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BIOCHEMISTRY

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Laboratory notes

GHIDURI ȘI ÎNDRUMĂTOARE DE LABORATOR

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L1. ORGANIZATION OF CHEMISTRY AND BIOCHEMISTRY LABORATORIES. GENERAL PROTECTION RULES.

Chemistry laboratories are equipped with installations and specific devices that must be permanently maintained and verified. The biochemistry laboratory is organized according to the same principles as chemistry laboratories, but with additional particularities due to the utilization of biological materials and specific devices, needed for the study and demonstration of metabolic processes.

General protection rules in the chemistry and biochemistry laboratory

As in other chemistry laboratories, in the biochemistry laboratory some operations may lead to serious accidents if performed by uninformed persons. To prevent these situations, some general work protection rules must be known and followed.

General rules of protection

1. All accidents, regardless of how minor they may appear, will be immediately reported to the lab assistant.
2. Access to the lab is permitted only to students wearing a clean lab coat. Protective gloves and goggles must be used for certain operations. The workplace will be maintained perfectly clean throughout the entire duration of the work. Any kind of liquids or solid substances accidentally spilled on benches, balances, the spectrophotometer or any other apparatus must be immediately cleaned. For this reason, students must always keep a cloth at hand.
3. All apparatus and tools to be used should be checked before starting work; any deficiency must be immediately reported to the lab assistant.
4. Electric apparatus may only be connected to the plugs with dried hands. After use, the electric apparatus must be disconnected from the power supply.
5. All the bottles containing chemicals must be properly labeled and should have appropriate stoppers. Do not use any chemical if the bottle is not labeled. Do not interchange the stoppers.
6. Acids, hydroxides, oxidant agents, papers, and broken glass must never be thrown into sinks.
7. Food and beverages are strictly prohibited in the laboratory area.

Flammable substances

Most laboratory fires are caused by flammable liquids (alcohol, ether, acetone, hydrocarbons, etc.). Do not work with such liquids close to an open fire. The fires aroused by solvents must be extinguished immediately by smothering the flames with cloths, sand or a fire extinguisher. Do not dispose of flammable liquids in the sewer. Once the fire has started, never attempt to stop it with water. Oxidant agents (chromic acid, chlorates, bromides, nitrates, nitrites, etc.) can easily catch fire in contact with organic substances.

- First-degree burns should be rinsed under running cool water.
- Second-degree burns should be rinsed under cool water and then treated with special powders (or ointments) and covered loosely with a sterile, non-stick dressing. Further medical consultation by a physician is obligatory.

Toxic and poisonous substances

Many substances used in the biochemistry laboratory are highly poisonous, even in small quantities. It is strictly forbidden to eat in the lab, to use lab vessels to drink water, to taste the chemicals.

Toxic reagents must be measured with pipettes using a teat, a pipetting ball or a long rubber tube. Glass pipettes used for biological liquids (blood, serum, plasma, urine) must be fitted with cotton wool, or else automatic pipettes should be used.

Smelling toxic substances (chloroform, ether, benzene, aniline, methanol, carbon monoxide, chlorine, ethylene oxide, etc.) is strictly forbidden.

Corrosive reagents

Examples of corrosive agents include concentrate acids (HCl, H_2SO_4, HNO_3, HF, CH_3COOH); alkali metal bases (NaOH, KOH, etc.), silver nitrate, hydrogen peroxide, bromine, phenols, etc.

Strong acids and bases are not only poisonous but also corrode clothes and skin. Do not pipette these substances by mouth; instead, use a teat, a pipetting ball, or vacuum aspiration.

Concentrated acids spilled on the bench should first be absorbed with a cloth, then the surface must be washed with plenty of water or a sodium bicarbonate solution.

Avoid skin contact with all chemicals. Wash off any spills immediately.

- Burns caused by acids should be rinsed with plenty of water and then with a sodium bicarbonate solution.
- Burns caused by bases should be rinsed with plenty of water, followed by 2% boric acid or 2% acetic acid solution.
- Burns from hydrobromic acid or bromine should be rinsed with 2% sodium thiosulfate solution.

Glassware and apparatus in the chemistry and biochemistry laboratory

Plumbing, lighting system, sewerage, and gas installation must function in optimal conditions and must serve all workstations.

Laboratory reagents must be kept in leak-proof bottles with labels and stored in appropriate lockers. Reagents labeled for storage at +4°C or -20°C must be kept in suitable refrigerators or freezers. For most laboratory works, the necessary solutions are already prepared and placed on working benches together with the required glassware and instruments.

Laboratory benches must be kept perfectly clean to avoid contact of skin and clothes with chemical substances.

Common laboratory equipment

- refrigerator
- heating bath
- peristaltic pump
- automatic titration system
- pharmaceutical balance
- water distillation unit
- drying cabinet
- hot plate
- table-top (clinical) centrifuge
- spectrophotometer
- analytical balance
- homogenizer
- magnetic stirrer with hot plate (and magnetic stirring bar)
- water thermostat with switch-contact thermometer (controlled temperature bath), etc.

Instruments

- base plate
- stand support
- stand rod
- universal bosshead
- double bosshead
- automatic pipette with tips
- pipette stand
- heater bath insert
- test tube rack (holder)
- double ended spatula

- sieve
- universal clamp, tubing clamp
- stand ring (with stem)
- tripod
- wire gauze
- wire triangle
- gas burner (Bunsen)
- thermometer
- stopwatch
- water jet pump
- safety cage
- pipetting aid
- labels
- waterproof pen
- plastic spatula
- scissors
- knife
- tweezers
- brush
- stoppers (rubber or plastic)
- dropper teat
- rubber tubing
- rubber bellows
- safety goggles
- protective gloves
- pipetting ball
- needle
- syringe

Glass Instruments and Vessels

- beaker
- Erlenmeyer (conical) flask
- test tube (and side-arm test tube)
- centrifuge tube
- watch glass
- Petri dish
- round-bottom flask
- flat-bottom flask
- evaporating dish
- glass tubing
- glass stirring rod
- funnel
- Büchner funnel
- separator (dropper) funnel
- glass filter crucible
- crystallizing dish
- spotting tile
- graduated cylinder
- graduated pipette
- bulb pipette
- burette
- piston pipette
- volumetric flask
- Pasteur pipette
- wash bottle
- dropping bottle
- suction bottle (Kitasato flask)
- compressed gas bottle
- mortar and pestle
- microscope slide
- vacuum desiccator
- narrow (or wide) neck bottle
- chromatography tank
- distilled water tank

Laboratory glassware is composed of glass objects needed for working with liquids, allowing manipulation, mixing, and measurement of solutions. The most used are:

- Glass or plastic tubes of various dimensions. Reagents are introduced, mixed, and then subjected to devices such as thermostats, centrifuges, or spectrophotometers.
- Glassware for volume measurement: graduated cylinders, volumetric flasks, pipettes, and burettes.

Graduated cylinders are made of glass or plastic and marked with volume gradations (cm^3). The total capacity is indicated at the top, along with the working temperature. Measurements are simple but less accurate, so they are mainly used for preparing solutions of approximate concentration.

Volumetric flasks are spherical or pear-shaped with a flat bottom and long neck. A circular graduation mark on the neck indicates the precise volume (5-2000 ml). They are calibrated at 20 °C and used to prepare solutions with very exact concentrations. Substances are weighed on an analytical balance, transferred into the flask using a funnel, and rinsed with solvent. After complete dissolution, the flask is allowed to reach room temperature, then filled to the mark with solvent.

Pipettes are used for rapid and accurate measurements of liquids. They may be manual or automatic. Based on calibration, they may have fixed or variable volumes. Fixed-volume pipettes measure only their calibrated volume (0.10-100 ml). Variable-volume pipettes have intermediate graduations (0.01-25 ml). Typical sizes: 0.10, 1.0, 2.0, 5, 10, and 25 ml.

Micropipettes are calibrated in microliters (µl) and have capacities between 0.01 and 0.02 milliliters (10-20 µl).

Burettes are graduated tubes that allow for controlled liquid release. For this, they have at their inferior part a device for stopping the liquid such as a stop-tap (stop-valve, stopcock) or a rubber tube with Mohr (pinchcock) clamp. Burettes are calibrated for volumes of 1, 2, 10, 25, 50, 100 milliliters.

Questions

1. List three general protection rules that must always be followed in a chemistry or biochemistry laboratory.

2. Explain the main difference in accuracy and purpose between a graduated cylinder and a volumetric flask.

3. Give two examples of corrosive acids and two examples of corrosive bases.

4. What should you do in case of spilling a solution of strong acid on your hand? What about a strong base?

5. What is the correct way to extinguish a fire caused by a solvent?

Lab Notes

Use this space for handwritten notes during the practical session.

L2. UNITS OF MEASUREMENT. MASS, VOLUME AND DENSITY MEASUREMENTS.

Units of measurement

The term physical **dimension (magnitude, quantity)** refers to everything that can vary in size, either increasing or decreasing. Any measurement must be expressed by a number and a unit. The unit identifies the type of the measured property (mass, volume, concentration, etc.) and the number indicates how many units of that property are present.

The International System of Units (**SI**, Système international d'unités) is derived from the metric system. It contains seven fundamental dimensions, eighteen derived dimensions, and additional units, covering most scientific needs (Table 1 and 2).

Table 1. SI base units of measurement

Physical quantity (dimension)	Unit of measurement	Symbol
Length	meter	m
Mass	kilogram	kg
Time	second	s
Electric current	ampere	A
Thermodynamic temperature	kelvin	K
Quantity of material	mole	mole
Light intensity	candela	cd

Table 2. SI derived units of measurements (examples commonly used in laboratory)

Physical quantity (dimension)	Unit of measurement	Symbol	Expression in other SI units	Expression in base SI units
Volume	cubic meter	m^3	-	m^3
Mass density	kilogram per cubic meter	kg/m^3	-	kg/m^3
Concentration*	mol per cubic meter	mol/m^3	-	mol/m^3
Frequency	hertz	Hz	-	s^{-1}
Force	newton	N	-	$m{\cdot}kg{\cdot}s^{-2}$
Pressure	pascal	Pa	N/m^2	$m^{-1}{\cdot}kg{\cdot}s^{-2}$
Energy, heat quantity	joule	J	$N{\cdot}m$	$m^2{\cdot}kg{\cdot}s^{-2}$
Power	watt	W	J/s	$m^2{\cdot}kg{\cdot}s^{-3}$
Electric potential, Difference of potential, electromotive force	volt	V	$W{\cdot}A^{-1}$	$m^2{\cdot}kg{\cdot}s^{-3}{\cdot}\ A^{-1}$
Catalytic activity	katal	cat	-	$mol{\cdot}s^{-1}$

* The symbol % should not be used for expressing concentration of solutions, but only the correct expression in the respective unit (mg/100mL, g/L, etc.).

Non-SI (additional, supplementary) units is a small category of units that are still coherent with SI but don't fall neatly into the first two groups. They are mainly used for angles (radian for plane angle, steradian for solid angle) and dimensionless quantities (%). Other units (liter for volume, tonne for mass) continue to be used for practical reasons (Table 3).

Table 3. Non-SI units of measurement (examples commonly used in laboratory)

Physical quantity (dimension)	Unit of measurement	Symbol	Value in SI units
Time	minute	min	1 min = 60 s
	hour	h	1 h = 3600 s
	day	d	1 d = 86400 s
Plane angle	degree	°	$1° = \pi / 180$ rad
	minute	′	$1′ = \pi / 10800$ rad
	second	″	$1″ = \pi / 648000$ rad
Volume	liter	L	$1L = 1dm^3$ or 10^{-3} m^3
Mass	ton	t	1t = 1000 kg

In practice, many measured quantities are either too small or too large to be conveniently expressed in base or derived SI units. To simplify their expression and avoid very large or very small numbers, the SI system uses standard prefixes that represent powers of ten (Table 4).

Table 4. Multiples and submultiples of units

Prefix	Symbol	Amplification factor	Numeric value of the units
exa	E	10^{18}	1 000 000 000 000 000 000
peta	P	10^{15}	1 000 000 000 000 000
tera	T	10^{12}	1 000 000 000 000
giga	G	10^{9}	1 000 000 000
mega	M	10^{6}	1 000 000
kilo	k	10^{3}	1 000
hecto∗	h	10^{2}	100
deca∗	da	10^{1}	10
deci∗	d	10^{-1}	0,1
centi∗	c	10^{-2}	0.01
milli	m	10^{-3}	0.001
micro	μ	10^{-6}	0.000 001
nano	n	10^{-9}	0.000 000 001
pico	p	10^{-12}	0.000 000 000 001
femto	f	10^{-15}	0.000 000 000 000 001
atto	a	10^{-18}	0.000 000 000 000 000 001

Commonly used units in laboratory

1. **Mass**. SI base unit = kilogram (kg), CGS base unit = gram (g).
   In chemistry and biochemistry, the gram (g) and its submultiples (mg, µg) are far more practical. Even smaller units, such as nanogram (ng, 10^{-9} g) or picogram (pg, 10^{-12} g), are important in molecular biology (DNA/RNA quantification). Distinction should be made between mass (property intrinsic to matter), and weight (property depending on gravity, W = m·g).

Table 5. Multiples and submultiples of mass units in SI

Denomination	Symbol	Value in basic unit
Kilogram	kg	10^3 g
Gram	g	1 g
Decigram	dg	10^{-1} g
Centigram	cg	10^{-2} g
Milligram	mg	10^{-3} g
Microgram	µg	10^{-6} g =10^{-3} mg
Nanogram	ng	10^{-9} g= 10^{-6} mg
Pictogram	pg	10^{-12} g = 10^{-9} mg
Femtogram	fg	10^{-15} g = 10^{-12} mg
Attogram	ag	10^{-18} g = 10^{-15} mg

2. **Length.** SI base unit = meter (m), CGS base unit = centimeter (cm).
   Laboratory practice relies on submultiples (cm, mm, µm). Ångström (Å = 10^{-10} m, 10^{-8} cm = 10^{-7} mm = 10 mµ), although not SI, is still widely used in crystallography, atomic and molecular physics to express wavelength. In microscopy and nanoscience, nm and µm are the most common scales.

Table 6. Multiples and submultiples of length units in SI

Denomination	Symbol	Value in basic units
Kilometer	Km	10^3 m
Hectometer	Hm	10^2 m
Decameter	Dam	10 m
Meter	M	1
Decimeter	dm	10^{-1}m
Centimeter	cm	10^{-2}m
Millimeter	mm	10^{-3}m
micrometer (micron)	µm (µ)	10^{-6}m =10^{-3} mm
nanometer millimicron)	nm (mµ)	10^{-9}m = 10^{-6} mm
picometer (micromicron)	pm (µµ)	10^{-12}m= 10^{-9} mm

3. **Surface (area).** SI base unit = square meter (m^2), CGS base unit = square centimeter (cm^2). Laboratory work usually deals with much smaller scales: cm^2, mm^2, μm^2. Applications: surface coverage, reaction surfaces, or thin-film measurements.

Table 7. Multiples and submultiples of surface units in SI

Denomination	Symbol	Value in basic units
square kilometer	km^2	10^6 m^2
square hectometer	hm^2	10^4 m^2
square decameter	dam^2	10^2 m^2
square meter	m^2	1 m^2
square decimeter	dm^2	10^{-2} m^2
square centimeter	cm^2	10^{-4} m^2
square millimeter	mm^2	10^{-6} m^2
square micrometer	μm^2	10^{-12} m^2 =10^{-6} mm^2

4. **Volume.** SI base unit = cubic meter (m^3), CGS base unit = cubic centimeter (cm^3).
In laboratory practice, the cubic meter is impractical instead, the following L and its submultiples are used. While mL and µL dominate in biochemical assays (enzymatic reactions, PCR, chromatography, etc.), L is commonly used for bulk (stock) solutions and gases.

Table 8. Multiples and submultiples of volume units in SI

Denomination	Symbol	Value in basic units
Cubic kilometer	km^3	10^9 m^3
Cubic meter	m^3	1 m^3
Cubic decimeter (liter)	dm^3 (L)	10^{-3} m^3
Cubic centimeter	cm^3	10^{-6} m^3 =10^{-3} dm^3
Cubic millimeter (milliliter)	mm^3 (mL)	10^{-9} m^3 =10^{-6} dm^3
Cubic micrometer	μm^3	10^{-18} m^3 =10^{-15} dm^3
Cubic nanometer cub	nm^3	10^{-27} m^3 = 10^{-24} dm^3

Table 9. Multiples and submultiples of Liter

Denomination	Symbol	Value in basic units
Hectoliter	hL	10^2 L
Liter = 1 dm^3	L	1 L
Deciliter	dL	10^{-1} L
Centiliter	cL	10^{-2} L
Milliliter = 1 cm^3	mL	10^{-3} L
Microliter	µL	10^{-6} L = 10^{-3} mL

5. **Time**. SI base unit = second (m^3).
   Submultiples such as ms are used in kinetics and spectroscopy, while µs and ns are used for fluorescence lifetimes, laser pulses. Multiples (min, h, d), although are not SI, are accepted for use with SI.

Table 10. Time units

Denomination	Symbol	Value in basic units
Hour	h	3600 s = 60 min
Minute	min	60 s
Second	s	1 s
Millisecond	ms	10^{-3} s
Microsecond	µs	10^{-6} s

6. **Temperature**. SI base unit = Kelvin (K) degree.
   Kelvin degree is the unit used in thermodynamics. Temperatures in Kelvin have no degree sign. Celsius (°C) is the most common laboratory scale (). Fahrenheit (°F) is rare in science but common in some regions. In the Fahrenheit scale, the water freezing point at 1 atm is 32 and the boiling point is 212 (the interval between these two points is divided into 180 degrees).

$$T(K) = T(°C) + 273.15; \qquad T(°F) = (9/5)\cdot T(°C) + 32$$

Table 11. Temperature units

Unit	**Symbol**	**Value of temperature**	
		At absolute zero	**Freezing water point**
Kelvin	K	0	273.15
Celsius degree	°C	-273.15	0
Fahrenheit degree	°F	-459.67	32.018

Mass measurements

Mass is the direct measure of the amount of matter in a substance. The mass of a sample provides a direct indication of the number of atoms or molecules it contains. Since chemical reactions occur in proportion to the number of reacting atoms or molecules, it is essential that the mass of reactant be accurately known. Weight, on the other hand, is a mass function representing the force exerted by gravity on a given mass. Thus, weight depends on location (e.g., Earth vs. Moon), while mass does not.

$$W = m \cdot g$$

W = the weight
m = the mass
g = the local gravitational acceleration.

The accurate determination of mass is one of the most fundamental laboratory techniques. Measurement of substances is carried out by **weighing**, using devices called **balances**. A good balance must meet three essential criteria:

- Sensitivity = the smallest mass difference it can detect.
- Precision = the reproducibility of repeated measurements of the same object.
- Accuracy = how close the measured value is to the true value.

Based on their sensitivity, balances can be classified as:

1. Technical balances, with a sensitivity of $1 \cdot 10^{-2}$ g
2. Pharmaceutical balances, with a sensitivity of $1 \cdot 10^{-3}$ g
3. Analytical balances, with a sensitivity of $1 \cdot 10^{-4}$ g - $5 \cdot 10^{-5}$ g
4. Semimicro balances, with a sensitivity of $1 \cdot 10^{-5}$ g
5. Micro balances, with a sensitivity of $1 \cdot 10^{-6}$ g
6. Ultramicro balances, with a sensitivity of $1 \cdot 10^{-7}$ g

There are various types of balances available, depending on their construction, appearance and precision. **Based on the counterbalance force**, balances can be divided into two categories:

- Mechanical balances: use standard weights for counterbalance.
- Electronic balances: use electromagnetic force compensation, providing rapid and highly accurate readouts.

Balances are usually installed in isolated, draft-free, and vibration-free rooms, away from direct sunlight and dust. Their performance is periodically checked with certified calibration weights, and a small error tolerance is accepted.

Good Practices in Weighing

Accurate weighing is critical for reliable experimental results. To minimize errors and protect sensitive balances, the following practices should always be observed:

- Always check that the display reads 0.000 g when the pan is empty. Use the *tare* or *zero* function to reset the reading before placing your sample container. If the balance cannot be zeroed, report the issue, do not continue using it and ask the instructor for help.
- All balances, but especially electronic ones, are very sensitive to moisture and chemical damage. Do not pour liquids in the vicinity of the balance. Immediately wipe away any spills in the balance area.
- No reagent chemical substance should ever be weighted directly on the pan of the balance. Ideally, reagents should be weighted directly into the beaker or flask in which they are to be used. Plastic weighing boats or watch glass may also be used if several reagents are required for an experiment. Choose the container size appropriate to the balance pan and avoid oversized glassware that may destabilize readings.
- For exact mass determinations, the object to be weighted must be at room temperature. If a hot or warm object is placed on the balance pan, it causes the air around it to become heated. Warm air rises, and its motion may be detected by the balance, lowering the value displayed by it.
- Try to find a place out of the wind to use the balance. Most analytical balances are equipped with draft shields, keep them closed during weighing. Avoid vibrations by placing balances on solid tables. Do not lean on or bump the bench while weighing.
- Use a clean, dry spatula for transferring powders. Avoid breathing directly over the pan; air currents from exhalation can disturb measurements. For volatile liquids, use containers with lids to reduce evaporation during weighing.
- After use, remove all containers and wipe the pan gently with a soft, dry tissue. Never use abrasive materials on the pan. Keep the area around it uncluttered and free of dust.
- Larger masses should be weighed on a top-loading balance to an appropriate degree of accuracy. Take care to note the limits for the balance; while most have devices to protect against overloading, you may damage the weighing mechanism permanently.

Electronic balances with digital readouts are widely used. They are easy to read, fast, and feature a tare function, which allows subtraction of the container mass automatically. The most common type offers accuracy down to 1 mg for ranges of 1 mg – 160 g, which is suitable for most chemical and biological applications.

Operating a Standard Self-Taring Balance

1. Level the balance. Adjust the feet until the bubble in the spirit level is centered (usually at the back of the machine). For accurate work, make sure a draught shield is on the balance.
2. Tare the container. Place an empty vessel on the balance pan and allow the reading to stabilize. Press the tare to reset to zero.
3. Add the sample. Place the chemical or object carefully in the vessel (powdered chemicals should be dispensed with a suitable sized clean spatula).
4. Remove excess carefully. If you add excess chemical, take great care when removing it. Record the mass. Allow the reading to stabilize and note the value. Don't place the excess back in the original container.
5. Record the mass. Allow the reading to stabilize and note the value.

Volume measurement

Many types of laboratory glassware are marked by the manufacturer to indicate the volume of liquid they contain when filled to a specific level. However, the precision of these graduations varies greatly depending on the type of glassware and its intended use. It is important to distinguish between situations where high precision is essential (e.g., titrations, preparing standard solutions) and those where only approximate volumes are acceptable:

- Beakers and Erlenmeyer flasks are marked with very approximate volumes, which serve merely as a rough guide to the volume of liquid in the container.
- Graduated cylinders, volumetric flasks, burettes, and pipettes, are marked much more precisely and are used when accurate volume determination is required.

The **temperature** (usually 20°C) employed for calibration is on most volumetric glassware. This matters because the volume of a liquid changes with temperature, which causes the density of the liquid to change. While a given pipette will contain or deliver the *same geometric volume* at any temperature, the *amount of substance* present in that volume will vary with temperature. At a quite high difference in temperature, glass can also contract or expand, given differences in volumes.

Some of the laboratory devices used to measure and transfer volumes of different substances are graduated cylinders, burettes, pipettes, syringes, volumetric flasks, etc.

1. Graduated cylinders

While graduated cylinders provide moderate accuracy, they are less precise than pipettes/burettes. They are used for many applications where exact precision is not critical (measuring solvent volumes).

It is important to perform a correct reading when measuring volume with graduated cylinders. When a solution is poured in a narrow glass container (such as a graduated cylinder), the liquid surface is not flat but rather curved, usually downward. This curved surface is called a meniscus and is caused by the interaction (adhesion) between the water molecules and the molecules of the glass container wall. For a correct reading, adjust the container with the liquid level at eye level, taking the lowest point of the meniscus as the correct reading.

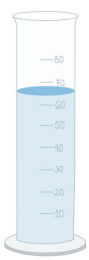


Figure 1. Graduated cylinder

2. Volumetric flasks

Volumetric flasks are glass or plastic bulbs with a flat base, round bult and a long, narrow neck. The volume they provide is fixed and marked on the bulb. A single calibration mark on the neck indicates the correct level when the meniscus touches it. The volumetric flasks are commonly used to prepare solutions of different molar, normal or weight/volume concentrations. The flasks must be used at the indicated temperature. Any variation may cause expansion or contraction of glass and introduce volume errors.

3. Burettes

A burette is a tall, narrow, graduated glass tube, fitted at the bottom with a stopcock/valve for controlling the flow of liquid. It is used to dispense variable but precisely measured volumes, especially in titrations. The precision permitted by a burette is ± 0.02 mL (for burettes of 10, 20, 50 and 100 mL) and ± 0.01 mL for micro burettes (1–5 mL). Manual burettes are gradually replaced by automatic ones, with digital displays of the volumes.

A burette must be scrupulously clean before use. Before used, the burette must be rinsed with several small portion of the solution to be used in the burette, discarding the flow-through. After use, the burette should be rinsed with distilled water.

It is not necessary to fill the burette with solution up until the 0.00 mark. The burette should be filled to a level that is comfortable for the user to read, based on the height. The volume of the liquid should be recorded initially, and at the end of the procedure. The final volume is determined by subtraction.

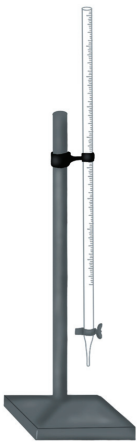


Figure 2. Burette

4. Pipettes

When a more precise determination of liquid volume is needed (cannot be provided by a graduated cylinder), a pipette may be used. Pipettes are especially useful if several measurements of the same volume are needed. Multiple types of pipettes are commonly available:

- Volumetric pipettes: can deliver only one fixed volume (barrel of the pipette).
- Graduated pipettes: can deliver variable volumes (up to the capacity of the pipette). Are calibrated along the stem with multiple graduations. There are 2 types: Mohr pipettes (graduations stop before the tip; liquid must be delivered only down to the last graduation mark, not to the tip) and serological pipettes (graduations extend all the way to the tip; designed to be emptied completely via blow-out).
- Pasteur pipettes: simple glass or plastic tubes with a bulb used for transfer of small, approximate volumes (not accurate). They are common in microbiology/qualitative work.

Pipettes are filled using a rubber safety bulb or a pipette aid. It is absolutely forbidden to pipette by mouth in the laboratory!

5. Micropipettors (Autopipettes)

Micropipettes are semiautomatic devices widely used in modern chemistry, biochemistry, and molecular biology. A micropipette must be fitted with the correct disposable tip before each use. For this, each manufacturer produces different tips to fit their own models.

The main parts of a micro pipettor are plunger button, tip-ejector button, volume adjustment dial, digital volume indicator, shaft, tip adjustment point.

6. **Syringes** are used for volumetric operations of very high precision in very small ranges. They are common in chromatography and electrophoresis. Syringes capacity is 1-500 µl, errors being up to 1% in the interval 5-500 µl and 2% in the interval 1-5 µl.

Density measurements

The density (ρ) of a substance is defined as the ratio of its mass and volume.

$$\rho = \frac{m}{V}$$

Density therefore represents the mass per unit volume (also called the specific mass of a substance). Density units of measurement are g/dm^3 (g/L) for gases and g/cm^3 (g/mL) for liquids and solids. Substances' densities vary with **temperature and pressure**. The pressure influence on liquids and solids is small (the compressibility coefficient is small), that's why, usually, is neglected. Temperature, instead, must be mentioned in all cases.

Density serves for substances characterization, being a **physical constant** that can be used to identify and verify the purity of a substance. Density is influenced by molecular structure. Increased branching generally decreases density because it reduces the efficiency of molecular packing. In contrast, the presence of double or triple bonds and cyclic structures often increases density by producing more compact molecules that occupy a smaller volume.

Density determination of liquids using a densimeter (hydrometer)

A hydrometer is an instrument used for determining the specific gravity (density relative to water) of liquids. It is usually made of glass and consists of a cylindrical stem and a bulb weighted with mercury or shot to make it float upright. The function of the hydrometer is based on Archimedes principle that a solid suspended in a liquid will be buoyed up by a force equal to the weight of the liquid displaced. Thus, the lower the density of the substance, the lower the hydrometer will sink.

The liquid is poured into a tall jar, and the hydrometer is gently lowered into the liquid until it floats freely. The point where the surface of the liquid touches the stem of the hydrometer is noted. Hydrometers usually contain a paper scale inside the stem, so that the specific gravity can be read directly. Specific gravity is a ratio of one density to that of the density of water. Therefore, specific gravity has no unit of measurement (dimensionless). Many industries have more than one set of hydrometers, 1.0-0.95, 0.95-0.9, etc., to provide more precise measurements of density.

Urine density (specific gravity) and its clinical significance

Urine density, more precisely called *specific gravity* (SG) or *relative density*, is a measure of how concentrated the urine is relative to water. It reflects the mass of solutes and water in the urine. Clinically, urine density is used as a surrogate marker of renal concentrating ability and hydration status.

$$specific\ gravity = \frac{density\ of\ urine}{density\ of\ water}$$

The normal urine specific gravity (*normostenuria*) is 1.015 - 1.025. The urine density varies directly proportional to the mass of substances dissolved in the urine and to the intensity of the color and inversely proportional to the amount of urine emitted.

Hypostenuria (density < 1.015) can result from a decreased ADH activity or a defective ability of the tubules to reabsorb water. Potential causes of reduced urine density are chronic kidney disease, pyelonephritis, acute tubular necrosis, diabetes insipidus, hyperhydration. At a density lower than 1.010 g/cm3, leucocytes and erythrocyte are being lysed which might give false negative results when analyzing the urinary sediment.

Hyperstenuria (density > 1.025) can be consequence of an increased reabsorption of water (often via antidiuretic hormone) or the presence of non-physiologic solutes (e.g., glucose, proteins) in urine. Potential causes of elevated urine density are nephrotic syndrome, glomerulonephritis, sweating, fever, exercise, hemorrhage, vomiting, diarrhea, uncontrolled diabetes mellitus, dehydration.

Questions

1. List the seven SI base units and their symbols.

2. Define sensitivity, precision, and accuracy in the context of balances.

3. What are the steps in weighing a substance in the laboratory?

4. A solution has a density of 1.25 g/mL. What is the mass of 2 L of this solution?

5. A hydrometer sinks deeper in liquid A than in liquid B. Which liquid has the higher density? Explain.

Lab Notes

Use this space for handwritten notes during the practical session.

