GENERAL LABORATORY PRINCIPLES

The objectives of this introductory laboratory course are:

- To familiarize you with the basic principles of laboratory work and safety in the laboratory
- To understand hypothesis testing using scientific methods
- To analyze the data you have obtained and draw conclusions
- To present the experiment, your data and conclusions in a clear and scientific fashion
- Read the experiment before coming to the laboratory session. It is important that you have a firm grasp of this material as the time available for you to carry out the experiment is limited.

1. Laboratory Rules

- Everyone must wear a lab coat or lab apron while in the laboratory.
- Record your observations and results directly in your lab notebook immediately after you obtain them. Do not put them on odd pieces of paper.
- Keep your bench and apparatus neat and clean. At the end of the laboratory period, clean and put away all apparatus, and wipe the bench.
- Never wear sandals or other open-toed shoes in the lab. Footwear should always cover the foot completely.
- Never wear shorts or skirts in the lab.
- Long hair must be tied back or put under the lab coat.
- Do not chew gum, drink, or eat in the lab.
- Do not work alone in the lab.
- If an instrument or piece of equipment fails during use, or isn't operating properly, report the issue to the laboratory assistants. Never try to repair the problem on your own.
- Never leave an ongoing experiment unattended.
- Never smell or taste chemicals.
- Do not pipette by mouth.

➢ If you notice any unsafe conditions in the lab, let your supervisor know as soon as possible.

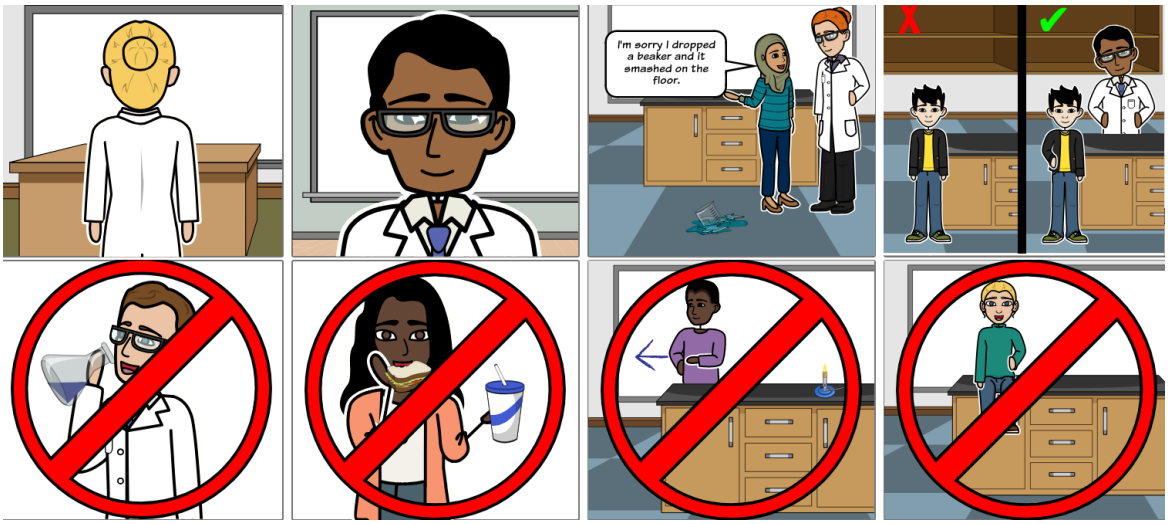

Figure 1: Laboratory safety rules.

➢ When working with equipment, hazardous materials, heat, and/or chemicals, always wear appropriate personal protective equipment (Figure 2).

➢ Before and after performing an experiment, you should always wash your hands with soap and water.

➢ When using lab equipment and chemicals, be sure to keep your hands away from your body, mouth, eyes, and face.

Figure 2: Personal protective equipment (PPE)

- Be sure to read all fire alarm and safety signs and follow the instructions in the event of an accident or emergency and know emergency phone numbers to use to call for help in case of an emergency.
- Make sure you know where your lab's safety equipment (first aid kit, fire extinguishers, eye wash stations, and safety showers) is located and how to properly use it.
- Chemicals or other materials should never be taken out of the laboratory.
- Chemicals should never be mixed or poured in sink drains.
- Flammable and volatile chemicals should only be used in a fume hood.
- All chemicals should always be clearly labeled with the name of the substance, its concentration, the date it was received, and the name of the person responsible for it.
- Always pour chemicals from large containers to smaller ones. Do not put unused chemicals back into their original container.
- Chemicals should never be mixed, measured, or heated in front of your face.
- Water should not be poured into concentrated acid. Instead, pour acid slowly into water while stirring constantly. In many cases, mixing acid with water is exothermic.
- Every chemical has a label and symbol containing information about risks, warnings, and precautions (Figure 3). Before handling a chemical these labels should carefully be read and blind spots must be asked to the laboratory instructor.

F (Flammable), F+ (Extremely Flammable)
Flammable and combustible (R10-R12). Liquids of Flash point below zero and boiling point maximum 35 degree. It should be kept away from fire, sparks and heat.

C (Corrosive)
Substances destroying living tissue or causes corrodes/corrosion of iron (R34, R35). They cause skin and eye damage. Special precautions should be taken to protect eyes and skin, vapor protective clothing should be worn and shouldn't be taken by inhalation. They should be kept away from metals.

Xi (Irritant), Xn (Sensitizing)
They cause skin and eye damage (R20–R22, R36-R38). Their vapor shouldn't be breathed. They shouldn't be contacted with the body. Special precautions should be taken to protect eyes and skin. Protective clothing should be worn. They harm the ozone layer.

N (Toxic to environment)
They harm to livings in water and nature. It shouldn't be spilled and released to nature.

H (Health effect)
It reflects serious longer term health hazards such as carcinogenicity and respiratory sensitization. (R40, R45-R47).Avoid body/skin contact and, ingestion and inhalation of these substances should be avoided.

G (Gas)
Contains gas under pressure. Evolving gas might be cold if heated, explosion might be occurred.

Figure 3: Some important symbols on chemicals.

2. Basic Tools is Used in Laboratory

There are many tools used in the biochemistry laboratory. It is not possible to introduce them all one by one. Instead, you will be introduced to basic tools that are common to most experiments in the Biochemistry laboratory classes. More specific instruments will be introduced during the experiments that involve their use.

The aim of this section for a biomedical engineer candidate is to recognize and know the necessary equipment for the basic biochemistry laboratory.

The materials you will encounter and use most frequently in laboratory studies are explained below under the headings of laboratory glasses, volumetric glassware and basic devices.

➢ Laboratory Glassware

Beaker: They are cylindrical glass materials used in many processes such as solution preparation, mixing, transferring, heating and crystallization. In addition, there is a grooved part in the mouth for the easy flow of liquid. It is made of high temperature resistant tempered glass. Tempered glass is about 5 times more durable than non-treated glass and when broken, it is divided into pieces in the size of a dice so that it reduces the risk of injury.

Figure 4: Beaker

Test Tube: These are cylindrical shaped, small diameter glass materials used to observe the interaction of substances with each other. There are various sizes. The most commonly used ones are the 15 x 1.5 cm size. They are generally used in qualitative analysis. Test tubes with narrow-pointed bottom test tubes are called centrifuge tubes and used in the centrifuge device to precipitate solutes in a mixture.

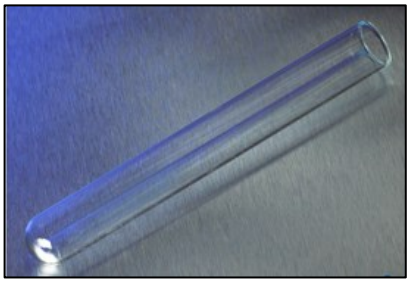

Figure 5: Test Tube

➢ **Volumetric Glassware**

Volumetric Flasks: · Volumetric flasks are often used for preparing accurate dilutions or for the preparation of standard solutions. There are 25, 50, 100, 250, 500 and 1000 m^3 volumetric flasks. They are used to prepare and store the solutions. There are also two- or three-mouth volumetric flasks that are required, especially when mixing, adding and gas passing are required at the same time. It can be round bottom, flat bottom or pear- shaped volumetric flasks that are used in different applications.

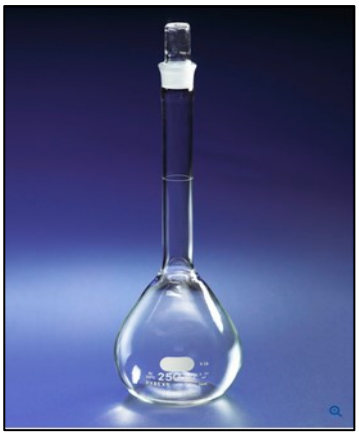

Figure 6: Volumetric Flask

Correct handling of volumetric flasks

- ✓ To prepare a standard solution, the exact amount of substance is weighed and added to the flask or an accurate amount of concentrated solution is poured into the flask.
- ✓ The flask should be then filled with distilled water (or the appropriate diluent) up to 50% capacity and shaken to ensure dissolution of the substance. The remaining volume of water/diluent should then be added to just below the ring mark.
- ✓ Using a wash bottle water/diluent should then be added carefully until the bottom of **the meniscus** is exactly at the ring mark (at eye level).
- ✓ The flask should then be closed and shaken to mix the contents.

Graduated Cylinder: It is a glass material used to measure the volume of pure liquids and solutions, with divisions in ml. They are used for measuring and transferring the approximate volumes of liquids because they are not very sensitive measuring tools. In general, it measures the volume of liquids between 50-1000 ml much better. It is used in the preparation of media and dyes in biochemistry laboratories.

Figure 7: Graduated Cylinder

Correct handling of graduated mixing cylinders

- ✓ Fill with the liquid until the bottom of the meniscus is exactly at the desired ring mark.
- ✓ The meniscus should be read at eye level.
- ✓ The inside cylinder wall should not be wetted above the mark.
- ✓ When finished, apply the stopper and mix by shaking the cylinder.

Pipettes: These are glass materials that are used to measure very sensitive and small amount of liquid volumes and transfer liquid materials from one container to another.

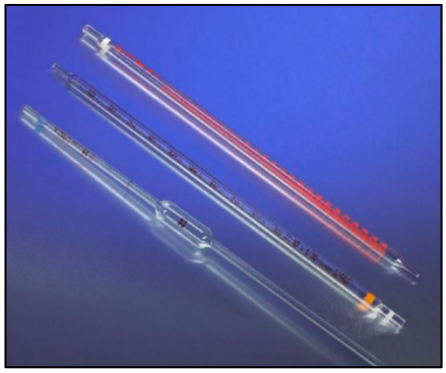

Figure 8: Pipettes

➢ **Basic Devices is Used in Laboratory**

Desiccator: These are glass materials that are generally used for holding or storing solid chemicals without moisture. Desiccators have moisturizing agents such as calcium chloride and silica gel. To ensure the continuity of the dry environment without moisture, the dehumidifying substance should be changed from time to time.

Figure 9: Desiccator

Fume Hood: Inhalation is a major route of entry of chemicals into the body. Chemical fumes and vapours can directly enter our bloodstream and small particles can lodge in the alveolar region of our lungs. A properly designed and operated fume-hood reduces exposure to hazardous fumes, vapours, gases and dusts. A fume-hood confines hazardous airborne material by diluting it with a large amount of air, drawing it through an exhaust system and then expelling the air in vents locate on the roof of building. Proper use of the fume-hood sash can also shield the worker from an uncontrolled reaction. Fume hoods are inspected and tested annually to assess performance standards but it is up to you to use a fume-hood safely.

Correct handling of fume hood

- ✓ Perform all work involving hazardous or volatile materials in a fume hood.
- ✓ Check that the fume-hood is operating correctly before you start work. To check the air flow a strip of paper, tissue, or ribbon can be taped to fume-hood sash.
- ✓ Avoid cross drafts and disruptive air currents in front of the fume hood. Ensure that windows and doors near the fume-hoods are closed.
- ✓ Always keep work at least 15 cm in from the opening of the fume hood.
- ✓ Use the sash as a safety shield when boiling materials or conducting an experiment with reactive chemicals.
- ✓ Always keep sash as low as possible. When the fume hood is not in use, ensure that all materials are in sealed containers.
- ✓ Connect all electrical devices outside of the hood to avoid sparks which may ignite a flammable or explosive chemical.
- ✓ Do not place your face or head inside the hood. Keep hands out as much as possible.
- ✓ Do not use a fume hood as a storage area, they should contain only working volumes of chemicals.
- ✓ Do not use fume hoods to vent or dispose of hazardous materials through air dilution.
- ✓ Do not place equipment in the hood that stops the sash from closing. A safer local exhaust ventilation method may exist and should be pursued.
- ✓ Do not modify fume hood or erect shelves in a fume hood for chemical or equipment storage.
- ✓ Do not place power boards, or other spark producing sources inside the hood.

Figure 10: Fume Hood

Precision Balance: It is one of the most important tools used in laboratories. Precision scales are scales that can weigh at least 1 mg.

Correct handling of precision balance

- ✓ Use incorrect weighing units can lead to accidents or experimental errors as a result of weighing errors. Check that the weighing units are correct before starting weighing.
- ✓ Avoid locations where the balance will be exposed to any air flow from an air conditioner, ventilator, door or window, extreme temperature changes, vibration, direct sunlight, dust, electromagnetic waves or a magnetic field.
- ✓ Do not use the balance outdoors or anywhere where it will be exposed to water.
- ✓ Treat the balance with care. Grasp it firmly with both hands to carry it.

Figure 11: Precision Balance

Hotplate/Magnetic Stirrer: It is a laboratory device that employs a wide circular table on which glass containers can be placed. It allows the solutions to be mixed by rotating magnetic field and heated at the same time. A magnetic fish is immersed in the solution which spins as a result of the rotating magnetic field. Spinning of magnetic fish creates a vortex in the solution, thus stirring it. The devices are available that both heat up and mix, or mix only (without a heater). In models with heating feature, temperature adjustment is carried out with an electronic thermostat.

Correct handling of hotplate/magnetic stirrer

- ✓ Always consider that the plate can be hot before touching it.
- ✓ Disconnect the magnetic stirrer from the main supply before cleaning.
- ✓ When the top plate is cool enough to touch, the outside of the hotplate stirrer should be cleaned regularly with a damp cloth or tissue paper.
- ✓ Do not use chemical cleaning agents or strong solvents. Only neutral agents or a mild soap liquid may be used for cleaning purpose.

Figure 12: Hotplate & Magnetic Stirrer

pH Meter: It measures the acidity or alkalinity of the solution. Fundamentally, a pH meter consists of a voltmeter attached to a pH-responsive electrode and a reference electrode. When they are immersed in a solution pH responsive electrode creates an electrical charge which is directly related to the pH of the solution. The voltmeter measures the potential difference between the pH responsive electrode and the reference electrode. This voltage difference is converted to a pH measurement and this measurement is displayed by the device.

