# Practical Manual *for*

## Nutritional Biochemistry Lab

FIRST EDITION, 2023

Institute of Home Science, University of Kashmir, Srinagar -190006

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# Institute of Home Science

# THE UNIVERSITY OF KASHMIR
# Hazratbal- 190006

**PHYSIOLOGY AND NUTRITIONAL BIOCHEMISTRY (Lab Course)**
**Code: HSC22103CR**
**Credits: 4**
**Periods/week: 8 Hours**
**Max. Marks: 100**

1. Qualitative Analysis of carbohydrates.
2. Qualitative Analysis of Proteins/Amino-acids.
3. Qualitative test for Fats, Cholesterol.
4. Qualitative test for Calcium and Phosphorus.
5. Determination of Saponification value of lipids, Acid number of fats and
6. Iodine number of fats.
7. Quantitative estimation of sugar by titrimetric method.
8. Use of pH meter and determination of pH value of dilute and strong acids and bases. Fruits and vegetable extracts.
9. Estimation of glucose in blood.
10. Estimation of soluble proteins in blood (Biuret method).
11. Estimation of calcium & phosphorus.
12. Estimation of bilirubin & cholesterol.
13. Estimation of creatinine & vitamin C.
14. Estimation of blood urea.
15. Microscopic examination of slides of various tissues.
16. Estimation of hemoglobin (Sahil's method).
17. Examination of Total blood count, differential count.
18. Determination of various blood group.
19. Examination of urine.

# A. Biochemistry an introduction

## 1. Introduction and scope of Biochemistry

Biochemistry is the branch of life science which deals with the study of chemical reactions occurring in living cells and organisms. The term Biochemistry was first introduced by the German Chemist Carl Neuberg in 1903. It considers the studies related to the nature of the chemical constituents of living matter, their transformations in biological systems and the energy changes associated with these transformations. Biochemistry may thus be treated as a discipline in which biological phenomena are analyzed in terms of chemistry. The branch of Biochemistry for the same reason, it has been variously named as "Biological Chemistry" or Chemical Biology. Modern biochemistry has two branches, descriptive biochemistry and dynamic biochemistry. Descriptive biochemistry deals with the qualitative and quantitative characterization of the various cell components and the dynamic biochemistry deals with the elucidation of the nature and the mechanism of the reactions involving these cell components. Many newer disciplines have been emerged from biochemistry such as enzymology (study of enzymes), endocrinology (study of hormones), clinical biochemistry (study of diseases), molecular biochemistry (study of biomolecules and their functions) etc. Along with these branches certain other specialties have also come up such as agricultural biochemistry, pharmacological biochemistry etc. Biochemistry is related to almost all the life sciences and without biochemistry background and knowledge, a thorough understanding of health and well-being is not possible. Those who acquire a sound knowledge of biochemistry can tackle the two central concerns of the biomedical sciences:

1) The understanding and maintenance of health.
2) The understanding and treatment of diseases.

### 1.1 Objectives of Biochemistry

The major objective of biochemistry is the complete understanding of all the chemical processes associated with living cells at the molecular level. To achieve this objective, biochemists have attempted to isolate numerous molecules found in cells, to determine their structures and to analyze how they function. Biochemical studies have illuminated many aspects of disease and the study of certain diseases has opened up new therapeutic approaches. In brief the objectives can be listed as follows:

- Isolation, structural elucidation and the determination of mode of action of biomolecules
- Identification of disease mechanisms
- Study of inborn errors of metabolism
- Study of oncogenes in cancer cells
- The relationship of biochemistry with genetics, physiology, immunology, pharmacology, toxicology etc.

Biochemistry is the study and application of substances, reactions and processes in animals, plants, bacteria and viruses. Biochemistry has vast scope. Biochemists work in hospitals,

industry, drug design and development, environmental sciences, forestry, agriculture, dietetics, hormone production, vaccine research, virology, immunology, microbiology, toxicology, food science, plant science and associated areas and in areas from marine biology to entomology not just to carry out R&D work and develop new products but also to monitor the production, quality and safety of the product. Biochemists provide diagnostic service, carrying out tests on blood, urine and other body fluids, while researching the underlying causes of disease and methods of treatment. They find jobs in pharmaceutical and agrochemical companies, food brewing and biotechnology industry. The postgraduate degree in biochemistry also provides opportunity for advanced teaching in universities, colleges, medical, dental and veterinary schools and consulting or allied work. Observational, organizational, computational skills, planning and team work are important for biochemists.

## 1.2 Scope and importance of Biochemistry

Biochemistry is the study of life; hence the scope it offers is as broad as life itself. The field has seen unprecedented growth, especially in the present times. Biochemistry offers a lot of scope in fields like:

a) **Medical Sciences:** There is a huge scope in areas like pathology, immunology, pharmacy, vaccine development, etc. People can also find a job as Biochemists, lab assistants, medical transcriptionists, clinical coordinators, marketing executives after having relevant degrees in any field of biochemistry. Another advanced scope of biochemistry is in Genetic Engineering or Recombinant DNA Technology.
b) **Agriculture:** Plant biochemistry offers enormous scope. Students of this branch can become agricultural scientists and develop agricultural crop variants that are high-yielding and disease-resistant. Knowledge of biochemistry can also help in setting up own farms or nurseries.
c) **Food Industry:** Nutritionists and dieticians help in monitoring and regulating the nutrient richness in the body. These roles have an enormous scope, especially in today's world where everyone is leading a sedentary lifestyle. Being a Food analyst or a Food security officer are other job prospects in this area.
d) **Academics and Research:** The field offers plenty of research opportunities. Every area underneath biochemistry is worth learning and researching.

At present, biochemistry is one of the most developing and critical areas of study in science. The field offers a significant avenue for research.

- Biochemistry helps understand the chemical aspects of different biological processes such as digestion, respiration, reproduction, excretion, the behaviour of hormones, contraction, and relaxation of muscles, and many more.
- It plays a vital role in health and nutrition.
- The field generally studies different body substances like enzymes, amino acids, carbohydrates, proteins, fats, DNA, RNA, Pigments, hormones, etc., at the fundamental level.

- Biochemists make use of chemical information and procedures to solve biological problems. Biochemistry solves fundamental problems in biology and medicine.
- Biochemistry provides interdisciplinary knowledge in science as it has many branches underneath, covering everything regarding organisms and their existence.
- It is essential in designing and manufacturing various chemical and biological products, clinical diagnosis, nutrition, treatment of diseases, agriculture, etc.
- Biochemistry ensures sustainability. It is a vast branch of science that offers endless possibilities- enormous scope for employment, hence reducing global poverty and starvation. Therefore, biochemistry is essential as a sustainable tool.

# B. General Laboratory Principles

## 2. General safety rules, requirements and regulations

Safety in the biochemistry laboratory involves a cautious attitude and an awareness of potential hazards. Usually potential accidents can be anticipated and prevented. If safety precautions are followed, fewer accidents will occur. The number of laboratory accidents can be reduced if every student follows all of the directions given for the experiment and by the instructor. Special note should be taken of specific instructions that are given in an experiment to eliminate recognized potential hazards. Total awareness of hazards and dangers and what to do in case of an accident is the responsibility of the student and the instructor. Work in the biochemistry laboratory involves the use of inflammable solvents, some corrosive and toxic chemicals, and apparatus which, if used improperly, can cause minor to severe injury. All work with solvents and chemicals must be performed in the fume hoods not on the bench top. Safety glasses and shoes must be worn at all times while in the laboratory. Here, are some rules which one should consider while working in the laboratory.

1. Prior to attending that laboratory session, the laboratory procedures must be read.
2. Laboratory smoking, eating and drinking are absolutely prohibited at any time in the laboratory.
3. Only closed-toe shoes should be worn in the lab. Due to the constant risk of cuts and infections from broken glass found on the laboratory floors and the possibility of chemical spills, sandals or open-toed or canvas shoes are not allowed.
4. Keep your face, nose, eyes, ears and mouth away from your hands and other objects. In the laboratory, the use of cosmetics in the laboratory is prohibited.
5. Before and after use, work areas or surfaces must be disinfected.
6. While in the laboratory, laboratory coats must be worn and buttoned. Outside of the laboratory, laboratory coats should not be worn.
7. When conducting any exercise or procedure in the laboratory, protective eyewear must be worn.
8. To minimize the fire hazard or contamination of experiments, long hair should be secured behind your head.

9. Prior to leaving the laboratory, hands must be washed.
10. Coats, books and other paraphernalia, such as purses, briefcases, etc., should be placed in specified locations when entering the laboratory and never on bench tops (except for your lab manual).
11. Never mouth-pipet anything (including water). Always use appliances for pipetting.
12. Label all materials with your name, date and any other information applicable (e.g., media, organism, etc.).
13. Waste disposal in its proper containers (see Biohazard Waste Disposal below).
14. Note the hazard code on the bottle when handling chemicals and take the appropriate precautions indicated.
15. Do not pour down the sink with chemicals.
16. Return to their appropriate places all chemicals, reagents, cultures, and glassware.
17. Do not pour fluids that are biohazardous down the sink.
18. It is necessary to wash the glassware with soap and water, then rinse it with distilled water.
19. Flame transfer loops, wires, or needles for transferring biological material before and immediately after use.
20. Do not walk around the laboratory with infectious matter containing transfer loops, wires, needles, or pipettes.
21. Around Bunsen burners, be careful. It is not always possible to see flames.
22. Turn off the incinerators before the laboratory leaves.
23. Report any broken equipment, report any broken glass, in particular those containing infectious materials immediately.
24. Contact your course instructor or TA immediately if you are injured in the laboratory.
25. In the event of further treatment being required, spills, cuts and other accidents should be reported to the instructor or TA.
26. Familiarize yourself with safety equipment and emergency escape routes in the laboratory.
27. Before putting it away, always wipe and clean your microscope's lenses. To this end, use the relevant tissue paper and cleaning solution.
28. With all biological fluids, apply appropriate universal precautions.
29. Without the written permission of the course instructor or TA, do not remove any materials from the laboratory.

## 2.1 Dealing with Solvents:

Never heat inflammable solvents, even small amounts, with or near a flame. As for refluxing or distillation, never place solvents in an open beaker. Pouring solvents in the vicinity of a flame is extremely hazardous. Use an oil bath, steam bath, water bath, heating mantle, or hot plate as a heat source whenever possible.

- Ethyl ether and Petroleum ether (bp 30-60°) are especially dangerous. Never heat them on a hot plate; always use a water or steam bath, and collect the distillate in an ice-cooled flask. In the case of Ethyl ether, the receiver should be a filtering or distilling flask connected to the condenser with a cork and with a piece of rubber tubing leading from side tube on the flask to the floor. This allows the heavy Ether vapours to spread along the floor instead of the desktop where they may be ignited by burners. Carbon disulphide is extremely hazardous. It has been known to ignite from hot steam pipes or electrical sparks, as from the thermostat on a hot plate, or the motor on a stirrer.
- If an inflammable solvent is spilled, have all workers at the desk turn off their burners and clean it up immediately using a cloth. Wring (squeeze)the solvent from the cloth into the solvent waste can and then rinse the cloth in the sink with much water. Use gloves.
- If Acetone is used to aid in drying glassware, use it sparingly and not near a flame.
- Inflammable solvents which you may have contact with are: Ether, Ligroin (Petroleum ether), Cyclohexane, Toluene, Xylene, Alcohols, Ethyl acetate, Carbon disulphide, Acetone, Dioxane etc. If in doubt about the in flammability of a solvent, assume that it is hazardous.
- Benzene and chlorinated solvents are toxic. In some cases, the toxic effect is cumulative. Avoid contact with the skin and inhalation of solvent vapours.
- Many organic solvents freely permeate latex gloves commonly used in laboratories, and are therefore inadequate protection of the skin from solvent vapours. Thicker neoprene or butyl rubber gloves are recommended.

## 2.2 Dealing with Chemicals:

- Especially corrosive substances which give off noxious fumes (e.g., Bromine, Acetyl chloride, Benzyl chloride, Phosphorus trichloride, Acetic anhydride, fuming Nitric and Sulphuric acids, Chlorosulphonic acid, Benzene sulfonyl chloride, etc.) should be handled in the hoods. Use proper gloves. Do not spill these chemicals on yourself or on the desktops. They will cause very painful burns. Do not put any of these in organic waste cans.
- Over the last several years a number of organic compounds have been confirmed as carcinogens and the list is steadily growing. It is best to assume that all chemicals are toxic, and possibly carcinogenic.
- Sodium and Potassium metals react explosively with water. They are rapidly corroded by the atmosphere and should be stored in kerosene or oil. These metals should not be allowed to come into contact with the skin. They may be handled with dry filter paper or tweezers. Unused pieces of metal may be destroyed by dropping into 95% Ethyl alcohol, or they may be returned to the bottle. Avoid all contact between chlorinated solvents and Sodium or Potassium.
- Concentrated acids and alkalis are corrosive to the desktops, clothing and skin. If there is a spill, first dilute with large volume of water. If an acid, neutralize with solid Sodium bicarbonate; and if a base, with 3% Acetic acid. Sulfuric acid is troublesome, since drops adhering to the tops of bottles tend to absorb moisture and run down the outside of the bottle.

- Mercury and its vapour are poisonous. Avoid spilling; the Institute should have special facilities for cleaning up mercury spills.

## 2.3 Handling Apparatus:

- Approved safety glasses, goggles, or a face shield must be worn at all times when in the lab. Normal prescription lenses are insufficient due to the possibility of explosion.
- When inserting tubing or thermometers into bored stoppers, it is wise to take some simple precautions. The tubing and stopper should be held by a towel, so that if the tubing breaks the towel will reduce the impact of the jagged edge. If the tubing does not enter the hole in the stopper easily, the hole may be made larger with a file (if a cork) or lubricated with water, alcohol, or Glycerine. Hold the tubing close to the stopper. In removing tubing from stoppers, follow the same technique. *Serious cuts have resulted from carelessness in inserting tubes in stoppers*.
- Closed systems are liable to explode if heated. Never carry out an atmospheric pressure distilled in a closed system.
- Do not support apparatus on books, boxes, pencils, etc. Use large, strong wooden blocks, rings, or lab jacks. Assemblies with a high centre of gravity (as when a reagent is added through the top of a condenser) should be assembled and operated with much care.
- Use glass stirring rods with care for breaking up solids. They are liable to break.
- Do not evacuate Erlenmeyer flasks larger than 50 ml (except filtering flasks). They may collapse.
- Oil baths and melting point baths can cause severe burns if spilled. Make sure they are well supported. Be especially careful not to get water into oil baths. Use electric heating mantles in preference to oil baths when possible.
- Dewar flasks and vacuum desiccators, implode easily when tipped over or dropped. Make sure the ones you use are wound on the outside with friction tape or are contained in protective shields, so they will not shower glass around the laboratory if broken.

## 2.4 Dealing with Accidents:

### 2.4.1 Fire:

Personal safety is most important. If a person's clothing catches fire, he/she needs immediate help. Prevent him/her from running. If he/she is close enough, put him/her under the safety shower because it is more effective than a blanket. If not, make him/her lie down and smother (obstruct) the flames by rolling, wrapping with lab coats, blankets, towels, etc. Never turn a Carbon dioxide extinguisher on a person. If a fire breaks out, turn off all burners and remove solvents if time allows. Carbon dioxide extinguishers are must in the laboratory, their positions and operation should be known. Point the extinguisher at the base of the flames. Very small fires can be put out with a damp towel

by smothering. The priority should be the safety of all than the matter of extinguishing the fire is considered. A few seconds delay can result in very serious injury, every person in the laboratory should plan in advance what he/she will do in case of such an emergency.

### 2.4.2 Chemicals:

If corrosive chemicals are spilled on the clothing, immediate showering (with clothing on) is the best remedy. Safety showers are located by each door. If chemicals are spilled on the skin, wash them off with large volumes of water. Bromine should be washed off with water and the skin then massaged with Ethanol or Glycerine. Do not apply a burn ointment. If the chemical is spilled in the eye, it should immediately be washed out thoroughly with water using the eyewash sprayer in the sinks. If acid was involved, a weak solution of Sodium bicarbonate in an eyecup should then be used. If a base, Boric acid is effective. If corrosive chemicals are spilled on the desk, dilute them with a large volume of water and then neutralize with Sodium bicarbonate if an acid, or dilute Acetic acid if a base.

### 2.4.3 First Aid in the Laboratory:

Accidents do not often happen in well-equipped laboratories if one of them understands safe laboratory procedures and are careful in following them. When an occasional accident does occur, it is likely to be a minor one. The following information will be helpful to you if an accident occurs.

- In accidental swallowing of acids or alkali, the mouth must be thoroughly rinsed with water. In the case of acid; rinse the mouth with dilute Sodium carbonate and in case of alkali; with dilute Citric acid. If proper swallowing has occurred, the person should be made to drink water followed by milk in case of acids and lemon juice in case of alkali.
- Skin burns should be washed under running water or ice water and then apply Petroleum jelly or burn ointment and cover with sterile gauge.
- Inhalation injury by toxic fumes is best treated by shifting the person to an open fresh air atmosphere. Irritation to throat can be soothened with hot water vapour inhalation or a warm drink.
- Chemical injury to eyes must be treated by thorough washing with water and then applying 2% Sodium carbonate drops till referred to a specialist.

### 2.4.4 Hazards in the Lab – Important terms:

- **Severe toxicity** - adverse effects of a substance that result either from a single exposure or from multiple exposures in a short space of time (usually less than 24 hours).
- **Irritant** - causes redness, inflammation
- **Corrosive** – "eats away" tissue gradually
- **Carcinogenic** - causes cancer
- **Flammable** - easily set on fire
- **Bio hazard** - substances that pose a threat to the health of living organisms, primarily that of humans.


### 2.4.5 Personal Protective Equipment (PPE):

Personal Protective Equipment or PPE refers collectively to equipment such as safety glasses, goggles, aprons, lab coats, gloves, protective shoes, respiratory protective equipment, ear defenders and similar equipment used to protect the person during their work.

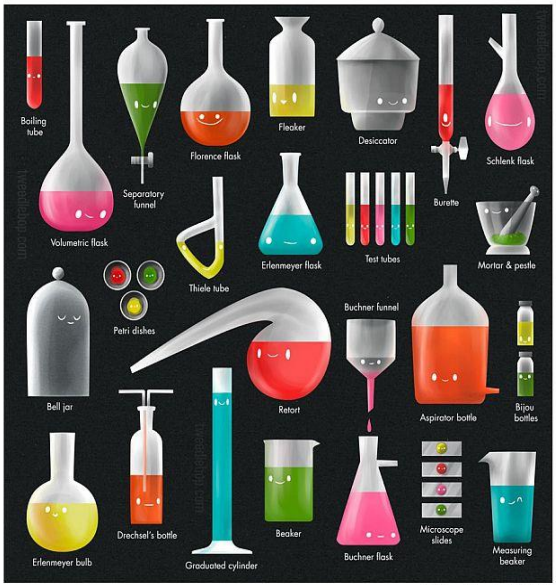

## 2.5 Laboratory Requirements

The basic requirements of a biochemistry laboratory include: good computer attached with a printer, temperature control or cold room, dark room, sinks, and instruments such as autoclave, microscope, water distillation units, hot air ovens, titration assembly, chemical fume hoods, laminar flow work stations, incubators, deep freezer, refrigerators, water baths, refrigerated centrifuges, micro centrifuge, pH meter, trans-illuminator equipped with a camera or electronic imaging system, photometric assembly, electrophoresis units, chromatography assembly, PCR , blotting apparatus, microwave oven, balances, and many other miscellaneous things.

The laboratory needs a variety of glassware including reagent bottles, beakers, measuring cylinders, conical flasks, standard flasks, test tubes, burettes, pipettes, watch glass, glass slides, cover slips, glass rods, petri-dishes, and other culture vessels. Other requirements include syringes, needles, forceps, scalpels, membrane filters (to sterilize heat labile liquids), magnetic stirrers, orbital shakers, inoculation loops, stop watches, nitro cellulose membranes, parafilm, saran wrap, aluminium foil, marker pens, filter paper, ice buckets, latex gloves, plastic boxes,

plastic bags, UV goggles, besides plastic bottles and containers etc. A good supply of chemicals is a very essential aspect of equipping a laboratory as are uninterrupted power and water supply.


**Laboratory Equipments**




**Laboratory Glassware**

## 2.6 Regulations in the Laboratory

- Safety glasses must be worn at all times by students and instructors. Visitors to the lab must be appropriately warned and safety glasses should be made available to them.
- Fume hoods must be used while working with chemicals that produce hazardous fumes.
- The location of fire extinguishers, safety showers, and eyewash stations must be known.
- There must be no unsupervised or unauthorized work going on in the laboratory.
- A laboratory is never a place for practical jokes or pranks.
- The toxicity of all the chemicals you will be working with must be known. Consult the instructor to aware on material safety data sheets (MSDSs), safety charts, and container labels for safety information about specific chemicals. Recently, many common organic chemicals, such as Benzene, Carbon tetra chloride, and Chloroform, have been deemed unsafe.
- Eating, drinking, or smoking in the laboratory is never allowed. Never use laboratory containers (beakers or flasks) to drink beverages.
- Shoes (not open-toed) must always be worn; hazardous chemicals may be spilled on the floor or feet.
- Long hair should always be tied back.
- Mouth pipetting is never allowed.

- Cuts and burns must be immediately treated. Use ice on new burns and consult a doctor for serious cuts.
- In the event of acid spilling on one's body, flush thoroughly with water immediately. Be aware that acid–water mixtures will produce heat. Removing clothing from the affected area while water flushing may be important, so as to not trap hot acid–water mixtures against the skin. Acids or acid–water mixtures can cause very serious burns if left in contact with skin, even if only for a very short period of time.
- Weak acids (such as Citric acid) should be used to neutralize base spills, and weak bases (such as Sodium carbonate) should be used to neutralize acid spills. Solutions of these should be readily available in the lab in case of emergency.
- Dispose all waste chemicals from the experiments according to your instructor's directions.
- In the event of an accident, report immediately to your instructor, regardless of how minor you perceive it to be.
- Always be watchful and considerate of others working in the laboratory. It is important not to jeopardize their safety or yours.
- Always use equipment that is in good condition. Any piece of glassware that is cracked or chipped should be discarded and replaced.

It is impossible to foresee all possible hazards that may manifest themselves in a laboratory. Therefore, it is very important for all students to listen closely to their instructor and obey the rules of their particular laboratory in order to avoid injury.

## 2.7 Keeping Records and Communicating Experimental Results

### 2.7.1 The Laboratory Notebook

All students need to maintain a laboratory notebook. The notebook should be used for the recording of laboratory data and calculations, and is critically important for writing your lab reports. The purpose of a laboratory notebook is to allow anyone with some biochemical knowledge to understand exactly what you did. You need to record the information in sufficient detail so as to be able to repeat it, and you must be able to understand exactly what your results were. You will need good notes to be able to write your lab reports; in addition, as your understanding of biochemistry improves, your notebook may allow you to figure out why some parts of your experiments did not work as expected.

A lab notebook should contain many things:

- A Table of Contents to aid navigation of the notebook.
- A date on each page.

- A written introduction / explanation to yourself of the importance of the experiment.
- Procedural notes (if following a published procedure, there is no need to copy the procedure. Just reference it, and note changes).
- Values collected (i.e., if a protocol called for using 5.0g of NaCl, how much did you actually use? 4.998 g?).
- All results collected along with observations (did the tube turn pink, and the protocol didn't mention that? That is an observation!)
- Analysis of the data – legible tables, graphs, and calculations
- Brief conclusions
- Answers to analysis and comprehension questions for the future.

Lab reports are a more formal presentation of your results. Instead, they focus on clearly explaining the significance of the experiment, and give a careful, well-reasoned and clearly worded analysis of the results, leading the reader to the conclusion.

## 2.8 Using Biochemical Reagents and Solutions

### 2.8.1 Water Purity

Water is the most common and widely-used substance in the biochemistry laboratory. Both the quality and quantity of water required must be considered for each lab application.

Applications of water usage include:

1) Solvent for preparing most buffer and reagent solutions.
2) Column chromatography.
3) High-performance liquid chromatography.
4) Tissue culture; and
5) Washing glassware.

Ordinary tap water contains a variety of impurities including particulate matter, dissolved organics, inorganics, and gases; and microorganisms and the natural degradation of microorganisms leads to the presence of by-products called pyrogens. For most laboratory procedures, it is recommended that some form of purified water be used. The purity of water is usually measured in terms of resistivity. Unit for resistivity is Ohm m **(Ω · m)**. There are five basic water purification technologies distillation, ion exchange, activated carbon adsorption, reverse osmosis, and membrane filtration. For most procedures carried out in the biochemistry lab, water purified by ion-exchange, reverse osmosis, or distillation is usually acceptable. Of these three processes, distillation is the slowest, least energy efficient, least pure and most high maintenance especially in areas with hard water (needs regular de-scaling). Distilled water must also be stored to prevent contamination by microbes. For special procedures such as buffer

standardization, liquid chromatography, and tissue culture, ultra-pure water, which is usually bottled and available commercially, should be used. Water that is purified only by ion-exchange will be low in metal-ion concentration, but may contain certain organics that are washed from the ion-exchange resin. These contaminants will increase the UV-absorbance properties of water. If sensitive UV-spectroscopic measurements are to be made, distilled water is better than de-ionized. If large volumes of high-purity water are required reverse osmosis should be the choice.

### 2.8.2 Cleaning Laboratory Glassware

The results of your experimental work will depend, to a great extent, on the cleanliness of your equipment, especially glassware used for preparing and transferring solutions. There are at least two important reasons for this:

1) Many of the chemicals and biochemicals will be used in milligram, microgram, or even nanogram amounts. Any contamination, whether on the inner walls of a beaker, in a pipette, or in a glass cuvette, could be a significant percentage of the total experimental sample.
2) Many biochemicals and biochemical processes are sensitive to one or more of the following common contaminants: metal ions, detergents, and organic residues. The preferred method for cleaning glassware is to begin with hot tap water. Rinse the glassware at least 10 times with this; then rinse 4–6 times with distilled or de-ionized water. Occasionally it is necessary to use a detergent for cleaning. Use a dilute detergent solution (0.5% in water) followed by 5–10 water rinses with distilled or de-ionized water. When you needed dry glassware in the organic laboratory, you probably rinsed the glassware with Acetone, which rapidly evaporated, leaving a dry surface. But, this technique coats the surface with an organic residue consisting of non-volatile contaminants found in the Acetone. Because this residue could interfere with your experiments, it is best to refrain from acetone washing. Never clean cuvettes or any optically polished glassware with ethanolic KOH or other strong base, as this will cause etching. All glass cuvettes should be cleaned carefully with hot tap water or 0.5% detergent solution, in a sonicator bath or in a cuvette washer, followed by thorough rinsing with purified water.

### 2.8.3 Solutions: Concentrations

The concentrations for solutions used in the biochemistry laboratory may be expressed in several different units. The most common units are:

- **Molarity (M):** concentration based on the number of moles of solute per litre of solution.

- **Percent by weight (% wt/wt):** concentration based on the number of grams of solute per 100 g of solution.
- **Percent by volume (% wt/vol):** concentration based on the number of grams of solute per 100 ml of solution.
- **Weight per volume (wt/vol):** concentration based on the number of grams, milligrams, or micrograms of solute per unit volume.

### 2.8.4 Preparing and Storing Solutions

In general, solid solutes should be weighed on weighing paper or plastic weighing boats, with the use of an electronic analytical or top-loading balance. Liquids are more conveniently dispensed by volumetric techniques; however, this assumes that the density is known. If a small amount of a liquid is to be weighed, it should be added to a tared flask by means of a disposable Pasteur pipette with a latex bulb. The storage conditions of reagents and solutions in the biochemistry lab are especially critical. It is good practice to store all solutions in a closed container. Often it is necessary to store some solutions in a refrigerator at 4$^{o}$C. This inhibits bacterial growth and slows decomposition of the reagents. Some solutions may require storage below 0$^{o}$C. Stored solutions must always have a label containing the name and concentration of the solution, the date prepared, and the name of the preparer. All stored containers, whether at room temperature, or below freezing, must be properly sealed. This reduces contamination by bacteria and vapours in the laboratory air (Carbon dioxide, Ammonia, HCl, etc.). Volumetric flasks, of course, have glass stoppers, but test tubes, Erlenmeyer flasks, bottles, and other containers should be sealed with screw caps, corks, or hydrocarbon foil (Parafilm). Remember that hydrocarbon foil, a wax, is dissolved by solutions containing non-polar organic solvents like Chloroform, Diethyl ether, and Acetone. Bottles of pure chemicals and reagents should also be properly stored.

## 2.9 Quantitative Transfer of Liquids

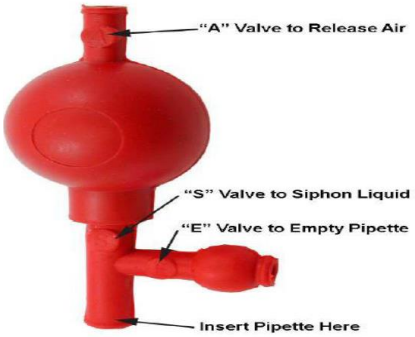
### 2.9.1 Pipettes Fillers:

The use of any pipette requires some means of drawing reagent into the pipette. Liquids should never be drawn into a pipette by mouth suction on the end of the pipette! Small latex bulbs are available for use with disposable pipettes. For volumetric and graduated pipettes, two types of bulbs are available. The features are special conical fitting that accommodates common sizes of pipettes.

Fillers

To use these, first place the pipette tip below the surface of the liquid. Squeeze the bulb with the left hand and then hold it tightly to the end of the pipette.
Slowly release the pressure on the bulb to allow liquid to rise to 2 or 3 cm above the top graduated mark. Then, remove the bulb and quickly grasp the pipette with your index finger over the top end of the pipette. The level of solution in the pipette will fall slightly, but should not fall below the top graduated mark. If it does fall too low, use the bulb to refill.