L3. SOLUTIONS. DILUTIONS. ISOTONIC SOLUTIONS. INFUSION SOLUTIONS.

Solutions

A solution is a homogeneous, monophasic mixture, made up of two or more substances, which do not chemically react with each other.

- The **solute (dissolved)** is the substance present in the smaller amount, dispersed at the molecular or ionic level.
- The **solvent (dissolvent)** is the substance present in the largest amount, which determines the physical state of the solution.

Depending on their physical state, there are 3 types of solutions:

- **Gaseous** solutions: air (O_2, CO_2, N_2, Ar).
- **Liquid** solutions: are the most common and can be *aqueous* (water as solvent, e.g., NaCl in water), *partially aqueous* (mixture of water and another solvent, e.g., ethanol-water) and *non-aqueous* (non-water solvent, e.g., iodine in ethanol, sugar in glycerol).
- **Solid** solutions: alloys (brass = Cu + Zn).

The fundamental parameter that characterizes a solution is its **concentration.** Concentration refers to the amount of solute in a solution; it is generally expressed in terms of the amount of solute in each volume of solution, but in some cases alternate expressions are used.

In analytical chemistry, solutions of known concentration are essential for determining the amount of unknown substances. Using solutions with known concentration one can determine the quantity of a substance in an analyzed solution, having an unknown concentration. There are multiple ways to express concentration, depending on the context:

Percent solution (percentage concentration, %) expresses the amount of solute relative to the whole solution, in terms of 100 parts. Unless otherwise indicated, percentage concentration refers to a weight/volume (w/v) solution.

- *A weight/volume percent (w/v %) solution* is a solution in which a specified number of grams of solute are contained in 100 ml of solution. Strictly speaking, this is a "hybrid", not a "true" percent solution since one refers to grams of solute in 100 ml of solution.

$$\%(w/v) = \frac{mass\ of\ solute\ (g)}{volume\ of\ solution\ (mL)} \cdot 100$$

- *A weight/weight percent (w/w %) solution* is a "true" percent solution in which a specified number of grams of solute is contained in 100 g of solution.
- *A volume/volume percent % (v/v %) solution* is likewise a "true" percent solution in which a specified number of ml of liquid are contained in 100 ml of solution.
- *A mg/100 mL (mg%) solution*, widely used in clinical chemistry, is again a "hybrid" and not a "true" percent solution. It is one in which a specified number of mg of solute are contained in 100 ml of solution.

Molarity (molar concentration, M) refers to the concentration of a solution expressed in terms of the number of moles of solute in 1 liter of solution. Note that *mole* (gram-molecular weight; molecular weight expressed in grams, mass of Avogadro's number of molecules) refers to the amount of solute, while *molarity* refers to concentration (amount of solute per given volume of solution).

$$M(moles/L) = \frac{moles\ of\ solute\ (mol)}{volume\ of\ solution\ (L)}$$

The symbol M (or mM, μM, nM) should never be used to represent amounts, only concentrations. In biochemistry, molarity is used even for substances, which do not exist as true molecules (such as NaCl) and for which formality is used in some branches of chemistry. Mass, molecular weight and moles are related as follows:

$$moles\ of\ solute(n) = \frac{mass\ of\ solute\ (g)}{molecular\ weight\ (g/mol)}$$

$$M(moles/L) = \frac{mass\ of\ solute\ (g)}{molecular\ weight\ (g/mol) \cdot volume\ of\ solution\ (L)}$$

Dilutions

A dilution is the process of decreasing the concentration of a solute in a solution by adding more solvent (usually water in the lab). Diluting a solution means adding more solvent to it. The amount of solute does not change, only the concentration decreases because the total volume increases. Since the solute amount stays constant before and after dilution, the dilution principle follows the equation:

$$c_1 \cdot V_1 = c_2 \cdot V_2$$

where: c_1 = concentration of the initial (stock, concentrated) solution
V_1 = volume of the initial solution
c_2 = concentration the of desired (final, diluted) solution
V_2 = volume of the desired solution

Dilution steps:

- start with a concentrated solution (called a stock solution).
- decide on the desired final volume and concentration.
- calculate how much of the stock solution is needed using the formula.
- add solvent (usually water) to reach the desired final volume.
- mix thoroughly so the solution is homogeneous.

Isotonic solutions

It is known that the osmotic pressure is directly proportional with the number of particles, which are dissolved in a volume of this solution, being independent both to the solvent nature and the solute. Isotonic (isosmotic) solutions have the same effective particle concentration (osmotic pressure) as a reference fluid (e.g., blood plasma). According to Avogadro's law, two equal volumes of different solutions, having the same osmotic pressure and temperature, will contain the same number of particles which can be molecules or ions. Vice versa, it can be said that molar solutions of electrolytes and nonelectrolytes, which contain the same number of particles (molecules or ions) have the same osmotic pressure.

Hence, a KCl solution that contains a gram-molecule of KCl in 1 liter of solution is isotonic with a NaCl solution that contains a gram-molecule of NaCl in 1 liter of solution, because they have the same number of particles.

$$NaCl \rightarrow Na^+ + Cl^-$$
$$KCl \rightarrow K^+ + Cl^-$$

Infusion solutions

Parenteral preparations are sterile pharmaceutical preparations intended to be administered by injection, infusion, or implantation through the skin or mucous membranes directly into the body using a syringe and needle. They must be sterile, pyrogen-free and preferably have an appropriate isotonicity, pH, and stability. There are 2 types of parental preparations:

- **Injection solutions** are designed for rapid administration of small volumes (<100 mL). The main routes of administration are intravenous, intramuscular, subcutaneous.
- **Infusion solutions** are designed for slow administration of large volumes (>100mL). They are always administered intravenously. The three types of intravenous solutions are isotonic, hypertonic and hypotonic (Table 1).

Isotonic solutions have the same osmolarity as serum/plasma. They expand extracellular fluid without altering cell size. They are used for rehydration or to replace the extracellular fluid losses (e.g., bleeding, dehydration from vomiting/diarrhea). The most used isotonic solutions are normal saline (NaCl 0.9%) and Ringer's lactate (Na^+, K^+, Ca^{2+}, Cl^-, lactate).

Hypotonic solutions have an osmolarity lower than serum/plasma. When administered, they cause water to enter the cells and the interstitial space, where the osmolarity is higher. Cells become hydrated and the volume of circulating liquid decreases. Hypotonic solutions are used when the patient is dehydrated intracellularly, such as after treatment with diuretics. They are not indicated to patients with cerebral edema, increased intracranial pressure, burns or low plasma proteins (due to malnutrition or liver problems). The most used hypotonic solution is the half normal saline (NaCl 0.45%).

Hypertonic solutions have an osmolarity higher than serum/plasma. When administered, they cause an increase of the initial serum osmolarity, which results in loss of fluids from inside the cells. Thus, the extracellular volume increases and the volume of cells decrease. They are administered in dehydration (caused by diarrhea and nausea), edemas and unstable blood pressure. Patients receiving hypertonic solutions should be monitored to prevent circulatory overload. They are not indicated for patients with cardiac or renal impairment. The most used hypertonic solutions are 10% dextrose (glucose) and hypertonic saline (e.g., 3% NaCl, 5% NaCl, used in severe hyponatremia).

Figure 1. Effect of different types of infusion solutions on erythrocytes

Table 1. Commonly used infusion solutions

Solution	Category	Utilization / advantage	Disadvantage
Solutions of sodium chloride			
NaCl 0,225%	Hypotonic	- Replacement of extracellular fluid loss when loss of $Cl^- \geq Na^+$ - Treatment of Na^+ depletion - The only solution that can be used with blood preparations	- Possible hypernatremia - Possible depletion of other electrolytes (K^+) - Possible circulatory overload
NaCl 0,45%	Hypotonic		
NaCl 0,9%	Isotonic		
NaCl 3%	Hypertonic		
Solution of dextrose			
Dextrose 5% in water (D5W)	Isotonic	- caloric intake as carbohydrates - treatment for hypercalcemia and dehydration	- Irritation of blood vessels, (high concentrations) - Dehydration caused by the rapid infusion of hypertonic solutions - Possible hyperinsulinemia - Incompatible with blood preparations and some treatments
Dextrose 10% in water (D10W)	Hypertonic		
Dextrose 20% up to 70% in water	Hypertonic		
Combinations of physiological saline solutions and dextrose			
Dextrose 5% + NaCl 0,225%	Isotonic	- Temporary treatment of hypovolemic shock - Replacement of nutrients and electrolytes - Treatment of dehydration and diuretic fostering	- Possible hyponatremia, acidosis and circulatory overload - Requires caution in patients with cardiac, renal or liver diseases
Dextrose 5% + NaCl 0,45%	Hypertonic		
Dextrose 5% + NaCl 0,9%	Hypertonic		
Electrolytes solutions			
Ringer Solution	Isotonic	- Treatment of dehydration; restoring the balance of fluids before and after surgery - Tolerated by patients with liver disease - Similarity with saline	- Reduced caloric intake - Exacerbation of sodium retention, renal failure
Ringer lactate Solution	Isotonic	- Treatment of dehydration, fluid losses in case of burns - Similarity electrolytes content as the extracellular environment - Precursor of bicarbonate	- Possible hypernatremia - Contraindicated in liver disease, hypovolemia, profound shock

In newborns, fluid and electrolyte needs are unique. At birth, there is excess of extracellular water that will be removed in the early days. A few days after birth, fluid and electrolyte requirements are higher, as the newborn begins to grow. Therefore, there must be a strict management of fluids and electrolytes in infants born before term, considering birth weight, gestational age and age after birth. Infants with respiratory syndrome need appropriate fluid replacement. However, excessive administration of fluids can lead to hyponatremia and volume overload, worsening lung condition and increasing the risk of bronchopulmonary dysplasia. Infusion solutions in neonates that are generally administrated are: saline, normal saline solution with 5% glucose and 0.45% NaCl solution with 5% glucose.

Depending on their purpose, infusions can be further divided in:

1. Infusions for fluid therapy

These infusions are used to restore and maintain blood volume (volemia), correct electrolyte and acid-base imbalances and provide prophylaxis during anesthesia/surgery. The fluid therapy can be achieved by the administration of colloid and crystalloid solutions.

- **Crystalloid solutions** contain low molecular weight molecules capable of crossing semipermeable membranes, such as the vascular endothelium. They can be *ionic* (NaCl) or *molecular* (glucose). Based on their osmolarity they can be isotonic, hypotonic or hypertonic. Many types of crystalloid solutions are used to replace the extracellular fluid (ECF) of the interstitial space and the intravascular space, being isotonic with them.
  The main element that defines these solutions is the concentration of Na^+ similar to Na^+ concentration the ECF, which results in their distribution only to the ECF level, proportional to the volume ECF of each compartment (interstitial and intravascular); the intracellular volume is not influenced.
  If they are used to replace blood loss there is a need for administration of a volume of 3-4 times greater than the lost volume, because only 1/3 to 1/4 of administered volume remains intravascular. In a healthy adult with normal hemoglobin values, for a lost blood volume representing 20% of the total blood volume (approx. 1000 ml) fluid therapy may be administered with 3000-4000 ml of crystalloid solutions without the occurrence of adverse effects. This type of solutions is preferred to solubilize drugs and parenteral administration of drugs in infusion.
  Glucose solution 5%, isotonic with plasma (252 mosm/l) is indicated for the treatment of dehydration and hypernatremia; glucose is taken up by cells, practically in the extracellular compartment remaining only the water that reduces the concentration of sodium and increases the volume of extracellular water.
  More concentrated glucose solutions, hypertonic, are indicated for the treatment of hypoglycemia, hyperkalemia and for the energetic intake. The rate of feeding glucose (solution flow rate) should not exceed 0.5 g/kg/hour.
- **Colloid solutions** contain high molecular weight molecules (proteins, polyglucides) unable to cross the vascular endothelium. Administrated intravenously, they remain for longer time in the intravascular space compared to crystalloid solutions and create a greater volemic effect; a higher colloid-osmotic pressure compared to plasma explains the effect. Commonly used colloidal solutions are derivatives of gelatin, a protein produced by partial and irreversible hydrolysis of collagen (oxypolygelatine), hydroxyethyl starch, and rarely dextrans (Dextran 70, Dextran 40).

2. Blood and blood preparations

The blood is the primary carrier of oxygen, nutrients, hormones, and other important substances in the body. Adult body contains about 5 liters of blood. When the patient suffers a decrease in circulating blood volume, it may be necessary to replace it. Any reduction in this volume produces electrolyte and body fluids imbalances. Administering blood and blood preparations restore fluid volume, improves oxygen transport function and replaces the deficient blood components, including clotting factors. Blood preparations are used alone or in combination, depending on the condition of the patient. Blood and blood preparations are administered only with saline solution and not glucose (which may cause hemolysis). The main examples are:

- Whole blood: restores oxygen transport + clotting factors.
- Red cell concentrate: for anemia or blood loss, less plasma.
- Platelet concentrate: for thrombocytopenia or bleeding disorders.
- Plasma (fresh frozen plasma): clotting factor deficiencies, liver disease.

3. Parenteral nutrition

Is a type of intravenous infusion through which all or part of nutrients are administrated intravenously to patients who cannot properly feed by intestinal route. Parenteral nutrition is indicated in malnutrition, severe digestive diseases, obstruction, perforation, etc. The main intravenous nutrients are:

- glucose - the main source of energy in parenteral nutrition.
- lipid emulsions - provides energy and essential fatty acids.
- amino acid solutions of 5.5%, 8.5%, 10%, 15% and 20%.

Table 2. Components of solutions for parenteral nutrition

Component	Aim
Dextrose or substituents	Maintain caloric intake
Amino acids	Keep protein deposits and prevent loss of muscle proteins
Electrolytes	Restore the balance of electrolytes (K^+, Mg^{2+}, Ca^{2+}, P and NaCl)
Vitamins	Cover the necessary of hydro- and liposoluble vitamins, biotin and folic acid
Micronutrients	Cover the needs of zinc, copper, chromium, selenium and manganese
Water	The required amount is determined based on individual needs
Insulin	Added to prevent hypoglycemia caused by high concentrations of glucose
Lipids	Administered separately to prevent or correct deficiency of fatty acids

4. Infusions for drug treatment

Various drug treatments (antibiotics, anticoagulants, antidiabetics, etc.) may be administered intravenously as such, in bolus, or they can be handled via other infusion solutions (5% glucose, saline solution).

Intravenous infusions involve the use of liquids already prepared, or requiring preparation to get the optimum dose to be administered. Preparation of intravenous infusions is based on calculating the concentration of the drug usually expressed in grams or gram submultiples per milliliters and the total volume of liquid to be administered. In certain situations, it is necessary to even calculate the dose per kilogram.

EXPERIMENTAL PART

1. Preparing solutions of various concentrations

Prepare 100 mL of NaCl solution of the following concentrations: 2%, 4%, 6%, 0.34M, 0.7M, 1M.

Steps:
- determine the amount of NaCl, solution mass/volume and volume of distilled water (for percentual solutions) that are needed for the experiment.
- weigh the necessary amount of NaCl using an electronic balance.
- add distilled water and homogenize
- check the concentration using a densimeter, knowing that the molecular weight of NaCl = 58.44 g/mol at 20°C.

% (w/w)	**M**	**ρ (g/cm^3)**
2	0,3465	1,0125
4	0,7028	1,0268
6	1,0691	1,0413

Percentual solutions, 100g – are prepared in beakers:
- pour the necessary amount of NaCl from the weighing vial into the beaker.
- measure the distilled water (solvent) volume using a graduated cylinder.
- use a small portion of solvent to rinse the weighing vial, adding the liquid into the beaker.
- add the remaining solvent into the beaker and homogenize the solution using a stirring wand.

Molar solutions, 100 mL – are prepared in a volumetric flask:
- pour the necessary amount of NaCl (g) from the weighing vial into the volumetric flask.
- wash the weighing vial using a wash bottle and add the liquid into the volumetric flask.
- add small portions of distilled water in the volumetric flask and mix well, so the NaCl dissolves completely.
- add distilled water until it reaches indicated level.
- attach the cap and homogenize the solution.

2. Diluting stock solutions to obtain new solutions of various concentrations

Using a stock solution of NaCl 2M, prepare 100 mL of NaCl solution of the following concentrations: 0.34M, 0.7M, 1.07M, 1.44M and 1.83M.

Solution	**Concentration (mol/L)**	**ρ (g/cm^3)**
Solution 1	0.34	1.0125
Solution 2	0.70	1.0268
Solution 3	1.07	1.0413
Solution 4	1.44	1.0559
Solution 5	1.83	1.0707

3. Preparing isotonic solutions

A. Preparation of phosphate solutions, isotonic with blood plasma

An isotonic solution has the same osmotic pressure as blood plasma, meaning it produces no net movement of water across cell membranes. For reference, an isotonic NaCl solution contains 8.6 g NaCl in 1 liter of solution (≈0.15 M NaCl, since NaCl dissociates into 2 particles). To prepare other isotonic solutions, we compare the number of dissolved particles (osmoles) produced by each solute with that of NaCl.

A.1. Isotonic monosodium phosphate (NaH_2PO_4) solution: When dissolved in water, both NaCl and NaH_2PO_4 dissociate into 2 particles, therefore a 1M NaCl solution is isotonic with a 1M NaH_2PO_4 solution. The quantity of $NaH_2PO_4 \cdot H_2O$ that will be dissolved in 1000 milliliters of distilled water in a volumetric flask can be calculated:

$$NaCl \rightarrow Na^+ + Cl^- \qquad NaH_2PO_4 \rightarrow Na^+ + H_2PO_4^-$$

1 mol NaCl ..... 1 mol $NaH_2PO_4 \cdot H_2O$
58.45 g NaCl ..... 138.01 g $NaH_2PO_4 \cdot H_2O$
8.6 g NaCl x

$$x = \frac{8.6 \cdot 138.01}{58.45} = 20.3$$

g $NaH_2PO_4 \cdot H_2O$ per 1L of water

A.2. Isotonic disodium phosphate solution (Na_2HPO_4): When dissolved in water, NaCl dissociates into 2 particles and Na_2HPO_4 in 3 particles, therefore a 3M NaCl solution is isotonic with a 2M Na_2HPO_4solution. The quantity of $Na_2HPO_4 \cdot 12\ H_2O$ that will be dissolved in 1000 milliliters of distilled water in a volumetric flask can be calculated:

$$NaCl \rightarrow Na^+ + Cl^- \qquad Na_2HPO_4 \rightarrow 2Na^+ + HPO_4^{2-}$$

3 mol NaCl ..... 2 mol $Na_2HPO_4 \cdot 12\ H_2O$
3·58.45 g NaCl ..... 2·358.18 g $Na_2HPO_4 \cdot 12\ H_2O$
8.6 g NaCl x

$$x = \frac{8.6 \cdot 2 \cdot 358.18}{3 \cdot 58.45} = 35.1$$

g $Na_2HPO_4 \cdot 12\ H_2O$ per 1L of water

A3. Isotonic trisodium phosphate solution (Na_3PO_4): When dissolved in water, NaCl dissociates into 2 particles and Na_3PO_4 in 4 particles, therefore a 2M NaCl solution is isotonic with a 1M Na_3PO_4 solution. The quantity of $Na_3PO_4 \cdot 12\ H_2O$ that will be dissolved in 1000 milliliters of distilled water in a volumetric flask can be calculated:

$$NaCl \rightarrow Na^+ + Cl^- \qquad Na_3PO_4 \rightarrow 3Na^+ + PO_4^{3-}$$

2 mol NaCl ..... 1 mol $Na_3PO_4 \cdot 12\ H_2O$
2·58.45 g NaCl ..... 380.17 g $Na_3PO_4 \cdot 12\ H_2O$
8.6 g NaCl x

$$x = \frac{8.6 \cdot 380.17}{2 \cdot 58.45} = 28.0$$

g $Na_3PO_4 \cdot 12\ H_2O$ per 1L of water

B. Preparation of glucose solution, isotonic with blood plasma: When dissolved in water, NaCl dissociates into 2 particles and glucose does not dissociate, therefore a 1M NaCl solution is isotonic with a 2M glucose solution. The quantity of glucose that will be dissolved in 1000 milliliters of distilled water in a volumetric flask can be calculated:

$$NaCl \rightarrow Na^+ + Cl^- \qquad C_6H_{12}O_6\,(glucose) \rightarrow C_6H_{12}O_6\,(glucose)$$

1 mol NaCl ..... 2 mol glucose
58.45 g NaCl ..... 2·180.16 g glucose
8.6 g NaCl x

$$x = \frac{8.6 \cdot 2 \cdot 180.16}{58.45} = 53.0$$

g glucose per 1L of water

4. Working with infusions for drug treatment

Example A. Phenobarbital for seizures.
A patient must receive 160 mg phenobarbital intravenously. The drug is supplied in ampoules of 1 mL, 40 mg/mL. How many ampoules are required?

40 mg phenobarbital ..... 1 mL solution 40 mg/mL
160 mg phenobarbital x
x = 160/40 = 4 mL. Since 1 ampoule has 1 mL => 4 ampoules are needed

Example B. Glucose infusion rate
A 70-kg patient requires glucose infusion at 0.18 g/kg/h for 2 h. The solution available: 5% glucose, 500 mL per vial. How many vials are needed?

Required dose of glucose for 1 hour (0.18 g/kg): 0.18 (g) · 70 (kg) = 12.6 g
Required dose of glucose for 2 hours (0.18 g/kg/h): · 12.6 = 25.2 g

5 g glucose ..... 100 mL solution 5%
25.2 g glucose x
x = (25.2·100)/5 = 504 mL. Since 1 vial has 500 mL => approximately 1 vial is needed

Example C. Dopamine infusion
A 70-kg patient requires dopamine at 5 µg/kg/min. Available solution: 200 mg dopamine in 500 mL 5% glucose. Calculate the infusion rate in mL/h.

Required dose of dopamine per minute (µg/kg/min): 5 (µg) · 70 kg = 350 µg
Required dose of dopamine per minute: 60 · 350 = 21,000 µg = 21 mg

200 mg dopamine ..... 500 mL solution glucose 5%
21 mg dopamine x
x = (21·500)/200 = 52.5 mL/h

Questions

1. Write the formula for calculating
    a. % (w/v) concentration.
    b. Molar concentration (M).

2. Give examples of hypertonic solutions. When can they be used?

3. What is the difference between colloid and crystalloid solutions? Give examples.

4. A stock solution of glucose has a concentration of 20% (w/v). How much of this solution is required to prepare 100 mL of 5% glucose?

5. How many grams of NaCl are needed to prepare 250 mL of 0.9% (w/v) solution?

Lab Notes

Use this space for handwritten notes during the practical session.

L4. ACIDS. BASES. TITRATION.

Water ionization

Water is an **amphoteric molecule**, functioning both as a proton donor (acid) and proton acceptor (base). Its dissociation produces hydronium (H_3O^+) and hydroxyl (HO^-) ions:

$$2H_2O \leftrightarrow H_3O^+ + HO^-$$

For simplicity, the hydronium ion will be referred to as the hydrogen ion (H^+):

$$H_2O \leftrightarrow H^+ + HO^-$$

The equilibrium constant, K_e, of this reaction is presented in equation (1), where the terms in brackets represent the molar concentrations:

$$K_e = \frac{[H^+] \cdot [HO^-]}{[H_2O]} (1)$$

In pure water, the concentration $[H_2O]$ is practically constant at ≈55 mol/L. By substituting this value into equation (1) we obtain equation (2), where **K_w (P_{H2O}) is the ionic product of water**.

$$P_{H_2O} = K_{H_2O} = K_w = [H^+] \cdot [HO^-] (2)$$

At 25°C, the ionic product of water is:

$$K_w = 1 \cdot 10^{-14} \Longrightarrow [H^+] \cdot [HO^-] = 1 \cdot 10^{-14} \Longrightarrow [H^+] = [HO^-] = 1 \cdot 10^{-7} M$$

Thus, in solutions, the concentration of H^+ and HO^- are reciprocally correlated. If $[H^+]$ increases, then $[HO^-]$ decreases, and vice-versa.

Acids and bases

Acids. In an ionolytic solvent (a solvent that dissociates into ions), an acid is any dissolved substance that dissociates into ions, producing a positive ion identical to the one resulting from the solvent's dissociation. In water, all substances that dissociate to give hydrogen ions (hydronium) are acids. In liquid ammonia, where ions NH_4^+ and NH_2^- exist, all ammonium salts are acids. The ionization reaction of any acid can be represented as:

$$HA \leftrightarrow H^+ + A^-$$

The equilibrium constant (K_a, acidity constant) for this system is:

$$K_a = \frac{[H^+] \cdot [A^-]}{[HA]}$$

Based on the acidity constant, acids can be strong or weak:

- Strong acids completely dissociate in water. In solution almost all acid molecules release protons, resulting in a very large K_a. Examples: HCl, HBr, HNO_3, $HClO_4$, H_2SO_4 (first ionization step).
- Weak acids partially dissociate in water. In solution only a fraction of acid molecules release protons, resulting in small K_a. Examples: CH_3COOH, H_2CO_3.

Bases. In an ionolytic solvent, a base is any dissolved substance that dissociates into ions, producing a negative ion identical to the one resulting from the solvent's dissociation. In water, all the substances that dissociate to give hydroxyl ions will be bases. The ionization reaction of any base can be represented as:

$$BOH \leftrightarrow B^+ + HO^-$$

The equilibrium constant (K_b, basicity constant) for this system is:

$$K_b = \frac{[B^+] \cdot [HO^-]}{[BOH]}$$

Based on the basicity constant, bases can be strong or weak:

- Strong bases completely dissociate in water. In solution almost all base molecules release hydroxyl ions, resulting in a very large K_b. Examples: alkali hydroxides (NaOH, KOH, LiOH), alkaline earth hydroxides ($Ca(OH)_2$, $Ba(OH)_2$)
- Weak bases partially dissociate in water. In solution only a fraction of base molecules release hydroxyl ions, resulting in small K_b. Examples: NH_3, $Al(OH)_3$.

Conjugate pairs. The ion formed when an acid donates a proton is its conjugate base. The ion formed when a base accepts a proton is its conjugate acid. The conjugate strength is inversely proportional: strong acids have weak conjugated bases (e.g., HCl = strong acid, Cl^- = weak conjugated base), whereas weak acids have strong conjugated bases (e.g., CH_3COOH = weak acid, CH_3COO^- = strong conjugated base). Same apply for bases: strong bases have weak conjugated acids and weak bases have strong conjugated acids.

pH introduction

pH is a measure of the hydrogen ion concentration of an aqueous solution. It is calculated as the negative decimal logarithm of the concentration in hydrogen ions.

$$pH = \log\left[\frac{1}{H^+}\right] = -\log[H^+]$$

According to the ionic product of water, $[H^+] \cdot [HO^-] = 10^{-14}$ M, therefore:

- In pure water: $[H^+] = [HO^-] \Rightarrow [H^+] = 10^{-7}$ M $\Rightarrow$ pH = 7
- In acidic solutions: $[H^+] > [HO^-] \Rightarrow [H^+] > 10^{-7}$ M $\Rightarrow$ pH < 7
- In basic solutions: $[H^+] < [HO^-] \Rightarrow [H^+] < 10^{-7}$ M $\Rightarrow$ pH > 7
- The pH range: $[H^+]_{min} = 10^0$ and $[H^+]_{max} = 10^{-14} \Rightarrow$ pH range = 0-14

Henderson - Hasselbalch equation

Starting from the acid dissociation equilibrium, taking logarithms, rearranging the terms and knowing that pH = -log[H^+] and pKa = -logK_a, we obtain the **Henderson-Hasselbalch equation**, widely used to calculate the pH of buffer solutions:

$$K_a = \frac{[H^+] \cdot [A^-]}{[HA]}$$

$$logK_a = log[H^+] + log\frac{[A^-]}{[HA]}$$

$$-log[H^+] = -logK_a + log\frac{[A^-]}{[HA]}$$

$$pH = pK_a + log\frac{[A^-]}{[HA]}$$

Titration

Volumetric analysis represents a group of quantitative analytic methods used to determine the quantity of an analyzed component = *analyte* (ion, radical, element, complex substance) by accurately measuring the volume of reagent solution = *titrant* (standard solution of known concentration) needed for the reaction.

Titration is a technique in which a reagent solution (titrant, R_1) is added gradually (drop by drop) to a known volume of another solution (analyte, R_2) to determine its concentration. For this, a burette is used to measure the exact volume of the reagent solution.

The reaction is complete when all analyte (R_2) has reacted with titrant (R_1). **The equivalence point** is the stage of titration at which stoichiometrically equivalent amounts of titrant and analyte have reacted (100% reaction). The concentration of the analyte is determined from:

- the known concentration of the titrant,
- the measured volume of titrant used,
- the balanced chemical equation.

Requirements for a good titration reaction:

- reaction must go to completion.
- reaction must be fast and selective.
- the equivalence point must be clearly detectable.

To determine the equivalence point, chemical **indicators** are used as a third component in titration. Indicators do not participate in the titration reaction itself but are added in the analyte solution to signal the end of the reaction. Most titration indicators are organic or inorganic substances that have the property to change their color depending on medium conditions. Some of the most frequently used indicators are:

- acid-base (pH) indicators: methyl-orange, phenolphthalein
- redox indicators: methylene blue, 2,4-dichlorindophenol
- complexometric indicators: calcon, murexide
- precipitation indicators: potassium chromate, diphenyl carbazone

Acid base titration

Acid base titration is based on the neutralization reaction. A neutralization reaction is the chemical reaction between an acid and a base, resulting in the formation of a salt and water. The general reaction is:

$$HA + BOH \leftrightarrow BA + H_2O$$

Acid-base (pH) indicators are organic substances that change color according to pH. Acid-base indicators are usually weak acids or bases, and they change color depending on their dissociation state. Each has a pKa and a transition pH range (about ±1 unit around pKa). Examples of pH indicators are presented in Table 1.

