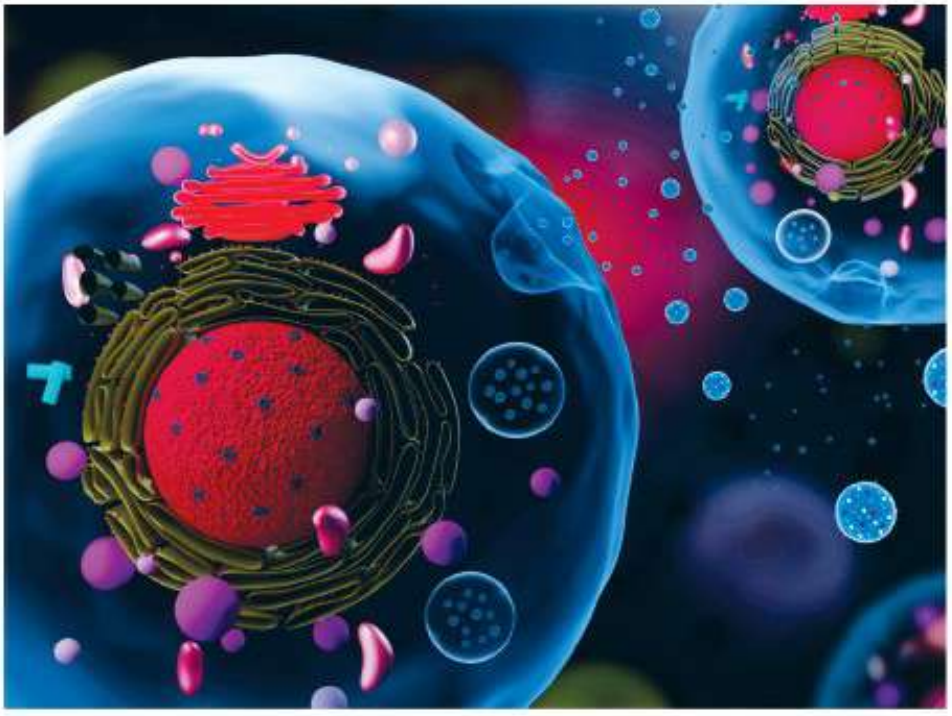
BIOCHEMISTRY LABORATORY HANDBOOK

by

Hayarpi Javrushyan

University of Traditional Medicine in Armenia



Biochemistry Laboratory handbook

by

Hayarpi Javrushyan

University of Traditional Medicine in Armenia

Yerevan

2021

UDC 577.1(07)

The publication is approved by
the scientific council of the
University of Traditional Medicine.

Editors:
M.Shahinyan: PhD,
R.Zakharyan: PhD,
T.Petrosyan: PhD,
N.Avtandilyan: PhD.

Javrushyan H. G.
Biochemistry Laboratory Handbook / H. G. Javrushyan.- Yerevan: Author's edition, 2021.- 164 p.

The manual is intended for conducting practical laboratory classes for students of medical and dental faculties of the University of Traditional Medicine. It includes the basic rules of laboratory safety, the main principles of using modern equipment, guidelines for the qualitative and quantitative analysis of biomolecules, and the skills of using various scientific platforms on the Internet. The manual also includes multiple-choice and discussion questions, that will assist the student to prepare for the current final examinations. The book can be useful for students of other medical institutions, as well as for postgraduate students, researchers and lecturers in the field of natural sciences.

ISBN 978-9939-0-3688-5

ՀՏԴ 577.1(07)
ԳՄԴ 28.072գ7
Ջ 360

Հրատարակության է երաշխավորվել
Ավանդական բժշկության համալսարանի
գիտական խորհրդի կողմից:
Գրախոսներ՝
Կենսաբանական գիտությունների թեկնածու Մ. Շահինյան
Կենսաբանական գիտությունների թեկնածու Ռ. Զաքարյան
Կենսաբանական գիտությունների թեկնածու Տ.Պետրոսյան
Կենսաբանական գիտությունների թեկնածու Ն.Ավթանդիլյան

Ջավրուշյան Հ. Գ.
Ջ 360 Կենսաքիմիայի լաբորատոր ձեռնարկ / Հ. Գ. Ջավրուշյան.- Եր.: Հեղ. հրատ., 2021.- 164 էջ:

Ուսումնամեթոդական ձեռնարկը նախատեսված է Ավանդական բժշկության համալսարանի Բուժական և Ստոմատոլոգիական ֆակուլտետների ուսանողների գործնական և լաբորատոր պարապմունքների իրականացման համար: Այն ներառում է լաբորատոր անվտանգության տեխնիկայի առաջնային կանոններ, արդի սարքավորումների կիրառման հիմնական սկզբունքներ, կենսամոլեկուլների որակական և քանակական վերլուծություն իրականացնելու մեթոդական ցուցումներ և համացանցի տարբեր գիտական հարթակներից օգտվելու հմտություններ: Ձեռնարկում ընդգրկված են նաև գիտելիքների ստուգման և ամփոփման տեստային աշխատանքներ և հարցեր քննարկման համար, որոնք կօգնեն ուսանողին ընթացիկ և ամփոփիչ քննություններին պատրաստվելուն: Գիրքը կարող է օգտակար լինել բժշկական այլ բուհերի ուսանողների, ինչպես նաև բնագիտական ոլորտի ուսանողների, ասպիրանտների, գիտաշխատողների և դասախոսների համար:

ՀՏԴ 577.1(07)
ԳՄԴ 28.072գ7

ISBN 978-9939-0-3688-5

Preface

TO THE STUDENT AND INSTRUCTOR

Biochemistry is a fundamental medico-biological subjects which is essential for general medical education regardless of physician specialization. Modern theories about pathogenesis of human diseases are focused on molecular aspects of the pathological states' growth. As a result, thorough understanding of the nature of diseases is unlikely without serious biochemistry studies.

A biochemistry laboratory course, now offered at most colleges and universities around the world, is an important component in the training of students for careers in biochemistry, molecular biology, chemistry, and related molecular life sciences such as cell biology, neurosciences, medicine, pharmacology and genetics. Biochemistry lab courses offer students the expertise and skills they need to participate in future research at the undergraduate and graduate levels, as well as to work in the biotechnological and pharmaceutical industries.

The aim of this book is to help students better understand the ideas, methods, and methodologies used in biochemistry teaching and research labs. Lab instructors are especially eager to introduce new student-centered education methods such as problem-based learning (PBL) and other “active-learning” styles into their labs.

This course is intended to introduce you to some of the most commonly used experimental methods in biochemistry, including information about the various types of some equipment frequently used in laboratories. The class is an opportunity to learn valuable skills each lab period, provide background information and an outline of the procedures to be performed.

Aside from that, the manual contains self-preparation and discussion questions, as well as examples of control tests and an examination questionnaire.

It will aid in the preparation of homework assignments and minimize stress levels during tests and examinations for students.

The author

ACKNOWLEDGEMENTS

We acknowledge with gratitude the generous help we have received from the University of Traditional Medicine in Armenia, Campus International LLC and the editors of this manual.
Further suggestions towards the improvement of this manual are welcomed.

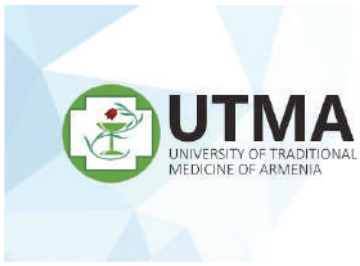


TABLE OF CONTENTS

Introduction

Biochemistry is the branch of life science that studies the chemical reactions that occur in living cells and organisms. Carl Neuberg, a German chemist, was the first to invent the word "biochemistry" in 1903. It considers research into the essence of living matter's chemical components, their transformations in biological systems, and the energy changes that accompany these transformations.

Biochemistry may thus be described as the study of biological phenomena through the lens of chemistry. For the same cause, the branch of Biochemistry has been termed "Biological Chemistry" or "Chemical Biology." Enzymology (the study of enzymes), endocrinology (the study of hormones), clinical biochemistry (the study of diseases), molecular biochemistry (the study of biomolecules and their functions), and other newer disciplines have arisen from biochemistry. Other specialties, such as agricultural biochemistry and pharmacological biochemistry, have emerged alongside these categories. Biochemistry is linked to almost all life sciences, and a comprehensive understanding of health and well-being is elusive without a biochemistry perspective and expertise. Many with a solid understanding of biochemistry will tackle the biomedical sciences' two key concerns: understanding and maintaining health and understanding and treating diseases.

OBJECTIVES OF BIOCHEMISTRY

Biochemistry's main goal is to fully comprehend all of the chemical processes that occur in living cells at the molecular level. Biochemists have attempted to isolate various molecules contained in cells, establish their structures, and examine how they behave in order to achieve this goal. Many aspects of disorders have been illuminated by biochemical experiments, and the study of specific diseases has formed the basis of new therapeutic approaches. In brief few objectives can be listed as follows:

- Biomolecule isolation, structural elucidation, and mode of action determination
- 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 and other disciplines.

Biochemistry is the study and application of substances, reactions and processes in animals, plants, bacteria and viruses. Biochemistry has an extensive range of applications. Biochemists work in a variety of settings, including hospitals, industry, environmental sciences, forestry, drug development, agriculture, dietetics, vaccine research, hormone production, virology, immunology, microbiology, toxicology, food science, plant science, and related fields, as well as marine biology and entomology. They provide diagnostic service, carrying out tests on blood, urine and other body fluids, while researching the underlying causes of disease and mechanisms of treatment.

Chapter 1. Safety

a. General Laboratory Principles

a1. Laboratory Safety Rules, Requirements and Regulations

Today, the "safety first" approach pervades the research community; working safely is more than just a habit; it entails routinely incorporating safety protocols into your work with hazardous materials. We'll go through the fundamentals of laboratory safety regulations, procedures, and equipment that all researchers should be aware of, consider, and apply to their research.

Biochemistry laboratory provides controlled environment in which scientific or technological research, experiments, and measurement may be performed. Because the needs of specialists in various disciplines of science and engineering varies, laboratories used for scientific study come in a variety of shapes and sizes.

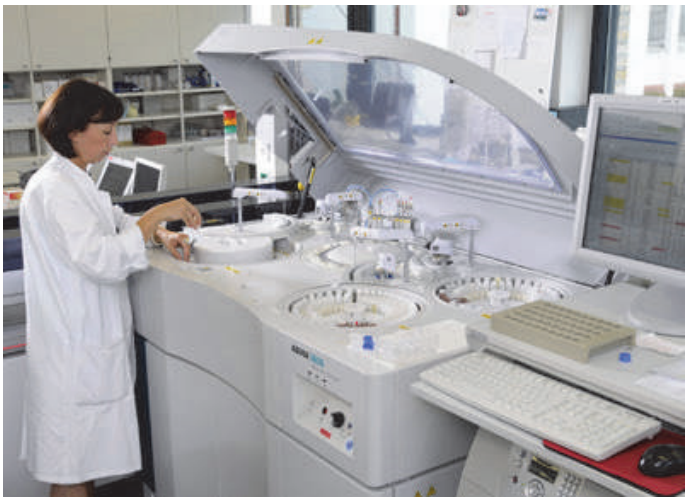


Fig.1. Biochemistry laboratory

a2. Safety in the Laboratory

Safety in the biochemistry laboratory involves a careful attitude, knowledge of possible risks and an awareness of potential hazards. Usually, potential accidents can be predicted and prevented. If safety precautions are followed, fewer accidents will occur. The number of laboratory accidents can be minimized 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. The student and the instructor are both responsible for complete understanding of hazards and dangers, as well as responsible in the event of an accident. The administration of flammable solvents, some caustic and poisonous substances, and apparatus in the biochemistry laboratory can result in mild to serious harm if handled incorrectly. Work with solvents and chemicals must be done in fume hoods rather than on the bench.

b. General Safety Procedures

1. Know the materials you'll be dealing with. (e. g. chemical, biological, radioactive)

a. Refer to your laboratory protocol and follow it

b. Regularly review the Material Safety Data Sheets (MSDS) for chemicals.

c. Consider the toxicity of the materials, the health and safety risks of each process, the laboratory staff's expertise and experience, and the safety equipment available. If you have any questions regarding how to handle the material, consult your PI or supervisor before doing so.

2. Know where safety equipment and emergency procedures are located in your region.

3. Always wear proper clothes (e.g. pants, shirts, shoes) and personal protective equipment (e.g. safety glasses, lab coats, gloves) when working in the laboratory. Open-toed shoes and sandals are unacceptable footwear; shorts and skirts are not permitted. The feet and lower legs are not protected from spills or shattered glass with these products. Before leaving the lab, remove your personal protective equipment and wash it.

4. Avoid working alone in the laboratory. While hazardous operations are performed, make sure another person is present in the lab. If you need to work alone in the laboratory (e.g. weekends, vacations, evenings) be sure your PI / supervisor is aware of your plans.

5. Use a fully functioning fume hood when working with hazardous chemicals. Air flows for university fume hoods are checked at least once every two years.

6. In the laboratory, you should not eat, drink, cook, or apply cosmetics.

7. Always keep your work area tidy and clean.

8. Do not leave experiments unattended, especially studies involving a heat source such as water baths, hot plates, etc.

9. Unauthorized visitors are not permitted to access the laboratory. All visits should be approved by the PI or supervisor, and visitors / guests should not be left unattended.

10. Procedures using hazardous substances should not be performed by anybody under the age of 18.

11. In any research laboratory, volunteer workers must be registered with Human Resources.

12. In university facilities, non-laboratory and non-assistance animals are not permitted.

c. Security

Security in the laboratory is a critical component of a successful safety program. Follow the procedures indicated below to provide a safe working environment.

1. When the lab is unoccupied, keep laboratory doors locked.

2. During off-hours or when the laboratory is empty, keep organisms and dangerous substances locked away.

3. Maintain an accurate inventory of chemicals, stocks, cultures, project materials, growth media, and other project-related things.

4. Examine any packages that arrive at the worksite. Do not open packages that appear to be suspicious. This includes packages from suppliers you're unfamiliar with and haven't bought products from, deliveries that aren't addressed directly to your laboratory, and packages that don't have a return address.

5. Before you depart, double-check that chemicals and biological substances have been appropriately and securely stored, and that the general workspace has been secured.

6. Request that individuals (i.e., people you don't know as coworkers or support personnel) identify themselves and leave the lab if they aren't authorized to be there.

d. Solvents

- Inflammable solvents should never be heated with or near a flame, even in tiny volumes. Never put solvents in an open beaker for refluxing or distillation. The act of pouring solvents near a flame is highly dangerous. 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 highly dangerous. Never heat them on a hot plate; instead, use a water or steam bath, and collect the distillate in an ice-cold 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 vapors 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 everyone at the desk switch off their burners and wipe it up with a towel as soon as possible. Wring (squeeze) the solvent out of the cloth into the solvent trash container, then thoroughly rinse it in the sink. Wear gloves.
- If acetone is used to aid in drying glassware, use it sparingly and keep it away from open flames.
- You may come into contact with flammable solvents such as ether, ligroin (petroleum ether), cyclohexane, toluene, xylene, alcohols, ethyl acetate, and carbon disulphide, acetone, dioxane etc. If you're unsure about a solvent's flammability, assume it's hazardous.
- Benzene and chlorinated solvents are toxic. The harmful impact might be cumulative in some situations. Avoid coming into touch with your skin and inhaling solvent fumes.
- Many organic solvents readily penetrate ordinary laboratory latex gloves, making them ineffective at protecting the skin from solvent vapors. Gloves made of thicker neoprene or butyl rubber are suggested.

e. Chemicals

- Bromine, acetyl chloride, benzyl chloride, phosphorus trichloride, acetic anhydride, fuming nitric and sulphuric acids, chlorosulphonic acid, benzene sulfonyl chloride, and other caustic chemicals that emit toxic fumes should all be handled in hoods. Wear the appropriate gloves. Do not get these chemicals on your skin or on your computers' desktops. They'll cause severe burns. None of these should be disposed of in organic trash cans.
- A variety of chemical compounds have been verified as carcinogenic in recent years, and the list is continuously expanding. It's better to consider that all substances are potentially cancerous and poisonous.
- 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 be avoided 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.
- Desktops, clothes, and skin are all corroded by concentrated acids and alkalis. If there is a spill, dilute it with a sufficient amount of water first. If the solution is an acid, neutralize it with solid sodium bicarbonate; if it's a base, neutralize it with 3 percent acetic acid. Sulfuric acid is bothersome, since drops adhering to the tops of bottles tend to absorb moisture and run down the outside of the bottle.
- Mercury, as well as its vapor, is toxic. Spills should be avoided, and the Institute/organization should have particular facilities for cleaning up mercury spills.

f. Apparatus

- When in the lab, approved safety glasses, goggles, or a face shield must be worn at all times. Due to the risk of explosion, regular prescription lenses are ineffective.
- It's a smart option to use caution while placing tubing or thermometers into bored stoppers. A cloth should be used to hold the tubing and stopper in place, so that if the tubing breaks, the jagged edge will be less noticeable. If the tube does not fit readily into the hole in the stopper, it can be enlarged using a file (if the stopper is made of cork) or coated with water, alcohol, or glycerin. Keep the tube as near to the stopper as possible. Follow the same procedure for removing tubing from stoppers. Carelessness in putting tubes into stoppers has resulted in serious wounds.
- If heated, closed systems are prone to explosion. In a closed system, never perform an atmospheric pressure distillation.
- Do not place equipment on books, cartons, pencils, or other similar items. Large,

sturdy wooden blocks, rings, or lab jacks should be used. Assemblies with a high center of gravity (as when a reagent is added through the top of a condenser) should be assembled and operated carefully.

- When breaking up solids, use glass stirring rods with caution. They're prone to breaking.
- Do not evacuate Erlenmeyer flasks larger than 50 ml (except filtering flasks). They may collapse.
- Oil baths and melting point baths can cause serious burns if spilled. Ascertain that they are well-supported. Avoid getting water into the oil baths. Use electric heating mantles instead of oil baths when possible.
- Dewar flasks and vacuum desiccators, implode easily when tipped over or dropped. Make sure the ones you use are wrapped on the exterior with friction tape or enclosed in protective shields so that if they break, they don't spray glass all over the lab.

g. First Aid in the Laboratory

Each laboratory should have a first-aid kit on hand. Oral medications (e.g., aspirin), topical creams, liquids, or ointments that might cause more suffering and/or obstruct medical care should not be included in first aid kits. Laboratories should verify the contents of first aid kits and replace any products that have passed their expiration date or are in need of replacement.

- Eye and face washes - instantly flush chemical droplets from the eyes and face with a big amount of tempered water for 15 minutes. Sinks used as eye and face wash stations must include a stay-open valve, which should be checked regularly to ensure adequate functioning and flush impurities from the pipes. Plastic eyewash bottles should not be used since they do not provide sufficient continuous flow. Emergency eyewash equipment should, in general, be within a 10-second walking distance of any safety risk and should not need leaving the laboratory to access. Furthermore, the path from the hazard to the equipment should be clear and as straight as possible; remember the victim may not be able to see.
- Door postings and other signs - hazard and emergency information signs should be posted on the laboratory door or wall facing the corridor. These signs are used by emergency response staff to identify hazards within the laboratory. In the case of an accident, chemical leak, fire, or personal harm, it is necessary to provide a point of contact.
- Open floor drains and sink traps – sometimes a sink trap or drain dries up. Sink traps and floor drains should be cleansed with one or two liters of water at least once a week to eliminate smells in buildings. If a laboratory area is not utilized for an extended length of time, it should be examined on a regular basis to ensure that the floor drains and sink traps are full of water. Any device that might block this periodic maintenance should not be placed over floor drains or sinks.

- Sharp containers and glass only boxes - Sharp containers are used for the disposal of hypodermic needles and syringes, razor blades and other sharp items. When ¾ full, sharp containers should be sealed and processed according to local unit procedures. While sharps containers may be used for contaminated broken glass, never “rebreak” such glass to facilitate placement in a sharp container. This contaminated glass box should be labeled and handled in a manner consistent with the contamination. “Glass Only” boxes are used for the disposal of “clean,” broken glass only. Sharp containers should be sealed and treated according to local unit standards when they are ¾ full. When the boxes are full, seal them and label them "Broken Glass" before throwing them away in a trash. Remember that biologically or chemically contaminated broken glass must be treated as a contaminated waste.
- Mechanical pipetting aids - Mechanical pipetting devices should be used. Mouth pipetting is intolerable.

h. Accidents

h1. Fire

Personal safety is most important. When a person's clothes catch fire, he or she need instant help. Stop him/her from running. Put him/her under the safety shower if he/she is close enough since it is more effective than a blanket. If not, force him or her to lie down and smother (obstruct) the flames by rolling, covering them with lab coats, blankets, towels, and so on. Never turn a carbon dioxide extinguisher on a person. If a fire breaks out, turn off all burners and, if time permits, remove any solvents. Carbon dioxide extinguishers must be present in the laboratory, and their locations and functions must be well known. Point the extinguisher at the base of the flames. Very small fires can be put out with a damp towel by smothering. The safety of everyone should come first, then the matter of extinguishing the fire is considered. A few seconds delay can result in very catastrophic injury, every person in the laboratory should plan in advance what he/she will do in case of such an emergency.

h2. Chemicals

If hazardous substances are spilled on clothing, the best treatment is to shower right away (while still wearing your clothes). Near each door, there should be a safety shower. If chemicals are spilt on the skin, thoroughly wash them away with plenty of water. Bromine should be washed off with water before applying ethanol or glycerin to the skin. Applying a burn ointment is not recommended. If a chemical is spilt in the eye, it should be rinsed out with water as soon as possible 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 significant amount of water and neutralize with sodium bicarbonate if the chemical is an acid, or dilute acetic acid if the chemical is a base.

- Accidents are rare in well-equipped laboratories if at least one of the members understands and follows safe laboratory procedures. When an accident does happen, it is almost often a minor one. If you are involved in an accident, the following information will be useful to you.
- The mouth must be properly cleansed with water if acids or alkalis are accidentally swallowed. In the case of acid; wash the mouth with dilute sodium carbonate and in case of alkali; with dilute citric acid. If proper swallowing has occurred, the person must drink water followed by milk in case of acids and lemon juice in case of alkali.
- Skin burns should be cleaned under running or cold water, then covered with sterile gauge and petroleum jelly or burn ointment.
- Inhalation injury by toxic fumes is best treated by shifting the person to an open fresh air. Irritation to throat can be soothe with hot water vapor 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 consulted to a professional.

Fig. 2. Types of glassware

i. Glassware Use

One of the most prevalent causes of laboratory injuries is broken glass. To reduce the chance of cuts or punctures, be careful when working with glassware. Inspect glassware for chips and cracks before use. Before using glassware, check for chips and cracks. These extra recommendations will assist in lowering the danger of injury:

• Never serve food or drinks in laboratory glassware.

• Handle and store glassware with care to avoid breaking it.

• Discard or repair any objects that are chipped or cracked.

• In containers with positive closures, leave a minimum of 10% air space.

• Use plastic or metal connections instead than glass connectors wherever available.

• After each use, thoroughly clean and sanitize glassware.

• Use thick-walled, round-bottomed glassware for vacuum operations. Flat-bottomed glassware is not as strong as round bottomed glassware. To avoid implosions, treat vacuum-jacketed glassware with care. Dewar flasks, vacuum desiccators, and other evacuated equipment should be taped or shielded and for vacuum work, use only glassware designed for that purpose.

Even though you take all the safeguards, glass may still break. Puncture wounds and the injection of hazardous substances can both be dangerous when broken glass is present.

• Never pick up shattered glass with unprotected or bare hands. Clean up shattered glass using a brush and a dustpan. Remove broken glass in sinks by suing tongs for large pieces and cotton held by tongs for small pieces and slivers.

• Glass contaminated with biological, chemical or radioactive material must be decontaminated before disposal.

j. Electrical Safety

Careless handling of electrical equipment is a common cause of laboratory fires. Stills, water baths, and other equipment in laboratories can overheat or generate electrical shocks. Decrease electrical safety risks by doing the following:

_ Before use, check all electrical apparatus for worn or defective insulation and loose or broken connections. Power cords should be thoroughly examined and replaced if they are found to be defective

_ All ground wires should be connected to clean metal, avoiding painted surfaces. Use three-prong grounded plugs whenever possible.

_ Keep electrical wires away from hot surfaces.

_ Don't allow water and other potentially destructive liquids to leak on electrical cables, switches and outlets.

_ Never touch a switch, outlet or other electric power source with wet hands.

_ Don't use handmade or improvised wiring, call an electrician for wiring.

Electrophoresis equipment is an example of a system which may be a significant source of electrical risk. This is because there is both high voltage and conductive fluid which presents a potentially lethal combination. Workers may be utterly clueless of the dangers of electrophoresis. At 25 milliamps, a typical electrophoresis running at 100 volts may give a fatal shock. Even a minor leak in the device tank might cause a severe electric shock. Besides the above, use these safety precautions to protect yourself from the hazards of electrophoresis and electrical shock.

• Always follow the manufacturer’s operating instructions.

• Use electrical interlocks and do not disable safety devices.

• Use warning signs to notify people about the possible electrical danger.

• Frequently check the physical integrity of the electrophoresis equipment.

• Turn the power off before connecting the electrical leads, opening the lid or reaching into the chamber.

• Use only insulated lead connectors and connect one lead at a time using one hand only. Make sure your hands are dry when connecting the leads.

• Keep the equipment away from water and other sources of water.

CHAPTER 2. HAZARDS IN THE LAB - KEY TERMS

- **Severe Toxicity-** adverse effects of a chemical caused by a single or numerous exposures in a short period of time (usually less than 24 hours)
- **Irritant-** causes redness, inflammation
- **Corrosive-** "eats away" tissue progressively
- **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.

Fig.3. Hazards in the Lab

Personal Protective Equipment (PPE)

Personal Protective Equipment or PPE refers collectively to variety of items and 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.

a. Safety in biochemical laboratory

Laboratory Safety Procedures

When you plan laboratory procedures, use that guide and these basic principles to help ensure the safety:

- Be familiar with the substances and dangers in your lab. Understand their flammability, reactivity, corrosivity, and toxicity potential.
- Be aware of what to do in a variety of emergency situations (e.g., fire, chemical spill, injury, etc.).
- Avoid working alone in a laboratory if possible.
- Don't underestimate risks; assume any mixture will be more hazardous than its most hazardous component and that all compounds of unknown toxicity are poisonous.
- All chemical exposures should be kept to a minimum. Few laboratory substances are completely risk-free. Use precautions when handling all laboratory chemicals. Wear personal protective equipment appropriate to the work. Do not wear contact lenses around chemicals, fumes, dust particles or other hazardous materials.
- Use extreme care when working with needles, blades and glass.
- Do NOT use tobacco products in the laboratory. Do not pipet with your mouth. Do not use ice from a laboratory ice machine for human consumption. Dedicate microwave ovens and other heating devices exclusively for food or for laboratory operations. Make sure that ovens are properly labeled to indicate their function.
- Provide adequate airflow/ventilation. The best way to prevent exposure to airborne substances is to vent them away from you. This is accomplished by using fume hoods and other ventilation devices. Avoid using dry ice in enclosed areas; it can produce elevated carbon dioxide levels. Dry ice mixed with isopropanol or ethanol may cause frostbite.
- Cleanup contaminated equipment and spills immediately. Avoid contaminating equipment with mercury and clean mercury spills immediately. Use non-mercury thermometers when possible.
- Keep hallways, corridors and exit ways clear. Do not place laboratory materials or equipment in these areas.

b. Laboratory and Personal Hygiene

Good personal hygiene will help minimize exposure to hazard chemicals Laboratory hygiene refers to procedures that are followed to avoid unintentional or accidental exposure to laboratory substances. A negligent employee can swiftly disseminate contamination across a laboratory if a little drop of fine powder occurs. Even minor exposures from some compounds might have negative consequences for people who work in laboratories every day. Your objective should be no skin contact with laboratory chemicals. Chemical pollution, unlike radioactive contaminants, is difficult to detect. When fluorescent dyes have been spilled in university buildings, it is unnerving to see how widely the contamination spreads. To keep yourself and others safe from accidental contamination, follow these steps:

• Do not touch things that are used by non-gloved hands (e.g., telephone, door knobs, etc.) if you are wearing gloves that have touched chemicals.

• Wash your hands in the lab regularly, especially after lab work and before eating, drinking, applying makeup or leaving the area.

• Routinely wash doorknobs, telephones, keyboards and desks.

• Don't keep food in refrigerators used to store chemical, radioactive or biological experiments. Do not use laboratory equipment to serve or store food or drinks.

• Never eat or beverages in labs.

• Remove contaminated clothing immediately and do not use the clothing again until it has been thoroughly decontaminated.

• Don't immerse your fingers or hand in liquids; use tongs or a tool.

• Chemicals should not be sniffed or tasted.

c. Chemical Disposal

Disposal of waste and harmful elements has become increasingly complicated. There are many chemicals that can be properly disposed of by discharge into the sanitary sewer after elementary neutralization or other chemical treatment method, by discharge into the sanitary sewer with no neutralization or other chemical treatment required, or directly into the normal trash. Campus incinerators do not meet the destruction standards for chemical waste, and are not allowed for chemical waste disposal. Label Your Waste!

Suitable Containers. Chemical mixtures, aqueous solutions, other liquids and reaction products should be placed in a suitable container. Empty containers in which the chemicals are supplied are usually satisfactory for removal. Make sure all containers are tightly closed and contain the material that they hold. Ensure any waste reaction mixture or cleaning solution is funished reacting and no longer producing a gas prior to securely capping, overpressure may produce a spill or explosion.

Chapter 2.1. Biological Safety

The hazards posed by biological materials, plus the risks of infections resulting from exposure to infectious materials, are essential concerns, when working in a biological laboratory. Several deaths have resulted from infections acquired in labs where etiological agents have been in use. Controlling exposures (and the resulting infections) requires an understanding of the factors involved in disease transmission in the laboratory. The most common routes of exposure are ingestion, inhalation, and self-inoculation. The development of an infection subsequent to an exposure to an infectious agent depends upon individual susceptibility, the size of the dose, and the pathogenicity of the organism. The only one of these three factors within the control of the investigator is the size of the dose. The risk of infection is significantly reduced if all exposures are limited below the infectious dosage. This is the fundamental basis for biological laboratory safety.

a. Basic precautions while working in a biological laboratory

- Use disposable, high cuff, latex or nitrile gloves when working with biohazards. Remember, latex gloves are permeable to organic solvents, including ethanol.
- Thin gloves provide minimal against cuts, bites, scratches, etc. Use the thickest gloves allowed by your work but do not sacrifice the dexterity you need to accomplish your job.
- Wearing two pairs of thinner gloves will allow the outer pair to be safely removed in the case of contamination.
- Always wear a lab coat while working in the laboratory. The lab coat should be buttoned and, if working with biological agents, the sleeves tucked into the gloves or otherwise restrained.
- Do not wear lab coats outside of the laboratory (e.g. breakroom, conference rooms, or outside the floor or building)
- Procedures which might produce aerosols should be performed in the rear third (i.e., toward the back) of the biosafety room.
- Avoid the use of needles, scalpels, or other sharp instruments whenever possible.
- Place sharps in a puncture-proof container.
- Use disposable glass or plastic-ware. If non-disposable glassware must be used, disinfect contaminated items before cleaning.
- Clean biological spills immediately with a fresh solution of chlorine bleach diluted 1:10 with water.
- Discard non-sharp disposable materials (e.g., gloves, pipettes, pipette tips, and plastic tubes) that are exposed to potentially infectious materials in polyethylene biohazard bags, for transport by medical waste transport.

- Treat blood and other potentially infectious fluids with 10% chlorine bleach and decant down the drain.
- Blood and sharps should not be disposed of in the usual laboratory garbage. Staff who work with human blood, blood products, secretions, or other potentially infectious materials are at an elevated risk of getting hepatitis. Anyone who works with blood or potentially HBV-containing material should get vaccinated against hepatitis B. The most essential approach for workers to protect themselves against Hepatitis B and other bloodborne diseases is to take universal precautions and treat all blood, blood products, secretions, and other potentially infectious material as if it were contagious.

b. Cell Lines

While particular cell lines are not identified, it is crucial to remember that there is no such thing as a "regular" cell line; many "normal" lines have viruses and potentially hazardous gene sequences. Handle these items with care as if they were contagious, and disinfect culture wastes before discarding. All cells should be fixed before subjecting them to an aerosol generating process (e.g., flow cytometers).

c. Viruses

Fluids, tissues, isolates and cell cultures containing infectious viruses pose a risk following exposure by ingestion, percutaneous or parenteral inoculation, and droplet or aerosol contamination of the mucous membranes of the eyes, nose, mouth, or broken skin. The aerosol risk from handling large volumes and concentrated stocks is great since some viruses are stable at ambient temperatures and withstand drying. Variation in viral structures results in differential susceptibility to “germicidal” agents and detergents; nevertheless, chlorine bleach treatment is typically effective.

d. Bacteria

Although many bacteria are widespread, some, such as Staphylococcus aureus and group A streptococci, cause severe illnesses in humans. Because spores are resistant to unfavorable or severe circumstances, aerosols are a serious problem when working with huge quantities or concentrated stocks, as well as harmful spore producing species. When handling bacteria, especially those that infect the conjunctiva, such as N. gonorrhea, safety glasses must be used. Prior to disposal, all wastes must be decontaminated; autoclaving or chlorine bleach treatments are effective.

e. Parasites

Human protozoal parasites' infectious stages can be found in blood, feces, lesion exudates, and infected arthropods. Accidental parenteral injection, transmission by arthropod vectors, skin penetration (including bites from infected animals), and ingestion are the most common laboratory risks, depending on the parasite. When working with cultures of Leishmania and Trypanosoma species, aerosol or droplet exposures to the mucous membranes of the eyes, nose, or mouth with trophozoites are possible risks. All exposures should be reported to the main investigator and managed as quickly as possible, for example, wiping a bite with 70% ethanol or irrigating an eye with distilled water. In general, protozoa are very fragile, sensitive to drying, and, with notable exceptions such as T. cruzi, lysed even by water; however, all spills and waste must be actively treated.

f. Fungi

In most cases, fungi do not cause disease in humans. It is extremely uncommon for fungal infections to spread from person to person. Fungal spores, on the other hand, are typically highly allergenic, and certain of the fungal components and by-products, such as aflatoxin B, may be quite poisonous. The more common hazardous fungi used in laboratories include: Blastomyces dermatitides, Coccidioides immitis, Cryptococcus neoformans, Histoplasma capsulatum, and Sporothrix schenckii.

g. Work with Laboratory Animals

IACUC approval is required for any vertebrate animal experiments. Animals should only be maintained in approved animal facilities and should not be kept in laboratories for longer than 24 hours. Virtually all laboratory animal species can contain diseases that are contagious to humans, therefore users of laboratory animals must be aware of this. Inoculated animals can easily spread viruses to their cage mates by inhalation and contact with urine, feces, sputum, and other body fluid. When working with any animal, take caution. Urine, excrement, and blood should all be cleaned up as quickly as possible.

h. Zoonoses

Despite the fact that animal illnesses seldom affect humans, a few do. The following are some of the most significant animal reservoirs:

• Primates: illnesses including tuberculosis, shigella, campylobacter, and salmonella can present major health risks. Rhesus, cynomolgus, and other old-world monkeys have the herpesvirus B, which can cause lethal encephalitis in humans.