Correct handling of pH meter

- ✓ Solutions used to clean electrodes must be handled with care. Cleaning solution should not involve corrosive substances.
- ✓ Do not allow the reference chamber of the electrode to dry out. Always keep it filled with the proper fill solution (KCL solution). Formation of KCl salt at the tip and side of the electrode is normal and should be rinsed off with warm water.
- ✓ Leave the tip of the electrode in an inch (25mm) of pH 7.0 or 4.0 buffer when it is not being used. Do not leave it in distilled water. For long term storage follows the electrode manufacturer's instructions.
- ✓ It should be known that each pH meter has a different calibration method. Check whether the pH meter has been calibrated. If it is not calibrated, it should be calibrated following the usage information given for that pH meter.
- ✓ The probe should be kept upright during measurement. Measured liquid should not be mixed with the probe, the probe must be fixed. A magnetic stirrer should be used for mixing. Care should be taken that the magnetic fish does not hit the probe.
- ✓ When the measurement cannot be made, it should be ensured that the probe is in the protection solution. Protection solution should not be poured; water should not be added on it. In order to keep the solution clean, the probe should be immersed in the solution after it is washed and dried.

Figure 13: pH Meter

Centrifuge: It applies a centrifugal force to separate mixtures based on the densities of the components. During the high speed rotation of the centrifuge, the heavier part of mixture in

the tube is collected at the bottom of the tube due to centrifugal force and separated from the part with low density.

Correct handling of centrifuge

- ✓ Broken and cracked tubes should not be used as containers to centrifuge the mixtures.
- ✓ Centrifuge tubes should be placed in the chamber and should be of equal weight.
- ✓ The weight on both sides of the centrifuge should be in **balance**.
- ✓ The tube should be filled up to 2 cm below the mouth.
- ✓ The fluid level in the tubes should be mutually equal.
- ✓ While the device is operating, its cover should not be opened and it should not be held by hand to stop, and it should be expected to stop by itself.
- ✓ When centrifuging, the tubes should be closed immediately when broken, and rinsed after cleaning.
- ✓ The instructions for use should be followed and cleaned as specified in the instructions for use.

Figure 14: Centrifuge

Oven: These are electrical devices that can be adjusted to maintain a certain temperature. Usually they are used in heating and drying processes. Microorganisms can be incubated in the ovens when they are set to the temperatures ideal for growing of the microorganisms. Oven is also used in sterilization process.

Figure 15: Oven

Water Bath: It is a water-filled container that allows chemicals or reactions in glassware to be kept at a certain temperature that can be adjusted. It is used to warm up solutions in a uniform manner.

Figure 16: Water Bath

Water Distiller: All analyzes carried out in laboratories should be done with purified water. Because there can be various organic and inorganic substances in tap water. This makes the analysis results to be error prone. For this reason, water distiller is used in laboratories. The working principle of the device is as follows: Tap water enters the water distiller device with a hose and water is boiled with the resistors inside. The heated water evaporates. As the water vapor passes through the pipes, it cools and condenses. Condensed water is discharged through the hose at the end of the tool. Since inorganic ions do not condense, the resulting water does not contain those ions. The water obtained, thus, is distilled from many substances in it and released.

Correct handling of water distiller

- ✓ Check the safety system and warning leds before any usage such as high water pressure, low water pressure, heater failure, half full storage tank, and full storage tank.
- ✓ The water level in the boiling tank can be controlled electronically. If there is not enough water in the boiler, the system will not work.
- ✓ In any water pressure decrease, when the water is cut off, the device will not work and the warning led will light up.

Figure 17: Water Distiller

3. Laboratory Cleaning

➢ Cleaning of Laboratory Glassware

Containers used in all chemical analyzes must be clean. If the containers are dirty, it results in wrong analysis results. For this, all kinds of glassware should be washed thoroughly, rinsed with distilled water and used after drying.

The cleaning stages of laboratory glassware are as follows:

- ✓ Cleaning of the equipment used in the laboratory should also be checked before each use. If the equipment is not clean, it should be cleaned in accordance with its instructions.
- ✓ Glassware to be cleaned is washed thoroughly (if necessary) with the help of tap water.
- ✓ Afterwards, glassware is cleaned thoroughly by using one of the suitable cleaning solutions suitable for dirt.

- ✓ Soapy water is used primarily for the cleaning of water-soluble chemicals. Chromic acid solution, basic permanganate solution or aqua regia is used to remove inorganic dirt that soapy water cannot remove.
- ✓ Dirt that chromic acid solution cannot remove should be removed using organic solvents.
- ✓ Rinse the glassware with tap water first until no soap or other cleaning solution is left. Then rinse well with distilled water. Drying of glass containers after cleaning is done either by leaving them on filter papers on the bench or in an oven.

➢ **Cleaning of Other Labware**

The same cleaning process is applied for the cleaning of materials other than glasswares. However, it is important to note that washing solutions do not cause corrosion and deterioration in the material. Plastic and metal containers used in the laboratory are cleaned only with detergent or soap solution. Porcelain materials used in laboratories should be cleaned just before use.

4. Laboratory Report Format

➢ The experiment reports should include the following sections:
(Font: Times New Roman – Font size: 12)

Title page

- Author name, student ID, experiment date and section, and experiment name.

1. Purpose of the Experiment (5 pt)

- Must answer what is the purpose of the experiment and why the experiment was carried out.

2. Theory (10 pt)

- Provides a brief summary of the background and theory pertaining to the experiment done. (This part should not exceed one page)

3. Materials (5 pt)

- Materials part must include information of used consumables, devices, chemical materials.

4. Methods (20 pt)

- Methods part is a step by step description of procedure in your own words.

5. Results (20 pt)

- State the direct outcome of the experiment or procedure.

Give all the data obtained.

- Show all the calculations (if there is any!), writing out the equations, and defining each variable.
- Each calculation result should contain unit.
- Figures and tables can be included to present the data generated by your experiment.
- Tables and figures must be numbered and titled.
- Table titles appear at the top, figure titles at the bottom.

6. Discussion (35 pt)

- Restate the purpose and findings of the experiment.
- This section is the explanation of the results section.
- Include explanations of unpredicted or inconsistent results and explain.
- Compare results with existing knowledge.
- Explain why you think the results mean.
- Discussion should give the audience a general conclusion about the results.
- (If there are), answer the questions - by searching through the sources and adding your own comments.

7. References (5 pt)

- List the sources you used to compose the lab report.
- As laboratory research relies on previous work, written reports must include references. All information or interpretations given in the introduction and discussion part should be supported by references. Inadequate or inappropriate referencing should be avoided. Include references to any journal articles, books, laboratory techniques manuals, etc. used to complete your lab reports both as parenthetical references (in the correct location of the text) AND in the bibliography.
- List references in APA format.

	YILDIZ TECHNICAL UNIVERSITY **BIOMEDICAL ENGINEERING DEPARTMENT** **BME2901- BIOCHEMISTRY COURSE** **2022-2023 FALL SEMESTER**

EXPERIMENT 1

BASIC BIOCHEMICAL TECHNIQUES AND MICROSCOPIAL TECHNIQUES

1. **PURPOSE OF THE EXPERIMENT:** To learn solution preparation methods, concentration units, to understand concepts of pH, to learn how buffer solutions are prepared and to understand microscopical techniques.

2. THEORETICAL KNOWLEDGE

2.1.SOLUTION & UNIT

Homogeneous mixtures of two or more substances are called "**Solution**".The disintegration medium in solutions is referred to as the solvent, and the disintegrating agent is called the solute. The solubility of a substance is determined by the maximum amount of dissolved in a solvent at a given temperature and pressure. Solubility of a solid or liquid material is expressed in grams of the material dissolved in 100 g of solvent. If the amount of dissolved substance is small, the solution is called dilute solution, and too, it is called concentrated solution.
Solutions containing less substances than the solvent can dissolve are called unsaturated solutions. If the substance is dissolved to the limit of solubility, such solutions are called saturated solutions.

The most commonly used solutions in chemistry according to solvent and soluble are:

Liquid in liquid: Water-alcohol solution
Solid in liquid: Salt water solution
Gas in liquid: Aqueous ammonia solution

The amount of dissolved substance in a given amount of solvent for any solution is called the concentration and is indicated by "C".

$$c = \frac{m_{solute}}{V_{solution}}$$

Here;
C = Concentration of solution
m_{solute}= Amount of solute

$V_{solution}$ = Amount of solute and amount of solvent.
The types of concentration are grouped as volume, mass and moles.

Volume Based Concentrations:
- Molarity (M),
- Normality (N),
- Mass/Volume Percent

Mass Based Concentrations:
- Mass percent,
- Molality (M),
- Millions (ppm),
- Billion (ppb)

Mole Based Concentrations:
- Mole percent
- Mole fraction (mostly used for physicochemical quantities)

Molar Solutions: Solutions containing 1 mole of substance per liter are called "**Molar solutions**". Molarity is the number of moles of material dissolved in 1 liter (1000 cm^3= 1000 ml) of solution. Molarity is defines as

$$M = \frac{The\ mole\ number\ of\ solute}{Volume\ of\ the\ solution} = \frac{n}{V(L)}$$

Molarity M; the number of moles of the solute n and the volume of the solution V.

For example; 1 M (or 1 molar) sodium chloride solution means 1 mole, ie 58.44 g NaCl, per liter of solution. The concentration of said NaCl solution is expressed in any of the terms 1 M, mol / L or 1 molar.

Molar (m) Solutions: In 1000 grams of solvent, it is called the number of moles of dissolved substance and is indicated by m. The most important difference from molarity is that the amount of solvent and solute is known but the solution volume is not known. For example, 3 molal NaOH solution is a solution prepared by dissolving 3 moles (3x40 = 120 g) of NaOH in 1000 grams of water.

$$m = \frac{Amount\ of\ solute\ (mol)}{Amount\ of\ solvent\ (kg)}$$

Example: 12 g of NaOH was dissolved in 120 g of water (120 ml of water). What is the molality of this solution?

12 g NaOH = (12/40) = 0,3 mol NaOH

$$m = \frac{Amount\ of\ solute(mol)}{Amount\ of\ solvent(kg)}$$

120 ml water = 120 g water = 0,120 kg water

$$m = \frac{0,3}{0,120} = 2,5molal$$

Normality (N): It is referred to as the number of equivalents of the solute in 1 liter of solution and is indicated by N. It is an important part of calculating the equivalent mass in the preparation of such solutions.
Equivalent weight is calculated by dividing the molecular mass by the valence.

$$GramEquivalent = \frac{(Solute)\ Mass\ of\ substance(m)}{Equivalent\ weight}$$

$$Equivalent\ Weight = \frac{Molecular\ mass(M_A)}{Valence}$$

Valence : The number of H^+ ions that the acids give to the environment and the number of OH^- ions that the bases give to the environment are called valence.

For example, for acids containing single H^+ ions such as HCl, HNO_3, CH_3COOH and single OH- ions such as NaOH, KOH, the equivalent weight is equal to the formula weight (effect value 1). Since H_2SO_4 contains two H^+ ions, the equivalent weight is equal to half of the formula weight (effect value 2).

In salt, the effect value is equal to the number of electrons given to or taken from the medium. For example, in salts such as NaCl, $AgNO_3$, the equivalent weight is equal to the formula weight (effect value 1). In the case of salts such as $BaCl_2$, $MgSO_4$, the equivalent weight is equal to half of the formula weight (effect value 2).

Example : 100 ml of concentrated H_2SO_4 (98%, d = 1.85) were diluted to 500 ml. What is the normality of this solution?

First, calculate how many grams of pure H_2SO_4 are present in 100 ml of concentrated sulfuric acid and find 108.32 g.

$$d = \frac{M}{V} \rightarrow M = d.V$$

M = 1,84 x 100= 184 g

$$\frac{100}{98} = \frac{184}{X} \rightarrow X = 180{,}32\ g\ pure\ H_2SO_4$$

Sulfuric acid gives water 2 hydrogen. Therefore, the effective value is 2. Its molecular mass is 98. The equivalent mass of sulfuric acid is 98/2 = 49 g. In this case, the number of equivalents of dissolved substances (180.32 / 49) = 3.68, the normality (3.68 / 0.5) = 7.36 N would be.

Percent Concentration: It is called the amount of substance dissolved in 100 units of solution and is indicated by the % sign. The percent can be expressed in three ways, mass percent, volume percent and mass/volume percent.

Mass Percent: 100 mass units (g, kg, mg, ton, etc.) indicate how many mass units are dissolved in the solution. With the following equation,

$$Mass\ Percent\left(\%\frac{m}{m}\right) = \frac{Mass\ of\ Solute}{Mass\ of\ Solution} x100$$

mass percent describes.

For example, 20% by mass sodium chloride solution means that there is 20 g of solid sodium chloride in 100 grams of sodium chloride solution or 20 kg of solid NaCl in 100 kg of NaCl solution.

Volume Percent: 100 Volume units (mL, L, m^3, etc.) indicate how many volume units are dissolved in the solution. With the following equation,

$$Volume\ Percent\left(\%\frac{v}{v}\right) = \frac{Volume\ of\ Solute}{Volume\ of\ Solution}x100$$

volume percent describes.

For example, 40% by volume alcohol solution means that there is 40 ml of pure alcohol in 100 ml of alcohol solution or 40 L of pure alcohol in 100 L of alcohol solution.

Mass/Volume Percent: 100 Volume units indicate how many weight units are dissolved in the solution. With the following equation,

$$\frac{Mass}{Volume}Percent\left(\%\frac{m}{v}\right) = \frac{Mass\ of\ Solute}{Volume\ of\ Solution}x100$$

mass/volume percent describes. This concentration is used for solutions of solids in water.

For example, 20% by volume-mass sodium chloride solution means that there is 20 g NaCl in 100 ml NaCl solution or 20 kg NaCl in 100 liters NaCl solution. Here the amount of the solution should be expressed in volume units and the amount of solute should be expressed in mass units.

Mole Fraction: The ratio of the number of moles of a component in the solution to the total number of moles is defined as the molar fraction of that component and is denoted by X.

For example, in a solution of components A, B, C ...

$$Mole\ fraction\ for\ A\ component,\ X_A = \frac{n_A}{n_A+n_B+n_C...}$$

$$Mole\ fraction\ for\ B\ component,\ X_B = \frac{n_B}{n_A+n_B+n_C...}$$

The sum of molar fractions of the components in the solution is one and can be expressed as $X_A + X_B + X_C + ... = 1$.

PPM and PPB Solutions: Sometimes in very sensitive analyzes the concentrations are so small that “ppm” or ppb ”are used as units.

Million (ppm): It is a concentration unit in terms of parts per million (ppm, abbreviated form of English part per million)

$$ppm = \frac{mg\ amount\ of\ solute}{kg\ amount\ of\ solution}$$

For example, when 2 ppm Hg^{+2} (mercury) solution is mentioned; It is understood that 1 kg of water contains 2 mg of mercury.

$$\frac{2mg}{1kg} = \frac{2mg}{10^6mg} = 2ppm$$

In very dilute solutions; the volume of the 1 kg solution is one liter (since the density of the water is 1 g / ml = 1 kg / liter). Accordingly, this unit in solutions,

$$ppm = \frac{amount\ of\ solute}{amount\ of\ solution}$$

describes in formula.

