+ formation, tryptophan is sometimes considered a provitamin for vitamin PP (niacin). In adults, the conversion of tryptophan into NAD^+ is sufficient to meet the body's needs, which explains why nicotinamide is not always regarded as an essential dietary vitamin.

Catabolism of Tryptophan

The principal catabolic route of tryptophan is the kynurenine pathway. The process begins with the oxidation of tryptophan to N-formylkynurenine by tryptophan dioxygenase. This is then converted into kynurenine, which undergoes further transformations to yield alanine and acetoacetyl-CoA. Alanine can be transaminated to pyruvate and enter gluconeogenesis, while acetoacetyl-CoA yields acetyl-CoA and acetoacetate, feeding into ketone body metabolism and the TCA cycle. Thus, tryptophan is both glucogenic and ketogenic.

Biosynthetic Pathways from Tryptophan

- **Serotonin and Melatonin:** Tryptophan can be hydroxylated by tryptophan hydroxylase (tetrahydrobiopterin/BH_4-dependent) to form 5-hydroxytryptophan, which is then decarboxylated by a PLP-dependent decarboxylase to yield serotonin (5-hydroxytryptamine, 5-HT). Serotonin acts as a neurotransmitter regulating mood, appetite, and intestinal motility. In the pineal gland, serotonin undergoes acetylation and methylation to form melatonin, which regulates circadian rhythm and sleep. This biochemical link explains why tryptophan-rich foods may promote sleepiness. Interestingly, high-protein meals may inhibit serotonin synthesis because of competition among amino acids for transport, whereas carbohydrate-rich meals increase tryptophan uptake into the brain (via insulin-mediated clearance of competing amino acids), thereby enhancing serotonin production.
- **Niacin and NAD^+/$NADP^+$:** Through the kynurenine pathway, tryptophan can also be converted into quinolinic acid, a precursor of niacin (vitamin B_3), which in turn is used for the synthesis of NAD^+ and $NADP^+$. Approximately 60 mg of tryptophan can produce 1 mg of niacin, a process that requires vitamin B_6. This pathway is clinically important because defects in tryptophan metabolism or vitamin B_6 deficiency may result in pellagra-like symptoms.
- **Indole Derivatives:** Intestinal bacteria metabolize tryptophan into indole compounds. These can be absorbed, modified in the liver, and excreted in urine, forming part of the normal excretory products of tryptophan catabolism.

Clinical significance: Pathology of Tryptophan Metabolism

- **Vitamin B_6 Deficiency:** The enzyme kynureninase, required in the kynurenine pathway, depends on vitamin B_6. In deficiency states, abnormal metabolites such as kynurenine and xanthurenic acid accumulate and appear in the urine, giving it a characteristic yellow-green color. This finding can be used diagnostically to indicate vitamin B_6 deficiency.
- **Hartnup Disease:** This hereditary disorder results from defective intestinal absorption and renal reabsorption of neutral amino acids, including tryptophan. The consequence is decreased tryptophan availability for NAD^+ synthesis and increased urinary excretion of metabolites such as indican. Clinically, this manifests with pellagra-like symptoms (dermatitis, diarrhea, dementia) due to secondary niacin deficiency.
- **Carcinoid Syndrome:** In tumors of enterochromaffin cells, large amounts of tryptophan are shunted into serotonin synthesis at the expense of niacin formation, which is further transformed into 5-hydroxyindoleacetic acid (5-HIAA) by oxidative deamination. Patients present with flushing, diarrhea, bronchospasm, and heart disease. Elevated urinary 5-HIAA is diagnostic.
- **Pellagra:** A classical condition caused by niacin deficiency, which may be dietary or secondary to impaired tryptophan metabolism. The clinical triad consists of dermatitis, diarrhea, and dementia (to which death may be added if untreated).

EXPERIMENTAL PART

1. Hyperphenylalaninemia identification by urine phenylpyruvic acid test

Hyperphenylalaninemia are a group of affections due to the alteration of the conversion mechanism of Phe to Tyr. In these conditions the Phe catabolism takes place following a secondary metabolic pathway, which main product is the phenylpyruvic acid. That one appears in enhanced quantities in plasma and urine, phenomenon called *phenylketonuria.* The precocious identification of the disease is essential to prevent mental retardation. The treatment consists in restrictive diet in Phe, so to maintain the Phe plasma level at 3 - 8 mg%. The test for urine phenylpyruvic acid has the role to identify the affection and to monitor the Phe diet.

Principle

The phenylpyruvic acid reacts with ferric chloride to give a blue-green colored compound (color persisting 2-4 minutes). A similar transitory color is given also by the homogentisic acid or the p-hydroxyphenylpyruvic acid.

Reagents

1. $FeCl_3$ solution: 10 g $FeCl_3.6H_2O$ are dissolved and brought to 100 ml with distilled water.
2. Precipitant agent for phosphate: 2.2 g $MgCl_2$.$6H_2O$, 1.4 g NH_4Cl and 2ml NH_3 are dissolved and diluted to 100 ml with distilled water.
3. Concentrated HCl

Procedure

2 ml urine and 0.5 ml precipitant agent are mixed in a test tube. The suspension is filtered and the filtrate is acidulated with 2-3 drops conc. HCl and mixed. 2-3 Drops of $FeCl_3$ solution are added. A positive test is considered if a blue-green color, which persists for 2-4 minutes, appears. The method sensitivity is 10 mg%. Urinary concentration of phenylpyruvic acid attends 50 - 100 mg% at suffering children, and 2 g phenylpyruvic acid per gram of creatinine are excreted.

2. Test for maple syrup urine disease

This affection is due to the defective catabolism of the branched chain amino acids: Leucine, Isoleucine and Valine. The normal catabolism presumes transamination to α-ketoacids, followed by the oxidative decarboxylation to result acyl-CoA derivatives.

The genetic defect of the decarboxylation step produces the accumulation in urine and blood of these amino acids and their α-ketoacid derivatives. The affected children seem normal at birth, but rapidly, as the protein intake increases, an acute ketoacidosis is installed which provokes vomiting, lethargy, weakness, coma, and, to survivors, mental retardation. The blood tests put in evidence an excess of branched amino acids, and in urine, high quantities of derivative ketoacids, which give to urine the specific smell of maple syrup.

Reagents

1. 2,4-dinitrophenylhydrazine solution 0.4 g / 100 ml in HCl 1M (DNPH)

Procedure

1 ml of filtrated urine is mixed with 1 ml of DNPH in a test tube. The mixture is left for 10 minutes at room temperature. The apparition of a yellow precipitate indicates the presence of an excess of α-ketoacids. The possible interference of acetone is eliminated by boiling first the urine.

3. Quantitative determination of 5-hydroxyindoleacetic acid (5HIAA)

5HIAA is formed by the oxidative deamination of serotonin (5-hydroxy-tryptamine), being its urine excreting product. Serotonin is synthesized from tryptophan at the level of argentaffin cells in the digestive tube and hypothalamus. It is a biogenic amine with vasoconstriction effects in the smooth muscle and neurotransmitter in brain. Because the plasma concentration of serotonin is very low, the urine determination of 5HIAA continues to be the most used method to evaluate serotonin metabolism.

Principle

The test is based on the formation of a purple diazo-derivative from 5HIAA, 1-nitroso-2-naphtol and nitrous acid (HNO_2).

Reagents

1. 1-nitroso-2-naphtol solution 1% in ethanol 95%
2. Nitrous acid solution: 0.2 ml of sodium nitrite solution 2.5% is mixed with 5 ml of sulfuric acid 2N. The solution must be freshly prepared.
3. Dichloroethane
4. Standard 5HIAA: 8 mg 5HIAA are dissolved and diluted to 100 ml with distilled water.

Procedure

Pipette the reagents in 3 centrifuge tubes as follows:

Reagents, ml	**Sample**	**Standard**	**Blank**
Urine	0.4	-	-
Standard	-	0.4	-
Water, dist.	1.6	1.6	2
1-nitroso-2-naphtol	0.5	0.5	0.5
Nitrous acid sol.	0.5	0.5	0.5
Mix, leave for 10 minutes at room temperature			
Dichloroethane	5	5	5

Shake the tubes strongly for 15 seconds and centrifuge them for 10 minutes at 3000 rpm. Read the absorbency of supernatant at 540 nm, zeroing the spectrophotometer with the blank.

Calculation

$$5HIAA\ (mg/24hours) = \frac{A_{sample}}{A_{standard}} \times 8 \times \frac{1}{100} \times V$$

Where V represents the urine volume (in ml) excreted in 24 hours.
If there is no standard, the calculation is done using the following formula:

$$\%HIAA\ (mg/24\ hours) = A(sample) \times F \times V$$

Where F = 0.32

Normal values: 2 - 9 mg 5HIAA / 24 hours

Pathology

Serotonin and implicit 5HIAA are produced in excess in the case of carcinogen tumors of argentaffin cells, when the urine excreted 5HIAA can be 100 - 220 mg / 24 hrs.

Questions

1. What is the difference between primary and secondary aminoaciduria.

2. Choose the correct precursor for each of the following compounds:

Melatonin	Tyrosine
Melanin	
Thyroid hormones	
Adrenaline	Leucine
Niacin	
Noradrenaline	
Serotonin	
Indole derivatives	Tryptophan

3. For each of the following pathologies, indicate the corresponding defective amino acid metabolism:

Tyrosinemias: _____
Vitamin B_6 Deficiency: _____
Maple syrup urine disease: _____
Phenylketonuria: _____
Alkaptonuria: _____
Hartnup Disease: _____
Carcinoid Syndrome: _____
Methylmalonic acidemia: _____
Pellagra: _____
Albinism: _____

4. Briefly describe 3 of the pathologies listed in exercise no 3.

5. Describe the Test for maple syrup urine disease, discussed at the practical part.

6. How can phenylpyruvic acid be identified in the urine?

7. Explain the principle of determination for 5-hydroxyindolacetic acid.

Lab Notes

Use this space for handwritten notes during the practical session.

L8. PROTEIN METABOLISM. DETERMINATION OF UREA

Ammonia metabolism

Ammonia is produced in all body tissues during the metabolism of nitrogen-containing compounds, particularly amino acids. Ammonia (NH_3) is a relatively strong base, and at physiological pH values it is mainly present in the form of the ammonium ion NH_4^+.

Major sources of ammonia include transamination followed by oxidative deamination of amino acids, catabolism of biogenic amines, breakdown of purine and pyrimidine nitrogenous bases, and intestinal bacterial activity, where urease converts urea into ammonia.

NH_3 and NH_4^+ are toxic, and at higher concentrations cause several problems, in particular brain damage. The reasons for the neurotoxic effects of ammonia have not yet been explained. It may disturb the metabolism of glutamate and its precursor glutamine in the brain. Thus, blood ammonia levels must remain very low; it has to be effectively inactivated and excreted. This is achieved primarily through conversion of ammonia into urea in the liver, which is then excreted in urine.

Therefore, amino acids participate in transamination reactions, which transfer their amino group to α-ketoglutarate, forming glutamate. Glutamate then undergoes oxidative deamination, releasing free ammonia for urea synthesis. Since free ammonia is toxic, peripheral tissues convert it into safer transport forms, primarily alanine and glutamine.

In the glucose-alanine cycle, pyruvate (produced by glycolysis in muscle) is transaminated to alanine, which enters the bloodstream and is taken up by the liver. There, alanine is converted back to pyruvate, and its amino group is used for urea formation.

Alternatively, glutamine serves as a non-toxic ammonia carrier throughout the body. In peripheral tissues, glutamine synthetase catalyzes the combination of glutamate and ammonia to form glutamine. Once transported to organs like the kidney and intestine, glutaminase breaks down glutamine, releasing ammonia and regenerating glutamate. This process also occurs in the liver, but to a lesser extent, and is notably stimulated by acidosis, as the released ammonia can help buffer excess protons.

Together, these pathways ensure that nitrogen waste is safely managed and eliminated, with minimal risk of ammonia accumulation in the bloodstream.

Urea cycle

The urea cycle runs only in the liver. It begins with the incorporation of ammonia into carbamoyl phosphate by the corresponding synthetase. This reaction occurs in three successive steps. The first step uses ATP to activate bicarbonate to carbamoyl phosphate, which then captures free ammonia to form carbamate. Another ATP-dependent step activates that intermediate to carbamoyl phosphate. The carbamoyl group will find its way into the urea that is produced by the urea cycle.

The subsequent reactions in the urea cycle are as follows:

- The carbamoyl group is transferred from carbamoyl phosphate to the δ-amino group of ornithine, a non-standard amino acid homologous to lysine, by ornithine transcarbamylase. This reaction yields citrulline.
- Citrulline and aspartate form argininosuccinate, catalyzed by argininosuccinate synthetase. This reaction again requires ATP, which is converted to AMP in the process.
- Argininosuccinate is cleaved to fumarate and arginine by argininosuccinase.
- Urea is released from arginine by arginase, which regenerates ornithine and closes the cycle.