• Dogs and cats: bite wound infections, cat scratch disease, visceral larva migrans and sarcoptic mange from dogs and toxoplasmosis and fungi’s (such as ringworm) from cats are common.

• Rodents: precautions should be taken against toxoplasmosis, lymphocytic choriomeningitis, salmonella, shigella, and ringworm. Toxoplasmosis is one of the most frequently acquired parasitic diseases in the laboratory.

• Rabbits, sheep, swine, and birds can be the source of tularemia, Q fever, erysipelas, and chlamydia (psittacosis), respectively.

h1. Some requirements for the use of animals:

• All users of laboratory animals must be vaccinated against tetanus and have additional immunization as required.

• Bites or scratches that break the skin should be washed well with soap and water and reported.

• Wearing a facemask, gloves and a lab coat is required of users of animals to prevent aerosol, direct contact or accidental oral and nasal exposure to contaminated materials.

• A full-face respirator is required for those at high risk.

• Lab coats should be changed and hands properly cleaned if an animal, its fluids, or feces is touched. Tyvek lab coats should be accessible.

• The most prevalent cause of human sickness linked to the use of animals in research is allergic reactions to laboratory animals. Allergies are caused by direct or indirect contact with allergens like as hair, dander, saliva, urine, serum, and so forth. Wheezing, sneezing, and rhinitis are common symptoms, as are itchy eyes and skin, visible rashes, and asthma. Do not dismiss the signs and symptoms. Continued exposure can cause anaphylaxis, which can be fatal.

• Employees who are pregnant should avoid exposure to excrement, dander, or biohazard regions, as well as work involving the handling of cats and monkeys. Likewise, pregnant women without immunity to toxoplasmosis should avoid cat contact to avoid the possibility of congenital disease and fetal death.

Chapter 2.2. General Biosafety Issues

a. Biological Stains

Fixatives and stains used to prepare tissues and cellular materials often have hazardous nature (e.g., methylene blue and trypan blue are teratogens), requiring the use of impermeable gloves and adequate ventilation. In addition, several dyes used in conjunction with flow cytometry and visualization of nucleic acids are suspect carcinogens. Be sure the precautions you are taking are adequate.

b. Incubators

Incubators can become the unintentional and unwanted reservoirs of microorganisms. Despite the fact that they may represent a danger to laboratory personnel, they are frequently a source of contamination in laboratory cultures. Besides the moist surfaces, rubber gaskets, the humidity trough (if present), and fan mechanism are areas in which contaminating microorganisms concentrate. In addition, the inner panels, trays, and the other removable parts should be autoclaved and the gaskets and non-removable parts properly cleaned with 70% ethanol every two months.

c. Freezer and Liquid Nitrogen Storage

Freezers containing potentially dangerous biological products and poisons should be appropriately labeled. To avoid the buildup of broken vials and excessive frost, these freezers should be defrosted at least once a year. Note that “frost-free” freezers allow small samples to thaw during warming cycles. Ethanol and other flammable solvents should not be stored in refrigerators or freezers that are not designed for flammable solvent storage. Moving the controls of a standard refrigerator or freezer to the outside is not acceptable, and does not allow for the storage of flammable solvents inside the altered unit. Upon thawing, screw cap vials may explode, producing an aerosol of glass and cell debris. If freezing manually, place ampules in the bottom of a beaker, cover with methanol and a dye, e.g., methylene blue, and transfer the entire beaker from refrigerator to freezer. The methanol provides even freezing and the dye will penetrate imperfectly sealed vials permitting their identification and elimination. When adding samples to liquid nitrogen storage repositories, be aware that the liquefied nitrogen may boil vigorously as warmer components are added. Use only in a well-ventilated area. Liquefied nitrogen is a cryogenic gas and expands 700-fold upon vaporization; this may result in a rapid displacement of air. Ampoules to be thawed should be dropped into a plastic beaker containing 70% ethanol at 37ºC within a spongy bucket and covered immediately. Verify the sample's identity.

d. Decontamination

Any waste management system must keep biological waste distinct from other waste sources. Biological waste should be handled and eliminated on-site wherever possible. Biological (medical) waste disposal is getting more regulated and expensive. All culture materials and biological specimens, including that from “normal” cultures and primary tissue, should be collected inside the biological safety cabinet. On a daily basis, these items should be autoclaved or otherwise chemically inactivated. Do not leave untreated garbage in a public space or corridor. Autoclaving hypochlorite or any other powerful oxidizing substance with organic materials such as paper, fabric, oils, or volatile solvents is not allowed. This might result in poisonous fumes or perhaps an explosion. As a result, never autoclave waste that has been bleached with chlorine. Materials contaminated with radioisotopes or hazardous substances should not be autoclaved. These compounds have the potential to volatilize, pollute the autoclave, and expose personnel. Before and after each session, clean off the biological safety cabinet with an appropriate disinfectant (see below).

e.Disinfectants

Alcohol - Isopropyl and ethyl alcohols in 70-90% concentrations may be germicidal against lipid containing agents but are not effective against spores and infectious DNA. It's worth noting that 100% ethanol isn't an effective disinfectant. Alcohols have the benefit of being fast acting, evaporating quickly, and leaving no residue. They can also be mixed with other disinfectants (quaternary, phenolics, and iodine) to produce tinctures that enhance bactericidal activity even more. Chlorine is a powerful disinfectant that kills a wide range of microorganisms, including gram-negative and gram-positive bacteria, bacterial spores, and most viruses. Disinfect media with a 10% solution of chlorine bleach (5.25% hypochlorite or 52,500 ppm) for 15 to 30 minutes. It's important to remember that solutions degrade with time and are quickly neutralized by organic substances. Its effectiveness may be enhanced by the addition of 0.1% solution of an ionic detergent. If used directly on a stainless-steel surface, rinse thoroughly with water to prevent tarnishing and decomposition. Chlorine solutions should not be autoclaved. *Iodophor* - Characteristics of chlorine and iodine are similar. Iodophors are effective against gram-positive and gram-negative organisms, mycobacteria, and some viruses, and are most effective in acid solutions. Organic matter reduces effectiveness, but iodophors are less affected than hypochlorite. Iodophors evaporate at 120°F, therefore don't autoclave them. Iodophors can be stored for a long time when they are kept cold and properly covered.

f. Regulated Medical/Bio-Hazardous Waste Management

The list below summarizes the disposal route for each of the biohazardous waste streams.

Sharps

• Syringes with or without needles

• Broken glass, contaminated by biological or chemical waste

• Scalpels, razors and lancets

• Glass pipettes

• Specimen tubes, slides,

Human or animal blood, blood-products, body fluids and tissues cultures, infectious agents and associated biologicals

• Used Petri plates containing culture agar

• Specimens from bottles, medical, pathology and research laboratories

• Discarded live and attenuated vaccines

• Wastes from the production of biologicals

• Culture flasks

Other laboratory wastes including but not limited to

• Surgical drapes and absorbents

• Protective gloves, disposable lab coats, or masks

• Specimen containers

• All microorganisms constructed using rDNA. Any medical equipment or disposables that have the appearance of medical wastes!

• Agarose gels

• PAGE gels

• Sample buffers (tubes containing small amounts of liquid)

• Membranes

• Any disposable tubes, plates, dishes, cuvettes, including centrifuge, micro-centrifuge and conical tubes

• Tips (of any kind)

Chapter 2.3. Laboratory Requirements

a. Simple 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 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, aluminum 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.

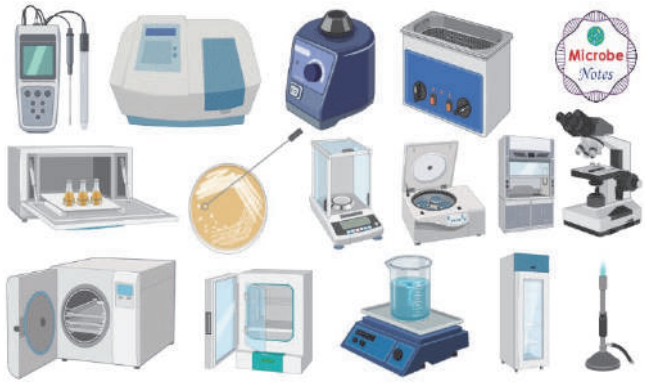


Fig.4. Laboratory Instruments

Fig.5. Laboratory Glassware

b. 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 vapors.

✓ 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 substances 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 considered hazardous.

✓ Hazardous chemicals may be spilled on the floor or on the feet, thus shoes (not open-toed) must always be worn.

✓ Long hair should always be tied back.

✓ Cuts and burns must be immediately treated. New burns should be treated with ice, and major wounds should be seen by a doctor.

✓ In the event of acid spilling on one's body, flush thoroughly with water immediately. Be aware that acid–water mixtures will generate 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 severe burns if left in contact with skin, even if only for a very brief period of time.

✓ Weak acids (such as citric acid) and weak bases (such as sodium carbonate) should be readily available in the lab in case of emergency.

✓ Dispose all waste chemicals from the experiments according to your instructor's directions.

✓ If you have an accident, report it to your instructor right away, no matter how minor you think it is.

✓ Always be watchful and considerate of others working in the laboratory. It's critical not to risk their or your own safety.

✓ Always use equipment that is in good condition.

It is difficult to anticipate all the dangers that may arise in a laboratory. To avoid harm, it is critical that all students pay careful attention to their instructor and follow the regulations of their specific laboratory.

CHAPTER 3. COMMUNICATING RESULTS FROM BIOCHEMISTRY RESEARCH

A scientific study isn't complete unless its findings are shared with scientists all around the world. The scientific paper, the oral presentation, and the poster are the three most significant ways or instruments for communication. Although the preparation for these three frequent techniques of delivering new biochemical knowledge differs greatly, they all have one thing in common: the exchange of experimental data and conclusions. Each method's specific principles and traditions will be discussed and compared below.

a. The Scientific Paper

A paper published in a biochemical journal is a formal way to report research results to colleagues in the international biochemical community. Before writing such a document, one must first determine the journal to which the article will be submitted. The best advice is to submit the manuscript to the most prestigious journal that has a large audience interested in his or her specialized topic. You write up the results from an experiment in the form of a journal article, so it is important to understand the conventions used in preparing a manuscript for publication. Most biochemical journal articles have the same basic organization: Title, Abstract, Introduction, Experimental Methods, Results, Discussion, and References. The specific requirements for each of these sections vary among the many journals.

b. The Oral Presentation

Preparing and publishing a paper is not the same as giving an oral presentation. In an oral presentation, you only have a few minutes to convey information and persuade your audience of the significance of your work. However, one advantage of giving an oral presentation over giving a paper is that it allows for more direct interaction with your audience, allowing for instant questions and comments. Presentations usually range from 15 to 60 minutes. Shorter presentations cover a much smaller unit of a research project, whereas 60-minutes talks (often called seminars) can give a broader exposure to the research area. Scientific presentations involve mixed media - oral and visual. The important verbal points are reinforced with the use of a visual aid such as a figure, graph, or other element. Scientific presenters today most often use power point, computer software that projects electronic slides onto a screen, although overhead transparencies are also acceptable and efficient. The slides in a visual aid must be carefully designed, with particular attention paid to the overall number of slides and the quantity of information on each. A presentation is structured similarly to a paper, with an introduction,

experimental techniques, findings, discussion, conclusions, questions/comments.

c. The scientific poster

The scientific poster is a hybrid communication strategy that combines features of an oral presentation (verbal expression and visual aids) with those of a paper (printed text and figures). At all professional conferences, including local, regional, national, and worldwide meetings, the poster has become the major medium for exchanging new scientific knowledge. In the absence of the presenter, the poster may be visible to readers for extended periods of time.

CHAPTER 4. USING BIOCHEMICAL REAGENTS AND SOLUTIONS

a. 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.

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 byproducts called pyrogens. For most laboratory procedures, it is recommended that some form of purified water be used. Resistivity is a common metric for determining the cleanliness of water. Unit for resistivity is Megohms-cm. There are five basic water purification technologies: distillation, ion exchange, activated carbon adsorption, reverse osmosis, and membrane filtration. 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. When doing sensitive UV-spectroscopic measurements, distilled water is preferable than de-ionized water. If you need a big amount of high-purity water, reverse osmosis is the way to go.

b. Cleaning Laboratory Glassware

The cleanliness of your equipment, particularly glassware used for preparing and transferring solutions, will have a significant impact on the outcomes of your experiments. 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. Starting with hot tap water is the best approach for washing glassware. 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 required dry glassware in the organic lab, you likely immersed it in acetone, which quickly evaporated and left a dry surface. However, this method leaves an organic residue on the surface, which is made up of nonvolatile pollutants present in acetone. It is recommended to avoid acetone cleaning because this residue may interfere with your study results. Cuvettes and other optically polished glassware should

never be cleaned with ethanolic KOH or any strong base, since this may induce etching. All glass cuvettes should be carefully cleaned in a sonicator bath or in a cuvette washer with hot tap water or a 0.5 percent detergent solution, followed by thorough washing with purified water.

c. 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 liter 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.

d. 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. Volumetric methods are more convenient for dispensing liquids; nevertheless, this assumes that the density is known. If a tiny amount of liquid needs to be weighed, a disposable Pasteur pipette with a latex bulb should be used to add it to a tared flask. In the biochemistry lab, the storage conditions of reagents and solutions are extremely important. All solutions should be kept in a completely closed container. Some solutions must often be kept in a refrigerator at 4°C. This delays the decomposition of the chemicals and limits bacterial growth. Some solutions may require storage at temperatures below 0°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 vapors 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.

CHAPTER 5. Statistical Analysis of Experimental Data

a. Statistical Analysis

An error in an experimental measurement is defined as a deviation of an observed value from the true value. There are two types of errors, determinate and indeterminate. Determinate and indeterminate errors are the two forms of errors. Determinate errors are those that the experimenter can control and are linked to equipment failure, poorly planned experiments, and changes in experimental circumstances. These are frequently referred to be human errors since they may be addressed or at least substantially minimized by proper experiment planning and construction. Random errors that the experimenter cannot control are known as indeterminate errors.

Two statistical terms involving error analysis that are often used and misused are *accuracy* and *precision.* Precision refers to the extent of agreement among repeated measurements of an experimental value. Accuracy is defined as the difference between the experimental value and the true value for the quantity. Because the true value is seldom known, accuracy is better defined as the difference between the experimental value and the accepted true value. Several experimental measurements may be precise (that is, in close agreement with each other) without being accurate. If an infinite number of identical, quantitative measurements could be made on a biosystem, this series of numerical values would constitute a *statistical population.* The average of all of these numbers would be the *true value* of the measurement. The alternative is to obtain a relatively small *sample of data,* which is a subset of the infinite population data. The significance and precision of these data are then determined by statistical analysis.

b. The Mean, Sample Deviation, and Standard Deviation

The arithmetic average, or mean, of the numbers is calculated by totaling all the experimental values observed for a sample (the counting rates, the velocity of the reaction, or protein concentration) and dividing the total by the number of times the measurement was made. *Sample deviation* is defined as the difference between the value for an observation and the mean value. A more useful statistical term for error analysis is *standard deviation,* a measure of the spread of the observed values.

c. Spreadsheet Statistics

A spreadsheet is a computer program that allows you to organize, analyze, and save data in a tabular format. For statistical analysis of biological data, computer spreadsheet applications are now widely used. Spreadsheets' graphing capabilities make it simple to check data for errors

and outliers, search for nonlinear relationships and non-normal distributions, and present the final conclusions. Even to use something like SPSS, SAAS and SyStat, there will be many times when it's easier to enter the data in to a spreadsheet first, inspect it for errors, sort and arrange it, then export it in to a format suitable for statistics package.

CHAPTER 5.1. USING THE COMPUTER AND INTERNET FOR RESEARCH IN BIOCHEMISTRY

a. What is research and how is it done in biochemistry?

Virtually all scientists are engaged in investigative activities that are broadly called **research.** It is a process of investigation. An examination of a subject from different points of view. It's not just a trip to the library to pick up a stack of materials, or picking the first five hits from a computer search. Research is a hunt for the truth. It is learning about a subject by reading about it, reflecting on it, experimenting with ideas, and selecting and following up on the areas that interest you. Research is the way you educate yourself.

The ultimate objective of all research is to discover the truth; nevertheless, the approach that is used can vary from discipline to discipline. For biochemists and other molecular life scientists, research is usually focused on elucidating the laws of nature or, in other words, addressing questions about natural phenomena.

b. The Scientific Method

One of the early originators of this organized body of techniques is believed to be Ibn al-Haythan (965–1039 A.D.; Latinized name, Alhazen), a physicist studying optics and living in Basra, Iraq. Many modifications of the process have been made over the centuries, but the true logical concept has stayed the same: data must be acquired by experiment and observation. Each of the stages is made up of a number of activities:

1. ***Select the topic.*** The topic for study is often one of strong interest to the scientist and one with which he or she may already have some familiarity. It is sometimes helpful to phrase the problem to solve as a question to which we do not yet know the answer. Some biochemistry study topics include: (a) what is the precise molecular mechanism for a certain enzyme? (b) what is the base sequence of a particular gene? (c) what is the function of a protein that has been newly isolated? (d) how can a new method for analysis of fatty acids in blood serum be designed? and (e) how does a certain medication work?

2. ***Collect current information and resources about the topic.*** This step always begins by reading the primary literature in the field of study. Primary literature refers to papers in scientific journals in which scientists describe their experiments, provide data, and analyze significant findings. When you do extensive background research, you start to become an expert in the field. The computer is often heavily used in this step. Background reading of the literature can also be done at a library by checking research journals; however, journals are expensive, thus libraries may not have all of them.

3. ***Propose hypotheses that may answer questions about your topic.*** The dictionary defines hypothesis as “a tentative assumption made in order to draw out and test its logical or empirical consequences.” In research, a hypothesis is an essential and helpful tool that may help you find answers to your concerns. Proposing a hypothesis involves guessing, but it is a "educated guess" since your knowledge has been strengthened by your reading of the literature. The most essential reason to have hypotheses is that they inform you which experimental paths to go in next on your project. A hypothesis cannot be proven by experimental data, only supported.

4. ***Design and perform experiments; collect data.*** For hypotheses to be tested properly, it is essential that you design experiments that provide results that are informative. The findings of the experiment must be observable, empirical, and measurable. The best experiments result from knowing your subject well and from asking good questions about what you need to find out about your research problem. It should be obvious that experiments must be completed with accuracy and care and that safe lab techniques are used. Before an experiment to be considered as valid, it must be repeated several times with the same results each time.

5. ***Analyze data.*** Experiment data might be either quantitative or qualitative. Both forms may be studied by constructing figures, tables, and graphs on a computer. If your findings are largely numerical, you'll need to do calculations followed by statistical analysis with Excel and other programs.

6. ***Interpret results and draw conclusions.*** Is at least one of your hypotheses supported by your experimental findings and interpretations? If that's the case, the evidence suggests that theory is correct. It might be a reasonable response to your first question. A theory can only be supported, not proven. Any theory that contradicts experimental findings must be dismissed. If the findings contradict all hypotheses, new hypotheses must be created and tested. This is referred to as an iteration, or a process that repeats itself. Research is a never-ending, ever-evolving process. It is always changing, but scientists believe that each step they take brings them closer to answering their original question.

7. ***Publish results in a scientific journal.*** This stage makes your work public, allowing scientists from all around the world to study it. Scientists may then reproduce your experiments, double-check their validity, and create additional experiments to explore the theory. Making sure your studies can be replicated by other scientists, especially in different labs, is a crucial part of the scientific process. A lack of objectivity is one issue that might arise while using the scientific method. Scientists might get biased, adopting a favored hypothesis and doing only tests that support it. Ethical behavior is another requirement that scientists must follow in addition to objectivity. Unethical activities, such as plagiarism, fabricating data, and other forms of scientific misconduct, are a threat to scientific and technological development. The currently controversial so-called “Climategate” issue is one that some scientists say lacks objectivity and is marred by unnecessary secrecy and a refusal to properly share data.

Chapter 5.2. USING COMPUTERS IN BIOCHEMISTRY

Human living has been revolutionized by the modern computer. Not unexpectedly, our approach to scientific research has altered with the computer as well. Computers have now become a vital instrument for investigating the structure, function, reactions and information of all aspects of biomolecules. The need for biochemical computers is increasing because of two factors: (1) the areas become more and more quantitative, and therefore computer compliance and its access to the Internet software and application programs are required in a complex and precise manner; and (2) because of the ease of determining protein and nucleic acid sequences and structures, there has been a proliferation of biological information that needs to be organized, stored, and made readily available to researchers. Initially, computers in biochemistry were used in the routine tasks of word processing, graphing, and statistical analysis of research data. However, computer use, when connected to the internet, is expanding greatly to include some of: (1) research of books and journal articles in the biochemical literary articles, (2) using software for analyzing experimental data, (3) accessing biological databases that provide nucleic acid and protein sequences and structures, (4) seeking research methodology, procedures, and protocols for lab work, and many more uses. This application of computer technology to the analysis, management, and manipulation of biochemical data is sometimes considered a part of the broad field of computational biochemistry. Bioinformatics, which is described as computer technology application for storing and using biological data, in particularly the sequence and structure of protein and nucleic acid, is a major computational subface of biochemistry. Your first encounter with a computer in the laboratory was probably while you were using an instrument that had a computer to control its operation, to collect data, and to analyze data. All major pieces of scientific equipment, including UV-VIS spectrophotometers, high-performance liquid chromatographs, gas chromatographs, nuclear magnetic resonance spectrometers, mass spectrometers, and DNA-sequencers, are now computer controlled. But your use of the computer will not end when you leave the lab, as you will use it to prepare laboratory reports including graphical and statistical analysis of your experimental data. This chapter mainly aims to understand how to solve problems in biochemistry using computer and software technology. It is important for your education and career that you become knowledgeable and skilled in the use of the computer and Internet. At the end of the chapter, you can find a word glossary related to computer terminology.

a. Accessing the Internet

Your instructor, supervisor or local computer service agency can answer questions relating to the utilization of your lab computer. Some suggestions are made in this book for certain hardware and software, but one must be aware that there are always new items and updates. Many of the listed software packages are free of charge. Microsoft Word is the most common application for word

processing (writing laboratory reports, etc.). Scientific and technical writing specialist software is available, although it is usually not essential at this level. Excel, Sigmaplot, SPSS, and SyStat are the main software tools for statistical analysis, graphics, and graphics sheets. You need a web browser, a web interface application that reads hypertext and displays web pages on your computer, to access the internet.

b. The World Wide Web

The World Wide Web (WWW, commonly known as the Web) is a heavily-used and fast-growing component of the Internet. Launched in 1992, this system allows the transmission of data as multimedia pages consisting of text, graphs, figures, audio and video. Each website that has its own address (URL) consists of Web pages. Every single website has its own address. Hypertext pointers connect the pages so the data stored on computers (servers) may be downloaded through the network via your computer. Web documents are written in a special coded language called Hypertext Markup Language (HTML). Type the URL into the dialog box for the browser to take you to the website homepage or starting point. One important feature you may note is that some text on the page is emphasized with underlining, in a different color, or perhaps highlighted in some other way. If you click the mouse on this text (called hyperlinks), your computer will connect to another related Web page that provides information on the hyperlink. This function increases the usage of the web considerably, as relevant websites are connected or linked together and may be reached simply by clicking the mouse instead of typing in a separate URL.

Chapter 5.3. WEB SITES USEFUL IN BIOCHEMISTRY

a. Directories, Library Resources, Databases, and Tools

Because of the complexity and the enormous number of Web sites available, one can easily become overwhelmed. These Web sites are especially valuable when seeking information and properties of biomolecules, including nomenclature, physical properties, structural data, reaction characteristics, spectroscopic information, and even chromatographic data. An annoying aspect of using Web sites is that they can disappear from the Internet. In fact, this may happen to some of the sites in this book. This process, sometimes called "Web rot," can happen when a link becomes broken or irrelevant over time. If the reason for a Web site disappearance is due to a new address, you will usually be directed to the new site. Millions of new sites are created every year. To access these new sites, you need the help of a search engine, a searchable directory that organizes Web pages by subject classification. The most widely used search engines are Google (http://www.google.com) and Yahoo (http://www.yahoo.com). Use of a search engine requires the input of a keyword or terms for searching. As you surf the Web, you may find sites you wish to save and review at a later date. Different Web browsers use different terms to define this saving process.

b. Viewing Structures of Biomolecules

Determining the biomolecular structure has now become a reasonably regular research laboratory task. Thousands of structures experimentally confirmed by X-ray crystallography, NMR, and electron microscopy are now available on certain Web sites. The structures include those of proteins, nucleic acids, and nucleic acid/protein complexes. Some of the most accessible and useful sites for viewing structures include:

• Protein Data Bank (http://www.rcsb.org)
• Molecules to Go (http://molbio.info.nih.gov/cgi-bin/pdb)
• PyMol Molecular Viewer (http://pymol.org)

The Protein Data Bank and Molecules to Go sites are freeware, however PyMol Molecular Viewer is a user-sponsored system and needs a subscription. Academic institutions may purchase a site license for an individual, lab and classroom, or complete department. To obtain a structure, access the Web site home page and type the name of the biomolecule desired into the dialogue box. Instructions for further analysis of the structure are given on all sites.

c. Searching the Biochemical Literature

Generating research ideas and maintaining a research lab require an deep understanding of the biochemical literature. Much of this literature is now accessible on the Internet, but occasionally it

may be necessary to use reference books in the library. The biochemical literature is massive and expanding rapidly. It is almost a full-time job just to maintain a current awareness of a specialized research area. The study of biochemistry has few disciplinary limitations. The biochemical literature is overlapping with biological, physical, medicinal and information sciences and technology. The intent of the following discussion is to bring order to the many sources of information including textbooks, reference books, research journals, and Internet Web sites that are available.

d. Textbooks

The student's first exposure to biochemistry is usually a formal class accompanied by the reading of a general textbook of biochemistry. By providing an in-depth survey of biochemistry, textbooks allow students to build a strong foundation of important principles and concepts. By the time most books are in print, the information is one to two years old, but textbooks should still be considered the starting point for mastery of the fundamentals of biochemistry. Many textbooks are now available online and may thus be updated by their writers from time to time or a new website can be added.

e. Reference Books

For more specialized and detailed biochemical information that is not offered by textbooks, reference books must be used. Reference works range from general surveys to specialized series. The best works are multivolume sets that continue publication of volumes on a periodic basis. Each volume usually covers a specialized area with articles written by recognized authorities in the field. It should be noted that reference articles of interest to biochemists are often found in publications that are not strictly biochemical. The best known and most widely used review publication is *Annual Review of Biochemistry*. Each volume in this series, which was introduced in 1932, contains several detailed and extensive articles written by experts in the field. For shorter reviews emphasizing current topics, *Trends in the Biochemical Sciences* (TIBS) is widely read. The active researcher has a continuing need for new methods and techniques. Several publications specialize in providing details of research methods, and many research methods are now available on the Web.

f. Research Journals

The core of the biochemical literature consists of peer-reviewed research journals (primary literature). A professional biochemist must maintain his knowledge of biochemical developments in his research field and adjacent fields. Scores of research journals are published with the intent of keeping scientists up to date. Some research journals have achieved an especially excellent reputation, and articles therein are considered to be of the highest quality. A recent ranking of the biochemical journals, based on the number of citations, produced the following order for the top six: *Journal of*

Biological Chemistry, Biochimica et Biophysica Acta, Biochemistry, Proceedings of the National Academy of Sciences of the United States of America, Biochemical Journal, and *Biochemical and Biophysical Research Communications.* The core journals used by an individual depend on the area of specialty and are best determined from experience. With the expansion of scientific information has come the need for efficient storage and use of research journals. Most publishers now provide journals on-line, and libraries pay the subscription cost for the journals they want. The articles may then be obtained free of charge by students, professors and researchers. If your institution does not subscribe to a journal you need; it is possible to read article titles and abstracts on journal Web sites at no cost. Usually, a fee is charged if the full text of an article is required.

g. Literature Searches on the Web

While you study and work in biochemistry, you will often need to cover a thorough literature search on some specialized field or topic. Practically, it is not easy to survey the hundreds of books, journals, and reports that may contain information related to the topic. Two publications that provide brief summaries of published articles, reviews, and patents are *Chemical Abstracts* and *Biological Abstracts.* If you are not familiar with the use of these abstracts, ask your instructor for assistance. Both of these abstracts are available on-line. Research articles of interest to biochemists may appear in many types of research journals. Research libraries have no finances to subscribe to each journal, nor do scientists have the time to survey every current journal copy for articles of interest. The computer revolution has reached into the chemical and biochemical literature, and most college and university libraries now subscribe to computer bibliographic search services. One such service is STN International, the scientific and technical information network. This on-line system allows direct access to some of the world's largest scientific databases. The STN databases of most value to life scientists include BIOSIS Previews/RN (produced by Bio Sciences Information Service; covers original research reports, reviews, and U.S. patents in biology and biomedicine) and CA (produced by Chemical Abstracts service, covers research reports in all areas of chemistry including biochemistry). These networks provide on-line service, and their databases can be accessed from personal computers in the office, laboratory, or library. The Library generally pays for subscription charges. Excellent websites are accessible as freeware to search for the biochemical literature using your own computer. The most widely used site is:

• **PubMed** from the National Center for Biotechnology Information (http:// www.pubmed.gov)

PubMed is a service of the NIH (National Library of Medicine). In addition to the direct URL listed above, it may be accessed through the NCBI site (http://www.ncbi.nlm.nih.gov), National Library of Medicine site (http://www.nlm.nih.gov) or many other databases. Search terms may be topics, author name, or journal name. Results are reported in a table, beginning with the most recent articles, with article titles, author(s), and journal reference.

g1. Study exercises:

a) Searching the biochemical literature on PubMed

To illustrate the use of this search service, point your Web browser to the appropriate URL above for the PubMed home page. Many features on display are available, but the most basic is the search capability. For bibliographic searching, you may enter in the dialogue box a search term, author name, or journal name. For example, type in “human alpha-lactalbumin,” a calcium-binding protein found in milk. Clicking on “Search” will then provide more than 1100 citations (or articles). The lists are composed of article title, author(s), and reference in reverse chronological order. By clicking on the highlighted title (in hypertext), you can retrieve the abstract of the article. Another useful and timesaving feature is the hypertext (see “Related Articles”). Clicking on this will provide a list of papers related to the specific citation. The 1100 papers or so that you obtained in your original search are too many to screen; you may change the search parameters to reduce the number. For example, in the citation listing you see some interesting articles describing those complexes formed between human alpha-lactalbumin and oleic acid have been reported to display apoptotic activity against tumor cells. You can narrow down the citation list by searching the topic “complexes between human alpha-lactalbumin and oleic acid.” This yields about 12 results, a reasonable number of abstracts to read. Practice using PubMed with some terms you find interesting.

b) Sequence Homology in Proteins

Another activity that is valuable in biochemical research is to seek proteins that have various functions, but similar sequences (sequence homology). This research may be carried out on a few websites:

• Protein Data Bank (http://www.rcsb.org)

• National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov)

Both Web sites compare biomolecules to seek to find regions of sequence similarity.

a. Virtual Biochemistry Laboratories

For some colleges and universities around the world, offering a “genuine” biochemistry laboratory course for students is not a possibility. Some causes are the absence of costly science instruments, equipment and reagents; the lack of suitable laboratory space; the absence of staff skills and the incapacity to adapt planned laboratory time to a narrow curriculum. Alternative student activities are now accessible in "virtual biochemistry laboratories" on the Internet (Table 1). Although simulated labs should never be viewed as a replacement or substitute for the real thing, especially for student majors in biochemistry, molecular biology, other biological sciences, and chemistry, they may be acceptable for some students who will never work in a lab or manipulate lab data. Furthermore, viewing experiments and procedures in virtual labs may actually offer benefits to students who will

perform similar experiments in the lab. The virtual laboratory may be seen as a tool for students to get to know equipment, methods and processes and to perform real experiments more effectively in their labs. This experience is particularly essential when students work with high-cost equipment and reagents. Virtual laboratories can also be seen as a safety feature — students will be trained to handle toxic substances and dangerous procedures.

Table 1. Virtual Biochemistry Labs

Name	Description	URL
Carnegie Mellon University	Biology labs	http://telstar.ote.cmu.edu/biology/lab/index.html
Lund University	Labs in bioanalytical chemistry	http://www.drylabs.com
Changbioscience	Virtual labs for biologists	http://www.changbioscience.com/virtualab.html
HHMI Interactive	Virtual labs in biology	http://www.hhmi.org/biointeractive

b. Glossary of Computer Terms

Bioinformatics: the use of computer technology to solve biological problems.

Biological databases: computer sites that organize, store, and disseminate files that contain information consisting of literature references, nucleic acid sequences, protein sequences, and protein structures.

Bookmark: a function to save a Web site address for later use (Netscape Navigator).

Computational biochemistry: the application of computer technology to the analysis, management, and manipulation of biochemical data.

Ethernet: a family of frame-based computer networking technologies for local area networks.

Favorites: the Internet Explorer form of a bookmark.

Freeware: software that is provided free of charge by its developer.