For example, 20 ppm Fe means 20 mg Fe^{+2} in 1 liter of solution.

Billion (ppb): Another concentration unit is used for very small concentrations. **ppb,** which means parts per billion (abbreviated parts per billion in English); liters is the amount of solvent expressed in micrograms.

$$ppb = \frac{mg\ amount\ of\ solute}{to\ namountofsolution} \qquad \text{or} \qquad ppb = \frac{ml\ amount\ of\ solute}{m^3\ amount\ of\ solution}$$

Dilution of Solutions: Solutions are generally prepared from **stock** solutions of known concentration. For this purpose, the solution taken from the stock solution is taken into the flask selected according to the desired volume and the solvent is added to the line indicating the volume. In this way a more dilute solution is prepared than the initial concentration.

The dilution calculations are based on the fact that the number of moles of solute taken from the stock solution is the same as the number of moles of the solute in the dilute solution and is expressed as (stock concentration) x (stock volume) = (desired concentration) x (desired volume). It should be careful that the concentration and volume units are the same on both sides of the equation.

Since most concentrations are expressed in molarity and normality,

In short, it can be written as $\mathbf{M_1V_1 = M_2V_2}$ **or** $\mathbf{N_1V_1 = N_2V_2}$.

$\mathbf{N_1}$, $\mathbf{M_1}$, $\mathbf{V_1}$ are the **initial** values of normality, molarity and volume, and $\mathbf{N_2}$, $\mathbf{M_2}$, $\mathbf{V_2}$ are the normality, molarity and **final** values of volume.

2.2. pH AND BUFFER SOLUTION

pH was defined as negative logarithm of molar concentration of hydrogen ion for the first time by Sorensen at 1909.

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

or

$$[H = 10^{-pH}]$$

For pure water at 25^o C, value of pH is 7.

pH= -log $[H^+]$= -log(10^{-7}) = - (-7)=7

As the pH value decreases, $[H^+]$ increases, and as the pH increases, $[H^+]$ decreases. Acids are proton donors, bases are proton acceptors. Strong acids (HCl, H_2SO_4) are completely ionized in the solution. Weak acids are partially ionized in their solutions. The same situation is valid for the bases. Most body fluids are weak acids.

pH Meter: The strength of acids or bases can be defined by pH values. The scale that shows the acidity or alkalinity (basicity) of substances is called a pH meter. The pH meter ranges from 0 to 14.

pH is often depicted on a graphical colour scale as shown below:

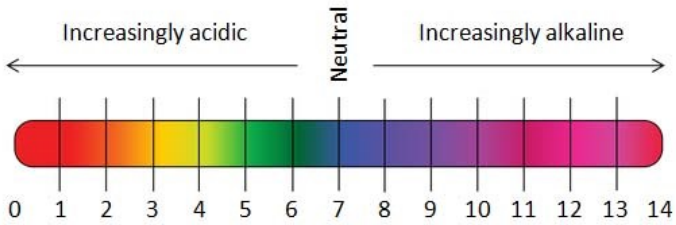

Figure 1. Graphical colour scale of pH

Accordingly, the table below shows the pH values of some substances:

Table 1 - pH values of some substances

SUBSTANCE	pH
1M HNO_3	0
Gastric juice	1.0-3.0
Lemon juice	2.4
Vinegar	3.0
Orange juice	3.5
Tomato	4.0- 4.4
Urine	5.0-7.0
Saliva	7.0-7.5
Pure water not in contact with air	7
Blood	7.35 – 7.45
Tear	7.4
Sea Water	8.5- 10.0
Ammonia used for household cleaning	11.5
1 M NaOH solution	14

Strength of Acids and Bases: In the nineteenth century, Arrhenius was the first to describe acids as substances that produce hydrogen ions (H^+) when they dissolve in water. On the other hand, bases are ionic compounds that dissociate into a metal ion and hydroxide ions (OH^-) when they are dissolved in water.

Acids are typically classified according to their ability to donate protons and the strength of the acids depends on the percentage of ionization. As the percentage of ionization increases, the strength of acidity increases. The percentage of ionization of an acid is related to the binding strength of the acidic hydrogen to the molecule. The weaker the bonding strength of the acid hydrogen is, the more easily it can be separated (ionizable) from the molecule. When dissolved in

water, acids that are 100% or nearly 100% ionizable are called strong acids. Acids that can ionize to a small extent when dissolved in water are called weak acids. Since HCl, a strong acid, is completely ionized in its solution, the H^+ molar concentration is equal to the HCl molar concentration.

Bases are classified in terms of their ability to accept protons. Strong bases have a strong attraction for protons, whereas weak bases have less attraction for protons. For example, the Arrhenius bases such as LiOH, KOH, NaOH, and $Ca(OH)_2$ are strong bases that dissociate completely (100%). In addition, the KOH molar concentration is equal to the OH^- molar concentration.

Indicators are substances that used to understand whether the medium is neutral, acidic or basic. Indicators can change color depending on the acidity or alkalinity of the medium. Also, they give different colors to the solution at different pH values. For example; if phenol red is added to a solution of pH range between 6.6 to 8.2, the solution color turns to orange. If the pH of the solution is less than 6.6, the solution turns to yellow.

The following table lists some of the acid base markers and the effective pH ranges.

Table 2 – Indicator list with the effective pH ranges

	Color		
Indicator	**Acid**	**Base**	**pH Ranges**
Thymol Blue	Red	Yellow	1.2 - 2.8
Methyl Orange	Orange	Yellow	3.1 - 4.4
Methyl Red	Red	Yellow	4.2 - 6.3
Bromothymol Blue	Yellow	Blue	6.9 - 7.6
Cresol Red	Yellow	Red	7.2 - 8.8
Phenolphthalein	Colorless	Pink Red	8.3 - 10.0

Neutralization: It is a reaction between an acid and a base to produce a salt and water.

In the neutralization reaction, H^+ from the acid reacts with OH^- from the base to form water, leaving the spectator ions from the salt in the solution.

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

Acidity Alkalinity Neutrality

As we understand from the above equation, 1 mole of H^+ ion is completely combined with 1 mole of OH^- ion to form 1 mole of water. In this case, numbers of H^+ ion and OH^- ion coming from the mixed solutions specify the acidity or alkalinity of the medium.

- When water has an equal concentration of H^+ ions and OH^- ions, it is said to be neutral (pH=7)

  $nH^+ = nOH^- \Rightarrow$ Solution is neutral. $pH = pOH = 7$

- When water has a greater concentration of H^+ ions, it is said to be acidic (pH<7)

  $nH^+ > nOH^- \Rightarrow$ Solution is acidic. $pH < 7 < pOH$

- When a solution has a greater concentration of OH^-, it is said to be alkaline (pH>7)

  $nH^+ < nOH^- \Rightarrow$ Solution is basic. $pH > 7 > pOH$

Buffer Solutions: Buffer solutions are those solutions pH of which does not change with a certain dilution or with the addition of a small amount of strong acid or base. Therefore, they are vital in

biochemical reactions. Even a slight change in pH of organisms is life-threatening. Thus, all organisms are naturally buffered to provide an appropriate metabolism.

Buffer solutions are those solution which contain a weak acid and its conjugate base or a weak base and its conjugate acid.

$$CH_3COOH_{(aq)} \leftrightarrow H^+_{(aq)} + CH_3COO^-$$

Weak Acid Conjugated Base

$$NH_{3_{(aq)}} \leftrightarrow NH^+_{4_{(aq)}} + OH^-_{(aq)}$$

Weak Base Conjugated Acid

$$pH = pKa + Log\frac{[A^-]}{[HA]} \qquad (\text{Handerson} - \text{Hasselbach Equation})$$

Buffer Capacity: Buffer capacity is defined as the concentration of H^+ or OH^- ions which the buffer solution can neutralize without much change in its pH. The buffer capacity is indicated by β. The capacity of a buffer does not only depend on the total concentration of the conjugated acid-base pair but also the ration between their concentrations. As the concentration ratio increases to the values greater than or less than 1, the decrease in buffer capacity increases. A good buffer;

- should not be toxic,
- should not give no absorption in the UV region,
- should be biologically and chemically inactive,
- pKa value should change to a minimum level with temperature and ion strength.

The pH range at which the prepared buffer solution can resist pH change by buffering against the added acid or base is calculated as follows:

pH capacity = pKa ± 1

2.3.MICROSCOPY

Light microscopy uses visible or ultraviolet light to illuminate an object. The light passes through several glass lenses that alter the path of the light and produce a magnified image of the object.

Basic Parts of a Compound Light Microscope:

- **Eyepiece (Ocular):** Usually contains a 10X lens.
- **Arm:** contains the housing for the fine and coarse adjustments and connects the base of the microscope to the nosepiece and ocular.

- **Nosepiece:** A rotating head that has the objective lenses attached to it. The lens to be used should "click" into position when the wheel is gently turned so that it is directly over the speciman slide.
- **Objective:** Basically housing for a lens. Microscopes have three objective lenses - 4X, 10X, and 40X or 100X.

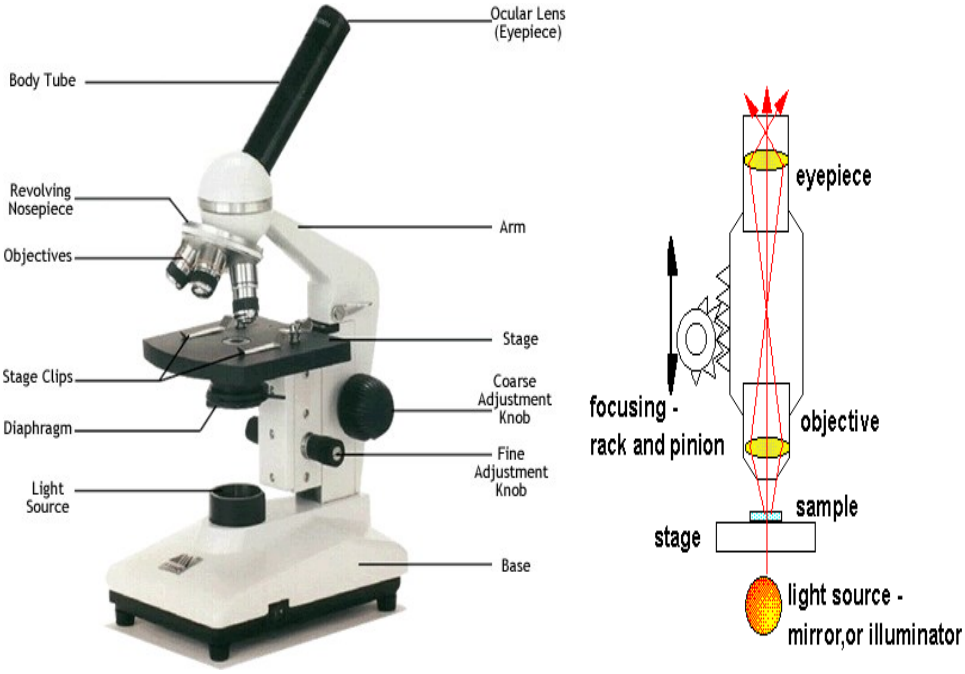

Figure 2. Basic parts of a compound light microscope.

- **Stage:** The speciman slides rests on this part of the microscope.
- **Coarse adjustment knobs:** The larger of two sets of knobs located on either side of the arm, just above the base. This adjustment is used to make large adjustments in focusing by moving the lenses up and down. *Never use this adjustment when using the 40X objective.*
- **Fine adjustment knobs:** The smaller of two sets of knobs located on either side of the arm. This adjustment is used to make small adjustments in focusing. It has a limited amount of movement and is most efficiently used after focusing with the 4X objective and coarse focus, then increasing magnification and making final adjustments with the fine focus knob.
- **Light source:** Located directly under the stage.
- **Adjustable diaphragm:** This rotating wheel on the underside of the stage allows the user to adjust the amount of light that passes through the specimen. As a general rule, the lowest intensity of light that allows you to resolve the structure of the object you are viewing should be used.

Magnification: The increase of an object's apparent size.

Total magnification is the product of the magnifying powers of the individual lenses. The magnifying capability of a microscope is the product of the individual magnifying powers of the two lenses;

1- Ocular lens (eyepiece): The lens nearest the eye to magnify object 10 times (10X)

2- Objective lens: The lens nearest the specimen to magnify object 4, 10, 40, and 100 times (4X, 10X, 40X, 100X)

Total magnification = ocular x objective

If the ocular lens enlarges by a factor of 10 (10X) and the objective lens enlarges by a factor of 40 (40X), the total magnification is the product of the two - 400X.

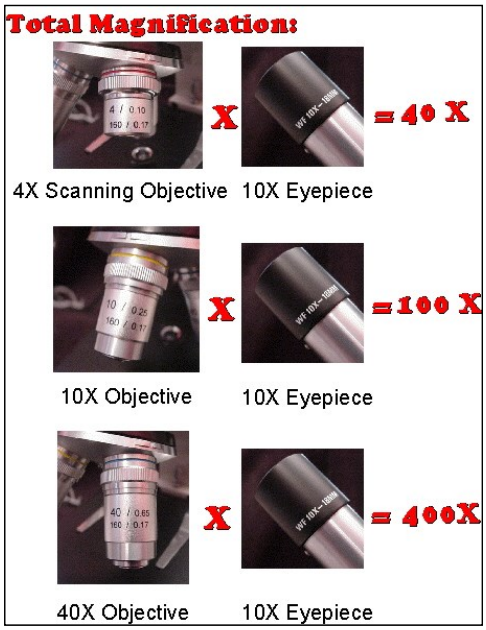

Figure 3. Examples of total magnification.

- **Field of view:** the area visible through the microscope lenses. Field of view decreases as magnification increases.
- **Resolution:** is the degree to which the detail in the specimen is retained in the magnified image. The ability to see in detail is essential lest everything appears as an unresolved blur. Magnifying object by using microscope is useful only if detail can be accurately preserved and observed.

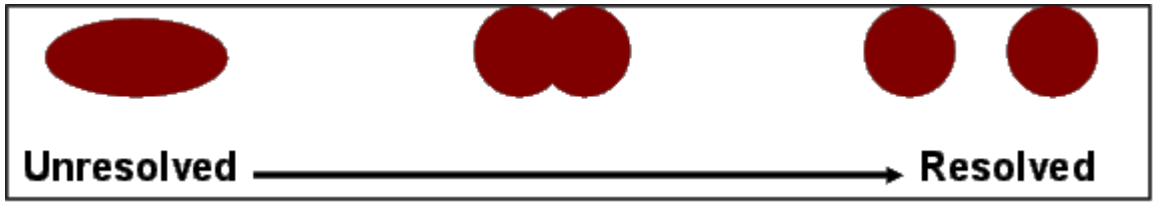

Figure 4. Resolution of objects.