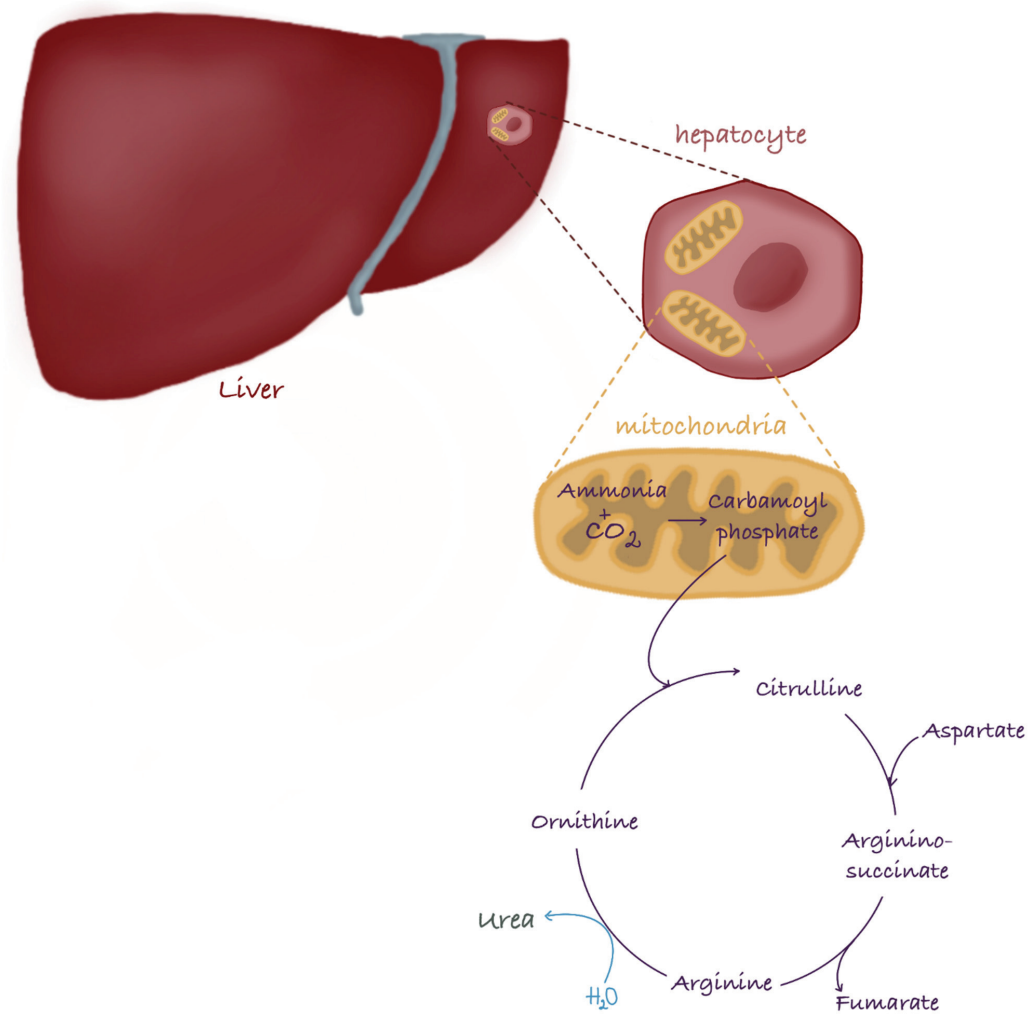


Figure 1. Urea Cycle

After synthesis, urea is secreted into plasma and transported to the kidney, where it is eliminated. Other minor ways of elimination are through the gut and through the sweat glands. These paths are exacerbated in pathological situations.

EXPERIMENTAL PART

Determination of blood and urine urea using the enzymatic method with urease (optical test)

Principle

After plasma deproteinization, numerous non-protein nitrogen-containing compounds remain in the blood. These are collectively referred to as non-protein nitrogen (NPN) or residual nitrogen. Substances in this category include urea (15-50 mg %), uric acid (2-8 mg %), creatinine (0.7-1.2 mg %), creatine, amino acids, choline, polypeptides, ammonia, glutathione, histamine etc.

Compounds of greatest clinical significance are urea, creatinine and uric acid, whose concentration in blood and urine are to be determined.

Urea is hydrolyzed to ammonia and carbon dioxide, in the presence of the enzyme urease. The resulting ammonia then reacts with α-ketoglutarate and $NADH,H^+$ to form glutamate and NAD^+, in the presence of glutamate dehydrogenase (GLDH). The reaction is monitored spectrophotometrically by measuring the decrease in absorbance at 340 nm, which corresponds to the oxidation of NADH to NAD^+. This decrease in absorbance per unit time is directly proportional to the urea concentration in the sample.

$$urea + H_2O + 2H^+ \xrightarrow{urease} 2NH_4^+ + CO_2$$

$$2NH_4^+ + \alpha - ketoglutarate + 2NADH, H^+ \xrightarrow{GLDH} H_2O + 2NAD^+ + glutamate$$

Reagents

1. Reagent 1 (buffer): contains Tris, 80 mmol/l, pH 8.0; α-ketoglutarate 5 mmol/l.
2. Reagent 2 (enzymatic): contains urease >100U/ml; GLDH 6000 U/l; NADH 0.32 mmol/l.
3. Reagent 4 (standard): urea 50 mg/l (8,325 mmoli/l).
4. Working reagent: prepare by adding 1 vial of Reagent 2 to a vial with Reagent 1 (buffer solution), then shake gently for 30 minutes.

Biological samples

- serum, plasma (collected on anticoagulant-heparin; fluoride-containing anticoagulants are not used
- inhibits the activity of urease, or ammonium ions - interferes with dosage);
- urine (collected over 24 hours).

Procedure

Pipette the reagents in 2 cuvettes as follows:

Reagents, µl	**Standard**	**Sample**
Working reagent	1000	1000
Standard	100	–
Sample (serum, diluted urine)	–	100

The kinetic method is used. After the mixing of the reagents, the absorbance is measured at 30 seconds and at 2 minutes and 30 seconds, at 340 nm, in 1 cm cuvettes. Both samples and standard are read in the same conditions, zeroing the spectrophotometer with distilled water.

Calculation

The urea concentration in the serum sample is calculated as:

$$\boldsymbol{serum\ urea\ (mg/dl)} = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times c_{standard} = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times 50$$

where: ΔA_{sample} = change in absorbance of the sample
$\Delta A_{standard}$ = change in absorbance of the standard
$C_{standard}$ = 50 mg/dl

The urea concentration in the urine sample is calculated as:

$$\boldsymbol{urinary\ urea\ (g\ urea/24h\ urine)} = \frac{\frac{\Delta A_{sample}}{\Delta A_{standard}} \times c_{standard}\ \frac{V_{urine\ (L)}}{V_{sample\ (L)}} \times 50}{1000}$$

$$= \left(\frac{\Delta A_{sample}}{\Delta A_{standard}} \times c_{standard} \times V_{urine\ (L)} \times 50\right) \div \left(V_{sample\ (L)} \times 1000\right)$$

where: ΔA_{sample} = change in absorbance of the sample
$\Delta A_{standard}$ = change in absorbance of the standard
$C_{standard}$ = 50 mg/dl = 500 mg/L
$V_{urine\ (L)}$ = total volume of 24-hour urine in liters
50 - urine dilution factor
1000 - conversion from mg to grams if standard is in mg

Normal values
Serum: 15 – 45 mg/dl (1.7 – 7.5 mmol/l) urea
Urine (24 h urine) : 20 – 36 g (333 – 600 mmol) urea

Clinical significance

Blood urea nitrogen (BUN) was the first endogenous compound used to evaluate kidney function through serum or plasma analysis. It is a key waste product of protein metabolism, with approximately 90% of urea excreted by the kidneys. Urea is freely filtered by the glomeruli but not actively secreted by the renal tubules. However, a significant portion - about 40 - 70% - is passively reabsorbed in the tubules. Because of this reabsorption, BUN levels may underestimate the actual glomerular filtration rate (GFR), especially in conditions of reduced renal perfusion, where more urea is reabsorbed back into the bloodstream.

Additionally, urea concentrations can be influenced by dietary protein intake, liver function, and various disease states, making it a less specific marker when used in isolation.
The serum and urinary urea value is dependent on the protein (food) intake, the volume of the excreted urine and the functional state of the kidney.

Modifications in the blood

- **Physiological variation in blood**

The variation of the blood urea as a function of the protein intake is illustrated in the following table:

Protein intake (g/kg body weight / day)	Normal urea value (mg urea/ 100 ml serum)
0.5	13 - 25
1.5	24 - 52
2.5	31 - 59

- **Pathological variation in blood**

Increased values are found in:

- Acute and chronic renal failure (in this case the urine urea is decreased)
- Extra-renal causes of anuria: cardiac deficiency, acute infectious diseases, gastro-intestinal hemorrhage, diabetes mellitus, Addison disease, dehydration by massive vomiting and diarrhea, etc.

Decreased values are found in:

- Advanced stages of uncompensated liver failure (e.g., hepatic coma), where hepatic function is severely impaired due to extensive damage to the liver parenchyma

Modifications in the urine

- **Physiological variation in urine**

Urinary urea excretion varies widely depending on diet. When considered alone, its value has limited clinical significance. The urinary elimination of urea is 20 - 35 grams/day for a normal (mixed) diet, and only 10 grams/day for a diet without any protein.

Increased values - high protein diet

Decreased values - vegetarian or low protein diet

- **Pathological variation in urine**

Increased values are found in protein hyper catabolism (e.g., in fever, trauma, burns, or hyperthyroidism)

Decreased values are found in chronic and acute renal failure and in severe hepatic insufficiency (uncompensated liver failure)

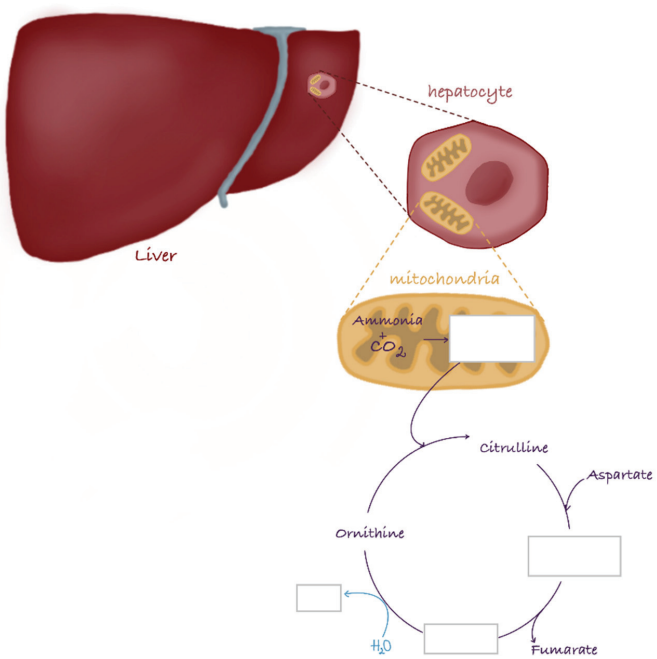
Questions

1. Which are the main sources of ammonia in human organism?

2. Explain the effect of acidosis on ammonia level.

3. Explain the role of glutamine.

4. Fill in the corresponding compounds in the figure below:



5. Which are the main factors that can influence serum urea level?

6. Why is the absorbance read at 340 nm in the case of determination of serum urea by the enzymatic method with urease?

7. What is the possible clinical significance of a value of 159 mg/dl for urea?

Lab Notes

Use this space for handwritten notes during the practical session.

L9. PROTEIN METABOLISM. DETERMINATION OF CREATININE IN SERUM AND URINE

Creatine is synthesized in the liver and kidneys from the amino acids arginine, glycine and S-adenosyl-methionine, then transported to skeletal muscles and the brain, where it is converted into the high-energy compound creatine phosphate.

Each day, a small proportion (1-2%) of creatine phosphate in muscles undergoes spontaneous, non-enzymatic dehydration to form creatinine. The daily creatinine production is proportional to an individual's muscle mass.

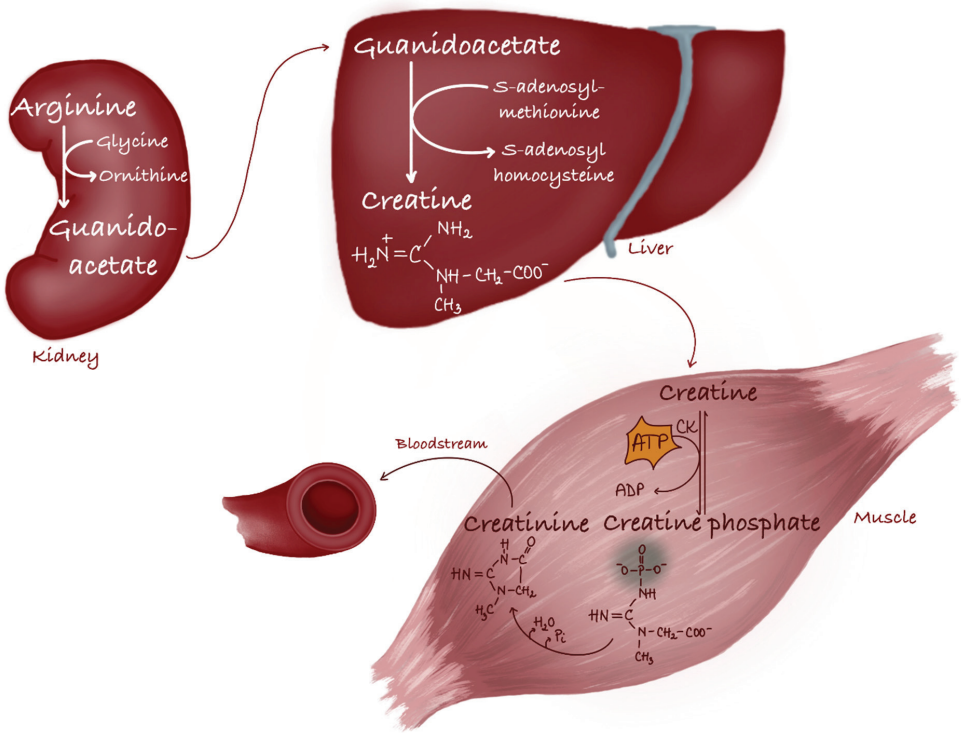


Figure 1. Creatinine synthesis

In the bloodstream, creatinine circulates unbound to plasma proteins, is freely filtered by the glomeruli, and excreted in the urine with minimal tubular reabsorption. A small fraction (less than 10%) of urinary creatinine may be secreted by the renal tubules. Decreased clearance by the kidney results in increased blood creatinine.