Home page: the beginning page for access to a Web site.

HTML: Hypertext Markup Language; a special, coded language that is used to write Web pages.

Hyperlink: link or connection between related Web pages.

Hypertext: a language that connects similar documents on the Web.

Internet: the worldwide matrix that allows all computers and networks to communicate with each other.

Multimedia: several forms of media including text, graphics, video, and audio.

Search engine: a searchable directory that organizes Web pages by subject classification.

Server: a computer that acts as the storage site for retrievable data.

URL: uniform resource locator; a standard address form that identifies the location of a document on the Internet.

Web site: a collection of documents (Web pages) on a server.

WWW: World Wide Web ("the Web"); a component of the Internet that uses a hypertext-based language to provide resources.

CHAPTER 6. Biochemistry Lab Techniques

6.1. Basic Instrumentation Practices

6.2. Mortar and Pestle

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, which usually made of wood, ceramic or stone. The pestle is an instrument in the shape of a large blunt club which is used to crush and grind. The substance to be ground is placed in the mortar and ground, crushed or mixed using the pestle.

Fig.6. Mortar and Pestle

6.3. Desiccator

Fig.7. Vacuum desiccators(left) & Desiccators (right)

Desiccators are sealable enclosures containing desiccants used for preserving moisture sensitive items. For desiccators, chemical products that are hygroscopic or that react with water are usually protected from humidity. Desiccators are used occasionally to remove water from an almost dry sample. Glassware is chilled in desiccators to avoid the absorption of humidity from the surrounding air. In case of insufficient drying agents alone, the sample may be dried using the Abderhalden's drying pistol at high temperatures.

a. Parts of a Desiccator

The most common desiccators in the laboratory use 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. A small coating of petroleum jelly or other lubricant must be grated over the base glass rim of the desiccator lid to ensure airproof closure. A stopcock may be involved to permit the desiccators to be evacuated. Such models are usually known as vacuum desiccators. When using a vacuum, it is a common practice to crisscross 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.

6.4. Micro Centrifuge

A micro centrifuge, also known as microfuge, is an essential lab equipment; it is used for spinning small (2ml or less) liquid samples at elevated speeds (generally tens of thousands of times g-force).

Fig.8. Micro Centrifuge

a. Working

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

b. Points to Remember

- Wipe the inner chamber and keep open for drying after centrifugation.
- Clean the rotor after every use. There are chances for any spill of liquids used.
- Observe if any voltage fluctuation occurs.
- It shows drive fault if any error occurs. In that case switch off and then enter the program.
- Always ensure that the necessary temperature is attained before the rotor starts.

6.5. Vortex mixer

A vortex mixer is a basic instrument often used in labs to mix small amounts of fluids in vials. 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.

Fig.9. Vortex mixer

a. 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-center. As the motor runs the rubber piece oscillates quickly in a circular motion.

b. Working

The motion is transferred to the fluid and a vortex is produced when a test tube or other corresponding container is pressed into the rubber cup (or touched to its edge). The majority of vortex mixers has several speed settings and may be configured to operate continuously or only if the rubber component receives downward pressure.

6.6. Rotary Shaker

Rotary Shakers are perfect for mixing and development of cultures, chemicals, solvents, assays, etc. and for production of basic chemicals, pathological work, and numerous other applications. The shaker is also widely used for shaking solutions in Erlenmeyer flasks. Rotary shaker extracting, dissolving slow-reacting samples; cultivation of cells; extraction of mineral oil from soil, tissue culture for analytical diagnostics; de-aeration of tested biodegradable materials and samples; rotating closed containers for dialysis in a circular fashion.

Fig.10. Rotary Shaker

a. Components of a Rotary Shaker

A basic shaker has a table board that oscillates horizontally, powered by an electric motor. The liquids to be stirred are held in beakers, jars, or Erlenmeyer flasks that are placed over the table; or, occasionally, in test tubes or vials that are placed into holes in the plate. Orbital shakers also exist, that shake the vessel circularly.

6.7. Homogenizer

A homogenizer is a piece of laboratory or industrial equipment used for homogenizing different kinds of material, such as tissue, plant, food, soil, and many others. Homogenization is a very common sample preparation step prior to the analysis of nucleic acids, proteins, cells, metabolism, pathogens, and many other targets.

Fig.11. Homogenizer

a. Principle

High speed mechanical and hydraulic shear forces are key to the success of this instrument. Rotor and stator generate a shearing action which ensures that materials being processed are subjected to thousands of shearing actions each minute

6.8. Fume hood

A fume hood or fume cupboard is a type of local ventilation equipment that is designed to limit exposure to hazardous or toxic fumes, vapors or dusts. A fume hood is typically a large piece of equipment enclosing five sides of a work area, the bottom of which is most commonly located at a standing work height. There are two primary types of fume hoods, ducted and recirculating (ductless).

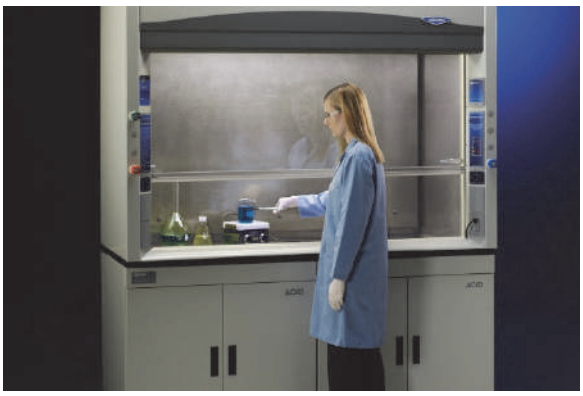


Fig.12. Fume hood

a. Principle

The speed of the air moving through the hood opening is known as face velocity. Air is drawn in from the front (open) side of the cabinet, and either expelled outside the building or made safe through filtration and fed back into the room.

Instrumentation

A fume hood is basically a cabinet, with an open side (or sides) for access to the interior of the hood. A transparent, movable sash, allows the user to restrict or enlarge the fume hood opening. The hood is connected, via ductwork, to an exhaust fan, usually located on the roof of the building in which the hood is placed. The exhaust fan extracts air from the hood's chamber by opening and exiting the ductwork.

6.9. Laminar Air Flow Cabinet

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

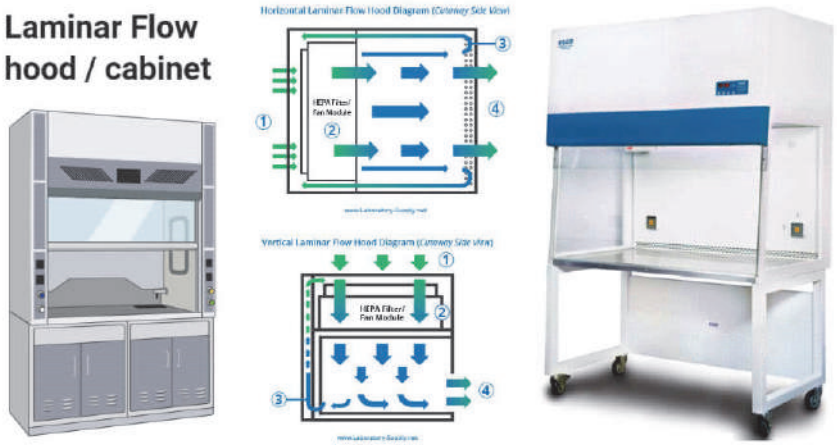


Fig.13. Laminar air flow cabinet

a. Principle

The principle behind laminar air flow is the passage of continuous air flow at uniform velocity. HEPA (High Efficiency Particulate Air) filters designed to create a particle free working environment and provide product protection. Air taken through the filtration system is then drained into the work surface. Generally, 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.

b. Instrumentation

A laminar flow hood includes a filter pad, a fan and a HEPA 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 area where you can do all your work without risk of contamination. A UVC (subtype of UV light) is responsible to sterilize the interior and contents. When not in use, it is necessary

to switch a UVC germicidal lamp off, to limit exposure to skin and eyes since stray ultraviolet light emissions can cause cancer and cataracts.

6.10. Electronic Weighing Balance

Balances are designed to meet the specific weighing requirement in the laboratory. These balances come in precision designs and operating characteristics enabling fast and precise measurements.

Fig.14. Electronic Weighing Balance

a. 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 gently according to the need. Wait until the "g" is stabilized near the displayed weight.

b. Note

Know the maximum and minimum quantities that can be weighed using balance. Never spill the chemicals on the weighing pan, if it happens wipe off with tissue or a soft brush. Switch off fan and close windows nearby when working with it since it may cause fluctuations in the value due to interaction with air density. Always use weigh boats or butter paper to weigh.

6.11. Magnetic Stirrer

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.

Fig.15. Magnetic Stirrer

Fig.15.1. Stir bars

A stir bar is the magnetic bar placed within the liquid that 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.

a. 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.

b. Principle

Principally, 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.

6.12. Water Bath

A water bath is constructed 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.

Fig.16. Water Bath

a. 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.

b. Working

It is composed of double walled, outer body made of MS (mild steel) sheet, powder coated, inner body made of stainless steel, and 304 quality sheet glass wool insulation, fitted with 30ºC to 110ºC 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 (alternating current) supply. It is accompanied with a digital display temperature controller. In general, they use water, but some baths use oil. Make sure it is clean and that the necessary accessories have been installed before use of the water bath. The steps mainly followed are:

- Fill the water bath with fluid to keep the temperature constant (water or oil). Once the containers to be heated are placed, ensure that the fluid level is between 4 and 5 cm from the top of the tank.
- Install the necessary control instruments, such as thermometers and circulators. Use additional mounts provided for this purpose. Verify the position of the thermometer's bulb or thermal probe to make sure that the readings are correct.
- If water is used as the warming fluid, verify that it is clean. Some manufacturers recommend addition of products that inhibit fungal or algae growth.

- Put the main switch in the ON position. Some manufacturers have incorporated controls with microprocessors which initiate auto-verification routines once the ON switch is activated.
- Choose the operation temperature using the menu buttons and the buttons for parameters to be adjusted.
- Select the cut-off temperature. 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.

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.

Avoid the use of the water bath with the following substances:

- Bleach
- High chlorine containing liquids
- Weak saline solutions (e.g. 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
- Deionized water, since it causes corrosion and perforation in the stainless steel

c. 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.
- While working with vapor generating, place the water bath under a chemical hood or in a well-ventilated area.
- Remember that liquids incubated in the water bath tank may produce burns if hands are unintentionally placed inside it.
- Please remember that the water bath is designed for use with a liquid inside the tank. If inside of the tank is dry, the temperature can get 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.

- Do not use the water bath if any of its controls is not working, (e.g., the temperature or limit controls).

d. Cleaning

Frequency: once a month (more if necessary)

- Switch off and disconnect the equipment. Wait until it cools to avoid the risk of burns andaccidents.
- Remove the heating fluid. It can be poured via a siphon if it is water. If it is oil; collect into a container with an adequate capacity.
- Remove the thermal diffusion grid placed at the bottom of the tank.
- Disassemble the circulator and clean to remove scale and potential algae present.
- Clean inside the tank with a mild detergent. Use chemicals for cleaning stainless steel if there is any evidence of corrosion. Rub lightly with synthetic sponges or similar to it. Avoid using steel wool to remove rust stains since these leave particles of steel that might speed up corrosion process.
- Do not bend or strike the temperature control capillary tube generally located at the bottom of the tank.
- Clean inside and outside of the water bath with clean water.

6.13. pH Meter

Fig.17. pH Meter

pH meter is used for measuring the pH potentiometrically, which is either the concentration or the activity of the hydrogen ions, of an aqueous solution. Usually, it has a glass electrode plus a calomel reference electrode, or combination electrode. pH meters are usually used to measure the pH of the liquids, however special probes are sometimes used to measure the pH of semi-solid substances.

a. 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 contain a simple electronic amplifier and a pairof probes, or a combination probe, and some form of display calibrated in pH. The probe is the primary component, generally constructed from glass, which has a rod-like shapes with the bulb at the bottom housing the sensor. Frequent calibration with solutions of known pH, perhaps before each use, may guarantee the best accuracy. To measure the pH of a solution, the probe is dipped into it.

b. 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 specifically designed, 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.

c. Working

- Turn on pH Meter. Lift up the electrode and clean the electrode tip by pressing with tissuepaper.
- 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 measure the pH.
- If there is an increase in pH, stabilize it with adding 0.1N HCl which decreases the pH. If the pH is low, 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 eachaddition.

d. Note

- Always keep the electrode immersed in 3 mol/l KCl solution, and never let it dry.
- Be attentive about the electrode level shown on the screen. It indicates the fitness of electrode. Any kind of unusual appearance can lead to 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 make fresh buffer solutions (preferably every month) for calibration.
- Always make buffer solutions in sterile water.
- Never mishandle the electrode like using it for stirring the solutions.
- Every time use sterile water to clean the electrode after immersing it in solutions.
- Do not place the electrode in solutions which are in extreme temperature conditions.
- No urgency should be present for reading the pH.
- The instrument and its premises should be cleaned, if there is any spill.

6.14. Microscope

A microscope is an equipment 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 frequently used microscope for general purposes is the standard compound microscope. It increases the size of the object by a complex system of lens arrangement.

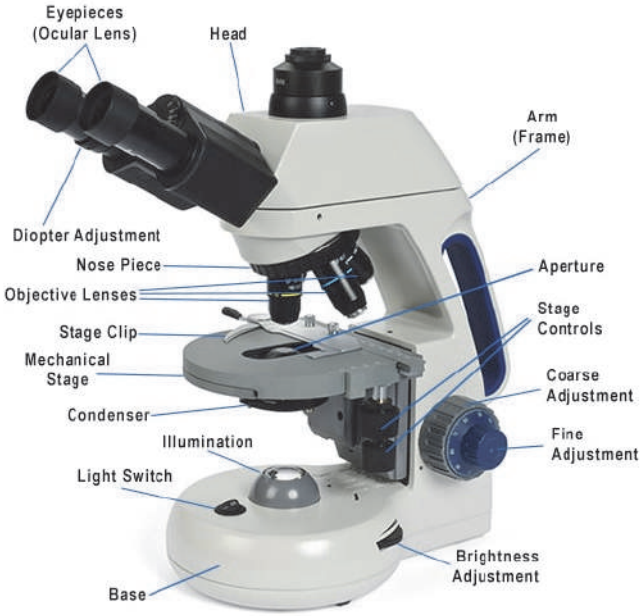


Fig.18. Microscope

a. Principle

It has a series of two lenses; (i) the objective lens near the observable object 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 eye through the eyepiece. As light passes directly from the source to the eye through the two lenses, the field of vision is brightly illuminated. Therefore; it is a bright-field microscope.

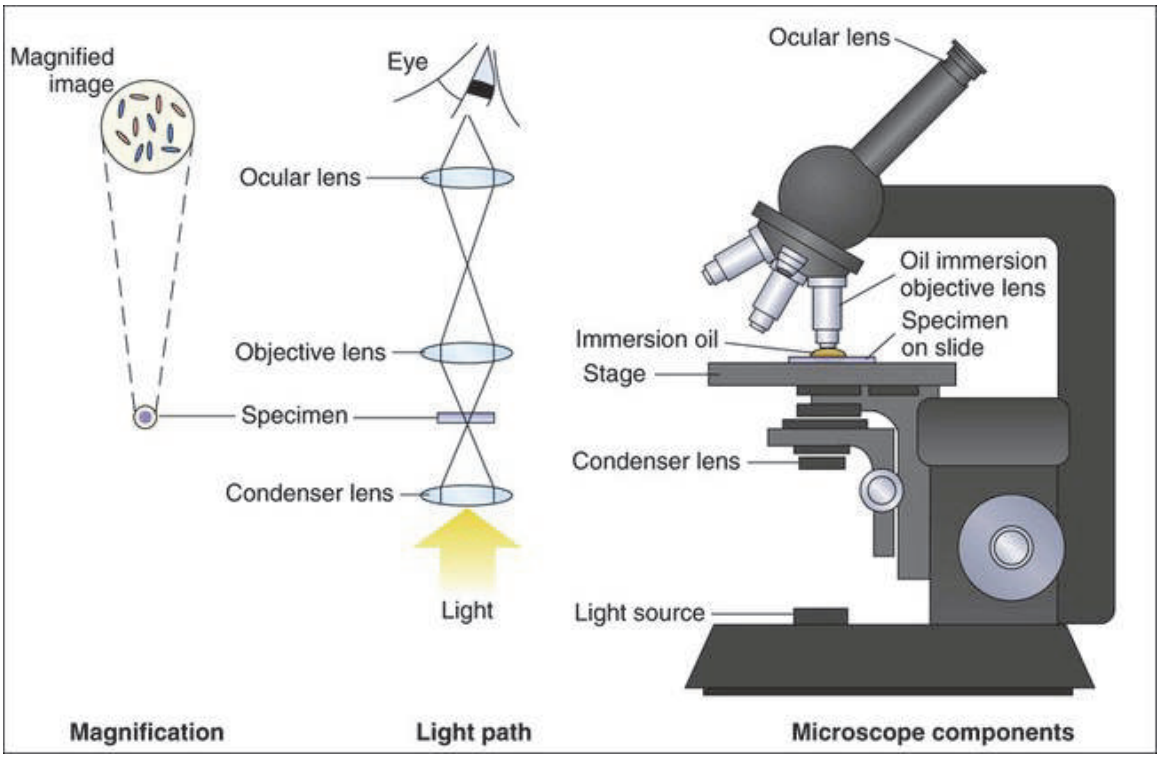


Fig.18.1. Working Principle of a Microscope

b. Parts of a Microscope

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

(II) **Base or Metal Stand:** The entire microscope lies on this base. Mirror, if present, is fitted to it.

(III) **Pillars:** It is a pair of elevations on the base, by which the body of the microscope is held to the base.

(IV) **Inclination joint:** It is a mobile 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 required by the observer, for easier observation.

(V) **Curved Arm:** It is a curved construction held by the pillars. It maintains the stage, body

tube, fine adjustment and coarse adjustment.

(VI) Body Tube: It is normally a vertical tube that holds the eyepiece at the top and the revolving nosepiece with the objectives at the bottom. The length of the draw tube is termed as 'mechanical tube length' and is usually 140-180mm (mostly 160 mm).

(VII) Draw Tube: It is the upper section of the body tube, slightly narrower, into which the eyepiece is slipped during observation.

(VIII) 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.

(IX) 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.

(X) Stage: It is a horizontal platform projecting from the curved arm. It has a hole at the center, 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.

(XI) 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.

(XII) 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 various magnifying powers. Based on the necessary magnification, the nosepiece is rotated in a way, so that only the objective specified for the required magnification remains in line with the light path.

(XIII) Optical Parts: These pass the light through the object and magnify its size.

(XIV) Light Source: Modern microscopes have in-built electric light source in the base. The source is connected to the mains via a regulator, which controls the brightness of the field.

(XV) Diaphragm: If light coming from the light source is too bright 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 placed under the condenser to control the amount of light entering into the condenser.

(XVI) Condenser: It's a sub-stage condenser positioned between the light source and the stage. It has a series of lenses to converge on the object. After passing through the object, the light enters into the objective. The 'light condensing', 'light converging' or 'light collecting' 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.

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

c. The objectives can be:

(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 immersionoil fills the space between the object and the objective.

6.15. Refractometer (Abbe's Hand Refractometer)

Fig.19. Refractometer

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.

a. Principle

The apparatus works on the idea of 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. Light enters from the left and passes through the liquid sample. When the light hits the prism at the bottom of the liquid, it sharply slows down more than in the liquid. The reason is that the prism has a higher refractive index. In the case of a refractometer, the light bends in proportion to the liquid's refractive index. While 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 rest is dark. The dividing line between light and dark is the region to read the scale. Calibration is accomplished by turning the calibration screw, which elevates or decreases the reticle (the scale) relative to the path of the light.

b. Working

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

6.16. Refrigerator

Laboratory refrigerators are utilized to cool samples or specimens for preservation. They vary from typical refrigerators in restaurants or households, because they must be entirely sanitized and completely reliable.

a. Principle

The refrigerating effect in refrigeration systems is based on the utilization and management of the phase transition processes of evaporation. As the refrigerant evaporates it absorbs energy (heat) from its surroundings and by placing an object in thermal contact with the evaporating refrigerant it can be cooled to low temperature.

Fig.20. Laboratory refrigerators

There are distinct melting and boiling points of different substances. For example, gold melts at 1064°C and chocolate at 26°C while most refrigerants melt around -100°C at temperatures. For a substance present in two of its phases at the same time or undergoing a phase change pressure and temperature, become dependent. If the two phases exist in a closed container

and the two phases are in thermal balance the condition is said to be saturated. If the temperature of the two-phase mixture is elevated the pressure in the container will also increase. The relationship between pressure and temperature for saturated conditions (liquid and vapor) is usually considered the vapor pressure curve. With a vapor pressure curve, the pressure for an evaporating or condensing process may be determined.

6.17. Deep Freezer

Fig.21. Deep Freezer

Deep freezer systems are beautifully constructed to satisfy the high standard in terms of pharmacy, medicine, industry, biotechnology and clinical performance and controlled temperature storage requirements.

a. Principle

The fundamental concept behind a freezer is evaporation. When a liquid evaporates, it causes the surrounding area to cool. However, water can't be used in freezer, since it evaporates at too high temperature. But certain liquids evaporate at very low temperatures. For example, isobutene (becoming more frequent in household freezers) evaporates at very low temperatures. This ability to evaporate at very low temperatures means that it cools surfaces which are already very cold.

b. Instrumentation

- **Evaporator**: Air pressure affects evaporation. The higher the pressure of the air, the less fluid evaporates.
- The **compressor** takes in the refrigerant (as gas); raise the air pressure which converts the refrigerant gas to liquid.

- As the refrigerant liquid flows from the compressor to the **expansion valve** the high air pressure prevent its evaporating and instead it gives off heat and becomes cooler. The refrigerant liquid passes through the expansion valve where the air pressure is significantly lower. This leads to the evaporation of refrigerant liquid which cause the pipe to become very cold within the freezer.
- One important element of the freezer is the **thermostat**. The thermostat senses the temperature inside the freezer and when it drops below a certain temperature it turns off the motor so the flow of the refrigerant liquid stops. When the temperature increases above a certain level the thermostat turns on the motor and the refrigeration process restart.

c. Refrigerant Liquids

Different freezers have different refrigerant liquids. The used liquid is important for two main reasons. Firstly, different liquids are more or less efficient for use in a freezer. Less efficient liquids will use more electricity and therefore cost you more to run your freezer. Secondly, some refrigerant liquids contribute significantly more than others to global warming and destruction of the ozone layer. Before 1990 many freezers used CFC (chlorofluorocarbon) which caused significant environmental damage to the atmosphere. This was then replaced with HCFC (hydro chlorofluorocarbon) which slightly damage the ozone layer but still does contribute to global warming. Nowadays, HFC (Hydrofluorocarbon) and isobutene are utilized as refrigerant liquids in most household freezers. Both are a good with isobutene being the best.

6.18. BOD (Biological Oxygen Demand) Incubator

BOD incubators are frequently called low temperature incubators, which in many research institutions, hospitals and other pharmaceutical laboratories are one of the main laboratory equipment. Bacteriological incubators are known as exceptional lab incubators specially designed for a variety of incubation and testing applications. The series of BOD incubator is ideal for BOD testing and other associated research applications.

Fig.22. BOD Incubator

a. Principle

The basic concept behind the BOD incubator is to maintain constant environment condition for anyparticular kind of study such as cell and microbiological cultures, etc.

6.19. Hot Air Oven

It is a dry heat sterilization unit. A dry heat cabinet is easy to install and has relatively inexpensive cost of operation; it is none hazardous, harmless to the environment and it is noncorrosive for metal and sharp instruments.

Fig.23. Hot Air Oven

a. 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 center of it, layer by layer. The whole system will finally reach the temperature required for sterilization. Dry heat does most of the damage by oxidizing molecules. The important cell components are destroyed and the organism dies. The temperature is maintained for almost over an hour to destroy the most difficult of the resistant spores. The most basic time-temperature relationships for sterilization with hot air sterilizers are:

1.170°C (340°F) for 60 minutes,

2.160°C (320°F) for 120 minutes,

3.150°C (300°F) for 150 minutes or longer depending upon the volume

b. Working

Main operating concept of hot air oven is the forced circulation of hot air within 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.

c. 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.

6.20. Autoclave

An autoclave is a massive pressure cooker. It is a moist sterilizer. 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 approximate 15–20 minutes depending on the size of the load and composition. It was invented by Charles Chamberland in 1879.

Fig.24. Autoclave

a. Parts of an autoclave

Autoclaves have four main parts

- **Water Intake-** An autoclave needs water to make the steam used for sterilization. A water intake hookup or hose, is allowing the user to pump water directly into the equipment.
- **Chamber-** The chamber is the space where the user loads items to sterilize. There are wire shelves in the chamber that keep different things upright or laying and allow steam from every angle to penetrate.
- **Control Panel-** The user may personalize the autoclaving process using the control panel. Some materials can withstand higher temperatures, while some must be autoclaved at lower temperatures for longer periods of time.
- **Machinery-** Air pump system must be installed on autoclaves to remove oxygen from the chamber, to generate a vacuum to fill the reservoir with pressed steam from the water. The water becomes heated either via a heating element inside the water reservoir or a heat-generating mechanism that completely surrounds the reservoir**.**

b. Principle

It works according to 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. The heating power of steam is mostly due to its latent vaporization heat (the amount of heat required to convert boiling water to steam).

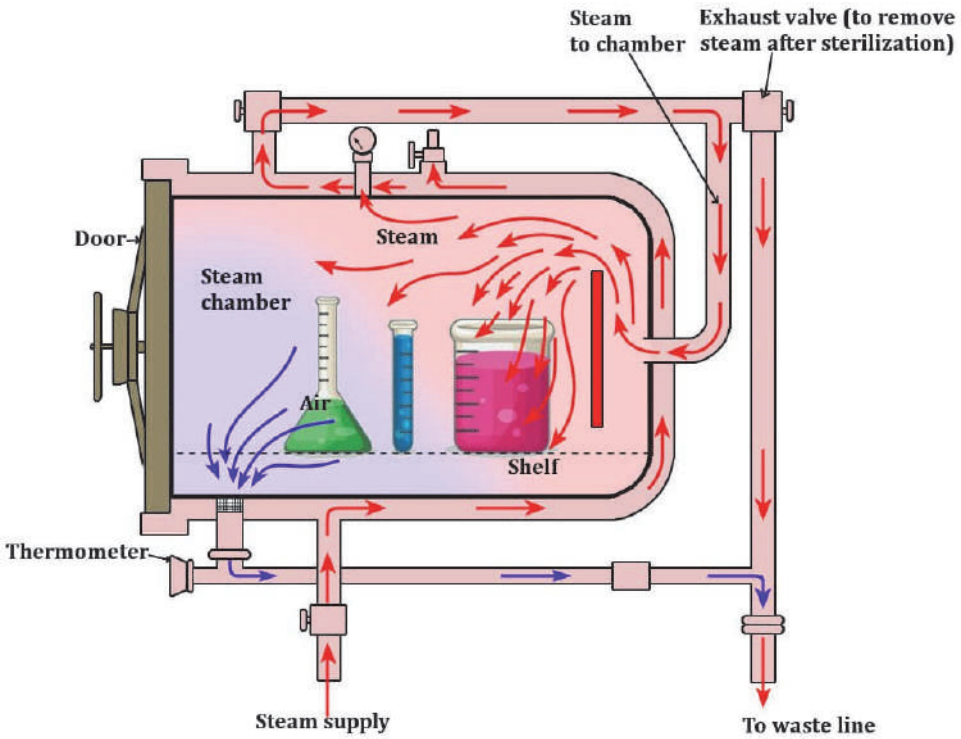


Fig.24.1. Principle of Autoclave

Steam is able to penetrate objects with cooler temperatures because once the steam meets a cooler surface; it instantly condenses to water, producing a concomitant 1,870-fold reduces in steam amount. This generates 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 are balanced, a saturated steam environment is created. Achieving high and even moisture content in the steam-air environment is crucial for providing appropriate 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, and hence steam is one of the most efficient transporters of heat. Steam therefore also results in the efficient killing of cells and the coagulation of proteins. Moist heat is supposed to destroy bacteria by coagulating the most important 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 destruction to all metabolic functions of the organism. Death rate is directly proportional to the concentration of microorganisms at any given time. The time necessary to kill a known bacteria population at a certain temperature in certain conditions is called the time of thermal death (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 115ºC/10psi, 121ºC/15psi and 132ºC/27psi. (Psi=pounds per square inch).

c. 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). A safety valve provides protection against overpressure. 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.

d. Note

Please remember that after loading and starting the autoclave, the processing time is measured after the autoclave reaches normal operating conditions of 121ºC (250ºF) and 15 psi pressure, not simply from the time you click the "on" button. Since autoclaves utilize steam, heat and pressure the risk of personal exposure and potential damage are considerable. Personnel should wear proper personal safety equipment (i.e., heat resistant gloves, eye protection and a lab coat) especially when unloading the autoclave. Periodically inspect the autoclave for proper operation. Do not presume that the temperature and pressure is down before opening the chamber. Look at the values of the gauges. Even if the pressure gauge shows "zero", open the chamber c cautiously; 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 objects rest for five minutes before handling. This will minimize the possibility of over-boiling and burns. Do not place sealed containers in an autoclave, since they might explode. This enables 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 your containers with a few inches of "head space". This way, it won't spray into your face if the object is boiled. 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 liter of media, you need to put it in a flask that hold at least 2 liters). 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.

6.21. Gel Documentation System

A gel doc, also called a gel documentation system, gel image system or gel imager, is equipment widely used in molecular biology laboratories for the imaging and documentation of nucleic acid and protein suspended within polyacrylamide or agarose gels. These gels are typically stained with ethidium bromide or other fluorophores such as SYBR Green (asymmetrical

cyanine dye).

Fig.25. Gel Documentation System

a. Principle

Principle of fluorescence with fluorescent staining of nucleic acids, a fluorescent substance that has bound to nucleic acids is excited by ultraviolet irradiation and emits fluorescent light. The fluorescent substance ethidium bromide binds specifically to nucleic acid and the amount of binding depends on the molecular weight and concentration of the nucleic acid. In other words, a band for a large molecular weight or large amount will shine brighter; conversely, fluorescence will be weaker for a band for a small molecular weight or small amount.

b. Instrumentation

- **Source of irradiation:** UV Transilluminator 20x20cm, 312nm (254nm selectable)
- **Base Plate:** A sample tray, a gel viewer
- A set of **filters**
- **Imaging device:** A camera (CCD with high resolution) unit integrated with darkroom, cameracontroller, video monitor and printer.
- **Readout system:** Computerized system

6.22. Centrifuge

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 are separated according to their size, shape, density, viscosity of the medium and rotor speed.

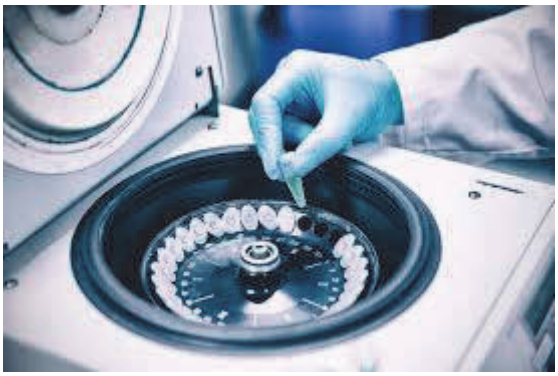


Fig.26. Centrifuge

a. 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 (CF), expressed as a multiple of the force of gravitational force. When a particle is subjected to centrifugal force, it will migrate away from the axis of rotation at a rate dependent on the particle's size and density.

b. Instrumentation

- The part that holds the centrifugation tubes is called the centrifuge rotor.
- Centrifuges are constructed in a way, 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.

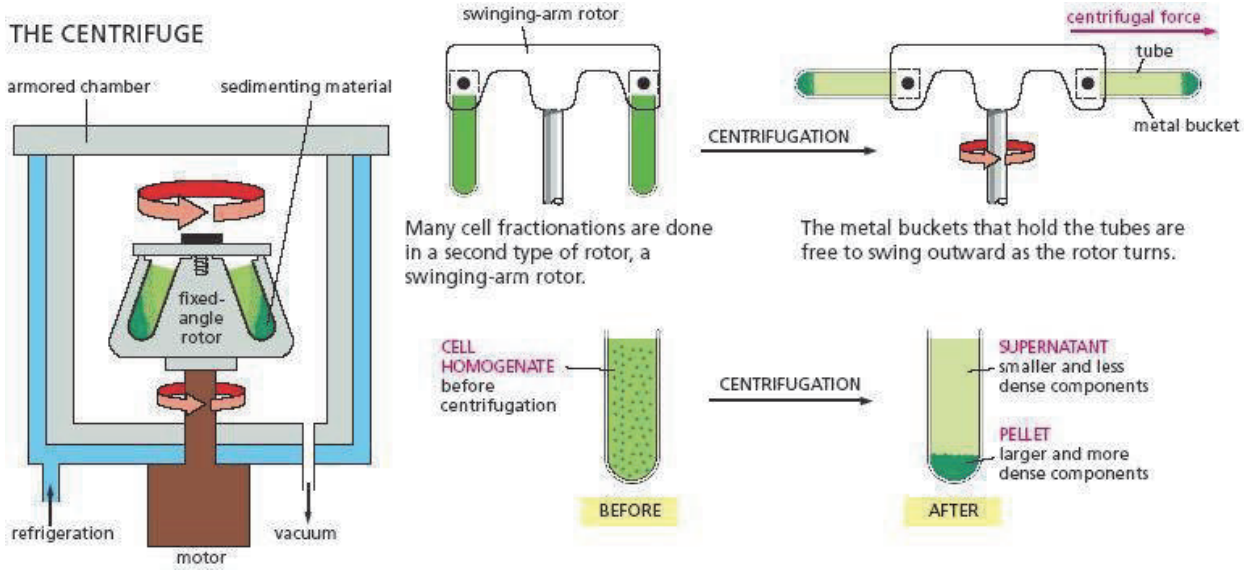


Fig.26.1. Rotors and Centrifugation

6.22.1. Differential Centrifugation

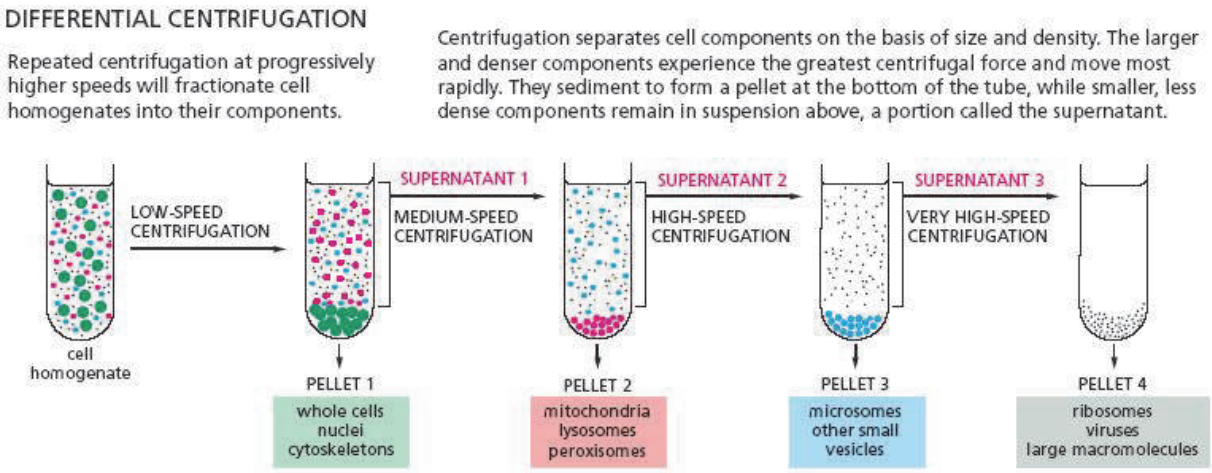


Fig.26.2. Differential Centrifugation

Differential centrifugation is one of two main 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 further centrifugations to make

pellets containing other cellular components of smaller size and / or density.