Brightfield Microscope: The most common type of microscope in biology is the brightfield microscope, a light microscope in which visible light is transmitted through the specimen. This microscope usually requires staining of specimens and rarely is used to observe live microorganisms. It has a light source, a condenser lens that focuses the light on the specimen, and two sets of lenses that contribute to the magnification of the image. The specimen generally appears dark on a bright background.

3. MATERIALS AND METHODS:

3.1.Materials to be used in the experiment: Precision balance, spatula, distilled water, graduated cylinder, 250 ml beaker, magnetic stirrer, and NaCl.

3.2.Experimental Method:

Preparation of NaCl solution:

1. Determine the amount of solute using the appropriate equations.
2. Weigh the calculated amount of NaCl on a precision balance.
3. Transfer the NaCl to the erlenmeyer flask.
4. Add 50 ml of water.
5. Mix until the magnetic stirrer becomes homogeneous.
6. Transfer your solution to a clean and dry solution bottle.

Microscopial Techniques

1. Setting up your microscope

1.1. Adjusting the light source

For microscopes with a built-in illuminator:

- Plug in your microscope and turn on the light.
- Set illumination knob to 5 or 6.

1.2. Focusing for the Both Eyes

- With the 10X objective in place, adjust the distance between the eye pieces so that only one clear circular luminous field is observed when looking into the microscope with both eyes (10X objective is easiest to work with).
- Place a specimen (mounted on a slide) on the stage within the illuminated area.
- Using the coarse stage adjustment knob (coarse focus knob), raise the stage up as far as possible without touching the objective. Watch the objective while doing this.
- Looking in one eyepiece with your dominant eye (your stronger eye), lower the stage slowly using the coarse adjustment knob until you see your specimen in focus. You can gently move the stage around to help you find your sample.

- Using the fine adjustment knob, focus the image for your dominant eye.
- Without touching the stage adjustment knobs, now focus for your other eye by turning the eyepiece. Check that you get a good focused binocular image.

1.3. Focusing the Condenser Lens

- Centering and focusing of the condenser lens has been done for you before the lab period. If modified by error, please call the instructor for assistance. You can, however adjust the iris diaphragm of the condenser lens to optimize contrast. Notice that you will need to open it more when using higher power objectives.

2. Specimen Observation

Observe specimens following the procedure below:

- Begin by using the low power objective (10X)
- Select a slide to be examined, locate a field containing the sample and observe.
- Record your observation (general appearance and organization of the specimen) Make a drawing of what you see at a scale that permits you to record any observable detail. Record the total magnification.
- Before moving to a higher power objective, center on the area that you want to observe in more detail, because the field of observation decreases when the magnification of the objective increases.
- Turn the revolving nosepieces without touching the objective until the 40X objective is in place. Check that it does not touch the slide while doing this.
- Using the fine focusing knob, correct the focus. It might be necessary to open the iris diaphragm and gently increase the light intensity.
- Again record your observation (e.g. arrangement of cells in a given area of your sample, some major features of intracellular structure) and draw what you observe. Record all relevant details.
- Center on the part of the specimen that you want to observe in even greater detail.

3. Preparation of Specimens The Letter "e"

- Using scissors cut out a letter "e" from a piece of newspaper or a printed page.
- Place the letter (e up) on a clean glass slide.
- Place a drop of water on the letter e.
- Gently lower a clean coverslip over the drop at an angle of 45 degrees. Try not to rap any air bubbles.

- To orient the specimen, place your slide with the letter “e” right side up on the stage with low power objective in the light path. Center the letter in your field of view and carefully bring it into focus.
- Draw the “e” as you see it through the ocular lens. Record the total magnification used. It may be preferable to use 40X objective.
- Move the specimen to the right while watching through the microscope. In which direction does the image move?
- Move the specimen away from you. Now in which direction does the image move? What can you conclude about the orientation of the image viewed through the microscope?

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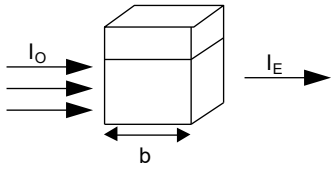
EXPERIMENT 2

SPECTROPHOTOMETRICAL ANALYSIS

1. THEORETICAL KNOWLEDGE

Spectrophotometry is the measurement of the interaction of light with matter. The most common application of this technique in biochemistry is in the measurement of the concentration of a compound in solution.

Compounds that absorb visible light appear colored. Many colorless compounds also absorb light in the region of the spectrum that is not visible to the naked eye. Light absorption of molecules is due to the nature of their chemical bonds. While molecules absorb some of the light that passes through a specimen, some of the light is transmitted. Spectrophotometer is the device that effectively "counts" the number of photons that enters a sample and compares it with the number of photons that exits a sample. Thus, it can detect the amount of light absorbed by molecules in a solution.



I_O = Intensity of incident light
I_E= Intensity of exiting light
b = path length of sample

$$T= I_0/I_E$$

Where:

T: Transmittance

$$A= - \log_{10} T$$

The quantitative relation for absorption as a function of concentration of an absorbing species was formulated by Beer and Lambert and known as Beer-Lambert Law. This law states that "the proportion of light absorbed by a medium is independent of the intensity of incident light" and "the absorbance of light is directly proportional to the concentration of the absorbing medium and the thickness or path length of the medium". It is formulated as the following equation:

$$A = \varepsilon \times b \times c$$

where:

A: absorbance

Ɛ : molar absorptivity (molar extinction coefficient)

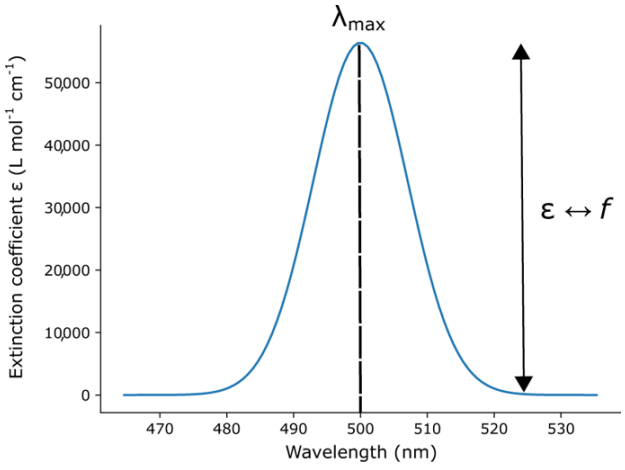
b: length of the path

c: concentration

It follows from this equation that absorbance is directly proportional to concentration. Thus, when Ɛ and b are known, the concentration of the absorbing species in a solution can be determined by measuring its absorbance.

However, the wavelength of light at which maximum absorption takes place (λ_{max}) differs for various molecules and should be used to detect the concentration of a specific type of molecule in a solution. For example, while DNA absorbs highest energy at 260 nm, proteins absorb at 280 nm, both in the range of ultraviolet light. Pigments and dyes, on the other hand, absorb visible light.

The spectrophotometer is also able to take white light and separate it into its constituent colors (i.e. somewhat like a prism), allowing the user to examine the absorption spectrum of the molecule and to find λ_{max}. Absorption spectrum of a molecule looks like below:



According to Beer-Lambert Law, the absorbance versus concentration curve should yield a straight line, the slope of which is E x b. The procedure used to achieve such a plot is called the Construction of a Calibration Curve. A calibration curve is constructed using known concentrations of the sample from which an unknown concentration can be derived using its absorbance value.

In this laboratory class, the absorption spectrum of a $KMnO_4$ solution will be found by a wavelength scan from 200 to 700 nm by 20 nm intervals. This will be done by taking absorbance values of the solution throughout the wavelength region between 200 and 700 nm. This absorption spectrum will give an estimate of the region where the λ_{max} will be. Wavelength scan will then be repeated around the maximum wavelength with 2 nm intervals. After this, λ_{max} will be found with a high precision. A calibration curve for $KMnO_4$ will be then constructed by measuring the absorbances of a series of $KMnO_4$ solutions of different concentrations at the predetermined λ_{max}. Finally, the concentration of $KMnO_4$ in an unknown solution will be calculated using this calibration curve.

2. MATERIALS

1) $4x10^{-4}$ M $KMnO_4$ Solution.

Take 0.0316 g $KMnO_4$ into a 500 mL volumetric flask, dissolve in water and dilute to the mark.

2) 0.5 N H_2SO_4.

Add 7 mL of concentrated H_2SO_4 (sp. gr. = 1.84 g/mL, purity= 96 %) on 493 mL of water slowly, while stirring continuously.

3. METHOD

3.1. Absorption Spectrum of $KMnO_4$

Take 5 mL of the $4x10^{-5}$ M $KMnO_4$ solution into a test tube, add 2 mL of 0.5 N H_2SO_4, and mix. Rinse and fill one of the spectrophotometer cuvettes with this diluted $KMnO_4$ solution and another with 0.5 N H_2SO_4. Measure the absorbance of $KMnO_4$ against H_2SO_4 at 20 nm intervals throughout the wavelength region between 400 and 660 nm. Take additional readings at 2 nm intervals around the peak value. Plot the absorption curve as absorbance versus wavelength.

3.2. Calibration Curve for $KMnO_4$

Prepare a series of $KMnO_4$ solutions with different concentrations as follows:

i) 2 mL $4x10^{-5}$ M $KMnO_4$ + 2 mL 0.5 N H_2SO_4 + 6 ml dH_2O

ii) 3 mL $4x10^{-5}$ M $KMnO_4$ + 2 mL 0.5 N H_2SO_4 + 5 ml dH_2O

iii) 4 mL $4x10^{-5}$ M $KMnO_4$ + 2 mL 0.5 N H_2SO_4 + 4 ml dH_2O

iv) 5 mL $4x10^{-5}$ M $KMnO_4$ + 2 mL 0.5 N H_2SO_4 + 3 ml dH_2O

v) 6 mL $4x10^{-5}$ M $KMnO_4$ + 2 mL 0.5 N H_2SO_4 + 2 ml dH_2O

vi) 7 mL $4x10^{-5}$ M $KMnO_4$ + 2 mL 0.5 N H_2SO_4 + 1 ml dH_2O

vii) 8 mL $4x10^{-5}$ M $KMnO_4$ + 2 mL 0.5 N H_2SO_4

Read the absorbances of these solutions at the chosen wavelength, and plot a calibration curve. Calculate Ɛ x b from the slope of your curve.

3.3. Determination of $KMnO_4$ Concentration in a Sample

Read the absorbance of the solution given at the chosen wavelength. Calculate the concentration of $KMnO_4$ in the sample using the calibration curve.

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BIOMEDICAL ENGINEERING DEPARTMENT

BME2901- BIOCHEMISTRY COURSE

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EXPERIMENT 3

PROTEIN QUANTIFICATION BY BRADFORD ASSAY

1. THEORETICAL KNOWLEDGE

Proteins are the molecular instruments through which the genetic information is expressed. They are structurally diverse and functionally sophisticated. There can function as structural proteins, motor proteins, transport proteins, gene regulatory proteins and as enzymes, signals, receptors. They are biomacromolecules that are composed of amino acids.

Amino acids have a carboxyl group, an amino group and H atom bonded to the same carbon atom (the α carbon) (Figure 1).

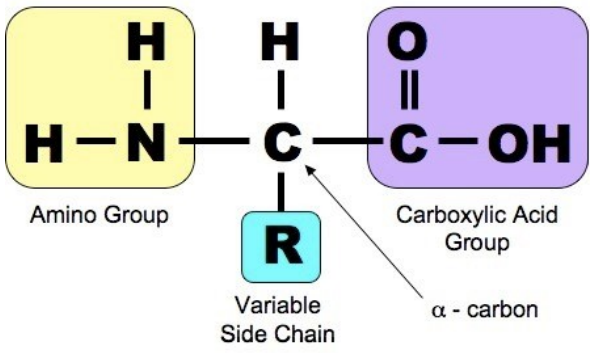

Figure 1. Structure of amino acid.

There are 20 different amino acids. They differ from each other in their side chains, or R groups, which vary in structure, size, and electric charge, and influence the solubility of the amino acids in water (Figure 2).

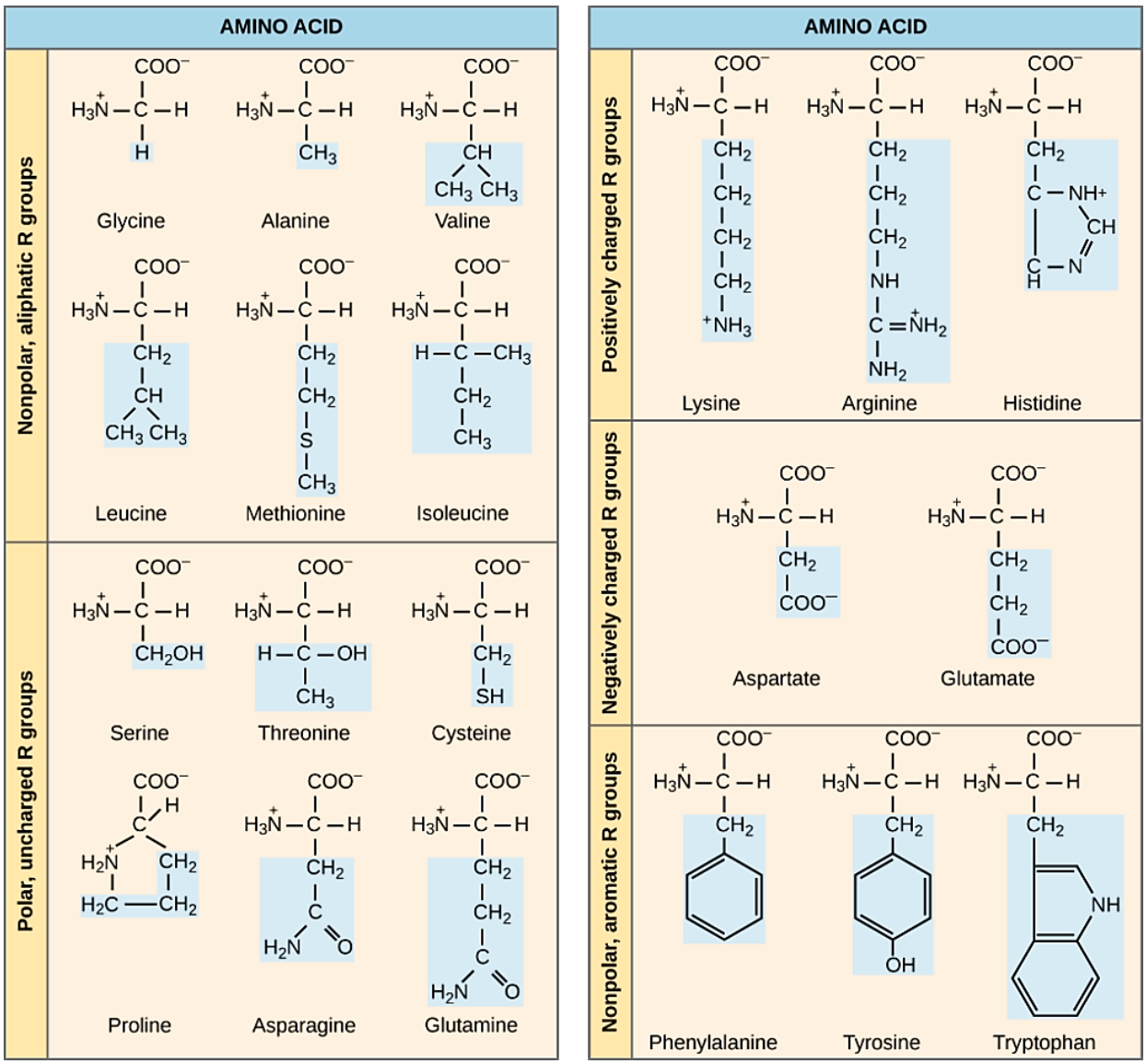

Figure 2. The 20 types of amino acids. The side chains are in blue.