### 2.9.1.1 Safety Pipette Fillers

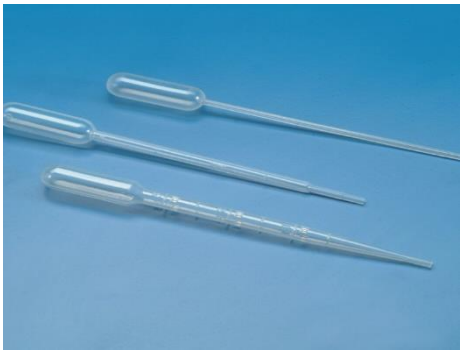



Mechanical pipette fillers (made of silicone and sometimes called safety pipette fillers, propipettes, or pi-fillers) are more convenient than latex bulbs. These fillers are equipped with a system of hand operated valves and can be used for the complete transfer of a liquid.
The three valves (A valve, S valve and E valve) release air, draw liquid in to the pipette, and accurately release liquid.
Never allow any solvent or solution to enter the pipette bulb! To avoid this, two things must be kept in mind:

**i.** Always maintain careful control while using valve S to fill the pipette.
**ii.** Never use valve S unless the pipette tip is below the surface of the liquid. If the tip moves above the surface of the liquid, air will be sucked into the pipette and solution will be flushed into the bulb.

### 2.9.2 Pipettes

### 2.9.2.1 Disposable Pasteur Pipettes

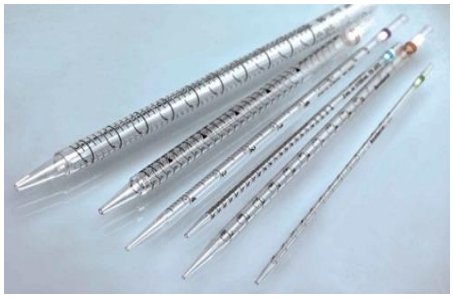


Often it is necessary to perform a semi-quantitative transfer of a small volume (1–10 ml) of liquid from one vessel to another. Because pouring is not efficient, a Pasteur pipette with a small latex bulb may be used. Pasteur pipettes are available in two lengths (15 and 23 cm) and hold about 2 ml of solution. Used disposable pipettes should be discarded in special containers for broken glass.

### 2.9.2.2 Calibrated Pipettes

When a quantitative transfer of a specific and accurate volume of liquid is required, some form of calibrated pipette must be used.

### 2.9.2.2.1 Volumetric Pipettes



Used for the delivery of liquids required in whole-millilitre amounts (1, 2, 5, 10, 15, 20, 25, 50, and 100 ml). To use these pipettes, draw liquid with a latex bulb or mechanical pipette filler to a level 2–3 cm above the fill line. Release liquid from the pipette until the bottom of the meniscus is directly on the fill line.

Touch the tip of the pipette to the inside of the glass wall of the container from which it was filled. Transfer the pipette to the inside of the second container and release the liquid. Hold the pipette vertically; allow the solution to drain until the flow stops, and then wait an additional 5–10 seconds. Touch the tip of the pipette to the inside of the container to release the last drop from the outside of the tip. Remove the pipette from the container. Some liquid may still remain in the tip. Most volumetric pipettes are calibrated as "TD" (To Deliver), which means the intended volume is transferred without final blow-out; that is; the pipette delivers the correct volume.

Fractional volumes of liquid are transferred with graduated pipettes, which are available in two types:

### 2.9.2.2.2 Mohr Pipettes

Mohr pipettes are available in long- or short-tip styles. All Mohr pipettes are TD, and they are available in many sizes (0.1 to 10 ml). The use of a Mohr pipette is similar to that of a volumetric pipette.

### 2.9.2.2.3 Serological Pipettes

Serological pipettes are similar to Mohr pipettes, except that they are graduated downward to the very tip and are designed for blowout. Their use is identical to that of a Mohr pipette except that the last bit of solution remaining in the tip must be forced out into the receiving container with a rubber bulb. This final blow-out should be done after 15–20 seconds of draining.

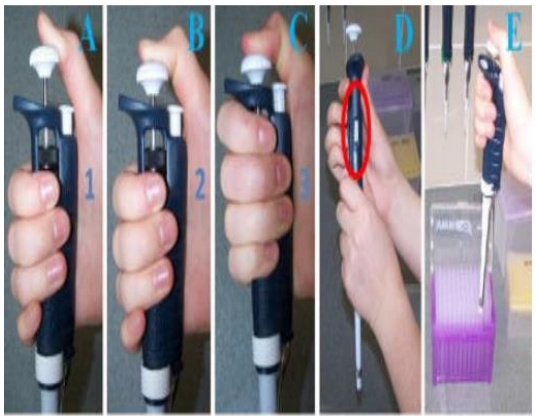
### 2.9.3 Cleaning and Drying Pipettes

Special procedures are required for cleaning glass pipettes. Immediately after use, every pipette should be placed, tip up, in a vertical cylinder containing warm tap water or a dilute detergent solution (less than 0.5%). The pipette must be completely covered with solution. This ensures that any reagent remaining in the pipette is forced out through the tip. If reagent solutions are allowed to dry inside a pipette, the tips can easily become clogged and are very difficult to open. After several pipettes have accumulated in the water or detergent solution, the pipettes should be transferred to a pipette rinser. Pipette rinsers continually cycle fresh water through the pipettes. Immediately after detergent wash, tap water may be used to rinse the pipettes, but distilled water should be used for the final rinse. Pipettes may then be dried in an oven.

### 2.9.4 Automatic Pipetting Devices / Micropipettes

For most quantitative transfers, including many repeated small-volume transfers, a mechanical microlitre pipettor (i.e., Eppendorf type, Pipetteman) is ideal. This allows accurate, precise, and rapid dispensing of fixed volumes from 1 to 10,000 µl ;0.001 to 10ml. The pipettes push-button system can be operated with one hand, and it is fitted with detachable polypropylene tips. The advantage of polypropylene tips is that the amount of reagent film remaining in the pipette after delivery is much less than for glass tips.

**How to use an adjustable pipetting device?**




A-C, three positions of the button of the pipette

D-H, liquid handling with pipettes

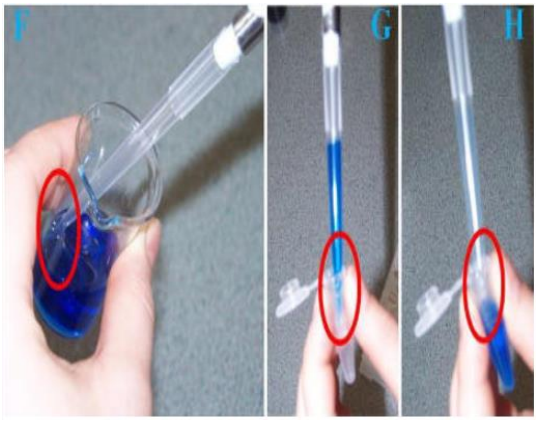
D, adjusting the volume

E, fastening an appropriate tip

F, drawing of the liquid

G, transferring the liquid into an Eppendorf tube

H, dispensing the liquid




Set the desired volume with the digital micrometer or plunger button. Attach a new disposable tip to the shaft of the pipette. Press tip on firmly with a slight twisting motion. Depress the plunger to the first positive stop, immerse the disposable tip into the sample liquid to a depth of 2–4 mm, and allow the

pushbutton to return slowly to the up position
and wait 1–2 seconds.

To dispense sample, place the tip end against the side wall of the receiving vessel and depress the plunge slowly to the first stop. Wait 2–3 seconds, and then depress the plunger to the second stop to achieve final blow-out. Withdraw the device from the vessel carefully with the tip sliding along the inside wall of the vessel. Allow the plunger to return to the up position. Discard the tip by depressing the tip ejector button.

# C. Biochemistry Lab Techniques

## 3. Basic Instrumentation Practices

### 3.1 Mortar and Pestle

General Description

A mortar and pestle can be used to prepare ingredients or substances by crushing and grinding them in to a fine paste or powder. The mortar is a bowl, typically made of hard wood, ceramic or stone. The pestle is a heavy and blunt club shaped object, the end of which is used for crushing and grinding. The substance to be ground is placed in the mortar and ground, crushed or mixed using the pestle.

### 3.2 Desiccator

General Description

Desiccators are sealable enclosures containing desiccants used for preserving moisture sensitive items. A common use for desiccators is to protect chemicals which are hygroscopic or which react with water from humidity. Desiccators are sometimes used to remove traces of water from an almost dry sample. To prevent adsorption of moisture from the surrounding air, glassware is cooled in desiccators.

Where, desiccators alone are unsatisfactory, the sample may be dried at elevated temperature using Abderhalden's drying pistol.

**Parts of a Desiccator**

In laboratory use, the most common desiccators are circular and made of heavy glass. There is usually a removable platform on which the items to be stored are placed. The desiccant, usually an otherwise inert solid such as Silica gel, freshly calcined quicklime or Anhydrous Calcium chloride to absorb water, fills the space under the platform. The

ground glass rim of the desiccator lid must be greased with a thin layer of Petroleum jelly or other lubricant to ensure an airtight seal. A stopcock may be included to permit the desiccators to be evacuated. Such models are usually known as vacuum desiccators. When a vacuum is to be applied, it is a common practice to criss-cross the vacuum desiccators with tape, or to place it behind a screen to minimize damage or injury caused by an implosion. To maintain a good seal, vacuum grease is usually applied to the flanges.

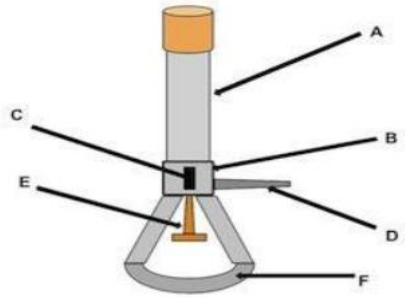
### 3.3 Bunsen burner

General Description

A Bunsen burner, named after Robert Bunsen, is a common piece of laboratory equipment that produces a single open glass flame, which is used for heating, sterilization and combustion. The gas can be natural gas which is mainly Methane or a liquefied petroleum gas, such as Propane, Butane or a mixture of both.

**Parts of a Bunsen burner**

A Bunsen burner is made entirely of metal. In order to function properly, Bunsen burners must have:




(A) **Barrel**, that's approximately five inches long

(B) **Collar** with

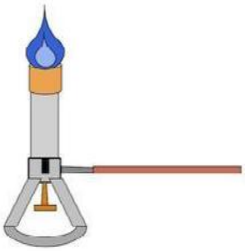
(C) **air holes**

(D)**Gas intake**

(E)**Gas valve**

(F)**Stand**, to keep all of the pieces from making a contact with a work surface.

**Principle**



The amount of air mixed with the gas stream affects the completeness of the combustion reaction. Less air yields an incomplete and thus cooler reaction, while a gas stream well mixed with air provides oxygen in an equimolar amount and thus a complete and hotter reaction. The air flow can be controlled by opening or closing the slot openings at the base of the barrel.

This figure shows the burner producing two sets of blue flames. The inner flame is a smaller and a brighter blue, while the other flame is larger and a darker blue. The flame is hottest between the tip of the smaller flame and the tip of the larger flame.

**Working**

Once connected to a source of fuel, usually Methane, the Bunsen burner can be ignited with a spark. Incoming gas reacts with oxygen in a one-to-three ratio to produce a blue flame that comes out of the top of the barrel.

Adjusting the gas valve and on the Bunsen, burner then changes the volume of gas flow; the more gas entering the burner, the larger the flame. The collar at the base of the barrel contains air holes to control the amount of oxygen reacting with the gas. The collar can be rotated to adjust oxygen intake. In general, the more oxygen present, the more intense and bluer the flame will be. Less oxygen lends to a weaker yellow flame. The more oxygenated blue flame is both hotter and more controlled than the oxygen-deprived flame; scientists generally prefer a controlled blue flame.

1. Air hole closed (safety flame used for lighting or default
2. Air Hole slightly open
3. Air hole half open
4. Air hole fully opens (Roaring blue flame)


### 3.4. Micro Centrifuge

General Description

A micro centrifuge, also called a microfuge, is an important piece of lab equipment; it is used to spin small (2 ml or less) liquid samples at high speeds (generally tens of thousands of times g-force).

**Working**

- Choose a flat area on your table.
- Put the line chord into a suitable 220 V 50 Hz power plug.
- Timer can be set in pulse mode. Timed cycles from 1-15 minutes in increment of 1 minute and continuous mode.
- The RESET- OFF key is to be pressed whenever time or rpm setting is to be changed.
- An audible beep sounds at the beginning and on completion of the cycle

**Points to Remember**

- After centrifugation wipe the inner chamber and keep open to be dried.
- Clean the rotor after use. There are chances for any spill of liquids used.

- Notice if any voltage fluctuation occurs.
- It shows drive fault if any error occurs. In that case switch off and then enter the program.
- Always make sure the required temperature is attained before the rotor starts.

### 3.5. Vortex mixer

General Description

A vortex mixer, or vortexer, is a simple device used commonly in laboratories to mix small vials of liquid. The vortex mixer was invented by the Kraft brothers (Jack A. Kraft and Harold D. Kraft). In a biochemical or analytical laboratory, they may be used to mix the reagents of an assay or to mix an experimental sample and a dilutant.

**Parts of a Vortex mixer**

It consists of an electric motor with the drive shaft oriented vertically and attached to a cupped rubber piece mounted slightly off-centre. As the motor runs the rubber piece oscillates rapidly in a circular motion.

**Working**

When a test tube or other appropriate container is pressed into the rubber cup (or touched to its edge) the motion is transmitted to the liquid inside and a vortex is created. Most vortex mixers have variable speed settings and can be set to run continuously, or to run only when downward pressure is applied to the rubber piece.

### 3.6 Laminar Air Flow Cabinet

General Description

A laminar flow cabinet or laminar flow closet or tissue culture hood is a carefully enclosed bench designed to prevent contamination of semiconductor wafers, biological samples, or any particle sensitive materials.

**Principle**

The principle behind laminar air flow is the passage of continuous air flow at uniform velocity. HEPA filters designed to create a particle free working environment and provide product protection. Air taken through the filtration system is then exhausted across the work surface. Commonly, the filtration system comprises of a prefilter and a HEPA filter. The laminar air flow cabinet is enclosed on the sides and constant positive air pressure is maintained to prevent the intrusion of contaminated room air.

**Instrumentation**

A laminar flow hood consists of a filter pad, a fan and a HEPA (High Efficiency Particulate Air) filter. The fan sucks the air through the filter pad where dust is trapped. After that the prefiltered air has to pass the HEPA filter where contaminating fungi, bacteria, dust etc. are removed. Now the sterile air flows into the working (flasking) area where you can do all your flasking work without risk of contamination. AUVC germicidal lamp to sterilize the interior and contents when not in use (It is important to switch this light off during use, to limit exposure to skin and eyes as stray ultraviolet light emissions can cause cancer and cataracts).

### 3.7. Electronic Weighing Balance

General Description

Balances are designed to meet the specific weighing requirement in the laboratory working environment. These balances come in precision designs and operating characteristics that allows making quick and accurate measurements.

**Principle**

Electronic weighing balance accurately measures the weight of chemicals. Calibrate the balance by internal calibration. Place the weighing boat and tare the weight. Wait till it becomes zero. Chemical should be weighed slowly according to the need. Wait till the symbol ‘g’ stabilizes next to the weight shown.

**Note**

Always have the knowledge about the maximum and minimum quantity which can be weighed using the balance. Never spill the chemicals on the weighing pan, if it happens wipe off with tissue. Switch off fan and close windows nearby when working with it as it may cause fluctuations in the value due to interaction with air density. Always use weigh boats or butter paper to weigh.

### 3.8 Magnetic Stirrer

General Description

Magnetic stirrer employs a rotating magnetic field to cause a stir bar (also called "flea") immersed in a liquid to spin very quickly, thus stirring it. The rotating field may be created either by a rotating magnet or a set of stationary electromagnets, placed beneath the vessel with the liquid.

A stir bar is the magnetic bar placed within the liquid which provides the stirring action. The stir bar's motion is driven by another rotating magnet or assembly of electromagnets in the stirrer device, beneath the vessel containing the liquid. Stir bars are typically coated in Teflon, or less often in glass.

**Components of a Magnetic Stirrer**

A stirrer magnet placed in the liquid and a magnetic drive located outside the vessel. Both, stirrer magnet and magnetic drive form a magnetic circuit. For trouble-free stirring in liquids with different viscosities the magnetic drive shall have a wide range of different speeds.

**Principle**

In principle, it is difficult to find the most effective magnetic stirring bar for a particular application, but important factors are the vessel shape and the viscosity of the stirring medium. The ideal configuration is where the magnet of the stirring bar and the magnet of the drive are of equal length and with a minimum distance between them.

### 3.9 Water Bath

General Description

A water bath is made from a container filled with heated water. It is used to incubate samples in water at a constant temperature over a long period of time. All water baths have a digital or an analogue interface to allow users to set a desired temperature.

Utilizations include warming of reagents, melting of substrates or incubation of cell cultures. It is also used to enable certain chemical reactions to occur at high temperature. Water bath is a preferred heat source for heating flammable chemicals instead of an open flame to prevent ignition. Different types of water baths are used depending on application. For all water baths, it can be used up to 99.9°C.

**Principle**

It is a system for the control of temperature in which a vessel containing the material to be heated is set into or over one containing water and receiving the heat directly.

**Working**

It has a double walled, outer body made of MS sheet, powder coated, inner body made of stainless steel, and 304 quality sheet glass wool insulation, fitted with 30$^{o}$C to 110$^{o}$C thermostat. By means of these, heat is transferred to the medium (water or oil) until reaching the temperature selected with a control device (thermostat or similar). It is

provided with concentric rings with a diameter of 75 mm suitable to work on 220V AC supply. It is accompanied with a digital display temperature controller. In general, they use water, but some baths use oil.

Before using the water bath, verify that it is clean and that accessories needed are installed. The steps normally followed are:

- Fill the water bath with fluid to keep the temperature constant (water or oil). Verify that once the containers to be heated are placed, the fluid level is between 4 and 5 cm from the top of the tank.
- Install the control instruments needed, such as thermometers and circulators. Use additional mounts provided for this purpose. Verify the position of the thermometers bulb or thermal probe to ensure that the readings are correct.
- If water is used as the warming fluid, verify that it is clean. Some manufacturers recommend adding products which prevent the formation of fungus or algae.
- Put the main switch No.1 in the ON position (the numbers identifying the controls herein correspond to those shown in the diagram). Some manufacturers have incorporated controls with microprocessors which initiate auto-verification routines once the ON switch is activated.
- Select the operation temperature using the menu No.2 buttons and the buttons for adjusting the parameters.
- Select the cut-off temperature (in water baths with this control). This is a safety control which cuts off the supply of electricity if it exceeds the selected temperature. This is selected also by using the menu button and is controlled by the parameter adjustment buttons.

**Avoid using the water bath with the substances indicated below:**

- Bleach
- Liquids with high Chlorine content
- Weak saline solutions such as Sodium chloride, Calcium chloride or chromium compounds
- Strong concentrations of any acid
- Strong concentrations of any salt
- Weak concentrations of Hydrochloric, Hydrobromic, Hydroiodic, Sulphuric or Chromic acids
- Deionised water, as it causes corrosion and perforation in the stainless-steel Safety
- Avoid the use of the water bath in environments where there are flammable and combustible materials. The equipment has components (resistors generating very high temperatures) which could start an accidental fire or explosion.

- Always connect the equipment to an electrical outlet with a ground pole to protect the user and the equipment from electrical discharges.
- Use the water bath exclusively with non-corrosive or non-flammable liquids.
- When working with substances that generate vapours, place the water bath under a chemical hood or in a well-ventilated area.
- Remember that liquids incubated in the water bath tank can produce burns if hands are inadvertently placed inside it.
- Consider that the water bath is designed for use with a liquid inside the tank. If the inside is dry, the temperature of the tank can become very high. Use the diffusing tray for placing the container inside of the filled tank of the water bath. This has been designed for distributing the temperature in a uniform way.
- Avoid using the water bath if any of its controls is not working, e.g. the temperature or limit controls.

**Cleaning**

Frequency: Monthly

- Turn off and disconnect the equipment. Wait until it cools to avoid the risk of burns and accidents.
- Remove the fluid used for heating. If it is water, it can be poured through a siphon. If it is oil; collect into a container with an adequate capacity.
- Remove the thermal diffusion grid located at the bottom of the tank.
- Disassemble the circulator and clean to remove scale and potential algae present.
- Clean the interior of the tank with a mild detergent. If there is any indication of corrosion, use substances for cleaning stainless steel. Rub lightly with synthetic sponges or equivalent. Avoid using steel wool to remove rust stains as these leave particles of steel which could accelerate corrosion.
- Avoid bending or striking the temperature control capillary tube generally located at the bottom of the tank.
- Clean the exterior and interior of the water bath with clean water.

### 3.10 pH Meter

General Description

pH meter used for potentiometrically measuring the pH, which is either the concentration or the activity of hydrogen ions, of an aqueous solution. It usually has a glass electrode plus a calomel reference electrode, or a combination electrode. pH meters are usually used to measure the pH of liquids, though

special probes are sometimes used to measure the pH of semi-solid substances.

**Components of a pH meter**

Basic potentiometric pH meters simply measure the voltage between two electrodes and display the result converted into the corresponding pH value. They comprise a simple electronic amplifier and a pair of probes, or a combination probe, and some form of display calibrated in pH. The probe is the key part; it is a rod-like structure usually made of glass, with a bulb containing the sensor at the bottom. Frequent calibration with solutions of known pH, perhaps before each use, ensures the best accuracy. To measure the pH of a solution, the probe is dipped into it.

**Principle**

pH measurement is based on the use of a pH sensitive electrode (usually glass), a reference electrode, and a temperature element to provide a temperature signal to the pH analyzer. The pH electrode uses a specially formulated, pH sensitive glass in contact with the solution, which develops a potential (voltage) proportional to the pH of the solution. The reference electrode is designed to maintain a constant potential at any given temperature, and serves to complete the pH measuring circuit within the solution. It provides a known reference potential for the pH electrode. The difference in the potentials of the pH and reference electrodes provides a millivolt signal proportional to pH.

**Working**

- Turn on pH Meter. Lift up the electrode and clean the electrode tip by pressing with tissue paper.
- Calibrate using buffer 4±0.01 and buffer 7±0.01
- The buffers should come to the room temperature before calibration.
- Place the electrode in the solution to know the pH.
- If there is an increase in pH, stabilize it with adding 0.1N HCl which lowers the pH. If there is a decrease in pH, stabilize it with adding 0.1N NaOH which increases the pH.
- Add acid or alkali drop by drop and always stir well the solution with glass rod after each addition.

**Note**

- Read the pH when √A appears.
- Always keep the electrode dipped in 3 mol/l KCl solution, never leave the electrode dry.

- Be cautious about the electrode level displayed on the screen. It symbolizes the fitness of electrode. Any kind of unusual appearance can cause pH fluctuation.
- Buffer solutions should always be clear without any turbidity or mycelia growth. Same is the case if the buffer is used to protect electrode.
- Always prepare fresh buffer solutions every month for calibration.
- Always prepare buffer solutions in sterile water.
- Never mishandle the electrode like using it for stirring the solutions.
- Always use sterile water to clean the electrode after dipping it in solutions.
- Never place the electrode in solutions which are in extreme temperature conditions.
- No hurry should be there for reading the pH.
- Do not forget to make entry with appropriate comments in the log book.
- The equipment and its premises should be clean if there is any spill.

### 3.11 Microscope

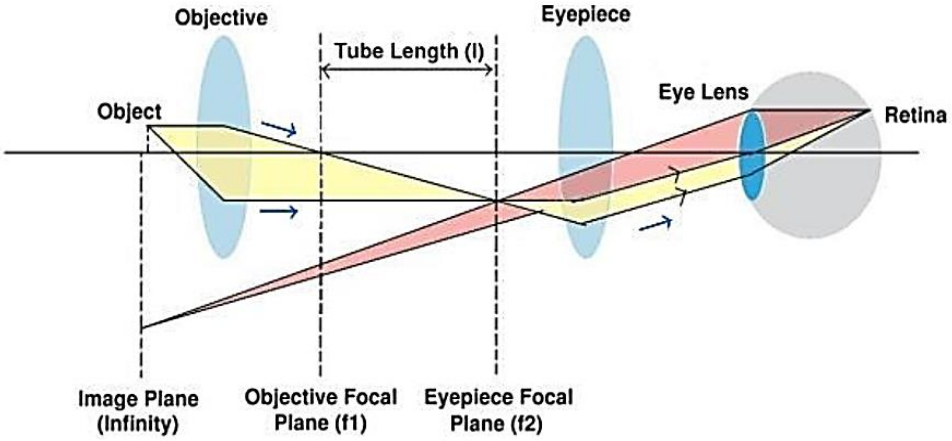
General Description

A microscope is an instrument used to see objects that are too small for the naked eye. The science of investigating small objects using such an instrument is called microscopy.

The most commonly used microscope for general purposes is the standard compound microscope. It magnifies the size of the object by a complex system of lens arrangement.

**Principle**

It has a series of two lenses; (i) the objective lens close to the object to be observed and (ii) the ocular lens or eyepiece, through which the image is viewed by eye. Light from a light source (mirror or electric lamp) passes through a thin transparent object. The objective lens produces a magnified 'real image' (first image) of the object. This image is again magnified by the ocular lens (eyepiece) to obtain a magnified virtual image (final image), which can be seen by the eye through the eye piece. As light passes directly from the source to the eye through the two lenses, the field of vision is brightly illuminated. That is why; it is a bright-field microscope.



## Parts of a Microscope

**(I) Mechanical Parts:** These are the parts, which support the optical parts and help in their adjustment for focusing the object.

- Base or Metal Stand: The whole microscope rests on this base. Mirror, if present, is fitted to it.
- Pillars: It is a pair of elevations on the base, by which the body of the microscope is held to the base.
- Inclination joint: It is a movable joint, through which the body of the microscope is held to the base by the pillars. The body can be bent at this joint into any inclined position, as desired by the observer, for easier observation.
- Curved Arm: It is a curved structure held by the pillars. It holds the stage, body tube, fine adjustment and coarse adjustment.
- Body Tube: It is usually a vertical tube holding the eyepiece at the top and the revolving nosepiece with the objectives at the bottom. The length of the draw tube is called 'mechanical tube length' and is usually 140-180 mm (mostly 160 mm).
- Draw Tube: It is the upper part of the body tube, slightly narrower, into which the eyepiece is slipped during observation.
- Coarse Adjustment: It is a knob with rack and pinion mechanism to move the body tube up and down for focusing the object in the visible field. As rotation of the knob through a small angle moves the body tube through a long distance relative to the object, it can perform coarse adjustment.
- Fine Adjustment: It is a relatively smaller knob. Its rotation through a large angle can move the body tube only through a small vertical distance. It is used for fine adjustment to get the final clear image.
- Stage: It is a horizontal platform projecting from the curved arm. It has a hole at the centre, upon which the object to be viewed is placed on a slide. Light comes from the light source below the stage passes through the object into the objective.
- Mechanical Stage (Slide Mover): Mechanical stage consists of two knobs with rack and pinion mechanism. The slide containing the object is clipped to it and moved on the stage in two dimensions by rotating the knobs, so as to focus the required portion of the object.
- Revolving Nosepiece: It is a rotatable disc at the bottom of the body tube with three or four objectives screwed to it. The objectives have different magnifying powers. Based on the required magnification, the nosepiece is rotated, so that only the objective specified for the required magnification remains in line with the light path.

**(II) Optical Parts:** These parts are involved in passing the light through the object and magnifying its size.

- Light Source: Modern microscopes have in-built electric light source in the base. The source is connected to the mains through a regulator, which controls the brightness of the field.
- Diaphragm: If light coming from the light source is brilliant and all the light is allowed to pass to the object through the condenser, the object gets brilliantly illuminated and cannot be visualized properly. Therefore, an iris diaphragm is fixed below the condenser to control the amount of light entering into the condenser.
- Condenser: The condenser or sub-stage condenser is located between the light source and the stage. It has a series of lenses to converge on the object, light rays coming from the light source. After passing through the object, the light rays enter into the objective.

The light 'condensing', 'light converging' or 'light gathering' capacity of a condenser is called 'numerical aperture of the condenser'. Similarly, the 'light gathering' capacity of an objective is called numerical aperture of the objective. If the condenser converges light in a wide angle, its numerical aperture is greater and vice versa.

There are three types of condensers as follows:

**A.** Abbe condenser (Numerical aperture=1.25): It is extensively used.
**B.** Variable focus condenser (Numerical aperture =1.25)
**C.** Achromatic condenser (Numerical aperture =1.40): It has been corrected for both spherical and chromatic aberration and is used in research microscopes and photomicrographs.

- **Objective**: It is the most important lens in a microscope. Usually three objectives with different magnifying powers are screwed to the revolving nosepiece.

**The objectives are:**

(a) Low power objective (X 10): It produces ten times magnification of the object.

(b) High dry objective (X 40): It gives a magnification of forty times.

(c) Oil-immersion objective (X100): It gives a magnification of hundred times, when immersion oil fills the space between the object and the objective.

The scanning objective (X4) is optional. The primary magnification (X4, X10, X40 or X100) provided by each objective is engraved on its barrel. The oil-immersion objective has a ring engraved on it towards the tip of the barrel.

## 3.12 Refractometer (Abbe's Hand Refractometer)

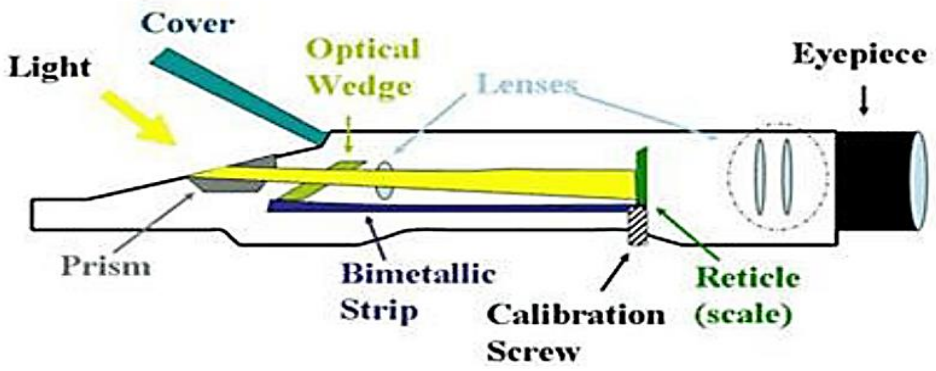
General Description

A refractometer is an optical instrument that is used to determine the refractive index of a substance, measuring how light is bent as it moves through the substance.

**Principle**

The instrument works on the critical angle principle, utilizing lenses and prisms to project a shadow line onto a small glass reticle inside the refractometer, which is then viewed by the inspector through a magnifying eyepiece.

The Fig. shows light enters from the left and passes through the liquid sample. When the light hits the prism at the bottom of the liquid, it suddenly is slowed more than in the liquid because the prism has a higher refractive index.