6.23. Refrigerated Centrifuge

It is widely used in chemistry, biology, and biochemistry for isolation and separation suspensions. It also provides the cooling system to maintain the uniform temperature throughout the operation of the sample.

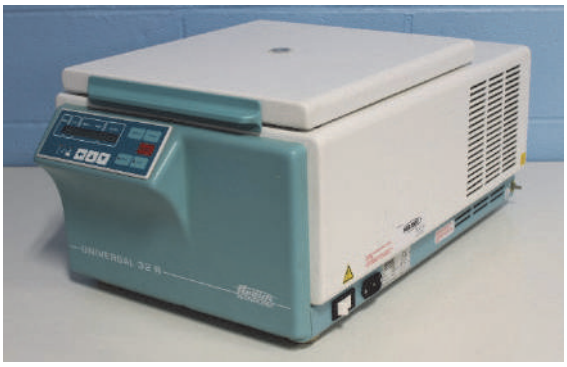


Fig.27. Refrigerated Centrifuge

a. Principle

It 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 revolutions 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.

b. Working

- Switch on the power supply. It will illuminate the power indicator.
- Press the PROG button. The required program number can be set by using the UP or DOWN.
- Timer settings: Press the PROG button once 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 increasing and DOWN arrow for decreasing the time. Set the necessary time and release the button for the program.
- Setting the temperature: Press the PROG. Choose the necessary 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 time for acceleration: Press the PROG button again. Use UP or DOWN arrow to set the acceleration time.
- Release the button. Setting the deceleration time: Press the PROG button again. Use UP or DOWN arrow to set the deceleration time. Release the button.
- Rotor Selection: Press the PROG button again. The rotor heads are numbered. Use arrow UP or DOWN select the specific number of the rotor head you plan to use. Release the button.

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. You can press the RETURN key to come back to Normal Display mode.

6.24. Thermal Cycler (PCR machine)

The thermal cycler (also is known as a thermo cycler, PCR machine or DNA amplifier) is an equipment used to amplify segments of DNA due to the Polymerase Chain Reaction (PCR). The device has a thermal block with holes where tubes holding the PCR reaction mixtures can be inserted. The cycle then raises and decreases the temperature of the block in discrete, controlled steps.

Fig. 28. Thermal cycler (PCR machine)

6.24.1. Polymerase Chain Reaction (PCR)

PCR stands for the Polymerase Chain Reaction and was developed in 1987 by Kary Mullis (which won him a Nobel Prize) and associates. This technique enables to make virtually limitless copies of a single DNA molecule even though it is initially present in a mixture containing many different DNA molecules. It can be used for the amplification of a specific DNA (target) sequence lying between known positions (flanks) on a double-stranded (ds) DNA molecule. The polymerase chain reaction can be applied to amplify both double and single stranded DNA.

a. Principle

The cycling reactions: There are three key steps in a PCR, which are repeated for 30 or 40 cycles. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

1. Denaturation: This step is the first regular cycling event and consists of heating the reaction to 94-98°C for 20-30 seconds. It causes DNA melting by separating the hydrogen bonds holding together complementary bases, yielding single-stranded DNA molecules.

2. Annealing: The reaction temperature is reduced to 50-65°C for 20-40 seconds allowing annealing of the primers to the single-stranded DNA template. This temperature should be low enough to allow for hybridization of the primer to the strand, but high enough for the hybridization to be specific (the primer should only bind to a perfectly complementary part of the template) If the temperature is too low, the primer could bind imperfectly. If it is too high, the primer might not bind. Typically, the annealing temperature is about 3-5°C below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and starts DNA formation.

3. Extension: The temperature at this point will depend on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75-80°C, and typically a temperature of 72°C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. At each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.

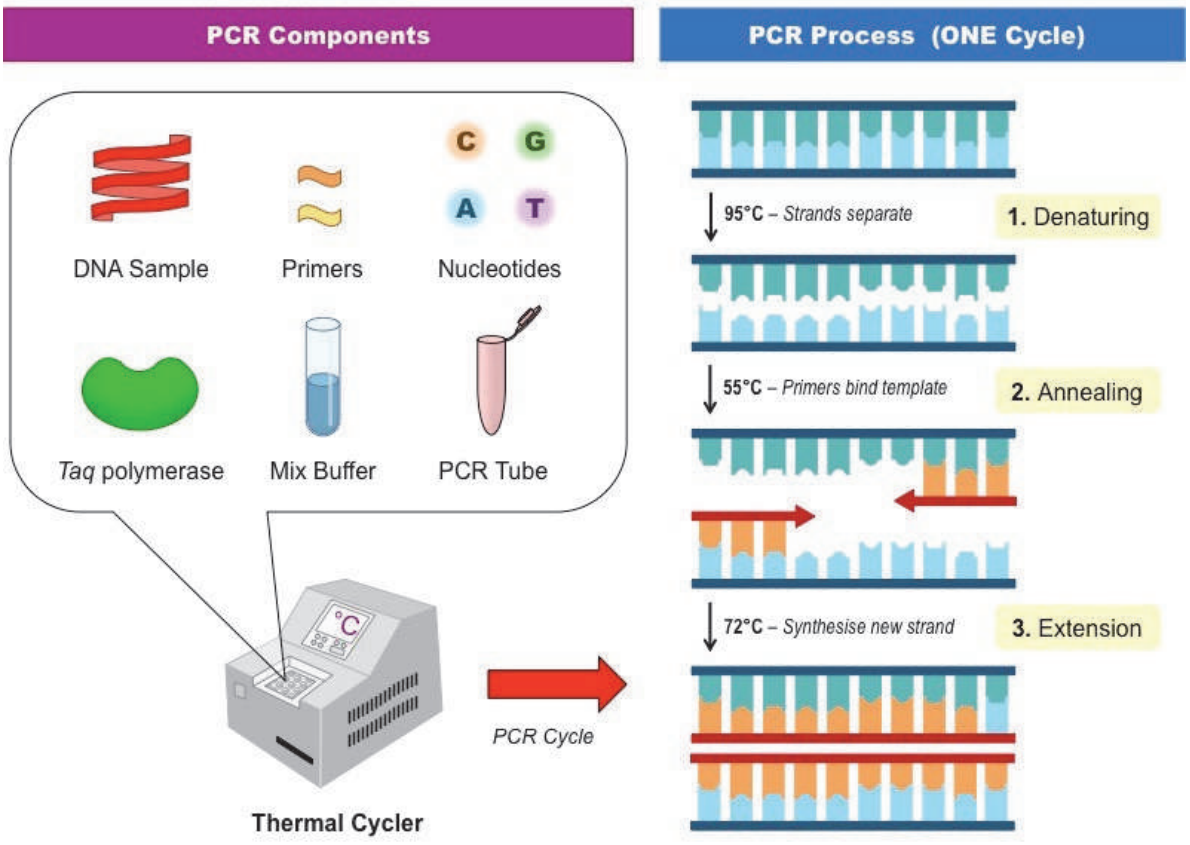


Fig.28.1. Principle of Polymerase Chain Reaction

4. Final elongation: This single step is occasionally performed at a temperature of 70-74°C for 5-15minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is completely extended.

5. Final hold: This step at 4-15°C for an indefinite time may be employed for short-term storage of the reaction.

b. Requirements

- **DNA template:** That contains the DNA region (target) to be amplified.
- **Two primers (Forward primer & Backward primer):** Which are complementary to the DNAregions at the 5' (five prime) or 3' (three prime).
- A **thermostable DNA polymerase**: Such as Taq polymerase.
- **Deoxy nucleoside triphosphates (dNTPs):** The building blocks from which the DNA polymerasessynthesize a new DNA strand.
- **Buffer solution:** Providing a suitable chemical environment for optimum activity and stability ofthe DNA polymerase.
- **Divalent cations:** Magnesium or manganese ions; generally, Mg2+ is used.
- **Nuclease free water**

c. Procedure

1. The DNA molecule carrying a target sequence is denatured by heat at 90-95ºC for 20 seconds. The two strands separate due to the breakdown of the hydrogen bonds. Oligonucleotide primers are added.
2. A reaction mixture containing all four deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP) and a thermostable DNA polymerase is added. A DNA polymerase (Taq) is used that is not denatured by the high temperature. It is usually sourced from *Thermus aquaticus*, a bacterium isolated from hot springs.
3. The mixture is allowed to cool to a lower temperature (50-65ºC). Each DNA strand is annealed with an oligonucleotide primer complementary to either end of the target sequence. Primer annealing takes 20 seconds.
4. The temperature is increased to 60-75ºC and primers are extended by the action of DNA polymerase for 30 seconds. The polymerase synthesizes complementary sequence the 5' to 3' direction away from each of the primers. If the template contains an A nucleotide, the enzyme adds on a T nucleotide to the primer. If the template contains a G, it adds a C to the new chain. Polymerization continues until each newly synthesized strand has proceeded far enough to contain the site recognized by the other primer. At this point there would be exactly two copies of the target DNA sequence.
5. The mixture is heated again at 90-95ºC to denature the molecules and separate the strands and the cycle repeated. Each new strand then acts as a template for the next cycle of synthesis. Thus, amplification proceeds at an exponential rate (amount of DNA produced doubles at each cycle) The amplified product at the end of PCR is called amplicon.

6.25. Blotting technique

Blotting is a method used to immobilize nucleic acids or proteins onto a solid support generally nylon or nitrocellulose membranes. Blotting of nucleic acid is the central technique for hybridization studies. Nucleic acid labeling and hybridization on membranes have established the basis for a range of experimental techniques involving understanding of gene expression, organization, etc. Blotting techniques are used to identify unique proteins and nucleic acid sequences. They have been developed to be highly specific and sensitive and have become significant instruments in both molecular biology and clinical research.

a. Principle

The method of blotting is rather simple and generally consist of four different separate steps: electrophoretic separation of protein or nucleic acid fragments in the sample; transfer to and immobilization on paper support; binding of analytical probe to target molecule on paper; and

visualization of bound probe. In a sample, molecules are first separated by electrophoresis and then transferred on to an easily handled support medium or membrane. This immobilizes the protein or DNA fragments, provides a faithful replica of the original separation, and facilitates subsequent biochemical analysis. After being transferred to the support medium the immobilized protein or nucleic acid fragment is localized by the use of probes, such as antibodies or DNA, that specifically bind to the molecule of interest. Finally, the position of the probe that is bound to the immobilized target molecule is visualized usually by autoradiography. Three major blotting techniques have been developed and are commonly known as Southern, Northern and Western blotting.

6.25.1. Southern blot

Southern blot is a method used to define the presence of a DNA sequence in a DNA sample. Themethod is named after its inventor, the British biologist Edwin Southern.

a. Procedure

- Restriction endonucleases are used to cut high molecular weight DNA strands into smaller fragments, which are then separated by electrophoresis on an agarose gel according to their size.
- If the DNA fragments are larger than 15 kb, then prior to blotting, the gel may be treated with an acid, such as dilute HCl, which depurinates the DNA fragments, breaking the DNA into smaller pieces, thus allowing more effective transfer from the gel to membrane.
- If alkaline transfer methods are used, the DNA gel is placed into an alkaline solution (containing NaOH) to denature the double-stranded DNA. The denaturation in an alkaline environment may improve binding of the negatively charged DNA to a positively charged membrane, separating it into single DNA strands for further hybridization to the probe and destroys any residual RNA that may still remaining in the DNA.
- A sheet of nitrocellulose (or nylon) membrane is placed on top of (or below, depending on the direction of the transfer) the gel. Pressure is applied evenly to the gel (either applying suction, or by placing a stack of paper towels and a weight on top of the membrane and gel), to maintain good and even contact between gel and membrane. Buffer transfer by capillary action from a region of high-water potential to a region of low water potential (usually filter paper and paper tissues) is used to move the DNA from the gel on to the membrane; ion exchange interactions bind the DNA to the membrane due to the negative charge of the DNA and positive charge of the membrane.
- The membrane should next be baked in a vacuum or standard oven at 80°C for 2

hours or exposed to ultraviolet radiation (nylon membrane) to permanently bind the transferred DNA to the membrane. The membrane is then exposed to a hybridization probe a single DNA fragment with a specific sequence whose presence in the target DNA is to be determined. The probe DNA is labeled so that it can be detected, generally by incorporating radioactivity or tagging themolecule with a fluorescent or chromogenic dye.

- After hybridization, excess probe is washed from the membrane and the pattern of hybridization is visualized on x-ray film by autoradiography in the case of a radioactive or fluorescent probe or by development of color on the membrane if a chromogenic detection technique is used.

Hybridization of the probe to a specific DNA fragment on the filter membrane indicates that this fragment contains DNA sequence that is complementary to the probe. The transfer step of the DNA from the electrophoresis gel to a membrane permits easy binding of the labeled hybridization probe to the size-fractionated DNA. Southern blots performed with restriction enzyme-digested genomic DNA may be used to determine the number of sequences (e.g., gene copies) in a genome. A probe that hybridizes only to a single DNA segment that has not been cut by the restriction enzyme will produce a single band on a Southern blot, whereas multiple bands will likely be observed when the probe hybridizes to several highly similar sequences (e.g., those that may be the result of sequence duplication). Modification of the hybridization conditions (i.e., increasing the hybridization temperature or decreasing salt concentration) may be used to increase specificity and decreasehybridization of the probe to sequences that are less than 100% similar.

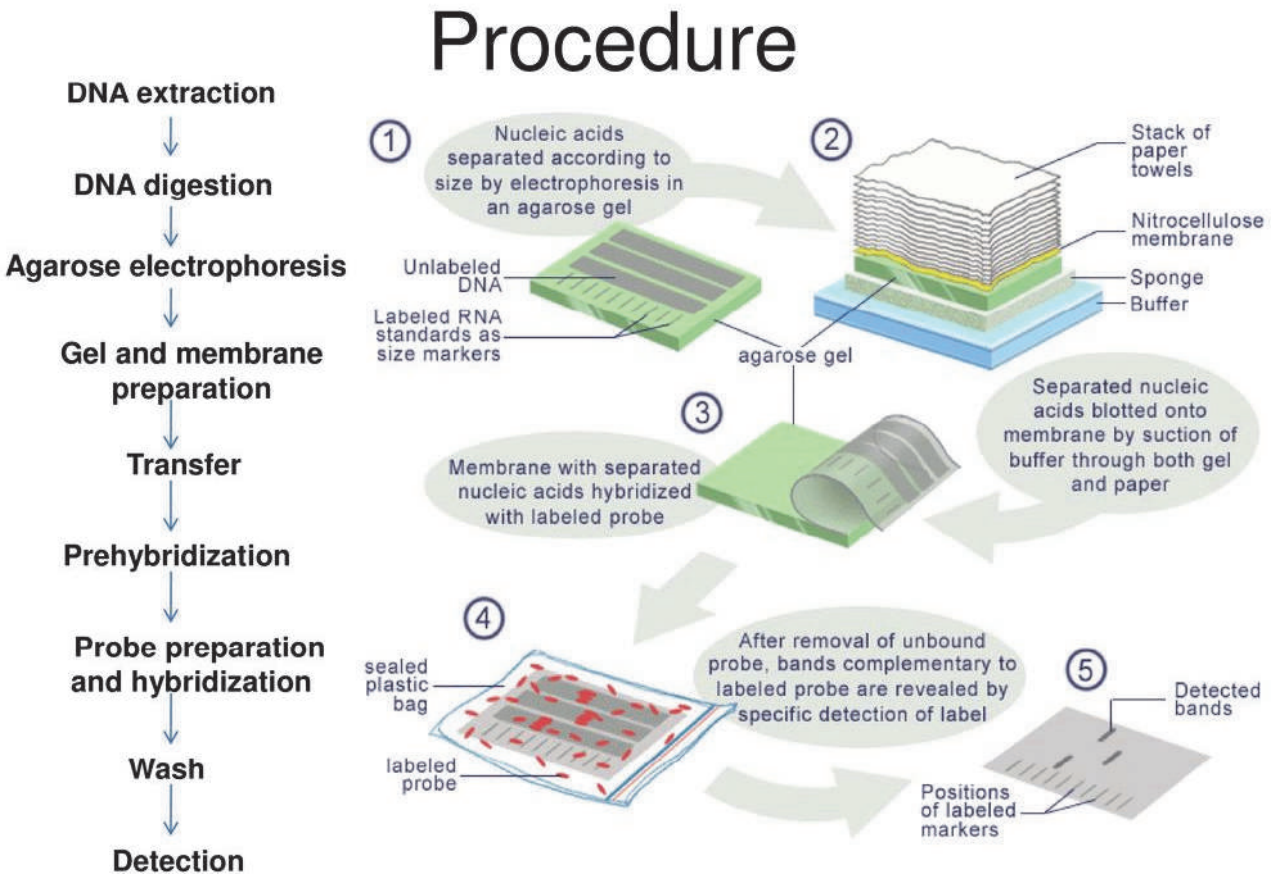


Fig. 29. Southern Blot

6.25.2. Northern blot

The Northern blotting method is used to evaluate the gene expression by detecting RNA (or isolated mRNA) in a sample. With Northern blotting it is possible to observe cellular control over structure and function by determining the particular gene expression levels during differentiation, morphogenesis, as well as abnormal or diseased conditions. This technique was developed in 1977 by James Alwine, David Kemp and George Stark at Stanford University. Northern blotting takes its name from its similarity to the first blotting technique, the Southern blot. The main difference is that instead of DNA RNA is analyzed in the Northern blot.

a. Procedure

The blotting procedure starts with extraction of total RNA from a homogenized tissue sample. The mRNA can then be isolated through the use of oligo (DT) cellulose chromatography to maintain only those RNAs with a poly (A) tail. RNA samples are then separated by gel electrophoresis. A nylon membrane with a positive charge is the most effective for use in Northern blotting since the negatively charged nucleic acids have a high affinity for them. The transfer buffer used for the blotting usually contains formamide because it lowers the annealing temperature of the probe-RNA interaction preventing RNA degradation by high temperatures.

Once the RNA has been transferred to the membrane it is immobilized through covalent linkage to the membrane by UV light or heat. After a probe has been labeled, it is hybridized to the RNA on the membrane. The membrane is washed to ensure that the probe has bound specifically. The hybrid signals are then detected by x-ray film and can be quantified by densitometry.

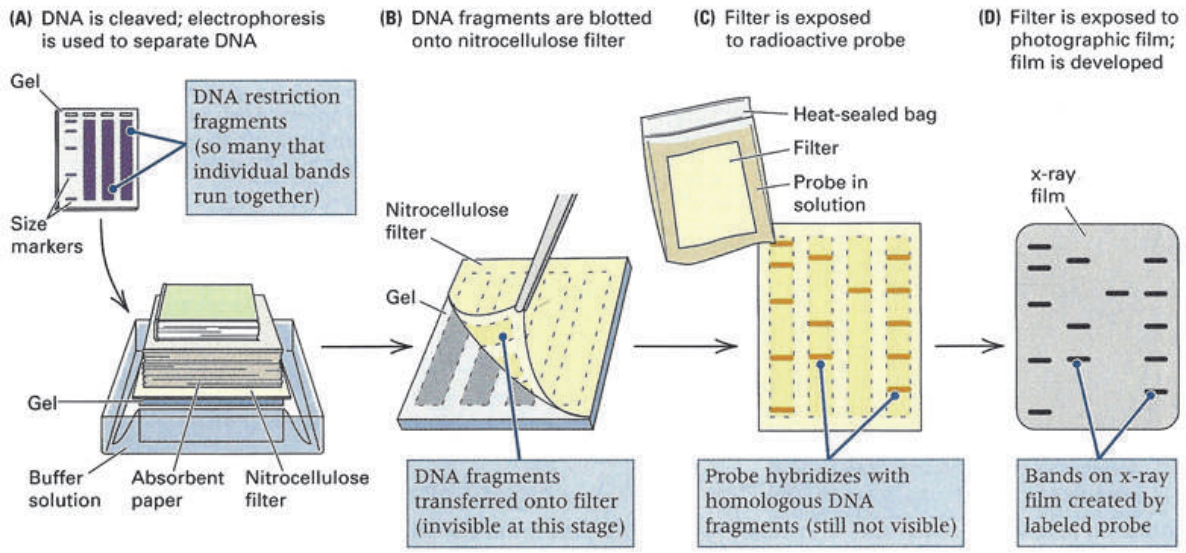


Fig.30. Northern Blot

6.25.3. Western blot

The Western blot (known also as immunoblot) is used to detect specific proteins in a given sample of tissue homogenate or extract. The method originated from the laboratory of George Stark at Stanford. The name Western blot was given to the method by W. Neal Burnette.

a. Procedure

The first step in a Western blot is to prepare the protein sample by mixing it with a detergent called sodium dodecyl sulfate, which makes the proteins unfold into linear chains and coats them with a negative charge. Further, the proteins are separated according to their sizes by gel electrophoresis. Following separation, the proteins are transferred from the gel onto a blotting membrane. Although this step is what gives the technique the name "Western blotting," the term is typically used to describe the entire procedure. Once the transfer is complete, the membrane carries all of the protein bands originally on the gel. Next, the membrane goes through a treatment called blocking, which prevents any nonspecific reactions to occur. The membrane is then incubated with an antibody called the primary antibody, which specifically binds to the protein of interest. After incubation, any unbound primary antibody is washed away, and the membrane is incubated yet again, but this time with a secondary antibody that specifically recognizes and binds to the primary antibody. The secondary antibody is linked to a reporter enzyme that generates color or

light, which allows it to be easily detected and imaged. These steps allow a specific protein to be detected from among a mixture of proteins.

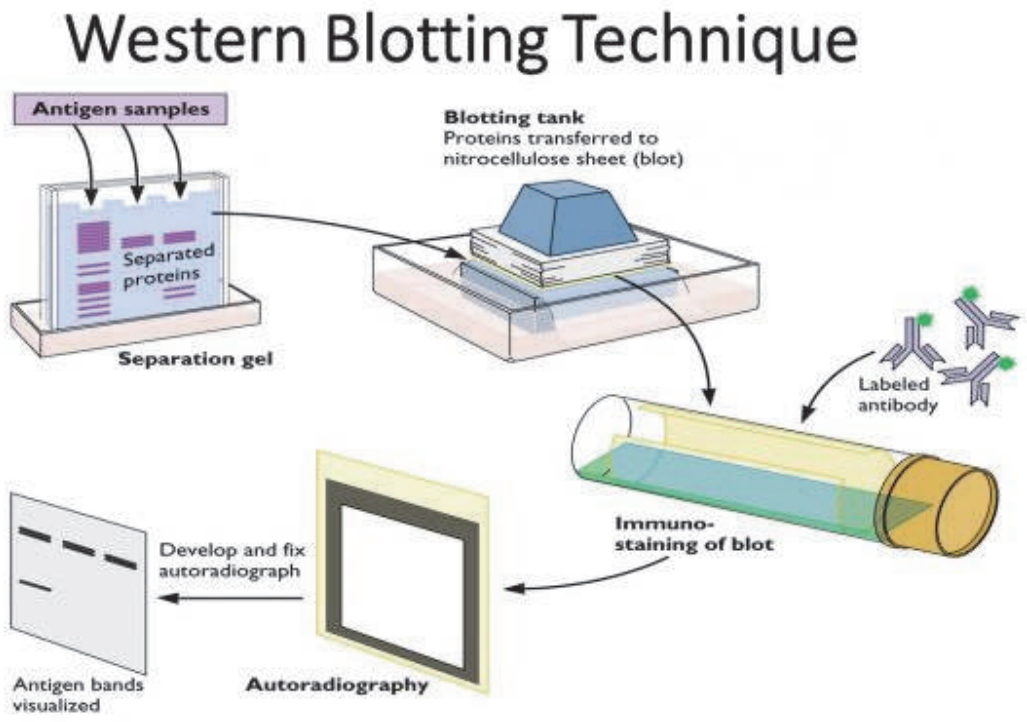


Fig. 31. Western blot

6.26. Electrophoresis

The term electrophoresis means Electro= electric field + Phoresies= migration. So as the name indicates, electrophoresis is a technique for separating charged molecules in an electric field. The charged molecules under the influence of electric field migrate towards oppositely charged electrodes. The molecules with positive charge travel towards cathode, while negatively charged molecules travel towards anode. The migration is due to charge on the molecules and potential applied across the electrodes.

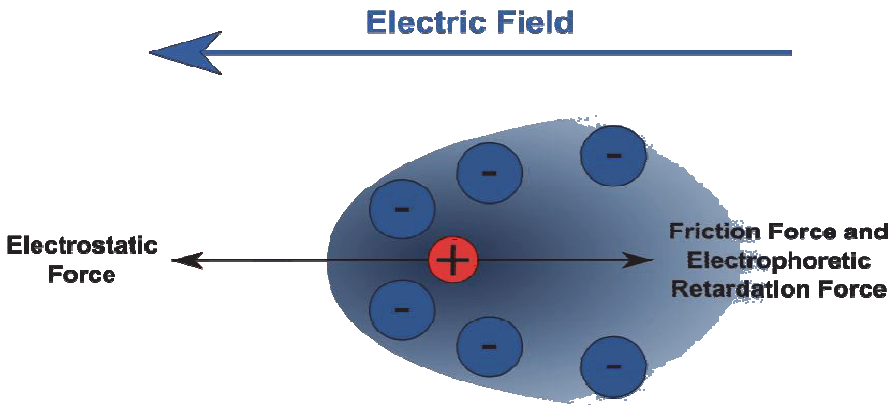


Fig. 32. Movement of charged particles in an electric field

a. Principle

The charged molecule moves to their counter charge electrodes but electric field is removed before it reaches the electrode. Movement of the charged species in an electric field gives differential mobility to the sample based on the charge and consequently resolves them. Movement of the charged particle is retarded with the addition a polymeric gel so that a sufficient time is available for resolving the sample. The polymeric gel is inert, uncharged and does not cause retardation by binding the molecule. Instead, it, forms pores of different size (depending on the concentration of polymer) and sample pass through this pore and therefore their electrophoretic mobility is decreased.

b. Electrophoretic techniques

Different types of electrophoresis techniques are designed depending upon whether it carried out in the presence or absence of a supporting media.

6.26.1. Moving boundary electrophoresis

In this method, the electrophoresis is carried in solution, without a supporting media. The sample is dissolved the buffer and molecules move to their respective counter charge electrodes. Moving boundary electrophoresis is carried out in a U shape tube with platinum electrodes attached to the end of both arms. At the respective ends, tube has refractometer to measure the change in refractive index of the buffer during electrophoresis due to presence of molecule. Sample is loaded in the middle of the U tube and then the apparatus is connected to the external power supply. Charged molecule moves to the opposite electrode as they pass through the refractometer, a change can be measured. As the desirable molecule passes, sample can be taken out from the apparatus along with the buffer.

6.26.2. Zone electrophoresis

In this technique, an inert polymeric supporting media is utilized between the electrodes to separate and examine the sample. The supporting media used in zone electrophoresis are absorbent paper, gel of starch, agar and polyacrylamide. The presence of supporting media limits mixing of the sample and that makes the analysis and purification of the molecule from the gel much easier than the moving boundary electrophoresis. This is of two types:

6.26.2.1. Paper electrophoresis

Is a technique which uses a Whatman filter paper No.1 which is moistened by a buffer and thenconnected at two ends to two opposite charged electrodes.

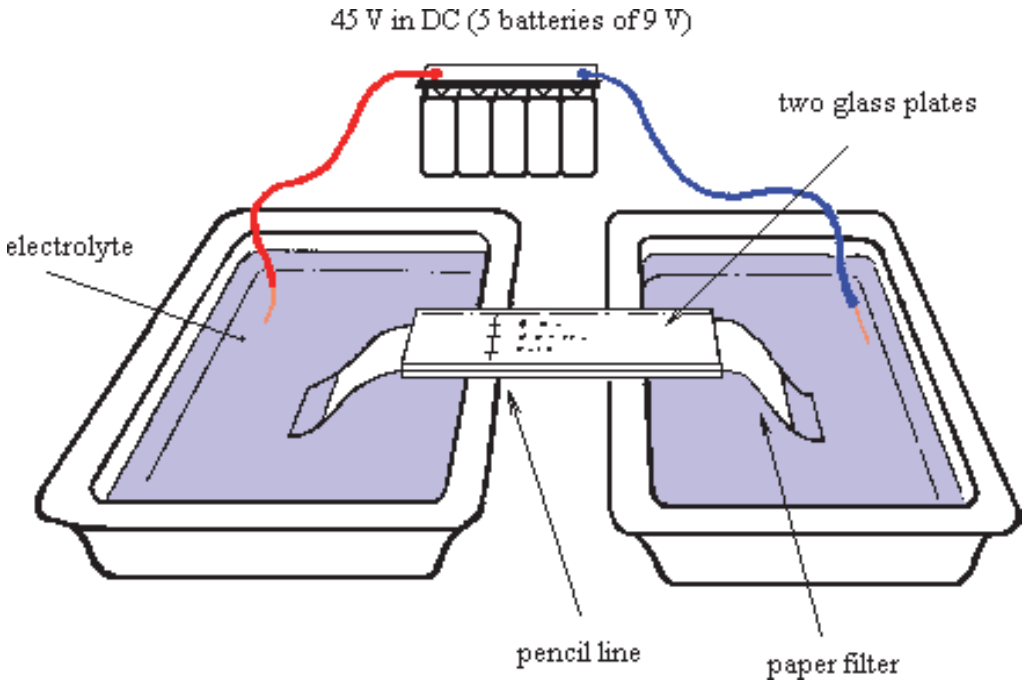


Fig.33. Paper electrophoresis

Then sample is applied on to one end and let for separation of components under electric field. Afterseparation, the paper is dried and stained to get colored bands.

6.26.2.2. Gel electrophoresis

6.26.2.2.1. Vertical Gel Electrophoresis

The electrophoresis in this system performed in a discontinuous way with buffer in the upper and lower tank connected by the gel slab.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

PAGE: Polyacrylamide gel electrophoresis (PAGE) is probably the most common analytical technique used to separate and characterize proteins. A solution of acryl amide and bis-acryl amide is polymerized. Acrylamide alone forms linear polymers. The bis-acrylamide introduces cross links between polyacrylamide chains. The 'pore size' is determined by the ratio of acrylamide to bis-acrylamide, and by the concentration of acryl amide. A high ratio of bis-acryl amide to acrylamide and a high acrylamide concentration causes low electrophoretic mobility. Polymerization of acrylamide and bis-acrylamide monomers is induced by ammonium per sulphate (APS), which spontaneously decomposes to form free radicals. TEMED (tetramethyl ethylenediamine), a free radical stabilizer, is generally included to promote polymerization.

SDS PAGE: Sodium dodecyl sulfate (SDS) is an amphipathic detergent. It has an anionic head group and a lipophilic tail. It binds non-covalently to proteins, with a stoichiometry of approximately

one SDS molecule per two amino acids. SDS leads to protein to denaturation and disassociate from each other (excluding covalent cross-linking). It also confers a negative charge. In the presence of SDS, the intrinsic charge of a protein is masked. During SDS PAGE, all proteins migrate toward the anode (the positively charged electrode). SDS-treated proteins have very similar charge-to-mass ratios, and similar shapes. During PAGE, the rate of migration of SDS-treated proteins is effectively determined by molecular weight.

a. Instrument

It contains two buffer chambers, upper chamber and a lower chamber. Both chambers are equipped with the platinum electrodes connected to the external power supply from a power pack which supplies a direct current or DC (Direct current) voltage. The upper and lower tank loaded with the running buffer is connected by the electrophoresis gel casted in between two glass plates (rectangular and notched). There are additional accessories required for casting the polyacrylamide gel such as comb (to prepare different well), spacer, gel caster etc.