While all proteins are assembled from the same set of 20 amino acids through peptide bonds, it is the length and sequence of an individual protein that determines its structure and activity. Cells can produce proteins with strikingly different properties and activities by joining the same 20 amino acids in many different combinations and sequences.

Tryptophan and tyrosine, and to a much lesser extent phenylalanine, absorb ultraviolet light at 280 nm due to the resonance in the aromatic ring structure in their R groups.

Amino acids are linked together by peptide bonds to form short peptides and longer proteins through a condensation reaction (Figure 3). Such a linkage is formed between the carboxyl group of one amino acid and the amino group of another.

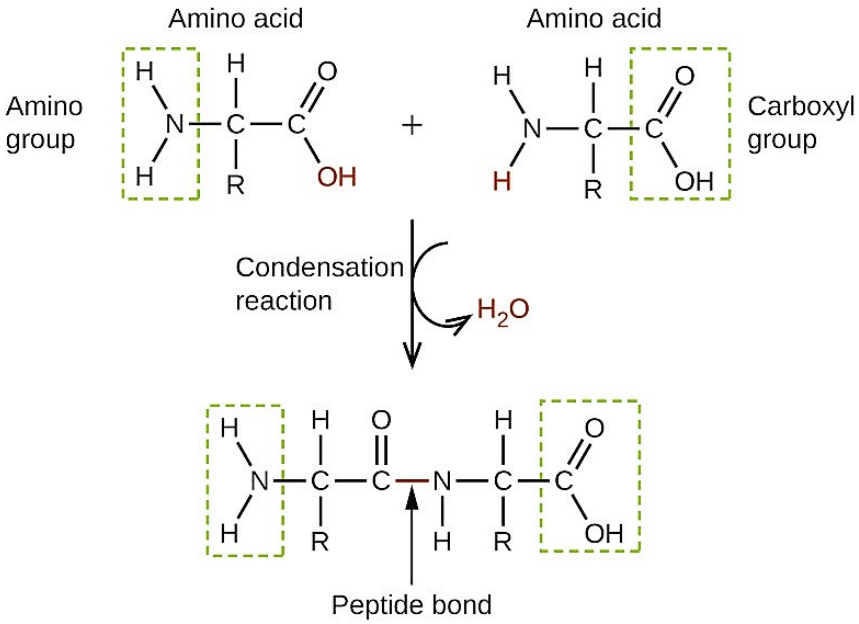

Figure 3. Formation of the peptide bond by condensation reaction.

The R groups or side chains simply extend out away from the backbone of the chain. The polypeptide chain extends from the first amino acid, which has a free amino group, to the last amino acid, which has a free carboxylic acid group. In a peptide, the amino acid residue at the end with a free -amino group is the amino-terminal (or N-terminal) residue; the residue at the other end, which has a free carboxyl group, is the carboxyl-terminal (C-terminal) residue (Figure 4).

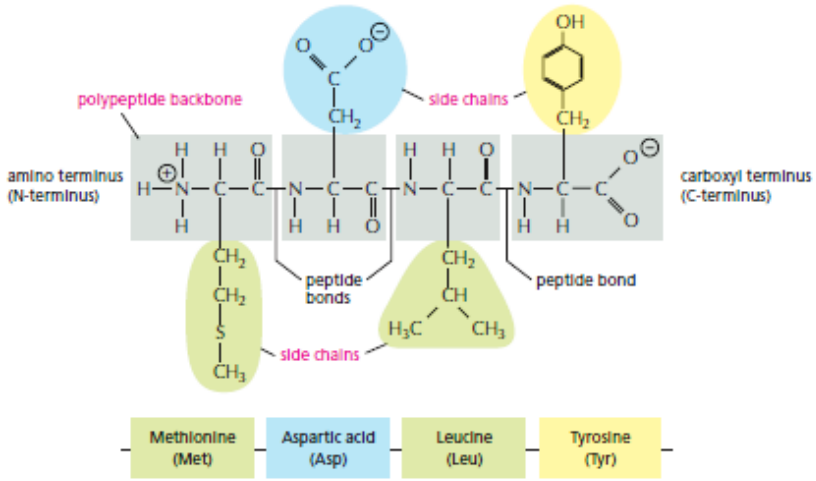

Figure 4. The polypeptide chain.

While a protein can be described in terms of its sequence of amino acids, proteins in a living cell do not exist as simple linear chains. Rather, each chain is folded through weak chemical bonds between the peptide bonds and R groups into a complex three-dimensional conformation.

Proteins absorb light at 280 nm mainly due to the presence of tryptophan in their structure. However, there are some proteins that do not involve tryptophan in their structure (such as Protein A). Also if there is a DNA (λ_{max}=260 nm) contamination in the protein solution it can also contribute to the absorbance. Thus, methods based on the measurement of the absorption of the protein solution by staining the proteins by a colored compound have been developed.

One such method, Bradford assay, is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs.

The absorbance at the 595 nm is proportional to the concentration of the protein solution and exact concentration can be found by preparing a calibration curve.

Determination of the total amount of protein in a given volume of solution has an important role in the selection of separation and purification processes and in controlling the protein yield and purity at protein isolation and purification processes.

2. MATERIALS

- BSA
- Bradford reagent
- Distilled water
- Microcentrifuge tubes
- Micropipette
- Spectrophotometer

3. METHOD

3.1.Preparation of a Calibration Curve Using Protein Standards

1. Dissolve 30 mg of BSA in 15 mL of distilled water to make a 2 mg /mL BSA stock solution.
2. Make dilutions of BSA using the volumes in the following table.

Vial	Volume of BSA solution	Volume of diluent (dH_2O)	BSA Concentration
A	1 mL of stock solution	-	... µg/ml
B	750 µL of stock solution	250 µL	… µg/ml
C	500 µL of stock solution	500 µL	… µg/ml
D	500 µL from Vial C	500 µL	… µg/ml
E	400 µL of Vial D	600 µL	… µg/ml
F	500 µL of Vial E	500 µL	… µg/ml

3. Transfer 10 µL of each vial to a microcentrifuge tube.
4. Add 500 µL Bradford reagent to each microcentrifuge tubes.
5. Pipette all solutions slowly.
6. Place the samples to cuvettes.
7. Read the absorbance of the solutions at 595 nm using the spectrophotometer.
8. Record the readings and construct the calibration curve.

* Make sure you include the appropriate blank solutions.

3.2.Determination of protein concentration in an unknown sample

1. Mix 10 µL of unknown sample with 500 µL of Bradford reagent.
2. Transfer this solution to a cuvette.
3. Read the absorbance of the solution at 595 nm using the spectrophotometer.
4. Record the reading and calculate the protein concentration in the unknown sample using the calibration curve.

* Make sure you include the appropriate blank solutions.

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EXPERIMENT 4
ENZYME KINETICS

1. PURPOSE OF THE EXPERIMENT

To learn the key equations and graphing methods for explaining and examining enzyme activity. To learn methods for spectrophotometrically measuring the kinetics of an enzymatic reaction.

2. THEORETICAL KNOWLEDGE

The activity of enzymes is important for the proper functioning of cells since the organism must be able to catalyze chemical reactions efficiently and selectively. In the context of energy flow in living organisms, enzymes catalyze most reactions in metabolic pathways. Acting in organized sequences, they catalyze the hundreds of stepwise reactions that degrade nutrient molecules, conserve and transform chemical energy, and make biological macromolecules from simple precursors. Through the action of regulatory enzymes, metabolic pathways are highly coordinated to yield a harmony in the interplay of the many activities necessary to sustain life. Thus, enzymes not only make most reactions possible in an intracellular environment, enzymes allow for the control and stabilization of these reactions.

The behaviour of enzymes and reaction rates in response to different concentrations of the reaction chemicals (both substrates and products) comprise the basic characteristics of each type of enzyme. This behaviour, referred to as enzyme kinetics, is responsible for much of the reaction control in biological systems.

A key factor affecting the rate of a reaction catalyzed by an enzyme is the concentration of substrate, [S]. However, studying the effects of substrate concentration is complicated by the fact that [S] changes during the course of an in vitro reaction as substrate is converted to product. One

simplifying approach in kinetics experiments is to measure the **initial rate** (or **initial velocity**), designated $\mathbf{V_0}$, when [S] is much greater than the concentration of enzyme, [E].

The effect on V_0 of varying [S] when the enzyme concentration is held constant is shown in Figure 1. At relatively low concentrations of substrate, V_0 increases almost linearly with an increase in [S]. At higher substrate concentrations, V_0 increases by smaller and smaller amounts in response to increases in [S]. Finally, a point is reached beyond which increases in V_0 are very small as [S] increases. This plateau-like V_0 region is close to the maximum velocity, V_{max}.

Leonor Michaelis and Maud Menten explained the relationship between [S] and V_0 and derived an equation called Michaelis-Menten Equation:

$$V_0 = \frac{V_{\max}[S]}{K_{\mathrm{m}} + [S]}$$

Where V_0 is the initial velocity, V_{max} is the maximum velocity, [S] is the substrate concentration and K_m is the Michaelis constant. Substances that stick tightly to the stationary phase move very slowly, while those that stick loosely or do not stick at all move rapidly.

The curve expressing the relationship between [S] and V_0 (Figure 1) is also called as Michaelis-Menten curve and has the same general shape for most enzymes (it approaches a rectangular hyperbola).

An important numerical relationship emerges from the Michaelis-Menten equation in the special case when V_0 is exactly one-half V_{max}. Then,

$$\frac{V_{\max}}{2} = \frac{V_{\max}[S]}{K_{\mathrm{m}} + [S]}$$

If we solve the equation we obtain,

$$K_{\mathrm{m}} = [S], \qquad \text{when } V_0 = \frac{1}{2}V_{\max}$$

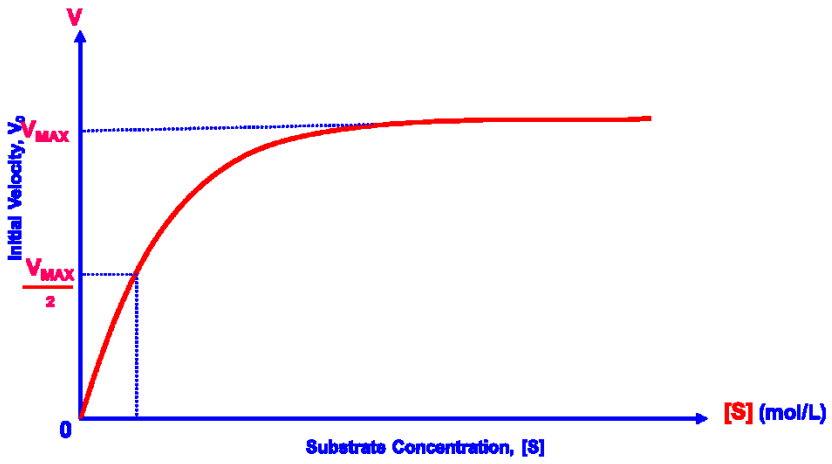

Figure 1. Michaelis-Menten Plot

It is not easy to determine V_{max} using the Michaelis Menten plot constructed with the experimental values obtained in an enzyme kinetics study. Only an approximation is possible.

Lineweaver and Burk simplified the Michaelis-Menten equation to:

$$\frac{1}{V_0} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}$$

A plot of $1/V_0$ versus 1/[S] (the "double reciprocal" of the V_0 versus [S] plot we have been using to this point) yields a straight line. This line has a slope of K_m/V_{max}, an intercept of $1/V_{max}$ on the $1/V_0$ axis, and an intercept of $-1/K_m$ on the 1/[S] axis. The double-reciprocal presentation, also called a Lineweaver-Burk plot (Figure 2), has the great advantage of allowing a more accurate determination of V_{max}.

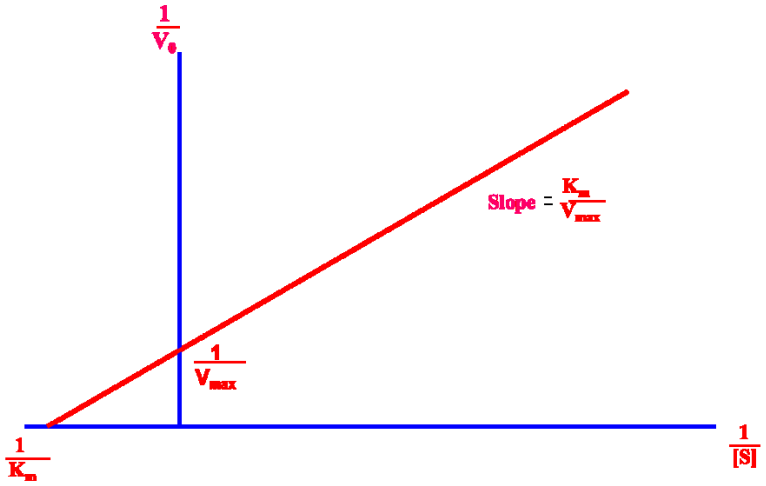

Figure 2. Lineweaver-Burk plot

In this experiment, we will study the kinetics of alkaline phosphatase (ALP) enzyme which is widely expressed in tissues like kidney, bone, liver and placenta. It cleaves phosphate from phosphate containing products and uses Zn^{++} and Mg^{++} as cofactor. It shows the best catalytic activity under alkaline conditions (pH = 9.0). In the experiment, we will use p-nitrophenly phosphate as the phosphate containing substrate of the ALP enzyme. The enzyme will convert p-nitrophenly phosphate into p-nitrophenol which is a yellow colored product (Figure 3). Product concentration can be calculated from the absorbance of the p-nitrophenol using Beer-Lambert's Law.