In the case of a refractometer, the light bends in proportion to the liquid's refractive index. As the light then travels down the refractometer, it passes through lenses and lands on a scale. The bending of the light at the liquid/prism interface sends the light higher or lower in the scale's grid. Observer then looks through the viewfinder on the other end and read where the light is falling on the scale. Light covers a portion of the scale, and the remainder is dark. The dividing line between light and dark is the place to read the scale. Calibration is accomplished by turning the calibration screw, which raises or lowers the reticle (the scale) relative to the path of the light.

**Working**

Operation consists of placing 1 or 2 drops of the water sample on the prism, closing a glass plate over the sample, then looking through the eyepiece for the reading. The water sample is sandwiched between the measuring prism and the cover plate. Light traveling through the sample is either passed through or totally internally reflected.

The net effect is that a shadow line is formed between the illuminated area and the dark area. It is at the point that this shadow line crosses the scale that a reading is taken.

### 3.13 Soxhlet Extractor

General Description

A Soxhlet extractor is a piece of laboratory apparatus invented in 1879 by Franz von Soxhlet. It was originally designed for the extraction of a lipid from a solid material. Typically, a Soxhlet extraction is used when the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. It allows for unmonitored and unmanaged operation while efficiently recycling a small amount of solvent to dissolve a larger amount of material.

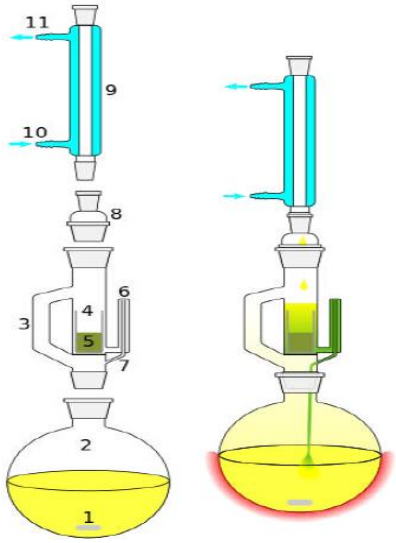
**Principle**

The solvent is heated to reflux. The solvent vapour travels up a distillation arm and floods into the chamber housing the thimble of solid. The condenser ensures that any solvent vapour cools, and drips back down into the chamber housing the solid material. The chamber containing the solid material slowly fills with warm solvent. Some of the desired compound dissolves in the warm solvent. When the Soxhlet chamber is almost full, the chamber is emptied by the siphon. The solvent is returned to the distillation flask. The thimble ensures that the rapid motion of the solvent does not transport any solid material to the still pot. This cycle may be allowed to repeat many times, over hours or days. During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the desired compound is concentrated in the distillation flask.

**Instrumentation**

A Soxhlet extractor has three main sections: A percolator (boiler and reflux) which circulates the solvent, a thimble (usually made of thick filter paper) which retains the solid to be laved, and a siphon mechanism, which periodically empties the thimble.

1. Stirrer bar
2. Still pot (the still pot should not be overfilled and the volume of solvent in the still pot should be 3 to 4 times the volume of the Soxhlet chamber).
3. Distillation path
4. Thimble
5. Solid
6. Siphon top
7. Siphon exit
8. Expansion adapter
9. Condenser
10. Cooling water in
11. Cooling water out




**Working**

Normally a solid material containing some of the desired compound is placed inside a thimble made from thick filter paper, which is loaded into the main chamber of the Soxhlet extractor. The Soxhlet extractor is placed onto a flask containing the extraction solvent. The Soxhlet is then equipped with a condenser. The solvent is heated to reflux. The solvent vapour travels up a distillation arm and floods into the chamber housing the thimble of solid. The condenser ensures that any solvent vapour cools, and drips back down into the chamber housing the solid material. The chamber containing the solid material slowly fills with warm solvent. Some of the desired compound will then dissolve in the warm solvent. When the Soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. This cycle may be allowed to repeat many times, over hours or days. During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the desired compound is concentrated in the distillation flask. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. After extraction the solvent is removed, typically by means of a rotary evaporator, yielding the extracted compound. The insoluble portion of the extracted solid remains in the thimble, and is usually discarded.

### 3.14 Muffle Furnace


General Description

A muffle furnace is a front-loading box-type oven or kiln for high temperature applications such as fusing glass, creating enamel coatings, ceramics and soldering

and brazing articles. They are used in order to determine what proportion of a sample is non-combustible and non-volatile that is called as ash.

**Principle**

Muffle furnaces are usually heated to desired temperatures by conduction, convection, or blackbody radiation from electrical resistance heating elements. Therefore, there is (usually) no combustion involved in the temperature control of the system, which allows for much greater control of temperature uniformity and assures isolation of the material being heated from the by-products of fuel combustion.

**Instrumentation**

The furnace chamber is heated by electric resistance elements and is insulated with ceramic fibre insulation. The controller is located under the furnace chamber and is well insulated from the heat generated in the furnace chamber. A door safety switch removes power to the heating elements whenever the furnace door is opened. The temperature is controlled by one of three types of controllers.

### 3.15 Titration Assembly

General Description

Titration, also known as titrimetry, is a common laboratory method of quantitative chemical analysis that is used to determine the unknown concentration of an identified analyte. Since volume measurements play a key role in titration, it is also known as volumetric analysis. A reagent, called the titrant or titrator is prepared as a standard solution. A known concentration and volume of titrant reacts with a solution of analyte or titrant to determine concentration. The volume of titrant reacted is called titration volume.

**Principle**

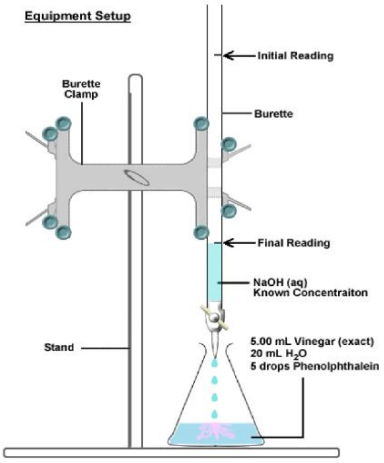
Titration is a process by which the concentration of an unknown substance in solution is determined by adding measured amounts of a standard solution that reacts with the unknown. Then the concentration of the unknown can be calculated using the stoichiometry of the reaction and the number of moles of standard solution needed to reach the so-called end point.

- The analyte: the solution of unknown concentration is known as the analyte. During titration the titrant is added to the analyte in order to achieve the equivalence point and determine the concentration of the analyte.

- The standard solution: the solution of known concentration. An accurately measured amount of standard solution is added during titration to the solution of unknown concentration until the equivalence or endpoint is reached.

**Components of a Titration Assembly**

The technique uses a particular set of apparatus with which volumes of solutions can be measured to an accuracy of greater than 0.1cm3.




| | |
|---|---|
| **1. Burette** | Used to deliver and measure the volume of a solution. |
| **2. Pipette** | Used to deliver an accurate volume (aliquot) of solution. |
| **3.Volumetric flask** | Used to prepare and store a standard solution of known concentration. |
| **4. Conical Flask** | Used to contain the reaction mixture (the initial pipetted aliquot of solution plus the addition of solution from the burette). |
| **(Erlenmeyer Flask)** | Used to facilitate pouring of solution into the burette. |
| **5.Glass Funnel** | Used to facilitate the acquisition of an aliquot of solution using the pipette, and, helps prevent possible contamination of stock solution. |
| **6. Beaker** | Perfect for dispensing liquids or for cleaning glasswares. |

In a titration the pipette is used to transfer 25cm3 (usually to ±0.05 cm3) of a solution in to a conical flask another solution reacts with the pipette solution in the conical flask is carefully added from a burette until it has all exactly reacted. This is called the end point of the titration (or equivalence point of the titration). There needs to be a way of knowing when the end point is reached. An indicator of some kind may be needed. For example, in the titration of strong acid and a strong base a few drops of Methyl orange or Phenolphthalein could be used.

**Procedure**

A typical titration begins with a beaker or Erlenmeyer flask containing a very precise volume of the analyte and a small amount of indicator placed underneath a calibrated burette or chemistry pipetting syringe containing the titrant. Small volumes of the

titrant are then added to the analyte and indicator until the indicator changes colour in reaction to the titrant saturation threshold, reflecting arrival at the endpoint of the titration. Depending on the endpoint desired, single drops or less than a single drop of the titrant can make the difference between a permanent and temporary change in the indicator. When the endpoint of the reaction is reached, the volume of reactant consumed is measured and used to calculate the concentration of analyte by,

$$Ca = \frac{Ct \times Vt \times M}{Va}$$

Where, Ca is the concentration of the analyte, typically in molarity; Ct is the concentration of the titrant, typically in molarity; Vt is the volume of the titrant used, typically in liters; M is the mole ratio of the analyte and reactant from the balanced chemical equation; and Va is the volume of the analyte used, typically in liters.

**Elements of Titration**

- The end point: It indicates once the equivalence point has been reached. It is indicated by some form of indicator which varies depending on what type of titration being done. For example, if a colour indicator is used, the solution will change colour when the titration is at its end point. The equivalence point is the ideal point for the completion of titration. At the equivalence point the correct amount of standard solution must be added to fully react with the unknown concentration.
- Equivalence point: Is when the moles of a standard solution (titrant) equal the moles of a solution of unknown concentration (analyte).
- The calibrated burette: it is the main piece of equipment required for a titration method. Calibration is important because it is essential for the burette to be as accurate as possible in order to dispense very precise amounts of liquid into the sample. A burette is a long cylindrical piece of glass with an open top for pouring in the titrant. At the bottom there is a carefully formed tip for dispensing. Burettes usually have a plastic stopper that can easily be turned to deliver mere fractions of a drop of titrant, if needed. Burettes come in many sizes and are marked in millilitres and fractions of millilitres.
- The Indicator: the use of an indicator is in performing a successful titration reaction. The purpose of the indicator is to show when enough standard solution has been added to fully react with the unknown concentration. Indicators must only be added to the solution of unknown concentration when no visible reaction will occur. Depending on the solution being titrated, the choice of indicator can become key for the success of the titration.

### 3.16 Distillation Assembly

General Description

In this equipment liquid is vaporized (turned to steam), re-condensed (turned back into a liquid) and collected in a container. Distillation is a process of separating the component substances from a liquid mixture by selective evaporation and condensation. Distillation may result in essentially complete separation (nearly pure components), or it may be a partial separation that increases the concentration of selected components of the mixture. In either case the process exploits differences in the volatility of mixtures components.

Laboratory scale distillations are almost exclusively run as batch distillations. The device used in distillation, sometimes referred to as a still, consists at a minimum of a reboiler or pot in which the source material is heated, a condenser in which the heated vapour is cooled back to the liquid state, and a receiver in which the concentrated or purified liquid, called the distillate, is collected.

Batch distillation refers to the use of distillation in batches, meaning that a mixture is distilled to separate it into its component fractions before the distillation still is again charged with more mixture and the process is repeated.

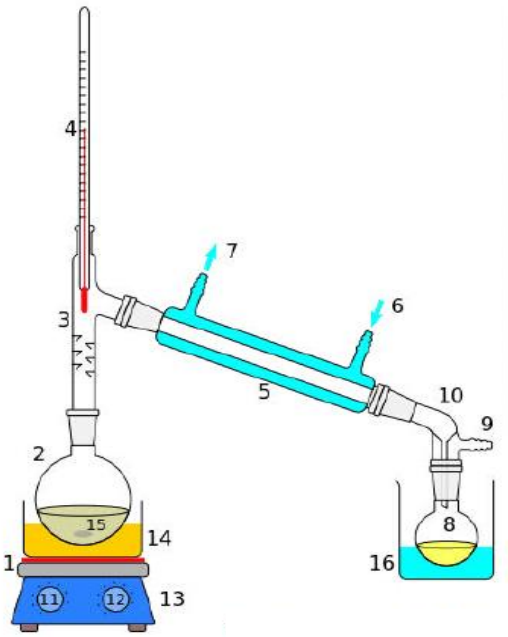
**Principle**

The separation of components from a liquid mixture via distillation depends on the differences in boiling points of the individual components. Also, depending on the concentrations of the components present, the liquid mixture will have different boiling point characteristics. Therefore, distillation processes depend on the vapour pressure characteristics of liquid mixtures. The liquid boils when its vapour pressure equalizes with the surroundings.

**Components of a Distillation Assembly**

1. A source of heat
2. Still pot
3. Still head
4. Thermometer/ Boiling point temperature
5. Condenser

6. Cooling water in
7. Cooling water out
8. Distillate/receiving flask
9. Vacuum/gas inlet
10. Still receiver
11. Heat control
12. Stirrer speed control
13. Stirrer/heat plate
14. Heating (Oil/sand) bath
15. Stirring means e.g. boiling chips or mechanical stirrer
16. Cooling bath.




### 3.17 Microwave Oven

General Description

Microwave ovens are used for heating and defrosting in laboratories. Microwave heating, which uses electromagnetic energy in the frequency range 300- 3000 MHz, can be used successfully to heat many dielectric materials.

However, improper use of a microwave can pose a number of hazards including:

- Ignition of flammable vapours
- Electrical shock from ungrounded or faulty units
- Ignition of materials being heated
- Pressure build-up in sealed containers
- Integrity of containers holding materials
- Sudden boiling of liquid in an open container following removal

**Principle**

A microwave oven heats samples by passing microwave radiation through it. Microwaves are radio waves. In the case of microwave ovens, the commonly used radio wave frequency is roughly 2,500 Megahertz (2.5 gigahertz). Radio waves in this frequency range have an interesting property: they are absorbed by water, fats and sugars. When they are absorbed they are converted directly into atomic motion and motion is converted into heat. Microwaves in this frequency range have another interesting property: they are not absorbed by most plastics, glass or ceramics. And

metal reflects microwaves, which is why metals cause spark in a microwave oven. The reason that metal reflects microwaves is that no electronic waves resident in inside of conductor because conductor's conductivity is infinity as we studied in our course. The property is possible because the frequency 2,500 megahertz is resonance frequency of water. Molecules of all food are consisting of a dipole and have positive charge in one side and have negative charge in another side. If we put electromagnetic fields in this, all molecules are rearranged: positive charge is to negative.

Pole and charge is to positive pole. In this process molecules heat is produced by Friction. The frequency of microwave oven is 2,500 megahertz as we saw before. Then Microwave of this frequency change the direction of electromagnetic fields 2,500,000,000 times in 1 second. Consequently, the heat efficiency of a microwave oven is high. In a conventional oven, the heat has to migrate (by conduction) from the outside of the food toward the middle. In microwave cooking, the radio waves penetrate the food and excite water and fat molecules pretty much evenly throughout the food. There is no "heat having to migrate toward the interior by conduction. There is heat everywhere all at once because the molecules are all excited together. There are limits of course. Radio waves penetrate unevenly in thick pieces of food (they don't make it all the way to the middle), and there are also "hot spots" caused by wave interference. The whole heating process is different. In a microwave oven, the air in the oven is at room temperature, so there is no way to form a crust. That is because it heats up foods by 'microwaves' instead of 'heat conduction'.

**Steps to Working Safely with Microwaves**

- Never attempt to heat, flammable liquids or solids, hazardous substances or radioactive materials in any type of microwave oven.
- Do not place metal items inside the microwave, including aluminium foil and plastic-coated magnetic stirrer bars.
- Do not modify the microwave in any way, including the removal of the grounding pin or change of the plug.
- Never use a laboratory microwave for food preparation (or kitchen microwave for laboratory materials).
- Do not heat-sealed containers in a microwave. Even a loosened cap or lid poses a significant risk since microwaves can heat material so quickly that the container explodes either in the oven or shortly after removal.
- Take care to avoid overheating liquids. It is possible to raise water to a temperature greater than normal boiling point; when this occurs, any disturbance to the liquid can trigger violent boiling that could result in severe burns.

**Additional safety measures must be considered when using a microwave to melt agar**

- Large amounts (e.g. 250 ml) of solidified agar should not be warmed in a microwave unless first chopped up with a sterile spatula. Not doing this can cause explosive vapourization in solid agar where vapour cannot escape.
- Use loose fitting sterile foam plugs or loose 'Kim wipe" plugs, rather than just relying on loosely placed cap.
- Ensure a good amount of headspace is available in the container above the material being heated.
- Set the power and timings correctly. Do not overheat.
- Thermal gloves and a face shield must be worn when removing a container from the microwave.
- Care should be taken when placing the container on the bench as not to cause unnecessary disturbance of the agar, which may cause the agar to boil over.

### 3.18 Hot Air Oven

General Description

It is a dry heat sterilization unit. A dry heat cabinet is easy to install and has relatively low operating costs; it is nontoxic and does not harm the environment and it is noncorrosive for metal and sharp instruments.

**Principle**

Sterilization by dry heat is accomplished through conduction. The heat is absorbed by the outside surface of the equipment, and then passes towards the centre of it, layer by layer.

The entire system will eventually reach the temperature required for sterilization. Dry heat does most of the damage by oxidizing molecules. The essential cell constituents are destroyed and the organism dies. The temperature is maintained for almost an hour to kill the most difficult of the resistant spores. The most common time-temperature relationships for sterilization with hot air sterilizers are:

1. 170$^{o}$C (340$^{o}$F) for 60 minutes,
2. 160$^{o}$C (320$^{o}$F) for 120 minutes,
3. 150$^{o}$C (300$^{o}$F) for 150 minutes or longer depending upon the volume

**Working**

Working principle of hot air oven is the forced circulation of hot air inside the chamber of oven. As it is a universal scientific fact that in any chamber hot air rises above, so by utilizing this principle when the hot air reaches the top of chamber it is circulated back to bottom by a fan installed inside the chamber and hence optimum amount of heat is achieved gradually inside the hot air oven.

There are two types of dry-heat sterilizers: one is the static-air type and the other is forced-air type. The static-air type is referred to as the oven-type sterilizer as heating coils in the bottom of the unit cause the hot air to rise inside the chamber via gravity convection. This type of dry-heat sterilizer is much slower in heating, requires longer time to reach sterilizing temperature, and is less uniform in temperature control throughout the chamber than is the forced-air type.

**Note**

Dry heat sterilization technique requires longer exposure time (1.5 to 3 hours) and higher temperatures than moist heat sterilization. Dry heat ovens are used to sterilize items that might be damaged by moist heat or that are impenetrable to moist heat (e.g., powders, petroleum products, sharp instruments).

The hot air oven is mounted on four rubber foots to prevent slipping and this protects the bench surface. The control panel houses a main ON/OFF switch indicator lamp and temperature setting knob. The scale is calibrated in 5°C steps.

### 3.19 Autoclave

General Description

An autoclave is a large pressure cooker. It is a moist sterilization unit. It is a pressure chamber used to sterilize equipment and supplies by subjecting them to high pressure saturated steam at 121°C (249°F) for around 15–20 minutes depending on the size of the load and the contents. It was invented by Charles Chamberland in 1879.

**Parts of an Autoclave**

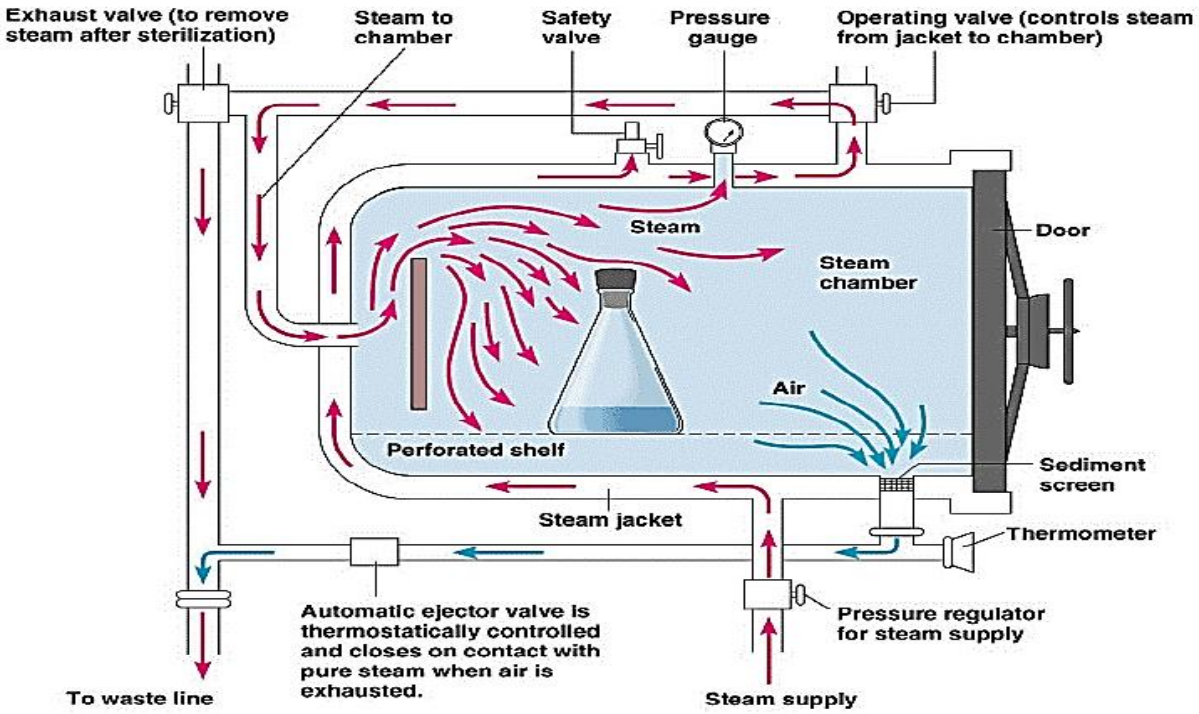
Autoclaves have four basic parts

- Water Intake: An autoclave needs water to make the steam used for sterilization. A water intake hook-up or hose, it allowing the user to pump water directly into the machine.

- Chamber: The chamber is the space where the user places items to sterilize. In the chamber are wire racks, which will hold various items upright or lying down and allow for steam penetration from all angles.
- Control Panel: The control panel allows the user to customize the autoclaving process. Some materials can withstand higher temperatures, while some must be autoclaved at lower temperatures for longer time.
- Machinery: Autoclaves must have an air pump system to remove the oxygen in the chamber and create a vacuum which then fills with pressurized steam created from the water in the reservoir. The water becomes heated either via a heating element inside the water reservoir or a heat-generating mechanism that completely surrounds the reservoir.

## Principle

It operates under the principle of steam under pressure as the sterilizing agent. High pressures enable steam to reach high temperatures, thus increasing its heat content and killing power. Most of the heating power of steam comes from its latent heat of vapourization (the amount of heat required to convert boiling water to steam).




Steam is able to penetrate objects with cooler temperatures because once the steam contacts a cooler surface; it immediately condenses to water, producing a concomitant 1,870-fold decreases in steam volume. This creates negative pressure at the point of

condensation and draws more steam to the area. A condensation continues so long as the temperature of the condensing surface is less than that of steam; once temperatures equilibrate, a saturated steam environment is formed. Achieving high and even moisture content in the steam-air environment is important for effective autoclaving. The ability of air to carry heat is directly related to the amount of moisture present in the air. The more moisture present, the more heat can be carried, so steam is one of the most effective carriers of heat. Steam therefore also results in the efficient killing of cells and the coagulation of proteins.

Moist heat is thought to kill microorganisms by causing coagulation of essential proteins. Another way to explain this is that when heat is used as a sterilizing agent, the vibratory motion of every molecule of a microorganism is increased to levels that induce the cleavage of intramolecular hydrogen bonds between proteins. Death is therefore caused by an accumulation of irreversible damage to all metabolic functions of the organism. Death rate is directly proportional to the concentration of microorganisms at any given time. The time required to kill a known population of microorganisms in a specific suspension at a particular temperature is referred to as thermal death time (TDT). All autoclaves operate on a time/temperature relationship; increasing the temperature decreases TDT, and lowering the temperature increases TDT.

Standard temperatures/pressures employed are 115¡C/10 psi 121¡C/15 psi and 132¡C/27 psi. (Psi=pounds per square inch).

**Working**

Steam enters the chamber jacket, passes through an operating valve and enters the rear of the chamber behind a baffle plate. It flows forward and down through the chamber and the load, exiting at the front bottom. A pressure regulator maintains jacket and chamber pressure at a minimum of 15 psi, the pressure required for steam to reach 121ºC (250ºF). Overpressure protection is provided by a safety valve. The conditions inside are thermostatically controlled so that heat (more steam) is applied until 121ºC is achieved, at which time the timer starts, and the temperature is maintained for the selected time.

**Note**

Please note that after loading and starting the autoclave, the processing time is measured after the autoclave reaches normal operating conditions of 121¡C (250¡F) and 15 psi pressure, not simply from the time you push the "on" button. Due to the fact that autoclaves utilize steam, heat and pressure the risk of personal exposure and potential harm is great. Personnel should wear proper personal protective equipment, i.e. heat resistant gloves, eye protection and a lab coat, particularly when unloading the

autoclave. Regularly inspect the autoclave for proper operation. Do not assume that the temperature and pressure is down before opening the chamber. Look at the gauges. Even if the pressure gauge shows "0", open the chamber carefully; crack the door to allow steam to dissipate (don't fling the door open, as steam might come out and burn you). After opening the door, let items sit for 5 minutes before handling. This will reduce the chance of boil-over and burns. Never place sealed containers in an autoclave they might explode. This allows for expansion during the cycle. Caps must be slightly loose so that pressure created during the cycle does not cause the vessel to break. For screw-cap containers, you can make the lid hand tight and then loosen the lid by one-half turn. Always leave a few inches of "head room" in your containers. That way, if the item boils, it won't spray out into your face. Liquids to be autoclaved must be in an autoclavable vessel that is at least twice as large as the volume to be autoclaved (i.e. If you are autoclaving 1 litre of media, you need to put it in a flask that hold at least 2 litres). Agar will clog the drain in the autoclave and break it.

Do not autoclave items containing solvents, volatile or corrosive chemicals (Phenol, Trichloro acetic acid, Ether, Chloroform, etc.) or any radioactive materials.

### 3.20 Centrifuge

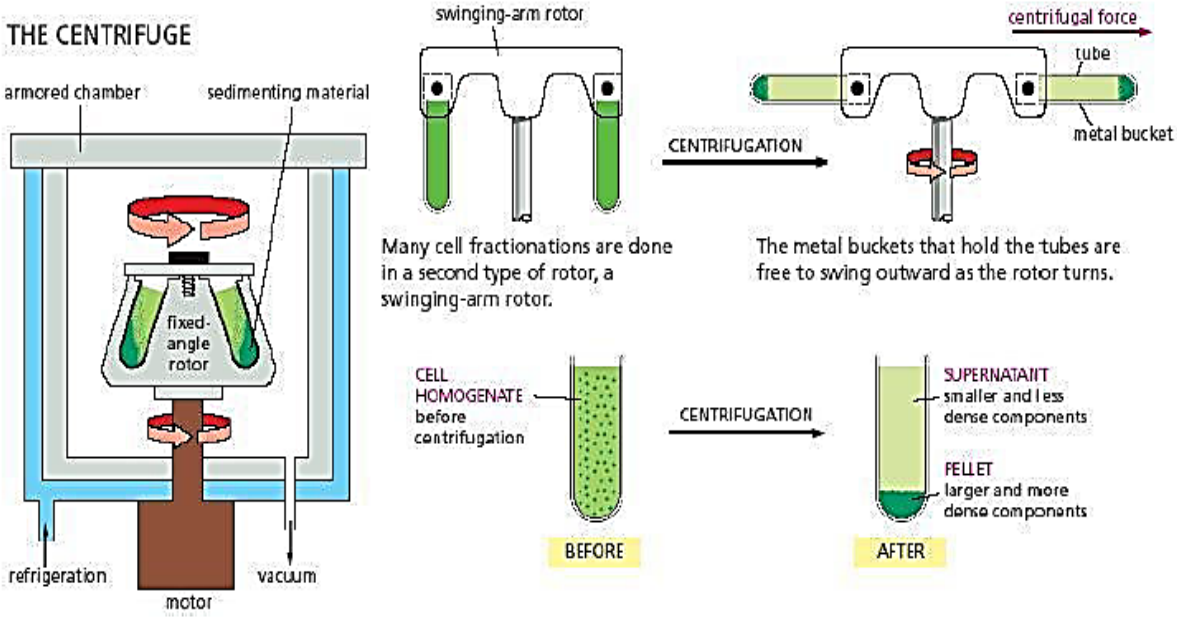
General Description

A centrifuge is the equipment generally driven by an electric motor that puts an object to rotate around fixed axis, and a perpendicular force is applied to axis. The particles get separated according to their size, shape, density, viscosity of the medium and rotor speed.


**Principle**

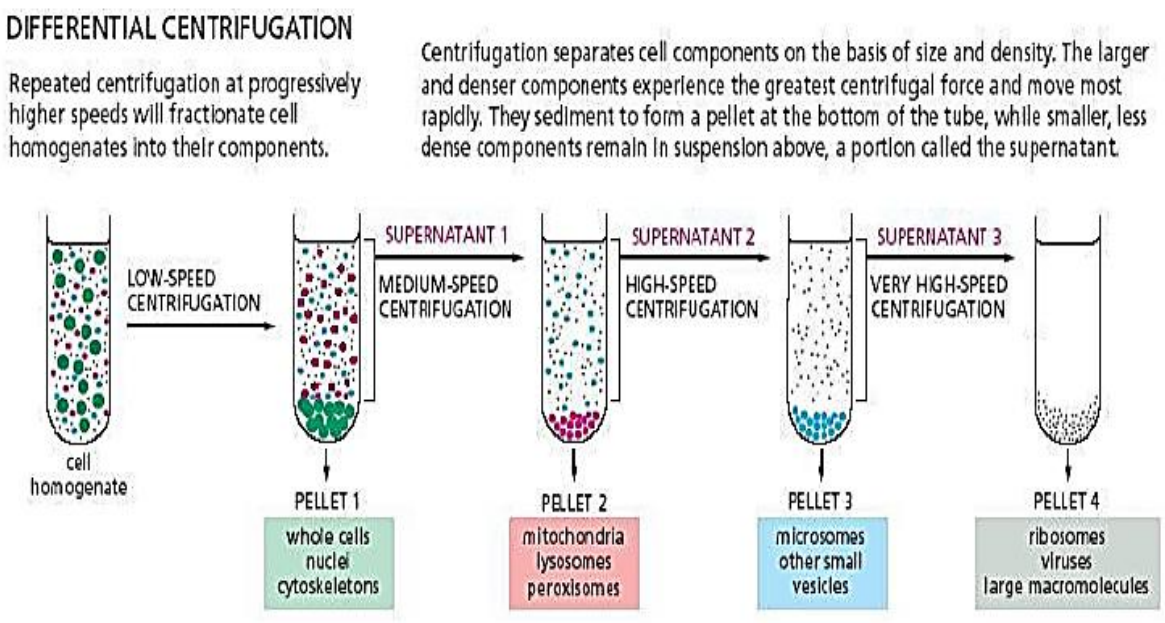
The centrifuge involves principle of sedimentation, where the acceleration at centripetal force causes denser substance to separate out along the radial direction at the bottom of the tube. In centrifugation, the lysate is rotated at a certain speed (expressed as rotations per minute (RPM)). This rotation imposes a force on the particles perpendicular to the axis of rotation. The force is called a relative centrifugal force (RCF), expressed as a multiple of the force of Earth's gravitational force (x g). When a particle is subjected to centrifugal force, it will migrate away from the axis of rotation at a rate dependent on the particles size and density.