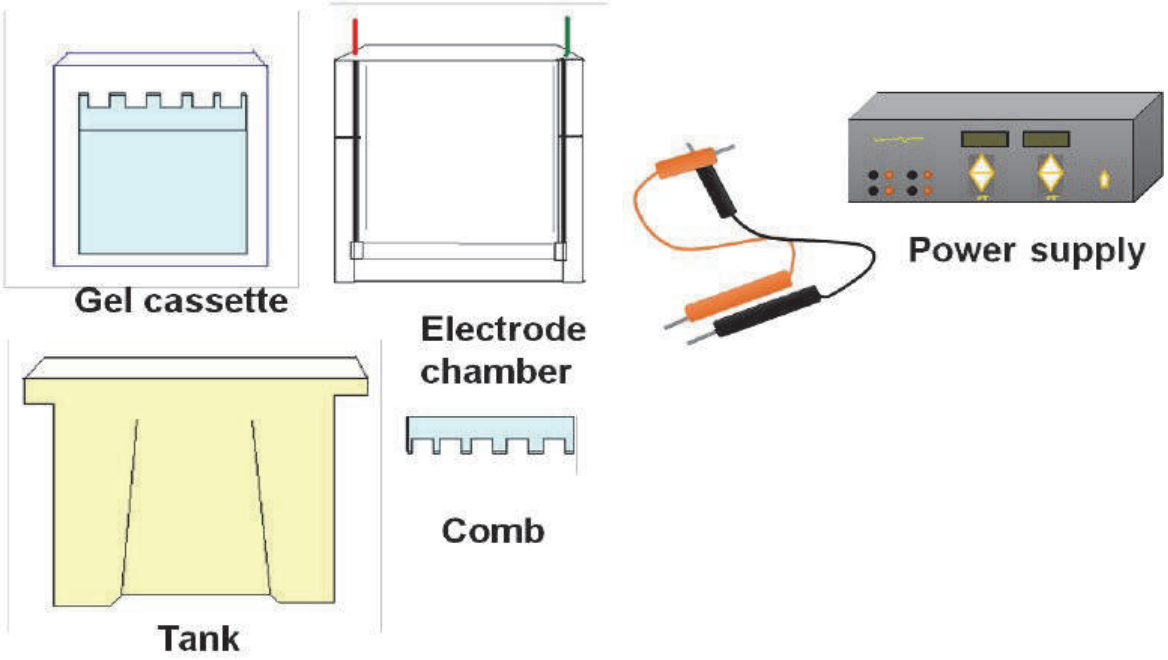


Fig.34. SDS-PAGE Instrument

b. Buffer and reagent for electrophoresis

- **N, N, N', N'-tetramethyl ethylenediamine (TEMED) -** catalyzes the acrylamide polymerization.
- **Ammonium persulphate (APS)-** initiator for the acrylamide polymerization.
- **Tris-HCl-** constituent of running and gel casting buffer.
- **Glycine-** component of running buffer.

- **Bromophenol blue**- tracking dye to monitor the progress of gel electrophoresis.
- **Coomassie brilliant blue R250-** it is used to stain the Polyacrylamide gel.
- **Sodium dodecyl sulphate-** used to denature and provide negative charge to the protein.
- **Acryl amide (a potent neurotoxin)-**monomeric unit used to prepare the gel.
- **Bis-acrylamide-** cross linker for polymerization of acrylamide monomer to form gel.

c. Casting of the gel

The acrylamide solution (a combination of monomeric acrylamide and a bifunctional cross linker bis-acrylamide) is mixed with the TEMED and APS and poured instantly in between the glass plate fitted into the gel caster. Ammonium persulphate in the presence of TEMED forms oxygen free radicals and induces the polymerization of acryl amide monomer to form a linear polymer. These linear monomers are interconnected by the cross linking with bis-acrylamide monomer to form a 3-D mesh with pores. The size of pore is regulated by the concentration of acryl amide and amount of bis-acrylamide in the gel.

In a vertical gel electrophoresis system, we cast two types of gels: stacking and resolving. First the resolving gel solution is made and poured into the gel cassette for polymerization. A thin layer of organic solvent (such as butanol or isopropanol) is layered to stop the entry of oxygen (oxygen neutralizes the free radical and slow down the polymerization) and make the top layer smooth. After polymerization of the resolving gel, a stacking gel is poured and comb is fitted into the gel for construction of different lanes for the samples.

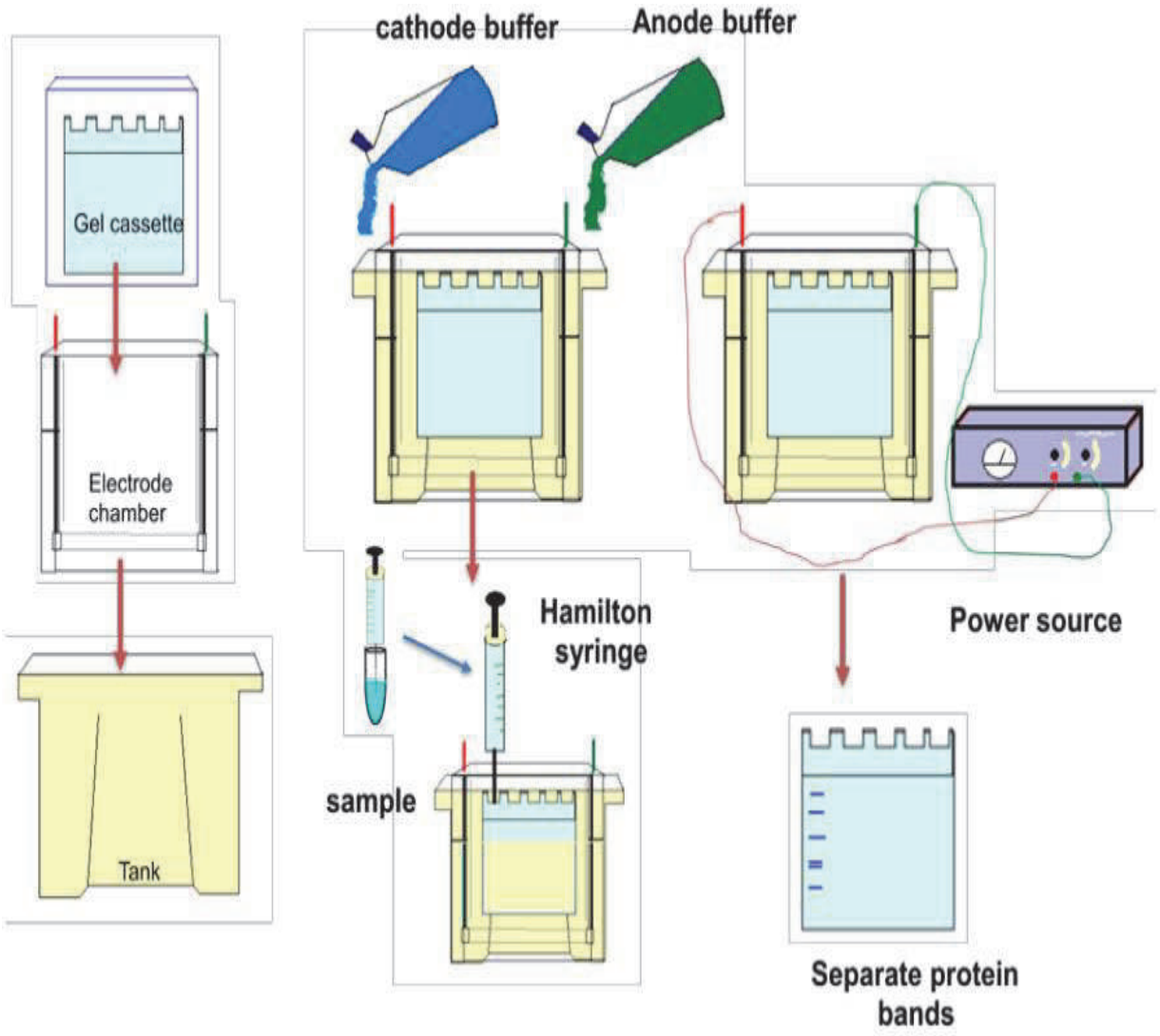


Fig.34.1 Casting of the Gel

d. Running of the gel

The sample is prepared in the loading dye containing SDS, β-mercaptoethanol in glycerol for denaturation of the sample. Glycerol enables loading of the sample in the well. As the samples are filled vertically there is a distance drift occurred between the molecules at the top and at the bottom in a lane. This problem is taken care once the sample runs through the stacking gel. The pH of the stacking gel is 6.8. At this pH, glycine is moving slowly in the front whereas Tris-HCl is moving fast. As a result, the sample gets sandwiched between glycine-Tris and get stacked in the form of thin band. As the sample enters into the resolving gel with a pH 8.8, the glycine is now charged, it moves fast and now sample runs as per their molecular weight (due to SDS they have equal negative charge). After tracking dye reaches to the bottom of the gel. The latter is taken out from the glass plate with the help of a spatula and it is stained with Coomassie brilliant blue R 250 dye. The dye stains protein present on the gel.

Fig.34.2. Running of the Gel

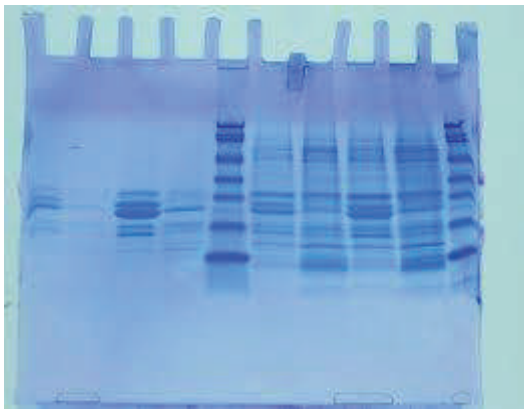


Fig.34.3. Stained Gel

6.26.2.2.2. Horizontal Gel Electrophoresis

The electrophoresis in this system is accomplished in a continuous way and the in the horizontal direction. The electrophoresis in this gel system is performed with both electrodes and gel cassette submersed within the buffer. The electrophoresis chamber has two platinum electrodes placed on the both ends are connected to the external power supply from a power pack which supplies a direct current or DC voltage. The tank loaded with the running buffer and the gel casted is submerged inside the buffer. There are additional accessories required for casting the agarose gel such as comb (to prepare different well), spacer, gel caster etc.

a. Buffer and reagent for electrophoresis

1. **Agarose-**polymeric sugar used to prepare horizontal gel for DNA analysis.
2. **Ethidium bromide (a potent carcinogen)-**for staining of the agarose gel for visualization

of the DNA.

3. **Sucrose-** For preparation of loading dye for horizontal gel.
4. **Tris-HCl-** The component of the running buffer.
5. **Bromophenol blue-**Tracking dye to monitor the progress of the electrophoresis.

b. Casting of the agarose gel

The agarose powder is dissolved in a TAE (TRIS-acetate-EDTA) or TBE (TRIS-borate-EDTA) buffer and heated to melt the agarose. Hot agarose is poured into the gel cassette and allowed it to set. A comb can be inserted into the hot agarose to cast the well for loading the sample. In few cases, we may add ethidium bromide within the gel so that it stains the DNA during electrophoresis.

c. Running and staining

The gel cassette is placed in the electrophoresis tank submerged completely and DNA loaded into the well with the help of pipet and run with a constant voltage. DNA runs from negative to positive end and ethidium bromide (EtBr) present in the gel stain the DNA. Observing the agarose gel in a UV-chamber shows the DNA stained with EtBr as orange colored fluorescence.

Fig.34.4. Running of the Gel

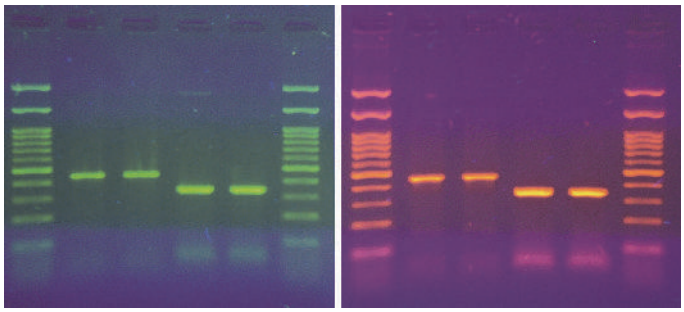


Fig.34.5. Visualization of the Gel

6.27. Chromatography

Chromatography is a physicochemical technique to separate complex mixtures.

a. Principle

Chromatography is a separation process that is achieved by distributing the components of a mixture between two phases, a stationary phase and a mobile phase. Those components held preferentially in the stationary phase are retained longer in the system than those that are distributed selectively in the mobile phase. As a result, solutes are eluted from the system as local concentrations in the mobile phase in the order of their elevating distribution coefficients with respect to the stationary phase: a separation is taking place.

6.27.1. Types of Chromatography

Based on the interaction of solute to stationary:

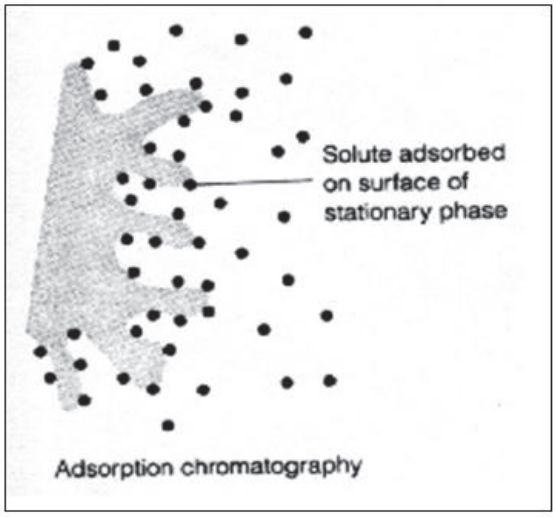


Fig.35. Adsorption Chromatography

6.27.1.1. Adsorption Chromatography

Adsorption chromatography utilizes a mobile liquid or gaseous phase that is adsorbed onto the surface of a stationary solid phase. The equilibration between the mobile and stationary phase accounts for the separation of different solutes. Here the molecules or components of the mixture travel with different rates due to differences in their affinity towards stationary phase. Adsorption means a physical attachment between the compound and the particles of stationary phase. Based on the nature, polar compounds adsorb with stronger or greater intensity to the polar stationary phase while non-polar compounds adsorb better to the non- polar stationary phase than polar components. Hence during separation of components, when we use a polar stationary phase, polar components elute out late due to greater adsorption and non-polar components get out of the column or elute out first. This is exactly reverse on using a non-polar stationary phase.

6.27.1.2. Partition Chromatography

This form of chromatography is based on a thin film formed on the surface of a solid support by a liquid stationary phase. Solute equilibrates between the mobile phase and the stationary liquid. Here the molecules get preferential separation in between two phases. i.e., both stationary phase and mobile phase are liquid in nature. Thus, molecules get dispersed into either phase preferentially.

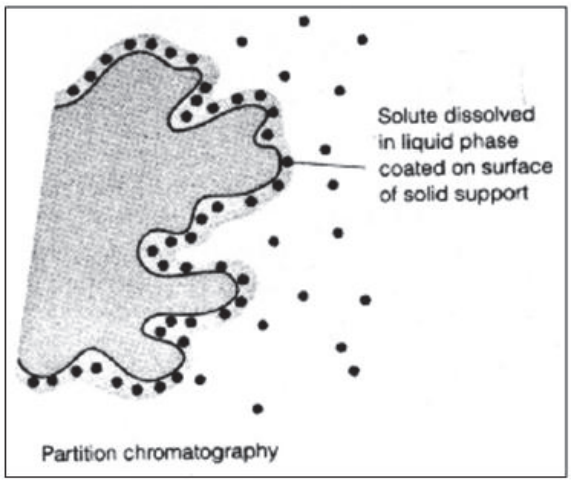


Fig.36. Partition Chromatography

Polar molecules get partitioned into polar phase and vice-verse. This type of partition chromatography applies to liquid-liquid, liquid-gas chromatography and not to solid-gas chromatography. Because partition is the phenomenon in between a liquid and liquid or liquid and gas or gas and gas. But not in solid involvement.

6.27.1.3. Ion Exchange Chromatography

In this type of chromatography, a resin (the stationary solid phase) is used to covalently link anions or cations onto it. Solute ions of the opposite charge in the mobile liquid phase are attracted to the resin by electrostatic forces. Ion exchange chromatography is mainly used to separate charged biological molecules as are proteins, peptides, amino acids or nucleotides. The amino acids that make proteins are zwitterionic compounds (contain both positively and negatively charged chemical groups). Depending on the pH of the environment, proteins may carry a net positive charge, a net negative charge, or no charge. The pH at which a molecule has no net charge is called its isoelectric point or PI. In a buffer with a pH greater than the PI of the protein of the interest, the protein will carry a net negative charge; therefore, a positively charged anion exchange resin is chosen to capture this protein.

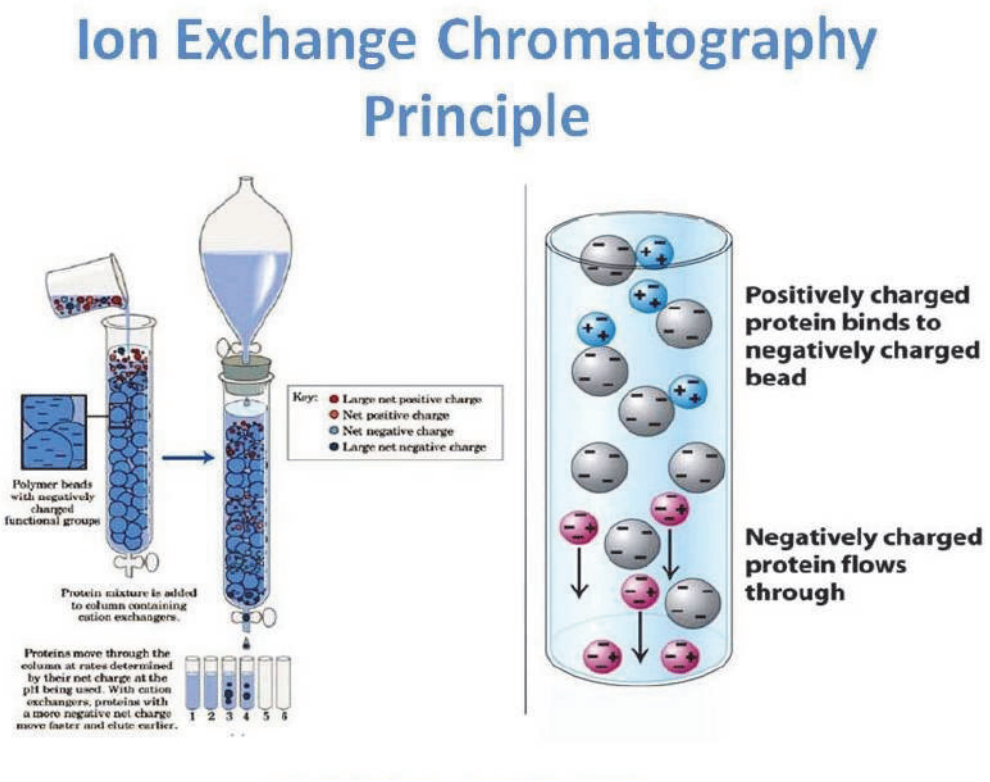


Fig.37. Ion Exchange Chromatography

In a buffer with a pH lower than the pI of the protein of interest, the protein will carry a net positivecharge; therefore, a negatively charged cation exchange resin is chosen.
When an ion exchange chromatography column is loaded with a sample at a particular pH, all proteinsthat are appropriately charged will bind to the resin.

6.27.1.4. Molecular Exclusion Chromatography

Also is called gel permeation or gel filtration chromatography. The liquid or gaseous phase passes through a porous gel which separates the molecules taking into account its size. The pores are typically small and eliminate the larger solute molecules, yet allow smaller molecules to penetrate the gel, causing them to flow through a larger volume. This causes the larger molecules to pass through the column faster than the smaller ones. Molecular exclusion chromatography separates molecules based on their size by filtration through gel. The gel consists of spherical beads containing pores of a specific size distribution. Separation occurs when molecules of different sizes are included or excluded from the pores within the matrix. Small molecules diffuse in to the pores and their flow through the column is retarded according to their size, while large molecules do not enter the pores and are eluted in the columns void volume. Thus, molecules are separated on the basis of their size as they pass through the column and are eluted in order of decreasingmolecular weight.

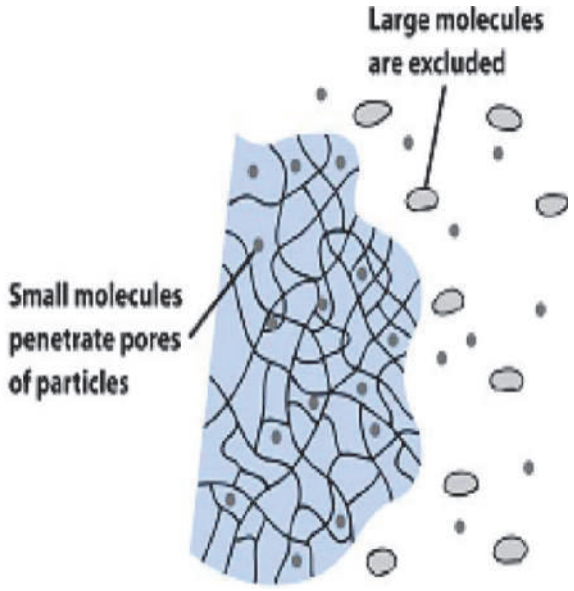


Fig.38. Molecular Exclusion Chromatography

6.27.2. On the basis of chromatographic bed

6.27.2.2. Column Chromatography

Column chromatography is a separation method in which the stationary bed is within a tube. The particles of the solid stationary phase or the support coated with a liquid stationary phase may fill the whole inside volume of the tube (packed column) or be concentrated on or along the inside tube wall leaving an open, unrestricted path for the mobile phase in the middle part of the tube (open tubular column). Differences in rates of movement through the medium are calculated to different retention times of the sample. In column chromatography, the stationary phase, a solid adsorbent, is placed in a vertical glass (usually) column. The mobile phase, a liquid, is added to the top and flows down through the column by either gravity or external pressure. Column chromatography is generally used as a purification technique: it isolates desired compound from a mixture. The mixture to be analyzed by column chromatography is placed inside the top of the column. The liquid solvent (or eluent) is passed through the column by the application of air pressure. An equilibrium is established between the solute adsorbed on the adsorbent and the eluting solvent flowing down through the column.

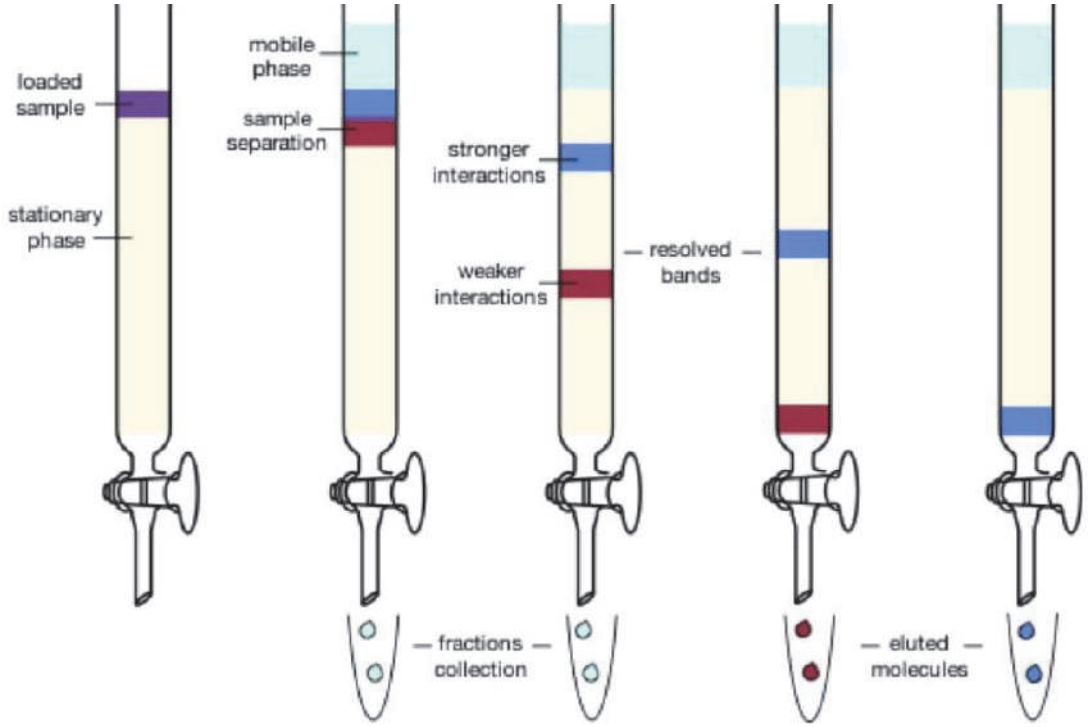


Fig.39. Column Chromatography

6.27.2.3. Planar Chromatography

Planar chromatography is a separation technique in which the stationary phase is present as or on a plane. The plane can be a paper, serving as such or impregnated by a substance as the stationary bed (paper chromatography) or a layer of solid particles spread on a support such as a glass plate (thin layer chromatography). Different compounds in the sample mixture travel different distances according to how strongly they interact with the stationary phase as compared to the mobile phase. The specific retention factor (R_f) of each chemical can be used to aid in the identification of an unknown substance.

6.27.2.4. Paper Chromatography

Paper chromatography is a technique that involves placing a small dot or line of sample solution onto a strip of chromatography paper. The paper is placed in a jar containing a shallow layer of solvent and sealed. As the solvent rises through the paper, it meets the sample mixture which starts to travel up the paper with the solvent. This paper is made of cellulose, a polar substance, and the compounds within the mixture travel farther if they are non-polar. More polar substances bind with the cellulose paper faster, and therefore do not travel as far.

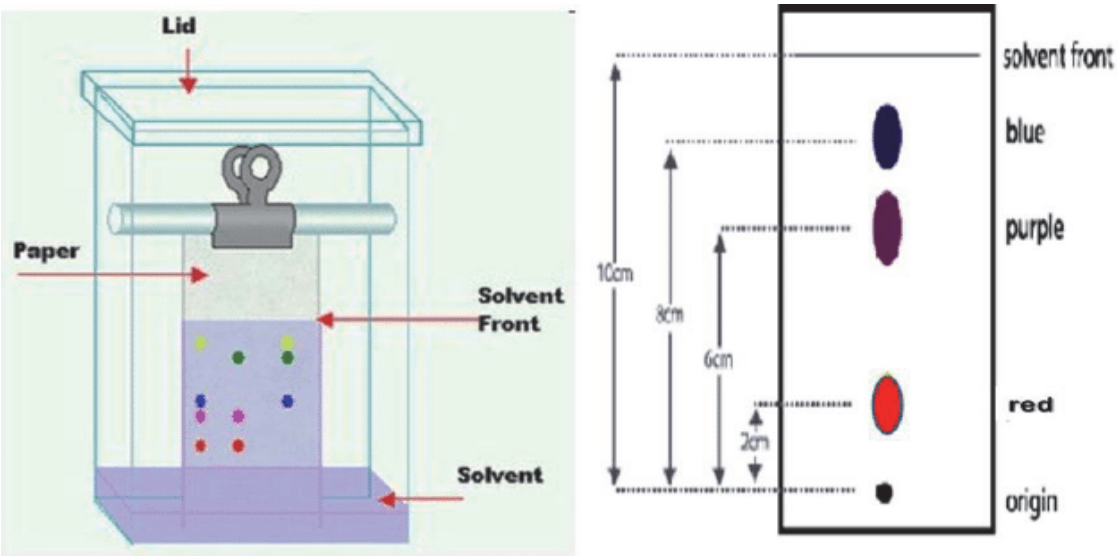


Fig.40. Paper Chromatography

6.27.2.5. Thin layer chromatography

Thin layer chromatography (TLC) is a widely employed laboratory technique and is similar to paper chromatography. However, instead of using a stationary phase of paper, it involves a stationary phase of a thin layer of adsorbent like silica gel, alumina, or cellulose on a flat, inert substrate. Compared to paper, it has the advantage of faster runs, better separations, and the choice between different adsorbents. For even better resolution and provide for quantification, high-performance TLC can be used.

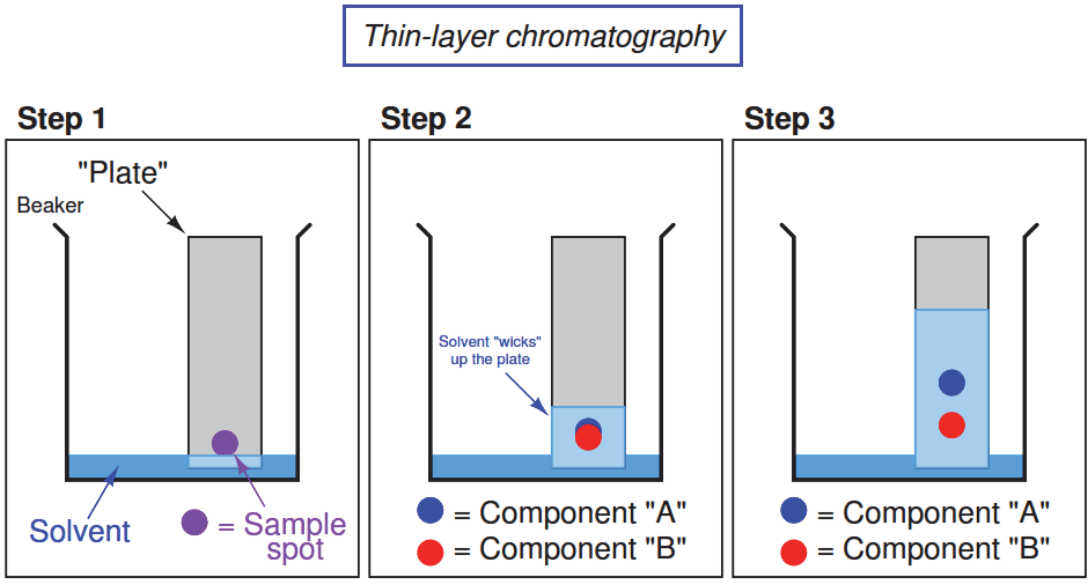
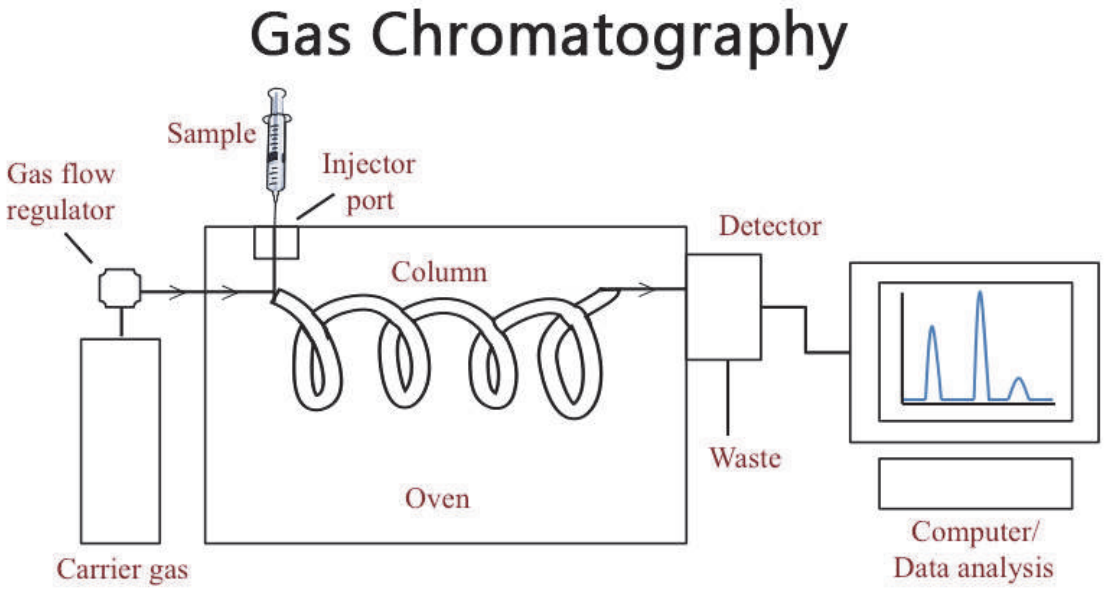


Fig.41.Thin Layer Chromatography

6.27.2.6. Gas Chromatography



a.

b.

Fig.42. Gas Chromatography (a, principle and b, equipment)

Gas chromatography (GC) is a separation technique in which the mobile phase is a gas. Gas chromatography is always carried out in a column, which is typically “packed” or “capillary”. GC is based on a partition equilibrium of analyte between a solid stationary phase (often a liquid silicone-based material) and a mobile gas (most often helium). The stationary phase is adhered to the inside of a small-diameter glass tube (a capillary column) or a solid matrix inside a larger metal tube (a packed column). It is widely used in analytical chemistry. Although the high temperatures used in GC make it unsuitable for high molecular weight biopolymers or proteins (heat leads to denaturation), frequently encountered in biochemistry, it is well suited for use in the

petrochemical, environmental monitoring, and industrial chemical fields. It is also widely used in chemistry research.

6.27.2.7. Liquid Chromatography

Liquid chromatography (LC) is a separation technique where the mobile phase is a liquid. Liquid chromatography can be carried out either in a column or a plane. Present day liquid chromatography that generally utilizes very small packing particles and a relatively high pressure is referred as high-performance liquid chromatography (HPLC).