Table 1. The most used acid-base indicators and their pH range

Indicator	pK_a	pH range	Color		
			Acidic form	Transition	Basic form
Thymol blue (1st transition)	1.7	1.2-2.8	Red	Orange	Yellow
Thymol blue (2nd transition)	8.9	8.0-9.6	Yellow	Green	Blue
Methyl orange	3.4	3.1-4.4	Red	Orange	Yellow
Bromophenol blue	3.9	3.0-4.7	Yellow	Green	Blue
Chlorophenol red	6.0	5.2-6.8	Yellow	Orange	Red
Bromothymol blue	7.1	6.0-7.6	Yellow	Green	Blue
Phenol red	7.8	6.6-8.0	Yellow	Orange	Red
Phenolphthalein	9.5	8.3-10.0	Colorless	Pink	Red/Fuchsia

The titration curve (pH vs volume of titrant) shows a sharp vertical change at equivalence (inflexion) point. The indicator must have a transition range that falls within this vertical region:

- Strong acid + strong base results in an equivalence at pH ≈ 7 => phenolphthalein or bromothymol blue.
- Strong acid + weak base results in equivalence at pH < 7 => methyl orange.
- Weak acid + strong base results in equivalence at pH > 7 => phenolphthalein.

Procedure of acid-base titration:

- Rinse burette with titrant solution, fill it, and note initial volume.
- Pipette a known volume of analyte solution into a conical flask. Add 2-3 drops of suitable indicator.
- Start titration: add titrant slowly, swirling, until indicator changes color (end point).
- Note final burette reading, representing the volume of titrant used.
- Calculate analyte concentration using stoichiometry:

$$c_{analyte} = \frac{c_{titrant} \cdot V_{titrant}}{V_{analyte}}$$

EXPERIMENTAL PART

Determining the concentration of an unknown acetic acid or oxalic acid solution by titration with standard sodium hydroxide solution.

Materials:

- Erlenmeyer flask (100 mL)
- Burette (50 mL)
- Pipette (10 mL) with filler
- 0.01 M NaOH solution = standard titrant
- Unknown acid solution: acetic acid or oxalic acid (10 mL) = analyte
- Phenolphthalein solution = indicator

Procedure:

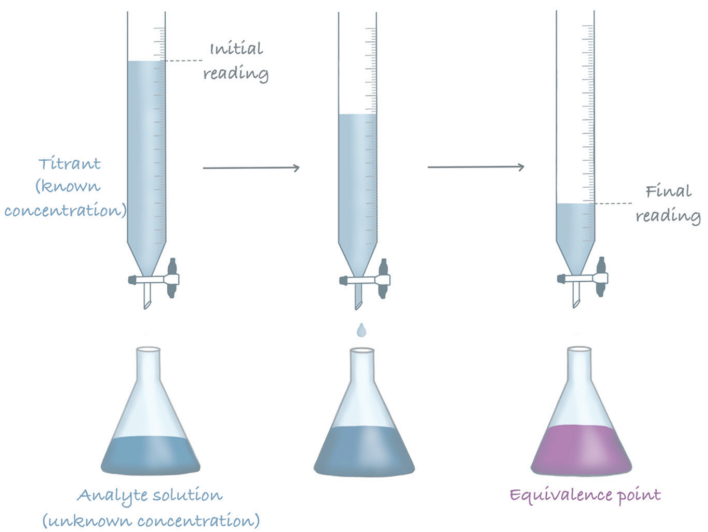
- Pipette 10.00 mL of the unknown acid solution into an Erlenmeyer flask.
- Add 3-4 drops of phenolphthalein indicator. Swirl to homogenize.
- Rinse and fill the burette with 0.01 M NaOH. Record the initial burette reading.
- Add NaOH solution gradually to the acid while swirling.
- As the endpoint approaches (color change visible), add NaOH drop by drop.
- The endpoint is reached when the solution turns pale pink that persists for at least 30 seconds. Read the equivalence volume (V_e).
- Calculate the concentration of the acid

$$CH_3COOH + NaOH \leftrightarrow CH_3COONa + H_2O$$

$$n_{CH_3COOH} = n_{NaOH} \Longrightarrow c_{CH_3COOH} = \frac{c_{NaOH} \cdot V_{NaOH}}{V_{CH_3COOH}} = \frac{0.01M \cdot V_e}{10mL}$$

$$HOOC - COOH + 2NaOH \leftrightarrow NaOOC - COONa + 2H_2O$$

$$2 \cdot n_{HOOC-COOH} = n_{NaOH} \Longrightarrow c_{HOOC-COOH} = \frac{c_{NaOH} \cdot V_{NaOH}}{2 \cdot V_{HOOC-COOH}} = \frac{0.01M \cdot V_e}{2 \cdot 10mL}$$



Questions

1. In pure water, what are the concentrations of hydrogen and hydroxyl ions?

2. Give two examples of strong bases and two examples of weak bases.

3. Write the general equation for a neutralization reaction between an acid and a base.

4. Why must the indicator's pH transition range overlap with the equivalence point?

5. In a titration, 25.0 mL of HCl was neutralized by 20.0 mL of 0.100 M NaOH. What is the concentration of the HCl?

Lab Notes

Use this space for handwritten notes during the practical session.

L5. THE CONCEPT OF pH. DETERMINING THE pH OF SOLUTIONS AND BIOLOGICAL FLUIDS.

Definition of pH

The pH of a solution is a measure of its hydrogen ion concentration. pH is defined as the negative decimal logarithm of the hydrogen ion concentration:

$$pH = \log\left[\frac{1}{H^+}\right] = -\log[H^+]$$

According to the ionic product of water, $[H^+] \cdot [HO^-] = 10^{-14}$ M, therefore:

- In pure water: $[H^+] = [HO^-]$ => $[H^+] = 10^{-7}$ M => pH = 7
- In acidic solutions: $[H^+] > [HO^-]$ (excess protons) => $[H^+] > 10^{-7}$ M => pH < 7
- In basic solutions: $[H^+] < [HO^-]$ (excess hydroxyls) => $[H^+] < 10^{-7}$ M => pH > 7

The pH range: $[H^+]_{min} = 10^0 = 1$ and $[H^+]_{max} = 10^{-14}$ => **pH scale = 0-14**. The relationships between hydrogen/hydroxyl ion concentrations and their exponents are summarized in Table 1:

Table 1. The relationship between pH, pOH and the concentration of protons and hydroxyl ions

$[H_3O^+]$	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	10^{-13}	10^{-14}
$[HO^-]$	10^{-14}	10^{-13}	10^{-12}	10^{-11}	10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	1
pH	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
pOH	14	13	12	11	10	9	8	7	6	5	4	3	2	1	0
Solution	← acidic							neutral				alkaline →			

In biochemistry, dissociation reactions are always written in the sense of proton donation:

$$H_2CO_3 \leftrightarrow H_2CO_3^- + H^+$$
$$NH_4^+ \leftrightarrow NH_3 + H^+$$
$$H_2PO_4^- \leftrightarrow HPO_4^{2-} + H^+$$

The dissociation constant for proton-related equilibria is always the acidity constant (Ka), therefore pK = pKa. For polyprotic acids (molecules with more than one dissociable proton), the pKs values are numbered in order of increasing pH. For example, for H_3PO_4:

$$H_3PO_4 \overset{pK_1}{\leftrightarrow} H_2PO_4^- + H^+$$
$$H_2PO_4^- \overset{pK_2}{\leftrightarrow} HPO_4^{2-} + H^+$$
$$HPO_4^{2-} \overset{pK_3}{\leftrightarrow} PO_4^{3-} + H^+$$

From the dissociation equilibrium and Henderson–Hasselbalch eq.: $pH = pK_a + log\frac{[A^-]}{[HA]}$, when $[A^-] = [HA]$ => $pK_a = pH$. Therefore, the pK_a is the pH value at which the concentrations of dissociated and undissociated forms are equal.

Calculation of the pH of solutions

To calculate the pH of acid/base solutions, the acidity/basicity constant equations are needed. We denote with c the initial concentration of the acid/base.

$$K_a = \frac{[H^+] \cdot [A^-]}{[HA]}, K_b = \frac{[B^+] \cdot [HO^-]}{[BOH]}$$

a. Solutions of strong acids or strong bases. Strong acids and bases are completely dissociated in aqueous solution. Therefore, the concentration of hydrogen ions will be equal to the analytical concentration (c) of the acid or base. In this case:

- For strong acids: $[H^+] = [HA]_i = c$
  $pH = -\log[H^+] \implies pH = -\log[HA]_i \implies \boldsymbol{pH = -\log(c)}$
- For strong bases: $[HO^-] = [BOH]_i = c$
  $pH = 14 - pOH = 14 + \log[BOH]_i \implies pH = 14 + \log[BOH]_i \implies \boldsymbol{pH = 14 + \log(c)}$

b. Solutions of weak acids or weak bases. Weak acids and bases are only partially dissociated. To calculate their pH, the acidity/basicity constant are needed:

- For weak acids: $[H^+] = [A^-]$ and $[HA]_{undisociated} = c - [H^+]$. Thus, the acidity constant becomes:
  $K_a = \frac{[H^+]^2}{c - [H^+]} \implies [H^+]^2 = K_a \cdot (c - [H^+]) \implies [H^+] = \sqrt{K_a \cdot (c - [H^+])}$
  Given that $c - [H^+] \approx c \implies [H^+] = \sqrt{K_a \cdot c} \implies \boldsymbol{pH = -\log\left(\sqrt{K_a \cdot c}\right)}$
- For weak bases, similarly: $\boldsymbol{pH = 14 + \log\left(\sqrt{K_b \cdot c}\right)}$

c. Solutions of salt. Hydrolysis reaction. When certain salts dissolve in water, their ions can react with the H^+ + HO^- ions from water dissociation. This reaction is called hydrolysis. Ions from strong acids or bases are neutral (no hydrolysis), but ions from weak acids or bases hydrolyze. Hydrolysis is a reversible reaction. The equilibrium position depends on the acid or base strength of the parent compounds, the degree of dissociation, the solubility or volatility of the hydrolysis products:

- *Neutral salts (strong acid + strong base):* Both the cation and anion come from strong acid and base; therefore, their conjugated pairs are weak species, unable to react with water. The concentration of protons and hydroxyl group remain unchanged, equal, so pH = 7.
- *Basic salts (weak acid + strong base):* The cation comes from a strong base and represents a weak conjugated acid; therefore, it does not react with water. The anion come from a weak acid and represents a strong conjugated base; therefore, it hydrolyses, generating hydroxyl ions and elevating the pH (> 7): $A^- + H_2O \leftrightarrow HA + HO^-$
- *Acid salts (strong acid + weak base):* The anion come from a strong acid and represents a weak conjugated base; therefore, it does not react with water. The cation comes from a weak base and represents a strong conjugated acid; therefore, it hydrolyses, generating protons and decreasing the pH (< 7): $B^+ + H_2O \leftrightarrow BOH + H^+$
- *Basic/acid salt (weak acid + weak base)*: Both the cation and anion come from weak acid and base; therefore, their conjugated pairs are strong species, able to hydrolyze. The pH depends on relative strength of the parent acid and base: $A^- + B^+ + H_2O \leftrightarrow HA + BOH$

pH determination

The pH of a solution can be determined using two main methods:

- pH indicator paper
- pH-meter

The **pH indicator paper** is impregnated with a mixture of pH-sensitive dyes and it changes color depending on the acidity/alkalinity of the solution. The color of the paper is compared with a standard scale to estimate the pH. It has the advantages that is simple, inexpensive, and rapid, but it is limited by the fact that it only provides approximate values (accuracy ~ ±0.5-1.0 units).

The pH meter is the preferred method whenever precise measurements are required. It is a potentiometer that measures the potential developed between a glass electrode and a reference one (calomel: $Hg/Hg_2Cl_2/KCl$/saturated solution), immersed in the solution with the unknown pH. The glass electrode has an end made of a special, very thin glass that is permeable only to hydrogen ions.

Currently, most devices use the **combined electrode** (the reference electrode and the pH dependent electrode are in the same glass tube). The use of such an electrode is relatively simple. There are, however, a series of precautions on which the proper functioning of the electrode depends. Thus, a periodic check of the level of the saturated KCl solution in the electrode is necessary. After use, the electrode should be stored in a 3M KCl solution. In addition, careful handling is important because the special glass end, permeable to hydronium ions, is very fragile.

Calibration of the pH-meter

Before performing a measurement, the pH-meter must be calibrated with buffer solutions (standardized standards with a well-established pH). The most common buffer solutions have a pH value of 4, 7 or 10 and an accuracy of ±0.02 pH units.

Working procedure

1. Remove the electrode from storage solution.
2. Rinse with distilled water and gently blot dry with filter paper (do not rub, to avoid damaging the glass membrane).
3. Immerse the electrode tip (including ceramic junction) in the solution to be measured.
4. Wait for the reading to stabilize, then record the pH value.
5. After measurement, rinse electrode again and place it back into the 3M KCl storage solution.

Isoelectric pH

The isoelectric pH (pI or pHi) is the pH at which a molecule carries no net electrical charge. At this point, the number of positive charges = the number of negative charges, the molecule's overall charge = 0, the solubility is minimum, and the migration in an electric field does not occur.

The isoelectric pH of amino acids

Amino acids are compounds that have both acidic and basic functions in their structure. The usual representation (I) containing an $–NH_2$ group and a –COOH group does not correctly represent their structure, due to the fact that the two groups can neutralize each other. Amino acids, in aqueous solution, have the structure of amphions or zwitterions/bipolar ions (II):

$$H_2N - R - COOH \text{ (I)} \qquad H_3N^+ - R - COO^- \text{(II)}$$

Because of this dual character of amino acids, they form salts with both acids and bases. In an acidic environment, amino acids act as bases, and in a basic environment as acids. If an acid is added to the solution of an amino acid, the protons will be neutralized according to equation (1); if a base is added, hydroxyl ions will be neutralized according to reaction (2). In both cases the pH of the solution does not change appreciably. The use of amino acids in buffer solutions is based on this property.

$$H_3N^+ - R - COO^- + H^+ \leftrightarrow H_3N^+ - R - COOH \text{ (1)}$$
$$H_3N^+ - R - COO^- + HO^- \leftrightarrow H_2N - R - COO^- \text{ (2)}$$

Consequently, in acidic solutions amino acids are cations and migrate towards cathode (–). In basic solutions amino acids are anions and towards anode (+). In amphionic form, amino acids do not contribute to the transport of current in the electric field, being equally attracted to both the cathode and the anode:

$$H_3N^+ - R - COOH\ (cation) \overset{pK_1}{\leftrightarrow} H_3N^+ - R - COO^-(amphion) \overset{pK_1}{\leftrightarrow} H_2N - R - COO^-(anion)$$

The isoelectric pH of an amino acid (pI) is defined as the pH of the solution at which the amino acid is completely present as bipolar ions, with zero global charge. At the isoelectric pH, the solubility of the amino acid is minimal, and it does not migrate in an electric field. Considering their 2 constants of acidity:

$$K_{a1} = \frac{[H^+] \cdot [H_3N^+ - R - COO^-]}{[H_3N^+ - R - COOH]}, K_{a2} = \frac{[H^+] \cdot [H_2N - R - COO^-]}{[H_3N^+ - R - COO^-]}$$

At the isoelectric pH: $[H_3N^+ - R - COOH] = [H_2N - R - COO^-]$, therefore:

$$\frac{[H^+] \cdot [H_3N^+ - R - COO^-]}{K_{a1}} = \frac{K_{a2} \cdot [H_2N - R - COO^-]}{[H^+]} \Longrightarrow [H^+]^2 = K_{a1} \cdot K_{a2}$$
$$[H^+] = \sqrt{K_{a1} \cdot K_{a2}} \Longrightarrow pH = -log\sqrt{K_{a1} \cdot K_{a2}} \Longrightarrow \boldsymbol{pI = \frac{1}{2}(pK_{a1} + pK_{a2})}$$

Several amino acids have more than one amino and carboxyl group and thus have 2 pKa values. The isoelectric point of different amino acid types is therefore:

- Neutral amino acids (monoaminomonocarboxylic): pI ≈ 6
- Acidic amino acids (monoaminodicarboxylic): pI < 6 (acidic range).
- Basic amino acids (diaminomonocarboxylic): pI > 7 (basic range).

The isoelectric pH in amino acids has physiological relevance as amino acids are part of polypeptide chains of proteins. The electric charge of an amino acid is positive in biological fluids with a pH lower than the isoelectric pH and negative if it is higher. At physiological blood pH (~7.4) aspartate & glutamate residues (-COOH groups) are negatively charged (–), whereas lysine, arginine, histidine residues (-NH_2 groups) are positively charged (+). This charge distribution influences protein folding, enzyme activity, and protein-protein and protein-DNA interactions

For example, glycine (Figure 1) has pK_{a1} = 2.34 and pK_{a2} = 9.60, therefore pI = 5.97. At a pH = 5.97, the net charge of the molecules in the solution is zero.

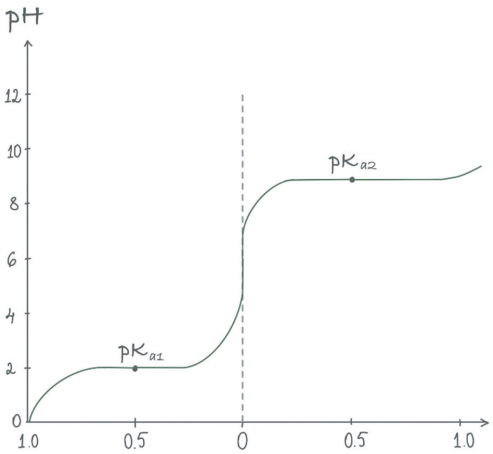


Figure 1. Titration curve of glycine

Determination of blood and urine pH

a. Blood pH

Blood pH determinations are useful for diagnosing some ailments as well as for assessing the functioning of various organs. The normal blood pH value is 7.35-7.45.

- The drop in blood pH below 7.35 is called acidosis (*decompensated diabetes*)
- The increase in blood pH above 7.45 is called alkalosis (*pulmonary hyperventilation, prolonged vomiting*)

Table 2. Modifications of pH, pCO_2, HCO_3^- in acidosis and alkalosis

CONDITION	pH	pCO_2	HCO_3^-
METABOLIC ACIDOSIS	↓	N / ↓ (compensation)	↓
RESPIRATORY ACIDOSIS	↓	↑	N / ↑ (compensation)
METABOLIC ALKALOSIS	↑	N / ↑ (compensation)	↑
RESPIRATORY ALKALOSIS	↑	↓	N / ↓ (compensation)

The introduction of the **ASTRUP** micro method in the exploration of acid-base balance represents an important advance in this field. Unlike the determination of plasma bicarbonates, the Astrup method considers the role of the buffer made by hemoglobin and the intervention of erythrocyte carbonic anhydrase. This method is based on the inverse proportional relationship that exists between the partial pressure of CO_2 (pCO_2) and the pH values in the blood. For the Astrup micro method, 3 drops of arterial or venous blood are sufficient from which blood pH, pO_2, pCO_2, base excess and current bicarbonate can be determined.

b. Urine pH

Urine pH can be determined with the help of urine strips (also called reactive strips). They semi-quantitatively analyze some products present in urine: urobilinogen, bilirubin, ketone bodies, ascorbic acid (some of them), glucose, proteins, red blood cells, nitrites, leukocytes, as well as urinary pH and density.

A strip consists of 4 superimposed layers: a protective nylon film, a paper impregnated with the corresponding reagent, an absorbent filter paper and a plastic support. The test strip contains several distinct zones, corresponding to a certain parameter to be tested. Inserting the strip into the urine will produce a color change in each area or not.

Reading the urine strips can be performed:

- manually: the changes will be compared with a standard scale (Figure 2), delivered with the test, in which the correlation is made between the concentration of the analyzed parameter and the intensity of the coloration
- automatically, with the help of devices called strip readers. These are portable photometers that can read a variable number of samples/hour and can be used in analytical laboratories, hospitals, ambulances and individual practices by family doctors or medical specialists.

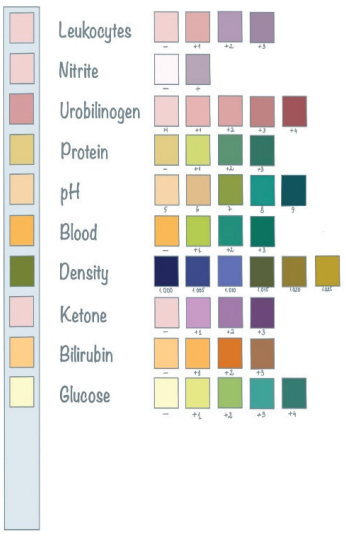


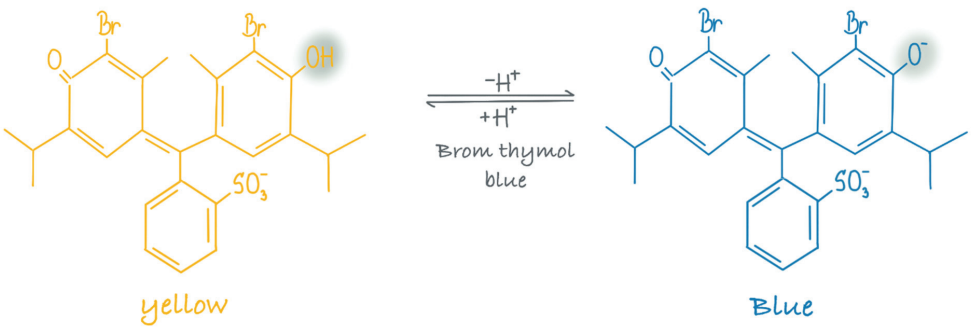
Figure 2. Urinary strips and scale

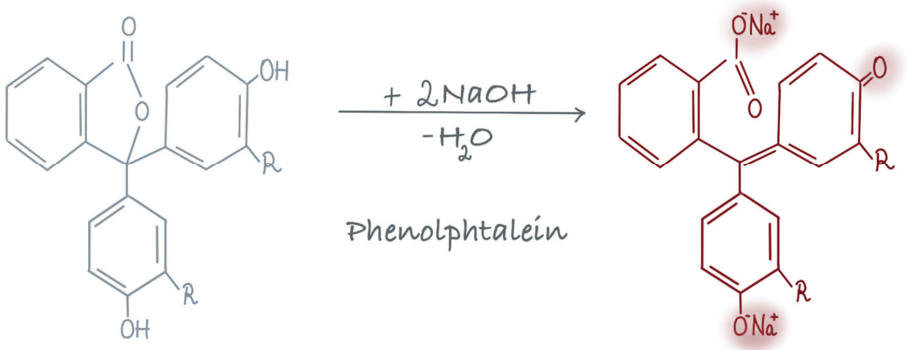
Procedure

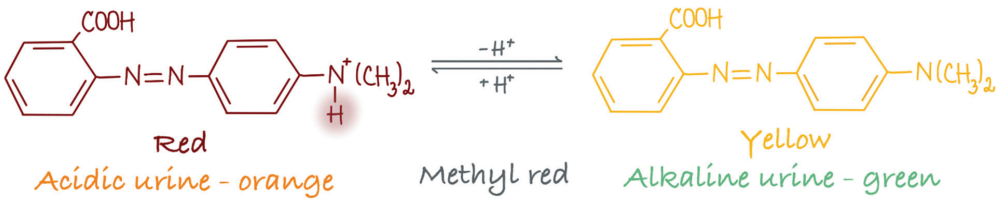
- Fresh uncentrifuged urine is used, usually the first urine in the morning.
- Urine is collected preferably in a sterile container (if the container is not sterile, nitrites are not interpreted).
- The strip is immersed for 2 seconds in the urine so that its entire surface (each reactive area) is completely covered by the liquid.
- Remove the excess liquid, sticking the edge of the strip to the edge of the urine container, then place the strip in a horizontal position.
- The reactive areas on the strip are compared with the corresponding ones on the standard scale in an interval of 60 seconds (for leukocytes the interval is 60-120 seconds).
- Two minutes will not be exceeded for comparing the results.

The principle of determining urine pH with urine strips

The reactive zone contains a mixture of three indicators: methyl red, bromothymol blue and phenolphthalein. In the pH range of 5-9, a color is obtained that varies gradually from orange-yellow-green to blue.







Error sources

In the case of urine samples that sit for a long time before being worked on, the pH can turn towards alkaline because of bacterial growth.

Clinical significance

Normally, urine has an acidic reaction. The pH of the urine is determined, to the greatest extent, by the proportion of dibasic (alkaline) and monobasic (acid) phosphates in the urine. As is known, the kidney is irrigated by blood, which has a pH of about 7.4 and forms urine with an acidic pH. This pH variation is achieved, in part, by changing the proportion between acid and alkaline phosphates. Through this mechanism, a large number of fixed bases of the body are spared, especially Na^+, but also K^+, Mg^{2+} şi Ca^{2+}.

Alkaline phosphate ratio in plasma (pH = 7.4) is:

$$\frac{NaH_2PO_4}{Na_2HPO_4} = \frac{1}{5}$$

The ratio of alkaline phosphates in the urine (pH = 4.5-5.5) reverses:

$$\frac{NaH_2PO_4}{Na_2HPO_4} = \frac{5}{1}$$

In cases where urine reaches maximum acidity, the ratio is equal to 50:1.

Urinary pH varies between 5.8-7.4 during the day. Morning urine has a pH of 5-6. By storing it for a longer time, the urine can become alkaline because, through contamination with microbial germs, urea is decomposed (under the action of urease) into ammonia. The pH values that exceed the value of 7 have an analytical importance because, in this case, the erythrocytes and leukocytes undergo rapid lysis, which explains the negative results obtained in the urine sediment in the conditions of positive results on the strip.

Physiological changes in urinary pH

- depending on the diet:
  - in a strictly carnal diet, urine is acidic (pH = 5.2 - 5.3)
  - in an exclusively vegetarian diet, urine is alkaline (pH = 7.0 - 7.5)
  - in the case of a mixed diet, the urine reaction is weakly acidic
  - it must be remembered that, after the administration of bicarbonates or treatment with mineral waters, the urine has an alkaline reaction
- depending on the nictemeral rhythm:
  - the most acidic urine is eliminated around midnight and the least acidic (sometimes alkaline) in the morning

Pathological changes in urinary pH

- **Low urinary pH values (acidic urine)** are found in starvation, various intoxications, renal failure, diabetic ketoacidosis.
- **Increased urinary pH values (alkaline urine)** are found in profuse vomiting of various etiologies, urinary tract infections (urethritis, cystitis, pyelitis, pyelonephritis), severe hypokalemia, various intoxications, fever of various etiologies.

Table 3. pH range of some biological fluids

Biological fluid	pH
Blood	7,35-7,45
Tears	7,20-7,40
Saliva	6,40-7,00
Gastric juice	1,50-3,00
Pancreatic juice	7,50-8,00
Urine	5,00-7,50
Cerebrospinal fluid	7,20-7,40

EXPERIMENTAL PART

Determination of the pH of solutions using the pH-meter.

Objectives:

1. Measure the pH of five prepared solutions using a pH-meter.
2. Compare the measured pH values with those of biological fluids in the human body.

SOLUTION 1 – pH ~ 2 (gastric juice)
SOLUTION 2 – pH ~ 5.5 (urine)
SOLUTION 3 – pH ~ 7. 4 (plasma)
SOLUTION 4 – pH ~ 7. 8 (urine)
SOLUTION 5 – pH ~ 8.2 (pancreatic juice)

Working Procedure with the pH-meter:

- Turn on the pH-meter (power button).
- Prepare the electrode: Rinse with distilled water. Gently blot dry with filter paper (do not rub the glass membrane).
- Measure the pH: Insert the electrode tip into the solution. Wait ~30-40 seconds until the reading stabilizes. Read and record the pH value from the display.
- Repeat the procedure for each solution, rinsing and drying the electrode between measurements.
- After all measurements, return the electrode to the storage solution (3M KCl).

Questions

1. What is the pH of pure water at 25 °C and why?

2. Acetic acid has Ka = 1.8×10^{-5}. Calculate the pH of a 0.1 M acetic acid solution.

3. Why do salts of weak acids + strong bases give basic solutions? (Give examples)

4. What is the physiological significance of the isoelectric point in proteins?

5. Given that alanine has $pKa_1 = 2.34$ and $pKa_2 = 9.69$ determine the pH at which it has minimum solubility.

Lab Notes

Use this space for handwritten notes during the practical session.

L6. BUFFER SOLUTIONS.

Buffer solutions

A buffer solution is a solution that contains a weak acid (HA) and its conjugate base (A^-) (or a weak base and its conjugate acid). It resists significant pH changes when small amounts of strong acid or strong base are added. Buffers are essential in both chemical and biological systems to maintain a stable pH.

If an acid (H^+) is added to a buffer solution it is consumed by the conjugate base (A^-), which converts into its corresponding acid (HA), as displayed in equation (1). If a base (OH^-) is added to a buffer solution it is consumed by the weak acid (HA), which converts into its corresponding base (A^-), as in equation (2). As a result, addition of either acid or base leads to a change in the A^- / HA ratio.

$$A^- + H^+ \leftrightarrow HA \text{ (1)}$$

$$AH + HO^- \leftrightarrow A^- + H_2O \text{ (2)}$$

Henderson - Hasselbalch equation. Buffer range and buffer capacity

Starting from the acid dissociation equilibrium, taking logarithms, rearranging the terms and knowing that pH = -log[H^+] and pKa = -logK_a, we obtain the **Henderson-Hasselbalch equation** (3). In the **Henderson - Hasselbalch equation**, the ratio [A^-]/[HA] is a logarithmic function, so even large changes in the concentrations of [A^-] and [HA] produce small absolute changes in pH.

$$K_a = \frac{[H^+]\cdot[A^-]}{[HA]} \Leftrightarrow logK_a = log[H^+] + log\frac{[A^-]}{[HA]} - log[H^+] = -logK_a + log\frac{[A^-]}{[HA]}$$

$$pH = pK_a + log\frac{[A^-]}{[HA]} \text{ (3)}$$

Buffer's range is determined by the pK value of the system. The most effective buffer range is **pH = pK_a ±1**. Outside this range, the solution loses buffering ability (too much acid/base form).

Buffer capacity (β) = the number of equivalents of either H^+ or OH^- (the number of moles of strong acid or base) that must be added to 1 L of buffer to change its pH by 1 unit. Buffer capacity increases with a higher total concentration of buffer components and a larger buffer volume.

The selection of biochemical buffers

Ideally, all the biochemical investigation must be done in aqueous buffer solutions. The natural medium of biomolecules and cellular organelles is under a strict pH control (pH ≈ 6-8). Extraction of biological components (proteins, nucleic acids, organelles) requires a controlled environment to maintain stability. Many enzymatic reactions consume or release protons (H^+), which would otherwise change the medium's pH; a buffer system is therefore essential to keep pH constant. Occasionally, buffers with other pH values are required (Table 1).