The primary limitation of using serum creatinine as a marker of glomerular filtration (GF) is its strong dependence on muscle mass. Creatinine production differs significantly between individuals - for example, a bodybuilder will naturally produce more creatinine than a cachectic patient. Additionally, muscle mass decreases with age, and women typically have less muscle mass than men, all of which affect serum creatinine levels. A high intake of meat can cause temporary increases in serum creatinine (up to 30%) due to the absorption of creatinine from the ingested meat. Similarly, intense physical exercise may lead to mild, transient elevations in serum creatinine levels.

As a result, estimating GF from serum creatinine in individuals with non-average muscle mass can lead to inaccuracies.

There are two main approaches to estimate GF from serum creatinine:

- Direct interpretation of serum creatinine concentration: This method is problematic because the reference range varies by age and sex, making it difficult for clinicians to assess kidney function accurately based on creatinine levels alone.
- Calculation-based estimation: More accurate GF estimates are obtained by using formulas that incorporate serum creatinine along with age, sex, and sometimes race (e.g., African Americans typically have greater muscle mass). These calculations provide results in standardized GF units (e.g., mL/s), facilitating clearer clinical interpretation. A widely used example is the MDRD (Modification of Diet in Renal Disease) equation, particularly the four-variable version that includes serum creatinine, age, sex, and race. The MDRD equation is recommended for population-level screening of reduced GF. However, it is not applicable to children or pregnant women.

Another important issue is the method used to measure creatinine. The conventional Jaffé method, which involves a reaction with picric acid in an alkaline environment, is susceptible to interference from several substances (such as acetoacetate, proteins, and glucose) which can lead to falsely elevated results. Various modifications of the method aim to reduce these interferences. An enzymatic method for creatinine determination has also been developed, offering the advantage of fewer interferences. Although this method tends to yield systematically lower values than the Jaffé method, the clinical significance of this difference is still under debate.

EXPERIMENTAL PART

Determination of creatinine in serum and urine - Jaffé method (variant Popper - Mandel - Mayer)

Principle

Creatinine in biological fluid reacts with picric acid in an alkaline medium, forming a red-orange compound. The absorbance of this complex at 520 nm is directly proportional to the creatinine concentration in the sample. However, several substances commonly present in serum (such as ascorbic acid, acetone, pyruvic acid, acetoacetic acid, glucose, uric acid, proteins, and certain antibiotics) can interfere with this reaction, leading to inaccurate results. To address this issue, various modified versions of the method have been developed to minimize interference. The method described below uses picric acid both as a deproteinizing agent and as the color reagent.

Procedure

Reagents, ml	Serum	Urine	Standard	Blank
Supernatant serum	0.2	-	-	-
Supernatant urine	-	0.2	-	-
Standard creatinine	-	-	0.2	-
Distilled water	-	-	-	0.2
Picric acid	0.6	0.6	0.6	0.6
NaOH	0.1	0.1	0.1	0.1

The tubes are mixed, left for 20 minutes at room temperature. The absorbances of Serum, Urine and Standard are measured at 530 nm, zeroing the spectrophotometer with the blank.

Calculation

The concentrations of creatinine in serum and urine are calculated with the formulas:

$$serum\ creatinine\ (mg/dl) = \frac{A_{serum}}{A_{standard}} \times c_{standard} = \frac{A_{serum}}{A_{standard}} \times 2$$

$$urinary\ creatinine\ (g/l) = \frac{A_{urine}}{A_{standard}} \times c_{standard} = \frac{A_{urine}}{A_{standard}} \times 2$$

Normal values

Serum: 0.4 - 1.5 mg/dl (45 - 95 µmol /liter)
Urine: 1 - 1.8 g / 24 hours (8.8 - 13.3 mmol / 24hours)

Pathological values

Higher than normal values appear in diseases like acute and chronic nephropathy, diseases affecting the muscle (progressive muscular dystrophy, myasthenia gravis).

Endogenous creatinine clearance

Because the creatinine quantity depends on the muscle mass and renal elimination by glomerular filtration, creatinine determination and calculation of the creatinine clearance too are methods for renal function investigation.

Creatinine clearance represents the volume of plasma (in milliliters) that is cleared of creatinine by the kidneys per minute. The result is often adjusted to a standard body surface area using the patient's weight and height. This test is frequently used to estimate the glomerular filtration rate (GFR), particularly in individuals with symptoms or a history of kidney disease, as well as in patients at risk of renal toxicity from medications such as certain antibiotics.

To perform this test accurately, a 24-hour urine collection is required.

Calculation of creatinine clearance

$$C_{cr}(ml/\text{min}) = \frac{U \times V}{P \times 1440}$$

where: U = creatinine concentration in urine (mg / ml)
V = urine volume (ml in 24 hours)
P = creatinine concentration in serum (or plasma) (mg / ml)
1440 = minutes in 24 hours

Normal values: 95 - 150 ml / min
Pathological values
In acute and chronic nephropathies, endogenous creatinine clearance decreases before the serum creatinine increases.

Questions

1. Fill in the blanks: Creatine is produced in the _____and _____from the amino acids: _____, _____and _____, then transported to _____and the _____, where it is converted into the high-energy compound called _____, which undergoes spontaneous, non-enzymatic dehydration to form _____.

2. Explain the difference between creatine and creatinine.

3. Which factors can influence serum creatinine level?

4. What does the following formula represent? Provide an explanation for each term.

$$C_{cr}(ml/\text{min}) = \frac{U \times V}{P \times 1440}$$

U =_____ P = _____
V =_____ 1440 = _____

5. Describe the principle of determining creatinine with the Jaffe method.

6. Which are the biochemical markers that you have studied are used to investigate renal function? Which one of them is better and why?

7. Which of the following sentences are True or False
   - Seum creatinine level is higher in an elder person compared to a young adult.
   - Clearence creatinine is low in a patient with kidney disfunction.
   - Serum creatinine level is higher in an adult which goes often to the gym compared to a sedentary individual.

8. A 70-year-old patient presents to the having frequent urination, especially at night in small quantities, dysuria. Laboratory tests indicate the following results: fasting blood glucose = 80 mg / dL (75-115 mg/dL), HbA1c = 5% (4-6%), Serum creatinine = 2.5 mg / dL (F<0,9 mg/dL; M<1,1 mg/dL), ALAT = 15 U/L (10-29 U/L), ASAT = 17 U/L (10-25 U/L).
Which organ might be affected and why.

Lab Notes

Use this space for handwritten notes during the practical session.

L10. PROTEIN METABOLISM. DETERMINATION OF HEMOGLOBIN AND BILIRUBIN

Heme consists of a *porphyrin* ring that holds a central iron ion.

Functions of heme:
Redox chemistry
• electron transport: cytochromes in the respiratory chain
• enzyme catalysis: cytochrome P450, cyclooxygenase, others

Reversible binding of gases
• O_2: hemoglobin and myoglobin (80-90% of all heme)
• NO: guanylate cyclase

In hemoglobin and myoglobin, the heme iron remains in the *ferrous* or Fe^{2+} state throughout the cycle of oxygen binding and release. In redox-active enzymes and in the respiratory chain, heme regularly goes back and forth between the ferrous and the *ferric* or Fe^{3+} states.

HEME BIOSYNTHESIS

Heme contained in the food is taken up from the intestines, but this is only relevant because of the iron it contains. The organic porphyrin ring is mostly synthesized from scratch. The synthetic pathway is split across two compartments; the initial and final steps occur in the mitochondria, while the intervening ones occur in the cytosol. Heme exerts feedback inhibition on the first step in the pathway.

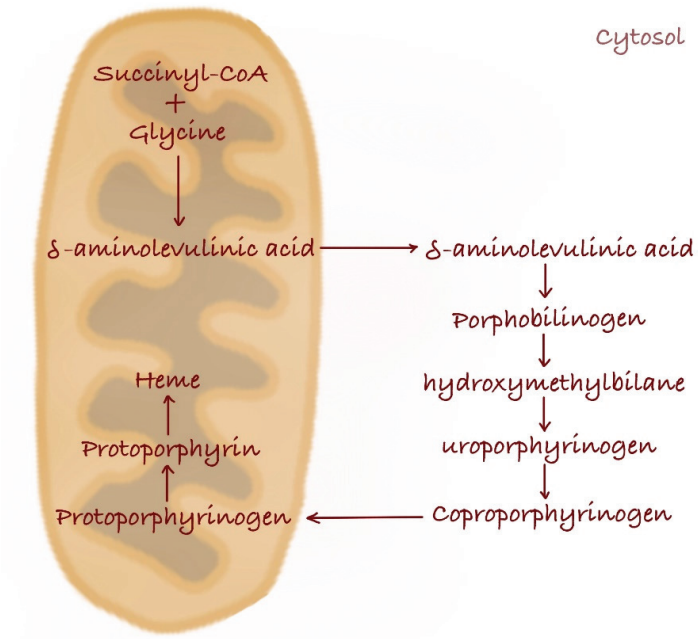


Figure 1. Heme biosynthesis

The first reaction in the porphyrin synthesis pathway occurs in the mitochondria. Glycine forms a Schiff base with pyridoxal phosphate (PLP) in the aminolevulinate synthase enzyme. The aminolevulinate synthase reaction is the committed step of heme synthesis and is subject to feedback inhibition by heme, the final product of the pathway. The reaction product, δ-aminolevulinic acid (ALA), is transported to the cytosol, where the next four reactions occur. Heme synthesis is completed by inserting the iron, catalyzed by ferrochelatase.

Disruptions of heme synthesis:
• hereditary enzyme defects (porphyrias)
• iron depletion
• vitamin B_6 deficiency-inhibition of aminolevulinate synthase
• lead poisoning-inhibition of porphobilinogen synthase

Hereditary deficiencies are known for each of the enzymes in the synthetic pathway. Clinical symptoms are due to both lack of heme and to the accumulation of biosynthetic intermediates. Heme exercises feedback inhibition on the first enzyme in the synthetic pathway, that is, δ-ALA synthase. A lack of heme therefore disinhibits this enzyme and amplifies the accumulation of intermediates upstream of the enzyme defect in question. Backed-up synthetic intermediates often undergo spontaneous conversion to aberrant products.

Inhibition of heme synthesis can also result from causes other than enzyme defects. The most common cause is iron depletion; another one is deficiency of vitamin B_6, which can result from malnutrition or, in inflammatory intestinal diseases, from malabsorption. Vitamin B_6 (pyridoxin) is the precursor of pyridoxal phosphate, the coenzyme in aminolevulinate synthase. Lead intoxication causes inhibition of porphobilinogen synthase.

EXPERIMENTAL PART

Hemoglobin determination as cyanmethemoglobin

Hemoglobin (Hb) determination is used to help diagnose anemia, hemorrhage, and other hematologic disorders. It provides more valuable information than red blood cell (RBC) count in assessing anemia. Normally, Hb levels below 12 g/dL in men and 10 g/dL in women indicate anemia. Low Hb may also occur in hemoglobinopathies, where abnormal Hb types (such as Hb S in sickle cell anemia) replace normal ones (Hb A, A_2, and F). In iron-deficiency anemia, Hb levels are reduced, and RBCs appear hypochromic (paler) and microcytic (smaller). During pregnancy, Hb concentration naturally decreases due to plasma volume expansion, a condition known as physiologic anemia of pregnancy.

Principle

The cyanmethemoglobin method is the preferred technique for measuring hemoglobin because it provides a stable standard, is easy to use, and is compatible with automated analyzers. In this method, the patient's blood is diluted with a solution containing potassium ferricyanide and potassium cyanide, which react with hemoglobin to form cyanmethemoglobin. The absorbance of this compound is then measured at 540 nm using a spectrophotometer and compared to a standard to determine the hemoglobin concentration.

Procedure

20 µL of blood are pipetted directly in **5 mL od Drabkin** solution. The samples are mixed and left for 20 minutes at room temperature. The sample absorbance is read at 540 nm, zeroing the spectrophotometer with the Drabkin solution.