## Instrumentation




- That part of the centrifuge that holds the centrifugation tubes is called the centrifuge rotor.
- Centrifuges are designed so that a number of different rotors can be used by the instrument. There are three types of centrifuge rotors: fixed-angle rotors, swinging-bucket rotors, and vertical rotors.
- Fixed-angle and swinging-bucket rotors are the most commonly used. In a fixed-angle rotor, the centrifuge tubes are spun at a fixed angle, which is usually approximately 35 degrees. They are most commonly used for pelleting cells and subcellular components.
- With swinging-bucket rotors, the tubes are free to swing out perpendicular to the axis of rotation as the rotor rotates. This rotor is particularly useful in density-gradient centrifugation schemes.

## Differential Centrifugation



Differential centrifugation is one of two major types of centrifugation schemes. Differential centrifugation is the sequential centrifugation of a cell lysate at progressively increasing centrifugation force, isolating cellular components of decreasing size and density. The separation of the cellular components is based solely on their sedimentation rate through the centrifugation medium, which, in turn, is dependent on the size and shape of the cellular components. In differential centrifugation, each centrifugation step results in the production of a pellet, usually containing a mixture of cellular components of the same size and/or density. The fluid resting above the pellet, the supernatant can be removed and subjected to additional centrifugations to generate pellets containing other cellular components of lesser size and / or density.

## 3.21 Refrigerated Centrifuge

General Description

It is extensively used in chemistry, biology, and biochemistry for isolating and separating suspensions. It additionally provides the cooling mechanism to maintain the uniform temperature throughout the operation of the sample.

**Principle**

Refrigerated centrifuge works on the concept of sedimentation principle by holding up the sample tubes with a capacity of 2ml, 10ml and 50ml in rotation around a fixed axis. In this, the centripetal force causes the denser substances to separate out along the radial direction in the bottom of the centrifuge tube. The rate of the centrifugation is calculated by the acceleration applied to the sample and it is typically measured in revolution per minute (RPM) or relative centrifugal force (RCF). The particles settling velocity during centrifugation depends on the function of their size and shape, centrifugal acceleration, the volume fraction of solids present, the density difference between the particle and the liquid, and the viscosity.

**Working**

- Switch on the mains: The power indicator will get illuminated.
- Setting the Program Number: Press the PROG button. The required program number can be set by using the UP or DOWN.
- Setting the timer: Press the PROG button again. The display panel will show Zero and the timer setting is initialized. The required time setting can be made by

pressing the UP arrow for the increment and DOWN arrow for decreasing the time. Set the time required for the program and release the button.

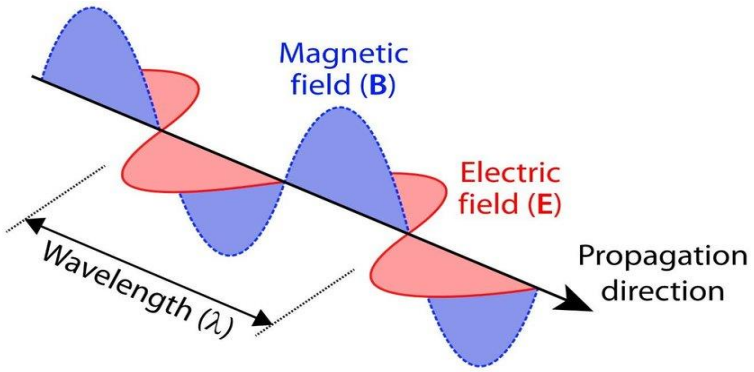
- Setting the temperature: Press the PROG again. Now the system is ready for temperature setting. Set the required temperature by pressing the UP or DOWN arrow till the required temperature is set. Release the button.
- Selection of RPM: Press the PROG button again. The option to select the display mode to read normal display in RPM\RCF can be selected by using the UP or DOWN
- Setting the rotor speed: Press the PROG button again. Use the UP or DOWN arrow to set the required speed. Release the button
- Setting the acceleration time: Press the PROG button again. Use UP or DOWN arrow to set the acceleration time within the range of 60-240 seconds. Release the button.
- Setting the deceleration time: Press the PROG button again. The system is ready to set the deceleration time. Use UP or DOWN arrow to set the deceleration time within the range of 45- 240 seconds. Release the button.
- Rotor Selection: Press the PROG button again. The system is ready to set the rotor selection. The rotor heads are numbered and select the specific rotor head number which you plan to use in this program. Use arrow UP or DOWN select the specific number of the rotor head you plan to use. Release the button, saving the setting and locking the program.
- Now you have completed the setting of the parameters for the first program and the same is automatically registered and saved as parameters of the set program number as program 1. Press the RETURN key to come back to Normal Display mode.

### 3.22 Photometry and Spectrophotometry

Photometry is the measurement of the luminous intensity of light or the amount of luminous light falling on a surface from such a source. Spectrophotometry is the measurement of the intensity of light at selected wavelengths. The term photometric measurement was defined originally as the process used to measure light intensity independent of wavelength. Modern instruments isolate a narrow wavelength range of the spectrum for measurements. Those that use filters for this purpose are referred to as filter photometers, where as those that use prisms or gratings are called spectrophotometers.

## Principle

Light can be described as a wave. This wave has an electric component and a magnetic component which are perpendicular to each other. Electromagnetic radiation exhibits a direction of propagation and wave-like properties (i.e., oscillations).

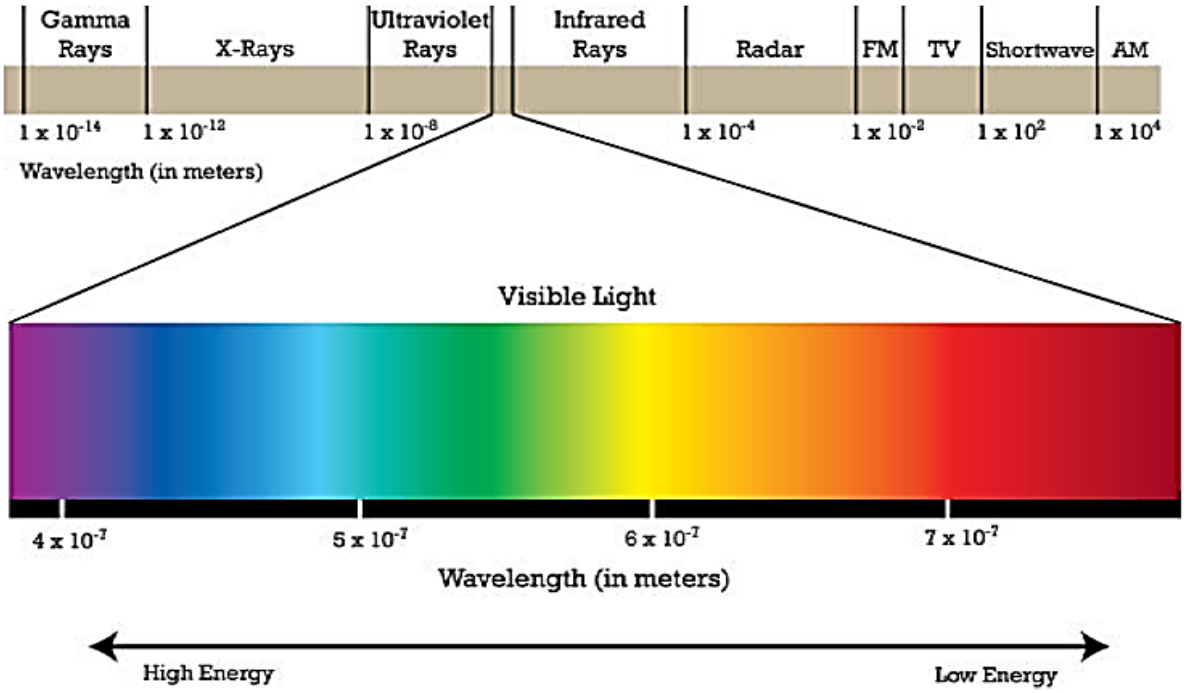


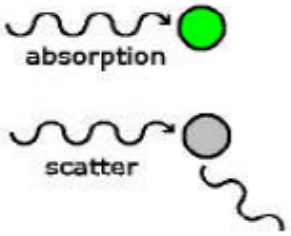

The energy of electromagnetic radiation is defined as:

$$E = hc/\lambda = h\upsilon$$

Where, E = energy, h = Plank's constant, c = the speed of light, λ = the wave length, and υ= frequency

Light behaves both as a wave and as a particle. The conceptual particle of light is called a photon and is represented by hυ. Electromagnetic radiation exhibits a wide spectrum and specific ranges of wavelengths have names. The energy of electromagnetic radiation is inversely proportional to its wavelength.






When a light wave encounters a particle, or molecule, it can be either scattered (i.e., direction changed) or absorbed (energy transferred). Molecules can only absorb discreet packets of energy, or quanta. Absorption occurs when the energy of the photon corresponds to differences between energy levels in that particular molecule. Absorption of the energy from the photon elevates the molecule to an excited state.

A molecule or substance that absorbs light is known as a chromophore. Chromophore exhibit unique adsorption spectra and can be defined by a wavelength of maximum adsorption, or λ max. A large number of biological molecules absorb light in the visible and ultraviolet (UV) range.

The net effect of absorption is that the intensity of the light decreases as it passes through a solution containing a chromophore. The amount of light absorbed depends on the nature of the chromophore, the concentration of the chromophore, the thickness of the sample, and the conditions (e.g.: pH, solvent, etc.)

Absorption is governed by the Beer-Lambert Law:

$$I = I_o 10^{-\varepsilon dc} \text{ or } \log(I/I_o) = -\varepsilon dc$$

Where,

$I_o$ = initial light intensity, I = final light intensity, ε = molar extinction coefficient, d = thickness, and c = molar concentration.

Absorption (A) will be defined by: $A = -\log(I/I_o) = \varepsilon dc$

### 3.23 Colorimeter

General Description

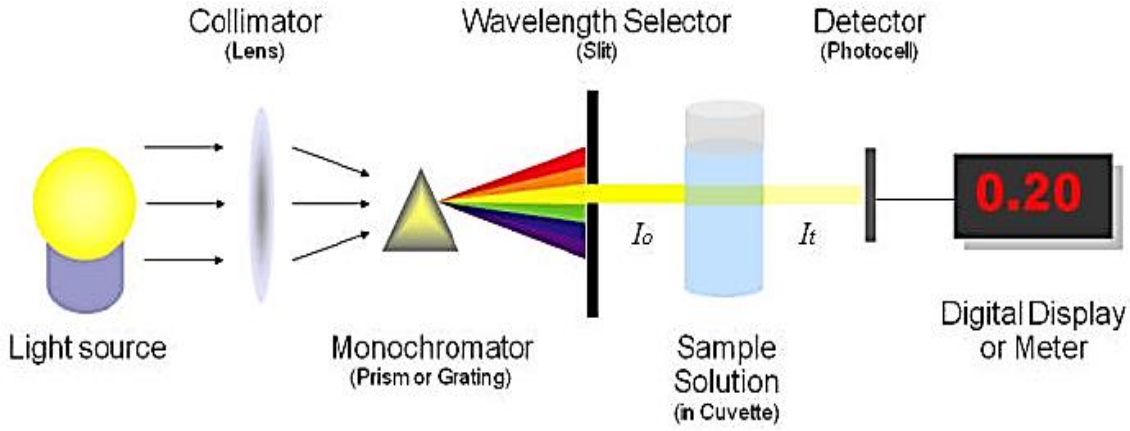
A colorimeter is a light sensitive device used for measuring the transmittance and absorbance of light passing through a liquid sample. The device measures the intensity or concentration of the colour that develops upon introducing a specific reagent into a solution.

**Instrumentation**

The three main components of a colorimeter are a light source, a cuvette containing the sample solution, and a photocell for detecting the light passed through the solution.

The instrument is also equipped with either coloured filters or specific LEDs to generate colour. The output from a colorimeter may be displayed by an analog or

digital meter in terms of transmittance or absorbance. In addition, a colorimeter may contain a voltage regulator for protecting the instrument from fluctuations in mains voltage. Some colorimeters are portable and useful for onsite tests, while others are larger, bench-top instruments useful for laboratory testing.




## Principle

The colorimeter is based on Beer-Lambert's law, according to which the absorption of light transmitted through the medium is directly proportional to the medium concentration.

According to Beers law when monochromatic light passes through the coloured solution, the amount of light transmitted decreases exponentially with increase in concentration of the coloured substance.

$$I_t = I_o^{e\text{-}KC}$$

According to Lamberts law the amount of light transmitted decreases exponentially with increase in thickness of the coloured solution.

$$I_t = I_o^{e\text{-}kt}$$

Therefore, together Beer-Lamberts law is:

$$I_E/I_o = e^{-KCT}$$

Where,

$I_E$ = intensity of emerging light, $I_o$ = intensity of incident light, e = base of neutral logarithm, K = a constant, T = thickness of the solution and C = concentration

### 3.24 Spectrophotometer

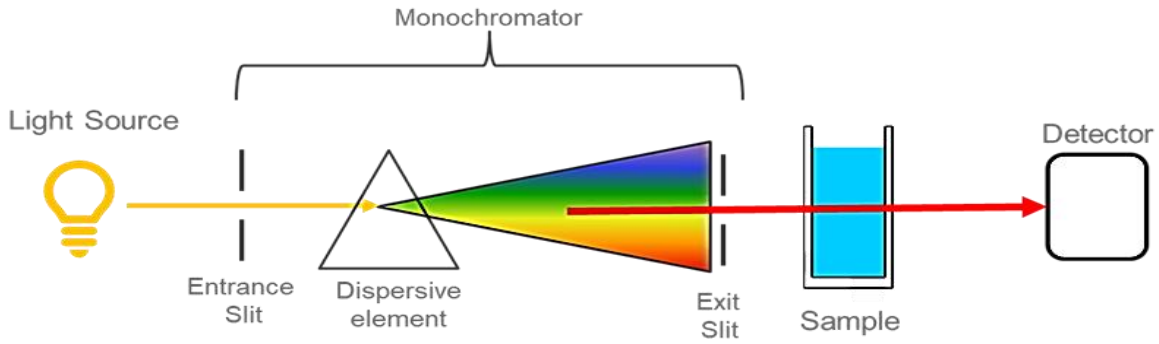
General Description

The spectrophotometer is an instrument which measures the amount of light of a specified wavelength which passes through a medium. They produce monochromatic light and then accurately measure the light intensity.

**Principle**

Spectrophotometer works with the principle of Beer-Lambert Law.

**Instrumentation**




The major components of a spectrophotometer are the light source, a monochromator, sample holder, a light detector (phototube), and a meter.

In most instruments a tungsten lamp is used for the visible range and either high pressure H2 or D2 lamps are used for UV range. Monochromatic light is generated by either

1. A movable prism,
2. A diffraction gradient, or
3. Filters.

**Working**

1. White light from a bulb (source) is focused into a narrow beam by passing it through a thin slit.
2. A prism is used to split the beam of white light into its component colours, in the same way that water droplets can split sunlight into its component colours to make a rainbow. Different colours of light have a different wavelength: the distance between the peaks of the light waves, measured in nanometres (where 1 nanometre is 10-9metres).
3. A second thin slit, just after the prism, can be moved from side to side to select just one colour of light to pass through to the sample.

4. The light passes through a container with the liquid sample inside (usually the light passes through 1 cm thickness of the liquid).
5. A light detector measures how much light is transmitted through the sample, and compares this with how much light was emitted by the source. The difference between these values gives a measure of how much light was absorbed by the sample: i.e., the absorbance (A), often also called the optical density (OD). The absorbance varies with wavelength, so measurements of this type always specify the wavelength of light that was shone through the sample.

# D. Practical Work

## 4. Introduction of carbohydrates

This experiment is intended to introduce you to one of the three major classes of macronutrients found in food, carbohydrates. You will also learn a variety of ways to categorize carbohydrates and several tests used in the analysis of carbohydrates. The experiment is composed of four parts that should be completed in the order listed below (background, prelab, experiment, post lab).

You may proceed directly to the section on carbohydrate tests if you are already familiar with carbohydrates.

$$nCO2 + n\ H2O + \text{energy} \longrightarrow CnH2nOn + nO2$$

It is possible to classify carbohydrates as simple or complex:

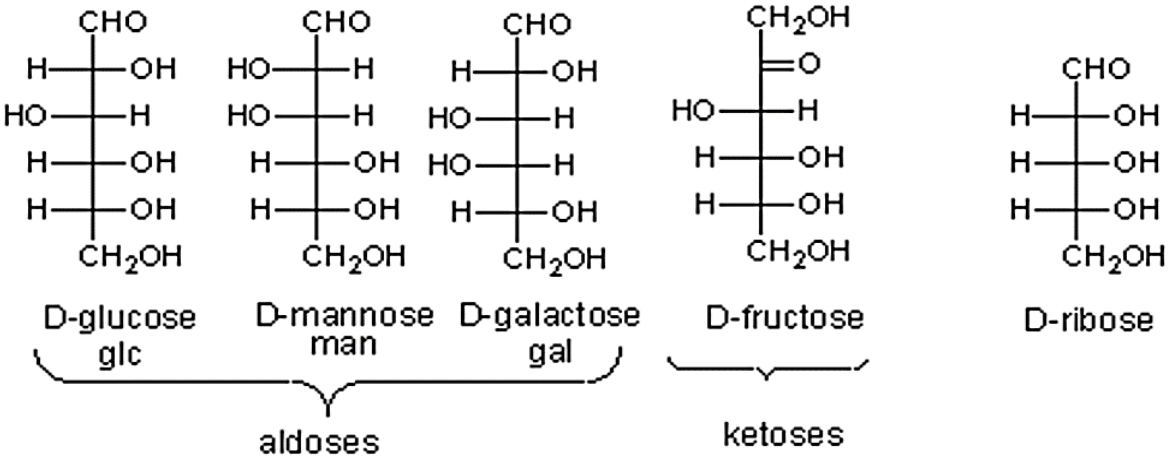
1. Simple carbohydrates: Often referred to as simple sugars or monosaccharides, contain one unit of saccharide and cannot be broken down into smaller carbohydrates.
2. Complex carbohydrates: Those containing more than one group of saccharides.
   - Disaccharides contain two units of monosaccharide.
   - Oligosaccharides have 3-6 units of monosaccharides.
   - Over 7 or more monosaccharide units may be contained in polysaccharides.

Through a process known as hydrolysis, complex carbohydrates can be broken down into smaller carbohydrate units.

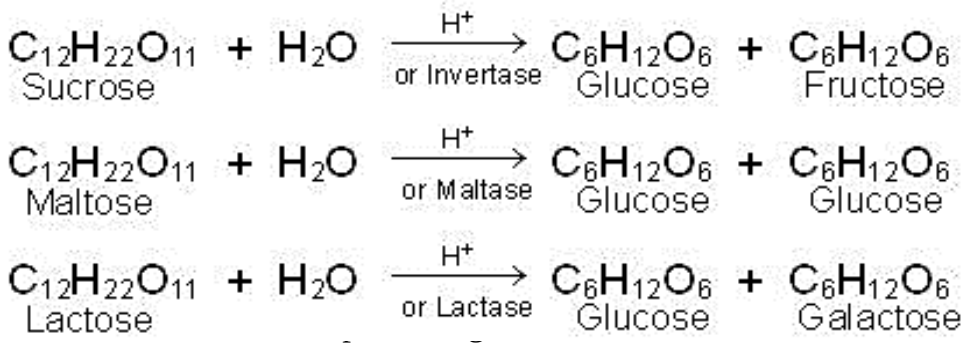
It is possible to classify monosaccharides in a number of ways.

- They can be categorized by the number of atoms of carbon they contain. While there are others, the most common are pentose (5 carbons) and hexose (6 carbons).
- Ketoses or aldoses may also be classified as monosaccharides. In addition to having one or more hydroxyl groups, ketose contains a carbonyl group. Besides the hydroxyl groups, an aldose contains an aldehyde group.

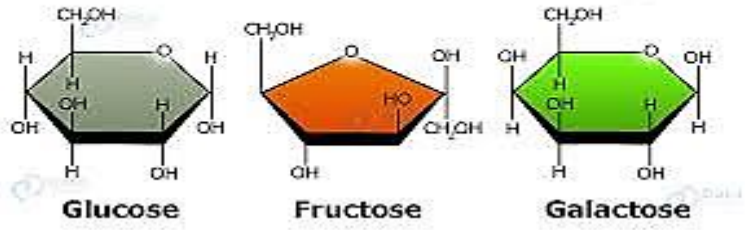
For an aldose or "aldohexose" for ketose, which is also a hexose, the two descriptors are usually combined into a single term like "ketohexose". See several examples of monosaccharides below.




Hydrolysis can break disaccharides into two monosaccharide units and lose one molecule of water.




Oligosacc ose one or more wat




Polysaccharides can be broken into 7 or more units of monosaccharides by hydrolysis and one or more molecules of water can be lost.

### 4.1 Qualitative Analysis of Carbohydrates

#### 1. Solubility

Due to the polar hydroxyl groups that form H-bonds with water, monosaccharides and oligosaccharides are readily soluble in water. However, due to their large molecular weight, the polysaccharides make translucent colloidal solutions.

## 2. Qualitative tests for carbohydrates

There are several difficulties in their qualitative as well as quantitative analysis when analyzing a sample containing a mixture of carbohydrates, particularly sugar. These problems are attributed to their structural and chemical resemblance, as well as their stereoisomerism. It is therefore necessary to determine, during biochemical investigations, whether or not a given sample contains carbohydrates. In order to establish the presence or absence of a sugar or a carbohydrate in a sample, several rapid tests are available. Such tests are based on specific typical color reactions for their group. It is advisable to carry out these tests with the individual in the laboratory rather than with a sugar mixture. The sensitivity of these tests can be confirmed by using different concentrations of sugar solutions (0.1- 1 percent).

### A. GENERAL TESTS FOR CARBOHYDRATES:

In order to detect the presence of carbohydrates in a solution, the most common tests used are: For all carbohydrates, whether free or in combined form, it is a group test. It is routinely used to detect the presence of carbohydrates, despite its limitations.

#### a. Molisch's Test

**Principle**

The response is based on the fact that $H_2SO_4$ concentrated catalyzes the dehydration of sugars to form furfural (from pentoses) or hydroxymethyl furfural (from hexoses). These furfurals then condense to give a purple or violet colored product with sulfonated alpha-naphthol. A+ve reaction is also provided by polysaccharides and glycoproteins. The acid first hydrolyses it into monosaccharides, which are then dehydrated to form furfural or its derivatives, in the case of the carbohydrate being a poly- or disaccharide.

**Reagents**

i) Conc.$H_2SO_4$
ii) Molisch's reagent: 5 percent (w/v) of alpha-naphthol in 95% ethanol.

**Procedure**

Take 2 mL of the unknown solution and add the contents to the 2-mixture. Incline the tube and pour 1-2 mL of conc.H2SO4 carefully down the side of the tube. Tube so that a layer beneath the aqueous solution forms the acid. The formation at the junction of two layers of a purple or violet ring or zone indicates the presence of carbohydrates.

**Precautions:**

i) The solution of alpha-naphthol is unstable and should be made fresh.

ii) Conc.$H_2SO_4$ should be carefully added along the sides of the test tube, causing the contents of the tube to be minimally disturbed.

**Limitations:**

This test is also performed in addition to carbohydrates, furfurals as such, certain organic acids, aldehydes and ketones. Secondly, due to the charring action of acid, a concentrated sugar solution can give a red colour instead of purple.

**b. Anthrone test**

**Principle**

Another general test for carbohydrates is anthrone reaction. Its principle is the same as that of Molisch's, except that furfurals and hydroxyl-methyl furfurals offer bluish green condensation products with anthrone.

**Reagents**

i) Anthrone reagent: in conc.$H_2SO_4$, 0.2% (w/v) solution.

**Procedure**

In a test tube, add about 2 mL of Anthrone reagent to approximately 0.5-1mL of the test solution and mix thoroughly. Watch if the colour changes to bluish green. If not, after keeping them in a boiling water bath for ten minutes, re-examine the tubes. A positive test indicates a blue-green color.

**B. SPECIFIC TESTS FOR CARBOHYDRATES:**

**a) Polysaccharide iodine test**

This test is performed to differentiate polysaccharides from mono and disaccharides.

**Principle**

With various polysaccharides, iodine forms coloured adsorption complexes. Because of iodine adsorption on the polysaccharide chains, these complexes are formed. The colour intensity depends on the length of the available unbranched or linear chain for the complex formation. Therefore, amylose gives a deep blue color and amylopectin, the unbranched helical component of starch, the branched component gives red color because the chains do not effectively coil. Glycogen, which is also highly branched, adds iodine to the red colour. This experiment is performed in acidic or neutral solutions.

**Reagents**

i. Iodine solution: prepare a 2% (w/v) KI solution in water to which a few iodine crystals are added until the solution is deep yellow.
ii. Starch solution: dissolve 1g of starch in about 10-20mL of boiling water and add saturated sodium chloride solution to a volume of 100mL.

**Procedure**

In a test tube, take 3 mL of the test solution and add 1-2 drops of dil. HCl. Mix and then add 1-2 drops of the solution of iodine. Mix and observe the change in colour. Heat the tube and again observe the colour. When heated, the blue colour disappears and reappears when cooled.

**b) Tests based on the reduction of carbohydrate properties**

Sugars with a free or potentially free group of aldehydes or ketones act as reducing agents, and this becomes the basis for the tests carried out to distinguish them from non-reducing sugars. These sugars have the ability to easily reduce alkaline metal solutions such as copper, bismuth, mercury, iron and silver. Aldo sugars are oxidized into the corresponding aldonic acids, whereas shorter chain acids are produced by keto sugars. If in the absence of sugar reduction, the alkaline copper solution is heated, the black precipitate of cupric oxide forms:

$$Cu(OH)_2 \xrightarrow{Heat} 2OH$$

However, the alkaline solution of copper is reduced to insoluble yellow or red cuprous oxide in the presence of a sugar reducer:

$$Sugar + 2\,Cu(OH)_2 \xrightarrow{Heat} 2O + 2\,H_2O$$

**A) Fehling's test**

In this reaction, Rochelle salt acts as a chelating agent:

CuSO4 + 2KOH ---------- 2 + K2SO4

$2Cu(OH)_2$ + Reducing Sugar ---------- 2O + Aldonic acid

**Reagents**

i. Fehling solution A: Dissolve 69.38 g of copper sulfate in distilled water and make 1 L of copper sulfate.
ii. Fehling's solution B: Dissolve 250 g of NaOH in DW, add 346 g of potassium sodium tartrate and add up to 1 L of volume.

Just before use, mix equivalent volumes of A & B solutions because mixing causes deterioration with time.

**Procedure**

2 mL of Fehling's reagent is added to the remaining 2 mL test solution. Mix the test tubes thoroughly and place them in a boiling water bath. The formation of yellow or red Cuprous Oxide precipitates indicates the presence of sugar reduction.

**Note**

- Leave the solution to stand for 10-15 minutes in the case of a mild reduction, then decant the supernatant. It is then possible to see a small amount of red or yellow precipitates adhering to the inner side of the tube.
- The Fehling test is only carried out with an alkaline solution
- Cuprous oxide is ammonia-dissolved. Small amounts of sugar reducers can therefore not be detected in fluids saturated with ammonium salts, such as urine.

**B) Benedict's Test**

Benedict modified the Fehling solution to produce a fairly stable, enhanced single reagent. As a chelating agent, Sodium Citrate works. It is very sensitive and sufficient precipitates are produced by even small amounts of sugar reduction (0.1 percent).

**Principle**

The Benedict's test is based on reduction of $Cu^{++}$ ions in alkaline solution. Complexing agent sodium citrate or potassium sodium tartarate, is added to form a deep blue coloured and soluble complex ion with $Cu^{++}$ as $CuCo_3$ solution. Sucrose does not reduce Benedict's solution because it has no free aldehyde or ketone group.

**Reaction**

$$Na_2CO_3 + 2H_2O \rightarrow 2NaOH + H_2CO_3$$

$$2NaOH + CuSO_4 \rightarrow Cu(OH)_2 + Na_2SO_4$$

$$Cu(OH)_2 \rightarrow CuO + H_2O$$

$$\text{D-Glucose} + 2\ CuO \rightarrow \text{D-gluconic acid} + Cu_2O$$

(Red ppt)

**Reagents:**

**Qualitative reaction of Benedict:**

Dissolve 173g of Sodium Citrate and 100g of Anhydrous Sod. In about 800mL of water, carbonate by gently heating the contents. Then, dissolve 17.3g of copper sulfate in about 100mL DW in a separate beaker. Slowly pour this solution into the Carbonate-Citrate mixture with constant stirring, and make up to 1 L with DW.

**Procedure:**

To add about 2 mL of Benedict's reagent, add 0.5-1 mL of the test solution. Keep the test tubes in a bath of boiling water. Note that green, orange, yellow or red precipitates are formed, indicating the presence of sugar reduction in the solution.

**Note:**

i. This test is particularly suitable for urinary sugar reduction detection because it is more specific than the Fehling test, which is also positive for non-reducing substances such as urates present in urine.
ii. This is a semi-quantitative test.

### C) Barfoed's Test:

This test is performed to differentiate between mono- and disaccharide reduction. Monosaccharides are more reactive reducing agents than disaccharides and thus react in about 1-2 minutes, while it takes 7-12 minutes for the reducing disaccharides to get hydrolysed and then react in the acidic solution. Therefore, it is possible to detect the difference in property reductions.