Tris [tris(hydroxymethyl)aminomethane] is one of the most used synthetic biochemical buffers (more than phosphate buffer). It is effective in the range: pH 7.5-9.0. Since it is a primary amine, it has minimal interference with biochemical processes. Moreover, it does not precipitate Ca^{2+} ions, it is chemically stable, and widely available. To prepare 1 L of 0.1 M Tris buffer: dissolve 12.11 g Tris in 950-975 mL distilled water, adjust the pH with concentrated HCl (to make Tris-HCl), and bring the final volume to 1 L with distilled water.

Table 1. Common buffer systems used in biochemistry

Compound	Abbreviation	MW	pK_1	pK_2	pK_3	pK_4
N-(2-acetamido)-2-aminoethansulfonic acid	ACES	182.2	6.90	-	-	-
N-(2-acetamido)-2-iminodiacetic acid	ADA	212.2	6.60	-	-	-
Acetic acid		60.1	4.76	-	-	-
Arginine	Arg	174.2	2.17	9.04	12.48	-
Barbituric Acid		128.1	3.79	-	-	-
N,N-bis(2-hydroxyethyl)-2-aminoethansulfonic acid	BES	213.1	7.15	-	-	-
N,N-bis(2-hydroxyethyl)glycine	BICINE	163.2	8.35	-	-	-
Boric acid		61.8	9.23	12.74	13.80	-
Citric acid		210.1	3.10	4.75	6.40	-
Ethylendiaminotetracetic acid	EDTA	292.3	2.00	2.67	6.24	10.88
Formic acid		46.03	3.75	-	-	-
Fumaric acid		116.1	3.02	4.39	-	-
Glycine	Gly	75.1	2.45	9.60	-	-
Glycylglycine		132.1	3.15	8.13	-	-
N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid	HEPES	238.3	7.15	-	-	-
N-2-hydroxyethylpiperasine-N'-3-propansulfonic acid	HEPPS	252.3	8.00	-	-	-
Hystidine	His	209.7	1.82	6.00	9.17	-
Imidazole		68.1	6.95	-	-	-
2-(N-morpholino)- ethansulphonic acid	MES	195.0	6.15	-	-	-
3-(N-morpholino)- propansulphonic acid	MOPS	209.3	7.20	-	-	-
Phosphoric acidc		98.0	2.12	7.21	12.32	-
Succinic acid		118.1	4.18	5.60	-	-
3-tris(hydroxymethyl)-aminopropansulphonic acid	TAPS	243.2	8.40	-	-	-
N-tris(hydroxymethyil)methyl-2-aminoethansulfonic acid	TES	229.2	7.50	-	-	-
N-Tris(hydroxymethyl)methylglycine	Tricine	179.0	8.15	-	-	-
Tris(hydroxymethyl)amino-methane	Tris	121.1	8.30	-	-	-

Ideal properties of biochemical buffers

1. Have a pKa between 6 and 8 (close to physiological pH).
2. Be highly soluble in water, but minimally soluble in organic solvents.
3. Contribute minimally to the ionic strength of the medium.
4. Not cross biological membranes (or have negligible effects if it does).
5. Have a minimal salt effect.
6. Not interact with metal ions of biological importance (or form stable, defined complexes only).
7. Have minimal absorbance in the visible and UV range (so it does not interfere with spectroscopic assays).
8. Be chemically stable over time and resistant to degradation.
9. Be easily available in pure form at reasonable cost.

Biological buffer systems

The H^+ concentration in the blood and extracellular space is approximately 40 nM. This corresponds to a **pH of 7.40**. The body tries to keep this value constant, as large shifts in pH are incompatible with life. The pH value is kept constant by buffer systems that cushion minor disturbances in the acid-base balance. In the longer term, the decisive aspect is maintaining a balanced equilibrium between H^+ production and uptake and H^+ release. If the blood's buffering capacity is not sufficient, or if the acid–base balance is not in equilibrium - e. g., in kidney disease or during hypoventilation or hyperventilation - shifts in the plasma pH value can occur. A reduction by more than 0.03 units is known as **acidosis,** and an increase is called **alkalosis.**

The most important buffer systems, which function permanently in the organism, are bicarbonate/carbonic acid, inorganic phosphates ($HPO_4^{2-}/H_2PO_4^-$), proteins and hemoglobin (unprotonated/protonated) systems.

1. Bicarbonate/carbonic acid

This is the most important extracellular buffer and the primary regulator of blood pH. It consists of water, carbon dioxide (CO_2, the anhydride of carbonic acid H_2CO_3), and hydrogen carbonate (HCO_3^-, bicarbonate). It represents an open system CO_2 can be exhaled via lungs and kidneys can excrete H^+ and regenerate HCO_3^-, allowing dynamic regulation.

The adjustment of the balance between CO_2 and HCO_3^- is accelerated by the zinc-containing enzyme carbonic anhydrase, that catalyses the hydration of CO_2, with the rapid formation of H_2CO_3. H_2CO_3 acts as a weak acid, neutralizing base excess and HCO_3^- as a weak base, neutralizing acid excess.

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$$

$$H^+ + HCO_3^- \longrightarrow H_2CO_3 \longrightarrow CO_2 + H_2O$$

$$HO^- + H_2CO_3 \longrightarrow HCO_3^- + H_2O$$

At the pH value of the plasma and a pK_a of 6.1, HCO_3^- and CO_2 are present in a ratio of about 20:1(24 mEq bicarbonate and 1.2 mEq carbonic acid). Instead of using 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.

2. Inorganic phosphates

$H_2PO_4^-/HPO_4^{2-}$ is important mainly in intracellular fluid and renal tubules. The $pK_a \approx 6.8$, so it buffers effectively around physiological pH. $H_2PO_4^-$ acts as a weak acid (neutralizes base excess) and HPO_4^{2-} as a weak base (neutralizes acid excess).

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

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

$$HO^- + H_2PO_4^- \leftrightarrow HPO_4^{2-} + H_2O$$

The ratio of dihydrogen-phosphate:monohydrogen-phosphate is 1:5 in blood and 5:1 in urine. In plasma phosphate buffer contributes only a small fraction of blood buffering capacity (much less than bicarbonate and hemoglobin), because plasma phosphate concentration is relatively low. In urine, phosphate buffering helps excrete H^+ and maintain systemic acid-base balance. In the intracellular fluid (ICF) phosphate is much higher than in plasma, and it plays a major role in buffering metabolic acids inside cells.

3. Proteins

Proteins are amphoteric molecules, they contain both acidic (–COOH) and basic ($–NH_2$) groups, plus ionizable side chains on certain amino acids. This allows them to either accept or donate protons (H^+) depending on the pH. At low pH, proteins act as bases (accept H^+), and at high pH, proteins act as acids (donate H^+). Histidine (imidazole group, pKa ≈ 6.8) is the most important amino acid for buffering at physiological pH.

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

Since it is the most abundant plasma protein (~60% of total protein) and carries many ionizable groups, albumin contributes significantly to plasma buffering capacity. Each albumin molecule has multiple residues of histidine, making it an effective buffer around physiological pH.

4. Hemoglobin

Hemoglobin (Hb) is a tetrameric protein in red blood cells, with each subunit containing a heme group (binds O_2) and a globin chain rich in histidine residues. These histidine imidazole groups are the main proton-binding sites.

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

Moreover, hemoglobin has an important buffering capacity thanks to the Bohr effect and its ability to bind H^+ generated during metabolism. In tissues (low O_2, high CO_2, acidic pH) CO_2 enters RBCs, producing H^+. H^+ binds Hb, stabilizing deoxyhemoglobin (HHb). This lowers Hb's affinity for O_2, promoting O_2 release. In lungs (high O_2, low CO_2, more alkaline pH): O_2 binds Hb, displacing H^+. H^+ combines with HCO_3^- to form CO_2, which is exhaled. This represents the Bohr effect. Hemoglobin functions in synergy with the bicarbonate buffer, therefore erythrocytes are the meeting point of these two systems.

Acid-base disorders

The normal pH values in blood are **7.35-7.45**. The pH of blood is mainly determined by the ratio of HCO_3^- and H_2CO_3 or pCO_2 (normally 20:1). Values of pH <6.9 or >7.9 represent the limits of survival.

- A decrease in HCO_3^- : pCO_2 ratio produces **acidosis** (pH<7.35).
- An increase in HCO_3^- : pCO_2 ratio produces **alkalosis** (pH>7.45).
- When the modifications of the acid-base equilibrium are caused by an initial modification of bicarbonates, we talk about **metabolic** acidosis or alkalosis.
- When the modifications of the acid-base equilibrium are caused by an initial modification of carbon dioxide, we talk about **respiratory** acidosis or alkalosis.

Metabolic acidosis represents the decrease in blood pH due to a primary decrease in bicarbonate (HCO_3^-). It can be caused by diabetic ketoacidosis (excess ketone bodies), lactic acidosis (shock, hypoxia), diarrhea (loss of HCO_3^-), chronic kidney disease (impaired H^+ excretion, HCO_3^- loss).

Metabolic alkalosis represents an increase in blood pH due to a primary increase in bicarbonate (HCO_3^-). It can be caused by prolonged vomiting (loss of gastric HCl), excessive antacid intake (alkali ingestion), treatment with diuretics (loss of Cl^-, K^+ leading to H^+ shift).

Respiratory acidosis represents a decrease in blood pH due to a primary increase in carbonic acid (↑pCO_2) from hypoventilation. It can be caused by chronic obstructive pulmonary disease (COPD), respiratory depression (drugs, anesthesia, head trauma), and airway obstruction.

Respiratory alkalosis represents an increase in blood pH due to a primary decrease in carbonic acid (↓pCO_2) from hyperventilation. It can be caused by anxiety, panic attacks (rapid breathing), high altitude (low O_2 → hyperventilation), early stages of pneumonia, pulmonary embolism, fever, sepsis.

EXPERIMENTAL PART

Preparing buffer solutions

Obtain 200 mL of acetic acid/sodium acetate buffer, pH=4.8, using acetic acid and sodium acetate solutions of 0.01M concentration, knowing that pK_a=4.76.

1. Prepare 200 mL of 0.1M sodium acetate solution, using pure solid sodium acetate:
   - Calculate the necessary amount of sodium acetate
   - Weigh using a weighing vial the necessary amount of sodium acetate
   - Transfer the substance in a 200 ml volumetric flask using a funnel and add distilled water
   - Homogenize the solution until the solid is completely dissolved
   - Add distilled water until you reach the mark on the volumetric flask

2. Prepare 200 mL of 0.1M acetic acid solution by diluting a 1M acetic acid solution:
   - Calculate the necessary volume of concentrated acetic acid solution (1M)
   - Measure the necessary volume using a graduated cylinder
   - Transfer the volume of concentrated acetic acid solution into a 200 ml volumetric flask, and add distilled water until you reach the mark on the volumetric flask
   - Homogenize the solution

3. Prepare 100 mL of CH_3COONa/CH_3COOH buffer solution of pH=4.8, using the prepared solutions at points 1 and 2.
   - Measure using graduated cylinders the necessary volumes of acetic acid and sodium acetate solutions and add them in the same 100 ml volumetric flask
   - Homogenize the mixture
   - Measure the pH of the buffer solution using a pH-meter

Buffering capacity:

Reagent	**Buffer solution**		**Distilled water**	
	1a	**1b**	**2a**	**2b**
Buffer solution (pH=4.8)	30 mL	30 mL	-	-
Distilled water	-	-	30 mL	30 mL
pH				
HCl 0,01M	0.5 mL	-	0.5 mL	-
NaOH 0,01M	-	0.5 mL	-	0.5mL
pH				

Questions

1. Define buffer capacity. List two factors that increase it.

2. Write the two reactions that explain how a buffer responds to added H^+ and added OH^-.

3. Acetate buffer, pKa = 4.76. What is pH when the ratio of sodium acetate to acetic acid is 1? How about when it is 2? But when it is 0.5?

4. Name the four major blood buffer systems and indicate which is quantitatively most important in plasma.

5. Explain how hemoglobin buffering links to the Bohr effect.

Lab Notes

Use this space for handwritten notes during the practical session.

L7. PREPARATION OF BIOLOGICAL SAMPLES FOR THE LABORATORY ANALYSIS. CENTRIFUGATION

Blood - is a specialized liquid, composed of a pale-yellow fluid, the plasma, and the cellular elements: erythrocytes (red blood cells), leukocytes (white blood cells), and thrombocytes (platelets).

Its primary role is transport: blood carries oxygen and nutrients to the tissues and removes carbon dioxide and metabolic waste products. In the systemic circulation, arterial blood is bright red because of its high oxygen content, while venous blood is dark red after oxygen has been delivered to tissues.

Collection techniques

Venous blood collection (venipuncture):

- Usually performed from a vein in the arm.
- The vein is made more prominent by applying a **tourniquet** and asking the patient to clench and unclench the fist.
- The puncture site is disinfected with alcohol and allowed to dry.
- A sterile needle is inserted into the vein, with the bevel facing upward. The vein is stabilized with the thumb to prevent rolling.
- Once blood begins to flow, the tourniquet is released. After the required volume is collected, the needle is removed, and the puncture site is covered with sterile cotton or gauze.

Capillary blood collection (micro-sampling):

- Used when only small quantities are required or when venous access is difficult.
- Obtained by puncturing the finger (usually the ring finger), the thumb (lateral side), the earlobe, or the heel in infants under 1 year.
- Indications include:
    - Very small or fragile veins (children, elderly patients).
    - Scar tissue from multiple venipunctures.
    - Severe obesity.
    - Bedside testing requiring minimal blood.

Pre-analytical considerations: Blood is collected after a fasting period of 8-12 hours (except emergency cases). The concentration of many metabolites is influenced by the food or drink intake.

Blood normally clots in 20-30 minutes at room temperature. During this process, fibrin, an insoluble protein, forms a meshwork that traps erythrocytes and other cells, producing a clot.

- For serum preparation, the collection tube is kept upright or at a 30-45° angle until clotting is complete and the serum has separated.
- If separation is incomplete, the sample can be centrifuged at about 1500 × g, which sediments the clot and erythrocytes, leaving clear serum.

Plasma and Serum

Plasma: the liquid component of non-coagulated blood, obtained by mixing whole blood with an anticoagulant (e.g., EDTA, heparin, citrate) before centrifugation. Of note: To separate plasma from blood cells, centrifugation is performed (usually 10 minutes at 1000-1200 × g)

Serum: the liquid component of coagulated blood, i.e., plasma without fibrinogen and clotting factors consumed during coagulation.

Thus:

- Blood = Plasma + Blood Cells
- Serum = Plasma – Fibrinogen

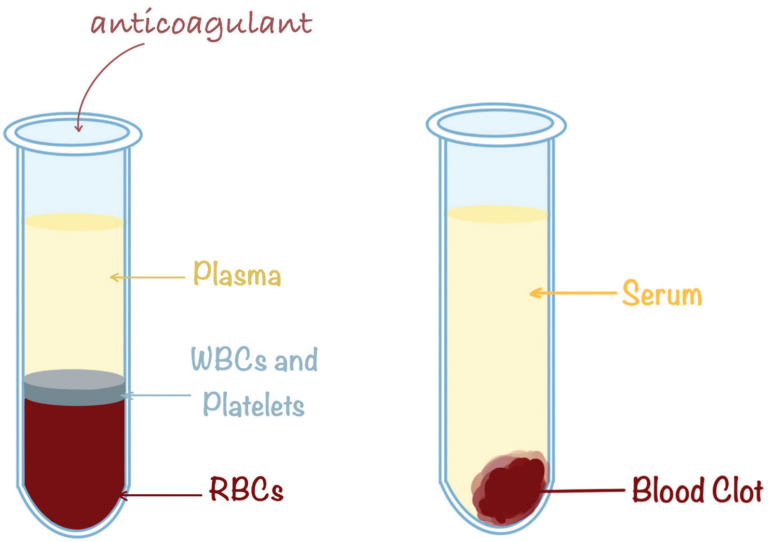


Figure 1. Blood serum (right) vs blood plasma (left)

Anticoagulants

In clinical practice and laboratory diagnostics, anticoagulants are used to prevent blood clotting when whole blood or plasma is required for analysis. The choice of anticoagulant depends on the test, since some interfere with biochemical reactions or may alter blood cell morphology. The following anticoagulants are commonly used for laboratory assays.

Na_2EDTA - (ethylenediaminetetraacetate) prevent the coagulation of blood by binding calcium ions (essential for blood coagulation) and forming a stable chelate. Preferred anticoagulant for hematology (complete blood count, differential count) because it best preserves cellular morphology without producing artifacts.

Of note: Not suitable for coagulation studies, as it irreversibly chelates calcium.

Sodium citrate binds calcium reversibly. It is the anticoagulant of choice for coagulation studies (e.g., prothrombin time, activated partial thromboplastin time). Blood must be mixed with citrate in a 9:1 ratio (blood:anticoagulant) to ensure accurate results.

Lithium or sodium heparin prevents blood clotting by enhancing the activity of antithrombin III, which inhibits thrombin and other clotting factors. Best anticoagulant for cytogenetic studies and some special hematology tests (e.g., osmotic fragility, plasma hemoglobin); also used in blood gas analysis.

Of note: Heparin is a naturally occurring anticoagulant; chemically, it is a sulfated glycosaminoglycan (polysaccharide).

Oxalates - generally potassium oxalate or sodium oxalate, binds to calcium, precipitating it as insoluble calcium oxalate thus, inhibiting coagulation. Formerly used in some chemical analyses and coagulation studies, but now largely replaced by citrate or EDTA because oxalates may distort blood cell morphology. Today is mostly used with sodium fluoride.

Sodium fluoride-potassium oxalate is a commonly used anticoagulant mixture for blood glucose determination. Sodium fluoride inhibits the glycolytic enzyme enolase, thereby preventing red blood cells and other blood cells from consuming glucose after collection. Potassium oxalate acts as the anticoagulant by binding calcium. This combination prevents both clotting and post-collection glucose loss.

Of note: In the presence of sodium fluoride, glucose concentration in the sample remains stable for up to 24 hours at room temperature (≈25 °C) and up to 48 hours when refrigerated (≈4 °C).

ACD-solution – contains 4.7 g citric **a**cid, 16.0 g Na_3**c**itrate, and 25.0 g **d**extrose (glucose) in 1000 ml distilled water. Citrate binds calcium ions reversibly in blood, which means clotting can be reinitiated if calcium is added back. Dextrose (glucose) serves as a nutrient for red blood cells, maintaining their metabolic activity (mainly glycolysis). This maintains red cell viability during storage. Citric acid helps maintain a slightly acidic environment, reducing metabolic activity and slowing down cell degradation.

It is used for the collection of blood for histocompatibility testing for transfusion and transplantation (e.g., blood bank testing and human leukocyte antigen (HLA) typing), and for DNA and paternity testing.

Figure 2. Vacutainers used for blood collection

Table 1. Order of draw - blood collection tubes

Order of collection	Tube Cap color	Anticoagulant / Additive	Main Uses
1	Blood culture bottles (Yellow SPS or bottles)	SPS (Sodium Polyanetholesulfonate)	Microbiology: blood cultures
2	Light Blue	Sodium Citrate (binds Ca^{2+}, reversible)	Coagulation studies: PT, aPTT, clotting factor assays
3	Red (plain) or Gold/" Tiger Top" (SST)	No anticoagulant / Clot activator ± Gel separator	Serum for chemistry, immunology, serology
4	Green	Lithium or Sodium Heparin (inhibits thrombin)	Clinical chemistry, plasma assays, cytogenetics
5	Lavander/Purple	K_2/Na_2 EDTA (chelates Ca^{2+})	Hematology: CBC, blood smears, HbA1c
6	Gray	Potassium Oxalate + Sodium Fluoride (fluoride inhibits glycolysis, oxalate prevents clotting)	Glucose testing
Special	Yellow (ACD solution)	Acid-Citrate-Dextrose	Blood bank (donor blood preservation), HLA typing, DNA studies
Special	Dark Blue (Royal Blue)	Special additives (EDTA, heparin, or none)	Trace elements, toxicology, heavy metals

PT = prothrombin time; aPTT = activated partial thromboplastin time; CBC = complete blood count; HbA1c = glycated hemoglobin

Blood, plasma or serum deproteinization

In some biochemical assays, proteins must be removed from the sample because they can interfere with reagents and lead to inaccurate results. Deproteinization is achieved by precipitating proteins as insoluble salts, followed by filtration to obtain a protein-free filtrate.

Types of protein-free filtrates:

a) Neutral protein-free filtrate, after Folin-Wo: proteins are precipitated with sodium tungstate and sulfuric acid. Neutral filtrates are used in the analysis of urea, uric acid, creatinine, glucose, amino acids, chloride.

b) Acidic protein-free filtrate is obtained by the protein precipitation with trichloroacetic acid (TCA). It is used in phosphorus, insulin, Na^+ tests.

c) Alkaline protein-free filtrate is obtained by protein precipitation with zinc sulphate ($ZnSO_4$) and sodium hydroxide (NaOH).

Urine

Except for urine obtained by catheterization or suprapubic aspiration, most specimens are collected directly by the patient during normal urination (micturition). Proper patient instruction is essential to ensure a reliable sample. Patients should be advised on clean collection techniques, hand hygiene, and general cleanliness to avoid contamination.

All urine specimens must be properly labeled with the patient's identification, as well as the date and time of collection, to ensure accurate interpretation of results.

Types of urine specimens

1. **Random specimen** – Collected at any time of day or night; commonly used for routine urinalysis.
2. **First morning specimen** – Collected immediately after waking; preferred for many tests because it is more concentrated.
3. **Timed specimen** – Collected over a defined period (e.g., 24 hours) or at a specific time after an event (e.g., 2 hours after eating for a postprandial glucose test).
4. **Midstream clean-catch specimen** – Requires careful patient preparation to minimize contamination from urethral flora; often used for microbiological culture.

Collection containers

- Urine should be collected in chemically clean containers.
- For urine cultures, the container must be sterile.
- If the urine will be tested for substances that degrade on exposure to light (e.g., bilirubin, porphyrins), it should be collected in a dark (amber) container or a container wrapped in aluminum foil.
- If a preservative is used, the container label should clearly state this, along with a "Caution" warning if the preservative is hazardous (e.g., hydrochloric acid, formalin).

Preservatives

Ideally, urine specimens should be examined immediately after collection. However, since this is not always possible, appropriate preservation methods are necessary to prevent chemical changes and bacterial growth.

For 24-hour urine collections, preservatives such as thymol (1-2 g), toluene, xylene, or chloroform (5-10 mL) may be added. Chloroform should not be used if the urine will be analyzed for glucose or ketone bodies, as it interferes with these tests.

If urine needs to be kept for longer than one hour, it should be refrigerated to slow bacterial activity and chemical degradation. Before analysis, the sample should be brought back to room temperature and mixed thoroughly to ensure homogeneity.

Method	Use	Disadvantage
Refrigeration	Preservation of all urines for 24 hours	Unsatisfactory for longer periods of time
Freezing	For prolonged storage	Destroys cells
Formalin	Good for preserving cells and casts	Interferes with urobilinogen and leukocyte esterase on reagent strip tests and bacterial culture
Chlorhexidine	Preservative for glucose	Interferes with protein test on reagent strip
Preservative tablets	Routine urinalysis and cells	Interferes with specific gravity, sodium, potassium, and hormone studies

Cerebrospinal fluid (CSF)

CSF is a secretion product of the choroid plexus at the level of cerebral ventricle. CSF investigation represents a real aid for clinicians for the diagnosis prognosis of central nervous system (CNS) diseases.

CSF collection is done by lumbar, sub-occipital or ventricular punction, in sterile recipients and with a special punction needle.

CSF composition: proteins, carbohydrates, urea, uric acid, organic acids, bilirubin, phosphate, carbonate, sulfate, calcium, magnesium, sodium, potassium, chloride.

CSF examination comprises physical examination (color, transparency, density), chemical examination (determination of proteins, glucose, sodium, etc), cytological, virological, serological examinations, etc.

CENTRIFUGATION

Centrifugation is a laboratory technique in which a suspension is subjected to **centrifugal force** to separate its components. This method is commonly used to collect precipitates, separate different phases (e.g., plasma from blood cells), or sediment macromolecules. The separation is based on differences in the **size, shape, and density** of the particles.

A centrifuge works by rotating tubes held in a rotor (head) around a fixed axis. As the rotor spins, a centrifugal force is generated, directed outward from the axis of rotation. The magnitude of this force increases with both the speed of rotation and the distance of the particle from the center.

Particles suspended in a liquid experience this centrifugal force, which pushes them outward, causing them to sediment. However, their movement is opposed by a frictional (drag) force, which depends on the size and shape of the particle as well as the viscosity of the medium. As the sedimenting particle accelerates, the frictional force increases until it eventually balances the centrifugal force.

At this point, the particle reaches a constant velocity of sedimentation. This steady rate of movement is what allows different particles to be separated efficiently during centrifugation.

Centrifuge classification

Centrifuges can be classified according to the speed of operation or the type of application for which they are designed.

By speed:

- ***Tabletop (clinical) centrifuges*** are low-speed centrifuges, generally providing speeds of up to about 3000 rotations per minute (rpm). They are commonly used in clinical laboratories for routine procedures such as separating serum or plasma from whole blood.
- ***High speed centrifuges*** provide speeds of up to about 20,000 rpm. They are useful for separating smaller particles such as organelles or large macromolecules.
- ***Ultracentrifuges*** provide speeds of up to about 60,000 rpm.

High-speed and ultracentrifuges are generally refrigerated to allow centrifugation of temperature-sensitive biological samples. Additionally, ultracentrifuges are operated under vacuum to eliminate the heat that would otherwise be generated by the friction between the rapidly spinning rotor and the air in the centrifuge chamber.

Centrifuges are also classified by type of application, as analytical or preparative:

- ***An analytical centrifuge*** is equipped with a variety of optical systems that permit the taking of photographs and the making of actual measurements during sedimentation.
- ***A preparative centrifuge*** is used primarily for the purification and fractionation of macromolecules.

Types of Rotors

Centrifuges are equipped with different types of rotors, chosen according to their intended use.

Swinging bucket rotors - the sample tubes are held in buckets that swing out to a horizontal position during centrifugation. **Advantages**: Particles travel a longer distance before sedimenting, which allows for better separation of components, especially in density gradient centrifugation. The horizontal orientation of the tubes makes it easier to remove the supernatant without disturbing the pellet.

In ***vertical rotors***, the sample tubes are positioned parallel to the axis of rotation. These are mainly used in high-speed centrifuges or ultracentrifuges and are often equipped with optical systems that allow real-time monitoring of sedimentation.

The fixed-angle rotor is the most widely used type, especially in preparative centrifugation. In this design, the tubes are held at a constant angle (usually between 25-45°) relative to the axis of rotation. **Advantages**: Particles travel only a short distance before reaching the tube wall, which speeds up sedimentation. This results in a shorter run time and greater efficiency compared to other rotor types.

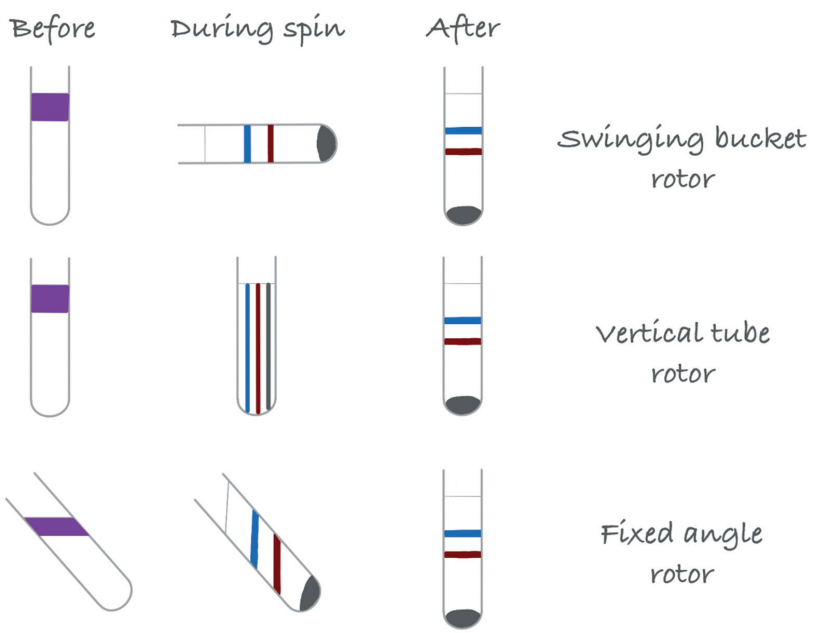


Figure 3. Types of rotors

Effect of Rotor Radius on Centrifugal Force

The centrifugal force depends not only on the speed of rotation (rpm) but also on the distance from the axis of rotation (radius). Thus, two rotors with different diameters, operated at the same rpm, will generate different centrifugal forces.

For this reason, centrifugation conditions are not reported in terms of rpm but rather as *relative centrifugal force (RCF)*, expressed as multiples of gravity (g). Using RCF ensures reproducibility across different rotors and centrifuges.

EXPERIMENTAL PART

Centrifugation of whole blood to separate plasma

Procedure: Blood collected in a vacutainer containing an anticoagulant will be centrifuged for 10 min at 2000 rpm.

The supernatant will be a transparent yellow liquid, called *plasma*.

Questions

1. Explain the differences between serum and plasma.

2. State the order of blood draw in different collection tubes.

3. Name three anticoagulants; indicate the color of the tube cap in which they are found.

4. Present the types of urine specimens.

5. Present the main centrifuge rotor types.

6. What is the difference between rpm and RCF?

Lab Notes

Use this space for handwritten notes during the practical session.

L8. PROTEINS. SEPARATION OF AMINOACIDS BY CHROMATOGRAPHY

Chromatography is a laboratory technique for separating complex mixtures into their individual components by exploiting differences in their affinity for a stationary phase and a mobile phase.

In chromatography, the *mobile phase* (a liquid or gas) moves the mixture through the system, while the *stationary phase* (a solid or liquid) interacts with the components to varying degrees. Because of these differences in interaction, the components travel at different speeds and become separated.

The term chromatography comes from the Greek words chrōma (‘color’) and gráphein (‘to write’). The name reflects the original use of the technique, which was developed to separate mixtures of biological pigments.