Calculation

In the absence of a cyanemethemoglobin standard, the Hb concentration is calculated using the following formula:

$$Hb(g/dl) = \frac{A_{sample} \times 16144}{11000 \times 1} \times 10^{-1} \times \frac{5.02}{0.02} = A_{sample} \times 36.8$$

Where:

- 11000 = the molar extinction coefficient of Hb ($l \times mol^{-1} \times cm^{-1}$)
- 16144 = the Hb molecular mass (g/mol)
- 5.02 = total volume added (ml)
- 0.02 = volume of sample added (ml)
- 1 = path length in cm
- 10^{-1} = conversion from l to dl

Normal values: Men: 14 - 16 g/dL; Women: 12 - 14 g/dL

Clinical significance

- **Hemoglobin decreases** in conditions such as anemia, Crohn's disease, chronic renal insufficiency, chronic glomerulonephritis, paroxysmal nocturnal hemoglobinuria, hyperhydration, and in cases of bone marrow infiltration or suppression.
- **Hemoglobin increases** in dehydration, polycythemia (including polycythemia vera), and secondary polyglobulia associated with chronic hypoxia, encephalitis or certain tumors (e.g., cerebral tumors, or conditions causing increased erythropoietin production).

HEME DEGRADATION

Heme is a porphyrin ring composed of four pyrrole subunits linked by methine bridges, with a central iron atom. Bilirubin is produced through a two-step enzymatic degradation process that occurs predominantly in cells of the reticuloendothelial system, particularly in the spleen, as well as in hepatic Kupffer cells and phagocytes. These cells internalize heme, where it is first acted upon by heme oxygenase, which represents the rate-limiting step in bilirubin formation. This enzyme catalyzes the oxidative cleavage of the α-methene bridge, releasing the chelated iron and generating equimolar carbon monoxide, which is subsequently exhaled via the lungs. The reaction also yields biliverdin, a green pigment, which is then reduced to bilirubin, a yellow-orange pigment, by the NADPH-dependent enzyme biliverdin reductase. The resulting bilirubin molecule, has a compact, hydrogen-bonded conformation, rendering it virtually insoluble in aqueous solutions at neutral pH.

In circulation, bilirubin is transported bound non-covalently but reversibly to serum albumin, facilitating its solubility and systemic distribution, and also preventing its diffusion into tissues or filtration by the kidneys.

Upon reaching the liver, the albumin-bilirubin complex is exposed to the highly permeable hepatic microcirculation, which facilitates its contact with the sinusoidal surface of hepatocytes. At this interface, bilirubin dissociates from albumin and enters hepatocytes via two primary mechanisms: passive diffusion and receptor-mediated endocytosis. This uptake process enables efficient transfer of bilirubin from the bloodstream into liver cells for further processing.

Conjugation is essential to render bilirubin water-soluble, allowing for its transport across the canalicular membrane and subsequent excretion into bile. Within hepatocytes, bilirubin is conjugated with glucuronic acid by enzymes of the uridine diphosphate-glucuronosyltransferase (UDP-glucuronosyltransferase/UDPGT) family. This glucuronidation process, an important detoxification pathway, typically results in the formation of bilirubin diglucuronide through esterification of two glucuronic acid residues to the propionic acid side chains of bilirubin. Under normal conditions, diglucuronide is the predominant form; however, when bilirubin production exceeds conjugation capacity, monoglucuronide forms may predominate.

Only conjugated bilirubin can be secreted into bile, which is then delivered to the small intestine; due to its hydrophilicity and large molecular size, conjugated bilirubin cannot be absorbed by the intestinal mucosa. Upon reaching the distal ileum and colon, conjugated bilirubin is deconjugated by bacterial *β*-glucuronidases and then reabsorbed, entering the *enterohepatic circulation*. Bilirubin can be also reduced by intestinal microbiota into colorless metabolites collectively known as urobilinoids, primarily urobilinogen and stercobilinogen. Most of these compounds are subsequently oxidized to urobilin, stercobilin which give the characteristic color to stool. Some urobilinogen will be reabsorbed, enters the *enterohepatic circulation*, and a small part of it, from the blood, will be excreted in the urine where it is converted to an oxidized form, urobilin, that gives urine its characteristic yellow color.

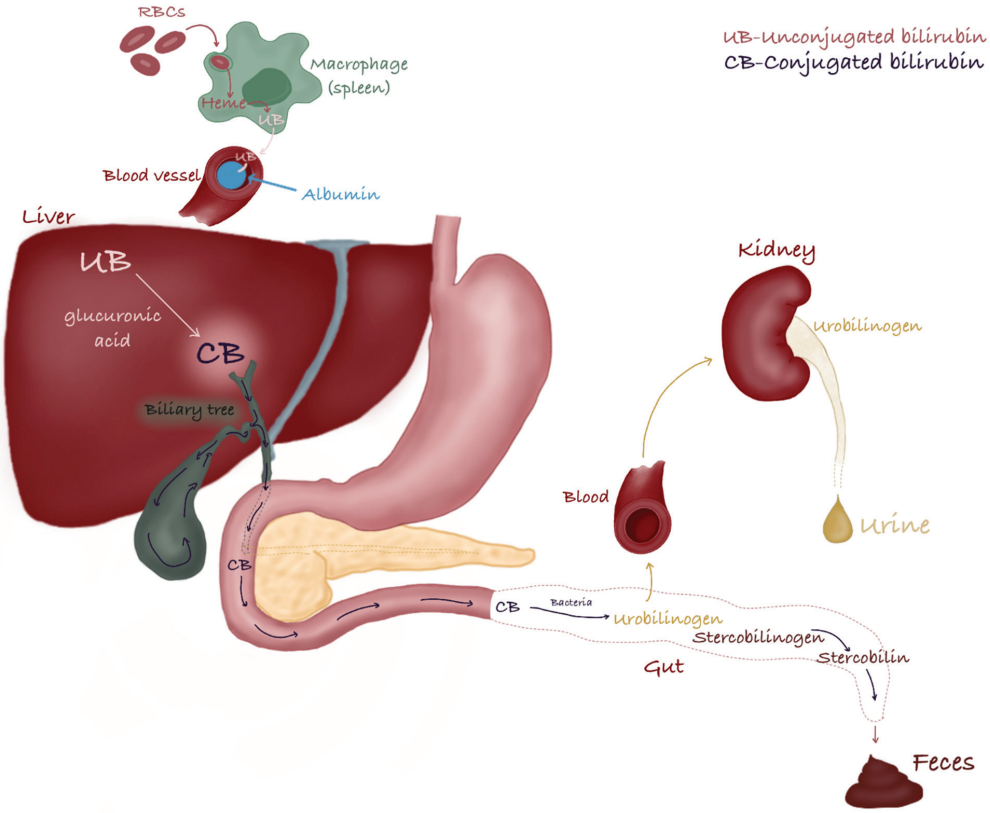


Figure 2. Bilirubin circuit

EXPERIMENTAL PART

Bilirubin determination

Principle

The methods commonly used for determining serum bilirubin are based upon Ehrlich's diazo reaction. In this reaction, bilirubin reacts with diazotized sulfanilic acid to form red-colored compounds known as azodipyrroles - a process referred to as the diazo reaction. Van der Bergh and Mueller first used this reaction to determine serum bilirubin, and since then there have been many modifications.

The method most widely accepted today is a modification of the Jendrassik and Grof diazo method. In this procedure, conjugated bilirubin (also known as direct bilirubin) reacts directly with diazotized sulfanilic acid. Total bilirubin is measured after the addition of an accelerator in an alkaline medium, which allows both conjugated and unconjugated bilirubin to react.
Unconjugated bilirubin (indirect bilirubin) is then calculated by subtraction of direct bilirubin from total bilirubin.

Reagents:

- R1 = sulfanilic acid 29 mmoles/l; HCl 0,17 moles/l;
- R2 = sodium nitrite 25 mmoles/l;
- R3 = caffeine 0.26 moles/l; sodium benzoate 0.52 moles/l;
- R4 = tartrate 0.93 moles/l; NaOH 1.9 moles/l;
- NaCl 0.9 % (physiological serum);

Interferences:

- Hemolysis leads to falsely low values because in many determinations the absorbance of the control sample increases.
- Lipemic sera give falsely high values because of the turbidity effect.
- Bilirubin is destroyed by light and heat, aspects that must be taken into account when making determinations.

Procedure for Direct bilirubin

Reagents (µl)	Sample	Blank
R1	200	200
R2	50	-
NaCl 0,9 %	2000	2000
Serum	200	200

Incubate 5 minutes at room temperature.
Measure the extinction of sample against the blank at 540 nm.

Calculation

$$direct\ bilirubin\ (mg/dl) = A_P \times 13.7$$

Procedure for Total bilirubin

Reagents (µl)	Sample	Blank
R1	200	200
R2	50	-
R3	1000	1000
Serum	200	200
Incubate 30 minutes at room temperature		
R4	1000	1000
Incubate 5 minutes at room temperature		

Measure the extinction of sample against the blank at 578 nm.

Calculation:

$$total\ bilirubin\ (mg/dl) = A_P \times 10.8$$

Unconjugated bilirubin (indirect bilirubin) is then calculated by subtraction:

$$Indirect\ bilirubin = Total\ bilirubin - Direct\ bilirubin$$

Normal values
Direct bilirubin: < 0,2 mg/dl
Total bilirubin: < 1,0 mg/dl
Indirect bilirubin: < 0,8 mg/dl

Clinical significance

Bilirubin is the end product of heme catabolism. Briefly, its metabolism involves three key steps: production (from heme breakdown), conjugation in the liver, and excretion into bile. Disruption of any of these steps can lead to hyperbilirubinemia, manifesting clinically as **jaundice** (accumulation of bilirubin in the body). The term jaundice originates from the French word *jaune*, meaning "yellow." Based on the underlying mechanism, jaundice is commonly classified into:

- pre-hepatic jaundice
- hepatic jaundice
- post-hepatic jaundice

A separate category is newborn jaundice, which has distinct physiological and pathological considerations.

1. Pre-hepatic (Hemolytic) Jaundice

This form results from increased bilirubin production due to excessive breakdown of red blood cells, overwhelming the liver's conjugating capacity. Causes include hemolytic anemias (e.g., hereditary spherocytosis, sickle cell anemia, glucose-6-phosphate dehydrogenase deficiency), autoimmune hemolysis, and hemolysis secondary to infections or toxins. Laboratory findings typically show elevated unconjugated (indirect) bilirubin in plasma, absence of bilirubin in urine (since unconjugated bilirubin is tightly bound to albumin in the bloodstream, it cannot pass through the glomerular filtration barrier under normal conditions and therefore is not excreted in urine - as a result, even in the presence of elevated serum levels, unconjugated bilirubin is absent from urine). Jaundice associated with unconjugated hyperbilirubinemia is referred to as acholuric jaundice due to the lack of urine discoloration. However, the liver conjugates as much bilirubin as it can, but the load is so high that more conjugated bilirubin is produced and excreted into bile. In the intestine, bacteria

convert much of this bilirubin into urobilinogen. Therefore, more urobilinogen is reabsorbed into the portal circulation and in the urine, raising urinary urobilinogen levels. Typically, stool has also a normal color.

2. Hepatic (Hepatocellular) Jaundice

This occurs when the liver's ability to conjugate or excrete bilirubin is impaired. Causes include viral hepatitis, alcoholic liver disease, drug-induced liver injury, cirrhosis. Depending on the predominant defect, both direct (conjugated) and indirect (unconjugated) bilirubin may be elevated. Urine may contain conjugated bilirubin (which will give a dark color to the urine) and variable (generally elevated) levels of urobilinogen. Stools may be normal or pale if excretion is significantly impaired.

3. Post-hepatic (Obstructive) Jaundice

This results from mechanical blockage of bile flow after conjugation, preventing bilirubin from reaching the intestine. Common causes include gallstones, pancreatic or biliary tumors, and biliary strictures. Laboratory features show a marked increase in direct bilirubin in plasma, bilirubinuria (dark urine), and absence (or very low levels) of urobilinogen in urine. Stools are typically pale or clay-colored due to the lack of stercobilin.

4. Neonatal Jaundice

Neonatal jaundice refers to the clinical manifestation of elevated total serum bilirubin, a condition termed neonatal hyperbilirubinemia, resulting from bilirubin deposition in the skin and other tissues. This is the most common medical condition observed during the first two weeks of life; it affects approximately 60% of term newborns and up to 80% of preterm infants within the first week after birth. In most cases, neonatal jaundice is mild, transient, and self-limiting, representing **physiologic jaundice**. This type of jaundice is caused by increased bilirubin production from hemolysis of erythrocytes and an immature bilirubin metabolic system. However, it is essential to distinguish this benign form from pathologic jaundice, which requires prompt evaluation and treatment. Neonatal hyperbilirubinemia is broadly classified into:

- Unconjugated hyperbilirubinemia - the most common type, usually associated with physiologic jaundice, breastfeeding jaundice, hemolysis, or impaired conjugation.
- Conjugated hyperbilirubinemia - always pathologic, indicating underlying hepatocellular disease or biliary obstruction, and requiring urgent investigation.

When jaundice is detected, identifying the underlying cause is crucial. While the majority of newborns present with unconjugated hyperbilirubinemia, conjugated hyperbilirubinemia must not be overlooked, as it signals a medical or surgical disorder such as biliary atresia, neonatal hepatitis, or metabolic disease. Failure to recognize and treat pathologic jaundice, especially severe unconjugated hyperbilirubinemia, can result in bilirubin encephalopathy and its irreversible neurological sequelae, known as kernicterus. Early differentiation between physiologic and pathologic jaundice, combined with appropriate monitoring and intervention, is essential to prevent complications and ensure optimal neonatal outcomes.