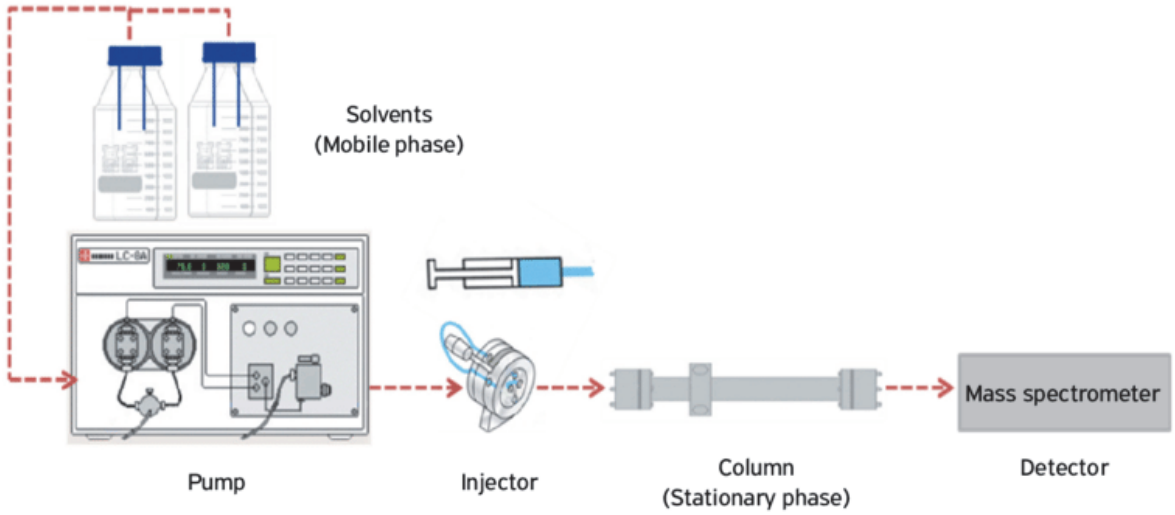
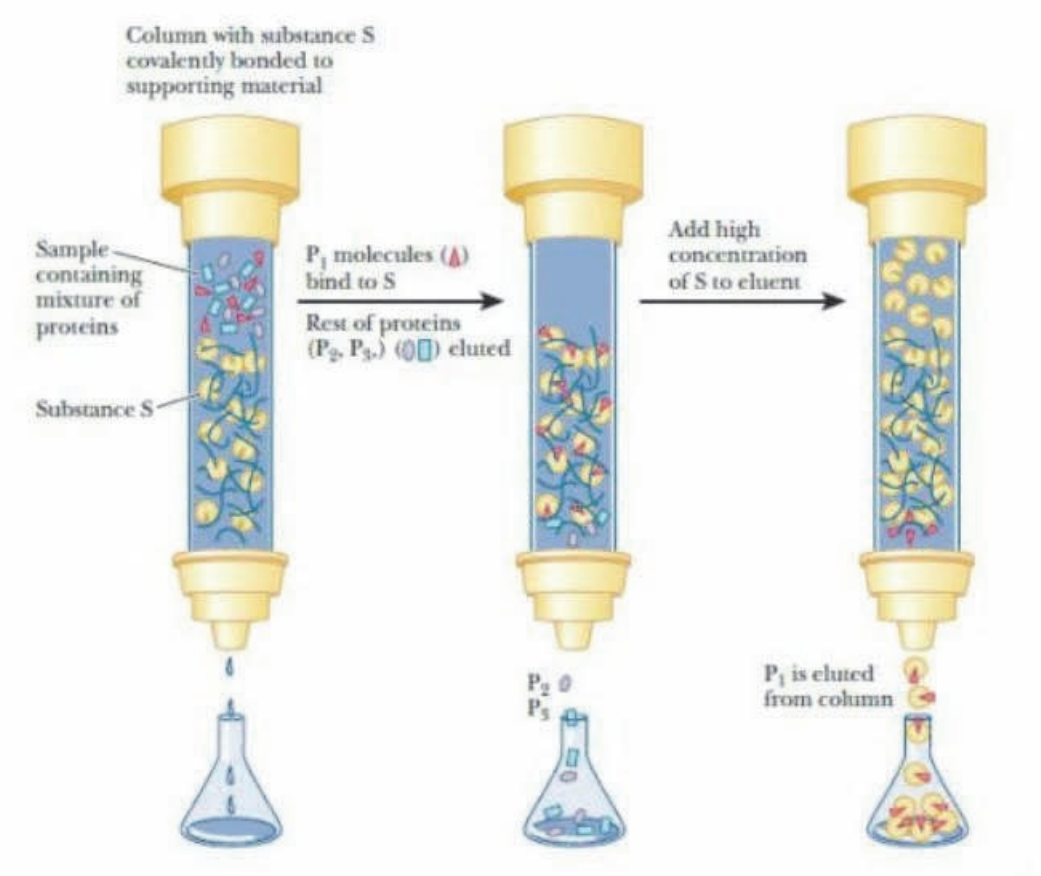


Fig.43. Liquid Chromatography

6.27.2.5.1. Affinity Chromatography

Affinity chromatography is based on selective non-covalent interaction between an analyte and specific molecules. Examples include antibody/antigen and enzyme/substrate interactions. It is very specific, but not very robust. It is often used in biochemistry in the purification of proteins bound to tags. These fusion proteins are labeled with compounds such as His-tags (polyhistidine-tag), biotin or antigens, which bind to the stationary phase specifically. After purification, some of these tags are usually removed and the pure protein is obtained. Affinity chromatography often utilizes a biomolecules affinity for a metal (Zn, Cu, Fe, etc.).



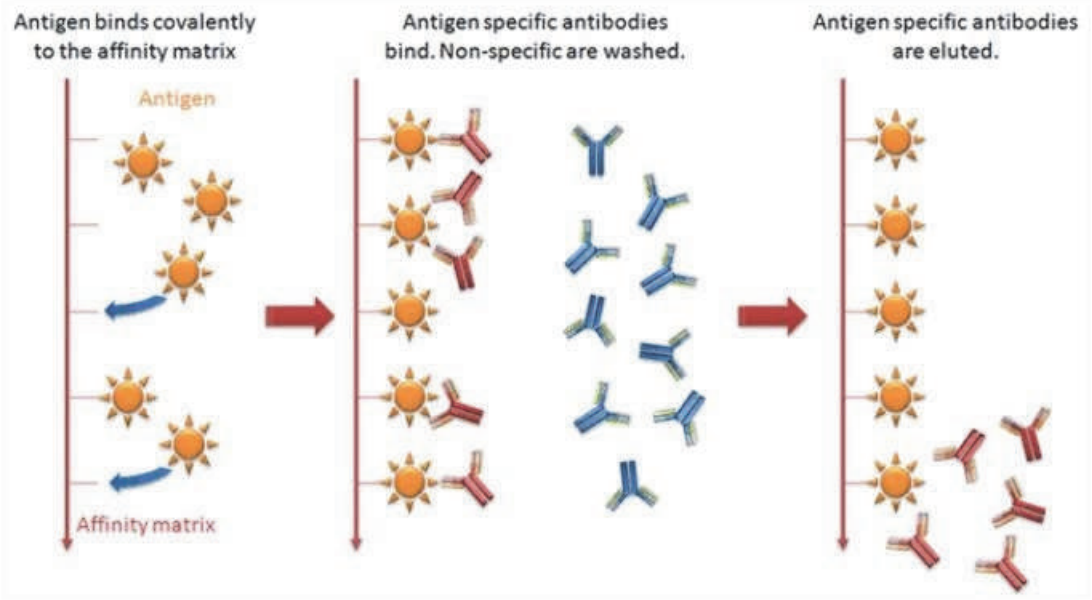


Fig.44. Affinity Chromatography

6.27.2.5.2. High Performance Liquid Chromatography (HPLC)

Fig.45. HPLC technique

HPLC instruments could develop up to 6000 psi of pressure and incorporated improved injectors, detectors and columns. With continued advances in performance during this time smaller particles, (even higher pressure) the acronym HPLC remains the same, but the name was changed to high performance liquid chromatography. HPLC is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantitate the compounds that are present in any sample that can be dissolved in a liquid.

a. Instrumentation

1. A **reservoir** (solvent delivery) holds the solvent (mobile phase).
2. A **high-pressure pump** solvent manager is used to generate and meter a specified flow rate of mobile phase, typically milliliters per minute.
3. An **injector** (sample manager or auto sampler) is able to introduce (inject) the sample into the continuously flowing mobile phase stream that carries the sample into the HPLC column.
4. The **column** contains the chromatographic packing material needed to influence the separation. This packing material is called the stationary phase because it is held in place by

the column hardware.

5. A **detector** is needed to see the separated compound bands as they elute from the HPLC column (most compounds have no color, so we cannot see them with our eyes). The mobile phase exits the detector and can be sent to waste, or collected, as desired.
6. When the **mobile phase** contains a separated compound band, HPLC provides the ability to collect this fraction of the eluate containing that purified compound for further study. This is known as preparative chromatography.
7. The **high-pressure tubing and fittings** are used to interconnect the pump, injector, column, and detector components to form the conduit for the mobile phase, sample, and separated compound bands.
8. The detector is wired to the **computer data** station, the HPLC system component that records the electrical signal needed to generate the chromatogram on its display and to identify and quantitate the concentration of the sample constituents.

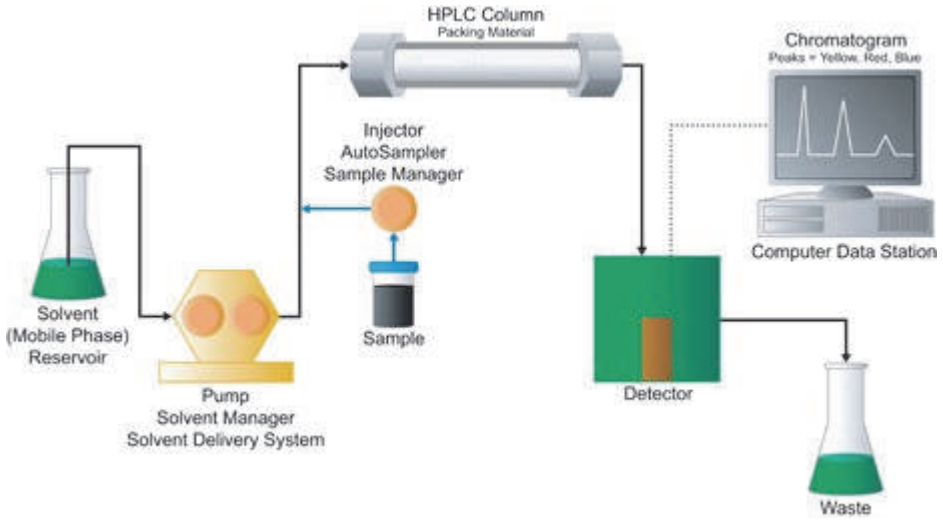


Fig.46. HPLC Instrumentation

Since sample compound properties can be very different, several types of detectors have been developed. For example, if a compound can absorb ultraviolet light, a UV-absorbance detector is used. If the compound fluoresces, a fluorescence detector is used. If the compound does not have either of these characteristics, a more universal type of detector is used, such as an evaporative-light-scattering detector (ELSD). The most powerful approach is the use multiple detectors in series. For example, a UV and/or ELSD detector may be used in combination with a mass spectrometer (MS) to analyze the results of the chromatographic separation. This provides, from a single injection, more comprehensive information about an analyte. The practice of coupling a mass spectrometer to an HPLC system is called LC/MS.

6.28. 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.

a. 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).

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

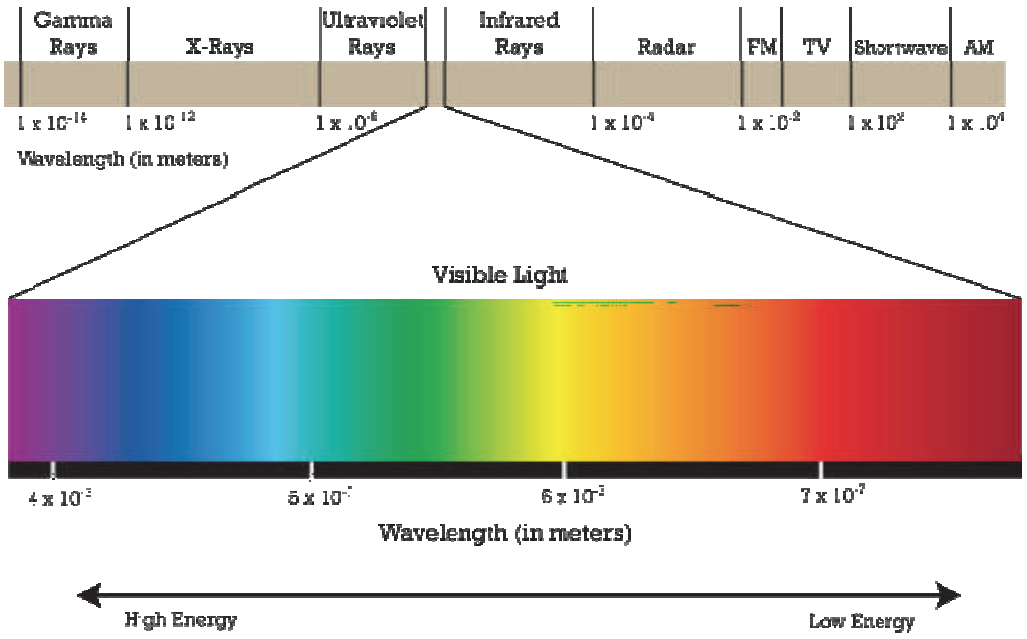


Fig.47. Electro Magnetic Spectrum

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 absorption spectra and can be defined by a wavelength of maximum absorption. 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.)

6.28.1. Colorimeter

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 of concentration of color that develops upon introducing a specific reagent into a solution.

Fig.48. Colorimeter

a. 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 colored filters or specific LEDs to generate color. 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.

b. 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 Beer's law when monochromatic light passes through the colored solution, the amount of light transmitted decreases exponentially with increase in concentration of the colored substance.

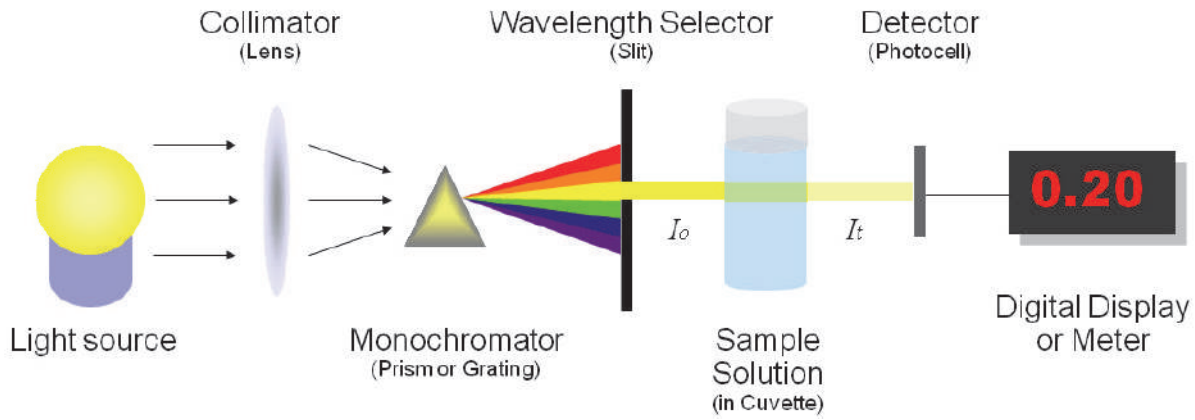


Fig.48.1. Instrumentation

6.28.2. Spectrophotometer

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

Fig.48.2. Spectrophotometer

a. Principle

Spectrophotometer works with the principle of Beer-Lambert Law.

b. 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 H_2 or D_2 lamps are used for UV range. Monochromatic light is generated by either

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

c. 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 colors. Different colors of lighthave a different wavelength.
3. A second thin slit, just after the prism, can be moved from side to side to select just one color of light to pass through to the sample.
4. The light passes through a container with the liquid sample inside.
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 should be specified the wavelength of light.

6.28.3. Mass spectrometer

Mass spectrometry is a powerful technique that is used to identify unknown compounds, to quantify known compounds, and to elucidate the structure and chemical properties of molecules. Detection of compounds can be accomplished with very minute quantities. The compounds can be identified at very low concentrations in chemical complex mixtures.

a. Principle

A mass spectrometer is an instrument that measures the masses of individual molecules that

have been converted into ions, i.e., molecules that have been electrically charged.

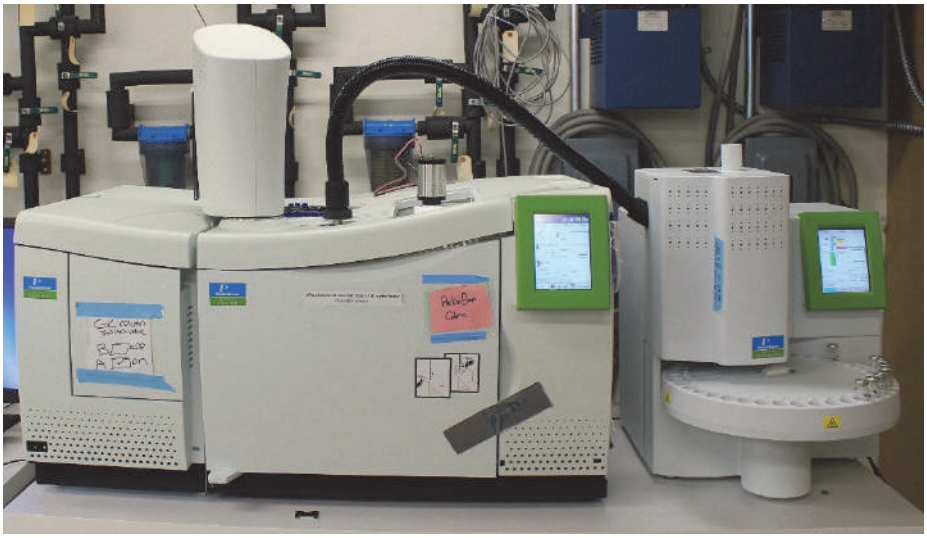


Fig.53. Mass spectrometer

b. Components of a Mass Spectrometer

Components of the mass spectrometer are:

- Inlet system
- Ion source
- Mass analyzer and
- Detector

Samples can be introduced to the mass spectrometer directly *via.* Solid's probe, or in the case of mixtures, by the intermediary of chromatography devices (e.g. gas chromatography, liquid chromatography). Once in the source, sample molecules are subjected to ionization. Ions formed in the source acquire some kinetic energy and leave the source. A calibrated analyzer then analyzes the passing ions as function of their mass to charge ratios. Different kind of analyzer can be used viz. Magnetic, Quadrupole, Ion trap, Fourier Transform, Time of Flight etc. The ion beam exiting the analyzer assembly is then detected and the signal is registered. The analyzer and detector of the mass spectrometer and often the ionization source too, are maintained under high vacuum to give the ions a reasonable chance of travelling from one end of the instrument to the other without any obstacle. The latter is under complete data system control on modern mass spectrometers.

c. Methods of Sample Ionization

Electron Ionization and Chemical Ionization

Electron Ionization (EI) is widely used in mass spectrometry for relatively volatile

samples that are insensitive to heat and have relatively low molecular weight. The spectra, usually containing many fragmentation peaks, are useful for structural characterization and identification. Small impurities in the sample are easy to detect. Chemical Ionization (CI) is used to enhance the abundance of the molecular ion. For both ionization methods, the molecular weight range is 50 to 800 Da.

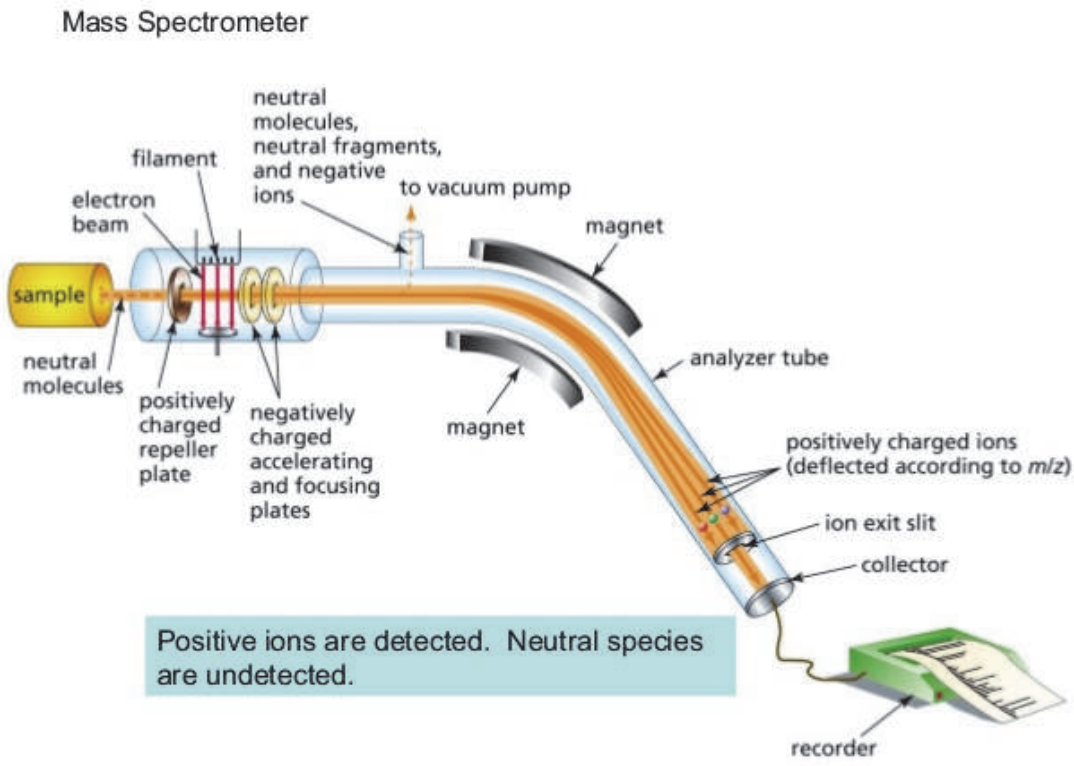


Fig.53.1. Instrumentation

d. Fast Atom Bombardment Ionization

Fast atom bombardment ionization is a softer ionization method than EI. The spectrum often contains peaks from the matrix, which is necessary for ionization, a few fragments and a peak for a protonated or deprotonated sample molecules. FAB is used to obtain the molecular weight of sensitive, non-volatile compounds.

Chapter 7. Methods: macromolecules

7.1. CARBOHYDRATES

7.1.1. Biomedical significance

Carbohydrates are polyhydroxy aldehydes or ketones, or substances that yield such compounds on hydrolysis. Many, but not all, carbohydrates have the empirical formula (CH2O)n; some also contain nitrogen, phosphorus, or sulfur. Carbohydrates are perhaps the most widespread biomolecule on the planet. Photosynthesis transforms around 100 billion metric tons of CO_2 and H_2O into cellulose and other plant compounds each year. Certain carbohydrates (sugar and starch) are the staple of the diet in most parts of the world, and the oxidation of carbohydrates is the central energy-yielding pathway in most none photosynthetic cells. In the cell walls of bacteria and plants, as well as in the connective tissues of animals, insoluble carbohydrate polymers serve as structural and protective elements. Other carbohydrate polymers lubricate skeletal joints and enable cells recognize and adhere to one another. More complex carbohydrate polymers covalently attached to proteins or lipids act as signals that determine the intracellular location or metabolic fate of these hybrid molecules, called glycoconjugates.

Lipopolysaccharides are the most prominent surface characteristic of the outer membrane of gram-negative bacteria such as *Escherichia coli* and *Salmonella typhimurium.* These molecules are significant determinants of the serotype of bacterial strains because they are major targets of the antibodies generated by the vertebrate immune system in response to bacterial infection (serotypes are strains that are distinguished on the basis of antigenic properties). The lipopolysaccharides of *S. typhimurium* contain six fatty acids bound to two glucosamine residues, one of which is the point of attachment for a complex oligosaccharide. *E. coli* has similar but unique lipopolysaccharides. Some bacteria's lipopolysaccharides are poisonous to humans and other animals; for example, they cause the dangerously low blood pressure that occurs in toxic shock syndrome, which is caused by gram-negative bacterial infections. Energy transformation is required for all physiological activities in living cells. Cells transform chemical bond energy into various forms of nutrients, such as an electrochemical gradient across the plasma membrane, muscle fiber action in the arm, or the building of complex molecules like DNA. These energy transformations can be divided into three principal phases: (1) oxidation of fuels (fat, carbohydrate, and protein), (2) conversion of energy from fuel oxidation into the high-energy phosphate bonds of adenosine triphosphate (ATP), and (3) use of ATP phosphate bond energy to drive energy-demanding processes.

7.1.2. Reactions of Monosaccharides

Carbohydrates are polyhydroxy aldehyde or ketone, or compounds that yield these derivatives on hydrolysis. They are classified into monosaccharides (single unit), disaccharides (two units), oligosaccharides (3 to 10 units) or polysaccharides (more than ten units). Monosaccharides can be further classified into trioses, tetroses, pentoses, hexoses, and so on, depending on the number of carbon atoms. They are also grouped into two classes, aldoses (having aldehyde group) or ketoses (having ketone group).

Sample: 1% glucose or 1% fructose solution given separately.

Experiment	**observation**	**inference**
i. Appearance. ii. Color iii. Odor iv. Reaction to litmus	Clear Colorless Odorless No change	 Neutral

7.1.2.1. Chemical Tests

1. ***Molish test***

Experiment	**observation**	**inference**
To 2ml of sample solution, add 1drop of α-naphthol in alcohol and 2ml conc. H_2SO_4 slowly and carefully along the side of the test tube	A purple ring develops	The sample contains carbohydrates

i. A strong dehydrating agent like conc. H_2SO_4 converts sugars to hydroxymethyl furfural. The furfural condenses with phenolic compounds like α-naphthol to give the colored ring.

ii. Molish test is given by at least five carbons.

iii. α-naphthol in alcohol must be prepared freshly.

iv. Water-acid interaction produces heat and can induce charring of carbohydrates, resulting in formation of a black ring. Therefore, acid should be layered extremely slowly and carefully to minimize this interaction.

Green ring formation may be caused by impurities in the reagent. That is negative test result.

v. Excess α-naphthol solution also may form green ring.

2a. Fehling's test

Experiment	**observation**	**inference**
Mix 1ml of Fehling's A solution to 1 ml of Fehling's B solution, boil, and add 1ml of sample solution (boil again if necessary).	Green-yellow to orange-red to brown ppt	Glucose and fructose reduce Cu^{2+} to Cu^{1+} in alkaline medium on heating

i. The tartrate from Fehling's (B) solution chelates cupric ion, releasing it slowly for reduction thus preventing the formation of black cupric oxide.

$CuSO_4 + NaOH \rightarrow Cu(OH)_2 + Na_2SO_4 \longrightarrow Cu_2O$ (red ppt). /Reducing sugar/

ii. Since uric acid and creatinine also gives a positive test; Fehling's test is no longer widely used nowadays.

2b. Benedict's test

Experiment	**observation**	**inference**
To 5ml of Benedict's reagent add 8 drops of sample solution, boil for 2 min	Green-yellow to brown or orange-red ppt	Glucose and fructose reduce Cu^{2+} to Cu^{1+} in alkaline medium on heating. In turn glucose is oxidized

i. Reducing sugars under alkaline conditions tautomerize and form enediols. The enediols are unstable and decompose to yield a variety of products. 1, 2-enediols will give formaldehyde and a pentose. The chain reaction continues to produce short chain aldehydes, which are powerful reducing agents. They can reduce cupric ion to cuprous form, which is the basis for the Benedict's (and Fehling's) reaction. In order to keep the hydroxide in solution, a metal chelator like citrate (or tartrate) is included in the solution.

ii. Benedict's reagent contains $CuSO_4$ (to provide cupric ions), Na_2CO_3 (to make the pH alkaline), and Na citrate (chelates Cuand releases it slowly for reduction), thus avoiding the production of black CuO. Sodium citrate acts as a stabilizing agent. Copper is reduced to produce green, yellow, orange or red precipitate.

iii. It is frequently used for detecting sugar in the urine of diabetic patients. Many reducing chemicals in urine, such as ascorbic acid, might provide a positive test result.

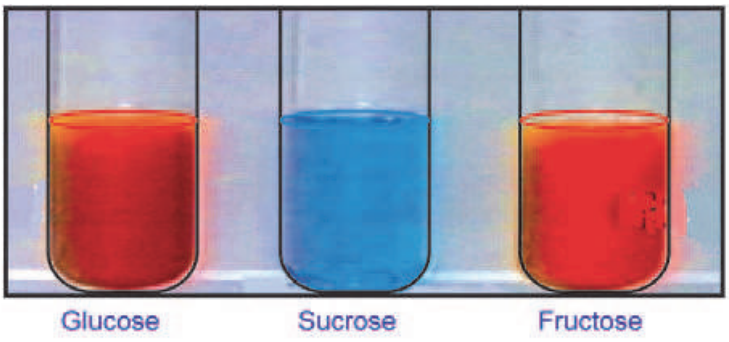


Fig. 54: Benedict's test

2. Barfoed's test

Experiment	observation	inference
To 5ml of Barfoed's reagent add 2ml of sample solution, keep in boiling water bath for exactly 2 min	Fine red ppt. clinging to the walls of the test tube; some settles down on cooling	Monosaccharides reduces Cu^{2+} to Cu^{1+} in acidic medium on boiling water bath for 2 min

The Barfoed's test is dependent on the sugar solution concentration and the amount of time it takes to boil. 5% disaccharides will also give Barfoed's test positive.

3. Osazone test

Experiment	observation	inference
To 5ml of sample solution add 5 drops of glacial acetic acid, a knife-point of phenyl hydrazine hydrochloride powder and two knife-point of sodium acetate, mix vigorously, place test tubes in boiling water bath for 30min, cool and take the crystals on a slide and observe under microscope	Long yellow or greenish yellow needle-shaped crystals	Glucose and fructose form identical osazones insoluble at high temperature.

i. When maintained at boiling temperature, all reducing sugars will produce osazone in the presence of excess phenyl hydrazine. Hydrazones are water soluble, whereas osazones are not.

ii. Osazone crystal shapes are unique to each sugar. Glucose, fructose and mannose form similar osazones as their 1st and 2nd carbon atoms are involved in the reaction during osazone formation.

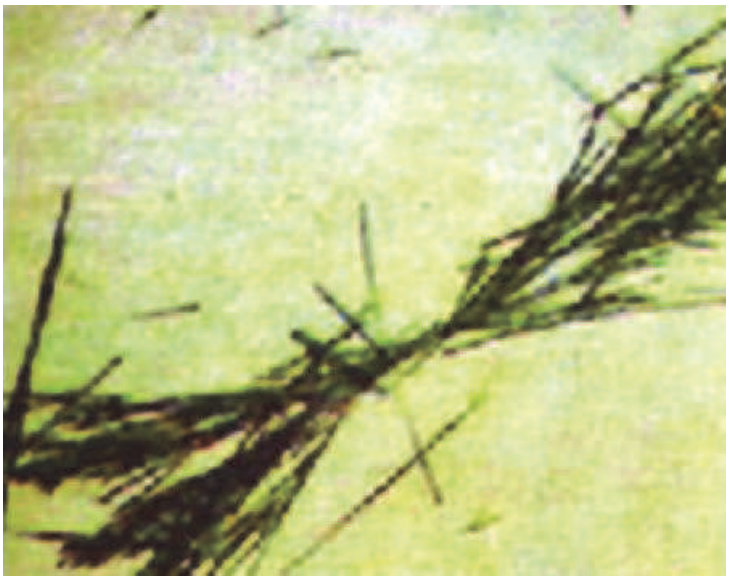


Fig. 55. Glucose osazone: needle-shaped crystals arranged like a broom

iii. Acetic acid and sodium acetate are useful as buffer to maintain pH 5, optimal for the formation of osazones.

iv. Osazones of monosaccharides are insoluble in hot solution, and will make crystals. Osazones of disaccharides are soluble in hot water. As a result, they will form crystals only when the test tube is cooled under running water (Fig. 55).

7.1.3. Analysis of Disaccharides

Glucose + Glucose (α-1,4 glycosidic linkage) = Maltose

Galactose + Glucose (β-1,4 glycosidic linkage) = Lactose

Glucose + Fructose (α-1,2 glycosidic linkage) = Sucrose

Maltose is the result of starch breakdown by enzymes. Lactose is a sugar that is present in milk.

1% lactose, 1% maltose, and 1% sucrose solutions are separately provided as sample solutions.

Physical Characters

Experiment	**observation**	**inference**
i. Appearance	Clear	
ii. Color	Colorless	
iii. Odor	Odorless	Neutral
iv. Reaction to litmus	No change	

7.1.3.1. Chemical Tests

Experiment	observation	inference
1. Molish test. To 2ml of sample solution, add 1drop of α-naphthol in alcohol and 2ml conc. H_2SO_4 slowly and carefully along the side of the test tube	Violet ring at the junction of two liquids	All three disaccharides are dehydrated to give furfural, which condense with α-naphthol to give the colored ring
2 a. Fehling's test	a. Red ppt in case of lactose or maltose b. No characteristic ppt for sucrose	a. Lactose and maltose are reducing sugars b. Sucrose is a non-reducing sugar
2 b. Benedict's test	a. Red ppt in case of lactose or maltose b. No characteristic ppt for sucrose	a. Lactose and maltose are reducing sugars b. Sucrose is a non-reducing sugar
3. Barfoed's test	No red ppt	Disaccharides do not respond to this test

i. Sucrose does not contain any free reducing group.

ii. Higher concentration of sugar tends to give a Barfoed's positive test.

iii. Prolonged boiling for more than 2 min in Barfoed's test may give positive test.

7.1.4. Analysis of Polysaccharides

Glycogen and starch are made up of amylase (1,4 glycosidic linkages) and amylopectin (1,6 glycosidic linkages). Compared to glycogen, which contains more branched chains with 1,6 glycosidic connections, starch has more 1,4 glycosidic links. Glycogen is a storage type of polysaccharide found in mammalian liver and muscles. Hydrolysis of starch by acid (conc. HCl) generates the following products.

Starch→ soluble starch → amylodextrins→ erythrodextrins → achrodextrins → maltose → glucose.

Maltose is the main end product of enzyme (amylase) breakdown. A one-percent starch solution is used for the experimental works described below:

Physical Character

Experiment	observation	inference
i. Appearance	Opalescent	
ii. Color	White	Neutral
iii. Odor	Odorless	
iv. Reaction to litmus	No change	

Chemical Tests

Experiment	observation	inference
1. a. To 3ml of sample solution, add 2 drops of 0.05N iodine solution	Deep blue color	Starch present
b. Heat the solution	Blue color disappears	Iodine is converted to sodium
c. Cool the solution	Color reappears	iodide and sodium
2. To 3 ml of sample solution, add 1 ml 40% NaOH, mix and add few drops of 0.1N iodine solution	No color	iodate. Hence, free iodine is not available for starch
To the aforementioned solution add 1 ml conc. HCl	Color reappears	Acid neutralizes the alkali to release free iodine, which can combine with starch

i. Iodine-starch absorption complex is blue in color. The complex breaks on heating, and reforms on cooling. Sometimes color may not reappear on cooling as small amounts of iodine added may evaporate during heating.