Essential Components of a Chromatography System

- **Stationary phase** – a solid (such as a gel) or an immobilized liquid bound to a support matrix.
- **Chromatographic bed** – the medium that holds the stationary phase; it may be packed into a glass or metal column, spread as a thin layer on a plate (glass or plastic), or adsorbed onto paper or cellulose fibers.
- **Mobile phase** – a liquid or gas that acts as a solvent, carrying the sample through the stationary phase and eventually eluting from the chromatographic bed.
- **Delivery system** – ensures continuous flow of the mobile phase through the chromatographic bed.
- **Detection system** – used to monitor and identify the separated substances.

During chromatography, each substance in the mixture interacts with the stationary phase to a different degree. Molecules that interact strongly with the stationary phase move more slowly, while those with weaker interactions travel faster with the mobile phase. These differences in migration or elution times allow for effective separation of the mixture’s components.

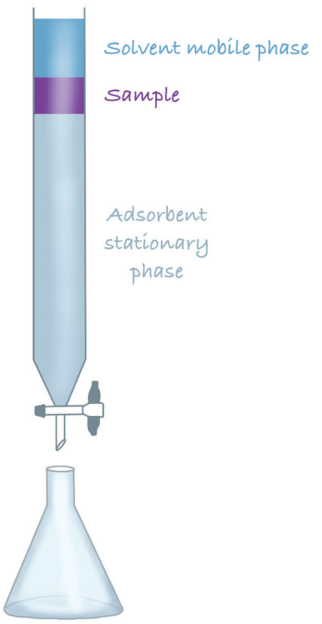


Figure 1. Chromatography system

Classification criteria

1. Classification according to the physical state of the two phases (stationary and mobile) in a chromatographic system

a) Solid stationary phase:
- mobile phase: gas → gas-solid chromatography (GSC)
- mobile phase: liquid → liquid-solid chromatography (LSC)

b) Liquid stationary phase:
- mobile phase: gas → gas-liquid chromatography (GLC)
- mobile phase: liquid → liquid-liquid chromatography (LLC)

2. Classification according to the nature of interactions between the components of the mixture and the phases (mobile and/or stationary)

The separation of molecules in chromatography is based on their distribution between the stationary and mobile phases. This distribution depends on one or more of the following fundamental processes: *adsorption, ion-exchange, partitioning,* and *size exclusion* (gel filtration/molecular sieving).

Thus, chromatography can be classified as:

Adsorption Chromatography

In adsorption chromatography, the stationary phase is a solid material, typically packed into a column in the form of very small beads with controlled diameter and porosity. Common stationary phases include silica gel and alumina (Al_2O_3)**,** which have large surface areas and active sites capable of interacting with sample molecules. The mobile phase can be either an aqueous solution or an organic (non-aqueous) solvent.

As the sample mixture flows through the column, molecules undergo repeated cycles of **adsorption (binding to the solid surface)** and **desorption (release back into the mobile phase)**. The overall rate of movement of each molecule depends on how strongly it interacts with (adsorbs to) the stationary phase:

- Molecules with stronger adsorption are retained longer and move more slowly.
- Molecules with weaker adsorption pass more quickly through the column.

This difference in migration rates enables the separation of components within the mixture. For example, adsorption chromatography is often used to separate amino acids, alkaloids, lipids, and pigments.

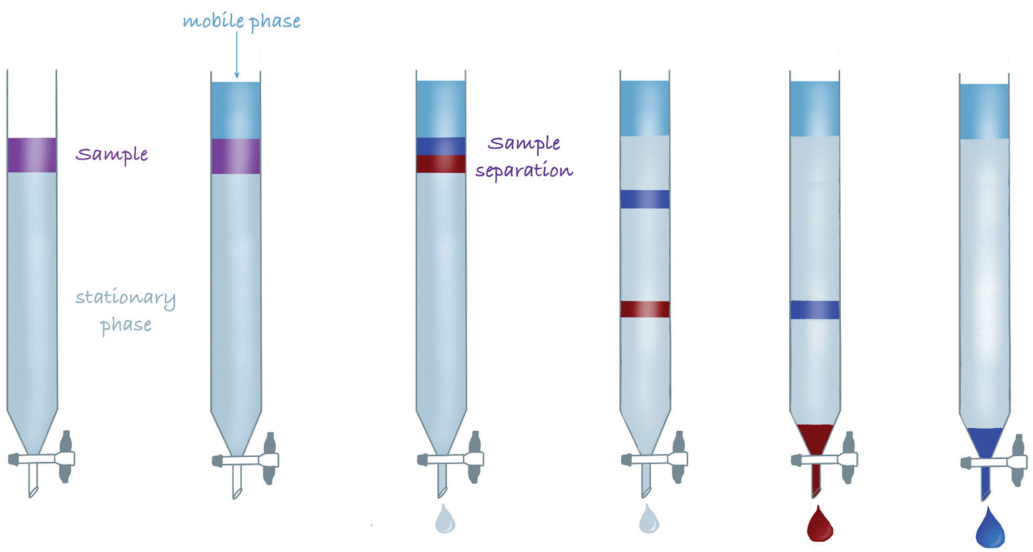


Figure 2. Adsorption Chromatography - The compounds to be separated interact with the stationary phase. Each compound "binds" (adsorbs) to the surface to a greater or lesser extent and therefore travels through the column with different speeds. Over a period, they separate completely as they exit the column.

Ion-Exchange Chromatography

In ion-exchange chromatography, molecules are separated according to their **net electrical charge**. The stationary phase consists of an ion-exchange resin, which is a cross-linked polymer containing multiple charged functional groups. The mobile phase is typically an aqueous buffer solution, and the method is most performed using a column format.

Separation occurs because molecules interact electrostatically with the oppositely charged groups on the resin. As the sample mixture passes through the column, charged molecules repeatedly undergo binding and release, which slows their movement to different extents.

For effective separation, the sample is applied at a pH where the molecules have a net charge opposite to that of the functional groups on the resin, ensuring that they bind to the column. Elution can then be achieved by: *changing the pH* of the buffer, which alters the net charge of the molecules, so they are no longer retained or *increasing the ionic strength* of the buffer (e.g., by adding salts), which disrupts electrostatic interactions and releases the bound molecules.

Two types of ion-exchange resins are commonly used:

- **Cation-exchange resins (Cationic exchangers)**: negatively charged resins that bind positively charged molecules (cations).
- **Anion-exchange resins (Anionic exchangers)**: positively charged resins that bind negatively charged molecules (anions).

This technique is widely used in biochemistry for the purification of proteins, peptides, nucleotides, and amino acids, as well as in clinical laboratories for the separation of hemoglobin variants (e.g., HbA1c) and water purification.

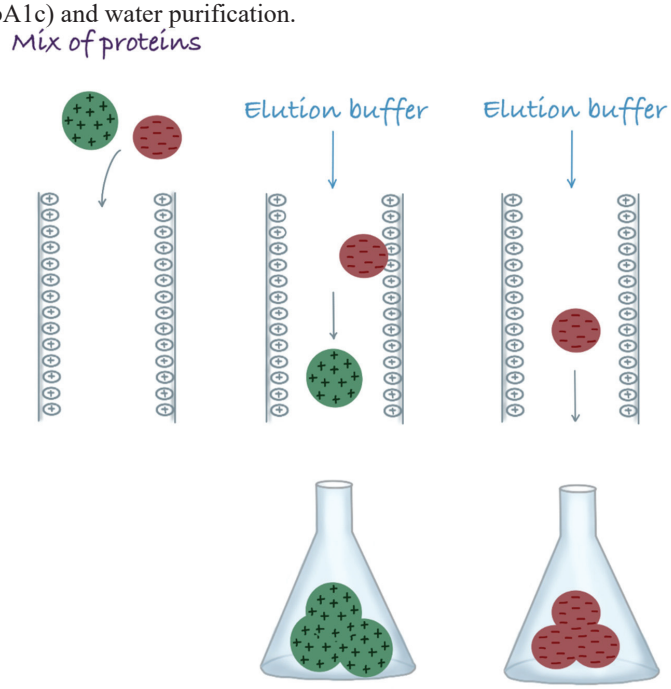


Figure 3. Ion-Exchange Chromatography – Separation occurs due to the differences in electrical charges. Negatively charged molecules are bound to the anion-exchange resin, while the positively charged molecules are separated first. After the addition of the elution buffer, negatively charged molecules are eluted lastly.

Partition Chromatography

Partition chromatography separates molecules based on their **relative solubility** in two immiscible phases: a mobile phase and a stationary liquid phase. A substance that is soluble in both phases will distribute, or "partition," itself between them according to its solubility.

The stationary liquid phase is held in place by a porous solid support, such as filter paper, giving rise to paper chromatography. In this method, the stationary phase consists of a thin layer of water molecules bound to the hydroxyl groups of cellulose in the paper (a polar phase). The mobile phase is typically a less polar organic solvent that moves along the paper by capillary action.

As the sample migrates with the solvent front, molecules repeatedly partition between the two phases. Non-polar molecules, being more soluble in the mobile phase, travel farther along the paper, while polar molecules, which interact more strongly with the stationary phase, migrate more slowly.

The movement of molecules in partition chromatography is expressed by the **retention factor (R_f value)**, calculated as:

$$R_f = \frac{distance\ moved\ by\ sample}{distance\ moved\ by\ solvent}$$

The R_f value is characteristic for each compound under defined conditions and is generally *proportional to the molecule's solubility in the mobile phase.*

There is also a reverse-phase partition chromatography in which the stationary phase is nonpolar (hydrophobic) and the mobile phase is relatively polar.

Partition chromatography is used for the separation of amino acids, pigments, and other small biomolecules.

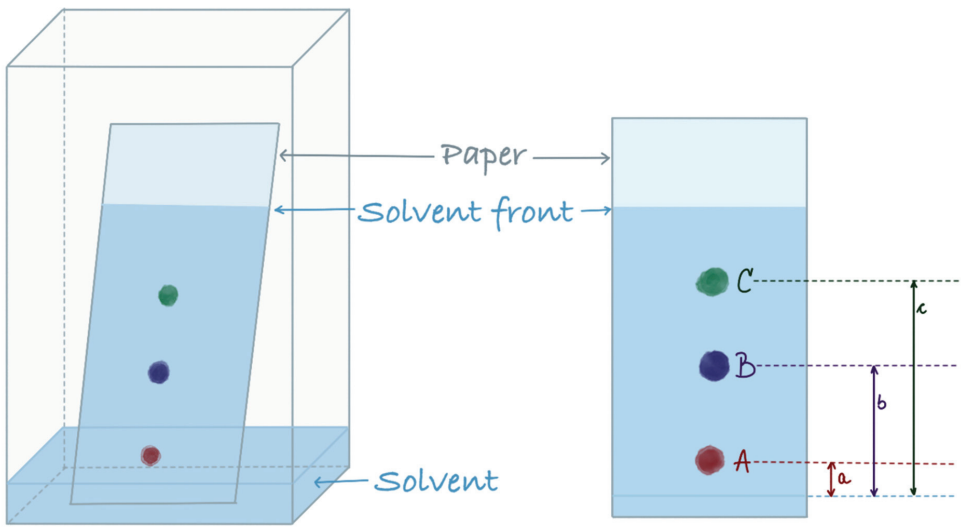


Figure 4. Partition Chromatography – Molecules are separated by their different relative solubilities. Molecules that are more soluble in the mobile phase travel farther (molecules C and B) and have a higher Rf. Molecules that are more soluble in the stationary phase migrate slower (molecule A) and have a lower R_f.

Gel-Filtration Chromatography

In gel filtration chromatography, also known as **size-exclusion or gel-permeation chromatography**, separation of molecules is based primarily on their **size and shape**. The stationary phase consists of spherical gel particles made of cross-linked polymers with controlled pore sizes, packed into a column. The mobile phase is a liquid that flows through the column and carries the sample.

As the sample mixture passes through the column, molecules interact differently with the pores of the gel particles:

- Small molecules can diffuse into the pores of the gel beads. Since they repeatedly enter and exit these pores, their passage through the column is retarded, and they elute later.
- Large molecules are excluded from entering the pores and move more quickly through the spaces between the beads, eluting earlier.
- Intermediate-sized molecules penetrate the pores to varying degrees, depending on their size and shape, and elute between the two extremes.

Thus, molecules are separated by size, with larger molecules eluting first and smaller molecules last. The elution volume (the volume of mobile phase required to elute a molecule) increases as molecular size decreases.

While molecular weight and size are the main factors determining separation in gel filtration, secondary factors such as charge interactions or adsorption to the gel surface may influence results under certain conditions.

Gel filtration chromatography is widely used for: purification of proteins, peptides, and nucleic acids; desalting and buffer exchange; estimating the molecular weight of biomolecules

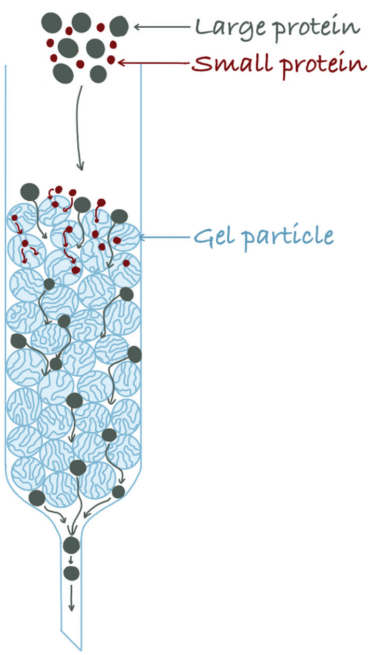


Figure 5. Gel-filtration Chromatography – Molecules are separated due to their different sizes. Larger molecules do not enter the pores of the gel and are eluted first (green molecules), whereas smaller molecules are eluted later because they are retained in the pores of the gel (red molecules).

Affinity Chromatography

Affinity chromatography is the most selective type of chromatography, relying on the **specific binding interactions** between a target molecule (often a protein) and a ligand. A ligand is defined as a molecule, ion, or group that binds, usually non-covalently, to another molecule or atom.

In this technique, the ligand is covalently attached to a solid support or matrix (such as agarose beads). The sample mixture is passed through the column, and only the molecules with a specific affinity for the ligand will bind to it, while all other components are washed away.

The ligands used can vary depending on the molecule to be purified:

- A substrate, product, or cofactor that naturally binds to an enzyme
- An antibody that specifically recognizes and binds to its antigen
- Other biomolecules engineered to selectively capture the target

After the non-bound molecules are washed out, the target protein (or molecule) can be eluted by changing the conditions of the mobile phase, such as altering pH, ionic strength, or adding a competitive ligand.

This high selectivity makes affinity chromatography a powerful tool for the purification of enzymes, antibodies, receptors, and nucleic acids.

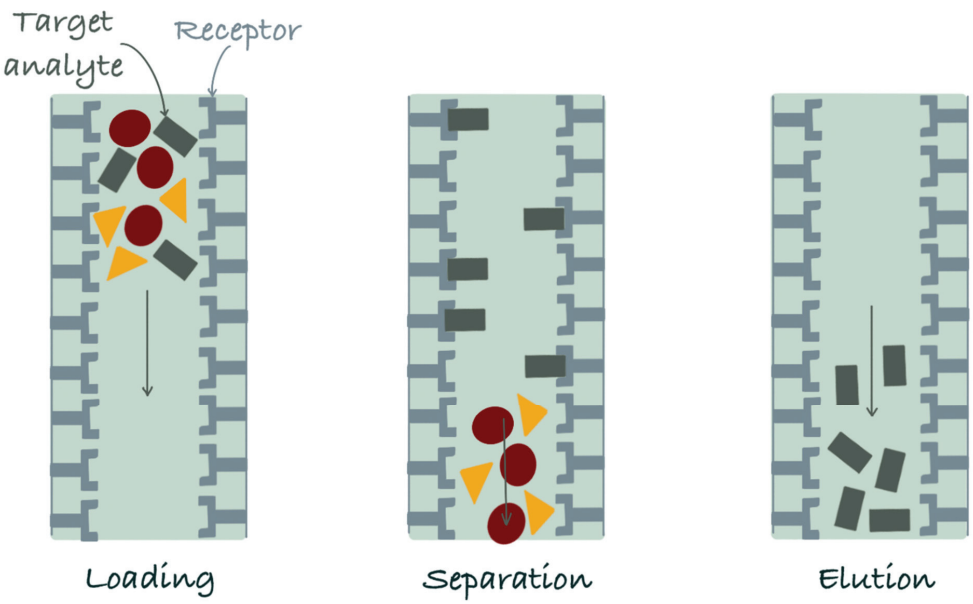


Figure 6. Affinity Chromatography – Molecules are separated due to their specific binding interactions. The target molecule (green) specifically binds to its antigen/receptor, while the other molecules are separated. The last elution fraction will contain only the target molecule.

3. Types of chromatography according to the form of the chromatographic bed

Thin layer chromatography (TLC) and paper chromatography

In this method, the sample is applied as a single small spot near one end of the chromatography sheet, using a microsyringe or microcapillary. After the spot has dried completely, the sheet is placed in a glass tank containing a shallow layer of solvent, ensuring that the spot is positioned above the solvent level. The solvent moves up the sheet by capillary action, carrying the sample components with it. The sheet is removed once the solvent front has traveled approximately 80-90% of its length. The movement of each substance can be quantified by calculating its relative frontal mobility (R_f value), as described in partition chromatography.

Column chromatography

In column chromatography, a glass or steel column is packed with the appropriate stationary phase, and the mobile phase is passed through the column, either by gravity or with the aid of a low-pressure pump. The sample is applied to the top of the column as a narrow band, after which the mobile phase carries it through the stationary phase.

If the substances in the mixture migrate at different rates, they will separate inside the column and elute at different times. The migration of each substance can be expressed as its retention time (t), or alternatively, as its elution volume (V_e) - the volume of mobile phase required to elute the compound from the column.

High-Pressure Liquid Chromatography (HPLC)

Originally, column chromatography used large, “soft” stationary phases that could not withstand high pressure. To avoid compressing the stationary phase, the mobile phase had to flow under low pressure. As a result, separations were slow and often of low resolution-these methods were considered “low performance.”

The development of High-Performance Liquid Chromatography (HPLC) greatly improved chromatographic resolution. In HPLC, the stationary phase consists of very small, uniformly sized beads that are tightly packed into the column. Because of this, high pressure (up to about 10 MPa, or hundreds of pounds per square inch) is required to push the mobile phase and sample mixture through the column. HPLC columns are usually made of stainless steel, and all components must be designed to withstand these pressures.

The advantages of HPLC include high speed, sensitivity, and versatility, making it the method of choice for separating many small biologically important molecules. Most often, separations are carried out using reverse-phase partition chromatography, where a nonpolar stationary phase and a polar mobile phase are employed.

For the separation of macromolecules such as proteins and nucleic acids, special biocompatible systems are preferred. These systems use materials such as titanium, glass, or fluoroplastics instead of stainless steel to avoid metal-induced denaturation. They also operate at lower pressures.

Gas chromatography (GC)

Modern gas chromatography (GC) commonly uses capillary columns with an internal diameter of 0.1-0.5 mm and lengths of up to 50 m. The stationary phase is typically a cross-linked silicone polymer, coated as a thin film on the inner wall of the capillary. At normal operating temperatures, this coating behaves like a liquid film but is far more stable and resistant to degradation.

The mobile phase (also called the carrier gas) is usually nitrogen or helium. Separation of compounds occurs because individual molecules partition differently between the gaseous carrier phase and the stationary silicone polymer phase.

The efficiency of separation in GC is strongly influenced by the column temperature. This may be kept constant throughout the analysis (isothermal operation, usually between 50-250 °C) or more commonly increased gradually in a programmed temperature ramp (e.g., from 50 °C to 250 °C at a set rate per minute). This allows efficient separation of compounds with a wide range of volatilities.

Samples are introduced through a sample injection port, located at the head of the column, which is sealed with a gas-tight septum.

The output from the column is monitored using sensitive detectors, commonly:

- Flame ionization detector (FID) – widely used for organic compounds.
- Electron capture detector (ECD) – highly sensitive for halogenated compounds.
- Thermal conductivity detector (TCD) – useful for general detection, including gases.

EXPERIMENTAL PART

Amino acid separation by paper chromatography

Principle

Paper chromatography is a type of partition chromatography, with a large use the years 1940-1960. Today it was practically completely replaced with *thin layer chromatography,* which uses a solid support laid as a thin layer on a plastic sheet or a glass.

The sample (amino acid solution) is deposited on a starting point on the paper sheet, in a very low quantity (1-10µl). The paper is dried and then eluted with a solvent system (usually up the paper due to its porosity, or capillarity). The amino acids will separate due to their different hydrophobic or hydrophilic properties. The substances are put in evidence by special color reagents (or UV light).

Reagents

1. Amino acid solution: 0.3% solution of phenylalanine and histidine.
2. Eluent: a mixture of n-buthanol, acetone, acetic acid and water at a volume ratio of: 3,5:3.5:1.0:2.0.
3. Ninhydrin solution: -it is used to put in evidence the migrated amino acids
4. Watman paper.

Materials and Apparatus

1. chromatographic chamber
2. spray, hair drier, hot plate
3. cylinder, pipettes, micropipette

Procedure

1. Introduce 10 ml of eluent in the chromatographic chamber. Attach the cover. Shake the chamber slowly and horizontally, to create an atmosphere saturated in eluent vapors.
2. Draw a line, with a **pencil**, on the chromatographic paper, at 1.5 cm from the base. Place a drop of the amino acid solution (approx. 5 µl) on the middle of the line, using the micropipette. Dry immediately the paper with the hair drier.
3. Suspend the dried paper in the chromatographic chamber so that it is immersed in the eluent for approx. 5 mm.
4. Maintain the paper in the covered chromatographic chamber till the eluent arrives at approx. 2 cm from the top of the paper. Then take out the paper and mark the eluent front with the pencil. Dry again the paper with the hair drier.
5. Spray the paper with the ninhydrin solution between the two marks and then dry it over the hot plate until a blue-violet color appears.

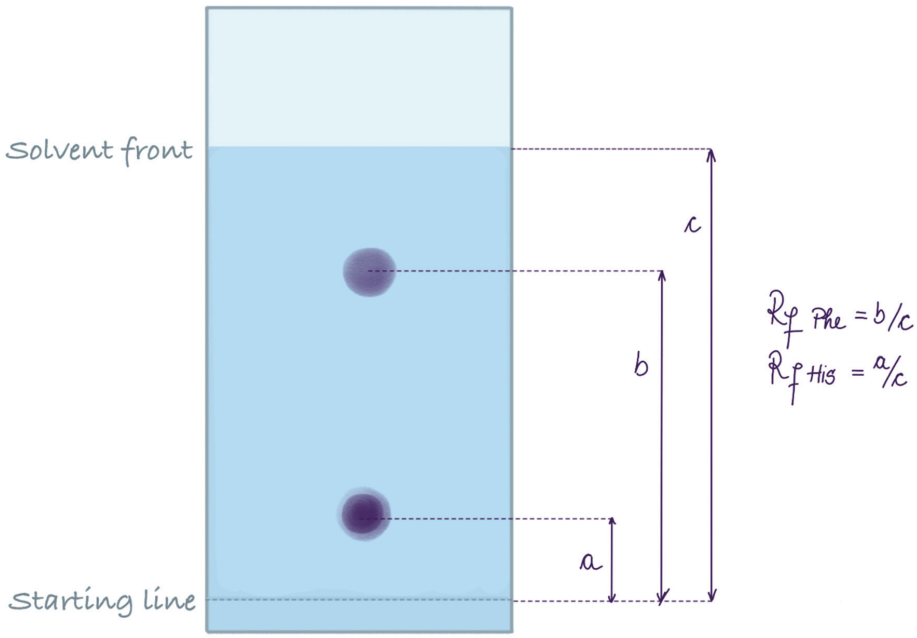


Figure 7. Experimental overview - Amino acid separation by paper chromatography

Calculation

The Rf values for the two amino acids will be calculated.

Based on their relative hydrophobicity, the migration order from the fastest to the slowest is: phenylalanine (more hydrophobic, moves further) followed by histidine (less hydrophobic, moves shorter distance).

Questions

1. Explain what is chromatography and which are the essential components of a chromatography system?

2. How can you classify chromatography according to the nature of interactions between the components of the mixture and the phases (mobile and/or stationary). Briefly describe each type.

3. Briefly present the experiment performed at the laboratory.

4. A mixture consisting of the following proteins is considered:
   A - with a molecular weight of 20,000 Da and a positive charge
   B - with a molecular weight of 100,000 Da and a positive charge
   C - with a molecular weight of 100,000 Da and a negative charge
   Which chromatographic methods can be used for their separation, and how is the elution of the proteins carried out? Explain.

5. A mixture of glutamic acid, tryptophan, and phenylalanine is separated by thin-layer chromatography. The stationary phase is hydrophobic, and the mobile phase is hydrophilic. Which of the amino acids has the highest R_f value? Explain.

Lab Notes

Use this space for handwritten notes during the practical session.

L9. PROTEINS. DIALYSIS. ELECTROPHORESIS

Proteins are fundamental biological macromolecules that play critical roles in virtually all cellular processes. They are polymers of amino acids linked by peptide bonds and folded into complex three-dimensional structures that determine their specific functions. Proteins serve as enzymes, structural components, hormones, transporters, and antibodies, making them essential for the maintenance of life. Their chemical and physical properties are determined by the nature of the amino acids they contain and the way these amino acids are arranged in space.

Properties of proteins

1. **Molecular weight -** Proteins are macromolecules with large molecular weights, typically ranging from about 5×10^3 to 1×10^6 Daltons (Da). Interestingly, the molecular weights of many proteins cluster around values close to multiples of 35,000 or 70,000 Da, reflecting evolutionary patterns in protein structure.
2. **Colloidal nature** - Due to their large size, proteins display colloidal behavior. Their diffusion rates in solution are slow, and they scatter light significantly, producing visible turbidity-a phenomenon known as the Tyndall effect.
3. **Denaturation -** refers to the loss of a protein's native structure and biological activity, often due to heat, pH changes, or chemical agents. Denaturation may be accompanied by coagulation, in which unfolded protein molecules aggregate and precipitate out of solution.
4. **Amphoteric nature -** Proteins, like amino acids, are amphoteric, meaning they can act as acids or bases depending on the pH of the environment. They carry a net charge that determines their migration in an electric field: towards the anode if net negative, or towards the cathode if net positive. At a specific pH, called the isoelectric point (pI), proteins have equal numbers of positive and negative charges, making the net charge zero. At this point, proteins do not migrate in an electric field and have minimal solubility, often leading to precipitation. This principle is widely used for protein purification.
5. **Ion Binding capacity** - Proteins can form salts with both cations and anions, depending on their net charge at a given pH. This property is important in many biological interactions and laboratory techniques.
6. **Solubility** - The solubility of proteins is highly influenced by pH. Solubility is lowest at the isoelectric point, since molecules carry no net charge and aggregate easily. Solubility increases in acidic or alkaline conditions because proteins acquire net charges (positive in acidic, negative in alkaline), leading to repulsion between molecules and improved solubility.
7. **Optical activity** - Most proteins are optically active, as they contain chiral amino acids (except glycine). In solution, proteins typically rotate the plane of polarized light, usually to the left (levorotatory). This property can be measured and used for concentration and purity analysis.

DIALYSIS

Osmosis is the process by which a solvent (usually water) moves across a semipermeable membrane separating two solutions of different solute concentrations. The solvent flows from the region of lower solute concentration (more dilute solution) to the region of higher solute concentration (more concentrated solution), with the goal of equalizing the osmotic concentrations on both sides of the membrane.

Osmotic pressure is the pressure exerted by the particles of a solution on the wall of a semipermeable membrane, to equalize osmolar concentrations on both sides of the membrane.

Dialysis is a separation technique that relies on the different rates of diffusion of the components of a mixture through a semipermeable membrane.

Semipermeable membranes are structures that separate two (or more) compartments. Simplistically, a membrane can be imagined as a surface acting as a barrier at the interface between two liquids. However, because of its permeability, the membrane also acts as a bridge between the phases it separates, and it serves as the site of complex transport processes.

The transport phenomena through a membrane depend on:

- the permeability of the membrane,
- the diffusion capacity of the substances,
- other physical forces (electrochemical gradient, osmotic pressure gradient, hydrostatic pressure gradient),
- and temperature.

Of note! The rate of dialysis increases with temperature, but higher temperatures can lead to protein denaturation.

Dialysis membranes are generally made from highly polymerized substances which, in the presence of solvents, swell and form molecular sieves. The pores of these sieves allow the passage of solvents and small molecules, but prevent the passage of large molecules (e.g., proteins). To be useful, the membrane must be chemically inert, so that no binding of sample components occurs. Modern dialysis membranes are often prepared from processed cellophane (cellulose).

Dialysis procedure

When applied to volumes of 1-100 milliliters, dialysis is usually carried out as follows:

1. The sample solution (dialysate) is placed in a small bag made of semipermeable membrane material.
2. This bag is suspended in a much larger volume of solvent, in a container maintained at the desired temperature.
3. Diffusible substances pass from the bag into the solvent, increasing their concentration in the surrounding solution.
4. Continuous agitation is required to disperse the diffusing molecules, thereby reducing their local concentration around the outside of the membrane.

Limitations of dialysis

- Relatively long time required to reach equilibrium between compartments.
- Ineffective for separating macromolecules, such as proteins bound to prosthetic groups or large ligands.