5. Genetic disorders causing jaundice

- **Gilbert Syndrome**

Gilbert syndrome is a common hereditary disorder of hepatic bilirubin metabolism, inherited in an autosomal recessive pattern. It results in mild to moderate unconjugated hyperbilirubinemia, typically manifesting as intermittent episodes of jaundice. The overall prognosis is excellent, as the condition does not progress to significant liver damage. However, affected individuals may exhibit an increased susceptibility to drug toxicity when exposed to medications that interfere with bilirubin conjugation or clearance. Biochemically, patients with Gilbert syndrome display isolated elevations of unconjugated bilirubin, while other liver function parameters remain within normal limits. The condition is usually asymptomatic and does not require therapeutic intervention. Nevertheless, certain physiological or environmental stressors, such as fasting, intercurrent illness, dehydration, or menstruation, can precipitate visible jaundice.

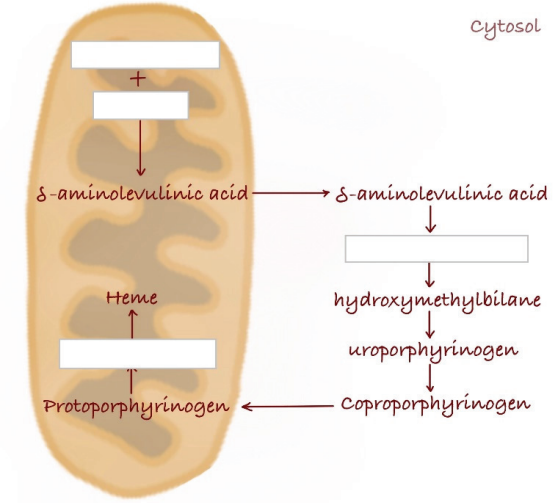
- **Crigler-Najjar Syndrome**

Crigler-Najjar syndrome is a rare, autosomal recessive congenital disorder characterized by nonhemolytic jaundice. The condition results from mutations in the UGT1A1 gene, leading to either a complete absence of the enzyme UDP-glucuronosyltransferase (type I) or a profoundly reduced enzymatic activity (type II). Because this enzyme is essential for the hepatic conjugation of bilirubin, its deficiency causes marked unconjugated hyperbilirubinemia, typically appearing shortly after birth. In its severe form (type I), bilirubin levels can rise to neurotoxic concentrations, posing a high risk of kernicterus and permanent neurological damage if left untreated. Early recognition and prompt intervention are crucial to prevent irreversible complications.

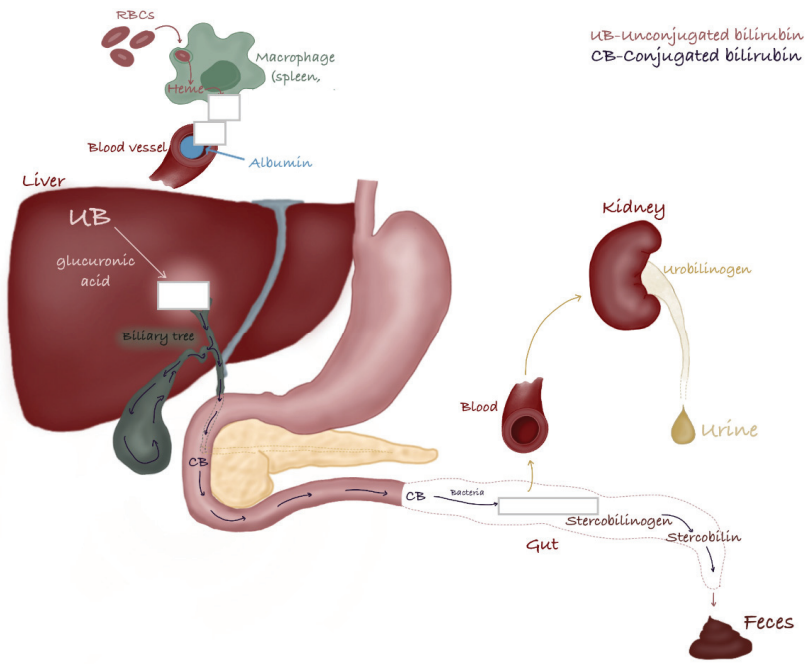
Questions

1. Provide the correct compounds to complete the figure below:

   Heme biosynthesis:



   Heme degradation:



2. What methods were employed in the laboratory for hemoglobin and bilirubin determination, and what are the underlying principles of these methods?

3. For each of the following pathologies, choose the associated type of jaundice:

Pathology	Type of jaundice
Hepatitis Drug-induced liver injury	Pre-hepatic jaundice
Gallstones Hemolytic anemia	Hepatic jaundice
Pancreatic cancer Glucuronytransferase deficiency	Post-hepatic jaundice

4. Fill in the following table with the appropriate information:

	PLASMA		URINE			FECES	
	Indirect Bilirubin	**Direct Bilirubin**	**Urobilinogen**	**Direct Bilirubin**	**Color**	**Stercobilin**	**Color**
Pre-hepatic jaundice						Normal	Normal
Hepatic jaundice	↑	↑					
Post-hepatic jaundice			↓ or absent	↑↑	Dark		

5. What clinical conditions could be suggested by a hemoglobin level of 7 g/dL in a 60-year-old patient?

6. What might a total bilirubin concentration of 10 mg/dl indicate in a newborn?

7. Under what circumstances may urobilinogen be absent from urine?

8. Which form of bilirubin can be detected in urine, and what does its presence suggest clinically?

9. What other biomarker modifications could be associated with prehepatic/hepatic/posthepatic jaundice?

Lab Notes

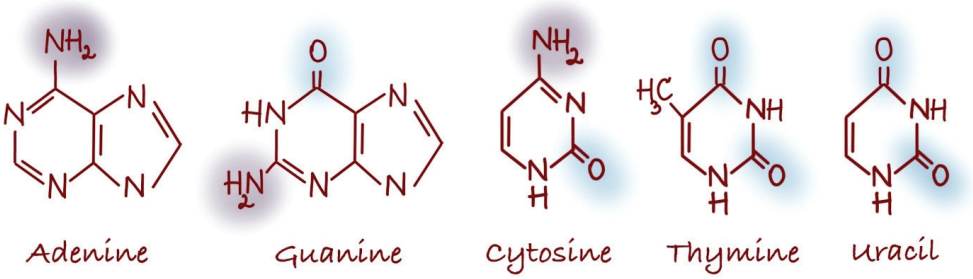
Use this space for handwritten notes during the practical session.

L11. NUCLEIC ACIDS METABOLISM. DETERMINATION OF URIC ACID

Nucleic acids are fundamental, universal components of all living organisms. As informational macromolecules, they play a central role in storing, transmitting, and expressing the genetic blueprint necessary for life.

Structurally, nucleic acids are high-molecular-weight polymers known as polynucleotides. These are composed of repeating monomeric units called nucleotides, linked covalently by 3'-5' phosphodiester bonds. Each nucleotide comprises three key components:

- **a nitrogenous base** - an aromatic heterocycle (purine: A, G and pyrimidine: C, T, U)
- **a pentose** (ribose or deoxyribose)
- **a phosphate group**



There are two primary types of nucleic acids: deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Both are essential to the structure, function, and regulation of cells, but they differ in several key aspects, including their chemical composition, structure, and biological roles.

DNA is the hereditary material in most living organisms. It stores and preserves the genetic instructions used in the development, functioning, and reproduction of all known life forms and many viruses. DNA is mainly located in the cell nucleus (with some found in mitochondria and chloroplasts) and is stable under cellular conditions, thus enabling long-term storage of genetic information. It is capable of self-replication, ensuring transmission to daughter cells.

The DNA molecule typically consists of two complementary polynucleotide strands. These strands are held together by specific hydrogen bonds between paired nitrogenous bases: adenine (A) pairs with thymine (T) via two hydrogen bonds, while guanine (G) pairs with cytosine (C) via three hydrogen bonds. Importantly, the two strands are antiparallel-one runs in the 5' to 3' direction, and the other in the 3' to 5' direction, and together they form a right-handed double helix around an imaginary central axis.

RNA plays a critical role in the expression of genetic information. It acts as the intermediary between DNA and protein synthesis, but also serves regulatory, structural, and catalytic roles. RNA is primarily found in the cytoplasm, though it is also present in the nucleus. RNA is ess stable than DNA, due to the reactive 2'-OH group, and it is versatile in function:

- mRNA (messenger RNA): Carries genetic information from DNA to ribosomes for protein synthesis.
- tRNA (transfer RNA): Brings amino acids to the ribosome during translation.
- rRNA (ribosomal RNA): A structural and functional component of ribosomes.
- regulatory RNAs: Such as microRNA (miRNA) and small interfering RNA (siRNA), involved in gene expression control.
- catalytic RNAs: Also known as ribozymes, capable of enzymatic activity.

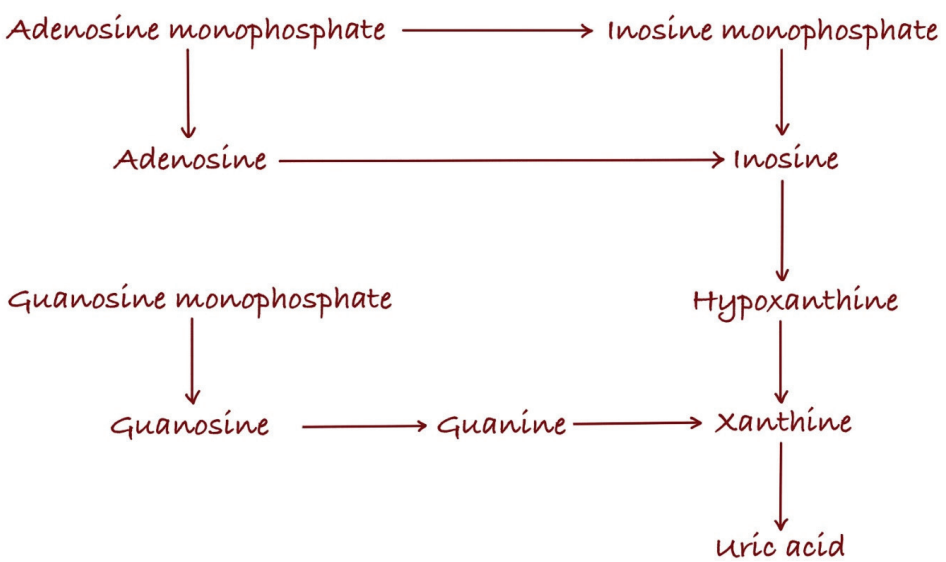
Catabolism of nucleic acids

The catabolism of nucleic acids involves their enzymatic breakdown into their constituent elements: nitrogenous bases, pentoses, and phosphoric acid. The main stages in nucleic acid catabolism are:

1. Hydrolysis of polynucleotides into nucleotides: **Deoxyribonucleases** (DNases) and **ribonucleases** (RNases) break down DNA and RNA into mononucleotides.
2. Degradation of nucleotides into nucleosides and free phosphate: **Nucleotidases** remove the phosphate groups, producing nucleosides.
3. Breakdown of nucleosides into nitrogenous bases and sugars: **Nucleosidases** or **nucleoside phosphorylases** then split the nucleosides into free nitrogenous bases and pentoses (ribose or deoxyribose).
4. Further catabolism of purine and pyrimidine bases into final excretory products.
    - Pyrimidines are broken down into leads to water soluble and easily excreted products: CO_2, H_2O, and NH_3.
    - Purines are degraded to uric acid (2,6,8-trihydroxypurine).

Catabolism of purines

Initially, the purine ring undergoes deamination, followed by progressive oxidation to hypoxanthine, xanthine, and finally uric acid. In humans and higher primates, uric acid is the end-product of purine catabolism. However, in most mammals, uric acid is further broken down into allantoin (via the enzyme uricase).



Uric acid, produced mainly in the liver from both endogenous and exogenous purines. The normal concentration of uric acid in serum is between 1–7 mg/100 mL, corresponding to a total body urate pool of approximately 1 gram. About two-thirds of uric acid is excreted by the kidneys into the urine via tubular secretion, while the remaining one-third is eliminated through the intestine.

Uric acid is a weak acid with a pKa of 5.75 in blood and 5.25 in urine. At the physiological pH of 7.4 in the extracellular compartment, about 98% of uric acid circulates in its ionized form, as the urate anion. Due to the high concentration of Na^+ in the extracellular fluid, urate is primarily found

as monosodium urate, which has a solubility limit of 6 mg/dL (360 μmol/L). When urate concentrations exceed this solubility threshold, the risk of monosodium urate crystal formation and precipitation increases. Pathologically, the urate pool can increase to 25-30 grams due to either impaired renal excretion (in 70-80% of cases) or increased production (20% of cases). This leads to elevated serum uric acid levels, and since uric acid is poorly soluble, it can deposit in soft tissues, causing a disease known as gout.