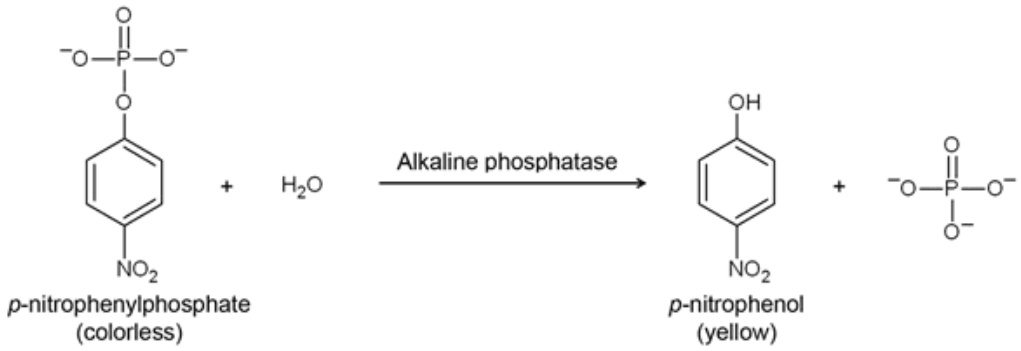

Figure 3. The reaction catalyzed by alkaline phosphatase.

3. MATERIALS AND METHODS

3.1.Materials

Alkaline Phosphatase from Human Placenta, p-nitrophenyl phosphate, Spectrophotometer, 96-well plates, Micropipettors, Pipette tips

3.2.Experimental Method:

Determination of V_0

1. Pipette 100 µL of the enzyme into a well on the 96-well plate.
2. Pipette 100 µL of the enzyme and 100 µL buffer solution to another well as the blank.
3. Add 100 µL of the substrate into the enzyme solution.
4. Start the timer.
5. Quickly mix the solution in the well by inversion and place in the spectrophotometer.
6. Read the absorbance at 405 nm beginning from the first 15th second after addition of substrate for 2 minutes with 15 seconds intervals.
7. Record the absorbance values and calculate the product concentration using Beer-Lambert's Law (Use $\varepsilon = 18.5\ mM^{-1}\ cm^{-1}$ and l = 0,56 cm).

8. Plot product concentration vs. time graph using Excel. Draw a best-line. Using the slope of the best-line determine the initial velocity (V_0).

Determination of K_m and V_{max}

1. Prepare the following substrate concentrations: 0.1 mM, 0.01 mM
2. Pipette 100 µL of the enzyme into 7 wells on the 96-well plate.
3. Pipette 200 µL of the enzyme solution to another well as the blank.
4. Add 100 µL of each substrate concentration on the enzyme solutions in different wells.
5. Start the timer.
6. Quickly mix the solution in the well by inversion and place in the spectrophotometer.
7. Read the absorbance at 405 nm beginning from the first 15th second after addition of substrate for 2 minutes with 15 seconds intervals.
8. Record the absorbance values and calculate the product concentration using Beer-Lambert's Law (Use $\varepsilon = 18.5\ mM^{-1}\ cm^{-1}$ and $l = 0.56$ cm).
9. Plot substrate concentration vs. time graph using Excel. Draw a best-line. Using the slope of the best-line determine the initial velocity (V_0) for each substrate concentration.
10. Plot Michaelis-Menten and Lineweaver Burke plot curves using V_0 and [S] values.
11. Determine V_{max} and K_m values.

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EXPERIMENT 5
PAPER CHROMATOGRAPHY

1. PURPOSE OF THE EXPERIMENT:
To understand concepts of chromatography and to learn identification of aminoacid by using paper chromatography.

2. THEORETICAL KNOWLEDGE

Chromatography is the physical separation of a mixture into its individual components. In chromatography methods, the components to be separated are distributed between two phases, a stationary and mobile phase. A mixture which contains the solutes is separated into pure components by passing it over the stationary phase (an insoluble material) to which the substances stick to varying degrees. The mobile phase, solvent (liquid or gas) is carrying the solutes over the stationary phase.

Separation by the chromatography is based on the different interactions of the compounds with the two phases. The movement of the components in the mobile phase is controlled by the significance of their interactions with the mobile and/or stationary phases: Substances that stick tightly to the stationary phase move very slowly, while those that stick loosely or do not stick at all move rapidly.

Chromatography steps separate the individual components of a complex mixture into *fractions* based on the properties of the protein—such as size, shape, or electrical charge.

There are different types of chromatography. However, most of them are based on column chromatography where a porous solid material with appropriate chemical properties (**the stationary phase**) is held in a column, while buffered solution (**the mobile phase**) carries the molecule of interest through it. According to the properties of the column which constitutes the stationary phase, compounds in a mixture are separated based on their size, shape, affinity or electrical charge.

Paper Chromatography is one of the chromatographycal methods which uses an adsorbent material usually paper as the stationary phase. Since it uses paper as the stationary phase this technique is generally called Paper Chromatography. It separates the compounds by taking advantage of their different rates of migration across sheets of paper. It is an inexpensive but powerful analytical tool that requires very small quantities of material.

In this technique, the compounds to be separated are applied on the paper as spots. Then the side of the paper where the sample is applied is immersed in a solvent which constitutes the mobile phase. The solvent penetrates the paper by capillary action and, in passing over the sample spot, carries along with it the various components of the sample. Thus, the compounds in the mixture migrate through the pores of the paper with a velocity depending on the differences in their affinity towards stationary and mobile phases under the capillary action of pores in the paper.

The distance a compound travels up the paper depends on a competition between how strongly the compound is attracted to the solvent and how strongly the compound is attracted to the stationary phase (i.e. paper). This attraction is related to the relative polarities of the sample, the paper and the solvent. Paper chromatography is especially useful in characterizing amino acids. The different amino acids move at differing rates on the paper because of differences in the polarities of their R groups.

When amino acids are analyzed using paper chromatography, they separate into colorless spots that are not visible to the eye. After the separation the paper will be treated with ninhydrin to make the spots visible. Ninhydrin reacts with the amino groups in the amino acids in the presence of heat and turns the spots purple.

The separated components of a mixture can be identified by comparison of their relative positions on the paper with the positions of known reference samples. The ratio of the distance traveled by a particular compound to the distance traveled by the solvent is called the Rf value of that particular compound (and is unique to that compound for the particular paper and solvent used).

Rf value = distance traveled by the compound / Distance traveled by the solvent

Rf values of aminoacids when isopropanol : water (70 : 30) (v / v) solution is used as the mobile phase are given in the table below:

Aminoacid	Rf value
Glycine	0.32
Alanine	0.37
Valine	0.45
Leucine	0.55
Isoleucine	0.53
Serine	0.35
Threonine	0.37
Aspartic acid	0.33
Asparagine	0.14
Glutamic acid	0.35
Glutamine	0.15
Lysine	0.03
Histidine	0.20
Arginine	0.02
Phenyl alanine	0.58
Tyrosine	0.57
Tryptophan	0.62
Cysteine	0.38
Cystine	0.32
Methionine	0.51
Proline	0.26
Hydroxy proline	0.34

3. MATERIALS AND METHODS:

3.1. Materials to be used in the experiment: Precision balance, Spatula, Distilled water, Graduated cylinder, L-glutamine, L-hydroxy proline, Amino acid mixture, Unknown amino acid, Phosphate buffered saline (pH = 7.4), Isopropanol, Distilled water, Ninhydrin solution (0.25% in acetone), Whatmann Paper No:1, 500 mL Beaker

3.2. Experimental Method:

1. Dissolve amino acids in PBS solution with a concentration of 200 mM.
2. Prepare a mixture of isopropanol : distilled water (70 : 30) (v / v).
3. Pour the solution into a 500 mL beaker.

4. Use a pencil to mark the Whatmann paper 1.5 cm from the sides and 2 cm from the bottom. Mark 4 spots on the bottom line for loading amino acids. Spots should be at least 1 cm apart from each other.
5. Load 2 μL of each amino acid and amino acid mixture to each spot.
6. Let the spots to dry.
7. Place the paper in the beaker. The solvent level should be below the line of spots.
8. Let the chromatogram to develop for 60 min.
9. After 60 min, mark the distance that the solvent travelled. Take the whatman paper from the set up.
10. Let chromatogram dry in the oven at 100°C for 30 min.
11. Spray ninhydrin solution on the paper to visualize the amino acids.
12. Let the colour to develop in the oven at 100°C for 5-10 min.
13. Measure the distance travelled by the solvent and by each amino acid.
14. Identify each amino acid on the chromatogram, calculate the Rf values of the amino acids.
15. Determine the unknown amino acid.

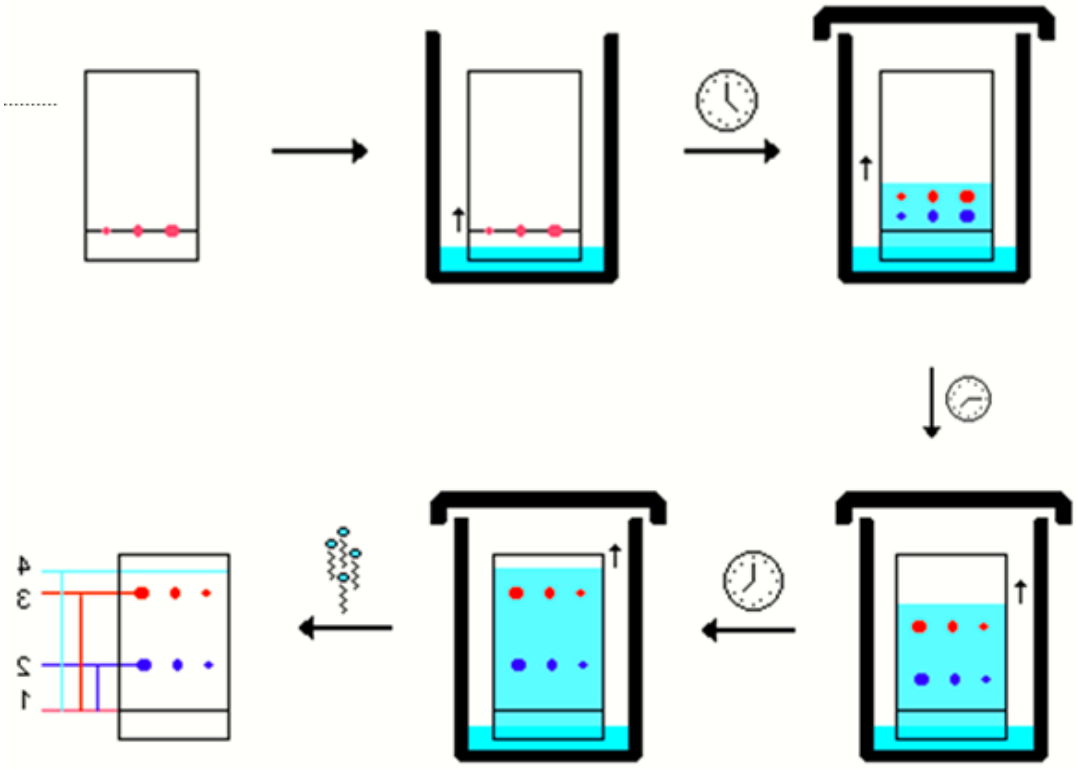

Figure 3.1 Experimental Procedure

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EXPERIMENT 6

DNA ISOLATION AND GEL ELECTROPHORESIS

1. THEORETICAL KNOWLEDGE

Deoxyribonucleic acid (DNA), "the master molecule," is a natural polymer which encodes the genetic information required for the growth, development, and reproduction of an organism. Found in all cells, it consists of chains of units called nucleotides. Each nucleotide unit contains three components: the sugar deoxyribose, a phosphate group, and a base with single or double ring structure. The base component can be any of four types: adenine, cytosine, guanine or thymine. DNA has a double helical structure with a negatively charged sugar-phosphate backbone and stabilized by hydrogen bonds between the bases attached to the two strands.

DNA can be isolated from a variety of sample sources including blood, buccal epithelial cells, cryopreserved cells, saliva, urine, hair, plants, fungi, bacteria etc. Choosing the source for DNA is important for the yield and for reduced risk of contamination. Anti-coagulated whole blood is the most convenient source of DNA since the yield is high due to the high number of lymphocytes in the blood. The risk of contamination is also very low. However, it is an invasive method since a syringe is puncturing your skin and vessels. DNA in hair, urine, saliva samples and a few cells that could be left behind by a criminal are generally preferred in forensic science. However DNA yield is generally very low due to the scarcity in the starting material. Buccal epithelial cells are generally used for paternity testing or comparison of the DNA isolated from a crime scene with the suspect's. The yield is high comparatively and the risk of contamination can be reduced by some precautions.

Many different methods and technologies are available for the isolation of genomic DNA. In general, all methods involve disruption and **lysis** of the starting material followed by the removal of proteins and other contaminants (**wash**) and finally recovery (**elution**) of the DNA.

Removal of proteins is typically achieved by digestion with proteinase K, followed by salting-out, organic extraction, or binding of the DNA to a solid-phase support (either anion-exchange or silica technology). Magnetic bead technology is also developed recently to isolate DNA more rapidly and with a higher yield.

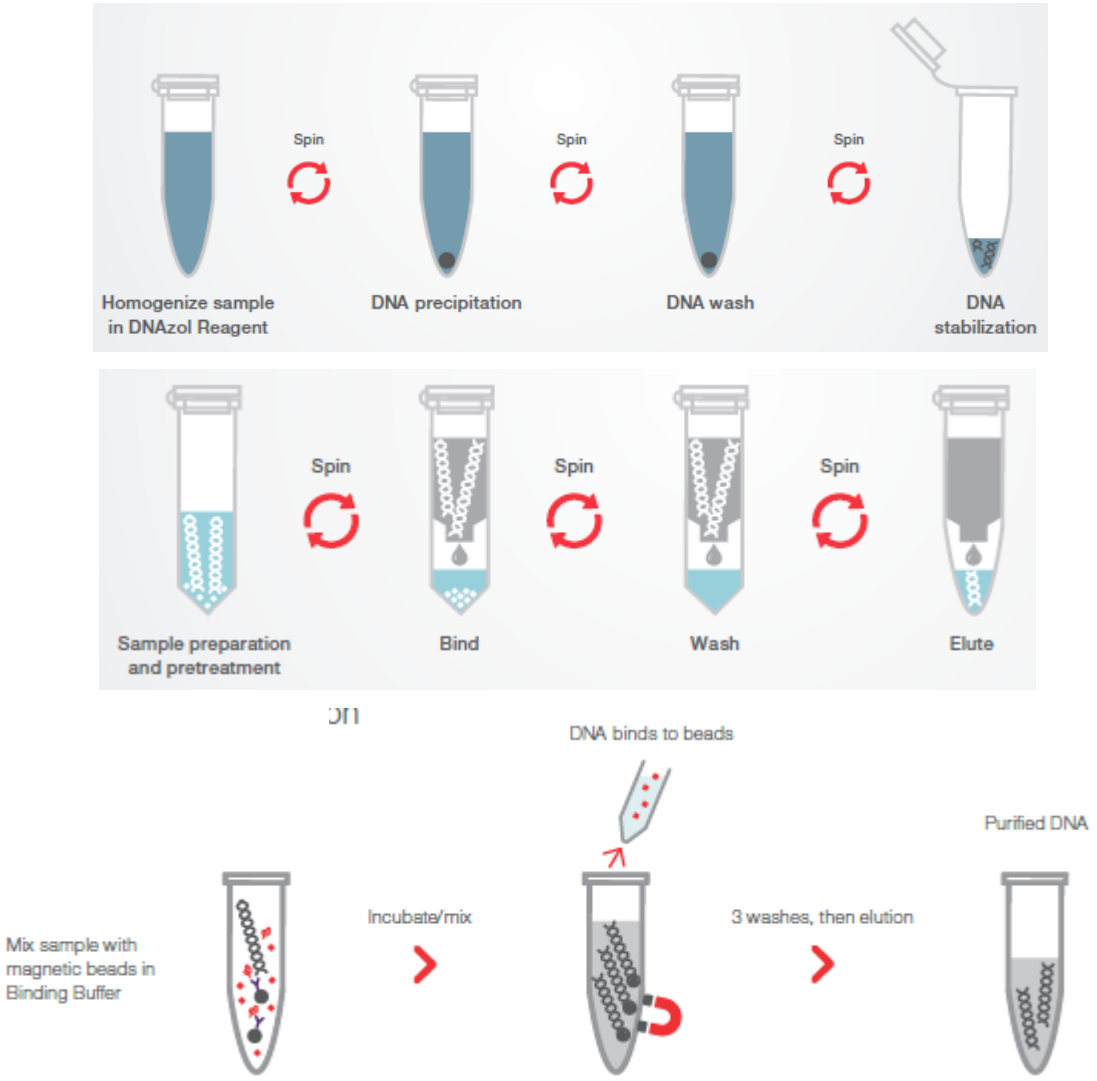

Figure 1. DNA isolation methods.