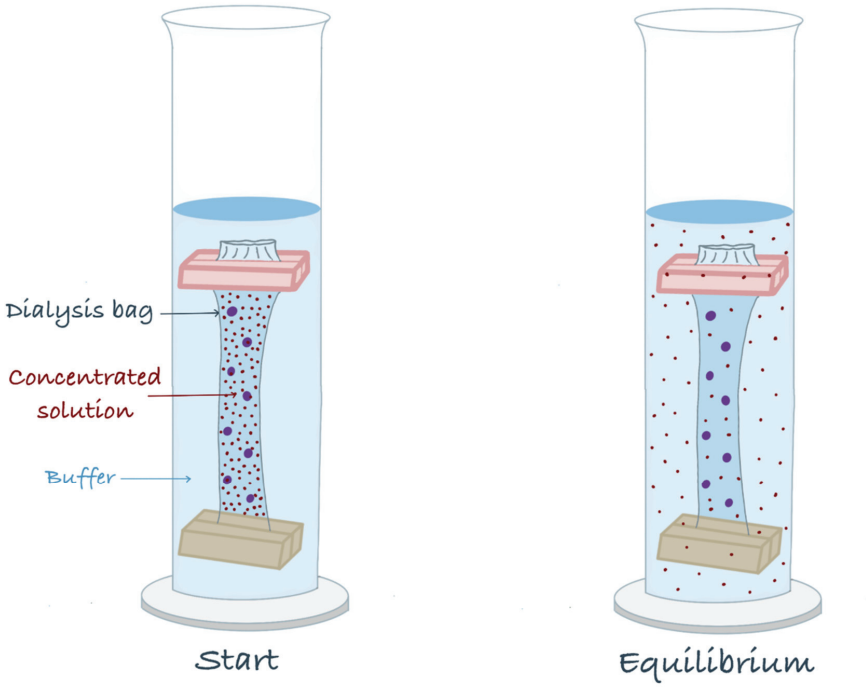


Figure 1. Dialysis

Applications of dialysis

Dialysis is a simple and widely used method in protein studies. It can be used for:

- **demonstrating the macromolecular nature of proteins** - Dialysis of a protein solution (e.g., albumin) against pure water shows that the protein does not pass through the membrane, while salts and other small solutes do. This demonstrates that proteins are large macromolecules.
- **determining and comparing protein diffusion properties -** Two proteins of different sizes (e.g., myoglobin vs. hemoglobin) can be placed in dialysis bags and monitored over time. The smaller protein diffuses more quickly, allowing their relative diffusion coefficients to be compared.
- **separating macromolecules from each other -** Separation of DNA from protein in a crude extract. A dialysis membrane with an appropriate cutoff can retain large DNA molecules while allowing smaller proteins or peptides to pass through.
- **removing small-molecular weight contaminants from proteins, nucleic acids, polysaccharides, etc. -** After purifying a protein using ammonium sulfate precipitation, dialysis is used to remove the excess ammonium sulfate ions, leaving only the pure protein solution. Similarly, nucleic acids can be dialyzed to remove salts, urea, or ethanol after extraction.

Clinical applications of dialysis

A major medical application of dialysis is the extracorporeal device, **"artificial kidney"**, which purifies blood by removing low-molecular weight compounds normally excreted in urine.

Hemodialysis is a medical procedure that uses an artificial kidney, or **dialyzer**, to purify the blood of patients with renal failure. The dialyzer is a transparent plastic cylinder, about 20 cm long and 5 cm wide, containing thousands of hollow fibers made of synthetic semipermeable membrane. These fibers form the blood compartment, through which the patient's blood flows. Surrounding the

fibers is the dialysate compartment, filled with dialysis solution. During treatment, blood is pumped from the patient's vascular access into one end of the dialyzer and distributed through the fibers, while the dialysis solution enters from another port and flows in the opposite direction around the fibers. This counter-current flow maximizes diffusion and ultrafiltration: waste products and excess fluid pass across the membrane into the dialysate, while essential components remain in the blood. Over a typical four-hour session, the entire blood volume circulates through the dialyzer multiple times, effectively reducing urea, creatinine, and correcting electrolyte imbalances.

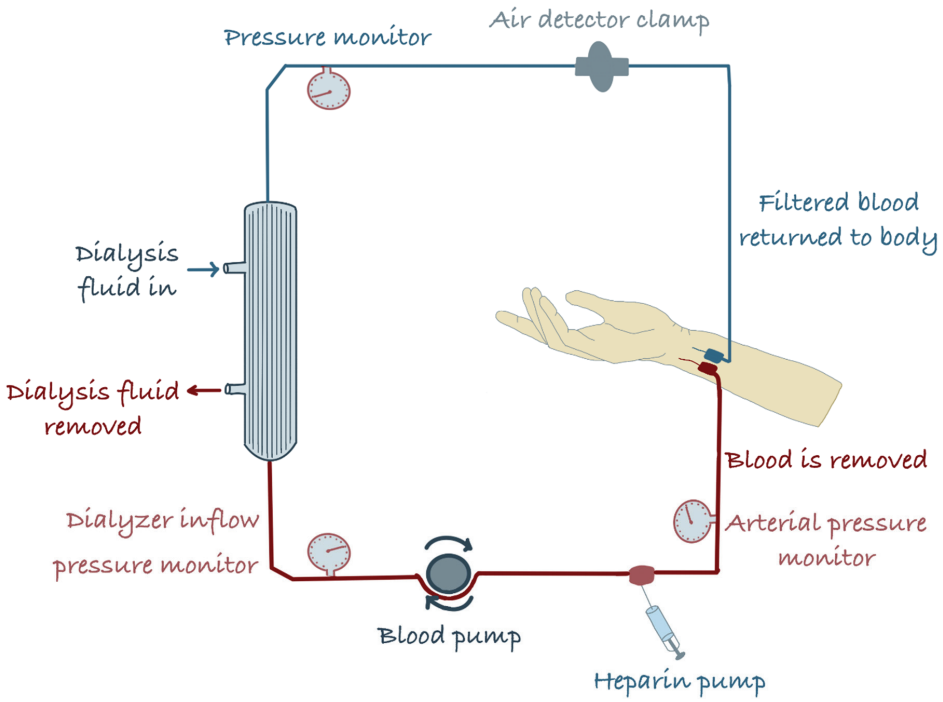


Figure 2. Schematic representation of hemodialysis

Peritoneal dialysis (PD) is a form of renal replacement therapy used in patients with end-stage kidney disease. The treatment involves introducing a sterile dialysis solution (dialysate) into the patient's peritoneal cavity through a catheter. The peritoneal membrane acts as a natural semipermeable filter, allowing waste products and excess fluids to move from the blood into the dialysate. The fluid is later drained and replaced with fresh solution. There are two main techniques:

1. **Continuous ambulatory peritoneal dialysis (CAPD)** - in which the patient infuses the dialysate fluid by himself.
2. **Automated peritoneal dialysis (APD)** - a machine performs the exchanges automatically, usually at night while the patient sleeps.

Both methods can be performed at home or at work, provided strict hygiene is maintained, offering patients greater independence compared to hemodialysis.

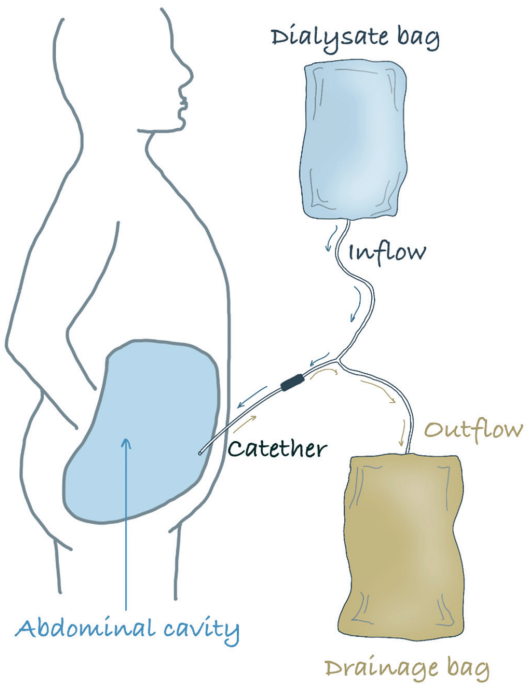


Figure 3. Schematic representation of peritoneal dialysis

ELECTROPHORESIS

Electrophoresis is the process by which electrically charged particles migrate through a liquid medium under the influence of an electric field (direct current). The particle moves at a constant velocity, meaning that the electric force (E · q) must balance the frictional force (f · v).

$$E \times q = f \times v$$

Where:
E = electric field (volts/cm)
q = net charge of the particle (electrostatic units)
f = frictional coefficient (depends on particle size and shape)
v = particle velocity (cm/sec)

The electric field **E** can be expressed either by voltage or by current intensity; therefore, in electrophoresis either the voltage or the current is kept constant.

The particle velocity **v** is defined as:

$$v = \frac{E \times q}{f}$$

If **E** is constant, velocity depends on the ratio between the particle's charge and its size/mass. Between two particles of the same shape, the one with the higher net charge (**q**) will migrate faster. Between two particles of the same mass and net charge, the more symmetrical one (closer to spherical shape, with an axial ratio nearer to 1.0) will migrate faster than the more asymmetrical (elongated) one, because the latter has a higher frictional coefficient.

Electrophoretic mobility (μ) is defined as the distance **d** traveled by a particle under the influence of the electric potential gradient **E** during the time interval **t**:

$$\mu = \frac{d}{t \times E} \quad or \quad \mu = \frac{v}{E}$$

This relation is only partially accurate, as it is influenced by several factors, particularly the effects of excess heat generated by increased current intensity.

The **electrophoretic migration velocity** depends on:

- the electric charge of the molecule,
- the size and shape of the molecule,
- the applied electric field strength,
- the nature of the solid support,
- the working temperature.

The electrophoretic mobility can also be expressed as:

$$\mu = \frac{k \times q}{r \times \eta}$$

Where:
μ = electrophoretic mobility, cm/(volt·second)
q = molecular charge
k = constant
r = ionic radius of the migrating particle
η = viscosity of the solution

Thus, electrophoretic mobility is directly proportional to the charge of the migrating protein and inversely proportional to its size and the viscosity of the medium (the buffer used in electrophoresis).

Types of electrophoresis

Depending on the nature of the medium, there are two main types of electrophoresis:

A. Electrophoresis in solution – in this method, particle migration takes place in a liquid solution. This system, developed by Tiselius, has low resolution and requires large amounts of material, so it is no longer used for analytical purposes, but only for electrophoretic mobility measurements.

B. Zone electrophoresis – in this method, the aqueous solution containing electrically charged particles is applied to a solid support or matrix. Zone electrophoresis produces **electropherograms**, where charged particles, usually proteins, are clearly separated from each other as distinct bands. This is the most widely used electrophoresis technique in biochemistry, especially for analyzing proteins from serum, urine, cerebrospinal fluid, as well as proteins from red blood cells and tissues.

According to the type of solid support, several variants exist:

- **Paper electrophoresis** - increasingly less used because of its low resolution and the long time required for separation. Moreover, electrophoretic separation is affected by chromatographic effects caused by adsorption of molecules onto the hydroxyl groups of cellulose, which slows migration and reduces separation quality.
- **Cellulose acetate electrophoresis** - an improved method compared to paper electrophoresis, since most hydroxyl groups in cellulose are acetylated, reducing adsorption of molecules to the support. This allows faster migration and better resolution. Additional advantages include:
    - requires smaller sample volumes,
    - transparent support, enabling direct quantification of bands by spectrophotometry,
    - solubility in various solvents, allowing easy and quick isolation of separated bands.
- **Gel electrophoresis** - the most common form of electrophoresis, using gels as the supporting medium. Gels are chemically inert, have uniform structures, and are easy to prepare reproducibly. The most widely used are **polyacrylamide gels**, which can be precisely modified to optimize separation. Polyacrylamide gels are formed by polymerization of acrylamide (monomer) and N,N′-methylene-bis-acrylamide (crosslinker) in the presence of free-radical generators (ammonium persulfate as initiator and tetramethylethylenediamine (TEMED) as catalyst). The concentrations of acrylamide and crosslinker determine gel properties such as density, elasticity, pore size, and mechanical strength. A very common variant, used for mostly proteins, is **SDS-PAGE** (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). In this method, separation occurs mainly by size. SDS denatures proteins, while reducing agents like β-mercaptoethanol break disulfide bonds, converting molecules into linear "rods." SDS also forms negatively charged micelles with proteins, eliminating their intrinsic charges. These complexes migrate toward the positive electrode, with the polyacrylamide gel pores acting as a molecular sieve: smaller molecules migrate faster than larger ones. **Agarose gels** can be also used, and they are mainly encountered in nucleic acid separation.

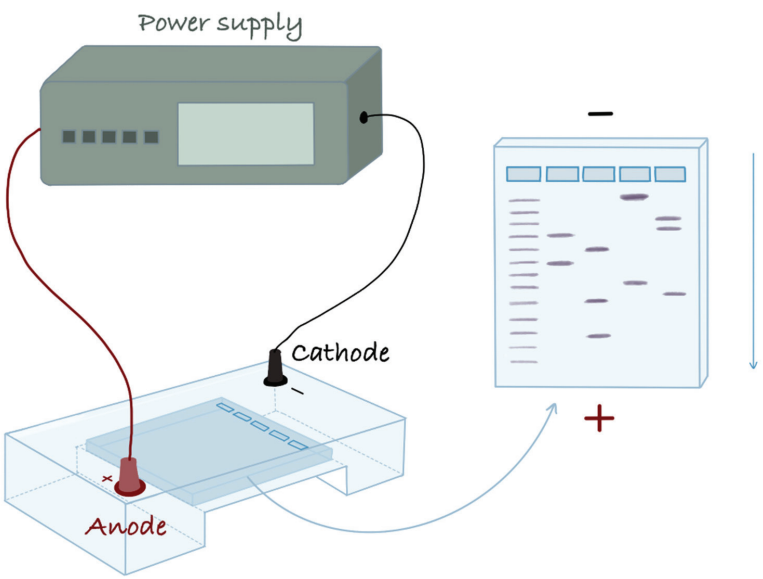


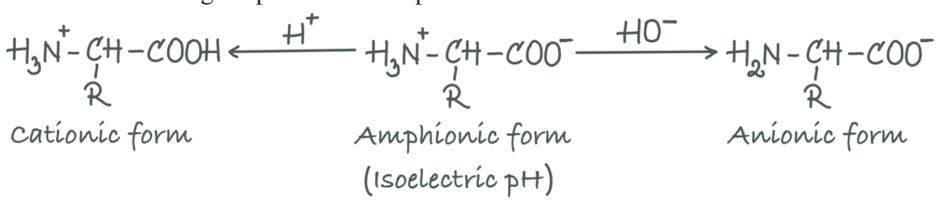
Figure 4. Gel electrophoresis

C. Isoelectric focusing (IEF) - electrophoresis in a pH gradient. This method is used to separate molecular species that differ only by net charge. Proteins migrate through the gradient as long as they carry a charge, stopping at the point where the pH equals their isoelectric point (**pI**). At this point, the net charge is zero, migration ceases, and proteins form distinct sharp bands. The pH gradient is created using amphoteric compounds of low molecular weight (ampholytes).

D. Capillary electrophoresis - here, particle migration occurs in very narrow capillary tubes (diameter 0.05-0.3 mm) filled with either liquid solution or a solid support. Because overheating is avoided, very high voltages (up to 10 kV) can be applied, significantly increasing migration speed.

Serum protein electrophoresis

The migration of proteins, in an electrical field, is due to their amphoteric nature, which permits an electric charge dependent on the pH of the solution:



Protein electrophoresis is widely used in medicine to evaluate proteins in the blood. The test is usually performed on serum rather than plasma, because plasma contains clotting factors, such as fibrinogen, which can interfere with the separation and analysis of proteins. Serum, being plasma without fibrinogen, provides a clearer profile for electrophoretic analysis. This method, known as serum protein electrophoresis (SPEP), separates proteins according to their size, shape, and electrical charge, allowing clinicians to detect abnormal or missing protein fractions.

An electric current is applied to a serum sample placed on a support medium (such as a gel or cellulose acetate). Proteins migrate at different speeds depending on their net charge and molecular size, forming distinct bands. The two main protein groups found in serum are **albumin** and **globulins**. Albumin, the most abundant serum protein, produces the largest peak on the electrophoretic pattern and migrates closest to the positive electrode. Globulins, which account for a smaller portion of total serum proteins, are the primary focus of interpretation in SPEP. They are divided into four categories: alpha-1, alpha-2, beta-1, and gamma, with the gamma fraction migrating nearest to the negative electrode.

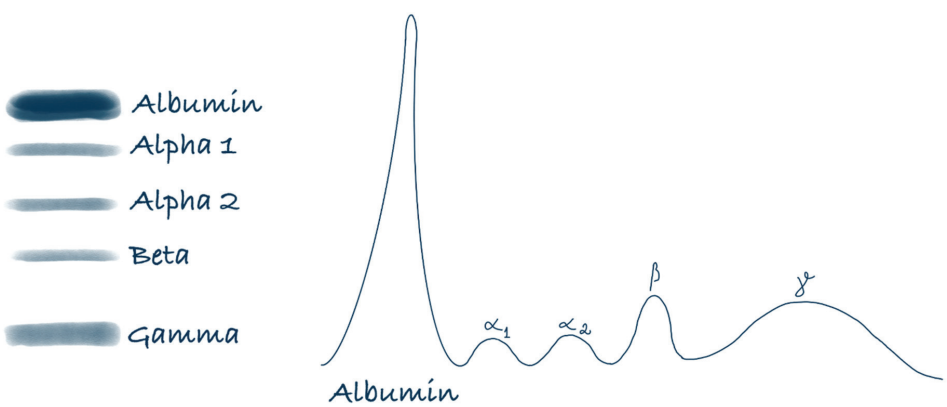
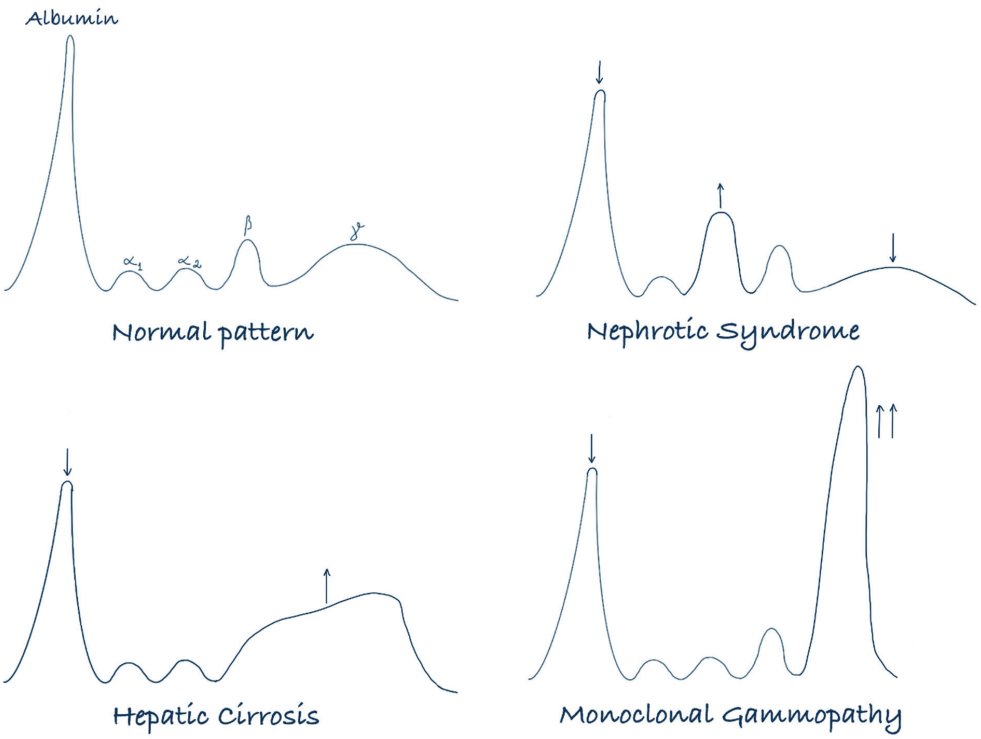


Figure 5. Serum protein electrophoresis - normal pattern

Table 1. Absolute and relative values of serum proteins

Protein fraction	Normal values (%)	Absolute values (for a total protein level of 7-9 g/dL)
Serum albumin	50 - 60	4.5 - 5.5
Serum globulin α_1	3.4 - 5.5	0.3 - 0.8
Serum globulin α_2	6.0 - 12.0	0.48
Serum globulin β	9.0 - 15.0	0.55 - 1.11
Serum globulin γ	15.0 - 21	0.64 - 1.28

Examples of serum protein electrophoresis:



Clinical applications

- Multiple myeloma: SPEP is a key diagnostic tool for detecting monoclonal proteins.
- Other protein abnormalities: It helps identify conditions like hypogammaglobulinemia (low antibody levels).
- Chronic diseases: Abnormal patterns may indicate inflammation, liver disorders, or kidney dysfunction, etc.

EXPERIMENTAL PART

Dialysis of a protein and ammonium sulfate solution

Principle

A dialysis bag (MWCO = 1000; MW = molecular weight; CO = cut-off) allows only molecules smaller than 1000 Da to pass through its pores.

A mixture of protein (albumin) and ammonium sulfate is placed in the dialysis bag. When the bag is immersed in a large volume of distilled water (the solvent), the semipermeable membrane enables the exchange of substances between the two phases (the dialyzed mixture inside the bag and the external solvent). Only components with a molecular weight below 1000 Da-namely, sulfate ions (SO_4^{2-}, MW = 96) and ammonium ions (NH_4^+, MW = 18) - diffuse out of the bag into the surrounding water. Meanwhile, water diffuses into the bag. Albumin (MW ≈ 47,000 Da) remains inside the dialysis bag.

This process is described as protein dialysis against water. The presence of NH_4^+ ions is detected with Nessler's reagent, SO_4^{2-} ions with barium chloride, and proteins with Coomassie Brilliant Blue (CBB).

Reagents

1. **Protein solution (1%) with ammonium sulfate**: Dissolve 1 g protein in 100 ml distilled water, then gradually add 10 g ammonium sulfate.
2. **Coomassie reagent**: Dissolve 60 mg Coomassie Brilliant Blue G250 in 1 L of 3% perchloric acid; filter to remove undissolved dye.
3. **Nessler's reagent**: Dissolve 50 g KI in a small volume of water. Add saturated $HgCl_2$ solution until a precipitate forms. Then add 200 ml of 5 N NaOH and dilute to 1000 ml. Allow the solution to settle and collect the clear supernatant.
4. **Barium chloride solution**: 5% ($BaCl_2$).

Materials

1. Semipermeable dialysis membrane (MWCO = 1000)
2. 400 ml Berzelius beaker, test tubes, pipettes
3. Clamps for sealing and support
4. Glass rod and magnetic stir bar
5. Magnetic stirrer

Procedure

1. Fill dialysis bags with the albumin + ammonium sulfate solution, seal the ends, and store them immersed in the protein-ammonium sulfate solution in the refrigerator until use.
2. Remove a dialysis bag from the refrigerator and rinse its exterior with distilled water.
3. Place 600 ml of distilled water in a 400 ml Berzelius beaker. Insert the dialysis bag (it floats) along with a magnetic stir bar. Put the beaker on a magnetic stirrer and start stirring at medium speed (setting 3-4).
4. In three separate test tubes, place 1 ml distilled water and test for: NH_4^+ ions (Nessler reagent), SO_4^{2-} ions ($BaCl_2$), and protein (CBB).
5. In three separate test tubes, place 1 ml of the initial dialysis mixture and test for: NH_4^+ ions, SO_4^{2-} ions, and protein, using the same reagents.
6. At hourly intervals, collect samples from inside the dialysis bag and test for NH_4^+, SO_4^{2-}, and protein.
7. At the same intervals, collect samples from the external water (1 ml each) and test for NH_4^+, SO_4^{2-}, and protein.

Identification reactions

- **Ammonium ions (NH_4^+):** Add 1-2 drops of Nessler's reagent to the test solution. A yellow-orange precipitate indicates NH_4^+.
- **Sulfate ions (SO_4^{2-}):** Add 2-3 drops of $BaCl_2$ solution. A white precipitate indicates SO_4^{2-}.
- **Proteins:** Add 1 ml Coomassie Brilliant Blue (CBB). A violet color develops, intensifying over time.

Results

The results are recorded in the following table, marking the presence ("+") or absence ("–") of the tested components:

Tested Component	Before dialysis		After 1 h		After 2 h	
	External (P_0)	Internal (P_0)	External (P_1)	Internal (P_1)	External (P_2)	Internal (P_2)
Protein						
Sulfate ions (SO_4^{2-})						
Ammonium ions (NH_4^+)						

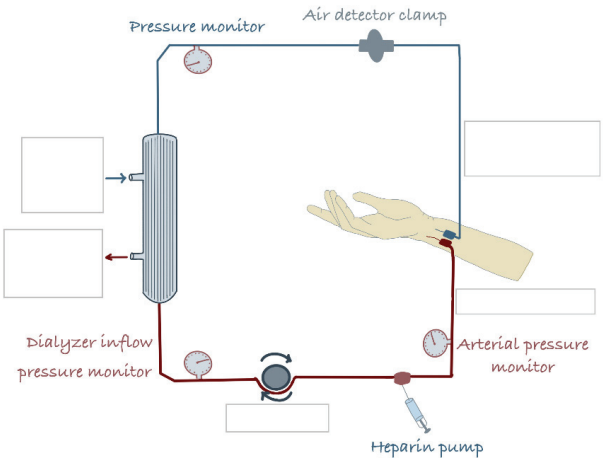
Observations

- Proteins are only present inside the dialysis bag, and absent in the external water
- Sulfate (SO_4^{2-}) and ammonium (NH_4^+) concentrations inside the bag decrease as these ions diffuse out into the surrounding water, demonstrating dialysis through the semipermeable membrane.
- Sulfate and ammonium concentrations in the external water increase over time.

Questions

1. Explain the principle of dialysis.

2. Fill in the following schematic representation of a hemodialysis procedure.



3. Briefly explain the experiment performed at the lab.

4. What is electrophoresis? Briefly describe the factors that influence the electrophoretic migration speed.

5. Describe the medical utility of electrophoresis.

Lab Notes

Use this space for handwritten notes during the practical session.

L10. OPTICAL METHODS OF ANALYSIS. DETERMINATION OF PROTEINS USING THE BRADFORD METHOD

Optical methods

Optical methods are based on the optical properties of the studied system, which are revealed during excitation and interaction with radiant energy across the electromagnetic spectrum.

Main characteristic: in most cases, both the input and output signals are of optical nature. These methods are defined by the type of interaction between the system and the incident energy, and the mode of observation of the resulting optical phenomena. Both characteristics depend on the nature of the input signal.

The differentiation between the optical methods is also done following the spectral domain in which the measurements are done. In this respect, there are: absorption spectroscopy in ultraviolet (UV), visible (VIS), infrared (IR) or X spectroscopy (absorption or emission), etc.

Optical methods of analysis are used for determining concentrations (clinical routine), clarifying reaction mechanisms, kinetic studies, and investigating structural aspects in biochemical research.

Spectroscopic methods

Measurements of the absorbance of light by substances in solution are widely used in biochemistry because many compounds of interest absorb light in the ultraviolet, visible, or near infrared domain. **Spectrophotometry** is the quantitative measurement and study of the electromagnetic spectra (including visible light, UV, and near-infrared - by absorption of matter).

Spectroscopic methods are based on the measurement and interpretation of absorption and emission spectra. The absorbance of light is characterized by two parameters, the wavelength of maximum absorption and the extent of absorption (extinction coefficient).

Electromagnetic radiation is described by wavelength (λ), frequency (ν), energy (E).

The relationships are:

- $\lambda \times \nu = c$, where c is the speed of light in vacuum (3×10^{10} cm/s).
- $E = h \times \nu$, where h is Planck's constant (6.62×10^{-27} erg·s).

Energy is inversely proportional to wavelength and directly proportional to frequency.

Spectral positions of absorption or emission may be expressed in terms of wavelength, frequency, energy.

Wavelength units:

- centimeter (cm),
- millimicron (1 mμ = 10^{-7} m),
- nanometer (1 nm = 10^{-7} cm),
- angstrom (1 Å = 10^{-8} cm),
- micrometer (1 μm = 10^{-4} cm).

Monochromatic radiation is a radiation composed of a single wavelength. In practice, it refers to a very narrow wavelength band isolated from a continuous spectrum (e.g., 600 ± 0.15 nm).

Visible spectrum is the relatively narrow spectral region perceived as light by the human eye, ranging from ~400 nm (violet) to ~760 nm (red).

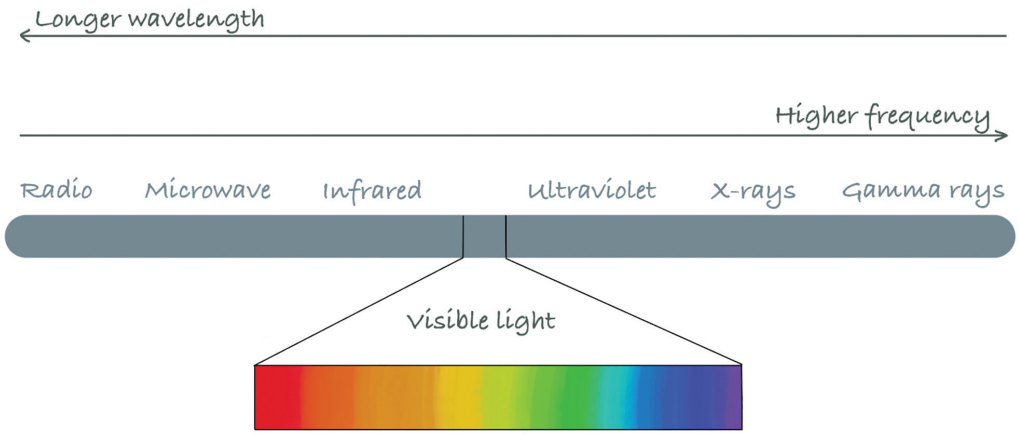


Figure 1. Electromagnetic radiation

Table 1. Visible domain with wavelength characteristics for some colors

Color	Peak wavelength (nm)
Blue	470
Cyan	525
Green	560
Yellow	585
Orange	600
Red	645
Deep red	700

laws of absorption of radiation

Absorbance (A) - It is also called optical density or extinction (E). It represents a measure of the capacity of a substance to absorb light of a specified wavelength. It can be calculated using the formula below:

$$A = log \frac{I_0}{I_t}$$

Where:
I_0 = intensity of the incident light
I_t = intensity of the transmitted light

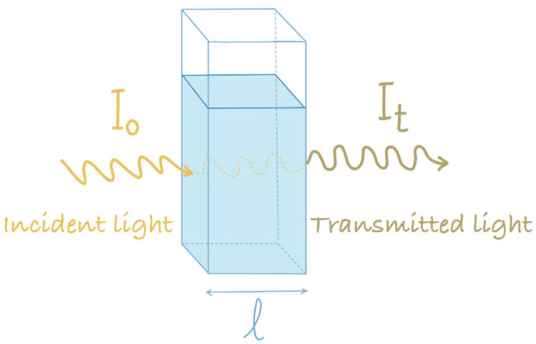


Figure 2. Light absorbance through s solution

Bouguer-Lambert law:

It describes how the intensity of a light beam decreases exponentially as it passes through a homogeneous medium, with the loss of intensity being proportional to the length of the path and the intensity itself. Therefore, as the thickness of an absorbing medium increases, the intensity of transmitted radiation decreases.

- The extinction coefficient characterizes the absorbing power of the medium, independent of path length (l) and incident intensity (I_0).
- It depends on the nature of the medium and on the wavelength of the incident radiation.
- The law is valid only for monochromatic radiation.