Gout is a common and painful form of inflammatory arthritis caused by the deposition of monosodium urate crystals in joints and soft tissues due to hyperuricemia. A typical acute gout attack is characterized by sudden, severe joint pain, often at night. Gout is often monoarticular but may become polyarticular in chronic cases. The classically affected joint is the first metatarsophalangeal joint, but in some cases other joints can suffer: ankles, knees, wrists, fingers. Concomitantly, swelling, redness, and warmth in the affected joint can also be present. Attack usually resolves in 7-10 without treatment. Chronic gout is characterized by recurrent attacks, formation of tophi (deposits of urate crystals under the skin), and joint deformity and damage. It can also lead to chronic kidney disease (gouty nephropathy).

The diagnosis is established by:

- Joint aspiration and microscopy: Presence of needle-shaped, negatively birefringent monosodium urate crystals under polarized light.
- Elevation of serum uric acid levels

EXPERIMENTAL PART

Determination of uric acid in serum or plasma - Colorimetric method

The determination of uric acid concentration can be performed using both chemical and enzymatic methods.

Principle

It is a method based on the reductive character of uric acid. Uric acid reduces phosphotungstic acid, in alkaline medium, forming a blue colored complex. The color intensity is directly proportional to the uric acid concentration in the analyzed sample. Ascorbic acid interferes with the reaction, but it is eliminated by the alkaline pH (10-11) of the buffer.

Procedure

Reagents, µl	**Sample**	**Standard**	**Blank**
Distilled water	900	900	1000
Serum	100	-	-
Uric acid standard (5 mg/dl)	-	100	-
Buffer solution	250	250	250
Mix and let for 5 minutes at room temperature, then add:			
Phosphotungstic reagent	100	100	100
Mix and incubate at room temperature for 15 minutes. Read the absorbances of sample and standard at 710 nm, zeroing the spectrophotometer against the blank.			

Calculation

$$uric\ acid\ (mg/dL) = \frac{A_{sample}}{A_{standard}} \cdot c_{standard} = \frac{A_{sample}}{A_{standard}} \cdot 5$$

Normal Values

1 - 7 mg/100 ml serum.

Clinical significance

- **Hyperuricemia** (elevated blood uric acid level) is caused by a variety of diseases or conditions. These include an increased synthesis of uric acid or a decreased excretion of uric acid. Deposition of uric acid in synovial as monosodium urate leads to ***gout***.
    - Overproduction of uric acid:
        - High-purine diet (red meat, organ meats, seafood)
        - Alcohol consumption (especially beer)
        - Tumor lysis syndrome (in cancer treatment, leukemia, myeloma, Hodgkin lymphoma)
        - Extended burns
        - Genetic disorders (e.g., Lesch-Nyhan syndrome)
    - Underexcretion of uric acid (most common):
        - Kidney disease
        - Certain medications (e.g., diuretics, aspirin/salicylates)
        - Dehydration
        - Lead toxicity

- **Hypouricemia** (decreased blood uric acid level) is not common. It may occur from severe hepatocellular disease or impaired renal tubular reabsorption of uric acid, in xanthine oxidase defect. Occasionally, it is a result of treatment for hyperuricemia.

Questions

1. Fill in the blanks: Nucleic acids are macromolecules composed of repeated units called _____, each made of a nitrogenous base, a _____ sugar, and a phosphate group. The catabolism of nucleic acids involves their enzymatic breakdown into _____, nucleosides, and nitrogenous bases. While pyrimidines are degraded into _____-soluble products like CO_2, H_2O, and NH_3, purines are ultimately converted into _____, which in humans is the end product of purine metabolism.

2. Match each enzyme with its role:

Xanthine oxidase	Hydrolyses of nucleotides to nucleosides and phosphate
Uricase	Converts xanthine to uric acid
Nucleotidase	Breaks nucleosides into free bases and sugars
Nucleosidase	Oxidizes uric acid to allantoin

3. What is the significance of the solubility threshold of uric acid (~6 mg/dL)?

4. What factors contribute to the supersaturation and crystallization of uric acid in soft tissues?

5. Describe the pathogenesis of a gout attack. What physical signs are typically seen?

6. What are the main causes of hypouricemia?

7. What is the principle of the colorimetric method used to determine uric acid in serum?

8. Which statements about uric acid are TRUE?
   A. It is the final product of pyrimidine catabolism in humans
   B. It circulates primarily as monosodium urate at physiological pH
   C. It is highly soluble in plasma at concentrations above 10 mg/dL
   D. It is produced from purine degradation via xanthine
   E. It is mostly excreted through the kidney

Lab Notes

Use this space for handwritten notes during the practical session.

L12. MINERAL METABOLISM. DETERMINATION OF pH. SERUM BUFFERING CAPACITY. DETERMINATION OF IONS IN BIOLOGICAL FLUIDS

I. pH DETERMINATION. SERUM BUFFERING CAPACITY

pH Notion

The pH of a solution is defined as the minus decimal logarithm of hydrogen ion concentration:

$$pH = -log\,[H^+]$$

- **Neutral solutions** have the hydrogen ions concentration equal to 10^{-7} and pH = 7.
- **Acidic solutions** have the hydrogen ions concentration higher than the hydroxyl ion concentration, and higher than 10^{-7}, and pH < 7 (**acidic pH**).
- **Alkaline solutions** have the hydrogen ions concentration lower than the hydroxyl ion concentration, and lower than de 10^{-7}, and pH < 7 (**basic pH**).

The arrangement of pH values on a scale from 0 to 14 permits the identification of hydrogen ion concentration in solutions.

pH scale has a zone with excess of hydrogen ions (acidic zone $0 \leq pH < 7$), a neutral zone at pH = 7 and a zone with hydroxyl ions excess (alkaline $7 < pH \leq 14$).

Blood pH measurement is valuable for diagnosing various diseases and assessing organ function. The normal blood pH ranges from 7.35 to 7.45.

A decrease in pH below 7.35 indicates acidosis, a condition that may occur in uncontrolled diabetes mellitus.

An increase in pH above 7.45 indicates alkalosis, which can result from prolonged vomiting or pulmonary hyperventilation.

pH Measurement

- pH measurement with colored indicators and pH indicating paper pH
- pH measurement with the pH-meter (electrometric)

Buffer solutions

- A buffer solution is a chemical mixture that helps maintain a constant hydrogen ion concentration (that means of the pH) when an acid or a base is added.
- A buffer system is formed of a mixture (in solution) of two substances with complementary actions:
    - **a weak acid + the salt of a weak acid with a strong base** or
    - **a weak base + the salt of a weak base with a strong acid**

- **Buffering capacity** = number of H^+ equivalents (respectively OH^-) needed to change the pH of a given buffer volume by a pH unit.
- The most used buffer systems contain an acid and its conjugated base;

- Buffer systems, mostly used in biochemistry, cover the interval pH = 6-8;
- During their functioning, the components of a buffer system pass from one form to the other one (A^-/AH);

  - when an acid is added (H^+): $A^- + H^+ \leftrightarrow HA$

  - when a base is added (OH^-): $AH + OH^- \leftrightarrow A^- + H_2O$
- In human organism, the buffer systems, together with other regulatory mechanisms, ensure that pH remains close to 7.4 ± 0.5.
- The most important buffer systems in the human organism are bicarbonate/ carbonic acid buffer system; protein buffer system; phosphate buffer system; hemoglobin buffer system.

Hemoglobin buffer system

In erythrocytes, hemoglobin is the primary buffering system, acting through the imidazole groups of histidine residues. Similar to what occurs in plasma, erythrocytes neutralize the H^+ ions generated from CO_2 produced by cellular metabolism. Hemoglobin buffers this excess of protons through two mechanisms:

1. **Direct buffering via ionizable groups** at the intracellular pH of 7.2. These include the four amino-terminal groups of the globin chains and the imidazole nitrogen of histidine side chains:

   $$Hb + H^+ \leftrightarrow HHb$$

2. **The Bohr effect**, in which the H^+ ions produced by the reaction:

   $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$, are taken up into erythrocytes and transported as HHb^+ to the lungs.

   There, the reverse reaction takes place: $HCO_3^- + H^+ \leftrightarrow CO_2 + H_2O$, allowing CO_2 to be exhaled through respiration.

Phosphate buffer system ($HPO_4^{2-}/H_2PO_4^-$)

The overall reaction of this buffer system is:

$$HPO_4^{2-} + H^+ \leftrightarrow H_2PO_4^-$$

To maintain a blood pH of 7.4, the **$HPO_4^{2-}/H_2PO_4^-$** ratio needs to be **4:1** (pKa = 6.8). Because extracellular fluid contains relatively low levels of phosphate, the phosphate buffer system primarily functions **intracellularly**, where, together with proteins, it serves as one of the major intracellular buffering systems.

In the kidneys, this buffer system contributes to the neutralization of hydrogen ions (H^+) actively secreted into the tubular fluid. Phosphate binds to these protons, helping to limit the drop in urinary pH and enabling continued H^+ secretion.

Protein buffer system

The protein buffer system is one of the most important buffering systems, alongside the bicarbonate system. The buffering capacity of proteins is due to the presence of acidic and basic side chains within the amino acid residues of the polypeptide chain, which can donate or accept protons (H^+).

Proteins act as buffers primarily due to the **amino and carboxyl terminal groups**, which can accept or donate H^+ ions. Plasma proteins (especially albumin) act as buffers at physiological pH (7.4) mainly through the histidine residues in their structure. These residues can accept a proton at the imidazole group (pKa = 7.3), according to the following reaction:

$$Protein^- + H^+ \leftrightarrow Protein \cdot H$$

In plasma, albumin can neutralize H^+ ions, including those derived from carbonic acid.

Carbonic acid - bicarbonate buffer system

The acid-base balance involves the bicarbonate buffer system. In chemical terms, the bicarbonate buffer system can be considered in the same way as any other chemical dissociation. Normally, the ratio of HCO_3^- and H_2CO_3 equivalents is 20/1, for example 24 mEq bicarbonate and 1.2 mEq carbonic acid. Instead of the ratio $[HCO_3^-]/[H_2CO_3]$, it is more practical to use the ratio **$[HCO_3^-]/pCO_2$**, where pCO_2 represents the partial pressure of carbon dioxide in the blood. A **decrease in this ratio leads to acidosis**, whereas an **increase results in alkalosis**.

The type of acid-base disorder is determined by the primary factor responsible for altering this equilibrium.

- **Metabolic acidosis or alkalosis** develops when the initial change involves **bicarbonate concentration (HCO_3^-)**.
- **Respiratory acidosis or alkalosis** develops when the initial change involves the levels of the partial pressure of **carbon dioxide (pCO_2)**.

Depending on the pH modifications, compensated or uncompensated acidosis or alkalosis will be produced. When pH value is not modified, we talk about a compensated acidosis or alkalosis. When pH decreases lower than 7.35, an uncompensated acidosis is installed; when pH increases higher than 7.45 we can talk about an uncompensated alkalosis. pH blood values lower than 6.9 or higher than 7.9 are incompatible with life.

Respiratory acidosis may occur in conditions associated with hypoventilation or impaired gas exchange, such as chronic obstructive pulmonary disease (COPD), resulting in a primary increase of pCO_2. This results in a decrease in pH. Compensation occurs via the renal system, which increases the reabsorption of bicarbonate, enhancing the buffering capacity and thereby assisting in the normalization of pH.

Respiratory alkalosis may occur in conditions associated with hyperventilation, such as anxiety, pain, high altitude or fever, resulting in a primary decrease in pCO_2. This loss of carbon dioxide causes an increase in pH. The renal system compensates by increasing the excretion of bicarbonate, which helps to restore pH levels to normal.

Metabolic acidosis may occur in conditions such as diabetic ketoacidosis, chronic renal insufficiency, or severe diarrhea, which leads to a primary decrease in plasma bicarbonate concentration. This results in a decrease in pH. Compensation is achieved through the respiratory system, which responds by hyperventilation, thereby lowering the partial pressure of carbon dioxide (pCO_2), which lowers carbonic acid concentration and raises pH toward normal values.

Metabolic alkalosis may occur in conditions such as prolonged vomiting or excessive bicarbonate administration, which leads to a primary increase in plasma bicarbonate concentration. As a result, there blood pH increases. The respiratory system compensates by hypoventilation, leading to an increase in pCO_2, which raises carbonic acid concentration and lowers pH toward normal values.

Table 1. Characteristic Changes in pH, pCO_2, and $[HCO_3^-]$ in Acid-Base Disorders

Disorder		**pH**	**pCO_2**	**$[HCO_3^-]$**
Respiratory acidosis	Uncompensated	↓	↑	**N**
	Compensated	**N (slightly ↓)**	↑	↑
Respiratory alkalosis	Uncompensated	↑	↓	**N**
	Compensated	**N (slightly ↑)**	↓	↓
Metabolic acidosis	Uncompensated	↓	**N**	↓
	Compensated	**N (slightly ↓)**	↓	↓
Metabolic alkalosis	Uncompensated	↑	**N**	↑
	Compensated	**N (slightly ↑)**	↑	↑

The **alkaline reserve** contains the entire amount of bases that the blood uses as buffer systems to neutralize the acids found in circulation, thereby maintaining a constant blood pH within physiological limits. Currently, we talk only about the quantity of bicarbonates in the blood (plasma bicarbonates).

Practically, bicarbonates are not determined as they are, but the CO_2 quantity in the bicarbonates (Van Slyke method). Van Slyke and Cullen defined alkaline reserve as the CO_2 quantity (milliliters) from 100 milliliters of plasma, at 0^0C and 760 mm Hg.