**Reagents:**

i. Barfoed's reagent: 66.5 g of Cupric acetate dissolved in approximately 900 mL DW. Add 9 mL of Glacial Acetic Acid and boil. Cool and use DW to cool the volume to 1 L and filter if necessary.

**Procedure:**

Take 2ml of test solution, add 2ml of Barfoed’s reagent to it. Keep the test tubes for only 2-5 min in a boiling water bath. Then cool under running tap water. Thin red precipitates indicate the presence of a reduction of monosaccharide at the bottom or sides of the tube.

**Note:**

i. The boiling should not be prolonged beyond 1-2min, otherwise the disaccharide reduction will respond to this test as well.
ii. This test is not effective in detecting urine sugar reduction due to the presence of chloride ions.

### D) Picric Acid Test:

This is another test for sugar reduction detection. To form a red colored Picramic Acid, the reducing sugars react with Picric Acid.

**Reagents:**

i. Picric acid saturation: Dissolve 13 g of picric acid in 100 mL of DW, boil and cool.
ii. Sodium Carbonate (10% solution).

**Procedure:**

Add 1 mL of the above-mentioned reagent to 1 mL of the test solution, followed by 0.5 mL of the 10% solution of sodium carbonate. In a boiling water bath, heat the test tube. The presence of sugar reduction in the solution indicates the appearance of a red color.

**E) Seliwanoff's Test**

**Principle:**

This test is a keto hexose-specific timed colour reaction. It is therefore used for the differentiation of aldoses from ketoses. Dehydration is carried out in the presence of HCl ketohexoses to yield 4-hydroxy methyl furfural more rapidly than aldohexoses. In addition, these furfural derivatives condense to form a cherry red coloured complex with resorcinol.

Sucrose, which is easily hydrolysed to glucose and fructose, give a (+ve) Seliwanoff's test. Upon continued boiling, aldoses give a red colour with the Seliwanoff's reagent, because of their gradual conversion to ketones by the HCl.

**Reagents:**

i. Seliwanoff's reagent: Dissolved 2.57 ml of HCl in 7.43 ml of distilled water and add 0.5 g of resorcinol reagent (Seliwanoff's reagent in a solution of resorcinol in HCl).

**Procedure:**

Add 1 mL of the test solution to about 2 mL of the Seliwanoff's reagent and warm it in a boiling water bath for 1 min. The presence of ketohexose indicates the appearance of a red colour (fructose).

**Note:**

i. Aldohexoses, e.g. glucose, also react if the boiling is prolonged because the catalytic action of the acid is converted into fructose.
ii. Sucrose and inulin are also tested because they are hydrolysed by fructose-giving acids.

### F) Bial's Test for Pentoses

**Principle:**

This test is specific to pentoses and to pentose-containing compounds and is therefore useful for pentose sugar determination. The reaction is due to the formation of furfural in the acid medium which in the presence of ferric ions, condenses with orcinol to give a blue-green complex.

**Reagents:**

i. Bial's reagent: Dissolve 1.5g of orcinol in 100 mL of Conc. HCL and add 20 drops of 10% solution of Ferric Chloride to improve the results. Make yourself fresh.

**Procedure:**

Add 4 to 5 drops of the test solution to about 2 mL of Bial's reagent. Heat until bubbles of gas rise to the surface in a boiling water bath. The presence of pentose sugar indicates the formation of the green solution and the precipitate.

### G) Test for sucrose:

This test is performed only if the Barfoed's test does not contain any precipitation.

**Principle:**

In an unknown solution, the sucrose present is hydrolyzed to glucose and fructose by acid. Then Seliwanoff's reagent forms the resulting fructose in the solution.

**Reagents:**

i. Conc. of HCL
ii. Seliwanoff's reagent
iii. Sodium carbonate

**Procedure:**

Add 1-2 drops of conc. HCl to approximately 2-3mL of test solution and boil for about 8-minutes in a water bath. The appearance of the red color indicates the presence of the hydrolytic sucrose product fructose.

**Note:**

Benedict's reagent can be tested for sugar reduction by acid hydrolyzed sample after cooling and then neutralizing with sodium carbonate.

### H) Mucic acid test for galactose

**Principle:**

This test is highly specific to galactose, which is either present in solutions independently or obtained by lactose hydrolysis. On heating with $HNO_3$, Galactose is converted to Saccharic acid (a strong oxidizing agent). Mucic acid (galactaric acid) formed from galactose due to the aldehyde & primary alcoholic group oxidation at

C1&C6. It is the only saccharic acid that is insoluble in cold water and thus assists in galactose identification.

**Reagents:**

i. Conc. of HNO3

**Procedure:**

Take 50 mg of galactose and 50 mg of glucose into the test tubes separately. Add to each tube 1mL DW and 1mL conc. $HNO_3$. Heat the tubes for about 1hr in a boiling water bath. Add 5mL of DW and slowly let the tubes stand and cool overnight. The presence of galactose will be indicated by colourless needles such as crystals.

**Note:**

This test will also be administered with lactose.

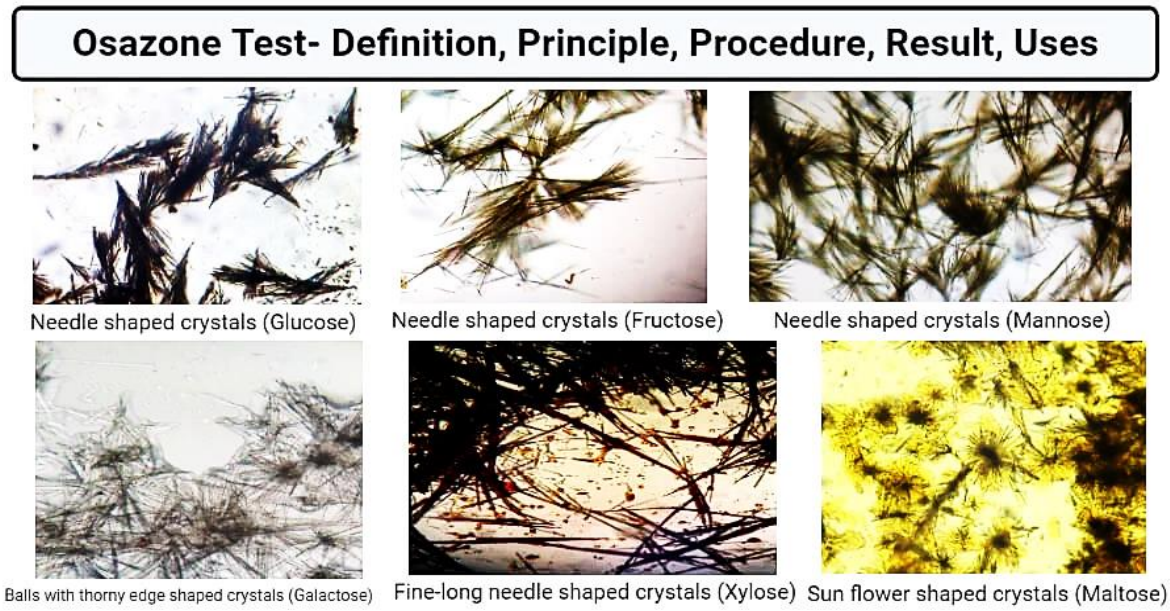
**I) Osazone test / Phenylhydrazine test**

This test is used to distinguish between lactose and maltose.

**Principle:**

The organic compound phenylhydrazine reacts to form osazones with the carbonyl carbon of sugar. The shapes and melting point, time of formation and solubility of these osazone crystals have yellow color characteristics. In the following table, the characteristic characteristics of osazone are given:

| S. NO. | Carbohydrate Osazone | Time of formation "Minutes" | Solubility in water | Crystalline Structure |
|---|---|---|---|---|
| **1.** | Fructosazone | 2 | Insoluble | Needle Shaped |
| **2.** | Glucosazone | 5 | Insoluble | Needle Shaped |
| **3.** | Galactosazone | 20 | Insoluble | Thorn Ball shape |
| **4.** | Maltosazone | 30-45 | Soluble | Sunflower/Star shape |
| **5.** | Lactosazone | 30-45 | Soluble | Cotton Ball/ Powder Puff Shape |




**Procedure:**

In a test tube, take 7-8 ml of carbohydrate solution and add a pinch of phenylhydrazine to that and double the amount of sodium acetate and 10 drops of acetic acid. Dissolve and allow slow cooling by shaking. Observe the shape of the crystal under low microscope power (10x).

**Observations and conclusion:**

Lactose forms crystals in the form of powder puffs, maltose forms sunflower-shaped or star-shaped crystals, while glucose and fructose form identical crystals in the form of needles.

## Colour reactions of Carbohydrates

| S. No. | Test | Observation | Inference |
|---|---|---|---|
| 01. | **Molisch Test (Ring test)** Aqueous or alcoholic solution of substances 10% alcoholic solution of α- naphthol shake + concentrated sulphuric acid along the side of the tube | Violet ring at the junction of two liquids | Carbohydrate present |
| 02. | **Solubility** Compound + water | Soluble<br><br>Insoluble | Mono & Disaccharides<br><br>Polysaccharides |

| | | | |
|---|---|---|---|
| **03.** | **Fehling's test**<br>2 ml of Fehling's A solution + 2 ml of Fehling's B solution + 2 ml of sugar solution. Boil | Yellow or brick red precipitate | Reducing sugars present |
| **04.** | **Benedict's test**<br>5 ml of benedict's reagent + 8 drops of sugar solution. Boil for 2 min. Cool | Green, yellow or red precipitate | Reducing sugars present |
| **05.** | **Tommer's test**<br>2 ml of tommer's reagent + 3 ml of sugar solution. Boil for 2 minutes | Yellow or red precipitate | Reducing sugars present |
| **06.** | **Barfoed's test**<br>2 ml of sugar solution + add 2 ml of Barfoed's test reagent. Boil on water bath. | Brick red precipitate at the bottom of the test tubes. | Monosaccharides present |
| **07.** | **Rapid furfural test**<br>1 to 2 ml of sugar solution + add 1 ml of α-naphthol solution (1% in alcohol) + add 5 ml of conc. HCl and Boil. | Deep purple colour | Ketoses like Fructose, Sucrose present |
| **08.** | **Osazone test**<br>0.2 gram of sugar + add 0.4 gram of phenyl hydrazine hydrochloride + 0.6 gram of sodium acetate + 4 ml of distilled water. Add 6-8 drops of glacial acetic acid. Heat on a water bath for 20 minutes. Cool and allow crystallization. Observe crystals under microscope | a) Greenish yellow needle shaped crystals arranged in fan-shape<br>b) Thin small needle shaped crystals appear luke ball of prickles<br>c) Sunflower like crystals | Glucosazone is glucose present<br>Lactose present<br>Maltose present |
| **09.** | **Iodine test**<br>Suspension or sugar solution of polysaccharides + add 1-2 drops of iodine solution | i) Blue violet colour develops<br>ii)Brown wine colour develops | Starch present<br>Glycogen present |

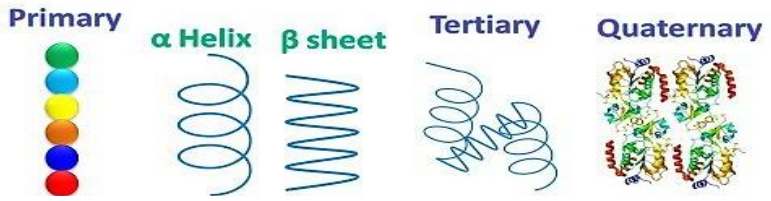
# 5. Introduction of proteins

Protein is an important macronutrient essential for survival. They are constituent of cells and hence are present in all living bodies. 10-35% of calories should come from protein. Protein is found in meats, poultry, fish, meat substitutes, cheeses, milk etc.

Proteins are large biological molecules of alpha-amino acids (amino acids in which the amino group is attached to alpha-carbon, which are crystalline in nature and exist as Zwitter ions). They contain carbon, hydrogen, oxygen, nitrogen, phosphorus and sulphur in some cases.

Molecules containing both the amino (NH2) and carboxylic (COOH) groups are amino acids. To form a specific type of linkage known as peptide linkage, amino acid molecules undergo a condensation reaction.

The products formed are classified as; depending on the number of amino acid molecules involved in the condensation reaction.




❖ **Dipeptide**

They are the products formed by the condensation of two molecules of alpha-amino acid.

❖ **Tripeptide**

They are formed by the condensation of three molecules of alpha-amino acid. If a large number of molecules of amino acids combine, the formed product is called a polypeptide. A protein is called a polypeptide that has a molecular mass greater than 10000 Da. Proteins differ primarily from each other in their amino acid sequence. There are about 20 + amino acids here. Some amino acids are not produced by the body and are delivered by diet. They are called amino acids, which are essential.

## 5.1 Qualitative Analysis of Proteins

### 1. Solubility Tests

The solubility of amino acids and proteins is largely dependent on the pH of the solution. The structural changes that occur at different pH values in an amino acid or protein change the molecule's relative solubility. Amino and carboxylic groups are both protonated in acidic solutions. Both groups are deprotonated in basic solutions.

In water, amino acids are essentially soluble. Depending on the structure of its side chains, their solubility in water, dilute alkali and dilute acid varies from one compound to another. Glycine, tyrosine, glutamic acid and cysteine will be used for this test.

**Procedure:**

The solubility of amino acids in water and alcohol should be noted by placing a small quantity in the test tube, adding a few mL of solvent and if necessary, warming up.

Use the litmus paper to determine whether the amino acid solution is acidic or basic, while testing the solubility in water. Using dilute HCl and dilute NaOH, repeat the solubility test.

WARNING: Avoid spilling ninhydrin solutions on your skin because it is hard to remove the resulting stains.

**2. Stability to Alkali**

Amino acids do not develop $NH_3$ or alkaline vapor when boiled with alkali, unlike amides and volatile amines. In order to differentiate amino acids from amines and amides, this method can be used. Apply this test to the amine or amide provided and to glycine as well.

**Procedure:**

Pipette 1 mL 1% glycine and the amide or amine solution into separate test tubes. Add 1 mL dilute NaOH to each test tube and boil. Test the vapor from each boiling tube with wet litmus paper.

## A. SPECIFIC REACTIONS FOR INDIVIDUAL AMINO ACIDS:

**a) Xanthoproteic Test:**

There are aromatic groups that are derivatives of benzene in some amino acids. Reactions that are characteristics of benzene and benzene derivatives can be experienced by these aromatic groups. The nitration of a benzene ring with nitric acid is one such reaction. The amino acids that have the benzene ring activated can easily undergo nitration. This nitration reaction forms a yellow product in the presence of the activated benzene ring. Apply this test to tyrosine, phenylalanine, tryptophan, and glutamic acid.

**Reagents:**

i. Conc. $HNO_3$
ii. NaOH, 40%

**Procedure:**

To 2 mL amino acid solution in a boiling test tube, add equal volume of concentrated HNO3. Heat over a flame for 2 min and observe the color.

Now, COOL THOROUGHLY under the tap and CAUTIOSLY run in sufficient 40% NaOH to make the solution strongly alkaline.

## B. OBSERVE THE COLOR OF THE NITRO DERIVATIVE OF AROMATIC NUCLEUS.

**a) Millon's Test:**

The test by Millon is specific to phenol that contains structures (tyrosine is the only common phenolic amino acid). Millon's reagent is a concentrated $HNO_3$ reagent that dissolves mercury. A red precipitate or a red solution is regarded as a positive test as a consequence of the reaction. A yellow HgO precipitate is NOT a positive response, but it usually shows that the solution is too alkaline. This test can be applied to tyrosine, phenylalanine.

**Reagents:**

i. Millon's Reagent: 150g of mercuric sulphate dissolved in 15 % volume / volume $H_2SO_4$
ii. $NaNO_2$, (0.5 %) = Sodium Nitrite

**Procedure:**

To 2 mL amino acid solution in a test tube, add 2-5 drops of Millon's reagent. Warm the tube in a boiling water bath for 10 min. cool it to room temperature and add 5 drops of NaNO2 solution. A brick red color is a positive reaction. Note that this is a test for phenols, and the ninhydrin test should also be positive if it is to be concluded that the substance is a phenolic amino acid.

**b) Hopkin's Cole Test:**

In the presence of concentrated $H_2SO_4$, the indole tryptophan group reacts with glyoxylic acid (glacial acetic acid, which has been exposed to light, always contains CHOCOOH glyoxylic acid as an impurity) to give a purple color. Apply this test to glycine, tyrosine and tryptophan.

**Reagents:**

i. Glacial acetic acid exposed to sunlight
ii. Conc. $H_2SO_4$

**Procedure:**

To a few mL of glacial acetic acid containing glyoxylic acid, add 1-2 ml of the amino acid solution. Pour 1-2 ml $H_2SO_4$ down the side of the sloping test tube to form a layer underneath the acetic acid. The development of a purple color at the interface proves a positive reaction for Tryptophan.

**c) Lead-Sulphide Test / Lead Acetate Test:**

When cystine is boiled with 40 % NaOH, sodium sulphide is covered with some sulphur in its structure ($Na_2S$). Using a sodium plumbate solution that causes the precipitation of PbS from an alkaline solution, $Na_2S$ can be detected. In order to carry out this test, the sodium plumbate solution must be prepared first. This test is applied to cysteine and cystine.

**Reagents:**

i. NaOH, 40%
ii. Lead Acetate, 2 %
iii. Sodium Plumbate Solution Preparation: Add 5 mL dilute NaOH to 2 mL dilute lead acetate. A white precipitate of lead hydroxide forms. Boil until the precipitate dissolves with the formation of sodium plumbate.

**Procedure:**

Boil 2 mL amino acid solution with a few drops of 40% NaOH for 2 min.
Cool and add a few drops of the sodium plumbate solution. A brown color or precipitate is a positive test for sulphides.

**d) Ehrlich Test:**

Aromatic amines and many organic compounds (indole and urea) provide this test with a color complex. This test is applied to tryptophan, urea and glycine.

**Procedure:**

Put 0.5 mL of the amino acid solution to a test tube. Add 2 mL Ehrlich reagent and observe the color changes. Repeat the test with urea solution.

**e) Sakaguchi Test:**

To test for a certain amino acid and proteins, the Sakaguchi reagent is used. The amino acid that this test detects is arginine. As arginine has a group of guanidine in its side chain, in the presence of an oxidizing agent such as bromine solution, it gives a red color with alpha-naphthol. Apply Arginine to this test.

**Reagents:**

i. NaOH, 5%
ii. α-naphthol, 1%
iii. Bromine-water, 1%

**Procedure:**

1 mL NaOH and 3 mL arginine solution is mixed and 2 drops of α- naphthol is added. Mix thoroughly and add 4-5 drops of bromine solution UNDER THE HOOD!! Observe the color change. Appearance of red colour indicates the presence of arginine.

**f) Nitroprusside Test:**

The nitroprusside test is specific to cysteine, the only sulfhydryl group amino acid containing cysteine (-SH). In the presence of excess ammonia, that group reacts with nitroprusside. This test is used for cysteine, cystine and methionine.

**Reagents:**

i. Sodium nitroprusside, 10%
ii. $(NH_4)_2\,SO_4$= Ammonium Sulphate
iii. Liquid Ammonia

**Procedure:**

Put 2 mL amino acid solution into the test tube. Add few solid crystals of ammonium sulphate, few drops of liquid ammonia & nitroprusside solution and shake thoroughly. Add 0.5 mL ammonium hydroxide. Observe the color change. Appearance of rose red colour indicates the presence of cysteine, cystine or methionine.

**g) Biuret Test:**

The Biuret Test recognizes positively the presence of proteins (not less than two peptides). The reaction in this test involves the complex formation in a strongly alkaline solution of the proteins with $Cu^{2+}$ ions. This test is applied to gelatin, casein and albumin.

**Reagents:**

i. Fehling's A solution (1/10 in water)
ii. NaOH, 40%
iii. Acetone

**Procedure:**

To 2 mL protein solution, add 5-6 drops of dilute $CuSO_4$ (Fehling's solution A diluted 1/10 with water). Add 3 mL 40% NaOH solution. Observe the color change.
If the protein tested is insoluble in water, then apply the procedure given below:
Measure 3 mL acetone and 1.5 mL water into a test tube. Add 1 drop of dilute NaOH and a little piece of protein to be tested. Boil continuously over a small flame for 2 min and cool.

**h) Ninhydrin Test:**

This test is given by only amino acids and proteins which contain free $–NH_2$ groups in their structure. Apply this test for all the proteins provided.

Ninhydrin is a powerful oxidizing agent reacts with amino acids producing $CO_2$, $NH_3$ and an aldehyde. The reduced ninhydrin then reacts with $NH_3$ in presence of a molecule of oxidizing ninhydrin forming a purple blue complex (which can also be measured optically at 570nm). In case of amino acid e.g., proline and hydroxy – proline different product is formed which is yellow in colour. This is because, proline has an imino group rather than amino group.

Proteins also give a blue colour with ninhydrin and so do some primary amines e.g., amino sugars. This is due to presence of N-terminal amino acids in proteins.

Amines other than amino acids react with ninhydrin without evolving $CO_2$ (Blue colour).

**Reagents:**

i. Ninhydrin solution, 0.2 %

**Procedure:**

Take 5 ml of amino acid and add 1ml of ninhydrin solution. Heat it for 1-2 minutes and then cool it. Blue or yellow colour indicates the presence of amino acids.

## C. PRECIPITATION OF PROTEINS:

The precipitation of a protein occurs in a stepwise process. The addition of a precipitating agent and steady mixing destabilizes the protein solution. Mixing causes the precipitant and the target product to collide. Enough mixing time is required for molecules to diffuse across the fluid.

**i. By Neutral salts**

The precipitation of a protein by neutral salt is commonly known as salting-out method. Addition of a neutral salt, such as ammonium sulfate [$(NH_4)_2 SO_4$], compresses the solvation layer and increases the protein-protein interaction. As the salt concentration of a solution is increased, more of the bulk water becomes associated with the ions. As a result, less water is available to take part in the solvation layer around the protein, which exposes hydrophobic parts on the protein surface. Therefore, proteins can aggregate and form precipitates from the solution. The amount of neutral salt required to cause protein precipitation varies with the nature of the protein and the pH of the solution. Apply this test to all the proteins provided.

**Procedure:**

Add solid ammonium sulfate to about 5 mL of protein solution in a test tube (the salt should be added in quantities of approximately 1 g at a time). Agitate the solution gently after each addition to dissolve the ammonium sulfate.

**ii. By salts of Heavy Metals:**

Heavy metal salts usually contain $Hg^{2+}$, $Pb^{2+}$, $Ag_{+}$, $Tl^{+}$, $Cd^{2+}$ and other metals with high atomic weights. Since salts are ionic, they disrupt salt bridges in proteins. The reaction of a heavy metal salt with a protein usually leads to an insoluble metal protein salt. Apply this test to all the proteins provided.

**Procedure:**
Treat 3 mL of the protein solution provided with a few drops of mercuric nitrate.
A white precipitate formation should be observed.

**iii.By Acid Reagents:**
The precipitation of a protein in the presence of acid reagents is probably due to the formation of insoluble salts between the acid anions and the positively charged protein particles. These precipitants are only effective in acid solutions. Apply this test to all the proteins provided.

**Procedure:**
Treat 3 mL of protein solution provided with a few drops of trichloroacetic acid solution. Note the protein precipitate formed.

## Colour reactions of Proteins

| S. No. | Test | Observation | Inference |
|---|---|---|---|
| 1. | **Biuret test**<br>Take 1 ml of albumin solution and add 1 ml of 5% sodium hydroxide then add 1- 2 drops copper sulphate solution and mixed well. | Violet colour is formed | Indicates albumin is protein |
| 2. | **Ninhydrin test**<br>Take 1 ml of albumin solution and add 5-10 drops of ninhydrin reagent. Heat to boiling. | Ruhemann's purple colour formed | Alpha amino acids are present |
| 3. | **Heat coagulation test**<br>Take 5 ml of albumin solution and heat flame slanting position, add 3 drops of 1% acetic acid glacial. | A white cloudy precipitate is formed | Albumin present |
| 4. | **Half saturation test**<br>Take 3 ml of test sample and add an equal vol. of saturated ammonium sulphate solution to make it half saturated. Mix well and wait for 3 minutes then filter the sample | No white precipitate is seen | Albumin is not precipitated by half saturation as it is a low Molecular weight protein. Hence, the biuret test is positive with the filtrate. |

| | | | |
|---|---|---|---|
| **5.** | **Biuret test with filtrate**<br>Take 2 ml of the filtrate of the half-saturation test and add an equal volume of 40% sodium hydroxide and 2 drops of 1% copper sulphate solution. Mix well | Violet colour is formed | Albumin is not precipitated by half saturation as it is a low Molecular weight protein. hence, the biuret test is positive with the filtrate. |

# 6. Introduction to amino acids

Amino acids are molecules containing an amine group, a group of carboxylic acids, and a side chain that varies from one amino acid to another. In some reactions, amino acids of the general formula RCH (NH2) COOH are amphoteric, acting as amines and in others as carboxylic acids. An amino acid has no overall charge at a certain pH, known as the isoelectric point, as the number of protonated ammonium groups (positive charges) and deprotonated carboxylate groups (negative charges) is equal.

For life, amino acids are critical. They have important functions in particular, such as being the building blocks of proteins and the intermediates in metabolism.

Amino acids are usually classified into four groups according to the properties of their side chain. The side chain can make a weak acid or a weak base of an amino acid, and if the side chain is polar, a hydrophobe or if it is nonpolar, a hydrophile.

Proteins (also known as polypeptides) are organic compounds arranged in a linear chain made of amino acids. The peptide bonds between the carboxyl and amino groups of adjacent amino acid residues are joined together by the amino acids in a polymer.

Proteins are essential parts of organisms, like other biological macromolecules such as polysaccharides and nucleic acids, and participate in virtually every process within cells.

Proteins are significant in:

- Catalyzing biochemical reactions (enzymes)
- Structural and mechanical functions (actin and myosin)
- Cell signalling
- Immune responses
- Cell adhesion
- Cell cycle

## 6.1 TESTS ON AMINO ACIDS

### A. Solubility Tests

The solubility of amino acids and proteins is largely dependent on the pH of the solution. The structural changes that occur at different pH values in an amino acid or protein change the molecule's relative solubility. Amino and carboxylic groups are both protonated in acidic solutions. Both groups are deprotonated in basic solutions. In water, amino acids are essentially soluble. Depending on the structure of its side chains, their solubility in water, dilute alkali and dilute acid varies from one compound to another. Glycine, tyrosine, glutamic acid and cysteine will be used for this test.

**Procedure:**

The solubility of amino acids in water and alcohol should be noted by placing a small quantity in the test tube, adding a few mL of solvent and if necessary, warming up. Use the litmus paper to determine whether the amino acid solution is acidic or basic, while testing the solubility in water. Using dilute HCl and dilute NaOH, repeat the solubility test.

**a) Millon's Test:**

The test by Millon is specific to phenol that contains structures (tyrosine is the only common phenolic amino acid). Millon's reagent is a concentrated $HNO_3$ reagent that dissolves mercury. A red precipitate or a red solution is regarded as a positive test as a consequence of the reaction. A yellow HgO precipitate is NOT a positive response, but it usually shows
that the solution is too alkaline. This test can be applied to tyrosine, phenylalanine.

**Reagents:**

i. Millon's Reagent: 150g of mercuric sulphate dissolved in 15 % volume / volume $H_2SO_4$
ii. NaNO2, (0.5 %) = Sodium Nitrite

**Procedure:**

To 2 mL amino acid solution in a test tube, add 1-2 drops of Millon's reagent. Warm the tube in a boiling water bath for 10 min. A brick red color is a positive reaction. Note that this is a test for phenols, and the ninhydrin test should also be positive if it is to be concluded that the substance is a phenolic amino acid.
WARNING: Avoid spilling ninhydrin solutions on your skin because it is hard to remove the resulting stains.

**b) Hopkin's Cole Test:**

In the presence of concentrated $H_2SO_4$, the indole tryptophan group reacts with glyoxylic acid (glacial acetic acid, which has been exposed to light, always contains CHOCOOH glyoxylic acid as an impurity) to give a purple color. Apply this test to glycine, tyrosine and tryptophan.

**Reagents:**

i. Glacial acetic acid exposed to sunlight
ii. Conc. $H_2SO_4$

**Procedure:**

To a few mL of glacial acetic acid containing glyoxylic acid, add 1-2 drops of the amino acid solution. Pour 1-2 mL $H_2SO_4$ down the side of the sloping test tube to form a layer underneath the acetic acid. The development of a purple color at the interface proves a positive reaction.

**c) Lead-Sulphide Test:**

When cystine is boiled with 40 % NaOH, sodium sulphide is covered with some sulphur in its structure ($Na_2S$). Using a sodium plumbate solution that causes the

precipitation of PbS from an alkaline solution, $Na_2S$ can be detected. In order to carry out this test, the sodium plumbate solution must be prepared first. This test is applied to cysteine and cystine.

**Reagents:**

i. NaOH, 40%
ii. Lead Acetate, 2 %
iii. Sodium Plumbate Solution Preparation: Add 5 mL dilute NaOH to 2 mL dilute lead acetate. A white precipitate of lead hydroxide forms. Boil until the precipitate dissolves with the formation of sodium plumbate.

**Procedure:**

Boil 2 mL amino acid solution with a few drops of 40% NaOH for 2 min. Cool and add a few drops of the sodium plumbate solution. A brown color or precipitate is a positive test for sulphides.

**d) Ehrlich Test:**

Aromatic amines and many organic compounds (indole and urea) provide this test with a color complex. This test is applied to tryptophan, urea and glycine.

**Procedure:**

Put 0.5 mL of the amino acid solution to a test tube. Add 2 mL Ehrlich reagent and observe the color changes. Repeat the test with urea solution.

**e) Sakaguchi Test:**

To test for a certain amino acid and proteins, the Sakaguchi reagent is used. The amino acid that this test detects is arginine. As arginine has a group of guanidine in its side chain, in the presence of an oxidizing agent such as bromine solution, it gives a red color with alpha-naphthol. Apply Arginine to this test.

**Reagents:**

i. NaOH, 5%
ii. α-naphthol, 1%
iii. Bromine-water, 1%

**Procedure:**

1 mL NaOH and 3 mL arginine solution is mixed and 2 drops of α-naphthol is added. Mix thoroughly and add 4-5 drops of bromine solution UNDER THE HOOD!! Observe the color change.