Experiment	observation	inference
3. Benedict's test 4. Rapid hydrolysis of starch and Benedict's test a. To 5ml of sample solution add 6 drops of conc. HCl, boil for 1 min, cool, add 6 drops of 40% NaOH b. To 5 ml of Benedict's reagent, add 1ml of above neutralized solution, boil	No colored PPT Red PPT	Starch is a non-reducing carbohydrate Acid hydrolyzes starch to produce glucose units.

7.1.5. Estimation of Glucose in Urine

PRINCIPLE

Benedict's quantitative reagent, which is $CuSO_4$ in alkaline medium, is reduced by glucose in urine to cuprous oxide, and combines with KCNS in the solution to form white ppt of cuprous thiocyanate preventing precipitation of Cu_2O as red ppt. On complete reduction, the blue color completely fades, leaving only a green color, which soon turns to colorless with an additional drop of urine. The reaction is:

$$CuSO_4 + Na_2CO_3 + H_2O \rightarrow Cu(OH)_2 \rightarrow CuO \text{ (black)}$$

(Prevented by Na-citrate)

$$Cu^{2+} + \text{Glucose} \rightarrow Cu_2O \text{ (red)}$$

Note: Na_2CO_3 neutralizes any urinary acidity and also liberates CO_2 during titration, which is to an extent useful in preventing reoxidation of cuprous ions.

Reagents

a. Benedict's qualitative reagent.

b. Benedict's quantitative reagent.

c. Na_2CO_3 powder.

Procedure

Part I: Perform Benedict's test with urine as described earlier using quantitative reagent. Based on the observed changes, dilute the urine sample with distilled water.

Observation	**Approx. amt of glucose**	**Dilution advice**		**Dilution factor (D)**
		Undiluted urine	**Distilled water**	
Green or yellow ppt	0.5–1 g%	50 ml	50 ml	2
Orange or red ppt	1–2 g%	20 ml	80 ml	5
Red ppt with colorless supernatant	> 2 g%	10 ml	90 ml	10

Part II: Pipette exactly 20 ml of Benedict's quantitative reagent in a conical flask. Add about 5 gm Na_2CO_3 powder and a few pieces of glass beads to avoid bumping of the solution on boiling. Boil the solution on a low oxidizing flame. Add the diluted urine initially at the rate 0.5 to 1ml at a time, wait for a few seconds to complete the reaction; and then continue with titration.

End point: Complete discharge of blue color; very faint green or white ppt.

Note: To avoid bumping, the boiling process should be continuous and moderate.

7.1.6. BASIC QUESTIONS FOR DISCUSSION

1. Structure, function and classification of carbohydrates
2. Digestion and absorption of carbohydrates
3. The main substrates of NAD-linked dehydrogenases.
4. FAD-linked dehydrogenases
5. Oxidative phosphorylation.
6. Oxidative decarboxylation of pyruvate and the tricarboxylic acid cycle (the Krebs cycle)
7. Pyruvic acid: the ways of the formation and utilization in the body. The significance of these processes. Acetyl CoA: the ways of the formation and utilization in the body. The significance of these processes. The aerobic degradation of glucose: the reaction sequence, physiological significance.
8. The anaerobic splitting of glucose: the reaction sequence, physiological significance.
9. The significance and regulation of gluconeogenesis.
10. The glucose-lactate cycle (the Cory cycle).
11. The glycogen synthesis and its degradation
12. The pentose phosphate pathway
13. Hormonal regulation of blood glucose level.
14. Regulation of carbohydrate metabolism.

7.1.5.a. The examples of the control task:

1.Two weeks after an episode of the flu, an 8-year-old boy with IDDM (Insulin-Dependent Diabetes Mellitus) is brought to the emergency room in a coma. His breathing is rapid and deep, and his breath has a fruity odor. His blood glucose is 36.5 mM (normal: 4–6 mM [70–110 mg/dL]). The physician administers IV fluids, insulin, and potassium chloride. A rapid effect of insulin in this situation is to stimulate

A. gluconeogenesis in the liver

B. fatty acid release from adipose

C. glucose transport in muscle

D. ketone utilization in the brain

E. glycogenolysis in the liver

2.An alcoholic has been on a 2-week drinking binge during which time she has eaten little and has become severely hypoglycemic. Which additional condition may develop in response to chronic, severe hypoglycemia?

A. Glycogen accumulation in the liver with cirrhosis

B. Thiamine deficiency

C. Ketoacidosis

D. Folate deficiency

E. Hyperuricemia

3. Glucose travels across the cell membrane on a transport protein. Hereditary deficiency of GLUT-1, insulin -independent transporter, results in decreased glucose transport. Patients manifest with intractable seizures in infancy and a developmental delay. GLUT-1 transporter is expressed in which of the following cell types?

A. Liver, kidney and pancreatic β cells

B. Brain (neurons)

C. Adipose tissue, skeletal muscle and cardiac muscle

D. Erythrocytes, brain, hepatic cells

E. Intestinal epithelium, Spermatozoa.

4.What is the end product of anaerobic glycolysis

A. Lactate

B. Pyruvate

C. Acetyl Co-A

D. acetone

5. During short run in the muscles of physically not trained person is observed hypoxia. To what process it can lead afterwards.

A. Ketoacidosis

B. Lactic acidosis

C. Hyperosmolar coma

D. Hyperglycosuria

7.2. PROTEIN

7.2.1. Biomedical significance

Proteins are the most abundant biological macromolecules, found in all cells and all parts of cells. Proteins also occur in great variety; thousands of different kinds, ranging in size from relatively small peptides to huge polymers with molecular weights in the millions, may be found in a single cell. Moreover, proteins exhibit enormous diversity of biological function and are the most significant final products of the information pathways. Proteins are the molecular tools that genetic information is expressed through. Relatively simple monomeric subunits provide the key to the structure of the thousands of different proteins. All proteins, whether from the most ancient lines of bacteria or from the most sophisticated forms of life, are formed the same omnipresent set of 20 amino acids, covalently bonded in characteristic linear sequences. Each of these amino acids contains a side chain with specific chemical features, and therefore this set of 20 precursor molecules may be regarded as the alphabet in which the language of protein structure is written. What's most amazing is that by combining the same 20 amino acids in a variety of combinations and sequences, cells can build proteins with a wide range of characteristics and functions. Different organisms can make a wide range of products from these building blocks, including enzymes, hormones, antibodies, transporters, muscle fibers, the lens protein of the eye, feathers, spider webs, rhinoceros' horn, milk proteins, antibiotics, mushroom poisons, and a wide variety of other biologically active substances. Among these protein products, the enzymes are the most diverse and specialized. Enzymes catalyze virtually all cellular processes. Proteins are dynamic molecules whose functions almost invariably depend on interactions with other molecules, and these interactions are affected in physiologically significant ways by sometimes subtle, sometimes striking changes in protein conformation. The importance of molecular interactions to a protein's function can hardly be overemphasized.

The function of a protein is determined by its structure. Regulative, signaling, mobility, protective, structural, transport, catalytic, storage, and many more functions are all performed by proteins. Under physiological conditions, the production of proteins is closely regulated, but alterations in the protein pattern may occur in a wide range of diseases and health disorders. The daily protein demand is increased by stress, disease, injury, and extended heat exposure. Having many essential physiological functions proteins are widely used in different biological fluids (blood, urine etc..) for diagnostic purposes. Life would be hardly possible without proteins.

7.2.2. Color Reactions of Proteins

Proteins are formed from amino acid residues linked by peptide bonds. Due to their polypeptide structure and various amino acid residues, protein reacts with different reagents and form colored products. These tests, known as color reactions of proteins, are important in qualitative detection and quantitative evaluation of proteins. Proteins and amino acids used in different experiments:

1. Egg albumin is a protein of egg, which is water soluble.
2. Casein is the main protein in milk. It is a phosphoprotein with phosphate groups attached to the hydroxyl groups or serine and threonine residues. It lacks cysteine.
3. Gelatin is formed from collagen, the connective tissue protein. It is a rich source of amino acid glycine. It is deficient in tyrosine, tryptophan and cysteine.
4. Metaproteins, proteoses and peptones are partially hydrolyzed products of proteins like albumins and globulins. Albumin has relatively low molecular weight.

EXPERIMENT 1. COLOR REACTIONS OF PROTEINS BASED ON PEPTIDE BONDS AND TYPE OF AMINO ACID RESIDUES

Solution: 10% Egg-white or albumin

Biuret Reaction

Experiment	observation	inference
To 2ml of sample solution, add 2ml 5% NaOH and 3 drops of 1% CuSO4. Repeat the test with distilled water (control)	Purple-violet or pink color in test Blue color in control	Peptide linkages present

i. The reaction is so named since biuret (NH2-CO-NH-CO-NH2) produced by the condensation of two molecules of urea when heated. CO-NH is the peptide linkage in biuret. A positive test requires at least two peptide links in the molecule. This test does not respond to individual amino acids or dipeptides.

ii. $CuSO_4$ is converted to $Cu(OH)_2$ which chelates with peptide linkage in proteins to provide the color.

iii. Strictly avoid excess addition of CuSO4. Magnesium and ammonium ions will interfere in this reaction.

iv. The color differs depending on the number of peptide bonds; albumin/globulin show violet, proteoses purple and peptones dark pink color indicating that albumin globulins have largest number of peptide linkages and peptones the least.

v. This reaction can be used for quantitative evaluation of proteins.

Ninhydrin Reaction

Experiment	**observation**	**inference**
To 1ml of sample solution, add 0.1% ninhydrin solution, boil and cool	Ruhemann purple color	Amino acid present

i. All α-amino acids give purple color. The imino acids, proline and hydroxyproline form yellow color. The colored complex is known as Ruhemann's purple. Glutamine and asparagine generate brown color.

ii. α-amino acid + ninhydrin → aldehyde + hydrindantin + NH_3 + CO_2;

hydrindantin + NH_3 + ninhydrin → Ruhemann's purple + $3H_2O$.

iii. Proteins will give a slight blue color.

iv. This reaction is often used to detect amino acids in chromatography.

v. Proteins do not react with ninhydrin to generate a genuine color, but the N-terminal amino group of a protein can react with it to produce a faint blue color.

Xanthoproteic Reaction (for Aromatic Amino Acids)

Experiment	**observation**	**inference**
To 2ml of sample solution, add 1ml conc. HNO3 and boil. Cool test tube and add 40% NaOH excess	Yellow precipitate Color of precipitate and the solution change to orange	Aromatic amino acids, i.e. tyrosine, tryptophan or phenylalanine present

Yellow color is due to the formation of nitro derivatives of benzene ring containing amino acids (tyrosine and tryptophan), the color turns orange due to ionization when alkali is added. This test typically yields positive results for all proteins. Nitric acid causes a yellow discoloration on the skin as a result of this interaction. Nitration of phenylalanine does not typically occur under these circumstances.

Sakaguchi Test for Guanidine Group (Reaction of Arginine)

Experiment	observation	inference
To 2 ml of sample solution add 2 drops of 1% α-naphthol in alcohol, 4 drops of 40% NaOH, and 8-10 drops of bromine water	Bright red color due to guanidium group	Arginine present

i. Instead of NaOH and bromine water, 8 to 10 drops of alkaline hypobromite (NaOBr) can be applied as a single reagent.

ii. Guanidino groups in arginine residues of proteins react with the α-naphthol and NaOBr to generate the colored complex.

iii. This test is given by albumin, globulin and gelatin also can be used as they contain arginine.

Modified Millon's Reaction (Cole's Test)

Experiment	observation	inference
To 2ml of sample solution, add 2ml 10% $HgSO_4$ in 10% H_2SO_4, boil, add 5 drops of 1% sodium nitrite, heat gently	Red PPT of mercury phenolate	Tyrosine present

i. The color is due to the formation of nitrated mercury phenolate ion of tyrosine (hydroxyphenyl group) present in proteins.

ii. Heat coagulable proteins form red PPT, whereas smaller molecules of proteins like peptones generate red colored solution without PPT.

iii. Because gelatin is poor in tyrosine, it doesn't give the test.

iv. Chloride interferes with this reaction; so, it is not suitable for urine test.

Pauly's Test for Imidazole Group and Phenolic Hydroxyl Group

Diazo benzene sulphonic acid reacts with the imidazole ring of histidine or the phenolic hydroxyl group of tyrosine to give the colored products in the alkaline medium.

Experiment	observation	inference
To 0.5 ml of 0.5% sulphanilic acid add 0.5ml 1% $NaNO_2$, mix, wait for 1 min, add 1ml of sample solution	Cherry red color	Histidine present
Then add 1ml of 10% Na_2CO_3	Orange red color	Tyrosine present

Tests Based on Precipitation Reactions of Proteins

Proteins are large molecules with variable sizes, shapes and charges. They can be classified as simple, conjugated and derived proteins. Colloidal solution is formed when most simple proteins, especially globular proteins, are dissolved in water. A colloid is a system in which the particles have diameters in the range of 1mm to about 200mm. The stability of a solution of a lyophobic colloid depends on the electrical charges on the surface of particles, which prevent their coagulation and precipitation. In case of lyophilic colloids, over and above the surface charges, the degree of hydration (shell of water molecules around the particles) also contributes to the stability. Polar groups of the proteins (-NH_2, COO–, OH– groups) tend to attract water molecules towards them to produce a shell of hydration. Albumin has a greater degree of hydration than globulins. Purification of enzymes and other proteins usually start with precipitating them from solution. Any factor, which neutralizes the charge or removes water of hydration will therefore cause precipitation of proteins. These characteristics of protein molecules depend upon their molecular weights, three dimensional structures and properties of the constituent amino acids.

PRECIPITATION BY SALTS

Supplied sample: 10% egg-white solution

Generally, proteins can be precipitated by the addition of salts. When an inorganic salt like ammonium sulphate is added to a solution of protein, it decreases concentration of water molecules available for stabilizing the protein solution and the protein is consequently precipitated. The process is known as "salting out". Albumin tenaciously holds a large number of molecules of water and, therefore, needs a much higher concentration of salt than globulin to get precipitated. This feature of proteins is

determined by the types of amino acids that make them up, as well as their sizes and structures, and it may be used to distinguish between proteins, such as albumin and globulins. Because globulins have a greater molecular weight, a smaller salt concentration is sufficient to precipitate it. Consequently, globulins are precipitated at half saturation of ammonium sulphate or 22% sodium sulphate, whereas albumin requires complete saturation of ammonium sulphate or 28% sodium sulphate. A protein's solubility is determined by the medium's ionic concentration. As a result, the addition of extremely tiny amounts of salts will enhance a protein's solubility by reducing protein-protein interaction. This is called “salting-in.”.

7.2.3. COAGULATION OF PROTEINS

10% Egg-White or 1% Albumin Solution

Proteins have unique structural organizations. The primary structure refers to the order of amino acids in the polypeptide chain of the protein. The three-dimensional conformation of the structure of a protein depends on its primary structure. The subunits of a protein, each possessing its own primary, secondary and tertiary structures, are united together to constitute the quaternary structure of a protein. The weak bonds, involved in the secondary, tertiary, and quaternary structures, are hydrogen bonds, hydrophobic bonds, van der Waals force, ionic bond and disulphide bonds. The disruption of secondary, tertiary and quaternary structures of a protein molecule is called denaturation. The aggregate of denatured proteins is called a coagulum, and the process is called coagulation. Denaturation is sometimes reversible, but coagulation is not. Some proteins when heated, though denatured, are still soluble. They may be precipitated by bringing to isoelectric pH.

Experiment	**observation**	**inference**
Take 10 ml of sample solution in a test tube. Heat the upper layer of the solution and add 1% acetic acid drop by drop	Cloudy white PPT	Albumin and globulin are coagulated by heat at its isoelectric pH

Heat may quickly coagulate albumin and globulin at or at their isoelectric point. On addition of acetic acid, there is a decrease in pH. When pH approaches the isoelectric pH of albumin/globulin, coagulation occurs spontaneously since the solution is pre-heated. This is called Heat and acetic acid test.

7.2.4. PRECIPITATION BY ORGANIC SOLVENTS

10% Egg-White Solution

Proteins in solution form hydrogen bonds with water. Organic solvents like acetone, ether or ethanol when added to a protein solution in water, they limit the amount of water molecules available to maintain the proteins in solution, resulting in fewer hydrogen bonds. The dielectric constant of the medium is also reduced causing aggregation, precipitation and denaturation of proteins. This denaturation does not occur to some proteins at low temperature.

Experiment	**observation**	**inference**
To 1ml of sample solution add 2ml ethanol and mix	Mild cloudy precipitate	Albumin/globulins are precipitated by organic solvents

PRECIPITATION BY HEAVY METALS

10% Egg-White Solution

When the pH of a protein solution is higher than the isoelectric pH of the protein (generally in an alkaline medium), protein molecules become negatively charged anions. Positively charged heavy metal cations may then bind with the negatively charged protein anion, causing their precipitation. Salts of iron, copper, zinc, lead, cadmium and mercury are toxic, because they tend to precipitate normal proteins of the gastro intestinal wall. Raw egg is sometimes used as an antidote for mercury poisoning.

Experiment	**observation**	**inference**
I. To 2ml of sample solution add 10% mercuric chloride solution drop by drop	White PPT	Albumin/globulins are precipitated by heavy metals like Hg, Pb and Fe
II. To 2ml of sample solution add 10% lead acetate solution drop by drop	White PPT	
III. To 2ml of sample solution add 10% ferric chloride solution drop by drop	White PPT	

i. If the sample solution is significantly alkaline, its pH should be adjusted to 7–7.5 to avoid formation of metal hydroxides, which interfere with the test.
ii. Avoid adding excess of heavy metal ions as this may redissolve the PPT due to absorption by the protein molecules, which will give them a positive charge.

7.2.5. PRECIPITATION BY ALKALOIDAL REAGENTS

10% Egg-White Solution

Tungstic acid, phosphotungstic acid, trichloroacetic acid, picric acid, sulphosalicylic acid and tannic acid are strong protein precipitating agents. These acids lower the pH of the medium, when proteins carry net positive charges. These protein cations are electrostatically complexed with negatively charged ions to form protein-tungstate, protein-picrate, etc. and thick flocculant precipitate is formed. Tanning in leather processing is based on the protein precipitating effect of tannic acid.
The test described in (I) is frequently used to identify proteins in body fluids, particularly in urine and CSF (Cerebrospinal fluid specimens).

Experiment	**observation**	**inference**
I. To 2ml of sample solution add 20% sulphosalicylic acid drop by drop	White flocculant precipitate	Albumin/ globulins are precipitated by alkaloidal reagents
II. To 2ml of sample solution add Esbach's reagent (Picric acid + citric acid) drop by drop	Yellow precipitate	
III. To 2ml of sample solution add 5% tannic acid drop by drop	Brown precipitate	

Esbach's reagent - Add 10 grams of picric acid and 20 grams of citric acid to 1000 ml distilled water and mix well.

7.2.6. PRECIPITATION BY STRONG MINERAL ACIDS

10% Egg White Solution

Heller's test

Experiment	observation	inference
I. To 2ml of sample solution add 2 ml conc. HNO_3 slowly along the test tube's side	White ring at the junction of two liquids	Albumin/globulins are precipitated by strong mineral acid
II. To 2ml of sample solution add 2ml conc. HCl slowly along the test tube's side	White ring at the junction of two liquids	

Test (I) is called Heller's test and is usually used to identify proteins in body fluids, particularly in urine.

7.2.a. BASIC QUESTIONS FOR DISCUSSION

1. Structure, classification and functions of proteins.
2. Amino acids as monomers of a protein molecule.
3. Denaturation of proteins.
4. Structural levels of proteins.
5. The kinds of natural ligands and peculiarities of their interactions with the proteins (prosthetic groups, cofactors, protomers, substrates, transported substances, allosteric effectors).
6. Digestion and absorption of proteins
7. Deamination of amino acids
8. Transamination of amino acids
9. Decarboxylation of amino acids
10. Enzymes as biological catalysts.
11. The mechanism of catalytic action of enzymes.
12. The principles of quantitative determination of enzyme activity.
13. The enzyme inhibitors: reversible, irreversible, competitive, noncompetitive. The role of enzymes in metabolism.
14. Determination of enzyme activities for the diagnosis of the diseases (LDH, creatine kinase, Aspartate aminotransferase).
15. Regulation of enzyme activity.

7.2.b. The examples of the control task

1.The sequence of amino acids in a polypeptide is called the

1. primary structure
2. secondary structure
3. tertiary structure
4. quaternary structure

2.HCl is produced in the stomach by

1. Parietal cells

2. Chief cells

3. Both

4. None

3.What statement is true

• Enterokinase converts the pancreatic zymogen trypsinogen to trypsin by removal of a hexapeptide from the NH2-terminus of trypsinogen

• Enterokinase converts the pancreatic zymogen chymotrypsinogen to chymotrypsin by removal of a hexapeptide from the NH2-terminus of trypsinogen

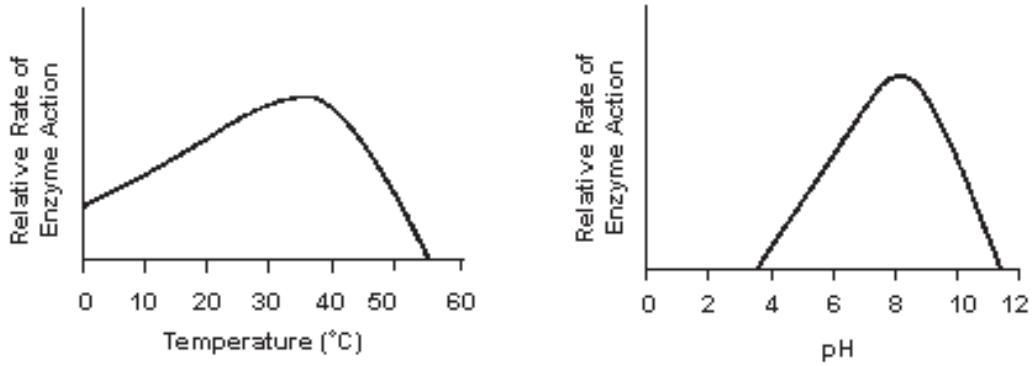
• Enterokinase converts the gastric zymogen trypsinogen to trypsin by removal of a hexapeptide from the NH2-terminus of trypsinogen

• Enterokinase converts the pancreatic zymogen trypsinogen to trypsin by removal of a heptapeptide from the NH2-terminus of trypsinogen

4.An 18-year-old man is involved in a severe motor vehicle crash. He is rushed to surgery for an emergent laparotomy. Unfortunately, he undergoes a complete pancreatectomy, which will have all of the following consequences except for which one?

A. Failure to convert pepsinogen to pepsin
B. Diabetes
C. Increased likelihood of duodenal ulcer formation
D. Decreased synthesis of chymotrypsin and trypsinogen
E. Steatorrhea

5.Which statement best describes the enzyme represented in the graphs?



A. This enzyme works best at a temperature of 35°C and a pH of 8.

B. This enzyme works best at a temperature of 50°C and a pH of 12.

C. Temperature and pH have no effect on the action of this enzyme.

D. This enzyme works best at a temperature above 50°C and a pH above 12.

7.3. LIPIDS

7.3.1. BIOMEDICAL SIGNIFICANCE

Lipids have a range of biological functions, some of which have only lately been discovered. They are the most common type of stored energy in most animals, as well as important components of cellular membranes. Extracellular and intracellular messengers (eicosanoids, phosphatidylinositol derivatives), pigments (retinal, carotene), cofactors (vitamin K), detergents (bile salts), transporters (dolichols), hormones (vitamin D derivatives, sex hormones), extracellular and intracellular messengers (eicosanoids, phosphatidylinositol derivatives), and membrane protein anchors are (covalently attached fatty acids, phenyl groups, and phosphatidylinositol). The ability to synthesize a variety of lipids is essential to all organisms. Fatty acids and triacylglycerols; glycerophospholipids and sphingolipids; eicosanoids; cholesterol, bile salts, and steroid hormones; and fat-soluble vitamins are the most common lipids present in the body. The molecular structures and activities of these lipids are quite varied. They do, however, share a feature in common: relative insolubility in water. Fatty acids are the body's primary source of energy. After eating, we store excess fatty acids and carbohydrates, that are not oxidized, but stored as triacylglycerols in adipose tissue. Between meals, these fatty acids are released and circulate in blood bound to albumin. Disturbances in dietary lipid digestion and absorption can be caused by a variety of factors, including inherited or acquired disorders of pancreatic lipase synthesis, obstruction of the bile excretory tract, and other factors that cause dietary lipid emulsification to be disrupted, inflammation processes in the small intestine, and so on. Eicosanoids are paracrine hormones, which operate solely on cells close to the source of hormone synthesis rather than being carried through the bloodstream to act on cells in distant tissues or organs. These fatty acid derivatives have a variety of dramatic effects on vertebrate tissues. They are known to be involved in reproductive function; in the inflammation, fever, and pain associated with injury or disease; in the formation of blood clots and the regulation of blood pressure; in gastric acid secretion; and in a variety of other processes important in human health or disease. Arachidonic acid, a 20-carbon polyunsaturated fatty acid from which all eicosanoids are produced (Greek eikosi, "twenty"), is the source of all eicosanoids. There are three classes of eicosanoids: prostaglandins, thromboxanes, and leukotrienes. Steroids are oxidized derivatives of sterols; they have the sterol nucleus but lack the alkyl chain attached to ring D of cholesterol, and they are more polar than cholesterol. Steroid hormones move through the bloodstream (on protein carriers) from their site of production to target tissues, where they enter cells, bind to highly specific receptor proteins in the nucleus, and trigger changes in gene expression and metabolism. Because hormones have very high affinity for their receptors, very low concentrations of hormones (nanomolar or less) are sufficient to produce responses in target tissues. The major groups of steroid hormones are the male and female sex hormones and the hormones produced by the adrenal cortex, cortisol and aldosterone. Prednisone and prednisolone are

steroid medicatios with strong anti-inflammatory activities, mediated in part by the inhibition of arachidonate release by phospholipase A2 and consequent inhibition of the synthesis of leukotrienes, prostaglandins, and thromboxanes. They are used to treat a number of medical conditions, including asthma and rheumatoid arthritis.

7.3.1.a. Work 1. Detection of the ketone bodies in urine.

The principle of a method

Acetone is detected in urine via its interaction with sodium nitroprusside, which results in the production of a violet-colored product. The qualitative revealing of acetoacetic acid is based on the formation of complex compound of iron with the enol form of acetoacetic acid.

The COURSE of WORK:

1. Express method of definition of acetone in urine (Lestrade's reaction). Put small quantity of powder containing sodium nitroprusside, ammonia sulfate and sodium carbonate on a surface of the Petry dish placed on a sheet of white paper. Then add 2-3 drops of the urine you've been studying. If acetone is present in the urine, the maximum cherry hue will develop in 1-2 minutes. If the urine color does not change, the test is considered negative, and if the distinctive violet color occurs in 1-3 minutes, it is considered positive.
2. The reaction with sodium nitroprusside for the detection of acetone: measure out 1 ml of urine in a test tube, then add 1-2 drops of sodium nitroprusside solution, next 3-4 drops of 10% NaOH solution. The red color will be observed. Then add 1-2 drops of 10% acetic acid. Red cherry color will be formed if acetone is present in the urine. If acetone is absent in the urine, red color will disappear after the addition of acetic acid.
3. The Gerhardt reaction for the detection of acetoacetic acid. Add 4-5 drops of iron chloride to 1-2 ml of the researched urine. The cherry-red color will appear, if acetoacetic acid is present in the urine.

7.3.2. LIPOPROTEINS of BLOOD PLASMA. TRANSPORT OF CHOLESTEROL.

The measurement of blood plasma lipoproteins is used to detect common lipid metabolic diseases such as hyper- and hypolipidemias. Blood of normal individuals contains three main classes of lipoproteins, such as HDL (high density lipoproteins), LDL (low density lipoproteins) and VLDL (very low-density lipoproteins). Chylomicrons are absent in blood plasma after 12-hr fasting. They appear in blood just after meal and have been utilized for the fasting time. Some pathological states may be accompanied by the appearance of IDL (intermediate density lipoproteins). **Hyperlipoproteinemia** is a condition in which the amount of specific lipoproteins in the blood plasma increases**.** The development of atherosclerosis is due to increase in VLDL and LDL content in blood plasma. Both of the fractions are termed **atherogenic lipoproteins**. The HDL fraction, which is antiatherogenic, is responsible for the reverse transfer of excess cholesterol to the liver.

The excess of dietary cholesterol and abnormal activation of its synthesis from dietary carbohydrates lead to the development of atherosclerosis accompanied by cholesterol deposit under the big blood vessel intima and atherosclerotic plugs forming.

7.3.2.a. Work 2. Quantitative determination of cholesterol in serum.

The EQUIPMENT: a FEC.

The PRINCIPLE of a METHOD.

In the presence of both acetic anhydride and sulfuric acid cholesterol gives green color (The Ilk method).

The COURSE of WORK.

Add very slowly (**Warning! See the instruction for students' safe work**) 0.1 ml of serum to 2.1 ml of the Ilk reagent in a test tube with a micropipette or dosator. Stir the contents of the test tube 10-12 times before placing it in a thermostat at 37°C for 20 minutes. The optical density is then determined using FEC. Use a red-light filter (630- 690 nm). The thickness of a cuvette is 5 mm. The colorimetry must be performed using distilled water. Using a calibrating graph, calculate the cholesterol concentration in the serum. Cholesterol levels in the blood should be between 3,0 and 8,8 mM/l.

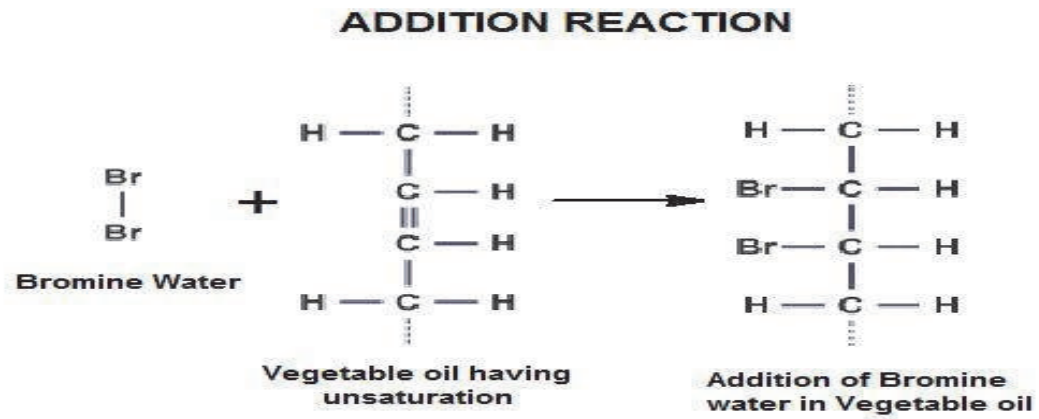
7.3.2.b. Work 3. Qualitative determination of unsaturated fatty acids in plant oils.

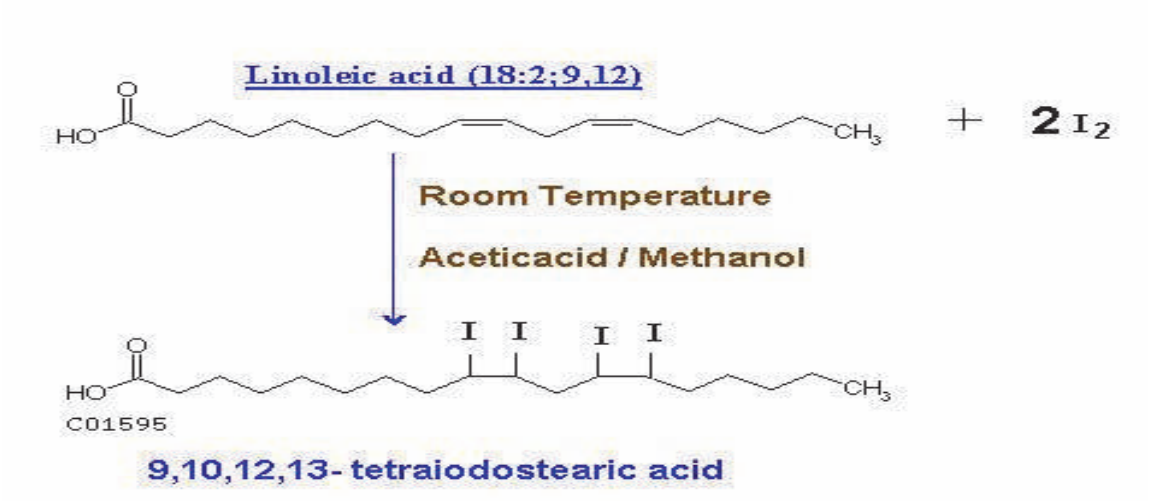
Fats and oils are glycerol and fatty acid esters. Fatty acids are made up of a long chain hydrocarbon with carbon-carbon double bonds. Then the substance is classified as unsaturated. Unsaturation is measured by the mass of iodine that interacts with the fat or oil. The iodine number of the fat or oil is determined by the quantity of iodine that reacts, and this value is used to determine the degree of unsaturation in the fat or oil.

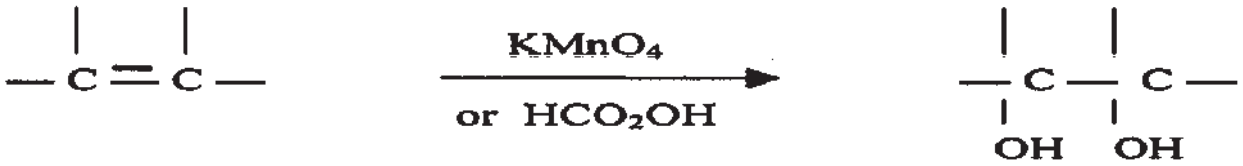
Chemicals

- Various vegetable oils and fats, as available
- Bromine water, approximately 0.02 M (HARMFUL)
- 1% iodine solution,
- 1% starch solution
- 1% $KMnO_4$

Reactions







Procedure

The experiment gives a rough comparison of the degrees of unsaturation of the oils and fats tested, although the results are not quantitatively comparable.