Bicarbonate determination is only an informative method, which can be interpreted only taking into account the rest of the clinical data.

The introduction of the Astrup micro-method for evaluating the acid-base equilibrium has represented a significant advancement in this field. The Astrup method also takes into account the buffering effect of hemoglobin as well as the role of carbonic anhydrase within erythrocytes. It is based on the inverse relationship between the partial pressure of carbon dioxide (pCO_2) and blood pH values. Notably, this technique requires only a few drops of blood to determine pH, pCO_2, base excess, and actual bicarbonate concentration.

II. ELECTROLYTE DETERMINATION IN BIOLOGICAL FLUIDS

Several chemical substances are dissolved in the human biofluids:

- organic compounds with low molecular weight (glucose, urea, amino acids, uric acid, fatty acids, triacylglycerols, cholesterol, etc.)
- macromolecular organic compounds (proteins)
- electrolytes with a role in the water distribution and retention, osmotic pressure, pH.

Mineral metabolism has a very important role in the maintenance of morph-functional integrity of the organism. Mineral elements are found as ions, having important functions as:

- role of Na^+ and K^+ in regulation of the hydro-electrolytic and acid-basic equilibrium.
- role of Ca^{2+} in blood coagulation and neuromuscular excitability.
- role in enzyme activation: Mg^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+}, etc.

Generally, any severe pathological condition is accompanied by hydro electrolytic disturbances, underscoring the importance for clinicians to be well acquainted with the principal ions, their interactions with general metabolism, and the normal serum levels of the major electrolytes.

The electrolyte quantity can be expressed in milliosmoles/liter:

$$\frac{mOsm}{l} = \frac{mg/l}{atomic\ weight}$$

or in milliequivalents/liter:

$$\frac{mEq}{l} = \frac{mg/l}{atomic\ weight}\ x\ valence$$

The normal concentrations (mEq/L) of the principal cations and anions in blood serum are illustrated in the Gamble diagram. As observed, the total cation number equals the total anion number.

It can be observed that the cellular liquid has as predominant cation K^+ and as anion HPO_4^-. In the extracellular space, the main cation is Na^+ and anion Cl^-. The osmogram is quite similar with the ionogram, with the difference that the values are in millimols. The normal plasma osmogram is approx. 300 mOsm/kg, and for urine, approx. 1200 mOsm/kg.

Table 2. Gamble diagram

Ions	Plasma	Interstitial liquid	Cellular liquid
Na^+	141	145.5	10 - 30
K^+	5	4.5	100 - 150
Ca^{2+}	5	3.5	0 - 4
Mg^{2+}	2	1.5 – 2.5	6 - 34
Total cations	153	155	*
Cl^-	101	112 - 117	0 – 70
Buffer bases			10 - 15
HCO_3^-	25	29	
Proteins	17		
Residual anions			
$H_2PO_4^-$	2	2,3	4 – 150
SO_4^{2-}	1	1	4 – 30
Organic acids	7	6.7	Variable
Total anions	153	156	*
Total mEq/l	306	311	*

A. Cations

a. Sodium (Na): normal values = 135 - 145 mEq/l

- is the main extracellular cation, participating in the regulation of the osmotic pressure, plasmatic volume (its concentration is higher comparing to the rest of cations) and acid-base equilibrium (the main buffer system is sodium bicarbonate/carbonic acid = 20/1)
- it participates in the regulation of the nervous system and muscle activity
- it participates in maintaining of the potential difference on one side and the other of the cell membrane, having an important role in nervous impulse transmission
- it has the role of activator of certain enzymes (alfa-amylase and beta-galactosidase)
- it has a role in the transmembrane transport (Na^+/K^+ - ATP-ase)
- sodium is eliminated through the kidneys, perspiration, intestine, with its regulation controlled by mineralocorticoid hormones (aldosterone).

Hyponatremia and hypernatremia are conditions influenced by water distribution across different body compartments.
Hyponatremia: severe diarrhea, massive perspirations, severe burnings, chronic nephritis, diuretics' administration, cortico-suprarenal insufficiency, diabetes mellitus with acidosis, after surgical interventions, renal insufficiency.
Hypernatremia: pyloric stenose, hyperaldosteronism, corticotherapy, prolonged fever states.

b. Potassium: **normal values = 3.8 – 5.4 mEq/l**

- it is the main intracellular cation
- it participates in the maintaining of the potential difference on one side and the other of the cell, having an important role in nervous impulse transmission (increasing of intracellular potassium produces the muscle excitability)
- during the muscle contraction, potassium passes in the extracellular compartment and sodium in the intracellular one
- It activates some enzymes (pyruvate kinase, carbamoyl phosphate synthase)
- It participates in the transmembrane transport (Na/K- ATP-ase)

Hypokalemia: it is accompanied by cardiac and neuro-muscular problems, and it is found in surgical syndromes (high intestinal obstruction, pyloric stenose), alkalosis, diabetic coma, hypoglycemic coma, Cushing syndrome, tubular nephropathies.
Hyperkaliemia: cell destructions (hemolytic syndromes, toxico-septic shock, extended burnings), renal insufficiency.

c. Calcium: normal values = 4.5 – 5.5 mEq/l (9 - 11mg/dl)

- In plasma it is found especially in two forms: 60% as free calcium (diffusible or nonionizable) and 40% as calcium proteinate (non-diffusible calcium)
- Calcium varies as a function of the acid-base equilibrium state (it increases in acidosis and decreases in alkalosis)
- It participates in the blood coagulation processes
- It is involved in the regulation of membrane permeability for Na^+ and K^+ (Ca^{2+} increased concentrations decrease cellular permeability for Na^+ and *vice versa*
- It is involved in muscle contraction (patients with decreased calcium present muscle fasciculations due to the Ca^{2+} decrease at the level of nervous junctions)

- Calcium ions participate in the regulation of excitability (at an alkaline pH, proteins liberate H^+, their negative charges increase, calcium is fixed to proteins, its concentration **decreases** in blood and the neuromuscular excitability increases; in acidic pH proteins fix H^+, calcium ions are liberated from proteins, its plasma concentration **increases,** and the neuromuscular excitability decreases)
- Calcium is fixed in bones as carbonic and hydroxylic apatites (99% of the organism calcium is fixed in bones and teeth) having role in maintaining calcemia

Hypocalcemia: appears in hyperparathyroidism (tetany, scurvy), hyperthyroidism, chronic renal insufficiency, increasing of calcitonin
Hypercalcemia: massive bone destructions, hyperthyroidism, D hypervitaminosis.

d. Magnesium: normal values = 1.9 – 2.5 mg/dl

- It is found preponderantly intracellularly
- It is an enzymatic activator (kinases, phosphatases)
- It participates in the nucleic acids' synthesis
- It is a constituent of bones and teeth
- Together with Ca^{2+}, Na^+ and K^+ participates in the regulation of neuromuscular excitability
- It can be an indicator of the renal function: it is filtered at the glomerulus's level and is reabsorbed at the tubular level; in the case of a deficient renal function its reabsorption increases and implicit its blood concentration

Hypomagnesemia: vomiting, diarrhea, cirrhosis, diabetic coma, scurvy
Hypermagnesemia: renal insufficiency, hyperthyroidism, Addison's disease

e. Iron: normal values = 50 – 180 μg/dl

- It contributes to the hemoglobin synthesis and of some redox enzymatic systems
- It is a constituent of Hb, myoglobin, catalase, peroxidase
- It is stored as ferritin
- It is transported in blood by a beta globulin – transferrin

Hyposideremia: iron deficiency anemia, post hemorrhagic anemia, acute infections.
Hypersideremia: idiopathic hemochromatosis, megaloblastic anemia, aplastic anemia, hemolytic anemia.

B. Anions

b. Phosphorus: normal values = 3 – 4.5 mg/dl

- It is, together with the proteins, the main intracellular anion
- It is found in serum combined with proteins (phosphoproteins, nucleoproteins), acido-soluble (inorganic phosphorus, glucidic esters, creatine phosphate, triphosphoric acids, phosphoenolpyruvate) and phospholipids (lecithins, cephalins, sphingomielins)
- It has a role in the formation of the bone tissue
- It is important for storing and transfer of energy in the organism
- It is important in glucose, lipid metabolisms, in maintaining of the acid-base equilibrium

Hypophosphatemia: scurvy, celiac disease, hyperparathyroidism.

Hyperphosphatemia: hypoparathyroidism, renal insufficiency, hypervitaminosis D.

c. Chlorine: normal values = 98 - 110 mEq/l

- It is the predominant anion in the extracellular space
- It is a component of the gastric juice
- It is involved in the equilibrium of liquids and electrolytes

Hyperchloremia: metabolic acidosis
Hypochloremia: metabolic alkalosis.

EXPERIMENTAL PART

Determination of ASTRUP Parameters

The epoc ASTRUP system consists of two components: epoc Reader and epoc Host.

Operation of the epoc ASTRUP device:

1. Connect the device to a power outlet.
2. Turn on the device.
3. Press and hold the Reader button (on the epoc Reader) → GREEN light appears.
4. Press the RED button on the epoc Host → The screen turns on, displaying the login page.
5. Enter a user ID and password, then press the Connect button → SEARCHING.
6. Tap the Discovery icon (Antenna - on the epoc Host component).
7. Press and hold Rdr 888 → "Perform blood analysis"!!
8. Enter patient information: The patient ID is entered via the keyboard (bottom right), along with additional settings related to respiratory therapy, age, and gender.
9. Using the GREEN arrow, navigate to Tab 2 → HEMODILUTION = select NO!!!
10. Press Tab 1.
11. OPEN the ANALYSIS CARD (starting from the notch).
12. Insert the analysis card:
    - Position the blue arrow label facing up and the sensor module towards the Reader.
    - Insert the card into the card slot in the epoc Reader until you feel slight resistance and hear a small "click"!
    - Push the analysis card slightly beyond this point to "lock" it in position.
    - Avoid sudden stops or uneven speed while inserting the card.
13. Wait → until the calibration process is complete.
14. On the epoc Host, the message "Inject the specimen…!!" appears.
15. Prepare the blood sample:
    - Open the blood syringe (remove the GREEN cap)!!
    - Remove ALL AIR BUBBLES from the blood syringe!!!
16. Inject the blood sample into the analysis card:
    - Apply gentle pressure and insert the tip of the syringe into the central hole of the blood sample port on the analysis card.
    - Continue inserting until you hear a small click, and the left side of the card fills with blood!
    - The epoc Reader emits a beep, and the analysis status indicator blinks green, confirming that sufficient blood has been received for analysis.
    - The epoc Host also displays the specimen acceptance message.
17. Close the blood syringe with the GREEN cap!!!
18. Wait ~170 seconds for the analysis to complete.
19. Once the Reader has finished the analysis, the status indicator will blink green, indicating that the analysis card can be removed.
20. CAUTION!!! Remove the analysis card carefully (risk of high temperature causing the card to stick to the device's interior)!!
21. Find the analysis results: Gases, Chemistry, Metabolites.
22. Save the results.
23. Print the results.
24. When all analyses with a Reader are completed and all data entries are done, close the session by tapping the RED X button (top right) to exit the Reader screen.

25. Log out the operator via the menu in the bottom left corner of the screen or press the "Log out operator" button.
26. Use the power button on the Host to turn off the device.

Reference values for ASTRUP parameters:

- **Gases:**
  - pH = 7.35 – 7.45
  - pCO_2 = 35 – 53 mmHg
  - pO_2 = 75 – 105 mmHg
  - $cHCO_3^-$ = 21 – 26 mmol/L
  - BE = -2 – 3 mmol/L
  - cSO_2 = 95 – 99%
- **Biochemical parameters:**
  - Na^+ = 128 – 145 mmol/L
  - K^+ = 3.6 – 5.1 mmol/L
  - Ca^{++} = 1.15 – 1.33 mmol/L
  - Cl^- = 98 – 109 mmol/L
  - $cTCO_2$ = 22 – 29 mmol/L
  - Hct = 38 – 51%
  - cHgb = 12 – 16g/dL
- **Metabolic parameters:**
  - Glucose = 74 – 100 mg/dL
  - Lactate = 0.56 – 1.39 mmol/L
  - Creatinine = 0.6 – 1.2 mg/dL

Questions

1. State the normal pH range of blood. What is the clinical significance if pH falls below 7.35 or rises above 7.45?

2. Differentiate between metabolic and respiratory acidosis in terms of primary disturbance and compensatory response.

3. List two causes of hyponatremia and two causes of hypernatremia.

4. What happens to calcium ion levels during acidosis and alkalosis, and how does this affect neuromuscular excitability?

5. What parameters can be measured using the Astrup micro-method?

6. A 58-year-old patient with chronic obstructive pulmonary disease (COPD) comes to the medical unit with shortness of breath and lethargy. Blood gases analysis: pH = 7.30, pCO_2 ↑, $[HCO_3^-]$ ↑.
- Identify the acid-base disturbance.
- What is the primary alteration, and what compensatory mechanism is observed in this case?

7. Based on the values provided in the table, suggest a possible diagnosis. Justify your answer by interpreting the acid-base status and relevant biochemical parameters.