**f) Nitroprusside Test:**

The nitroprusside test is specific to cysteine, the only sulfhydryl group amino acid containing cysteine (-SH). In the presence of excess ammonia, that group reacts with nitroprusside. This test is used for cysteine, cystine and methionine.

**Reagents:**

i. Sodium nitroprusside, 10%
ii. $(NH_4)_2 SO_4$ = Ammonium Sulphate
iii. Liquid Ammonia

**Procedure:**

Put 2 mL amino acid solution into the test tube. Add 0.5 mL nitroprusside solution and shake thoroughly. Add 0.5 mL ammonium hydroxide. Observe the color change.

## Colour test for Amino acids and proteins

| S.NO. | Test | Reactions | Responsible Amino acid | Colour formation |
|---|---|---|---|---|
| **1.** | **Biuret** | Alkaline Copper sulphate | Peptide proteins | Purple colour formation |
| **2.** | **Ninhydrin** | Ninhydrin | All Amino Acids, peptide proteins | Purple |
| **3.** | **Millon's test** | Silver nitrate with nitrous acid | Tyrosine | Brick red colour |
| **4.** | **Xanthoproteic test** | Boiling with Conc. Nitric acid | Tyrosine, Tryptophan, phenylalanine | Yellow in acid which turns orange yellow |
| **5.** | **Pauly's test** | Diazotized Sulphanilic acid in Alkaline solution | Tyrosine, Tryptophan, Histidine | Red colour |
| **6.** | **Hopkins Cole test** | Glyoxylic acid in Conc. Sulphuric acid | Tryptophan | Violet ring at interface |
| **7.** | **Sakaguchi test** | A-naphthol and sodium hypochlorite | Arginine | Red |
| **8.** | **Lead Sulphide test** | Lead acetate and NaOH | Cysteine | Black colour |

# 7. Introduction to lipids

Lipids are defined as a group of fatty nature, which are insoluble in water but soluble in non-polar solvents like ether, chloroform etc. Lipids thus include fat, oil, waxes and related compounds. Lipids are classified as Simple and Complex lipids.

**Simple Lipids**

Simple Lipids are those, which do not contain fatty acids. They are called as non-saponifiable lipids (they cannot form the soaps) e.g., steroids, terpenes, prostaglandins.

**Complex/Compound Lipids**

Compound Lipids are those, which contain fatty acids e.g., triacylglycerols, phospholipids, sphingolipids.

## 7.1 QUALITATIVE TESTS FOR LIPIDS

A large number of heterogenous compounds are referred to as lipids including fats, steroids, waxes, and related compounds, which are related more by their physical than their chemical properties.

**They have the common property of being:**

1. Relatively insoluble in water
2. Soluble in polar solvents such as ether and chloroform.

Fatty acids are aliphatic carboxylic acids. If the aliphatic chain contains no double bond then it is called saturated and if it contains one or more double bond it is called unsaturated. Most naturally occurring unsaturated fatty acids have cis-double bonds. Some of the most common fatty acids are palmitic acid and stearic acid. Palmitic has 16 carbon atoms and stearic has 18 carbon atoms.

As it is clear from the formulae, both are saturated fatty acids. Some fatty acids like oleic acid may be unsaturated. Naturally occurring animal fats consist largely of mixed glyceride of oleic, palmitic and stearic acids. They are usually mixture of individual fats. Fats have more saturated fatty acids whereas oils have more of unsaturated ones.

Lipids are simple, complex or derived. Simple lipids are esters of fatty acids with various alcohols, e.g., fats (esters of fatty acids with glycerol) and waxes (esters of fatty acids with higher molecular weight of monohydric alcohols). Complex lipids are esters of fatty acids containing groups in addition to an alcohol and a fatty acid, e.g., phospholipids or glycolipids etc. Derived lipids include fatty acids, glycerol, steroids, other alcohols, fatty aldehydes, and ketone bodies, lipid soluble vitamins, and hormones.

Phospholipids yield in addition to alcohol and fatty acids, phosphate and a nitrogenous base like choline, ethanolamine, etc. Lecithin's and cephalous are representatives of the phospholipids. Similarly, glycolipids contain carbohydrates, and sulpholipids contain sulphate. Lipoproteins are combinations of lipids with proteins.

**I. Physical Test:**

**1) Grease spot test:**

Take a small amount of oil on a piece of paper, a greasy spot penetrating the paper will be formed. This happens because lipid does not wet paper unlike water.

**2) Test for free fatty acids:**

Take a few drops of phenolphthalein solution in a test tube and add to it one or two drops of very dilute alkali solution, just sufficient to give the solution a pink colour. Now add a few drops of the oil and shake. The colour will disappear as the alkali is neutralized by the free fatty acids present in the oil.

**3) Emulsification:**

Oil or liquid fat becomes finely divided and is dispersed in water when shaken with water to form emulsification. Emulsification is permanent and complete in the presence of emulsifying agent. The important emulsifying agents are bile salts, proteins, soaps, mono- and diglycerides. Emulsification is important in the processes of fat digestion in the intestine. Emulsifying agents lower surface tension of the liquid.

**Procedure:**

Take 2 clean and dry test tubes, in one test tube added 2 ml water and in other 2ml dilute bile salt solution. Now to each tube added 2 drops of mustard oil and shaken vigorously for about one minute. Allow the tubes to stands for two minutes and note that the water, oil is broken in small pieces and floats on the surface; where as in the bile salt solution, the oil can be seen in minute droplets suspended in the liquid (permanent emulsification).

**4) Saponification test:**

Esters can be hydrolysed by alkali to yield the parent alcohol and salt. When the fatty acid possesses a long chain, the salt formed is a soap which we commonly use. This process is called saponification. Oils and fats usually contain long chain fatty acids and are, therefore, the starting materials for the preparation of soap.

**Procedure:**

Take 1 ml of the oil in a test tube and add an equal amount of alcoholic KOH solution, mix them thoroughly and keep the mixture during the course of warming and shake up gently with a little distilled water. Appearance of some oil drops will indicate the incomplete saponification. After complete saponification no oil drops will appear.

**5) Tests for unsaturation of fatty acids:**

Unsaturated fatty acids like oleic acid can react with halogens like bromine and iodine due to presence of double bonds as shown below.

$CH_3 (CH_2)_7CH = CH (CH_2)_7COOH + Br_2 \rightarrow CH_3 (CH_2)_7CHBr\text{-}CHBr (CH2)7COOH$

The amount of $Br_2$ or $I_2$ taken up will indicate the amount of unsaturation present in a particular acid. Approximate idea about the unsaturation in a different oils and fats can be obtained by the following test.

**Procedure:**

Set up four clean and dry test tubes each containing 5 ml of $CCl_4$. To the first, add one drop of shark liver oil, to the second, one drop of coconut oil, to the third, a drop of vegetable ghee and add nothing to the fourth tube. Now test for the unsaturation of the added oil by adding bromine water drop by drop to each tube followed by shaking.

Record the number of drops required to obtain a permanent yellowish red colour in each tube and infer the relative unsaturation in the three samples used. It may be

mentioned here; vegetable ghee is prepared by hydrogenating vegetable oil. Hydrogenation means saturation of unsaturated fatty acid by hydrogen.

**6) Isolation of free fatty acids from soap:**

Take a few ml of 20% $H_2SO_4$ in a test tube and gradually add 5 ml of some soap solution. The fatty acids will separate out in a distinct layer due to the hydrolysis of the soap.

$$RCOONa + H2O \rightarrow RCOOH + NaOH$$

Cool the solution which will become hot and skim off the surface layer and wash it several times with water till free from $H_2SO_4$. Then dissolve it in some water and add alkaline phenolphthalein solution and shake. The pink colour will be discharged indicating the presence of free fatty acids.

**Calcium soap formation:**

To a small amount of the soap solution in a test tube add $CaCl_2$ solution. A white precipitate will be formed. The white precipitate is due to insoluble calcium salt of fatty acid. This is referred to as calcium soap.

**Lead soap formation:**

To a small amount of the soap solution in a test tube add lead acetate solution, a white precipitate will appear. The white ppt is due to insoluble lead salt of fatty acids. This is referred to as lead soap.

**7) Tests for Glycerol:**

**I. Acrolein test:**

Take pure glycerol in a dry test tube; add to it a few crystals of potassium hydrogen sulphate. Warm gently to mix and then heat strongly. A very pungent odour of acrolein is produced. Acrolein is formed due to removal of water from glycerol by potassium hydrogen sulphate.

**II. Dichromate Test:**

Take in a dry test tube 3 or 4 ml of glycerol solution, to it add a few drops of 5% potassium dichromate solution and 5 ml of conc. $HNO_3$, mix well and note that the brown colour is changed to blue. This test is given by the substances containing primary and secondary alcohol groups. The chromic ions oxidize the glycerol and, in this process, they are reduced to chromous ions which give the blue colour. This test is also given by reducing sugars, so before confirming glycerol be sure that the reducing sugars are not present.

## 7.2 QUALITATIVE TESTS FOR CHOLESTEROL

Cholesterol is a lipid with a structure quite different from that of phospholipids. It is a steroid, built from four linked hydrocarbon rings. A hydrocarbon tail is linked to the steroid at one end, and a hydroxyl group is attached at the other end. In membranes, the molecule is oriented parallel to the fatty acid chains of the phospholipids, and the hydroxyl group interacts with the nearby phospholipid head groups.

Cholesterol is absent from prokaryotes but is found to varying degrees in virtually all animal membranes. It constitutes almost 25% of the membrane lipids in certain nerve cells but is essentially absent from some intracellular membranes.
The main test for cholesterol is known as Liberman-Burchard test. This is carried in the following way.

**1. Liberman-Burchard test:**

**Procedure:**

In a dry test tube take a small amount of solution of cholesterol in chloroform. Add 1 ml of acetic anhydride and 1 drop of conc. $H_2SO_4$. Mix and observe that a purple colour is formed which soon changes to green. It may take 15-30 min for full development and it is advisable to put the tube in dark during this time.

**2. Enzymatic Methods:**

Assays have been developed in which cholesterol oxidase obtained from the bacterium Nocardia erythropolis is used to convert cholesterol into cholest-4-en-3-one with the formation of Hydrogen peroxide. The cholest-4en-3-one formed has been measured by reading at 240 nm after extracting into isopropanol. Alternatively, hydrogen peroxide has been quantified by formation of chelate complex with quadrivalent titanium and xylenol orange.

**OTHER TESTS FOR CHOLESTEROL:**

**3. Salkowski's Test ($H_2SO_4$ Test):**

**Procedure:**

Dissolve cholesterol in 2 ml of chloroform in dry test tube. Add equal amount of conc. $H_2SO_4$. Shake gently. The upper layer turns red and the sulphuric acid layer shows a yellow colour with a green fluorescence.

**4. Formaldehyde- $H_2SO_4$ Test:**

**Procedure:**

Add 2 ml of formaldehyde-sulphuric acid solution (1 part of 40% formaldehyde to 50 parts of the acid) to 2 ml of chloroform solution in a dry test tube. The cherry colour is developed in the chloroform. Pour off the chloroform in another test tube and add 2-3 drops of acid anhydride. The blue colour develops.

# 8. CONFIRMATORY TESTS FOR (Ca, P & NaCl)

## 8.1 Tests for Calcium

### a. Colour Test

Observe the colour of the salt carefully, phase, colour and shape of the crystal. $CaCO_3$ compound usually taken under analysis. It's a white powder.

### b. Dry Heating Test

- Take, about 0.1 g of the dry salt in a clean and dry test tube.

- Heat the above test tube for about one minute and observe the colour of the residue when it is hot and also when it becomes cold. Observation of changes.
- Compound may condense into a liquid indicated compound is hydrated.
- It may evolve gases.
- Gas may have been coloured or colourless. And might have an Odour.
- Calcium carbonate is colourless and Odourless.

**c. Flame Test**

The chlorides of several metals impart characteristic colour to the flame because they are volatile in non-luminous flame. This test is performed with the help of a platinum wire as follows:

i) Make a tiny loop at one end of a platinum wire.
ii) To clean the loop dip it into concentrated hydrochloric acid and hold it in a non-luminous flame
iii) Repeat step (ii) until the wire imparts no colour to the flame.
iv) Put 2-3 drops of concentrated hydrochloric acid on a clean watch glass and make a paste of a small quantity of the salt in it.
v) Dip the clean loop of the platinum wire in this paste and introduce the loop in the non-luminous (oxidising) flame
vi) Observe the colour of the flame first with the naked eye and then through a blue glass and identify the metal.
vii) Calcium compounds gives Brick Red flame (Orange Red)

**d. Solubility Test**

Calcium carbonate (a calcium compound) is insoluble in water. And shows a reaction when dissolved in $HNO_3$.

$$CaCO_{3\,(S)} + 2H^{+}_{(aq)} \longrightarrow Ca^{2+}_{(aq)} + H_2O + CO_{2\,(g)}$$ (Odourless & Colourless)

**e. Reaction with ammonium oxalate**

Take 10 drops of salt solution, add aqueous ammonia to make the solution basic. Then add $[(NH_4)_2C_2O_4(NH_4)_2C_2O_4]$ = Ammonium oxalate solution dropwise. A white precipitate should form.

## 8.2 Tests for Phosphorus

Two Procedures may be used:

1. The Organic compound is heated with an Oxidising agent to oxidise phosphorus to phosphate. The solution is then boiled with concentrated $HNO_3$ and treated with ammonium molybdate. Yellow precipitate confirms the presence of phosphorus.

2. Phosphorus is detected by fusing the organic compound with an oxidising agent, i.e., sodium peroxide when phosphorus is oxidised to sodium phosphate. The fused mass is extracted with water. The aqueous solution is boiled with conc. $HNO_3$, and then ammonium molybdate solution is added. The appearance of

yellow precipitate or colouration due to the formation of ammonium phosphomolybdate indicates the presence of phosphorus.

### 8.3 Tests for Sodium Chloride

Sodium chloride (NaCl) consists of sodium (Na) and chloride (Cl) ions.

**Test for chloride ions:**

- Add a few drops of an aqueous solution of silver nitrate or $AgNO_3$ (which is colourless) to an aqueous solution of sodium chloride or NaCl (which is also colourless). You will observe a curdy white precipitate, silver chloride, which is insoluble in cold water and dilute nitric acid.
- The precipitate formed is dissolved in excess ammonium hydroxide solution to give a clear, colourless solution of diamine silver chloride.

**Test for sodium ions:**

- Dip a platinum wire in concentrated HCl and hold it in the blue Bunsen burner flame until the flame no longer changes colour. Next, dip the wire in HCl again, pick up some NaCl powder/paste with the wire and hold it in the flame. An intense golden-yellow or orange-yellow colour confirms the presence of sodium ion.
- If the substance answers both above tests, it is sodium chloride.

# 9. Lipid/ Fat Tests

### 9.1 Determination of Saponification value of Lipids

**Principle**

On refluxing with alkali, glyceryl esters are hydrolyzed to give glycerol and the potassium salts of the fatty acids (soaps). The saponification value is the number of milligrams of KOH required to neutralize the fatty acids resulting from the complete hydrolysis of 1 g of fat. The saponification value gives an indication of the nature of the fatty acids in the fat since the longer the carbon chain, the less acid is liberated per gram of fat hydrolyzed.

**Materials**

- Fats and oils (tristearin, coconut oil, corn oil, and butter),
- Fat solvent (equal volumes of 95% ethanol and ether),
- Alcoholic KOH (0.5 mol/L),
- Phenolphthalein (10 g/L in alcohol),
- HCl (0.5 mol/L)

**Protocol**

- Weigh 1 g of fat in a tarred beaker and dissolve in about 3 mL of the fat solvent.
- Quantitatively transfer the contents of the beaker to a 250 mL conical flask by rinsing the beaker three times with a further addition of solvent; add 25 mL of 0.5 mol/L alcoholic KOH and attach to a reflux condenser.
- Set up another reflux condenser as blank with everything present except the fat, and heat both flasks on a boiling water bath for 30 min.
- Leave to cool to room temperature and titrate with 0.5 mol/L HCl and phenolphthalein indicator.

- The difference between the blank and test reading gives the number of millilitres of 0.5 mol/L KOH required to saponify 1 g of fat.

**Results**

The molecular weight of KOH is 56, and since three molecules of fatty acid are released from a triglyceride

### 9.2 Determination of the acid value of a fat

**Principle**

During storage, fats may become rancid as a result of peroxide formation at the double bonds by atmospheric oxygen and hydrolysis by microorganisms with the liberation of free acid. The amount of free acid present, therefore, gives an indication of age and quality of the fat. The acid value is the number of milligrams of KOH required to neutralize the free fatty acid present in 1 g of fat.

**Materials**

- Olive oil, butter and margarine,
- fat solvent (equal volumes of 95% v/v alcohol and ether neutralized to phenolphthalein),
- Phenolphthalein (10 g/L in alcohol),
- KOH (0.1 mol/L)

**Protocol**

Accurately weigh out 10 g of the test compound and suspend the melted fat in about 50 mL of fat solvent. Add 1 mL of phenolphthalein solution, mix thoroughly, and titrate with 0.1 mol/L KOH until the faint pink color persists for 20–30 s. Note the number of milliliters of standard alkali required and calculate the acid value of the fat.

**Results**

The acid value present in the given fat sample is ……g/ml.

### 9.3 Determination of Iodine Number:

**Principle**

The iodine number of a fat is the amount in gm. of iodine taken up by 100 gm. of fat. Not only iodine but also equivalent amounts of other halogens will add at double bonds; so, bromine is often used instead of iodine because it is more reactive. The halogenating reagent used in this method is pyridine sulphate di-bromide.

**Materials**

This reagent can be prepared by adding carefully 8.1 ml pyridine in 20 ml glacial acetic acid and making the volume up to 1 litre with glacial acetic acid.

**Protocol**

Weigh the bottle containing sample of oil plus a medicine dropper and then transfer about 0.1 to 0.3 gm. of oil to a flask. Reweigh the bottle containing oil and dropper to find out the exact quantity of the sample transferred. Add 10 ml of chloroform and then 25 ml of the pyridine sulphate di-bromide reagent.

Shake thoroughly; allow standing for 5 minutes and then determining the residual bromine. To do this, add 10 ml of 10% KI and titrate the equivalent amount of iodine liberated by the residual bromine with the help of 0.1 (N) Na2S2O3 (sodium thiosulphate). The titration can be done by adding sodium thiosulphate solution through a burette to the flask.

When the colour of the solution in flask becomes light yellow add 1 ml of starch solution. It will become blue. Slowly add the thiosulphate solution again till it becomes colourless. Note the total volume of thiosulphate used.

The total amount of bromine originally added is found by titrating 25 ml of the pyridine sulphate di-bromide reagent with thiosulphate after adding KI as in the previous case. The amount of bromine taken up by the fat sample can be determined by the difference between the two titers and then the iodine number can be calculated.

Suppose with a sample of 0.2 gm. oil the data obtained are as follows:

- (N) $Na_2S_2O_3$ used for titration of blank = 47.0 ml
- (N) $Na_2S_2O_3$ used for titration of sample = 27.0 ml
- (N) $Na_2S_2O_3$ equivalent to iodine absorbed by the sample = 20.0 ml

**Calculations**

As 1 ml 0.1 (N) $Na_2S_2O_3$ = 1.0 ml of 0.1 (N) Bromine = 1 ml of 0.1 (N) Iodine

Hence, 20 ml of 0.1 (N) $Na_2S_2O_3$ = 20 ml of 0.1 (N) Iodine = 20×12.7/1000 gm Iodine = 0.254 gm Iodine.

Thus 0.2 gm of oil can take up 0.254 gm of iodine. Therefore, iodine number of oil used = 127.

## 10. Estimation of reducing sugar by titrimetric method (using benedict's reagent)

**Principle**

- The ability of monosaccharide and some disaccharides to reduce Benedict's solution in hot alkaline solution.
- If the amount of standard Benedict's solution reduced by the sugar is known, it's concentration can be estimated.

Benedict's quantitative reagent is a modification of qualitative. It contains copper sulphate, sodium acetate and sodium carbonate. It also contains potassium thio cyanate and small amount of potassium ferricyanide. The inclusion of acetate prevents the precipitation of copper carbonate by chelating $Cu^{3+}$ion. The thiocyanate causes the precipitation of white cuprous thio cyanate rather than red cupric oxide. On reduction of $Cu^{3+}$ion which enables the end point of the titration, i.e., the transition from blue to white to be readily observable. Methylene blue will be used as an additional indicator. The small amount of potassium ferricyanide prevents the re-oxidation of copper. A non-stoichiometric reaction is on which does not follow a defined pathway and cannot be described by an equation either quantitatively or qualitatively. The reduction of $Cu^{3+}$ion by sugar is a non-stoichiometric equation and is only constant over a small range of sugar concentration. To obtain accurate results the volume of sugar added must be within 6- 12

ml for 10 ml of benedict's reagent. If the preliminary titre value Falls outside this range the sugar solution must be titrations are repeated.

**Reagents Required:**

- **Standard Glucose Solution:** 200 mg of glucose was weighed accurately and made upto 100 ml with distilled water (concentration: 2 mg / ml)
- **Benedict's Quantitative Reagent:** 100 ml of solution acetate, 37.5 g of sodium carbonate and 62.5 g of potassium thiocyanate were dissolved in 300 ml of distilled water by warming gently and filtered. 9 g of copper sulphate is dissolved in 50 ml of water, added with continuous stirring. 2.5 ml of potassium ferricyanide is added and the volume is made upto 500 ml with water.
- **Anhydrous Sodium Carbonate**

**Procedure**

100 ml of benedict's reagent was pipetted out into a clean conical flask. About 600 mg of anhydrous sodium carbonate was added to provide the required alkylating with A few porcelain bits and heated to boiling over a moderate flame. The standard glucose solution is taken in the burette when the benedict's solution Boils, glucose solution is added drop by drop (one drop per second) till the last trace of blue colour disappears. The volume of glucose rundown is noted and the titrations are repeated for concordant value.

The given unknown sugar solution was made upto 100 ml in a standard flask with distilled water. Then the burette was filled with unknown sugar solution and the benedict's reagent was titrated as before. The volume of sugar solution rundown was noted and titrations are repeated for concordant values.

## Estimation of Reducing Sugar by Benedict's Method

**Titration 1**

Standardisation of Benedict's Reagent Vs Standard Glucose Solution

| S. No. | Volume of Benedict's reagents(ml) | Burette Readings | | Volume of standard glucose(ml) | Indicator |
|---|---|---|---|---|---|
| | | Initial ml | Final ml | | |
| | | | | | Self |

**Titration 2**

Estimation of Glucose Standardised Benedict's Reagent Vs Unknown Glucose

| S. No. | Volume of Benedict's reagents(ml) | Burette Readings | | Volume of unknown glucose(ml) | Indicator |
|---|---|---|---|---|---|
| | | Initial ml | Final ml | | |
| | | | | | Self |

**Calculation:**

The standard glucose solution 2 mg / ml

5 ml of Benedict's solution react with……. ml of the standard glucose solution.

…….ml of standard glucose solution which contains x 2 = mg

5 ml of Benedict's solution reacts with……. mg of unknown glucose 100 ml of unknown glucose contains is 100 x…….

**Result:**

The amount of glucose present in 100 ml of given unknown solution is…….

## 11. Use of pH Meter, determination of pH value of dilute and strong acids and bases. Fruits and Vegetable extract.

**Principle**

pH of a solution indicates acidity or alkalinity of a solution. It is defined as negative logarithm (base 10) of hydrogen ion concentration.

$$pH = - \log (H^+)$$

Since, all solution contain $H^+$ and $OH^-$ ions due to dissociation of water in practice the hydrogen ion conc. Is used to determine the pH of a solution. Thus, a substance which donates hydrogen ion is known as acid and the substance which accepts hydrogen ion is called as a base.

The dissociation of water can be written as:

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

And thus, $K_w = [H^+] [OH^-] / [H_2O]$

Where, $K_w$ is the dissociation constant of water. The ionic product of water has been found to be $10^{-14}$ moles / L or

$$[H^+] + [OH^-] = 10^{-7} + 10^{-7}$$

Since, the amount of $[H^+]$ and $[OH^-]$ is equal.

Therefore, $[H^+] = [OH^-] = 10^{-7}$ moles /L

So, pH of water can be represented as:

$$pH = -\log [H^+] = 7$$

In order to express the actual conc. of $[H^+]$ in any solution pH scale was devised by Sorensen. Accordingly, when the conc. of hydrogen in any solution is higher than 10, pH will be less than 7 and hydrogen ion conc. is lower than $10^7$, pH will be higher than 7. It may be noted that pH of an aqueous solution will depend upon the dissociated ion and therefore pH of two solutions having the same normality will differ because of different H dissociation capacity.

**Measurement of pH**

The most useful and accurate method of measuring the pH of a solution is by the use of pH meter. Which measures electromotive force (e.m.f) produced by $H^+$ around the electrode. A pH meter consists of two electrodes – a glass electrode which is sensitive to $H^+$ and a reference electrode called as calaml electrode, which has a constant e.m.f and is not sensitive to $H^+$ ions. The calaml electrode in a test solution acts as a half cell and the circuit is complete when it is connected through the potentiometer to the reference electrode. Some, of the pH meters have both electrodes combined in one unit and not as separate entities. The e.m.f of the complete cell (E) formed by the lining of these two electrodes is therefore

$$E = E - E \text{ glass}$$

E ref is the potential of the reference electrode which is constant and E glass is the potential of a glass electrode which depends on the pH of the solution under test.

**Operation of the pH meter:**

The electrode of a pH meter is very delicate and therefore have to be handled carefully. The following points are to be considered before the use of pH meter.

1. New glass electrode should be soaked in 0.1 mol/l of HCl or distilled water for several hours before use.
2. The solution must be thoroughly stirred before measuring the pH.
3. The electrode must be washed with distilled water before and after use and should not be touched with hands.
4. The pH meter is calibrated before use by means of standard solution. A solution whose pH is close to the test solution should be used for calibration.
5. After use the electrode must be dipped in distilled water and should not be allowed to dry.

| List of some Fruits and their pH Range | | |
|---|---|---|
| **S. No.** | **Fruit** | **pH Range** |
| **1.** | Lemon Juice | 2.00–2.60 |
| **2.** | Limes | 2.00–2.80 |
| **3.** | Blue Plums | 2.80–3.40 |
| **4.** | Grapes | 2.90–3.82 |
| **5.** | Pomegranates | 2.93–3.20 |
| **6.** | Grapefruits | 3.00–3.75 |
| **7.** | Blueberries | 3.12–3.33 |
| **8.** | Pineapples | 3.20–4.00 |
| **9.** | Apples | 3.30–4.00 |
| **10.** | Peaches | 3.30–4.05 |
| **11.** | Oranges | 3.69–4.34 |
| **12.** | Tomatoes | 4.30–4.90 |
| **13.** | Lemon Juice | 2.00–2.60 |
| **14.** | Limes | 2.00–2.80 |

| List of some Vegetables and their pH Range | | |
|---|---|---|
| **S. No.** | **Vegetable** | **pH Range** |
| **1.** | Beans | 6.0 – 7.5 |
| **2.** | Broccoli | 6.0 – 7.0 |
| **3.** | Cabbage | 6.0 – 7.5 |
| **4.** | Carrot | 5.5 – 7.0 |
| **5.** | Cauliflower | 5.5 – 7.5 |
| **6.** | Cucumber | 5.5 – 7.5 |
| **7.** | Garlic | 5.5 – 7.5 |
| **8.** | Kale | 6.0 – 7.5 |
| **9.** | Lettuce | 6.0 – 7.0 |
| **10.** | Onion | 6.0 – 7.0 |
| **11.** | Pea | 6.0 – 7.5 |
| **12.** | Potato | 4.5 – 6.0 |
| **13.** | Pumpkin | 5.5 – 7.5 |
| **14.** | Spinach | 6.0 – 7.5 |
| **15.** | Tomato | 5.5 – 7.5 |
| **16.** | Turnip | 5.5 – 7.0 |
| **17.** | Pea | 6.0 – 7.5 |
| **18.** | Potato | 4.5 – 6.0 |
| **19.** | Pumpkin | 5.5 – 7.5 |
| **20.** | Spinach | 6.0 – 7.5 |

# 12. Estimation of glucose in blood

**Introduction:**

For all practical purposes, glucose is the only sugar that is present in the blood. Glucose is absorbed by the body cells and is the major source of cellulose energy.

- Normal fasting level = 60-90mg/100ml of blood
- Glucose level half an hour (after meal) (post prandial) = 120-150mg/100ml of blood

In normal healthy individuals the peak glucose level (at any time of the day) = 60-110 mg/100ml of blood is considered normal. Normal HbA1c levels and range.

**Clinical significance of blood sugar level:**

Blood glucose level increases in diabetes mellitus, acute stress, hyperthyroidism and chronic liver disease. And it decreases in Addison's disease, hypothyroidism and cancer of the pancreas.

The increase in the blood glucose level is called hyperglycaemia and decrease in blood glucose level as hypoglycaemia. People suffering from diabetes mellitus need to get their blood glucose tested frequently.

**Methods used to measure blood glucose level**

Although a number of methods are used for glucose determination, commonly used two methods are discussed here.

These can be grouped into two categories- chemical and enzymatic.

❖ Chemical method: Folin-Wu method, Ortho-Toluidine method

❖ Enzymatic method: GOD-POD method. (Glucose oxidase method)

**Chemical method to estimate blood glucose:**

- Folin-Wu method:

  It is based on the principle that glucose when heated with an alkaline copper solution, reduces cupric ions to cuprous ions. The cuprous ions are then measured photometrically (calorimetrically) by adding phosphomolybdic acid which gets reduced to molybdenum blue. In this method, whole blood is used and the blood glucose value is determined by the intensity of blue color. This one is the time-consuming method and also is a non-specific method and also measures the Fructose.

- Ortho-Toluidine method:

  The O-toluidine method is an older method of blood glucose estimation. This method is no longer used today because O-toluidine is believed to be a carcinogen and is replaced by enzymatic methods. This method is still popular because of its simplicity, sensitivity and accuracy. It is based on the principle that the aldose sugar i.e. glucose on condensation with ortho-toluidine in glacial acetic acid gives a green colour that can be measured spectrophotometrically.

For glucose estimation from any material, blood is collected in fluoride containing vial.

- Fluoride inhibit glycolysis by inhibiting enolase enzyme.
- In CSF, bacteria & other cells are also present so analyzed immediately.

- For glucose estimation from urine, add 5ml glacial acetic acid as preservative to inhibit bacterial growth.