Combined Bouguer-Lambert-Beer law:

Bouguer-Lambert law was later extended by Beer to include the concentration of the absorbing substance, forming the Beer-Lambert Law. It is fundamental principle in spectroscopy where absorbance is directly proportional to both concentration and path length, enabling quantitative analysis of solutions - equations 1 and 2.

$$\boldsymbol{I_t = I_0 \cdot 10^{-aCl} \quad or \quad A = a \cdot l \cdot c} \quad \mathbf{(1)}$$

Where:
a = absorptivity,
c = concentration of the absorbing substance,
l = length of the light path.

When expressed in molar terms: $\boldsymbol{A = \varepsilon \cdot l \cdot c}$ $\mathbf{(2)}$
Where: $\boldsymbol{\varepsilon}$ = molar absorptivity (molar extinction coefficient), with units $L \cdot mol^{-1} \cdot cm^{-1}$.

Of note: - a and ε are wavelength-dependent.
- the molar extinction coefficient is a characteristic constant of an absorbing substance and can be considered analogous in significance to molecular mass.

As mentioned, the ***extinction coefficient*** is a key parameter of absorption; it may be defined as the *absorbance of a solution having unit concentration and measured with a unit light path.*

The measurement units of the extinction coefficient can be many, but they must be always related to the units of the concentration and the length of the light path, so that Ɛlc is a dimensionless quantity. This must be the case since absorbance, a logarithmic function of the ratio of light intensities, has no measurement units.

Deviations from the Beer-Lambert law

The Bouguer-Lambert-Beer law is valid at any wavelength and in any homogeneous medium, provided that the decrease in radiation intensity is caused solely by absorption, whether the medium is a gas, liquid, solid, or solution. In practice, most analytical absorption measurements are performed with solutions. These solutions behave ideally and obey the law as long as their optical properties, expressed by absorptivity (a) or molar absorptivity (ε), remain independent of concentration. The range in which the law is valid must be determined experimentally, typically by plotting absorbance (A) against concentration (c) and checking for a straight-line relationship.
The law can fail with:
- concentrated solutions,
- very dilute (weakly absorbing) solutions,
- very thin path lengths (l)

Deviations occur when this relationship is nonlinear. Causes include:

- Experimental factors: temperature, pressure, solvent effects.
- Instrumental factors: radiation dispersion, source stability, detector performance, wavelength selector precision, slit control, optical/electronic stability.
- Chemical factors: shifts in chemical equilibrium (e.g., pH changes, complexation, competing reactions), and concentration effects.
- Non-monochromatic radiation.
  Deviations may be positive (+) or negative (–).

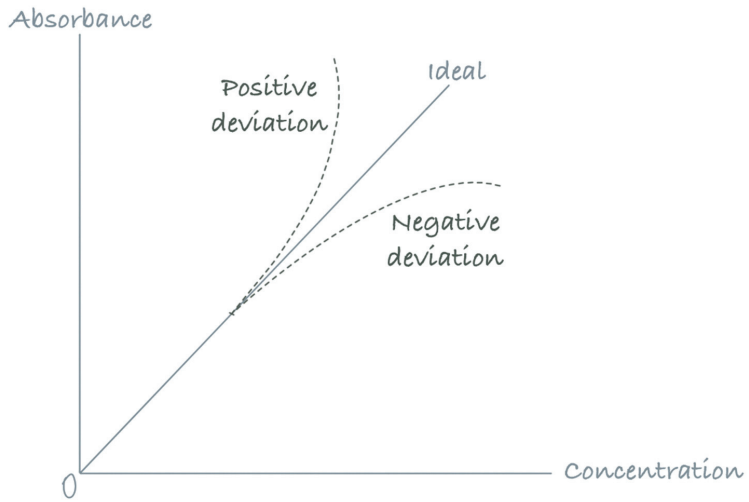


Figure 3. Deviations from Lambert-Beer Law

Although absorption measurements can be made across the electromagnetic spectrum, quantitative determinations are primarily carried out in UV, visible (VIS), and infrared (IR) spectrometry, where the Beer–Lambert law is extensively applied.

Apparatus

Measurements of the absorbance of light are made using a **photometer**, an instrument for the direct measurement of light intensities. There are two basic types, a filter photometer and a spectrophotometer. The former uses filters for the isolation of relatively broad bandwidths, and the latter uses a monochromator, composed of prisms or diffraction gratings, for the isolation of very narrow bandwidths. The basic parts of a spectrophotometer are a light source (often an incandescent bulb for the visible wavelengths, or a deuterium arc lamp in the ultraviolet), a holder for the sample (cuvette), a monochromator to separate the different wavelengths of light, and a detector. The detector is typically a photodiode. Photodiodes are used with monochromators, which filter the light so that only light of a single wavelength reaches the detector.

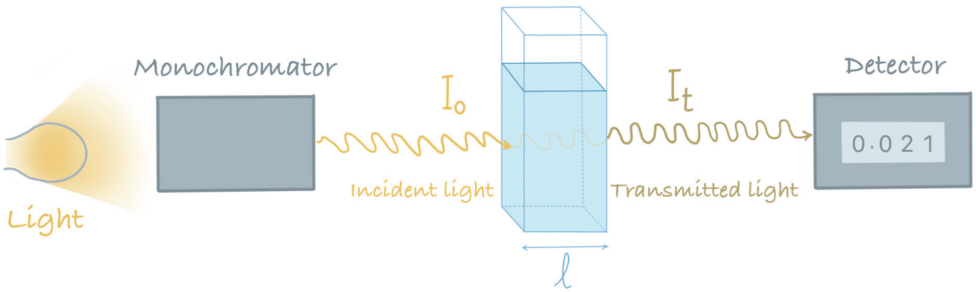


Figure 4. Features of an absorption spectrophotometer

The cuvettes for the samples must be made from substances transparent for the domain of analysis and their optical length must be reproducible or very exact.

Absorption measurements applied in quantitative determinations

The concentration of substances could be determined based on measurements of the light absorbance by substances in solution especially in the visible domain (color reactions) in several situations:

- The analyzed substance has a characteristic color;
- The analyzed substance leads to a colored compound which absorbs light;
- The analyzed substance, reacting with a colored compound, modifies that color.

For the analysis of a given compound by measurements of light absorbance:

- The obtained absorbance (the color) depends only on the analyzed compound;
- The color must be stable in time;
- The color development is independent of the ambient conditions;
- The Lambert-Beer law could be applied;
- The measurements of the light absorbance should be made at maximum of absorption or other specific λ.

The following methods can be used:

- Standard curve method;
- The method of reporting to a single standard;
- The molar extinction coefficient.

Standard curve method

The method involves the use of five or more standard solutions with known concentrations. Both the standards and the sample undergo the same specific color reaction and are processed identically. After the reaction is complete, the absorbance of each solution is measured using a spectrophotometer, which is first zeroed with a blank solution. The blank contains all reagents used for color development, except the analyte, so that their absorbance is optically cancelled and does not contribute to the final measurements. The absorbance values of the standards are then plotted against their known concentrations (Figure 5). According to the Lambert-Beer law, this yields a straight-line calibration curve. The concentration of the unknown sample is determined by locating its measured absorbance on the curve and identifying the corresponding concentration value.

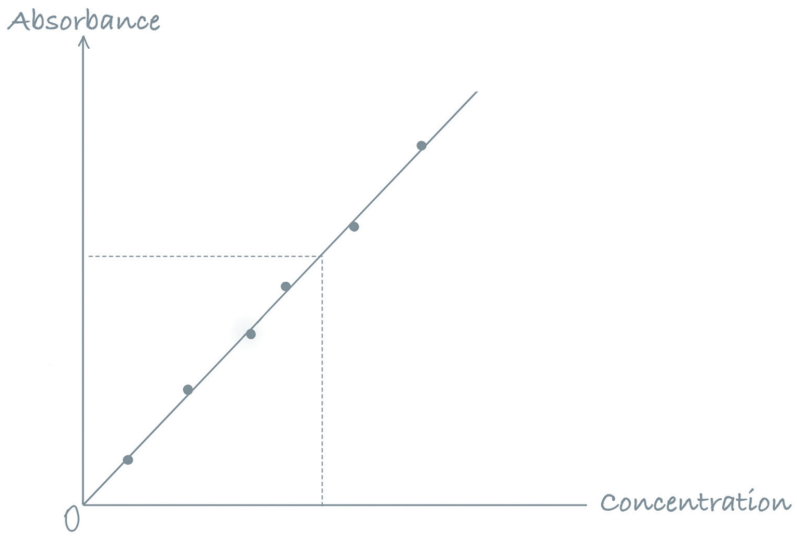


Figure 5. The standard curve representation

Reporting to a single standard method

From the set of standard solutions used in the experiment, a single standard can be chosen as a reference. Let this reference solution have a known concentration **Cs** and a measured absorbance **As** (for example, standard tube 3). For the sample with an unknown concentration **Cx** and a measured absorbance **Ax**, the following calculation can be applied:

This formula is based on the proportional relationship between concentration and absorbance described by the Lambert–Beer law

$$C_s \ldots\ldots\ldots\ldots\ldots A_s$$
$$C_x \ldots\ldots\ldots\ldots\ldots A_x$$

$$C_x = \frac{A_x \times C_s}{A_s}$$

If the analyzed solution has a dilution coefficient D then:

$$C_x = \frac{A_x}{A_s} \times C_s \times D$$

Reporting to the extinction coefficient

This approach is applied only when no full set of standards is available to construct a standard calibration curve. However, it should be noted that the Lambert-Beer law holds true only within the specific concentration range being investigated. To ensure accuracy, precisely calibrated pipettes must be used for reagent measurement, and cuvettes with a well-defined and consistent path length must be employed. Absorbance (A) measurements are carried out at a carefully selected wavelength; therefore, concentration can be calculated as follows:

$$C_x(moles/l) = \frac{A_x}{\varepsilon \cdot l} \times \frac{V_F}{V_A}$$

Where:
A_x - sample absorbance
ε - molar absorptivity
l - length of the light path
V_F - final sample volume
V_A - the volume of biological fluid analyzed

This simplified calculation method is commonly used for determining enzyme activity or for estimating sample concentrations in cases where appropriate standards are not available.

EXPERIMENTAL PART

Determination of proteins using the Bradford method

Principle of determination

Proteins react with the Bradford reagent (Coomassie Brilliant Blue G250) in an acidic medium and form a blue colored compound, whose absorbance of radiation at 595 nm is proportional with the protein concentration in the sample.

For the calibration curve, 5 solutions of BSA (bovine serum albumin) must be obtained, using a stock solution of 2 mg/ml concentration. The diluted solutions must be prepared at a volume of 1 ml, in Eppendorf vials, at the following concentrations:

P1 = 0.2 mg/ml
P2 = 0.4 mg/ml
P3 = 0.6 mg/ml
P4 = 0.8 mg/ml
P5 = 1.0 mg/ml

After the diluted solutions were obtained, the color reactions are assessed according to the working protocol:

For the BSA diluted solutions:

Reagent, µl	
Diluted BSA sample	50
Bradford reagent	1500

For the unknown sample:

Reagent, µl	
Unknown sample	50
Bradford reagent	1500

For the blank:

Reagent, µl	
Distilled water	50
Bradford reagent	1500

Homogenize the samples and incubate at room temperature for 20 minutes. Read the absorbance of the diluted solutions and unknown sample against the blank at 595 nm.

With the obtained values, draw the calibration curve (A vs concentration) and then find the concentration of the unknown sample.

Normal values for Serum proteins: 6.2-8.0 g/100 ml.

Pathological values

Pathological variations in total serum proteins have limited clinical significance because of their relatively low incidence and poor specificity.

Hyperproteinemia refers to an increase in total serum protein levels above 8 g/100 ml. It may be relative, occurring in cases of hemoconcentration following dehydration (e.g., nausea, vomiting, diarrhea), or true, resulting from a marked increase in one or more immune system components (such as in multiple myeloma, collagen diseases, or leukemia), or from the excessive presence of abnormal proteins.

Hypoproteinemia is defined as a decrease in total serum proteins below 6.2 g/100 ml and is always associated with a negative nitrogen balance. It occurs in conditions involving excessive renal protein loss, liver diseases that impair protein synthesis, immunodeficiency syndromes (where protein synthesis is reduced due to decreased antibody production), and in cases of burns or trauma, where accelerated protein catabolism is observed. Clinically, protein deficiency caused by malnutrition or impaired protein absorption leads to “hunger edema”, a condition marked by reduced albumin levels and decreased osmotic pressure. Such patients may also present with lowered immunity, anemia, osteoporosis, and gastrointestinal disorders.

Questions

1. What does Lambert-Beer law state?

2. Briefly describe the deviations from the Beer-Lambert law.

3. Briefly explain the standard curve method

4. Under what conditions is it recommended to use the extinction coefficient method?

5. Describe the experiment performed in the laboratory.

6. Under what conditions can hypoproteinemia or hyperproteinemia occur?

Lab Notes

Use this space for handwritten notes during the practical session.

L11. ENZYMATIC COFACTORS. THE OPTICAL TEST METHOD

Enzymatic cofactors

Enzymes are organic biocatalysts characterized by high specificity. Most enzymes have a protein structure, while only a few possess a polynucleotide structure.

According to their composition, enzymes are divided into two main classes:

- **Holoenzymes** - composed solely of protein
- **Heteroenzymes** - consist of a protein part (called the apoenzyme) and a non-protein component known as the **cofactor**. Without the cofactor, the enzyme is catalytically inactive. Cofactors can be further divided into three categories:

  - **Metal ions**, directly participate in the catalytic process of the enzyme (e.g. Cu^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Mo^{2+}, Se^{2+}, K^{+}, Zn^{2+}, etc.)

  - **Coenzymes** - organic, non-protein molecules that are loosely bound to the enzyme and can easily dissociate. They are sometimes referred to as secondary substrates or cosubstrates. Most coenzymes are vitamins (e.g., ascorbic acid) or vitamin derivatives, such as:
    - Thiamine pyrophosphate - TPP (from thiamine - B_1)
    - Nicotinamide adenine dinucleotide - NAD^+ (from niacin - B_3)
    - Nicotinamide adenine dinucleotide phosphate - $NADP^+$ (from niacin - B_3)
    - Coenzyme A (from pantothenic acid - B_5)
    - Tetrahydrofolate - THF (from folic acid - B_9)
    - Tetrahydrobiopterin - BH_4 (from GTP)
    - ATP/GTP/UTP (from nucleotides)

  - **Prosthetic groups** - organic or inorganic molecules covalently bound to the enzyme's protein component. They are regenerated in situ during the catalytic cycle. Many prosthetic groups are vitamins or vitamin derivatives, such as:
    - Flavin mononucleotide - FMN (from riboflavin - B_2)
    - Flavin adenine dinucleotide - FAD (from riboflavin - B_2)
    - Pyridoxal phosphate - PLP (from pyridoxine - B_6)
    - Biotin (from biotin - B_7)
    - Deoxyadenozylcobalamin and methylcobalamin (from cobalamin - B_{12})
    - Lipoic acid (non-vitamin compound)

Among these cofactors, only the derivatives from niacin (NAD^+, $NADP^+$ and their reduced forms NADH, respectively NADPH) are routinely used for the quantitative determination of the enzyme activity, due to their characteristic optical properties.

Determination of enzymatic activity

Two main types of determinations are used in the laboratory practice for the determination of the enzymatic activities:

- ***The methods in two points***: In this approach, a blank sample (containing the inactivated enzyme) and a test sample (with the active enzyme) are used. After a fixed incubation period, the enzyme in the test sample is also inactivated. The difference between the test and blank samples is evaluated based on the concentration of either the substrate or the reaction product. This type of determination does not allow the assessment of the kinetics of substrate.
- ***The kinetic methods***: In these methods, the reaction is monitored continuously, either digitally or using recording instruments. Kinetic determinations are simpler, faster, and provide real-time data on enzyme activity. The cost of reagents is generally not a limiting factor when analyses are performed on a small scale.

Optical test

The optical test is a widely used kinetic method, first introduced by Warburg and Christian in 1936 for determining enzymes that catalyze hydrogen transfer reactions involving the coenzymes NADH or NADPH. In principle, the method measures either the oxidation of NADH + H^+ or the reduction of NAD^+, as these reactions are accompanied by a measurable change in absorbance.

$$XH_2 + NAD(P)^+ \longrightarrow X + NAD(P)H + H^+$$

The oxidation or the reduction can be observed directly because NADH absorb the light strongly at 300-370 nm and NAD^+ absorb the light very little at this wavelength.

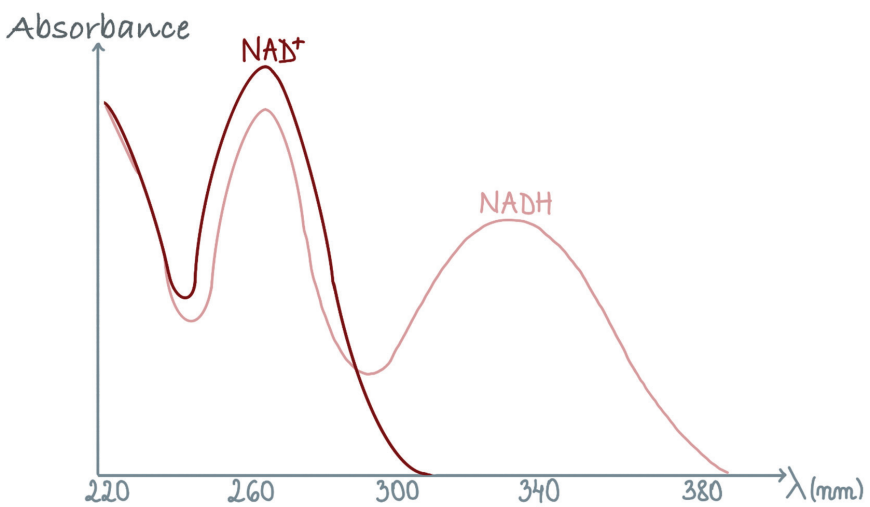


Figure 1. The absorption spectra of NAD^+ and NADH

Usually, the experiment is done at 340 nm or 366 nm.

The **optical test represents the determination in UV of the NAD^+ or $NADP^+$ dependent enzymes.** Practically, three variants of the optical test are used, making possible the determination of almost all enzymes in the clinical laboratory.

Technical variants of the optical test

1. **Simple optical test** - If the enzyme to be determined uses $NAD(P)^+$ or NAD(P)H as a coenzyme, the reaction occurs between the substrate, the enzyme (present in the serum), and the coenzyme, while the absorbance of light is measured over a specific time interval.

- ***Determination of lactate dehydrogenase (LDH)***: The enzyme LDH catalyzes the reaction:

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

The enzyme is present in all cells, has a high activity and is determined almost exclusively by the optical test in both directions of the reaction.

- ***Determination of sorbitol dehydrogenase (SDH)***: The enzyme SDH catalyzes the reaction:

$$D-fructose + NADH + H^+ \overset{SDH}{\longleftrightarrow} D-sorbitol + NAD^+$$

The enzyme is present in liver and seminal glands. This determination is used as a test of the hepatic function.

In all these types of tests, the change in absorbance over time is measured. At the beginning of the test, when the reduced coenzyme (NADH) is added, the absorbance increases rapidly and then stabilizes after approximately one minute. At this point, the addition of the substrate solution to the reaction mixture initiates the enzymatic reaction. The subsequent decrease in absorbance is recorded, and this decline is directly proportional to the enzyme activity. After about 3-4 minutes, the NADH coenzyme is completely consumed. Therefore, the rate of change in absorbance per minute (ΔE/min) is directly proportional to the enzyme activity.

2. **Optical test coupled with an indicator reaction** - Some enzymes do not use NADH as a coenzyme. However, their activity can still be determined using the optical test, coupled with a specific indicator reaction. In this case, the product formed in the primary reaction (catalyzed by the enzyme under investigation) serves as the substrate for a secondary, indicator reaction. This second reaction is catalyzed by an NADH-dependent enzyme, allowing the overall process to be monitored spectrophotometrically. For example:

- ***Determination of alanine aminotransferase-ALAT (glutamate-pyruvate transaminase-GPT)***

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

The resulted pyruvate participates in a second reaction (the indicator reaction), catalyzed by a NADH dependent enzyme - lactate dehydrogenase (LDH):

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

- ***Determination of pyruvate aminotransferase-ASAT (glutamate-oxalacetate transaminase-GOT)***

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

The resulted oxaloacetate participates in a second reaction (the indicator reaction), catalyzed by a NADH dependent enzyme - malate dehydrogenase:

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

A transaminase (or aminotransferase) is an enzyme that catalyzes the transfer of an amino group from one molecule to another, playing a key role in amino acid metabolism. Aspartate aminotransferase (AST) is similar to alanine aminotransferase (ALT) in that both enzymes are associated with liver parenchymal cells. However, ALT is found predominantly in the liver, with clinically insignificant amounts present in other tissues, whereas AST is distributed in the liver, heart (cardiac muscle), skeletal muscle, and other organs, though usually in smaller quantities.

As a result, ALT is a more specific marker of liver inflammation than AST, although both enzymes are routinely measured as part of liver function tests.

Furthermore, AST levels may also be elevated in diseases affecting other organs, such as myocardial infarction or musculoskeletal disorders.

Transaminase elevations may occur secondarily in pathological conditions affecting organs other than the liver, heart, or skeletal muscle; however, in these cases, the enzymes are not considered primary diagnostic markers for those organs.

3. **Optical test coupled with an auxiliary reaction and an indicator one**. If the product of the first reaction can't be the substrate for an NADH dependent enzyme, an intermediary reaction is used. The second product will be the substrate in an indicator reaction. For example:

- ***Determination of creatine kinase (CK).*** The catalyzed reaction is:

$$creatine + ATP \overset{CK}{\leftrightarrow} creatine - phosphate + ADP$$

The auxiliary reaction (second step) uses ADP as substrate, and is catalyzed by pyruvate kinase (PK):

$$phosphoenolpyruvate + ADP \overset{PK}{\leftrightarrow} pyruvate + ATP$$

The indicator reaction (third step) uses pyruvate as substrate, and is catalyzed by lactate dehydrogenase (LDH):

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

To obtain a direct proportionality between the rate of disappearance of creatine and the formation of NAD^+, all the reagents are added in excess.

Creatine kinase (CK) is an enzyme found in the heart, brain, and skeletal muscle that helps regulate energy by converting creatine into phosphocreatine and back. CK is a dimeric enzyme consisting of 2 catalytic subunits called M (muscle) and B (brain). The 3 isoenzymes resulting from the association of the two subunits are: CK-BB (CK1, containing 2 B chains), CK-MB (CK2, containing a B chain and an M chain), CK-MM (CK3, containing 2 M chains).

From the clinical point of view, elevated blood levels of CK indicate tissue damage, primarily in cardiac conditions and skeletal muscle injury or rhabdomyolysis.

Calculation of the enzymatic activity

There are different modalities for the calculation of the enzyme activity:

- the simultaneously use of a standard
- the use of the molar coefficient of extinction
- the graph method

The first method uses commercial reagents of standard series, with a known enzymatic activity or purified enzymes. Practically this system is not very often used because the enzyme activity is not constant in time and the commercial standard series are quite expensive.

More practical is to use a standard quantity of substrate and to pursue the consumption of this one in time (or the generation of the product). The formula for the calculation of the enzyme activity in that case is:

$$UI = \frac{E_P}{E_S} \cdot C_M \cdot 10^6 \cdot \frac{1}{t} \cdot \frac{V_F}{V_A} \quad \mu mols \cdot min^{-1} \cdot L^{-1}$$

Where: V_A = volume of the analyzed material, ml
V_F = final volume, ml
C_M = substrate (product) concentration, mol/1000 mL
E_P = sample extinction, E_S = standard extinction (absorbance)
t = incubation time, minutes

The kinetic methods note the absorption at different intervals of time, and the absorption variation per minute is calculated. The calculation formula is:

$$UI = \frac{\Delta E/min}{d \cdot \varepsilon} \cdot 10^6 \cdot \frac{V_F}{V_A} \quad \mu mols \cdot min^{-1} \cdot L^{-1}$$

Where: $\Delta E/min$ = variation of sample absorption per minute
V_A = volume of the analyzed material, ml
V_F = final volume, ml
d = cuvette length, cm
ε = molar coefficient of extinction of substrate (product)

EXPERIMENTAL PART

Determination of serum lactate dehydrogenase activity (LDH)

Introduction

Lactate dehydrogenase (LDH) catalyzes the reversible transformation of pyruvate to lactate. Pyruvate is reduced to form lactate, and NADH is oxidized to NAD^+. Actually, total LDH activity is the result of the activity of several enzymes which have very similar structures, and which operate on the same substrates, in different organs. These LDHs are called *isoenzymes*. LDH has two subunits, H (heart) and M (muscle). By combining the 2 subunits, 5 isoenzymes result: LDH_1 (HHHH), LDH_2 (HHHM), LDH_3 (HHMM), LDH_4 (HMMM) and LDH_5 (MMMM).

The reaction takes place at pH 7.5, so the pyruvate is reduced to lactate. The disappearance of NADH is followed by the decrease in absorbance at 340 nm, which is measured for several minutes either continuously or at frequent intervals. The change in the absorbance per minute (ΔA/min) is related directly to micromoles of NADH oxidized and, in turn, to micromoles of substrate transformed per minutes (International Units).

Backwards, transformation of lactate to pyruvate takes place at pH = 9.

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

Procedure:

- set the wavelength at 340 nm;
- calibrate the spectrophotometer against distilled water;
- in a cuvette, pipet 100 µl of serum (contains LDH) and 1000 µl of working reagent, and homogenize gently by pipetting up and down;
- put the cuvette in the spectrophotometer, and read the A at 0, 1, 2 and 3 minutes.

Calculation:

$$\frac{\Delta A}{min} = [(A_0 - A_1) + (A_1 - A_2) + (A_2 - A_3)]/3$$

$$Activity = \frac{\Delta A}{min} x\ 4127$$

Normal values for LDH: 135-225 U/l

Clinical significance:

Serum LDH is increased in hepatic, cardiac, skeletal muscle, renal disease, and some hematopoietic and neoplastic diseases.

Liver diseases (*viral hepatitis, cirrhosis*) cause increases to approx. twice upper reference range (increased LDH_5 isoenzyme).

Cardiac disease (*myocardial infarction-MI*) causes about the same degree of increase (LDH_1 and LDH_2). The serum LDH begins to increase ~ 12 hours after the MI and rises until it peaks at about 72 hours. The level returns to normal in about 10 days unless a second MI occurs.

Renal and pulmonary infarcts also caused increased LDH activity.

The hematological diseases of *pernicious anemia and acute lymphoblastic leukemia* also result in significant increases of serum LDH.

Questions

1. Write the differences between a coenzyme and a prosthetic group. Give examples for both categories.

2. What is the optical test. Explain why the absorbance determination is done at 340 nm and not 260 nm.

3. How many technical variants of optical test do you know. Present them briefly.

4. Briefly, present the experiment performed at the laboratory.

5. What is the clinical utility of LDH, ALAT, ASAT, CK determination.

Lab Notes

Use this space for handwritten notes during the practical session.

L12. ENZYME KINETICS. DETERMINATION OF K_M AND V_{max}

Chemical reactions proceed at varying rates - some occur spontaneously and very rapidly (though never instantaneously), while others are so slow that they require an activating factor, such as increased temperature or the presence of a catalyst, to proceed within a practical timeframe.

Enzymes are proteins that serve as biological catalysts, accelerating biochemical reactions within living cells. They function by binding to the substrate molecule to form an enzyme-substrate complex (ES), which lowers the activation energy needed to convert the substrate into the product. For a simple reaction involving a single substrate, the process can be represented as follows:

$$S + E \underset{k_2}{\overset{k_1}{\longleftrightarrow}} ES \xrightarrow{k_3} P + E \quad (1)$$

The initial rate of an enzymatic reaction can be determined by measuring the amount of substrate converted or product formed per unit of time. Most commonly, the reaction rate is expressed as the number of micromoles of product generated per minute (μmol/min).

The initial velocity (v) of the enzymatic reaction represents the instantaneous rate of product formation (P) or substrate disappearance (S) as a function of time and can be expressed:

$$v = -\frac{d[S]}{dt} = \frac{d[P]}{dt}$$

Figure 1 illustrates the variation of reaction rate over time for a typical enzymatic reaction. It is evident that the reaction rate decreases as the reaction progresses. This decline can be attributed to several factors, including enzyme denaturation, product inhibition, reduced substrate concentration (and thus decreased enzyme saturation), coenzyme inactivation, and the onset of the reverse reaction as the product accumulates. Therefore, it is essential to measure the reaction rate not at any random time point, but specifically at the very beginning, when these interfering factors are minimal. For this reason, the initial rate is used in enzymatic studies. In Figure 1, the initial rate corresponds to the slope of the curve at the origin.

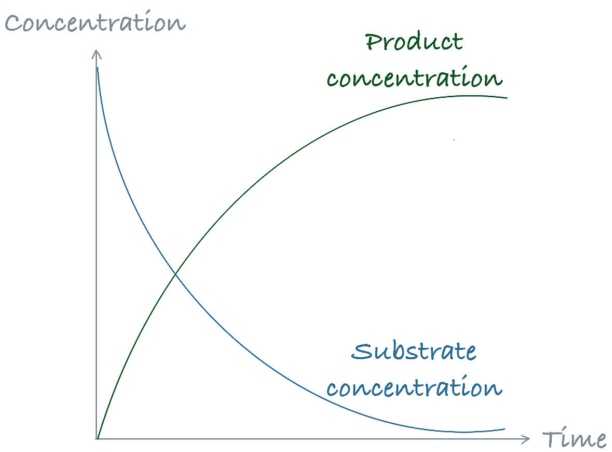


Figure 1. Dependence of substrate and product concentrations in time

In practice, the initial velocity can be calculated using the formula below, which illustrates that it is determined at the beginning of the reaction, when the rate of substrate disappearance or product formation is linear with respect to time.

$$v = \frac{\Delta[P]}{\Delta t} = -\frac{\Delta[S]}{\Delta t}$$

Figure 2 illustrates the variation of the reaction rate as a function of substrate concentration for an enzymatically catalyzed single-substrate reaction

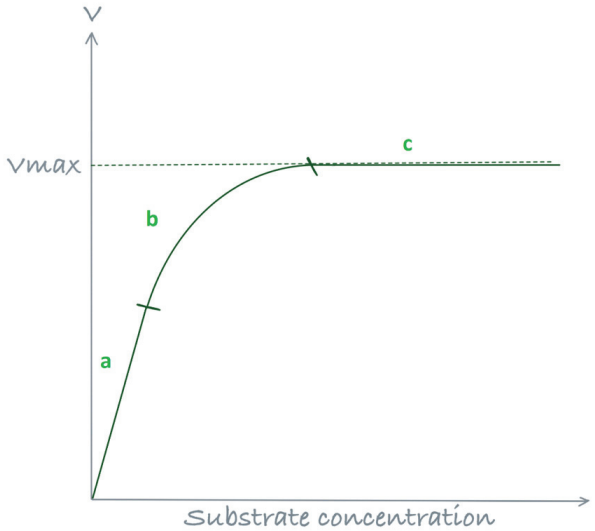


Figure 2. Variation of reaction rate as a function of substrate concentration

The analysis of the curve presented in Figure 2 leads to the following observations:

1. **Region "a"** - At relatively low substrate concentrations, the reaction is approximately *first-order* with respect to the substrate. This is because there are many more available enzyme active sites than substrate molecules; thus, each substrate molecule is rapidly transformed, and every increase in substrate concentration leads to a proportional increase in reaction rate.
2. **Region "b"** - As the substrate concentration increases, the initial reaction rate rises more slowly and is no longer linearly proportional to the substrate concentration. In this region, the reaction exhibits *mixed-order kinetics*.
3. **Region "c"** - At high substrate concentrations, the reaction rate becomes completely independent of substrate concentration and asymptotically approaches a constant value. In this phase, the reaction is *zero-order*, as the enzyme is saturated with substrate, meaning all active sites are occupied. At this substrate saturation point, the *maximum velocity (V_{max})* of the enzymatic reaction is reached, and further addition of substrate will not increase the reaction rate.