Parameter	**Value**		**Parameter**	**Value**
pH	7.34		K^+	3.5 mmol/L
pCO_2	33.8 mmHg		Ca^{2+}	1.18 mmol/L
HCO_3^-	15 mmol/L		cHb	13.3 g/dL
BE (ecf)	0.4 mmol/L		Glucose	348 mg/dL
cSO_2	91%		Lactate	1.45 mmol/L
Na^+	137 mmol/L			

Lab Notes

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L13. INTEGRATIVE METABOLIC ANALYSIS. DETERMINATION OF BIOCHEMICAL PARAMETERS USING THE PICCOLO XPRESS AUTOMATIC SYSTEM

Introduction

Metabolism encompasses the entirety of chemical reactions within living organisms, encompassing both catabolic processes, which break down complex molecules to release energy, and anabolic processes, which synthesize new molecules from simpler precursors. Although these pathways are often studied in isolation (carbohydrate metabolism, lipid metabolism, protein and amino acid metabolism, nuclei acid metabolism), in the human body they function as an integrated network. The flow of metabolites is dynamic, adapting constantly to changes in nutrient availability, hormonal signals, and energy demands. Understanding integrative metabolism allows the clinician to predict how a disturbance in one pathway will influence the entire metabolic network, a concept that is particularly relevant in disorders such as diabetes, metabolic syndrome, liver failure, and inborn errors of metabolism.

The Central Role of Energy Molecules

The goal of catabolic pathways is the production of ATP, the universal energy currency of the cell, and reducing equivalents (NADH, NADPH, $FADH_2$) that drive oxidative and reductive reactions.

- ATP provides energy for mechanical work, active transport, and biosynthesis. It is generated primarily by the oxidation of glucose, fatty acids, and amino acids through glycolysis, the citric acid cycle (TCA), and oxidative phosphorylation.
- NADH and $FADH_2$ are produced during oxidative catabolism and feed electrons into the mitochondrial electron transport chain to generate ATP.
- NADPH, by contrast, is used for reductive biosynthetic reactions such as fatty acid and cholesterol synthesis, and for maintaining reduced glutathione, a key antioxidant.

Mitochondria serve as the metabolic crossroads, where fuels derived from carbohydrates, lipids, and proteins converge as acetyl-CoA and enter the TCA cycle. This centralization ensures that energy production is coordinated and efficient.

Carbohydrate-Lipid-Protein Interrelationships

Carbohydrates are the body's most immediate energy source. In the fed state, glucose from the diet is oxidized to produce ATP or stored as glycogen in liver and muscle. When glucose is abundant, excess amounts are converted into fatty acids via *de novo* lipogenesis, which are then stored as triacylglycerols in adipose tissue. During fasting, hepatic glycogenolysis releases glucose into the blood, and when glycogen stores are depleted, gluconeogenesis takes over, using lactate from anaerobic glycolysis, glycerol from lipolysis, and glucogenic amino acids from muscle protein breakdown.

Lipids are the most concentrated energy store. In the fed state, dietary fats (mostly triacylglycerols) are transported as chylomicrons and stored in adipocytes. During fasting or prolonged exercise, lipolysis releases free fatty acids, which are oxidized in the liver and muscle to produce ATP. In prolonged fasting, the liver converts excess acetyl-CoA from β-oxidation into ketone bodies, which serve as an alternative fuel and help preserve muscle protein.

Proteins and amino acids serve primarily as structural and functional molecules, but they also act as metabolic substrates when carbohydrate and lipid reserves are insufficient. In the fed state, amino acids are used for protein synthesis or transaminated into intermediates for the TCA cycle. In fasting, muscle proteolysis releases amino acids, particularly alanine and glutamine, that support gluconeogenesis and ureagenesis in the liver.

Hormonal Coordination

Metabolic integration depends heavily on hormonal regulation:

- Insulin dominates the fed state, stimulating glucose uptake (via GLUT4 in muscle and adipose tissue), promoting glycogen and lipid synthesis, and favoring protein anabolism.
- Glucagon predominates during fasting, activating hepatic glycogenolysis, gluconeogenesis, and lipolysis in adipose tissue.
- Catecholamines (epinephrine and norepinephrine) mobilize fuel stores during acute stress and exercise.
- Cortisol and growth hormone play supportive roles in modulating substrate availability over longer time frames.

Metabolic States and Adaptations

In the fed (early postprandial) state, nutrient abundance promotes energy storage. During fasting, glycogenolysis is the primary source of blood glucose, with gluconeogenesis gradually increasing in importance. In prolonged fasting or starvation, ketone body production rises sharply, providing up to 60-70% of the brain's energy needs.

During exercise, energy demand spikes, and fuel selection depends on intensity, phosphocreatine and anaerobic glycolysis dominate early and at high intensities, while fatty acid oxidation contributes more during prolonged moderate activity.

Clinical Correlations

A key application of integrative metabolism is in understanding disease mechanisms:

- In type 1 diabetes, insulin deficiency leads to unchecked lipolysis, ketogenesis, and proteolysis.
- In type 2 diabetes, insulin resistance alters carbohydrate - lipid balance, promoting hyperglycemia and dyslipidemia.
- Metabolic syndrome represents a cluster of conditions characterized by insulin resistance, elevated triglyceride levels, and central obesity.
- Inborn errors of metabolism, such as glycogen storage diseases or defects in β-oxidation, highlight how disruption in one enzyme can affect multiple interconnected pathways.

EXPERIMENTAL PART

Determination of biochemical parameters using the Piccolo Xpress automatic system

Piccolo Xpress is a portable diagnostic analyzer offering a full complement of blood chemistry tests at the point of care. It allows the performance of various routine biochemistry panels using 100 µL of whole blood, serum, or plasma, using dry chemistry. The system uses single-use reagent discs that can contain up to 14 biochemical tests. For one rotor, results are obtained in approximately 12 minutes.

The Piccolo Xpress includes a integrated process called iQC (Intelligent Quality Control). iQC checks the analyzer, the reagent rotor, and the sample during every run to ensure proper electronic and chemical performance. The integrated iQC system sets standards that prevent incorrect measurement results.

The Piccolo Xpress has 13 available panels. However, below are presented only 3 panel types:

Comprehensive Metabolic Panel:
Alanine aminotransferase (ALT), Albumin (ALB), Alkaline phosphatase (ALP), Aspartate aminotransferase (AST), Calcium (Ca^{2+}), Chloride (Cl^{-}), Creatinine (CRE), Glucose (GLU), Potassium (K^{+}), Sodium (Na^{+}), Total bilirubin (TBIL), Total carbon dioxide (tCO_2), Total protein (TP), Blood urea nitrogen (BUN).

General Chemistry 13:
Alanine aminotransferase (ALT), Albumin (ALB), Alkaline phosphatase (ALP), Amylase (AMY), Aspartate aminotransferase (AST), Calcium (Ca^{2+}), Creatinine (CRE), Gamma-glutamyl transferase (GGT), Glucose (GLU), Total bilirubin (TBIL), Total protein (TP), Uric acid (UA), Blood urea nitrogen (BUN).

Lipid Panel Plus:
Total cholesterol (CHOL), High-density lipoprotein (HDL), Triglycerides (TRIG), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Glucose (GLU). Calculated parameters include LDL, VLDL, and the total cholesterol/HDL ratio.

Procedure

Operation of the Piccolo Xpress multiparameter system:
1. Connect the device to the socket.
2. Open the device.
3. Press the ANALYSE option → the hatch opens, where the rotor is placed with the white side facing up.
4. !!Before inserting the rotor into the machine, inject the blood sample using the special syringe (inject approx. 120 µl sample), so that the blood sample fills the crescent in the middle of the rotor.
5. CAREFUL - If the rotor is splashed with blood, it must be cleaned with a tissue (risk of contamination).
6. After placing the rotor on the device arm, press the CLOSE key.
7. Choose the PATIENT option on the device screen → enter an ID (number or name) for the sample to be analyzed.
8. Press the DONE key, at which point the device starts analyzing the sample (the analysis takes approx. 12 min).

9. When the analysis is complete, the message *Analysis complete* appears, after which the OPEN key is pressed to remove the rotor from the instrument. The result of the analysis is printed automatically.
10. Press the CLOSE key.
11. Press the HOME key.
12. Turn Off the device.

Normal values:

- Na^+ = 128 – 145 mmol/L;
- K^+ = 3.6 – 5.1 mmol/L;
- Cl^- = 98 – 108 mmol/L;
- tCO_2 = 18 – 33 mmol/L;
- CA = 8.0 – 10.3 mg/dL;
- GLU = 73 – 118 mg/dL;
- BUN = 7 – 22 mg/dL;
- CRE = 0.6 – 1.2 mg/dL;
- ALP = 42 – 141 U/L;
- ALT = 10 – 47 U/L;
- AST = 11 – 38 U/L;
- TBIL = 0.2 – 1.6 mg/dL;
- ALB = 3.3 – 5.5 g/dL;
- TP = 6.4 – 8.1 g/dL.

Piccolo Xpress automatic system can detect if the biological sample is:

- Hemolyzed (HEM: 0, +.....4+);
- Lipemic (LIP: 0, +.....4+);
- Jaundiced (ICT: 0, +.....4+).

Questions

1. Briefly describe the main processes that generate ATP and indicate the key cellular processes in which ATP is consumed.

2. Explain the interconnections between carbohydrate and lipid metabolism, highlighting the major molecules, pathways, and enzymes involved.

3. Describe how carbohydrate and protein metabolism interact through gluconeogenesis and the TCA cycle. Include the role of alanine, glutamine, and transamination reactions.

4. Describe the links between carbohydrate metabolism and energy metabolism, identifying the molecules, pathways, and enzymes that establish this connection.

5. Explain how protein metabolism is integrated with energy metabolism, focusing on the roles of specific molecules, pathways, and enzymes.

6. Acetyl-CoA is a central metabolic intermediate. Identify the pathways in which acetyl-CoA appears and explain how it serves as a hub connecting different metabolic processes.

7. Present the major metabolic processes that occur in the liver and their physiological significance.

8. Explain the effects of insulin, glucagon, and epinephrine on carbohydrate, lipid, and protein metabolism. How do these hormones coordinate energy supply during exercise and stress?

9. Compare the predominant metabolic pathways in the fed state (early postprandial) versus the prolonged fasting state. Explain the hormonal regulation by insulin and glucagon.

10. Compare the roles of skeletal muscle and adipose tissue in energy metabolism. How do these tissues differ in their responses to insulin and glucagon?

11. Which biochemical tests related to carbohydrate metabolism have you studied?

12. Which biochemical tests related to lipid metabolism have you studied?

13. Which biochemical tests related to protein metabolism have you studied?

14. Which biochemical tests related to nucleic acid metabolism have you studied?

15. Indicate the parameters that can be measured with a Comprehensive Metabolic Panel (CMP) using the *Piccolo Xpress* analyzer.

Lab Notes

Use this space for handwritten notes during the practical session.

REFERENCES

1. Marian C, Anghel A, Şeclăman E, Kaycsa A, Sîrbu IO, Tămaş L, Biochemistry of Metabolism for medical students, Victor Babeş Publishing House, 2016.
2. Anghel A, Tămaş L, Şeclăman E, Metabolic biochemistry with clinical applications, Eurostampa Publishing House, 2017.
3. Anghel A, Şeclăman E, Tămaş L, Motoc M, Şişu E, Kaycsa A, David D, Sîrbu IO, Marian C, Samoilă C, Grecu D, Sfrijan F, Georgescu A, Marcu A, Buzatu R, Bonţe D, Gurban C, Alexa A, Bujor G, Mărieş L, Mihala A, Sala-Cârtog M, Practical works of medical chemistry and biochemistry for medical students, Victor Babeş Publishing House, 2019.
4. Devlin TM, editor. Textbook of biochemistry with clinical correlations. John Wiley & Sons; 2010.
5. Bishop ML, Fody EP, Schoeff LE. Clinical Chemistry: Techniques, Principles, and Correlations. The Netherlands: Wolters Kluwer; 2018.
6. Meisenberg G, Simmons WH. Principles of Medical Biochemistry E-Book: Principles of Medical Biochemistry E-Book. Elsevier Health Sciences; 2016.
7. Berg JM, Tymoczko JL, Gatto Jr. GJ, Stryer L, Biochemistry, 9th Edition, 2019.
8. Ciaccio M, editor. Clinical and laboratory medicine textbook. Springer Nature; 2024.
9. Nelson DL, Cox MM. Lehninger Principles of Biochemistry. 7th ed. New York: W.H. Freeman; 2017.
10. Abali EE, Carman R, Spicer DB. Biochemistry Behind the Symptoms. 1st ed. Wolters Kluwer Health; 2023.
11. Lee M, editor. Basic skills in interpreting laboratory data. 6th ed. American Society of Health-System Pharmacists; 2017.
12. Murphy M, Srivastava R, Deans K. Clinical biochemistry: an illustrated colour text. 7th ed. Elsevier; 2023.
13. Ahmed N, editor. Clinical biochemistry. 3rd ed. Oxford University Press (Fundamentals of Biomedical Science); 2025.
14. Marshall WJ, Lapsley M, Day AP, Ayling RM. Clinical biochemistry: metabolic and clinical aspects. 3rd ed. Elsevier; 2014.
15. https://www.globalpointofcare.abbott/us/en/product-details/apoc/piccolo-xpress-chemistry-analyzer.html