In this experiment DNA is adsorbed onto the silica membrane. Salt and pH conditions in the lysate ensure that protein and other contaminants, which can inhibit PCR and other downstream enzymatic reactions, are not retained on the silica membrane.

DNA is usually recovered from the membrane by precipitation using ethanol or isopropanol and then solubilized in the solution by the elution buffer.

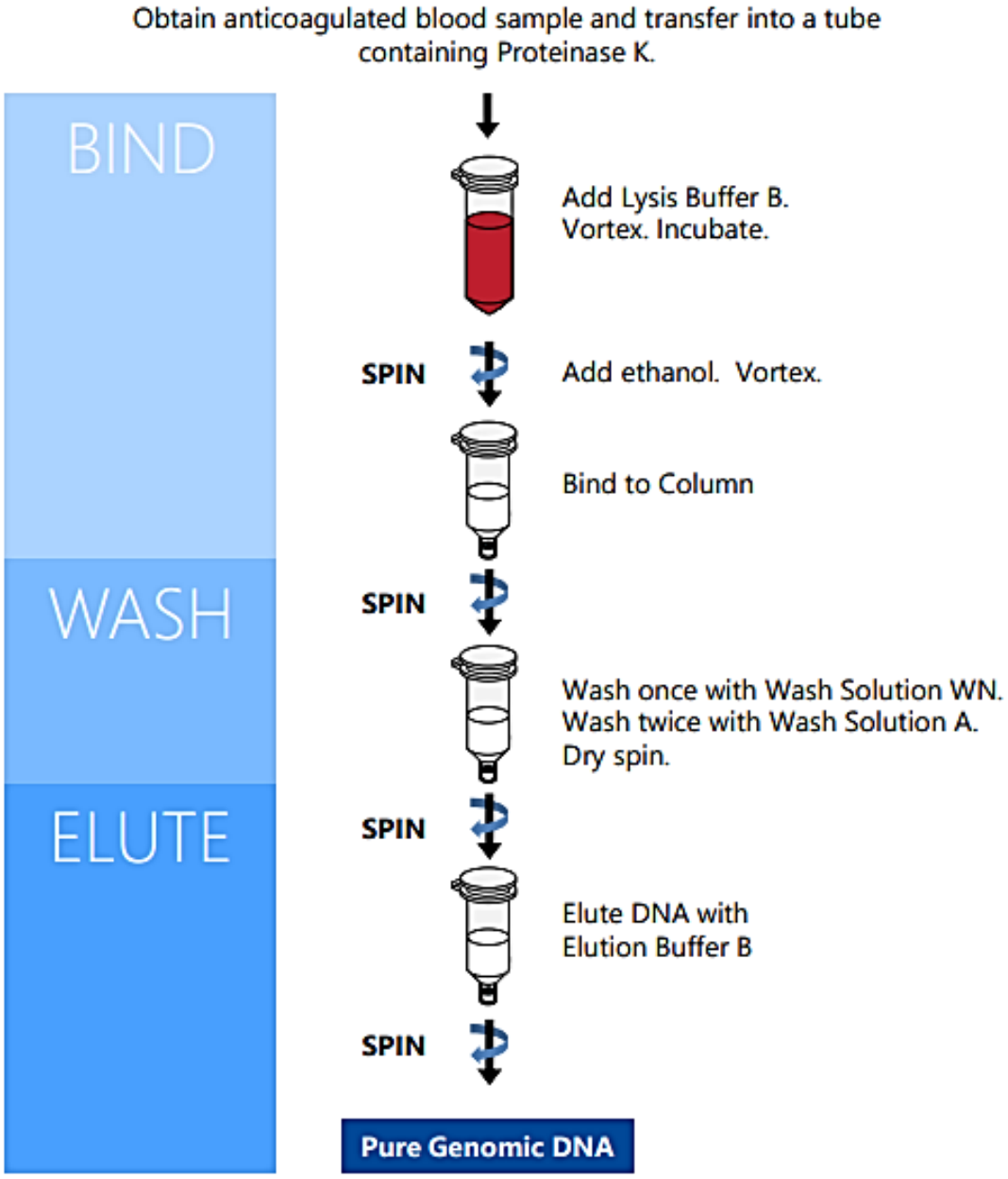

Figure 2. Silica column-based DNA extraction from blood sample.

The choice of a method depends on many factors: the required quantity and molecular weight of the DNA, the purity required for downstream applications, and the time and expense.

➢ <u>There are some necessary precautions for DNA isolation and purification:</u>

1. Blood should be handled with the appropriate precautions to avoid exposure to infectious agents.

2. Anticoagulant should be used. Anticoagulant is a substance that prevents blood from clotting or thickening of blood (e.g. EDTA, heparin, sodium citrate, sodium oxalate). Blood samples older than one week may produce poor yields and/or poor quality DNA unless they have been stored frozen.

3. Yield is dependent on the white cell count of the sample. One ml whole blood has about 10 million white cells and yields ~100 µg DNA suitable for ~200 amplification reactions.

4. Polypropylene tubes and tips should be used for isolating DNA; other plastic products may absorb DNA.

5. The need to store prepared DNA for long times should be considered in the choice of a protocol. For example, reference DNA used to monitor the specificity of primers and probes may be utilized over a long period of time. Therefore, a protocol that produces a more purified DNA preparation should be selected.

Agarose gel electrophoresis is a method of gel electrophoresis used in biochemistry, molecular biology, genetics, and clinical chemistry to separate a mixed population of macromolecules such as DNA or proteins in a matrix of agarose, one of the two main components of agar. The proteins may be separated by charge and/or size (isoelectric focusing agarose electrophoresis is essentially size independent), and the DNA and RNA fragments by length. Biomolecules are separated by applying an electric field to move the charged molecules through an agarose matrix, and the biomolecules are separated by size in the agarose gel matrix. DNA as well as RNA are normally visualized by staining with ethidium bromide, which intercalates into the major grooves of the DNA and fluoresces under UV light. The intercalation depends on the concentration of DNA and thus, a band with high intensity will indicate a higher amount of DNA compared to a band of less intensity

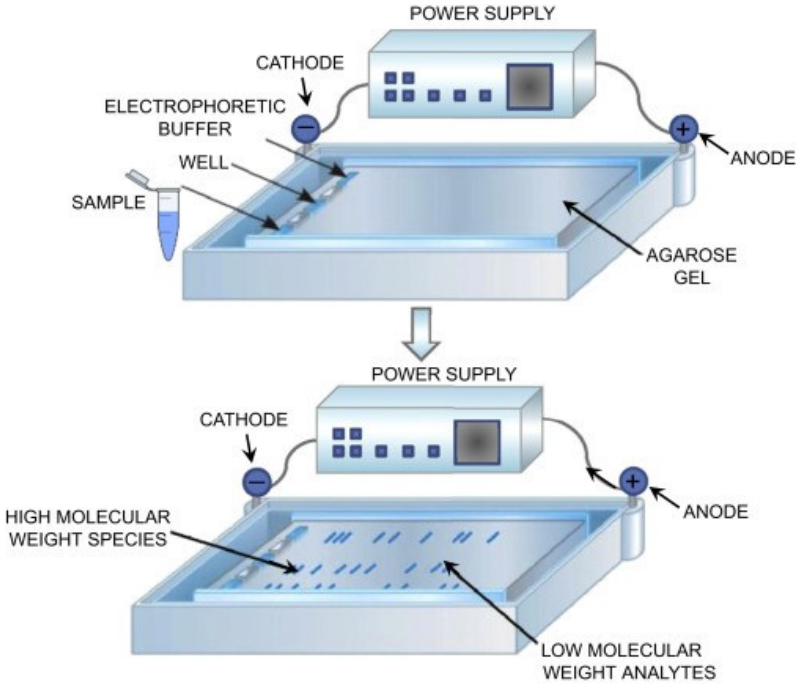

Figure 3. Gel electrophoresis system.

2. MATERIALS

* The GeneJET™ Genomic DNA Purification Kit. It is designed for rapid and efficient purification of high quality genomic DNA from various mammalian cell culture and tissue samples, whole blood, bacteria and yeast. The kit utilizes silica-based membrane technology in the form of a convenient spin column. Depending on the starting material, samples are digested with Proteinase K in either the supplied Digestion or Lysis Solution. The lysate is then mixed with ethanol and loaded on the purification column where the DNA binds to the silica membrane. Impurities are effectively removed by washing the column with the prepared wash buffers. Genomic DNA is then eluted under low ionic strength conditions with the Elution Buffer.

GeneJET Genomic DNA Purification Kit (Proteinase K, lysis/binding buffer, washing buffers, elution buffer, spin column and 2 mL collection tubes are provided in this kit)

* Whole Blood
* Minicentrifuge
* 1.5 mL microcentrifuge tubes
* Vortex
* Micropipettes and pipette tips
* Ethanol (96-100 %)
* 10X Tris/Acetate/EDTA buffer (TAE)
* Beaker
* Ethidium Bromide
* Agarose
* DNA loading dye
* Gel Tank
* Power supply
* UV camera

3. METHOD

3.1.DNA Isolation

* Add 400 µL of Lysis Solution and 20 µL of Proteinase K Solution to 200 µL of whole blood, mix thoroughly by vortexing or pipetting to obtain a uniform suspension.

* Incubate the sample at 56 °C while vortexing occasionally until the cells are completely lysed (10 min).

* Add 200 µL of ethanol (96-100 %) and mix by pipetting or vortexing.

* Transfer the prepared lysate to a GeneJET Genomic DNA Purification Column inserted in a collection tube. Centrifuge the column for 1 min at 6000 x g. Discard the collection tube containing the flow-through solution. Place the GeneJET Genomic DNA Purification Column into a new 2 mL collection tube.

* Add 500 µL of Wash Buffer I. Centrifuge for 1 min at 8000 x g. Discard the flow-through and place the purification column back into the collection tube.

* Add 500 µL of Wash Buffer II to the GeneJET Genomic DNA Purification Column. Centrifuge for 3 min at maximum speed (≥12000 x g). Optional. If residual solution is seen in the purification column, empty the collection tube and re-spin the column for 1 min. at maximum speed. Discard the collection tube containing the flow-through solution and transfer the GeneJET Genomic DNA Purification Column to a sterile 1.5 mL microcentrifuge tube.

* Add 200 µL of Elution Buffer to the center of the GeneJET Genomic DNA Purification Column membrane to elute genomic DNA. Incubate for 2 min at room temperature and centrifuge for 1 min at 8000 x g.

* Discard the purification column. Use the purified DNA immediately in downstream applications or store at -20 °C.

3.2.Agarose Gel Electrophoresis

* Preparation of 1X TAE: To prepare 500 ml of 1X TAE buffer add 50 ml of 10X TAE buffer to 450 ml of sterile distilled water. Mix well before use.
* Preparation of agarose gel: To prepare 100 ml of agarose gel, add 1g agarose to 100 ml 1X TAE buffer in a glass beaker. Heat the mixture until agarose dissolves completely. Allow the solution to cool down to about 55-60 °C. Add 5 µl Ethidium bromide, mix well and pour the gel solution into the gel tray. Allow the gel to solidify for about 30 minutes at room temperature.
NOTE: Ethidium bromide is a powerful mutagen and is very toxic. Appropriate safety precautions should be taken by wearing latex gloves.
* Loading of the DNA samples: To prepare sample for electrophoresis, add 2 µl of 6X gel loading buffer to 10 µl of DNA sample. Mix well by pipetting and load the sample onto the well. Load the Control DNA after extracting the DNA sample.
* Electrophoresis: Connect the power cord to the electrophoretic power supply according to the conventions: Red -Anode and Black- Cathode. Electrophorese at 100-120 volts and 90 mA until dye markers have migrated an appropriate distance, depending on the size of DNA to be visualized.

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EXPERIMENT 7
REACTIONS SPECIFIC TO CARBOHYDRATES

1. PURPOSE OF THE EXPERIMENT

To identify the presence of sugar in different solutions by taking advantage of the reductive properties of free ketone or aldehyde groups in the structure of sugars.

2. THEORETICAL KNOWLEDGE

Carbohydrates, which are one of the energy-providing compounds in the body, are found free or combined with other substances.

Glucose, fructose, ribose, deoxyribose, xylose, glyceraldehyde, dihydroxy acetone, lactose in milk, blood glucose, fructose in seminal fluid, and glycogen in liver and muscle cells are examples of free carbohydrates.

Ribose and deoxyribose in the structure of nucleic acids; galactose in the structure of cerebrosides found in muscle and nerve cell membranes; galactose found as prosthetic groups in glycoproteins; amino sugars, such as glycosamine and galactosamine which are incorporated into the structure of heteropolysaccharides, are examples of compound carbohydrates.

Carbohydrates are defined as polyhydroxy alcohols containing potentially active aldehyde or ketone groups in their structure, or as substances which, when hydrolysed, give these products.

Carbohydrates can be classified into four main groups;

a) Monosaccharides

They are the most simple and non-hydrolysable sugars. They are named according to their carbon numbers and the functional group (aldehyde or ketone) in the composition.

b) Disaccharides

They are clinically important sugars formed by the coupling of two monosaccharide units to each other with glycosidic bonds. When disaccharides hydrolyzed, they are separated into monosaccharides that form them.

c) Oligosaccharides

They consist of short chains (3-10 monosaccharide units) of monosaccharide units, or residues, joined by characteristic linkages called glycosidic bonds. The most abundant are the disaccharides, with two monosaccharide units.

d) Polysaccharides

Polysaccharides are carbohydrates (e.g. starch, cellulose, or glycogen) whose molecules consist of a number of sugar molecules bonded together. Homopolysaccharides contain only a single type of monomer such as glycogen, starch, cellulose; heteropolysaccharides such as heparin, hyaluronic acid, chondroitin sulphate contain two or more different kinds of monomers. When polysaccharides are hydrolysed, they are broken down into the monosaccharide units that form them.