The mathematical relationship between the initial reaction velocity, substrate concentration, and enzyme characteristics was established by L. Michaelis and M.L. Menten and later refined by E.G. Briggs and J.B.S. Haldane. The resulting equation, known as the **Michaelis-Menten equation**, is expressed as follows (Equation 2):

$$v = \frac{V_{max}[S]}{K_M + [S]} \qquad (2)$$

K_M represents the substrate concentration at which the enzymatic reaction rate reaches half of its maximum velocity ($V_{max}/2$) in reactions involving a single substrate. At this point ($v=V_{max}/2$ and $[S]=K_M$), the enzyme is bound to the substrate in half of its available active sites, meaning half of the enzyme molecules exist as enzyme-substrate (ES) complexes. Therefore, K_M corresponds to the dissociation constant of the ES complex and reflects how tightly the enzyme binds its substrate.

Therefore, K_M provides a quantitative measure of an enzyme's *affinity* for its substrate - the lower the K_M value, the higher the enzyme's affinity.

For example, considering an enzyme E that acts on three different substrates (S_1, S_2, and S_3), the relationship between reaction rate (v) and substrate concentration ([S]) can be graphically represented as shown in Figure 3.

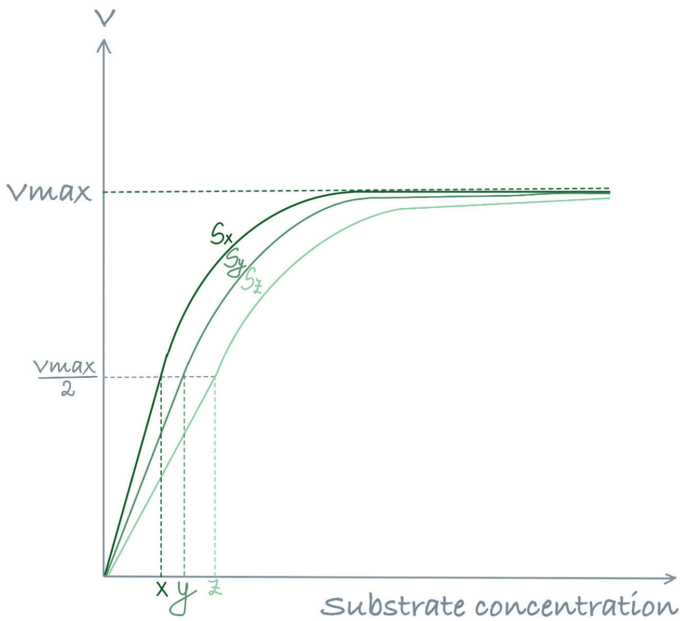


Figure 3. Representation of v=f[S]

By comparing the calculated K_M values from the graph with the corresponding $V_{max}/2$ points, the following relationship is obtained: $K_{M1} < K_{M2} < K_{M3}$. This indicates that the enzyme's affinity for the substrates decreases in the order $S_1 > S_2 > S_3$. In other words, for substrate S_1, a smaller concentration is required to reach half of the maximum velocity (Vmax/2) compared to S_2 and S_3. Therefore, an enzyme with a low K_M value achieves maximum catalytic efficiency at lower substrate concentrations, reflecting a higher substrate affinity.

Typically, K_M values range between 10^{-1} and 10^{-8} M. Enzymes with K_M values between 10^{-1} and 10^{-2} M exhibit *low affinity* for their substrates, whereas those with K_M values between 10^{-5} and 10^{-8} M show *high affinity*.

Both K_M and V_{max} are determined experimentally from the plot of v = f([S]). The maximum velocity (V_{max}) is estimated by extrapolating the asymptote of the hyperbolic curve, and K_M is then calculated from the corresponding point, as illustrated in Figure 4.

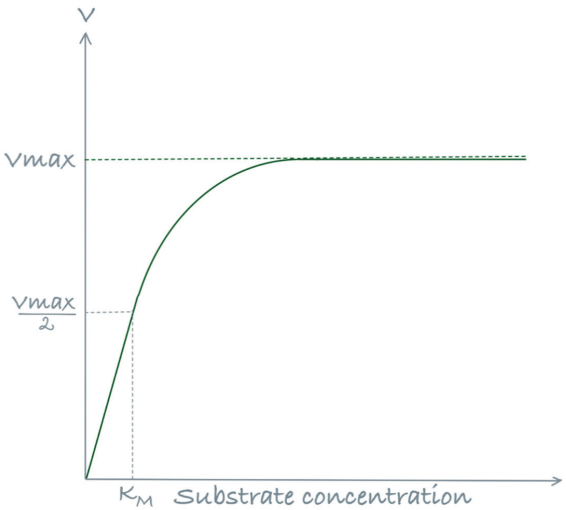


Figure 4. Determination of V_{max} and K_M

The determination of the two constants can be performed with greater accuracy by using a mathematical transformation. If the Michaelis-Menten equation is valid, then its reciprocal form must also hold true. By taking the reciprocal of both sides, the **Lineweaver-Burk equation** is obtained:

$$\frac{1}{v} = \frac{K_M}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

The graphical representation of 1/v as a function of 1/[S] yields a straight line, allowing for a more precise determination of V_{max} and K_M, as shown in *Figure 5*. On this plot, the line intersects the x-axis at $-1/K_M$ and the y-axis at $1/V_{max}$.

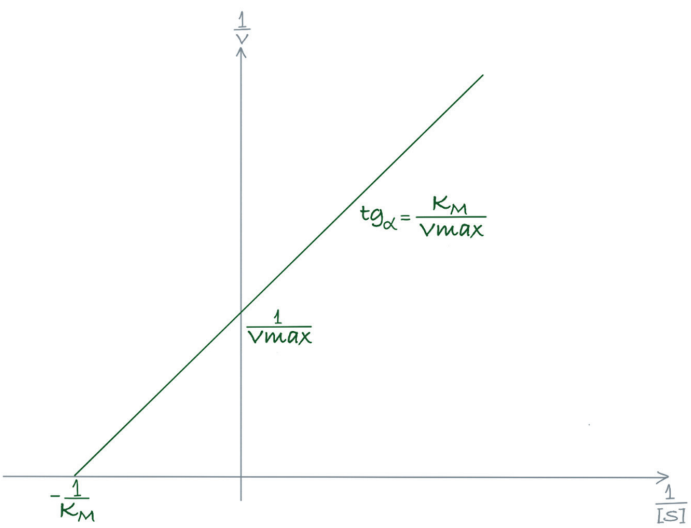


Figure 5. Lineweaver-Burk representation

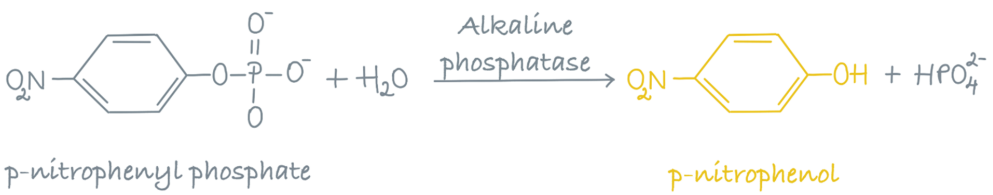
EXPERIMENTAL PART

Influence of substrate concentration on the reaction rate, catalyzed by alkaline phosphatase

Principle

Serum alkaline phosphatase (ALP) is an enzyme found in various tissues, including the liver, bones, kidneys, intestines, mammary glands, and placenta. Elevated ALP activity occurs in liver disorders (such as biliary obstruction or hepatic metastases), bone diseases (including rickets, osteomalacia, and bone metastases), as well as in certain neoplastic conditions. Conversely, decreased ALP activity may be observed in cases of anemia and malnutrition.

Alkaline phosphatase (ALP) catalyzes the hydrolysis of phosphate esters, removing a phosphate group from substrate molecules. A commonly used substrate in colorimetric assays is p-nitrophenyl phosphate (p-NPP). In this reaction, ALP catalyzes the conversion of p-nitrophenyl phosphate into p-nitrophenol (a yellow compound) and inorganic phosphate:



The variation of the absorbance of the sample over time, measured at 405 nm, is directly proportional to the serum ALP activity. Sodium p-nitrophenolate (resulting in alkaline medium) has a molar absorptivity of 18,496 x $10^3 M^{-1}$ cm^{-1}.

The aim of this experiment is to evaluate the influence of substrate concentration (pNPP) on the reaction rate for the determination of K_M and V_{max} of the enzymatic reaction.

The method of analyzing the enzymatic activity (reaction rate) is a kinetic method, by continuously monitoring over time the absorbance changes of the reaction mixture at λ=405 nm.

Materials and equipment:

1. Micropipette of 20-200 µL;
2. Micropipette of 100-1000 µL;
3. 1.5 mL Eppendorf tubes;
4. 1 mL spectrophotometer cuvettes;
5. Spectrophotometer.

Reagents:

1. R1: 1M Diethanolamine Buffer, pH = 9.80: Dissolve 100 mg $MgCl_2$ and 97 ml diethanolamine in 800 ml bidistilled water. Adjust the pH with a 10 M HCl solution to 9.80, then make up to a final volume of 1 liter with bidistilled water in a graduated flask.
2. R2: Substrate solution - 2mM para-nitrophenyl phosphate solution (pNPP)
3. R3: R3- working reagent is obtained by combining R1 with R2 according to the protocol in the table below
4. R4: Alkaline phosphatase solution (from blood serum)

	S1	S2	S3	S4	S5	S6
R1 (µL)	599	598	597	580	460	250
R2 (µL)	1	2	3	20	140	350
Substrate concentration (mmole/L)						

Procedure:

Each group performs a Michaelis-Menten curve containing six points. Six increasing concentrations of substrate (S1-S6) are prepared, for which the reaction rates are determined according to the protocol below.
- In the 6 Eppendorf tubes (noted S1 to S6) the volumes described in the table for the buffer solution (R1) and the substrate solution (R2) are pipetted. The solutions R1 and R2 together form a total volume of 600 µL.

- Set the wavelength to 405 nm at the spectrophotometer.
- Insert a cuvette with buffer solution into the spectrophotometer and set zero the spectophotometer.
- 600 µL of S1 + 100 µL R4 is pipetted in another cuvette (mixing easily with the pipette for homogenization).
- Insert the cuvette into the spectrophotometer and read the absorbance A_0 at time 0 (reading is done when the absorbance value is stabilized).
- Start the stopwatch and after 3 minutes read the absorbance A_3 at time 3 minutes. The same is done for S2-S6 solutions.

Determination of reaction rates

For each of the S_1-S_6 samples, the reaction rates V_1-V_6 are calculated according to the equation below:

$$Reaction\ rate\ (\mu mol/\text{min}) = \frac{\Delta A}{min} \times \frac{V_F}{V_A \cdot \varepsilon} \times \frac{10^6}{10^3}$$

Where:
ΔA / min - variation of sample absorption per minute;
V_F - final volume (mL);
V_A - volume R3 (serum) (mL);
ε - molar absorption coefficient (L M^{-1} cm^{-1}) = 18496;
l = 1 cm

$$Reaction\ rate\ (\mu mol/\text{min}) = \frac{\Delta A}{min} \times \frac{0.7}{0.01 \cdot 18496} \times \frac{10^6}{10^3}$$

$$Reaction\ rate\ (\mu mol/\text{min}) = \frac{\Delta A}{min} \times 3.7846$$

The substrate concentration for [S1]-[S6] is calculated considering the volume of pNPP concentration 2 mM added in each sample and the final mixture (700 µL). Example of calculation of substrate concentration [S] (mM) for sample S_1:

2 mmole pNPP ..... 10^6 µL
x mmole pNPP ..... 1 µL

x mmole pNPP/sample = 2 x 10^{-6}
2 x 10^{-6} mmole pNPP ..... 700 µL
y mmoles pNPP ..... 10^6 µl

y = 2 x 10^{-6} x 106/700 = 0.00286 mmol / L
[S] (mM) = (mmol pNPP / sample x 10^6) / 700

The results are shown in the table:

Substrate	**A_0**	**A_3**	**$\Delta A = A_3 - A_0$**	**Reaction rate (µmoles/min)**	**[S] (mM)**	**1/Reaction rate(min*µmol)**	**1/[S] (mM^{-1})**
S1							
S2							
S3							
S4							
S5							
S6							

To investigate the influence of the substrate concentration on the enzymatic activity, the Michaelis-Menten curve is plotted either according to the parameters [S] and V, or according to the parameters 1/V = f (1/[S]).

The importance of determining the constant K_M

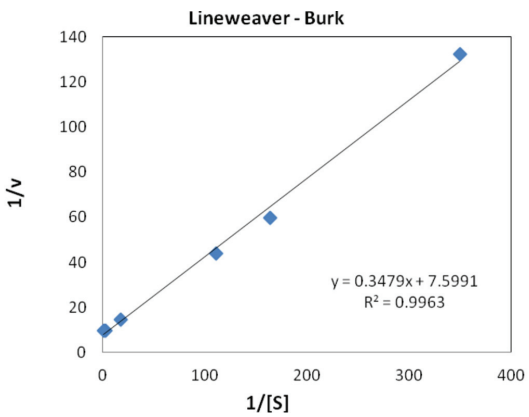
K_M represents a measure of the enzyme affinity for a substrate. Thus, K_M is inversely proportional to the affinity for the substrate. A **lower K_M** value indicates a **higher substrate affinity**, while a **higher K_M** reflects a **lower affinity**.

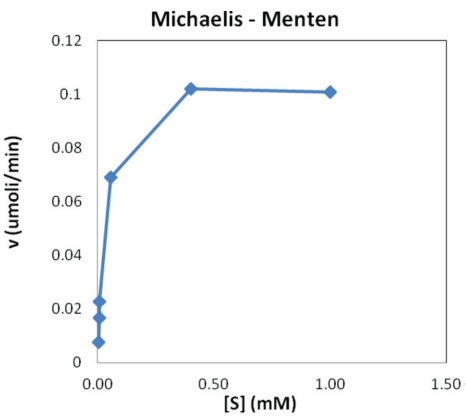
For example, in the physiological control of glucose metabolism, there are two types of hexokinases, one with high K_M (from the liver) and one with low K_M for glucose (from glucose-dependent tissues, such as brain and muscles). both of which contribute to maintaining the steady state of glucose in the blood. These two enzymes work together to maintain a stable blood glucose concentration.

A well-known example illustrating the physiological significance of K_M involves the lower alcohol tolerance in many Asian populations. Compared to Europeans, individuals of Asian descent often experience vasodilation (facial flushing) and tachycardia after consuming smaller amounts of alcohol. These effects are due to the accumulation of acetaldehyde, which is produced when alcohol dehydrogenase converts ethanol in the liver. Normally, mitochondrial aldehyde dehydrogenase (ALDH2) oxidizes acetaldehyde to acetate. However, many people of Asian origin possess a variant of ALDH2 with a high K_M (low affinity) for acetaldehyde, resulting in incomplete metabolism and accumulation of acetaldehyde, the compound responsible for the characteristic flushing and other symptoms.

The results of a similar experiment are presented below:

Substrate	A_0	A_3	$\Delta A = A_3 - A_0$	Reaction rate (µmoles/min)	[S] (mM)	1/Reaction rate ($min*\mu mol^{-1}$)	1/[S] (mM^{-1})
S1	0.049	0.055	0.006	0.757×10^{-2}	0.00286	132.100	349.65
S2	0.041	0.055	0.014	1.67×10^{-2}	0.00571	60.024	163.93
S3	0.037	0.055	0.018	2.27×10^{-2}	0.00857	44.038	111.11
S4	0.130	0.183	0.053	6.69×10^{-2}	0.05710	14.957	17.51
S5	0.751	0.832	0.081	10.22×10^{-2}	0.40000	9.787	2.50
S6	1.537	1.617	0.080	10.09×10^{-2}	1.00000	9.909	1.00





Using the equation:

$$1/V = 34.753 \times 1/S + 773.73$$

When
x=0, $1/V_{max}$ = 0.775 x 103, V_{max} = 0,13 µmoles/min
y = 0 -1/K_M= -22,263, K_M = 4,46 x10-5 M

Influence of temperature on the enzymatic activity of serum alkaline phosphatase

Principle

Being proteins, enzymes are highly sensitive to temperature. Each enzyme has an optimal temperature, the temperature at which its catalytic activity is maximal, typically between 35°C and 40°C. At temperatures above 50-60°C, enzymatic activity decreases or stops entirely due to denaturation of the protein structure.

The colorimetric reaction used in this experiment involves alkaline phosphatase (ALP) catalyzing the conversion of p-nitrophenyl phosphate (p-NPP) into p-nitrophenol (a yellow compound) and phosphate. The intensity of the yellow color formed during the reaction is proportional to the ALP activity.

The activity of ALP will be measured at 0°C (ice bath), 37°C, and 95°C to observe the influence of temperature on enzyme activity.

Reagents, ml	**0°C**	**37°C**	**95°C**
pNPP solution 2mM	1	1	1
Maintain at corresponding temperature for 10 min			
ALP solution	0,2	0,2	0,2
Maintain at corresponding temperature for 15 min			
NaOH	1	1	1

When the reaction takes place at 37°C, the color is more intense compared to the other 2 test tubes. The intensity of yellow color is proportional with ALP activity.

Influence of pH on ALP activity

Principle

Enzymes exhibit activity only within a limited pH range. Each enzyme has an optimum pH, the specific pH value at which its catalytic activity reaches a maximum. The optimum pH depends on various factors such as ionic strength, temperature, and substrate concentration. Deviations from this pH alter the enzyme's structure and charge distribution, gradually reducing its activity and eventually leading to enzyme inactivation.

The intensity of the yellow color produced during the hydrolysis of p-nitrophenyl phosphate (p-NPP) is proportional to the activity of alkaline phosphatase (ALP). In this experiment, ALP activity will be measured at different pH values using three buffer systems: diethanolamine buffer (pH 9.8), barbiturate buffer (pH 7), and acetate buffer (pH 4).

Reagents, ml	**pH 9.8**	**pH 7**	**pH 4**
pNPP 2mM	1	1	1
Buffer pH =10	2	-	-
Buffer pH = 7	-	2	-
Buffer pH = 4	-	-	2
ALP solution	0,20	0,20	0,20
Incubate at 37°C for 15 minutes			
NaOH 1M	1	1	1

It can be observed that the most intense color was obtained in the test tube where the pH value was 9.8.

Influence of effectors on enzymatic reaction rates

Enzymes can modify their catalytic activity in the presence of various compounds within the reaction medium. These compounds may bind either to the active site or to other amino acid residues of the enzyme. As a result, the enzyme's conformation changes, which can alter or inhibit its enzymatic activity.

In the case of metalloenzymes, the addition of chelating agents can further affect activity by binding to the metal ions that are essential for their structure and function.

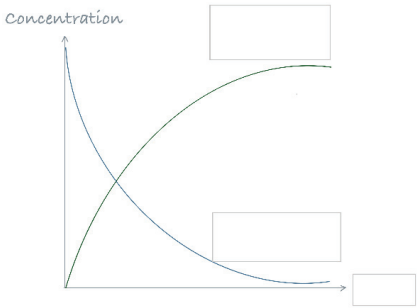
Alkaline phosphatase (ALP) contains zinc ions (Zn^{2+}) as cofactors. When EDTA is introduced, it chelates these zinc ions, leading to a decrease or loss of enzymatic activity. The colorimetric reaction used to assess this effect involves the ALP-catalyzed conversion of p-nitrophenyl phosphate (p-NPP) into p-nitrophenol, a yellow compound whose intensity reflects the enzyme's activity.

Reagents, ml	**Without EDTA**	**With EDTA**
pNPP 10mM	1	1
EDTA solution	-	0,20
H_2O	0,20	-
ALP solution	0,20	0,20
Incubate at 37°C for 15 minutes		
NaOH 1M	1	1

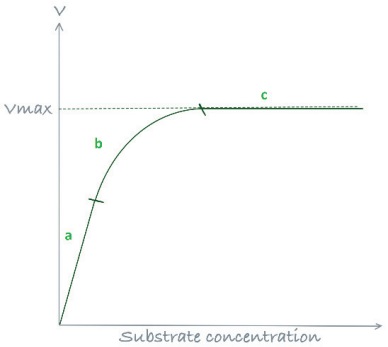
Based on the obtained results, the influence of EDTA on ALP activity will be commented. In the test tube where EDTA was absent, the yellow color is more intense compared to the other one. This is because EDTA chelated the Zn ion, so the enzymatic activity of ALP was affected.

Questions

1. Fill in the boxes in the image, and briefly interpret the figure

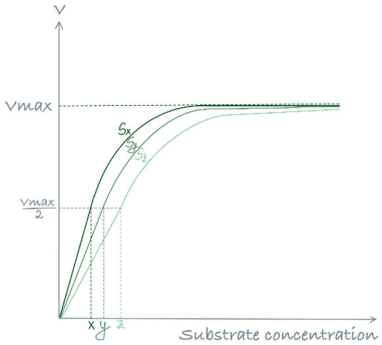


2. Interpret “region c” from the following figure.



3. Write the Michaelis-Menten equation and explain the significance of K_M. Illustrate the graphical representation of K_M and V_{max}.

4. Interpret the following graph



5. Illustrate the Lineweaver-Burk representation.

6. Briefly explain the Influence of temperature, pH and effectors on the enzymatic activity of serum alkaline phosphatase

Lab Notes

Use this space for handwritten notes during the practical session.

L13. VITAMINS DETERMINATION

Vitamins are bioorganic compounds required in small amounts to maintain the normal physiological functions of the human body. Most vitamins are obtained primarily from the diet, such as vitamins A, B_6, B_{12}, C, E, thiamine, riboflavin, folic acid, and pantothenic acid. Some can also be synthesized endogenously, including vitamin D and niacin. In addition, the intestinal flora contributes to the production of vitamins, particularly vitamin K and biotin.

A large proportion of vitamins act as enzymatic cofactors, playing essential roles in vital metabolic pathways. Based on their solubility, vitamins are divided into two major classes:

- Water-soluble vitamins: the B-complex group (thiamine - B_1, riboflavin - B_2, niacin - B_3, pantothenic acid - B_5, pyridoxine - B_6, biotin – B_7, folic acid/folate - B_9, cobalamin - B_{12}) and vitamin C.
- Fat-soluble vitamins (isoprenoid derivatives): vitamins A, D, E, and K.

The daily requirement for vitamins is very small. When intake is insufficient, metabolic disturbances occur; in severe cases, deficiency may lead to life-threatening conditions. Maintaining adequate vitamin levels depends on a balanced diet or, when necessary, supplementation with pharmaceutical formulations. However, excessive intake should be avoided, since toxic effects can develop, particularly with fat-soluble vitamins that accumulate in body stores.

Both deficiency and excess can be detected by laboratory analysis of vitamin levels in blood. Because of their structural complexity, purely chemical methods of determination are time-consuming and impractical. Therefore, in modern practice, they are usually replaced by high-performance physicochemical methods (e.g., chromatography, spectrometry).

Vitamin C

Vitamin C, also known as **ascorbic acid**, is a water-soluble vitamin essential for human health. Unlike many animals, humans cannot synthesize vitamin C due to the absence of the enzyme *L-gulonolactone oxidase*. For this reason, it must be obtained exclusively from the diet.

Sources

The main dietary sources of vitamin C are fresh fruits and vegetables. Citrus fruits such as oranges, lemons, and grapefruits are particularly rich in this vitamin. Other important sources include berries, kiwi, guava, peppers, tomatoes, broccoli, cabbage, and spinach. However, vitamin C is sensitive to heat, light, and long-term storage, so its content may be reduced during cooking or prolonged exposure.

Absorption

After ingestion, vitamin C is absorbed primarily in the small intestine by active transport and simple diffusion. In the blood, it circulates mostly in free form. Because vitamin C is water-soluble and not stored in significant amounts, excess quantities are rapidly excreted in the urine.

Biological roles

Vitamin C has several crucial biological roles. It acts as a cofactor for hydroxylation reactions, especially in collagen synthesis, where it is necessary for the hydroxylation of proline and lysine residues. This makes it vital for maintaining the integrity of connective tissue, proper wound healing, and bone health. It also functions as a powerful antioxidant, scavenging free radicals and regenerating

vitamin E from its oxidized form. Vitamin C supports the immune system by enhancing leukocyte function and resistance to infections. Additionally, it promotes iron absorption in the intestine by reducing ferric iron (Fe^{3+}) to the more absorbable ferrous form (Fe^{2+}), and it plays a role in neurotransmitter synthesis, including the conversion of dopamine to norepinephrine.

Daily dose

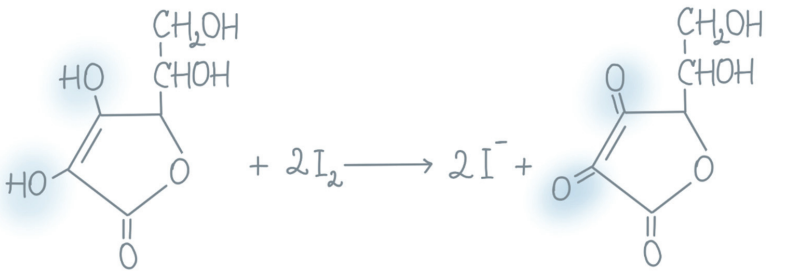
The daily requirement for vitamin C in adults is around **75-90 mg/day**, with higher needs in smokers because of increased oxidative stress. A deficiency of vitamin C leads to **scurvy**, a condition characterized by fatigue, irritability, bleeding gums, impaired wound healing, bone pain, perifollicular hemorrhages, and anemia due to impaired iron absorption. In severe, untreated cases, scurvy can be fatal.

Although vitamin C is generally considered safe, excessive intake can cause side effects since high doses overwhelm intestinal absorption and renal excretion. Common symptoms of excessive intake include gastrointestinal upset such as diarrhea and nausea. Chronic overconsumption may also lead to kidney stone formation due to increased oxalate excretion.

EXPERIMENTAL PART

Vitamin C determination in fruit juices by iodimetry

To determine the vitamin C (ascorbic acid) content of various fruit juices or soft drinks, a titration method with iodine solution can be used. In this reaction, iodine (I_2) oxidizes ascorbic acid to dehydroascorbic acid, while itself being reduced to iodide ions (I^-). The overall reaction is:



Ascorbic acid
Reduced form

Dehydroascorbic acid
Oxidized form

Ascorbic acid is easily oxidized by atmospheric oxygen, especially in neutral or alkaline conditions. To prevent this, the titration is carried out in an acidic medium, achieved by adding acetic acid.

During the titration, starch is added as an indicator. Starch forms a characteristic blue complex with iodine. Since iodine is consumed first by ascorbic acid, the blue color appears only when all the ascorbic acid has been oxidized and excess iodine remains in the solution. Thus, the appearance of the blue color marks the endpoint of the titration.

The purpose of this experiment is to compare the vitamin C content of fresh orange juice with that of commercially available orange juice.

Reagents

1. Standard ascorbic acid solution, 1 mg/ml.
2. Fresh juice obtained from an orange.
3. Commercially available orange juice.
4. Iodine solution 0,005 M.
5. Acetic acid solution 6M.
6. Starch solution 1%.

Procedure

1. Determination of the volume of iodine solution required for the titration of a standard ascorbic acid solution

Add the following solutions in two Erlenmeyer flasks:

- 10 ml distilled water
- 1 ml acetic acid solution 6M
- 5 ml standard ascorbic acid solution
- 1 ml starch solution 1%.

Titrate slowly with the 0.005M iodine solution, stirring the solution in the Erlenmeyer flask, observing the appearance of a blue color. When the blue color persists for 30 seconds, the titration is stopped, and the final titration volume is written down.

2. Determination of the volume of iodine solution required for the titration of an ascorbic acid sample

a. Fresh juice obtained from an orange is considered the first sample - solution A of ascorbic acid (unknown concentration)

b. Commercially available orange juice is considered the second sample - solution B of ascorbic acid (unknown concentration)

Add the following solutions in two Erlenmeyer flasks:
- 10 ml distilled water
- 1 ml acetic acid solution 6M
- 5 ml ascorbic acid solution of unknown concentration (A, respectively B)
- 1 ml starch solution 1%.

The titration procedure is similar with the one for the standard ascorbic acid solution. The obtained volumes are noted: V_A and V_B.

Calculation

Vitamin C concentration from the two samples is calculated using the following formulas:

$$C_A(mg/ml) = \frac{V_A}{V_{std}} \times 100$$

$$C_B(mg/ml) = \frac{V_B}{V_{std}} \times 100$$

Questions

1. Which are the main water-soluble vitamins and lipo-soluble vitamins.

2. Name the “vitamins” that can be synthesized by the human organism.

3. Briefly, present the main roles of vitamin C.

4. Briefly, explain the determination of vitamin C from juice using titration.

5. What it the role of starch during the experiment that you have performed.

Lab Notes

Use this space for handwritten notes during the practical session.

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