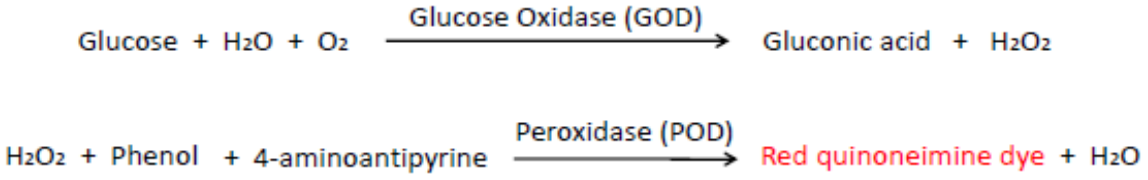
**GOD-POD Method for Glucose Estimation**

Being more specific, easier, and more accurate, enzymatic methods are preferred these days. Among them, the GOD-POD method is the most common method of glucose estimation.

**Principle:**

In the presence of atmospheric oxygen, glucose present in the specimen is oxidized by the enzyme glucose oxidase (GOD) to gluconic acid and hydrogen peroxide ($H_2O_2$).

Thus, formed $H_2O_2$ oxidatively couples with 4-aminoantipyrine and phenol in presence of peroxidase (POD) to form red-colored quinoneimine dye, which is measured colorimetrically at 540nm. The intensity of the color is directly proportional to the concentration of glucose present in the specimen.




**Requirements:**

- Specimen: Serum, or plasma free of hemolysis. Sodium fluoride is preferred as an anticoagulant due to its antiglycolytic activity.
- Reagents:
  - Glucose standard (100 mg/dl)
  - GOD-POD reagent: Enzyme reagent mixture containing glucose oxidase (GOD), peroxidase (POD), 4-aminoantipyrine, phenol, and phosphate buffer (pH≈7.0), some stabilizers and activators.
- Instruments:
  - Test tubes
  - Pipettes, disposable tips, rack
  - Water bath
  - Colorimeter

**Procedure:**

1. Label three clean, dry test tubes as Blank (B), Standard (S), and Test (T).
2. Pipette as follows:

| | Blank | Standard | Test |
|---|---|---|---|
| **GOD-POD Reagent** | 1 ml | 1 ml | 1 ml |
| **Distilled water** | 10 µl | – | – |
| **Glucose standard** | – | 10 µl | – |

| | | | |
|---|---|---|---|
| **Sample** | – | – | 10 µl |

3. Mix well and incubate at 370C for 10 minutes. Or, at room temperature (250C) for 30 minutes.
4. Measure the absorbance of the standard and test sample at 540nm (green filter) against blank within 60 minutes.

**Calculation:**

Calculate the concentration of blood glucose in the specimen using the following formula:

$$\text{Conc. of Glucose in the specimen (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times 100$$

# 13. Estimation of proteins:

## 13.1 By Lowry's method:

**Principle:**

Protein in the given solution when treated with alkaline copper sulphate and Folin's phenol reagent produces a blue colored complex. The intensity of the colour is directly proportional to the concentration of protein present in the given sample solution.

**Reagents Required**

- Stock Solution: Bovine serum albumin of 100 mg is weighed accurately and dissolved in 100ml of distilled water in a standard flask. (Concentration:1mg/ml)
- Working Standard: The stock solution of 10 ml is diluted to 100 ml with distilled water in a standard flask. (Concentration:100mg.ml)
- Folin's Phenol Reagent: Folin's phenol reagent is mixed with distilled water in the ratio of 1:2
- Alkaline $CuS0_4$ Reagent:
  Solution A: Sodium carbonate of 2% in 0.1N sodium hydroxide.
  Solution B: Sodium Potassium tartrate of 1%
  Solution C: Copper sulphate of 0.5%
  Solutions A, B, C are mixed in the proportion of 50: 1: 0.5
- Unknown Preparation: The given protein is made up to 100ml with distilled water.

**Procedure:**

Working standard of 0.2 to 1.0 ml is pipetted out into clean test tubes labelled as S1 to S5. Test solution of 0.2 and 0.4 ml is taken in test tubes labelled as T1 and T2. The volume is made up to 1.0 ml with distilled water. Distilled water of 1.0ml serves as blank. To all the test tubes 4.5 ml of alkaline copper sulphate reagent is added and it is incubated at

room temperature for 10 minutes. To all the test tubes 0.5 ml of Folin's Phenol reagent is added. The contents are mixed well and the blue colour developed is read at 640 nm after 15 minutes. From the standard graph the amount of protein in the given unknown solution is calculated.

| **Estimation of Protein by Lowry's Method** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **S. No.** | **Reagents** | **B** | **$S_1$** | **$S_2$** | **$S_3$** | **$S_4$** | **$S_5$** | **$T_1$** | **$T_2$** |
| **1.** | Volume of working standard (ml) | - | 0.2 | 0.4 | 0.6 | 0.8 | 1.0 | - | - |
| **2.** | Concentration of working standard (mg) | - | 20 | 40 | 60 | 80 | 100 | - | - |
| **3.** | Volume of unknown solution (ml) | - | - | - | - | - | - | 0.2 | 0.4 |
| **4.** | Volume of distilled water (ml) | 1.0 | 0.8 | 0.6 | 0.4 | 0.2 | - | 0.8 | 0.6 |
| **5.** | Volume of alkaline copper reagent (ml) | 4.5 ml in each | | | | | | | |
| **6.** | Volume of Folin's phenol reagent | 0.5 ml in each | | | | | | | |
| *The contents are mixed well and kept at room temperature for 10 minutes. The blue colour developed is read at 640nm* | | | | | | | | | |
| **7.** | Optical density 640nm | | | | | | | | |

**Calculation:**

……of unknown solution corresponds to…….x OD

OD corresponds of ……. mg of protein

i.e. 0.2ml of unknown solution contains …… of protein 100ml of unknown solution contains 100 x ----

**Result:**

The amount of protein present in the given solution is……

**OR**

## 13.2 By Biuret method:

**Introduction:**

This test was developed first by Allan G. Gornall and his *co-workers* in 1945 for the determination of serum proteins and is still widely used.

## Principle

This is a general test for all compounds, which contain two or more amide linkages (peptide bonds). The name of the test is derived from the fact that the compound 'Biuret' formed by heating urea at 180°C gives the same test. The cupric ion in the reagent reacts with peptide bond / nitrogen to form coloured complex. Proteins and polypeptides having three or more constituent amino acids give a positive test.

## Reagents Required

- Biuret reagent: Dissolve 9 g of potassium sodium tartarate in 500 ml of 0.2 N NaOH. Add 3 g of $CuSO_4.5H0$ and dissolve. Make up the total volume to 1 lit. with 02 N NaOH.
- Standard protein solution (BSA, 10 mg/ml): Dissolve 100 mg of BSA in double distilled water and make the total volume to 10 ml.

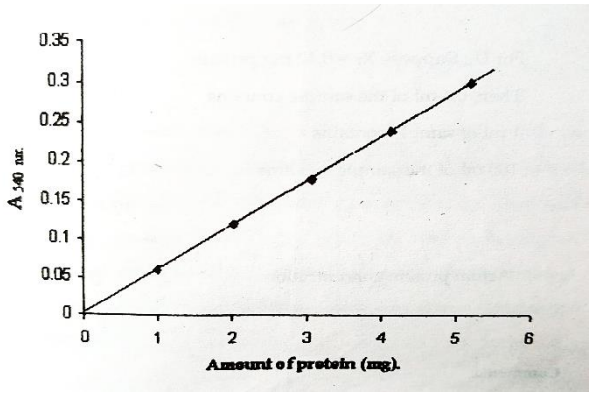
## Experimental Procedure

| | | **Test tube number (Standard BSA Protein)** | | | | | | | **Unknown Protein** | |
|---|---|---|---|---|---|---|---|---|---|---|
| **S. No.** | **Steps** | **B** | **1** | **2** | **3** | **4** | **5** | **6** | **$U_1$** | **$U_2$** |
| 1. | Protein solution (ml) | - | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 | 0.6 | 0.2 | 0.4 |
| 2. | Distilled Water (ml) | 1.0 | 0.9 | 0.8 | 0.7 | 0.6 | 0.5 | 0.4 | 0.8 | 0.6 |
| 3. | Biuret Reagent (ml) | 4ml in each | | | | | | | | |
| **Vortex the mixture for few sec. and then incubate for 20 min. at 37°C** | | | | | | | | | | |
| 4. | $A_{540nm}$ | - | 0.06 | 0.12 | 0.18 | 0.24 | 0.30 | 0.36 | $A_1$ | $A_2$ |
| 5. | Amount of Protein (mg) | - | 1 | 2 | 3 | 4 | 5 | 6 | $X_1$ | $X_2$ |

B represents blank, tubes numbered (1 – 6) represent standard BSA, $U_1$ and $U_2$ represent test solution.

***Calculations***

Plot a graph between absorbance and protein concentration.




Using standard curve, extrapolate the amount of BSA (assume it equals the amount of protein) in the sample. Horizontal lines are drawn from absorbance values A1 and A2 of U1 and U2 to intersect the standard curve. Vertical lines drawn from intersection points on the X- axis give protein concentration.

For U1: Suppose X1 = 2mg

Then, 0.2 ml of sample contains = 2mg protein

1ml of sample contains = 2 / 0.2

100ml of the sample contains = 2/0.2 × 100

= 1000 mg / 100 ml

= 1mg %

For U2: Suppose X2 = 0.42 mg protein

Then, 0.4 ml of the sample contains = 4.2 mg protein

1ml of sample contains = 4.2 / 0.4

100 ml of the sample contains = 4.2 / 0.4 × 100 ml

= 1050 mg / 100 ml

= 1.05 g%

Actual protein concentration = (1+1.05)! 2

= 1.025 g%

**Comments**

i. The substances which interfere with this assay include buffers, which are amino acids or peptides such a *Tris and Good's* buffers because they give positive test.
ii. The disadvantage of this method is lack of its sensitivity. Its lower limit is 1 mg.

# 14. Estimation of Calcium and Phosphorus

## 14.1 Estimation of Calcium by EDTA Titrimetric Method

The amount of calcium in any sample can be estimated by EDTA titrimetric method. The principle behind this method is described next.

**Principle**

In this test, Eriochrome dye is used. This dye is blue is colour. In unknown solution, calcium carbonate is ionized into calcium ($Ca^{2+}$) and carbonate ($CO_3^-$). The calcium will form a complex with Eriochrome (Eriochrome - $Ca^{2+}$ complex) and colour will change to pink, yellow or reddish. While titrating against standard EDTA, there will be a competition between EDTA and Eriochrome for $Ca^{2+}$. Since, EDTA is having high affinity with calcium compared to Eriochrome. Therefore, EDTA will chelate or bind with $Ca^{2+}$ leaving behind Eriochrome. The blue colour regenerated will be directly proportional to presence of $Ca^{2+}$ in the unknown solution.

OR

Calcium at pH 10 in presence of dye eriochrome black T (indicator) is wine red. The affinity of ethylene diamine tetraacetate (EDTA) for calcium is greater than the affinity of the indicator for calcium and so the indicator releases its metal to EDTA. When completely complexed (chelated) with EDTA, the indicator is released from its combination with calcium. This free indicator gives a blue colour to the solution indicating that the end point is reached. Magnesium must be present for satisfactory end point and is added as Mg EDTA. End point sharpness increases with pH but high pH may cause precipitation of Ca (OH), and cause colour changes of dye. A pH of 10.0 + 0.1 is a satisfactory compromise. A $NH_4OH$ - $NH_4CI$ buffer is used to maintain this pH. Limit of 5 minutes for titration minimizes precipitation.

The reaction involved in this estimation is presented herewith:

| Caln | + | EDTA | | CaEDTA | + | ln |
|---|---|---|---|---|---|---|
| (Calcium indicator) (red) | | (colourless) | | (colourless) | | (blue) |

So, in the estimation of calcium, the solution containing calcium is titrated against EDTA solution. EDTA can form a chelate complex with calcium. It is a six-coordinate Mg-EDTA complex. A small amount of indicator (In) is added to the $Ca^{2+}$ to form a red complex as indicated in the reaction above. As EDTA is added, it reacts first with free colourless $Ca^{2+}$ and then with the red Caln complex. The EDTA binds to $Ca^{2+}$ better than the indicator binds to $Ca^{2+}$, The indicator thus releases its metal to EDTA. The change from the red Caln to the blue of unbound indicator (In) signals the end point of titration. It is important to note that the colour of the free indicator is pH dependent. Hence, most indicators can be used only in certain pH range. In order to maintain pH, we therefore use buffers.

A metal ion indicator is a compound which changes its colour on binding to metal ion.

The detailed procedure involved in the estimation of calcium is described in Experiment I later in this practical. Read the procedure carefully before you start the experiment. Next, we shall study about phosphorus and the methods employed for its estimation.

**Apparatus**

- Burette - 50 ml
- Pipettes 5 ml, 10 ml
- Conical flasks 100 ml
- Volumetric flasks 100 ml
- Measuring cylinders
- Beakers
- Glass marker

**Chemicals Used**

- Calcium Carbonate ($CaCO_3$) = 100mg %
- Sodium- ethylenediaminetetraacetic acid (Na-EDTA) = 0.01M = 1.8 g in 500ml
- Ammonium Chloride ($NH_4Cl$)
- Ammonium Hydroxide ($NH_4OH$)
- Magnesium Chloride ($MgCl_2$)
- Sodium Chloride (NaCl)
- Eriochrome black-T

**Reagents Prepared**

1. *Buffer solution*: Dissolve l6.9 g $NH_4CI$ in 143 ml $NH_4OH$. To this add 50 ml of solution containing 1.17g $Na_2EDTA.\ 2H_20$ and 0.644 g $MgCl_2.6\ H_20$ and dilute to 250 ml with distilled water.
2. *Indicator:* This contains a mixture of 0.5 g Eriochrome black T and 100 g NaCl. It is used in a dry form. OR = 125mg + 25g NaCl
3. *EDTA solution (0.01 M):* Weigh 3.723 g $Na_2$ EDTA.$2H_20$ and dilute to one litter with distilled water.
4. *Standard calcium solution:* Calcium carbonate solution containing 1mg/ml of calcium carbonate.
5. *Distilled water*

**Procedure**

Carry out the experiment following the steps enumerated herewith:

1. Standard titration - Pipette 10 ml standard calcium solution into a 250 ml conical flask. To this add 50 ml of distilled water. Add 2 ml buffer solution and 150-200 mg indicator powder. Titrate with given EDTA solution stirring continuously until the reddish tinge disappears and the colour becomes blue which is the end point. The titration should be completed within 5 minutes from the time of addition of buffer

solution. Repeat this procedure at least three times or until concordant values are obtained.

2. Sample titration - Dilute the given calcium solution in 100 ml volumetric flask to the mark with distilled water. Shake well. Pipette 10ml of this dilute solution into a 250 ml conical flask and proceed just as you did for standard titration.

**Precautions**

1. Rinse all your glassware with distilled water.
2. Complete the titration in 5 minutes

**Method of Calculation**

While conducting the experiment record the titration reading in the format given herewith and do the calculations as enumerated.

**1. *Standard titration:***

1) Strength of standard $CaCO_3$ solution = 1 mg/ml
2) Volume of standard $CaCO_3$ solution = 10 ml
3) Volume of standard EDTA solution required = 10 **P** ml

| S. No. | Burette Reading (ml) | | |
|---|---|---|---|
| | Initial | Final | Difference |
| Pilot | | | |
| 1. | 50ml | 60ml | 10 ml |
| 2. | 60ml | 70ml | 10ml |
| 3. | 70ml | 80ml | 10ml |

Titter value = …10…. (**P**) ml

4) 1 ml of standard solution contains l mg $CaCO_3$

Therefore, 10 ml of standard solution contains 10 mg $CaCO_3$

i.e., 10 mg of standard $CaCO_3$ solution chelates with (P)..10....ml standard EDTA solution.

or (P)..10..... ml of standard EDTA solution chelates with 10 mg $CaCO_3$

**2. *Sample titration:***

5) Given $CaCO_3$ solution no .....(enter your flask no.) diluted to 100 ml =R mg
6) Volume of dilute sample $CaCO_3$ solution (Unknown) =10ml
7) Volume of standard EDTA solution required = 9ml (Qml)

| S. No. | Burette Reading (ml) | | |
|---|---|---|---|
| | Initial | Final | Difference |
| Pilot | | | |
| 4. | 80ml | 89ml | 9ml |
| 5. | 89ml | 98ml | 9ml |

Titter value = …9…. (**P**) ml

8) Q ml of EDTA chelates with R mg of $CaCO_3$.

9) R mg Can be calculated by:

$R = Q \times 10 / P$ mg

$R = 9 \times 10 / 10$ mg

$R = 9$ mg

10) Therefore, 9ml of EDTA chelates with 9mg of $CaCO_3$.
11) 9mg of $CaCO_3$ is present in 10 ml of unknown $CaCO_3$
12) 1mg of unknown $CaCO_3$ solution contains = 9/10 mg of Calcium carbonate.
= 90 mg of $CaCO_3$
13) 100mg of $CaCO_3$ contains = 40mg of $Ca^{2+}$
14) Therefore, 1mg of $CaCO_3$ contains = 40/100 mg of $Ca^{2+}$
15) 90mg of $CaCO_3$ contains = $40/100 \times 90$ mg $Ca^{2+}$
= 36mg of $Ca^{2+}$ or 36mg %

## 14.2 Estimation of phosphorus by the Fiske and Subbarrow's method

**Introduction:**

An adult body contains about 1 kg phosphate and is found in every cell of the body. About 80 % of it occurs in combination with calcium in the body skeleton. About 10 % of it is found in muscles and in blood in association with proteins, carbohydrates and lipids. The remaining 10 % of it, is widely distributed in various chemical compounds.

In blood phosphorus exists mainly in three different fractions:

i. Inorganic phosphate
ii. Lipid phosphorus (phospholipids)
iii. Organic acid soluble phosphate esters.

Phosphorus is involved in various biochemical functions:

i) For development of bones and teeth.
ii) For formation of energy rich compounds such as ATP, GTP, creatinine phosphate.
iii) In synthesis of DNA and RNA.
iv) Activation of several enzymes and proteins.
v) As phosphate buffer system is important for the maintenance of pH in the blood as well as in the cells.
vi) Phosphate is necessary for the absorption and metabolism of carbohydrates.

**Principle**

The estimation of phosphorus is based on the principle that, "proteins and phospholipids containing phosphorus from the blood sample or urine if present are precipitated by TCA and the precipitate obtained is digested with 60 % per chloric acid (PCA). The inorganic phosphate so released reacts with acid molybdate to form phosphomolybdic acid. The phosphomolybdic acid is heated with a reducing agent 1, 2, 4 amino naphthol - sulphonic

acid (ANSA) reagent to produce a blue colour of reduced molybdenum, which is read at 660 nm".

## Materials required

- 10 % trichloro acetic acid (TCA).
- 60 % per chloric acid (PCA).
- 4 % ammonium molybdate (prepared by heating at 60$^{o}$C
- ANSA reagent: 12 g of sodium bisulphite, 2.4 g of sodium sulphite and 0.2 mg of 1 - amino - 2 - naphthol 4 - sulphonic acid are dissolved in water and the volume is made to 250 ml. After 3 hrs., filter the solution to remove any un dissolved material. Store in a brown bottle at 4$^{o}$C.
- Standard stock solution: 4.391 g / lit. of $KH_2PO_4$.
- Working standard solution: Diluted to 1: 100 standard stock solution, which corresponding to 10 µg phosphorus / ml.

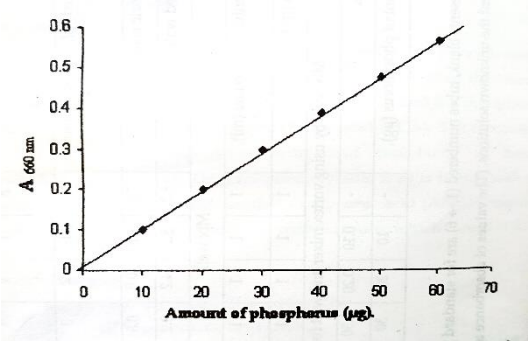
## Experimental Protocol

| S. NO. | Steps | B | 1 | 2 | 3 | 4 | 5 | 6 | $U_1$ | $U_2$ |
|---|---|---|---|---|---|---|---|---|---|---|
| **1.** | Standard Phosphorus solution (ml) | - | 1 | 2 | 3 | 4 | 5 | 6 | 2 | 4 |
| **2.** | Perchloric acid (ml) | **0.8 ml in each** | | | | | | | | |
| **3.** | Distilled water (ml) | 6.2 | 5.2 | 4.2 | 3.2 | 2.2 | 1.2 | 0.2 | 4.2 | 2.2 |
| Mix Well. | | | | | | | | | | |
| **4.** | Ammonium molybdate (ml) | **1 ml in each** | | | | | | | | |
| **5.** | ANSA (ml) | **1 ml in each** | | | | | | | | |
| Mix well, by using vortex mixer and wait for 10 – 20 min. | | | | | | | | | | |
| **6.** | $A_{660nm}$ | - | 0.10 | 0.20 | 0.30 | 0.39 | 0.48 | 0.55 | $A_1$ | $A_2$ |
| **7.** | Amount of Phosphorus (µg) | - | 10 | 20 | 30 | 40 | 50 | 60 | $X_1$ | $X_2$ |

B represents blank, tubes numbered (1 - 6) are for standard phosphorus solution, $U_1$ and $U_2$ represent the unknown solution. (The values of absorbance are arbitrary).

***Calculations:***

Plot a graph between the amount of phosphorus and absorbance which gives the straight line.




Using the standard curve, extrapolate the amount of phosphorus (assume it equals the amount of phosphorus) in the sample. Horizontal lines are drawn from absorbance values $A_1$ and $A_2$ of $U_1$ and $U_2$ to intersect the standard curve. Vertical lines drawn from intersection points on the X – axis give phosphorus concentration as:

For $U_1$: Suppose $X_1$ = 30 µg

Then, 2ml of sample contains = 30 µg of phosphorus

1ml of sample contains = 15 µg of phosphorus

100ml of the sample contains = 15 × 100 µg

= 1500 µg / 100ml

= 1.5 mg %

For $U_2$: Suppose $X_2$ = 58 µg

Then, 4ml of sample contains = 58 µg of phosphorus

1ml of sample contains = 58/4

= 14.5 µg

100ml contains = 14500 µg

= 1.45 mg %

Actual Phosphorus concentration = (1.5 + 1.45) / 2

= 1.475 mg %

**Comments:**

i. Same method is used for the estimation of phosphorus in serum or urine, but from serum, phosphorus is released in the form of phosphate from proteins, phospholipids

by precipitating the proteins and phospholipids by TCA and then digested with PCA. For this, following few more steps are required.

To 3 ml of distilled water, add 0.2 ml of serum. Shake well and add 1.5 ml of 10 % TCA drop wise and then add 1.5 ml of TCA rapidly. Mix, centrifuge and after 10 min decant off the supernatant. Add I ml of 60 % of perchloric acid to the residue and keep on a sand bath till the fluid becomes colourless (about for1 hr.) Make the volume to 7 ml with distilled water and add 1 ml of 4 % ammonium molybdate. Shake and then add 1 ml of ANSA solution. The colour developed is measured at 660 nm and the concentration can be determined from standard curve as given in calculations.

ii. If one wants to determine phospholipid content. Then multiply above obtained value by a factor 25.

iii. The estimation should be made on either serum or plasma separated soon after with-drawing the blood. The inorganic phosphorus increases when blood is allowed to stand, since ester phosphate present in the red cells are hydrolyzed with formation of inorganic phosphate.

iv. Normal serum phosphorus is 3 - 4.5 mg / 100 ml serum while in case of children it is 4-7 mg/ 100 ml. Increased levels of phosphorus has been found in case of hypo para thyroidism, acromegaly, child hood, high intestinal obstruction.

Decreased levels of phosphorus has been found in conditions like, alcoholism, acute gout, diabetic mellitus, anabolic steroids, post insulin and glucose administration, hypokalaemia, hyper para thyroidism.

v. *Kultner and Lichtenstein* used stannous chloride as reducing agent. Their method is simpler than the above one (i.e., *Fiske and Subbarrow's*), since the stock stannous chloride solution keeps almost indefinitely in contrast to the ANSA and gives a deeper colour, so that it is the method most easily adopted to use small quantities of serum. However, the colour produced appears to be rather variable with time. This method has been widely used although it is not quite reliable as that of the *Fiske and Subbarrow's* method.

# 15. Estimation of bilirubin from serum.

## 15.1 Estimation of bilirubin by using Diazotized Sulphanillic acid.

Bilirubin is a catabolic product of hemoglobin of red blood cells. Serum bilirubin is chiefly present in two fractions. Conjugated bilirubin one of the major fractions comprising of about 76%, conjugated with glucuronic acid and rest is unconjugated (free bilirubin). The normal level of serum bilirubin is (direct 0.1 - 0.4 mg/100ml and indirect 0.2- 0.7 mg /100 ml). The increased levels of bilirubin are found in hepatocellular disease, biliary duct obstruction and hemolytic disease (jaundice). The estimation of bilirubin can be carried out by the method of (*Van-den Bergh and Snapper -1913*).

## Principle

The method of bilirubin estimation is based on the principle that, "both conjugated and unconjugated fractions form purple coloured complex as azobilirubin when coupled with diazotized Sulphanillic acid". Conjugated bilirubin, because of the presence of glucuronic acid, is water-soluble and reacts quickly (direct reaction), whereas unconjugated (free) bilirubin is not soluble in water and requires a solubilizer, such as methanol (indirect reaction). However, both are soluble in alcohol and when the reaction is carried out in alcohol, total serum bilirubin is estimated.

## Materials required

1. Solution A: - Dissolve 1 g Sulphanillic acid and 15 ml of concentrated HCl in 1 lit. of distilled water.
2. Solution B: 0.5 g of sodium nitrite in 100 ml of distilled water.
3. Diazo reagent: Prepare freshly before use by adding, 0.3 ml of solution - B to 10 ml of solution - A.
4. Diazo blank: Mix 15 ml of concentrated HCI in 1 lit. of distilled water.
5. Methanol
6. Bilirubin standard: 10 mg % in chloroform.

| S. No. | Steps | Standard Bilirubin | Standard control | Control | Test sample |
|---|---|---|---|---|---|
| **1.** | Standard Bilirubin (ml) | 0.2 | 0.2 | - | - |
| **2.** | Serum (ml) | - | - | 0.2 | 0.2 |
| **3.** | Distilled water (ml) | 1.8 ml | | | |
| **4.** | Diazo reagent (ml) | 0.5 | - | - | 0.5 |
| **5.** | Diazo blank (ml) | - | 0.5 | 0.5 | - |
| **6.** | Methanol (ml) | 2.5 ml | | | |
| Mix well and keep the tubes for 30 minutes in dark | | | | | |
| **7.** | $A_{540nm}$ | $A_S$ | $A_{SC}$ | $A_C$ | $A_T$ |

**$A_S$** = Absorbance of standard bilirubin
**$A_{SC}$** = Absorbance of standard control
**$A_C$** = Absorbance of control
**$A_T$** = Absorbance of test sample

## Calculations

Serum bilirubin [Total] = $(A_T - A_c) / (A_s - A_{sc}) \times 10$
= =X µg / 100 ml.

The experimental protocol for determination of conjugated (direct), bilirubin is same except that instead of 2.5 ml methanol, 2.5 ml of water is added.

Conjugated serum bilirubin = $(A_T - A_c) / (A_s - A_{sc}) \times 10$
= [Y µg/ 100 ml]

Unconjugated bilirubin (indirect) = Total – conjugated
= X – Y = Z (µg/100 ml).

**Comments**

(i) Standard bilirubin is prepared in chloroform and refluxed for several hrs. in order to achieve its complete dissolution.

(ii) For serum isolation blood is collected in a clean container without any anticoagulant and allowed to clot. Serum can be separated from the clot as soon as possible, taking care to avoid hemolysis of RBC's.

## 15.2 Estimation of cholesterol by Zlatki's method

**Principle:**

This method of cholesterol estimation was introduced by *Zlatki's etal*. (1952) and is based on the fact that cholesterol on reaction with concentrated $H_2SO_4$ undergoes oxidation and dehydration, the resultant product in presence of ferric chloride gives an intense colour, which absorbs strongly at 560 nm.

**Reagents**

- Ferric chloride - Acetic acid reagent: - 0.05 % solution of $FeCl_3$. $6H_2O$ in glacial acetic acid.
- Concentrated sulphuric acid.
- Cholesterol standard (4 mg / 100 ml): - Dissolve 40 mg of cholesterol in 10 ml of warm Ferric chloride - Acetic acid reagent. Take 1 ml of this solution and make it to 100 ml with Ferric chloride - Acetic acid reagent.

**Experimental Protocol**

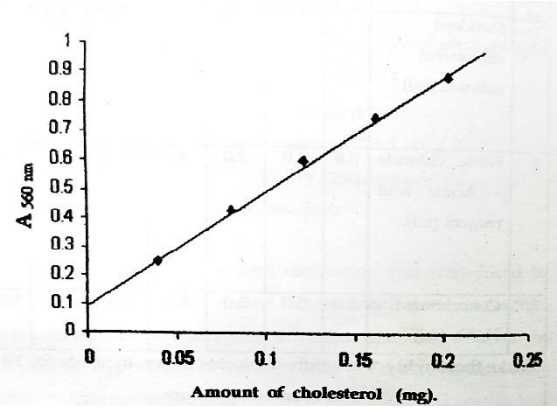
| S. NO. | Steps | B | 1 | 2 | 3 | 4 | 5 | U 1 | U 2 |
|---|---|---|---|---|---|---|---|---|---|
| 1) | Standard Cholestero l solution (ml) | - | 1.0 | 2.0 | 3.0 | 4.0 | 5.0 | 2.0 | 4.0 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **2)** | Ferric Chloride – Acetic acid reagent (ml) | 5.0 | 4.0 | 3.0 | 2.0 | 1.0 | - | 3.0 | 1.0 |
| **3)** | Concentrated $H_2SO_4$ (ml) | 5.0 ml in each | | | | | | | |
| **Shake thoroughly, but gently vortex mixer. Incubate for 10 min. at room temperature** | | | | | | | | | |
| **4)** | **$A_{560\ nm}$** | - | 0.25 | 0.43 | 0.60 | 0.75 | 0.89 | $A_1$ | $A_2$ |
| **5)** | **Amount of Cholesterol (mg)** | - | 0.04 | 0.08 | 0.12 | 0.16 | 0.20 | $X_1$ | $X_2$ |

B represents blank, tubes numbered (1 – 5) are for standard cholesterol, U1 and U2 represent test solutions. (The values of absorbance are arbitrary).