Carbohydrates present in a solution can be easily identified by performing certain tests in the laboratory. The important tests for carbohydrate detection are shown in Table 1.

All monosaccharides, some disaccharides, oligosaccharides and polysaccharides, having the free aldehyde or ketone groups in their structures as a reducing group. Carbohydrates with free or potentially free reducing groups easily reduce metal like copper (Cu), Ba, Hg (mercury), Iron (Fe) & silver (Ag) in alkaline solution when blue alkaline cupric oxide or hydroxide suspended in alkaline medium is heated it forms blue precipitate of cupric oxide (CuO) but in presence of reducing substances, e.g reducing sugars having free or potentially free aldehyde or ketonic group upon heating blue cupric hydroxide converted into insoluble brownish red cuprous oxide (Cu_2O) suspensions of metal hydroxide, used in metal reduction test and to precipitate in alkaline medium to check that organic compound having more than one alcoholic groups are added to give free metals.

All monosaccharides are reducing sugars as they all have a free reactive carbonyl group. Some disaccharides like maltose have exposed carbonyl groups and are also reducing sugars but less reactive than monosaccharides.

Test	Procedure	Observation	Inference	Reaction
Molisch's Test	2-3 drops of betanaphthol solution is added to 2ml of the test solution. Very gently added 1ml of Conc. H2SO4 along the side of the test tube.	A deep violet coloration is produced at the junction of two layers.	Presence of carbohydrates.	This is due to the formation of an unstable condensation product of betanaphthol with furfural (produced by the dehydration of the carbohydrate).
Iodine test	4-5 drops of iodine solution is added to 1ml of the test solution and mixed the contents gently.	Blue colour is observed.	Presence of polysaccharide	Iodine forms coloured adsorption complexes with polysaccharides.
Benedict's test	To 5 ml of Benedict's solution, add 1ml of the test solution and shake each tube. Place the tube in a boiling water bath and heat for 3 minutes.Remove the tubes from the heat and allow them to cool.	Formation of a green, red, or yellow precipitate.	Presence of reducing sugars	If the saccharide is a reducing sugar it will reduce Copper [Cu] (11) ions to Cu(1) oxide, a red precipitate.
Barfoed's test	To 2 ml of the solution to be tested added 2 ml of freshly prepared Barfoed's reagent. Place test tubes into a boiling water bath and heat for 3 minutes. Allow to cool.	A deep blue colour is formed with a red ppt. settling down at the bottom or sides of the test tube.	Presence of reducing sugars [appearance of a red ppt as a thin film at the bottom of the test tube within 3-5 min is indicative of reducing monosaccharide. If the ppt formation takes more time then it is a reducing disaccharide	If the saccharide is a reducing sugar it will reduce Cu (11) ions to Cu(1) oxide.
Seliwanoff test	To 3ml of of Seliwanoff's reagent, add 1ml of the test solution, boil in water bath for 2 minutes.	A cherry red colored precipitate within 5 minute is obtained.	Presence of ketoses [Sucrose gives a positive ketohexose test	When reacted with Seliwanoff reagent, ketoses react within 2 minutes forming a cherry red condensation product.
		A faint red colour produced.	Presence of aldoses	Aldopentoses react slowly forming the coloured condensation product.
Bials test	Add 3ml of Bial's reagent to 0.2ml of the test solution. heat the solution in a boiling water bath for 2 minutes.	A blue-green product.	Presence of pentoses	The furfurals formed produces condensation products with specific colour.
		A muddy brown to gray product.	Presence of hexoses,	

Table 1. Some examples of test for carbohydrate detection.

One of the most popular tests used for the estimation or detection of reducing sugars and non-reducing sugars is the Fehling's test. The test developed by German chemist H.C. Von Fehling is also used to differentiate between ketone functional groups and water-soluble carbohydrates.

The working principles of Fehling, Benedict and Barfoed tests based on the reducing properties of carbohydrates are based on the same principle. The only difference is that the medium is alkaline in the first two experiments, while the medium is slightly acidic in the Barfoed experiment.

The common principle of experiments is sugars with common aldehyde or ketone groups show the ability to reduce heavy metal hydrates (Cu, Bi, Ag) in an alkaline environment with the effect of heat. During the experiment, sugars are oxidized and give off sugar acids (aldonic acids).

3. MATERIALS AND METHODS

Fehling Test

Fehling test is a method used in the determination of compounds having reducing properties. Carbohydrates containing free aldehyde and ketone groups have reducing properties in alkali solutions. In addition, monosaccharides act as reducing agents in weak acid solutions.

3.1.Materials

$CuSO_4$ (Copper Sulphate), Na-K tartrate, NaOH (Sodium Hydroxide), Glucose, Distilled water, Fruit Juice, BSA, Water Bath, Test Tube, Pipette.

Preparation of Markers

Fehling 1 solution:

The compound dissolved in 7% $CuSO_4$ (34.6 crystalline copper sulfate) by gently heating in 300 ml of distilled water. Then, 200 ml of distilled water are added to complete the solution to a volume of 500 ml.

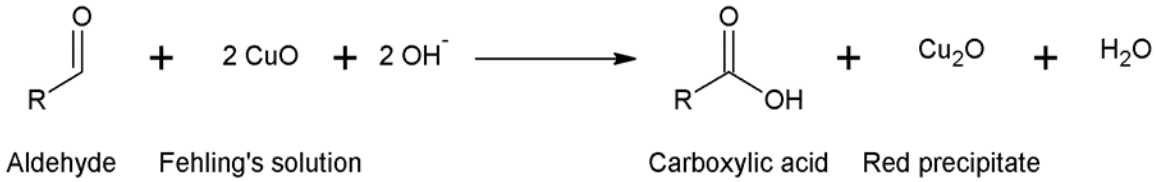
Fehling 2 solution:

Mixture of 35 g Na-K tartrate and 10 g NaOH is dissolved with some distilled water. After that, the mixture volume is complete to 100 ml with distilled water.

3.2.Experimental Method:

1. Equal volumes (1 ml) of Fehling I and Fehling II solution are transferred in a test tube.
2. The mixture is shaken. A dark blue colour solution appears. This solution is called “Fehling reagent”.
3. The Fehling reagent is placed in a water bath at 60 ^oC until boiling starts. If the colour of the solution does not change, it means that the reagent is clean and does not contain reducing agents.
4. After this control, 1 mL unknown concentrations of glucose are added to Fehling reagent while boiling is continued. If there is excess sugar in the solutions, turbidity with yellow colour or precipitate is seen.

5. If the amount of sugar in the tested solution is small, an amount of solutions equal to the volume of the Fehling reagent is added. If a yellow or red colour precipitate occurs, there is sugar. If no precipitate is formed, it is understood that there is no sugar in the examined solutions.



Aldehyde Fehling's solution Carboxylic acid Red precipitate

EXPERIMENT 8

LIPID EXTRACTION AND DETERMINATION

1. THEORETICAL KNOWLEDGE

Lipids are biomolecules having a diverse group of organic compounds that include fatty acids, waxes, phospholipids, glycolipids, and sterols. These compounds are insoluble or poorly soluble in water due to the presence of long hydrocarbon chains in their structures. They are soluble in organic solvents such as benzene, ether or chloroform.

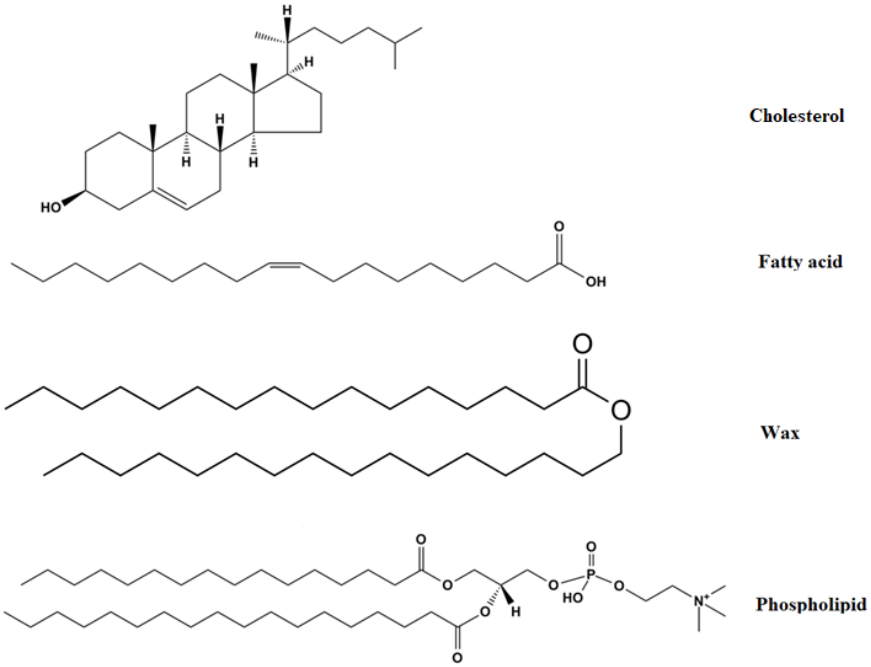


Figure 1. Structures of some common lipids.

Lipids perform a diverse range of functions within the cells of living organisms, such as 1) acting as a storage form of metabolic fuel or energy, e.g. fats 2) being an integral component of the biological membrane, e.g. phospholipids and cholesterol 3) signaling molecule, e.g. hormones, acting as 4)

cofactors, e.g. vitamins, 5) pigments, chlorophyll and 4) protective coat in bacteria, plants, insects, and invertebrates, e.g. waxes.

Cholesterol, the major sterol in animal tissues, is amphipathic, with a polar head group and a nonpolar hydrocarbon body. It has a short and rigid structure so that it fills the spaces between neighboring phospholipid molecules making the membrane less flexible. Similar sterols are found in other eukaryotes: stigmasterol in plants and ergosterol in fungi, for example. Bacteria cannot synthesize sterols; a few bacterial species, however, can incorporate exogenous sterols into their membranes.

The principle physicochemical characteristics of lipids used to distinguish them from the other components in foods are their solubility in organic solvents, immiscibility with water, low density, unique physical and spectroscopic properties. The analytical techniques based on these principles can be categorized into three different classes; solvent extraction, non-solvent extraction and instrumental methods.

Solvent extraction procedures are one of the most commonly used methods of isolating lipids from any working material, such as food, animal and plant tissues, bacterial cells, fungi and yeast cells. The fact that lipids are soluble in organic solvents, but insoluble in water, provides the analyst with a convenient method of separating the lipid components in the working material from water soluble components, such as proteins, carbohydrates and minerals.

The ideal solvent for lipid extraction would completely extract all the lipid components from the material, while leaving all the other components behind. In practice, the efficiency of solvent extraction depends on the polarity of the lipids present compared to that of solvent. Polar lipids (glycolipids or phospholipids) are more soluble in polar solvents than in non-polar solvents. On the other hand, non-polar lipids (triacylglycerols) are more soluble in non-polar solvents than in polar ones. In addition to the above considerations, a solvent should also be inexpensive, have a relatively low boiling point (so that it can be easily removed by evaporation), be non-toxic and be nonflammable.

Folch method, is one of the standard procedures to isolate total lipid fractions from biological matrices based on a solvent system consisting of chloroform/methanol/water. It is important that the ratio of chloroform, methanol and saline solution in the final mixture be close to 8:4:3. On the other hand, some modifications in the approach to reach the optimum concentrations can be applied. In this method, the endogenous water in the tissue was considered as a component of the extraction system.

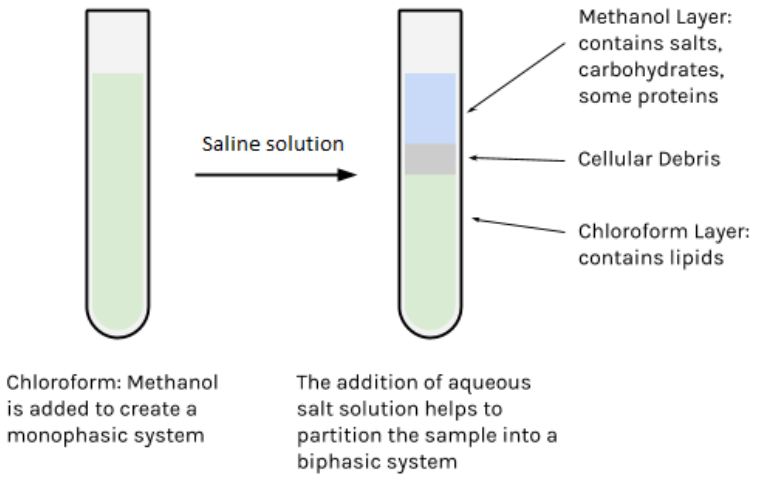


Figure 2. Folch extraction method.

Salkowsky experiment is a test which is applied for the qualitative analysis of cholesterol in a given liquid sample. It depends on the red coloring of the sterols with concantrated sulfiric acid as a result of the unsaturated lipid branches in their structures.

2. MATERIALS

- Egg
- Butter
- Olive oil
- Saline solution
- Methanol
- Chloroform
- Concentrated sulfuric acid
- Beakers
- Centrifuges tubes
- Centrifuge
- Magnetic stirrer

3. METHOD

3.1. Lipid Extraction

1. Test tubes 1-6 are weighed out and marked.
2. The egg is cracked open and the yolk is placed into a 100 ml beaker while the white is placed into another 100 ml beaker. Each part is homogenized by using a magnetic stirrer.

3. 1 ml of the yolk is measured out into test tube 1 by using the 10 ml graduated cylinder. This process is repeated with the white and placed into test tube 2. Each of these is diluted with 4 ml of saline.
4. 1 ml of each of two diluted solutions is measured out, the yolk being placed into the test tube 3 and white into test tube 4.
5. 1 ml of methanol and 2 ml of chloroform are added into both of the tubes 3 and 4. Tubes are centrifuged for 5 min at 2000 RCF.
6. After centrifugation, the bottom layer which is composed of lipids and chloroform is placed into test tube 5 (coming from test tube 3- the yolk) and test tube 6 (coming from test tube 4- the white).
7. These tubes are allowed to evaporate to ensure total evaporation.

3.2. Salkowsky Experiment for Lipids

Name four test tubes as positive control (butter and olive oil), negative controls (distilled water), sample 1 (egg yolk) and sample 2 (egg white). Transfer 1 ml of each sample into their named tubes. Then add 1 ml of chloroform into each test tube and add 500 µl of concentrated sulfuric acid. Mix the tubes and detect red-purple color appearance.