***Calculations***

Plot a graph between absorbance and the amount of cholesterol.




Using standard curve, extrapolate the amount of cholesterol (assume it equals the amount of cholesterol) in the sample. Horizontal lines are drawn from absorbance values $A_1$ and $A_2$ of $U_1$ and $U_2$ to intersect the standard curve. Vertical lines drawn from intersection points on the X - axis give cholesterol concentration.

For $U_1$: Suppose $X_1$ = 0.03 mg

Then, 2 ml of sample contains = 0.03 mg cholesterol

1 ml of sample contains = 0.03/ 2

100 ml of the sample contains = 0.015 x 100 mg

=1.5 mg/ 100 ml

=1.5 mg %

For $U_2$: Suppose $X_2$ = 0.06 mg

| | |
|---|---|
| 4 ml of sample contains | = 0.06 mg of cholesterol |
| 1 ml of sample contains | = 0.06 / 4 |
| | = 0.015 x 100 mg |
| 100 ml contains | = 0.015 x 100 |
| | = 1.5 mg % |
| Actual cholesterol concentration | = (1.5 + 1.5) /2 |
| | = 1.5 mg % |

# 16. Estimation of creatinine & Vitamin C by Jaffe's method

## 16.1 Estimation of creatinine By Jaffe's Method.

**Introduction**

Creatinine is a waste product from Muscles & Brain. And can be present in Urine. It (Creatine) originates from Kidneys, liver and pancreas and is transported via blood to other organs like Brain and muscles where it gets phosphorylated by *creatinine phosphatase* to form Phosphocreatine. Which is a good source for energy and gets used up by Muscles during contraction to form Creatinine. It can be determined by Jaffe's Method and is also used to diagnose Kidney functioning by checking Creatinine clearance. If levels in urine are low (Means, creatinine is accumulated in blood due to poor clearance of Kidneys).

**Normal Levels**

- Serum Creatinine Levels:
  - Adult Male = 0.74 – 1.35 mg / dl
  - Adult Female = 0.59 – 1.04 mg / dl
- Urine Clearance (24 hours collection):
  - 500 -2000 mg / day (4,420 – 17,680 mmol / day)

**If Elevated Levels**

- Shortness of breath
- Nausea
- Vomiting
- Dry Skin

This is all because of impaired Kidney's functioning.

**Principle**

Creatinine reacts with picric acid in alkaline solution to form a reddish coloured complex. The reaction is commonly known as the *Jaffe reaction* and the red coloured product as the *Janovski Complex.*

**Reagents Required**

- Alkaline medium: 0.75 N NaOH = &5 ml of 1N NaOH in 100ml distilled water.
- Picric Acid Reagent: 0.04M Picric Acid = 9.16 g of Picric Acid powder in 1L of distilled water.
- Stock Creatinine Standard: 100mg of Creatinine powder in 100ml of 0.1N HCl.
- Working Standard of Creatinine: Dissolve 5ml of Stock Creatinine into 500ml of Distilled water = 50 µg/ml
- Sample: Take 1 ml of your sample (In conical flask) & dilute it to 100ml with distilled water.

**Experimental Protocol:**

| Test Tubes | Working Solution of Creatinine (ml) | Amount of Creatinine (µg) | Volume of water (ml) | Volume of Picric Acid (ml) | Volume of NaOH (ml) | Incubation Time | Volume of Water | $A_{540nm}$ |
|---|---|---|---|---|---|---|---|---|
| 1. | 0.0 | Blank | 2.0 | 1 ml in each | 0.2 ml in each | Allow to stand for 15 min. | 5.8 ml in each | |
| 2. | 0.2 | 10 | 1.8 | | | | | |
| 3. | 0.4 | 20 | 1.6 | | | | | |
| 4. | 0.6 | 30 | 1.4 | | | | | |
| 5. | 0.8 | 40 | 1.2 | | | | | |
| 6. | 1.0 | 50 | 1.0 | | | | | |
| 7. | 1.0 | $U_1$ | 1.0 | | | | | |
| 8. | 1.0 | $U_2$ | 1.0 | | | | | |

## 16.2 Estimation of Vitamin 'C' by using DCIP.

Vitamin 'C', chemically named as ascorbic acid, is one of the water-soluble vitamins widely distributed in nature. It occurs in especially high concentration in citrus fruits and green plants such as green peppers and spinach. The fundamental role of an ascorbic acid in metabolic processes is not well understood. It has been found to be involved in hydrogenation reaction. Its function in these fundamental processes appear to be related to the ability of vitamin 'C' to act as reducing agent. The RDA of vitamin C is 70 mg/ day. Some scientists and physicians have suggested doses up to 1 - 3 g/ day in order to help resist the common cołd. t has found to be mainly responsible for formation of intracellular substances such a collagen of fibrous tissue structures, the matrices of bone, cartilage and dentin. Its deficiency leads to swollen joints and eventually scurvy.

**Principle**

The estimation of ascorbic acid is based on the principle of redox titration between a blue dye 2, 6-dichloro phenol indophenol (DCIP) and ascorbic acid. Although the original DCIP solution is blue in colour, it becomes light red in the acid solution. Upon reaction with ascorbic acid in the sample it becomes colourless. Titration is continued until, a very slight faint pink colour remains in the acid solution, indicating the end point.

**Materials required**

- 2, 6-Dichloro phenol indophenol (50 mg / lit. of solution).
- Ascorbic acid – 20 mg / lit. of solution (freshly prepared).
- Glacial Acetic Acid
- β-chloromercuri benzoic acid / oxalic acid (4%)

**Reaction**

Ascorbic Acid + Dye ⟶ Dehydroascorbic acid + Colourless in acid

**Method**

To 10 ml of test sample (unknown solution) in a conical flask, add 1 ml glacial acetic acid and titrate with dye (added from burette) to permanent faint pink colour. Volume of DCIP used is determined. The process is repeated for standard solution of Vitamin 'C'.

***Calculations***

**Volume of DCIP used for test sample.**

| S. NO. | Initial reading | Final Reading | Volume of DCIP used (ml) |
|---|---|---|---|
| **1.** | 0 | 14.5 | 14.5 |

| | | | |
|---|---|---|---|
| **2.** | 14.5 | 29.5 | 15.0 |
| **3.** | 29.5 | 44.5 | 15.0 |

**Concurrent Reading** = 15ml of DCIP

$V_T$ = 15 ml

**Volume of DCIP used for standard sample.**

| S. NO. | Initial reading | Final Reading | Volume of DCIP used (ml) |
|---|---|---|---|
| **1.** | 0 | 9.5 | 9.5 |
| **2.** | 9.5 | 19.5 | 10.0 |
| **3.** | 29.5 | 29.5 | 10.0 |

**Concurrent Reading** = 10ml of DCIP

$V_S$ = 10 ml

**Concentration of unknown solution of vitamin 'C'**

*= (Volume of DCIP used for standard solution) / (Volume of DCIP used for test sample) × 20 mg / lit.*

= VS / VT × 20 mg / lit.

= 10 / 15 × 20 = 13.3 mg / lit

**Comments**

1. The dye is decolourized by other compounds as well as by ascorbic acid, but the specificity can be increased to some extent by carrying out the reaction in an acid solution where interfering substances react only slowly.

2. Determination of vitamin 'C' in biological fluids such as blood and urine are more difficult because only small amounts of vitamin are present and many interfering reducing agents are present. The substances containing sulfhydryl groups, sulphite and thiosulphate are common in biological fluids and react with DCIP, but much more slowly than ascorbic acid. The interference by sulfhydryl group is often minimized by adding β – chloromercuri benzonic acid.

3. It is difficult to see the end point when a test solution is highly coloured. So, 1ml of chloroform is added to the reaction mixture and the end point is obtained when a permanent pink colour is seen in the organic phase.

# 17. Estimation of blood Urea by Diacetyl monoxime (DAM) method

**Introduction:**

Urea is a waste product formed in liver following the breakdown of proteins. It passes into the blood, is filtered out of the kidneys and excreted in urine. Thus, determination of blood urea is the most widely used screening test for the evaluation of kidney function. There are various methods used for the estimation of urea in a laboratory. Diacetyl monoxime (DAM) method is an older method.

**Principle:**

Proteins are first precipitated by trichloroacetic acid. The urea present in the protein-free filtrate reacts with diacetyl monoxime in a hot acidic medium in presence of ferric/cadmium ions and thiosemicarbazide to form pink or red colored complex- diazine. The intensity of the color developed is measured photometrically at 530nm, which is directly proportional to the concentration of the urea present in the fluid.

**Requirements:**

- Apparatus:
  - Colorimeter
  - Conical flasks and test tubes to hold 20ml
  - Pipettes: 50ul, 0.1ml, 0.5ml, 5 ml
  - Measuring cylinder, 50 ml
  - Water bath at 100°C
- Reagents:
  - Benzoic acid
  - Ferric Chloride
  - Diacetyl monoxime
  - Orthophosphoric acid
  - Thiosemicarbazide
  - Trichloroacetic acid
  - Urea
- Specimen:
  - Serum, heparinized plasma or fluoride plasma.

**Preparation of Reagents**

1. Reagent 1: Trichloroacetic acid, 50g/l (5%) solution
   - Trichloroacetic acid = 10g
   - Distilled water = upto 200ml

2. Reagent 2: Diacetyl monoxime (2,3-butanedione monoxime) solution
   - Diacetyl Monoxime = 2g
   - Distilled water = upto 500ml
3. Reagent 3: Acid reagent
   - Concentrated sulfuric acid = 44ml
   - Orthophosphoric acid (H3PO4), 85% = 66ml
   - Cadmium sulfate = 1.6 g
   - Thiosemicarbazide = 50mg
   - Distilled water = upto 500ml
4. Reagent 4: Colour reagent
   - Acid reagent (Reagent 3) = 50ml
   - Diacetyl monoxime reagent = 50ml
5. Reagent 5: Benzoic acid solution 1 g/l
   - Benzoic acid = 1 g
   - Distilled Water = 1000ml.
6. Reagent 6: Urea stock reference solution, 125mmol/l
   - Urea = 750mg
   - Benzoic acid, 1 g/l (0.1%) solution = upto 100ml
7. Reagent 7: Urea working reference solution, 10mmol/l
   - Urea stock reference solution = 8ml
   - Benzoic acid (C7H6O2), 1 g/l (0.1%) solution = upto 100ml

**Preparation of Sample**

To obtain protein free filtrate, take 50 ul of whole blood/serum/plasma in a centrifuge tube. Add 1 ml of TCA solution and mix. Centrifuge at high speed (3000 g) for 5 minutes to sediment the precipitated proteins and obtain a clear supernatant fluid. Do same for standard/control sample.

**Procedure**

1. Take three (or more if needed) large test-tubes and label as follows:
   - Blank tube (B)
   - Standard tube (S)
   - Test tube (T)
2. Pipette into each tube as follows:

| S. NO. | Reagents | Test | Standard | Blank |
|---|---|---|---|---|
| **1.** | Color Reagent (Reagent 4) | 3 ml | 3 ml | 3 ml |
| **2.** | Protein Free Filtrate | 0.1 ml | – | – |

| | | | | |
|---|---|---|---|---|
| **3.** | Urea Standard 10 mmol/l | – | 0.1 ml | – |
| **4.** | Distilled water | – | – | 0.1 ml |

3. Mix the contents of each tube. Place all the tubes in the water-bath at 100°C for exactly 15 minutes to allow the red color to develop.
4. Remove the tubes and allow them to cool in a beaker of cold water for 5 minutes.
5. Measure the colour produced in a colorimeter at a wavelength of 530nm.

**Calculations**

Calculate the concentration of urea in the blood specimen using the following formula:

$$\text{Concentration of urea in the specimen, mmol/l} = \frac{\text{O.D of Test}}{\text{O.D of Standard}} \text{ X10}$$

# 18. Estimation of haemoglobin by Sahli's/Acid Hematin method

**Principle:**

Blood is mixed with N/10 HCl resulting in the conversion of Hb to acid hematin which is brown in color. The solution is diluted till it's color matches with the brown colored glass of the comparator box. The concentration of Hb is read directly.

**Equipment required**

- Hemocytometer which consists of z comparator box which has brown colored glass on either side
- Hb pipette which is marked upto 20$mm^3$(0.02ml blood) z Tube with markings of Hb on one side z glass rod
- Dropper

**Reagents required**

- N/10 HCl Distilled water Sample:
- Venous blood collected in EDTA

**Procedure**

1. Add N/10 HCl into the tube upto mark 2 g %
2. Mix the EDTA sample by gentle inversion and fill the pipette with 0.02ml blood. Wipe the external surface of the pipette to remove any excess blood.
3. Add the blood into the tube containing HCl. Wash out the contents of the pipette by drawing in and blowing out the acid two to three times. Mix the blood with the acid thoroughly.

4. Allow to stand undisturbed for 10 min.
5. Place the hemoglobinometer tube in the comparator and add distilled water to the solution drop by drop stirring with the glass rod till it's color matches with that of the comparator glass. While matching the color, the glass rod must be removed from the solution and held vertically in the tube.
6. Remove the stirrer and take the reading directly by noting the height of the diluted acid hematin and express in g%.

# 19. Determination of:

## 19.1 Total Erythrocyte count

For RBC count, a method devised by Yokayama (1974) and later modified by Christensen et al., (1978) was followed. Hayem's diluting fluid, which had the following composition, was used for RBC count.

**Chemicals:**

- Mercuric chloride: 0.5gm
- Sodium chloride: 1.0gm
- Sodium sulphate: 5.0 gm
- Distilled water: 200ml

**Procedure**

An improved Neubauer's counting chamber was used for counting RBC (Baker and Silverton, 1982). Using RBC pipette, the blood was drawn up to 0.5 mark and the diluting fluid to the mark 101.

Although fluid is drawn to the mark 101 but the real dilution is 0.5:100 or 1:200 because the fluid in the capillary tube is discarded before the count.

**Calculations**

The number of RBC's per sq. mm was calculated as follows:

Area of a small square : 1/400 sq. mm

Depth of the counting chamber : 1/10mm

The volume of Small Squareis : 1/4000 cu mm

The dilution of the blood is : 1/200

Total RBC = cu mm

N = No of cells in 80 small squares

**Result**

The total RBC present in the given sample is …….

## 19.2 Total leucocyte count

A white cell count (TLC) estimates the total number of white cells in a cubic millimetre of blood. WBC diluting fluid or Turk fluid contains a weak acid to lyse the red blood cells and Gentian violet stain for staining the nucleus of white blood cells.

**Chemicals:**

The Turks fluid with following composition was used for TLC:

- Glacial acetic acid:1.5ml
- 1% aqueous solution of Gentian violet:1 ml
- Distilled water:100ml

**Procedure**

Neubauer's hemocytometer (Baker and Silverton, 1982) was used for counting of leucocytes. Using white cell pipette, the blood was drawn upto 0.5 mark and the diluting fluid to 11 mark, thus the dilution was 1:20.

**Calculations**

The number of RBC's per sq. mm was calculated as follows:

Area of a small square : 4 sq. mm

Depth of the counting chamber : 1/10mm

The dilution of the blood is : 1/20

$$\text{Total WBC} = \frac{N \times 20/10}{4} \text{ cu mm}$$

**Result**

The total WBC present in the given sample is …….

## 19.2 Differential leucocyte count

Differential Leucocyte Count in blood was determined by the method Ghai, (1993). A dried blood film stained with Leishman's stain, was examined under an oil immersion objective and the different type of white blood cells were identified. The percentage of distribution these cells was then determined.

**Reagents**

- Leishman's stain (Eosin and methylene blue dyes dissolved in acetone-free absolute methyl alcohol).

**Procedure**

A blood film was prepared, dried and placed on the staining rack and was covered with Leishman's stain, allowed to stand for 2 minutes. Then equal volume of distilled water was added and mixed by the slide first one way and then other. Allowed to stand it for 6 minutes. Drained off diluted stain in a stream of distilled water from a wash bottle for about 20 seconds and allowed the slide to remain on the staining rack for 1-2 minutes with the last wash covering it. Then kept the slide against a support in an inclined position, stained smear facing down and allowed it to dry. Then the stained slides were studied under low and high power objectives for differential leucocyte count by placing two drops of glycerol on the stained smear and using oil-immersion lens.

**Calculations**

Differential leucocyte was expressed as percentage.

$$\text{DLC (\%)} = \frac{\text{Number of type cells}}{\text{Total number of leukocytes}} \times 100$$

## 20. To determine the blood group and Rh factor of an individual.

**Introduction:**

Blood grouping is the classification of blood based on the presence or absence of two inherited antigenic substances on the surface of red blood cells (RBCs). The ABO and Rh are the major, clinically significant and the most important of all the blood group systems. The ABO blood group system was first discovered by Karl Landsteiner in 1900. The human ABO blood group system is divided into the following four major groups depending on the antigen present on the surface of their red blood cells:

1. "A" group
2. "B" group
3. "AB" group
4. "O" group

| Antigens on the surface of Red Blood Cells | Antibodies in the Serum | ABO Blood Group | Genotype |
|---|---|---|---|
| A | Anti B | A | AA or AO |
| B | Anti A | B | BB or BO |
| A and B | Neither Anti A nor Anti B | AB | AB |
| Neither A nor B | Anti A, Anti B, Anti AB | O | OO |

The Rhesus system (Rh) is the second most important blood group system in humans. The most significant and immunogenic Rhesus antigen is the RhD antigen. The individuals carrying the Rh antigen are considered to have positive blood group whereas those individuals that lack this antigen are considered to have negative blood group.

**Principle:**

The ABO and Rh blood grouping system is based on agglutination reaction. When red blood cells carrying one or both the antigens are exposed to the corresponding antibodies they interact with each other to form visible agglutination or clumping. Blood group A individuals have A antigens on RBCs and anti-B antibodies in serum. Similarly, blood group B individuals have B antigens on RBCs and anti-A antibodies in serum. Blood group AB individuals have both A and B antigens on RBCs and neither anti-A nor anti-B antibodies in serum. Whereas, blood group-O individuals have neither A antigens nor B antigens, but possess both anti-A and anti-B antibodies in serum.

The Rh antigens are transmembrane proteins in which the loops exposed on the surface of red blood cells interact with the corresponding antibodies.

**Materials**

Blood group kit

- Reagents: 70% Alcohol/ Spirit, Blood Grouping Kit

Blood Grouping Teaching Kit is stable for 6 months from the date of receipt without showing any reduction in performance. Store the Anti A Sera, Anti B Sera and Anti Rh-D Sera at 2-8ºC. Other contents can be stored at room temperature (15-25ºC).

- Other requirements: Cotton, lancet.

**Important Instructions:**

**Procedure:**

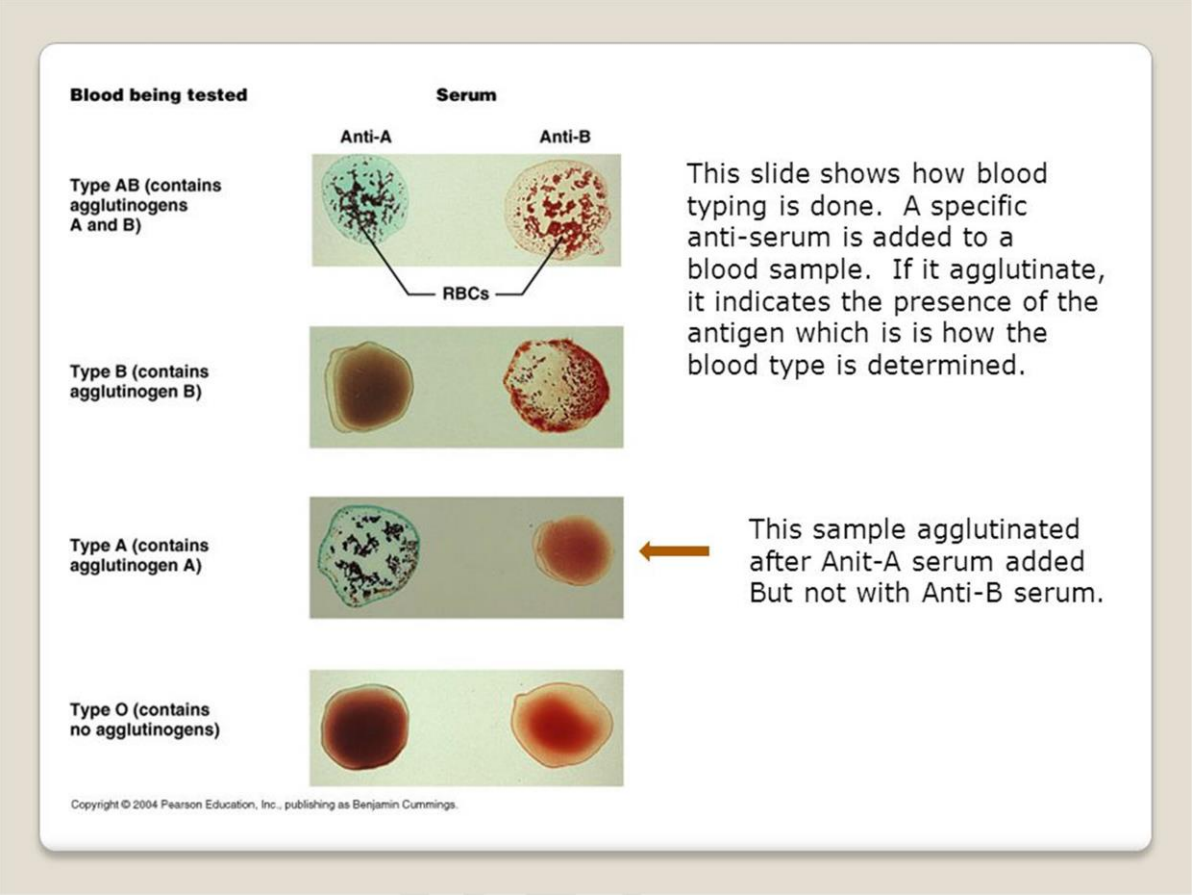
1) Dangle the hand down to increase the flow of blood in the fingers.

2) Clean the fingertip to be pierced with spirit or 70% alcohol (usually ring or middle finger).
3) With the help of the sterile lancet, pierce the fingertip and place one drop of blood in each of the cavities.
4) Add one drop of antiserum into each cavity as shown below:
   - Before starting the experiment, the entire procedure has to be read carefully.
   - Always wear gloves while performing the experiment.
   - Ensure the slide is clean and dry prior to use.
   - Do not allow the antisera reagent dropper to touch the blood sample.
   - The result of the reaction should be interpreted immediately after mixing.
   - Avoid intermixing of the antisera reagents while performing the experiment as it may give false result.
   - Mix each blood drop and the antiserum using a fresh mixing stick.
   - Observe agglutination in the form of fine red granules within 30 seconds. Anti RhD takes slightly longer time to agglutinate compared to Anti A and Anti B.

**Note:** Proper care should be taken while disposing the lancet and mixing sticks.

**Interpretation:**

- If agglutination is observed when blood is mixed with Anti A reagent, then the individual is said to have blood group "A".
- If agglutination is observed when blood is mixed with Anti B reagent, then the individual is said to have blood group "B".
- If agglutination is observed when blood is mixed with Anti A and Anti B reagent, then the individual is said to have blood group "AB".
- If no agglutination is observed when blood is mixed with Anti A and Anti B reagent, then the individual is said to have blood group "O".
- If agglutination is observed when blood is mixed with Anti RhD reagent, then the individual is said to have "+ve" Rh factor.
- If no agglutination is observed when blood is mixed with Anti RhD reagent, then the individual is said to have "-ve" Rh factor.




# 21. Examination of Urine

## Introduction:

A urinalysis (also known as a urine test) is a test that examines the visual, chemical and microscopic aspects of your urine (pee). It can include a variety of tests that detect and measure various compounds that pass through your urine using a single sample of urine. Healthcare providers often use urinalysis to screen for or monitor certain common health conditions, such as liver disease, kidney disease and diabetes, and to diagnose urinary tract infections (UTIs). While, several different aspects of your health can be tested with a urine sample, your healthcare provider will choose which tests to order under a urinalysis depending on your symptoms and situation.

It includes several different tests in a urinalysis. Depending on your symptoms, existing health conditions, and/or situation, your provider will choose which urine tests to order under a urinalysis. In general, a healthcare provider or laboratory technician can examine a urine sample for the following broad aspects:

1. Color and appearance.
2. Chemical findings.

3. Microscopic findings.

**1. Urine color and appearance**

For most urinalysis tests, a healthcare provider examines how the urine sample looks to the "naked eye." They check if it's clear or cloudy and if it's pale, dark yellow or another color.

Normal urine color usually has some shade of yellow and can range from colourless or pale yellow to deep amber, depending on how concentrated or diluted (watery) your urine is.

Many things can affect the color of your urine, including certain medications and supplements and certain foods you eat, such as beets. However, an unusual urine color can also be a sign of disease. For example, red-colored urine can happen when blood is present in your urine and can be an indicator of disease or damage to a part of your urinary system.

Cloudy urine doesn't always indicate unhealthy urine. For example, sperm and skin cells are harmless and could make your urine appear cloudy. Other substances that can make your urine cloudy, such as red blood cells, white blood cells and bacteria, may indicate several different medical conditions, including:

- Dehydration.
- Urinary tract infection (UTI).
- Sexually transmitted infections (STIs).
- Kidney stones.
- Diabetes.

**2. Urine chemical findings**

To examine chemical aspects of a urine sample, healthcare providers or lab technicians often use special test strips called dipsticks to test for certain chemical substances in the urine sample. The strips have pads of chemicals that change color when they come in contact with specific substances.

The degree of color change on the dipstick can give an estimate of the amount of substance present. For example, a slight color change in the test pad for protein may indicate a small amount of protein present in the urine sample, whereas a deep color change may indicate a large amount.

Common types of tests that use a dipstick that providers may include in a urinalysis include:

- Protein urine test: A protein urine test measures the presence of proteins, such as albumin, in your urine. Higher-than-normal urine protein levels may indicate several different health conditions, such as heart failure, kidney issues and dehydration.
- Urine pH level test: A urine pH test measures the acid-base (pH) level in your urine. A high urine pH may indicate conditions including kidney issues and a urinary tract

infection (UTI). A low urine pH may indicate conditions including diabetes-related ketoacidosis and diarrhoea.

- Ketones urine test: Ketones build up when your body has to break down fats and fatty acids to use as fuel for energy. This is most likely to happen if your body does not get enough sugar or carbohydrates as fuel. Healthcare providers most often use ketone urine tests to check for diabetes-related ketoacidosis.
- Glucose urine test: A glucose urine test measures the amount of sugar (glucose) in your urine. Under regular circumstances, there shouldn't be glucose in your urine, so the presence of glucose could be a sign of diabetes or gestational diabetes.
- Bilirubin urine test: Bilirubin is a yellowish pigment found in bile, a fluid produced by your liver. If you have bilirubin in your urine, it may indicate liver or bile duct issues.
- Nitrite urine test: A positive nitrite test result can indicate a urinary tract infection (UTI). However, not all bacteria are capable of converting nitrate (a substance that's normally in your urine) to nitrite, so you can still have a UTI despite a negative nitrite test.
- Leukocyte esterase urine test: Leukocyte esterase is an enzyme that's present in most white blood cells. When this test is positive, it may indicate that there's inflammation in your urinary tract or kidneys. The most common cause for white blood cells in urine is a bacterial urinary tract infection (UTI).
- Urine specific gravity test: A specific gravity test shows the concentration of all chemical particles in your urine. Abnormal results may indicate several different health conditions.

**3. Urine microscopic findings**

A lab technician may examine a urine sample under a microscope to look for tiny substances in the urine, including:

- Cells.
- Cell fragments.
- Urinary casts
- Mucus.
- Bacteria or other germs.
- Crystals.

Microscopic tests that providers may include in a urinalysis include:

- Red blood cell (RBC) urine test: An elevated number of RBCs indicates that there's blood in your urine. However, this test can't identify where the blood is coming from. For example, contamination with blood from haemorrhoids or vaginal bleeding can't be distinguished from a bleed somewhere in your urinary system. In some cases, higher-than-normal levels of red blood cells in your urine may indicate bladder, kidney or urinary tract issues.
- White blood cell (WBC) urine test: An increased number of WBCs and/or a positive test for leukocyte esterase may indicate an infection or inflammation somewhere in your urinary tract.

- Epithelial cells: Epithelial cells are cells that form the covering on all internal and external surfaces of your body and line body cavities and hollow organs. Your urinary tract is lined with epithelial cells. It's normal to have some epithelial cells in your urine, but elevated numbers of epithelial cells may indicate infection, inflammation and/or cancer in your urinary tract.
- Bacteria, yeast and parasites: Sometimes, bacteria can enter your urethra and urinary tract, causing a urinary tract infection (UTI). The urine sample can also become contaminated with bacteria, yeast and parasites, especially for people with a vagina. Yeast can contaminate the sample for people who have a vaginal yeast infection. *Trichomonas vaginalis* is a parasite that may also be found in the urine of people who have a vagina. It's the cause of an STI called trichomoniasis.
- Urinary casts: Casts are tiny tube-like particles that can sometimes be in your urine. They're formed from protein released by your kidney cells. Certain types of casts may indicate kidney issues, while others are completely normal.

Urinalysis tests are very common. They're a simple and non-invasive way to check several different aspects of your health. Your provider may order a urinalysis for you for one or more of the following reasons:

- As part of your routine medical exam to screen for early signs of certain health conditions.
- If you're experiencing and signs and symptoms of certain health conditions, such as diabetes or kidney disease.
- To monitor certain health conditions you're receiving treatment for, such as diabetes or kidney disease.
- To diagnose a urinary tract infection (UTI).
- If you've been admitted to a hospital.
- As a preparatory check-up for surgery.

To diagnose urinary tract infections and other infections specifically a urine culture is recommended. A urine culture involves growing bacteria from a urine sample in a lab. Urine cultures are not part of routine urinalysis tests. Like a urinalysis, a urine culture sample must be obtained by the clean catch method or by inserting a catheter through the urethra into the bladder.

If your urinalysis results indicate a UTI is likely, your healthcare provider may order a urine culture for a laboratory to run off the urine sample you gave for the original urinalysis. A urine culture can determine the type of bacterium that caused the UTI.

Urinalysis can include several different tests, measurements and assessments of aspects of your urine.

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