Add 1 ml of vegetable/plant oil in 3 different tubes. Fill the 1st tube with 0.5ml bromine water, in the 2nd add 1ml of water and 2 drops of iodine solution. In 3rd tube add 5 drops of $KMnO_4$ solution. After put on shaker for 3 min. In 2nd tube add starch solution. If there is no blue coloration then there is no free J left in 3rd tube.

If students find the color change to a permanent yellow difficult to distinguish, a variation can be tried using potassium manganate (VII) solution, $KMnO_4$ instead. The purple solution turns colorless while unsaturation is still present. The procedure is the same as for bromine water.

Warming fats using a beaker of hot water aids the fat dissolve and also accelerates the reaction.

The iodine value is a measure of the amount of unsaturation (number of double bonds) in a fat.

7.3.3. BASIC QUESTIONS FOR DISCUSSION

1. Structure, function and classification of lipids
2. Digestion and absorption of lipids
3. Lipolysis. The hormonal regulation of this process.
4. Oxidation of fatty acids. The role of carnitine.
5. The pathway of β-oxidation, the reaction equations, the net ATP yield.
6. Biosynthesis of ketone bodies.
7. Transport of acetyl CoA from mitochondria to cytoplasm. Regulation of this process.
8. The structure of fatty acid synthase complex. The fatty acid synthesis, reactions.
9. Regulation of lipogenesis.
10. Elongation of fatty acids. Desaturation of fatty acids.
11. Synthesis of triacylglycerols. Synthesis of triacylglycerols from glucose
12. Synthesis of cholesterol. Regulation of this process.
13. The role of acetyl CoA in the integration of carbohydrate and lipid metabolism.
14. Structure and function of lipoproteins
15. Metabolism of endogenous and exogenous lipids

7.3.4. The examples of the control task:

1.HDL particles are synthesized in:

1. the liver
2. blood plasma
3. the small intestine
4. the liver and blood plasma

2.The main function of LDL is

1. transport of exogenous triglycerides
2. transport of endogenous triglycerides
3. transport of cholesterol from the liver to the peripheral tissues
4. transport of phospholipids
5. transport of cholesterol from the peripheral tissues to the liver

3.Choose the functions of cholesterol.

1. Power
2. Transport
3. Structural
4. Regulative

5.Serves as a source to the synthesis of the biologically active molecules (vitamins, hormones)

4.Which of the following plasma lipoproteins is the largest in size?

1. HDL
2. VLDL
3. LDL
4. CM

5.Choose the VLDL functions.

1. Transport of cholesterol from the liver to the peripheral tissues
2. Transport of exogenous triglycerides
3. Transport of endogenous triglycerides
4. Transport of cholesterol from the peripheral tissues to the liver

7.4. NUCLEIC ACIDS

7.4.1. BIOMEDICAL SIGNIFICANCE

An organism must be able to store and maintain its genetic information, as well as pass it on to future generations and express it as it goes through all of life's activities. The fundamental dogma of molecular biology illustrates the key stages required in processing genetic material. The basic sequence of DNA molecules stores genetic information. Finally, during the gene expression process, this information is employed to create all of an organism's proteins. Traditionally, a gene is a DNA unit that codes for a certain protein or RNA molecule. Although our understanding of the various ways in which genes may be expressed has complicated this concept, it remains helpful as a working definition.

Inherited alterations of the primary structure of DNA result in either stopping of the protein synthesis encoded by the affected gene or the synthesis of "incorrect" proteins. Gene mutations may be either beneficial or harmful. The useful mutations provide more successful adaptation of the organism to the changing conditions of the environment in the evolution process. Harmful mutations cause the development of molecular pathology or death of a cell or even the entire organism. E.g., sickle cell anemia, hemophilia, enzymopathies of metabolism of amino acid, carbohydrate, and lipid belong to typical inherited diseases. Approximately 90% of human malignant tumors are induced by harmful chemical substances or physical mutagens. Many antibiotics are used as the specific inhibitors of the processes of DNA replication, transcription or translation of genetic information in prokaryotic cells (bacteria, Protista) for treatment of infectious diseases. In malignantly transformed eukaryotic cells antibiotics are used for chemotherapy of cancer.

7.4.2. Quantification of nucleic acid concentrations using UV light

The concentration of DNA, RNA, oligonucleotides, and even mononucleotides can be detected directly in aqueous solutions. Low-ion-concentration aqueous buffers (such as TE buffer) are excellent. The concentration is determined by measuring absorbance at 260 nm (subtracting the "blank" absorbance) and then simply calculating concentration via a standard factor as per the Beer-Lambert law. The absorption of 1.0 is equivalent to approximately:

50 µg/mL double-stranded DNA (dsDNA)

33 µg/mL single-stranded DNA (ssDNA)

40 µg/mL single-stranded RNA

30 µg/mL for ssDNA oligonucleotides.

The purity of a nucleic acid sample can be assessed by calculating the ratio between absorbances at 260nm and 280nm. Because proteins absorb more strongly at 280 nm, this ratio (A260/A280) is used to evaluate purity. The ratio for pure DNA should be about 1.8, whereas the ratio for pure RNA should be around 2.0. Absorption at 230nm indicates the presence of contaminants in

the sample, such as carbohydrates, peptides, phenols, or aromatic chemicals. For pure nucleic acid samples, the A260/A230 ratio should be about 2.2.

Experimental Procedures:

Part I. UV Spectrum for Nucleotide Bases

The aromatic bases: Adenine, guanine, cytosine, and thymine of DNA as well as uracil of RNA all absorb ultraviolet light but with different spectra. You will take a wavelength scan to acquire the spectrum of mononucleotides from 300nm down to 220nm in this experiment. The concentration of nucleotides will be 0.05 mM, resulting in a maximum absorbance of less than 1. Quartz cuvettes are required for these scans.

Part II. UV Assay of DNA and RNA

Run an ultraviolet absorbance spectrum from 220nm to 300nm on both a 0.04 mg/ml DNA solution from E. coli and an RNA sample both in 0.1x SSC. SSC is 0.105 M sodium citrate, 0.15 M sodium chloride, pH 7.0. Remember, you must use quartz cuvettes because you are in the UV range.

Part III. Effect of Heat on DNA and RNA

- At room temperature measure and record the absorbance of a 0.04 mg/ml DNA solution at 260 nm.
- Measure and record the absorbance of a 0.04 mg/ml RNA solution at 260 nm at room temperature.
- Heat about 5 mL of each of the DNA and RNA solutions in a screw cap plastic tube to 50^{o}, 70^{o}, and boiling.
- Carefully pour about 1 mL of the heated solutions in separate measurements to the quartz cuvette and record the absorbance at 260nm for each.
- Take another mL of the heated solutions into separate glass tubes and plunge into an ice water bath and immediately chill the solution. After cooling for ~5 minutes, read the absorbance at 260 nm.

Part IV. Effect of NaOH on DNA

- On ice add 0.2mL of 2M NaOH to 2 mL of 0.04 mg/ml DNA.
- Add 0.2mL of 2M NaCl to 2mL of 0.04 mg/ml DNA as a control.
- Measure the absorbance of each solution at 260 nm.
- Warm each tube to 50^{o}C and measure the absorbance once again. If no significant elevation is observed in either tube, increase the temperature up to 70^{o}C and measure the absorbance again.

Part V. Effect of DNase on DNA and RNA

- To 1mL of 0.04mg/mL DNA add DNase (from a 10mg/mL stock) to a final concentration of 7.5 µg/mL and MgCl2 (from a 25 mM stock) to a final concentration of 25µM. Incubate at 37ºC for 10minutes and measure the absorbance at 260 nm.
- Use a 0.04 mg/mL RNA sample to repeat the process.

Information for the lab report

Part I. Please include the following:

- Wavelength scan, in overlay mode, for each of the nucleotides.
- For each nucleotide, indicate the wavelength of maximum absorption.

Part II: Please include the following

- Wavelength scan of both DNA and RNA on the same graph.
- What is the wavelength of maximum absorbance for DNA? RNA?
- Is the spectrum similar to the spectrum of the individual nucleotides? Why do you think that is?
- Calculate the extinction coefficient for DNA and RNA in mg/ml (remember the Beer-Lambert law?)

Part III. Please include the following:

- Data table
- Calculate the percentage increase in absorbance of the samples after heating to 50º, 70º, and boiling.
- Tell the difference between the hyperchromic effects of DNA and RNA, if any exist.
- Did the samples plunge on ice return to their pre-heated absorbances? Explain.

Part IV. Please include the following:

- Data table
- What impact did the NaOH have on the absorbance of the DNA sample at various temperatures?
- Compared to the NaCl, did the NaOH cause a hyperchromic shift?

7.4.3. Work 1. Quantitative determination of DNA in various tissues.

The EQUIPMENT: a FEC.

The PRINCIPLE of a METHOD.

The method is based on the determination of the amount of phosphorus which is integrated into the structure of DNA. After DNA mineralization, free inorganic phosphate is released. DNA is previously isolated from 10 mg of certain tissue (the spleen, liver, pancreas, skeletal muscle) and then is subjected to mineralization. A colorimetric technique is used to determine the mineralized tissue's inorganic phosphorus. The color reaction with ammonium molybdate in the presence of any reducing agents is used in this method. The final product of the reaction has dark blue color. The intensity of the color is directly proportional to the amount of phosphorus in a test.

The COURSE of WORK.

1. Add the reagents for the colorimetric determination of inorganic phosphate (0.5ml of 2.5% molybdic acid and 0.5ml of 10% ascorbic acid) to the whole volume of the ready mineralized tissue from the spleen, liver, pancreas or muscle.
2. In 10 minutes measure the optical density of the colored solution with a FEC (the thickness of cuvettes is 5mm) with the red light-filter (670 nm).
3. Calculate the quantity of phosphorus using calibrating graph built with the data of determination of optical density of the standard solutions of chemically pure KH_2PO_4. The value of the result, found with the calibrating graph, multiply by ten because DNA concentration is expressed as mg of phosphorus per 100 mg of the tissue.

7.4.4. The examples of the control task:

1.A sample of human DNA is subjected to increasing temperature until the major fraction exhibits optical density changes due to disruption of its helix (melting or denaturation). A smaller fraction is atypical in that it requires a much higher temperature for melting. This smaller, atypical fraction of DNA must contain a higher content of

a. Adenine plus cytosine

b. Cytosine plus guanine

c. Adenine plus thymine

d. Cytosine plus thymine

e. Adenine plus guanine

2.Which pyrimidine base contains a methyl group

A. Uracil

B. Adenine

C. Thymine

D. Cytosine

3.Nucleic acids show maximum absorbance at …. nm (pH7)

A. 260nm

B. 290nm

C. 300nm

D. 450nm

4.According to Chargaff's analysis

A. T=A

B. G=C

C. A+G=T=C

D. All the mentioned

5.Enzyme responsible for the formation of deoxyribonucleotides is

A. Ribonucleotide polymerase

B. ribonucleotide reductase

C. deoxyribonucleotide reductase

D. deoxy thioredoxinase

7.4.5. BASIC QUESTIONS FOR DISCUSSION:

1. Nucleic acids. The definition of the term. The chemical composition of nucleic acids, differences between DNA and RNA.

2. The primary and secondary structure of DNA. Nucleotides as monomers of a polynucleotide chain. The double helix. Complementarity of the nitrogen bases.

3. The structural organization of DNA in chromosomes. The chemical composition of chromatin. Proteins of chromatin. Histones, their classification, common physicochemical properties (charge, ability to form oligomers). Nucleosomes as the kind of DNA packing in chromosomes.

4. The primary and spatial structure of RNA. Functional classification of RNA and the distribution of different kinds of RNA in the cell compartments.

5. Biosynthesis of purine and pyrimidine bases

6.Difference between salvage and de novo pathways

7. Degradation of purine bases. Gout.

8. Degradation of pyrimidine bases. Orotic aciduria.

9. Regulation of de novo pathway for purine and pyrimidine bases.

10. Formation of deoxyribonucleotides.

11. Antimetabolites for the biosynthesis of purine and pyrimidine nucleotides.

7.5. HEMOGLOBIN

Hemoglobin is a conjugated iron containing a metalloprotein with four heme molecules linked to the protein portion called "globin." Globin part consists of 4 polypeptide chains. Normal hemoglobin consists of 2 alpha and 2 beta chains. Oxygen is bound to the ferrous (Fe2+) atoms of the heme to form oxyhemoglobin. Defect in the synthesis of hemoglobin results in the formation abnormal hemoglobin. The binding of oxygen to hemoglobin can be dramatically altered by a small group of substances called allosteric effectors. Hydrogen ions (protons), carbon dioxide, and 2, 3-bisphosphoglycerate are effectors that can promote the release of oxygen by favoring the deoxygenated form of hemoglobin. Hemoglobin can be differentiated by electrophoresis. Heme synthesis takes place in mitochondrion and cytosol which depends on succinyl CoA, glycine, ALA synthase and iron. Catabolism of heme results in bilirubin.

Mutations in the globin chain are associated with the hemoglobinopathies, such as sickle cell disease and thalassemia. The reduction in the globin chain synthesis results in thalassemia. The abnormal hemoglobin of sickle cell anemia was first demonstrated by Linus Pauling in 1949. Structurally, each globin chain has its own genetic locus. The individual chain of hemoglobin is under genetic control. The name derived from the Greek word, *Thalassa* which means "sea." Greeks inherited this disease present around Mediterranean Sea. Absence or diminished synthesis of one of the polypeptide chains of human hemoglobin is characterized as "thalassemia." The reduction in the α-chain synthesis is called α thalassemia and decreased synthesis of β-chain synthesis is called β-thalassemia. The α-thalassemia is more common.

Clinical problems associated with heme metabolism are of two types. Disorders that arise from defects in the enzymes of heme biosynthesis are termed the porphyrias and cause elevations in the serum and urine content of intermediates in heme synthesis.

The inherited disorder of bilirubin metabolism results in hyperbilirubinemia. Bilirubin is potentially toxic waste product of heme catabolism. The body eliminates bilirubin by transporting it to the liver bound to albumin in the serum. In the liver, it is conjugated with glucuronate which renders it water soluble. The glucuronide conjugate is then excreted in the bile. Persons with extreme elevation in unconjugated bilirubin are susceptible to bilirubin encephalopathy, also referred to as kernicterus.

Accumulation of bilirubin in the plasma and tissues results in jaundice. Gilbert's syndrome and Crigler-Najjar syndrome result from predominantly unconjugated hyperbilirubinemia. Dubin-Johnson syndrome and Rotor's syndrome result from conjugate hyperbilirubinemia. The porphyrias are both inherited and acquired disorders in heme synthesis. These disorders are classified as either erythroid or hepatic, depending upon the principal site of expression of the enzyme defect. Eight different porphyrias have been classified.

Decreased levels of hemoglobin, with or without an absolute decrease of red blood cells, leads to symptoms of anemia, that has many different causes. Absence of iron decreases heme synthesis. Red blood cells in iron deficiency anemia are hypochromic (lacking the red hemoglobin pigment) and microcytic (smaller than normal). Other anemias are rarer.

7.5.1. Work 1. Benzidine test for heme group of hemoglobin.

The PRINCIPLE of a METHOD.

The reaction is caused by a hemoglobin ability to catalyze the oxidation of benzidine (colorless substance) with hydrogen peroxide to the dark blue colored product.

The COURSE of WORK.

Add 5 drops of 0.2% alcohol solution of benzidine to 5 drops of diluted blood and then several drops of hydrogen peroxide. The liquid becomes dark blue or green colored.

7.5.2. Work 2. Teichmann test

This test determined if there is blood in the sample present or not. In most vertebrates, the hemoglobin molecule is an assembly of four globular protein subunits. Each subunit is composed of a protein chain tightly associated with a non-protein prosthetic heme group. Each protein chain arranges into a set of alpha-helix structural segments connected together in a globin fold arrangement. This folding pattern contains a pocket that strongly binds the heme group. Dry blood heating with glacial acetic acid is splitting hemoglobin into globin and hematin. At the presence of NaCl hematin is converting into hemin chloride derivative, which is making crystals after decreasing the temperature. The hematin crystals begin to form when heated blood is mixed with glacial acetic acid in the presence of salt. Test produces light brown/dark brown colored crystals that can reach up to 10 micrometers in size. Teichman's test is used in forensic science for determination of blood /blood traces/ in sample. In this sample hem is different from hemin, which contains Fe^{3+} and chloride. Twelve-year-old stains are capable of giving a positive result when tested by the Teichmann. Bloodstains /over 20 years were also able to give a positive with this test/. The sensitivity and specificity of the Takayama and the Teichmann tests are similar: both give positive results with as little as ml of blood or 0.1 mg of hemoglobin.

Equipment and reactive: blood /animal/, glacial CH_3COOH, condensed solution NaCl, glass rods, glass slides, spirit lamp, microscope.

Procedure: Fresh blood drop place on the glass slide. Dry it carefully on spirit lamp, hold the glass slide high, do not let sample boiling /low flame/. Add a few drops of glacial acetic acid to the dry sample, blend carefully with glass rod. Close the sample with glass coverslip and dry it again on the spirit fire. Add to the dry sample condensed NaCl /a few crystals/. If required, then dry it once more. Let the sample a few minutes to cool, after that watch under the microscope. The hemin chloride rhombic-shaped crystals formed due to the degradation of hemoglobin. If the crystals are not seen clearly or are not seen at all, open the glass slide, add a few drops of glacial acetic acid again and repeat the procedure.

If the blood trace is old, then the sample is prepared from the grater of the tissue where it’s found or together with that tissue /surface/.

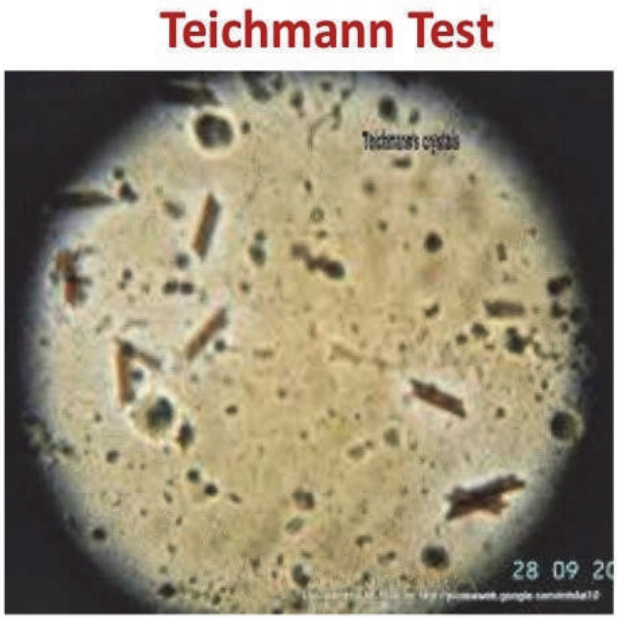


Fig. 56. Hemin chloride rhombic-shaped crystals formed due to the degradation of hemoglobin

7.5.3. BASIC QUESTIONS FOR DISCUSSION

1. Serum proteins, their functions.
2. The properties of serum proteins:
    a) albumin, its role in the organism,
    b) globulins, their features.
3. Synthesis of the blood proteins. The diagnostic significance of determination of protein concentration in blood. Pathological deviations of the normal blood plasma protein content.
4. Hemoglobin as the main protein of erythrocytes, its molecular organization.
5. The role of hemoglobin in the transport of oxygen and carbon dioxide. The mechanism of regulation of the affinity of hemoglobin to oxygen.
6. Abnormal varieties of hemoglobin, hemoglobinopathies.
7. Biosynthesis of heme.
8. Blood plasma enzymes, the diagnostic significance of enzymes activity detection in blood.
9. Degradation of heme.
10. Regulation of heme metabolism

7.5.4. The examples of the control task:

1. Heme synthesis starts in

1. Cytoplasm
2. Mitochondria
3. Both
4. None of the mentioned

2. Lead inhibits

1. ALA dehydratase
2. Ferrochelatase
3. Both
4. None of the mentioned

3.Which is NOT a hemoprotein

1. Catalase
2. NO-synthase
3. Cytochrome P 450
4. Aspartate aminotransferase

4.Heme synthesis intermediates are absorbing the wavelength around

1. A. right answer is missing
2. B.300-400 nm
3. C.700-800 nm
4. D.500-600 nm

5.Coenzyme for the amino levulinate synthase serves

1. Vit. B12
2. Vit. B6
3. Vit. B9
4. Vit. B1

Appendix

Appendix I

Properties of Common Acids and Bases

Compound	*Formula*	*Mol. weight*	*Specific gravity*	*% by Weight*	*Molarity (M)*
Acetic acid, glacial	CH_3COOH	60.1	1.05	99.5	17.4
Ammonium hydroxide	NH_4OH	35.0	0.89	28	14.8
Formic acid	HCOOH	46.0	1.20	90	23.4
Hydrochloric acid	HCl	36.5	1.18	36	11.6
Nitric acid	HNO_3	63.0	1.42	71	16.0
Per chloric acid	$HCLO_4$	100.5	1.67	70	11.6
Phosphoric acid	H_3PO_4	98.0	1.70	85	18.1
Sulfuric acid	H_2SO_4	98.1	1.84	96	18.0

Appendix II

Approximate pH and bicarbonate concentration in extracellular fluids

Fluid	*pH*	*meq HCO_3^-/liter*
Plasma	7.35-7.45	28
Cerebrospinal fluid	7.4	25
Saliva	6.4-7.4	10-20
Gastric secretion	1.0-2.0	0
Tears	7.0-7.4	5-25
Aqueous humor	7.4	28
Pancreatic juice	7.0-8.0	80
Sweat	4.5-7.5	0-10

Appendix III

PHOSPHATE BUFFER PREPARATION TABLE – 0.2 M SOLUTION

Potassium Phosphate Monobasic Anhydrous g/L	Sodium Phosphate Dibasic Heptahydrate g/L	23 °C pH	Potassium Phosphate Monobasic Anhydrous g/L	Sodium Phosphate Dibasic Heptahydrate g/L	23 °C pH
22.4	3.49	5.7	10.80	29.51	6.9
22.08	4.29	5.8	9.36	32.73	7.0
21.60	5.37	5.9	7.92	35.95	7.1
21.05	6.60	6.0	6.72	38.63	7.2
20.40	8.05	6.1	5.52	41.31	7.3
19.56	9.93	6.2	4.56	43.46	7.4
18.60	12.07	6.3	3.84	45.07	7.5
17.64	14.22	6.4	3.12	46.68	7.6
16.44	16.90	6.5	2.52	48.55	7.7
15.00	20.12	6.6	2.04	49.09	7.8
13.56	23.34	6.7	1.68	49.89	7.9
12.24	26.29	6.8	1.27	50.81	8.0

"

Appendix IV

CITRIC ACID – Na_2HPO_4 BUFFER PREPARATION, PH 2.6–7.6

Citric acid monohydrate, $C_6H_8O_7 \cdot H_2O$, MW 210.14; 0.1 M contains 21.01 g/L. Na_2HPO_4, MW 141.98; 0.2 M contains 28.40 g/L, or $Na_2HPO_4 \cdot 2H_2O$, MW 178.05; 0.2 M contains 35.61 g/L. x mL 0.1 M-citric acid and y mL 0.2 M-Na_2HPO_4 mixed

pH	x mL 0.1 M-citric acid	y mL 0.2-Na_2HPO_4
2.6	89.10	10.90
3.0	79.45	20.55
3.6	67.80	32.20
3.8	64.50	35.50
4.0	61.45	38.55
4.4	55.90	44.10
4.8	50.70	49.30
5.0	48.50	51.50
5.4	44.25	55.75
5.6	42.00	58.00
6.0	36.85	63.15
6.2	33.90	66.10
6.8	22.75	77.25
7.0	17.65	82.35
7.4	9.15	90.85
7.6	6.35	93.65

Appendix V

CITRIC ACID – SODIUM CITRATE BUFFER PREPARATION, PH 3.0-6.2

Citric acid monohydrate, $C_6H_8O_7 \cdot H_2O$, MW 210.14; 0.1 M contains 21.01 g/L. Trisodium citrate dihydrate, $C_6H_5O_7Na_3 \cdot 2H_2O$, MW 294.12; 0.1 M contains 29.41 g/L.
x mL 0.1 M-citric acid and y mL 0.1 M-trisodium citrate mixed

pH	x mL 0.1 M-citric acid	y mL 0.1 M-trisodium citrate
3.0	82.0	18.0
3.2	77.5	22.5
3.6	68.5	31.5
3.8	63.5	36.5
4.0	59.0	41.0
4.2	54.0	46.0
4.4	49.5	50.5
4.8	40.0	60.0
5.0	35.0	65.0
5.2	30.5	69.5
5.4	25.5	74.5
5.6	21.0	79.0
5.8	16.0	84.0
6.0	11.5	88.5
6.2	8.0	92.0

Appendix VI

SODIUM ACETATE – ACETIC ACID BUFFER PREPARATION, PH 3.7–5.6

Sodium acetate trihydrate, $CH_3COONa \bullet 3H_2O$, MW 136.09; 0.2 M contains 27.22 g/L. x mL 0.2 M-NaOAc and y mL 0.2 M-HOAc mixed

pH, 18 °C	x mL 0.2 M-NaOAc	y mL 0.2 M-HOAc
3.7	10.0	90.0
3.8	12.0	88.0
4.0	18.0	82.0
4.2	26.5	73.5
4.4	37.0	63.0
4.6	49.0	51.0
4.8	59.0	41.0
5.0	70.0	30.0
5.2	79.0	21.0
5.4	86.0	14.0
5.6	91.0	9.0

Appendix VII

NA_2HPO_4 – NAH_2PO_4 BUFFER PREPARATION, PH 5.8-8.0 AT 25 °C[1]

$Na_2HPO_4 \cdot 2H_2O$, MW 178.05; 0.2 M contains 35.61 g/L. $Na_2HPO_4 \cdot 12H_2O$, MW 358.22; 0.2 M contains 71.64 g/L. $NaH_2PO_4 \cdot H_2O$, MW 138.01; 0.2 M contains 27.6 g/L. $NaH_2PO_4 \cdot 2H_2O$, MW 156.03; 0.2 M contains 31.21 g/L.

x mL 0.2 M-Na_2HPO_4, y mL 0.2 M-NaH_2PO_4; diluted to 100 mL with H_2O

pH, 25 °C	x mL 0.2 M-Na_2HPO_4	y mL 0.2 M-NaH_2PO_4
5.8	4.0	46.0
6.0	6.15	43.85
6.2	9.25	40.75
6.4	13.25	36.75
6.6	18.75	31.25
6.8	24.5	25.5
7.0	30.5	19.5
7.2	36.0	14.0
7.4	40.5	9.5
7.6	43.5	6.5
7.8	45.75	4.25
8.0	47.35	2.65

Appendix VIII

IMIDAZOLE (GLYOXALINE) – HCL BUFFER PREPARATION, PH 6.2–7.8 AT 25 °C
Imidazole, $C_3H_4N_2$, MW 68.08

25 mL 0.2 M-imidazole (13.62 g/L), x mL 0.2 M-HCl, diluted to 100 mL with H_2O

pH, 25 °C	x mL 0.2 M-HCl
6.2	21.45
6.4	19.9
6.6	17.75
6.8	15.2
7.0	12.15
7.2	9.3
7.4	6.8
7.6	4.65
7.8	3.0

"

Appendix IX

SODIUM CARBONATE – SODIUM BICARBONATE BUFFER PREPARATION, PH 9.2-10.8

$Na_2CO_3 \cdot 10H_2O$, MW 286.2; 0.1 M contains 28.62 g/L. $NaHCO_3$, MW 84.0; 0.1 M contains 8.40 g/L.
x mL 0.1 M-Na_2CO_3 and y mL 0.1 M-Na_2HCO_3 mixed

20 °C	**37 °C**		
x mL 0.1 M-Na_2CO_3	**y mL 0.1 M-Na_2HCO_3**		
9.2	8.8	10	90
9.4	9.1	20	80
9.5	9.4	30	70
9.8	9.5	40	60
9.9	9.7	50	50
10.1	9.9	60	40
10.3	10.1	70	30
10.5	10.3	80	20
10.8	10.6	90	10

BUFFER PREPARATION FORMULAS AND EQUATIONS

Percentage by weight (w/v)

(% buffer desired / 100) × final buffer volume (mL) = g of starting material needed.

Molar Solutions

desired molarity × formula weight × solution final volume (L) = grams needed

Henderson-Hasselbach Equation

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

The EXAMINATION QUESTIONS

1. Proteins, their types and classification.
2. Amino acids as monomers of a protein molecule.
3. Simple and complex proteins.
4. Chemical composition of nucleoproteins, lipoproteins, glycoproteins, phosphoproteins, chromoproteins, metalloproteins.
5. The primary structure of proteins. Peptide bond. The kinds of intramolecular bonds in proteins.
6. Denaturation of proteins.
7. Allosteric enzymes.
8. Biological functions of proteins.
9. Specific interactions of proteins with the ligands. The kinds of natural ligands and peculiarities of their interactions with the proteins (prosthetic groups, cofactors, protomers, substrates, transported substances, allosteric effectors).
10. Enzymes as biological catalysts.
11. The mechanism of catalytic action of enzymes.
12. Specificity of enzyme action. The enzyme cofactors.
13. The dependence of the velocity of enzyme reactions on substrate and enzyme concentrations, temperature, and pH.
14. Kinetics of enzyme reactions. The principles of quantitative determination of enzyme activity.
15. The enzyme inhibitors: reversible, irreversible, competitive, noncompetitive.
16. The role of enzymes in metabolism. Diversity of enzymes.
17. Determination of enzyme activities for the diagnosis of the diseases (LDH, creatine kinase, Aspartate aminotransferase).
18. Regulation of enzyme activity. The enzyme modification: partial proteolysis, chemical modification, allosteric regulation.
19. The primary and secondary structure of DNA. The primary and secondary structure of RNA.
20. The kinds of RNA: the peculiarities of their structure, size, form, cell location, and function.
21. Biochemistry of digestion. The enzymes of digestion of carbohydrates, lipids, proteins, nucleic acids.
22. The enzyme composition of various digestive juices (saliva, gastric, pancreatic intestinal juices).
23. The mechanism of action of digestive enzymes.
24. Absorption of the digestion products.

25. ATP and the other high energy compounds. Basic pathways of ATP phosphorylation and ATP utilization.
26. NAD-linked dehydrogenases. The structure of oxidized and reduced forms of NAD. The main substrates of NAD-linked dehydrogenases.
27. NADH dehydrogenase and the electron carriers of an inner membrane of mitochondria.
28. FAD-linked dehydrogenases: succinate dehydrogenase, acyl-CoA dehydrogenase.
29. The further way of electrons in a respiratory chain. Oxidative phosphorylation.
30. The structural organization of a mitochondrial respiratory chain: the enzyme complexes of a respiratory chain.
31. The tricarboxylic acid cycle (the TCA cycle, the Krebs cycle): the reaction sequence, relation to a respiratory chain, regulation.
32. Acetyl CoA: the ways of the formation and utilization in the body.
33. The aerobic degradation of glucose: the reaction sequence, physiological significance.
34. The anaerobic splitting of glucose: the reaction sequence, physiological significance.
35. The glucose biosynthesis (gluconeogenesis): the probable precursors, reaction sequence. The significance and regulation of gluconeogenesis.
36. The glucose-lactate cycle (the Cory cycle).
37. The glycogen synthesis and its degradation, biological significance.
38. The regulation of the phosphorylase activity and glycogen synthesis. The glycogen metabolism disturbances.
39. The pentose phosphate pathway of the glucose transformation
40. The role of the pentose phosphate pathway.
41. Regulation of the blood glucose concentration. The origin of blood glucose.
42. The main lipids of the body, their structure. Classification of lipids.
43. Fatty acid oxidation. The sequence of the oxidation reactions.
44. The physiological significance of the fatty acid oxidation.
45. The lipid biosynthesis in the liver and in adipose tissue.
46. Resynthesis of neutral fat in intestine cells.
47. The transport blood lipoproteins, specificity of their structure, composition, and function. CM, VLDL, LGL, HDL. Their role in the fat and cholesterol metabolism.
48. Apoproteins, their classification, participation in the construction and metabolism of lipoproteins.
49. Storage and mobilization of neutral fat in adipose tissue. Physiological significance of the fat storage.
50. Biosynthesis and utilization of ketone bodies.

51. The cholesterol synthesis: the reaction sequence, regulation of the biosynthesis. The role of lipoproteins in the cholesterol transport.
52. The integration of the carbohydrate, lipid, and protein metabolism.
53. The role of the pentose phosphate pathway of glucose metabolism to the lipid synthesis.
54. The mechanisms of transport of substances across a membrane.
55. Transamination of amino acids. The specificity of aminotransferases. The significance of the reactions of transamination.
56. Catabolism of amino acid.
57. The formation and detoxication of ammonia. The urea biosynthesis, the reaction sequence.
58. The phenylalanine and tyrosine metabolism. The tyrosine utilization for the catecholamines.
59. Decarboxylation of amino acids and their derivatives. energy metabolism.
60. Absorption of the dietary amino acids. Essential and non-essential amino acids.
61. A purine nucleotides biosynthesis: the origin of C- and N-atoms of a purine ring and the role of 5'-phosphoribosyl-1-pyrophosphate (PRPP).
62. The catabolism of purine nucleotides. Hyperuricemia and podagra (gout).
63. The biosynthesis and catabolism of pyrimidine nucleotides.
64. The biosynthesis of deoxyribonucleotides.
65. The classification of hormones according to their chemical structure: protein and peptide hormones, derivatives of amino acids, steroid hormones.
66. The mechanism of transference of hormone signal in a cell.
67. Trimeric G-Protein coupled receptors
68. The adenylate cyclase pathway
69. The phosphatidyl inositol pathway
70. The most important mechanisms of detoxication of poisoning substances in the liver
71. The degradation of heme. the formation of bilirubin and bilirubin glucuronide. The ways of the bilirubin and other bile pigments excretion.
72. Synthesis of heme. The most important proteins of extracellular matrix: collagen and elastin. Posttranslational modifications of collagen, the formation of fibrillar structures. The participation of vitamin C in the collagen synthesis.
73. Transport through cell membranes, simple diffusion
74. Transport through cell membranes, facilitated diffusion
75. Transport through cell membranes, active transport
76. Regulation of heme synthesis
77. Impairment of cell signaling during neoplasms
78. Chemical composition of mineralized tissues. Apatites.
79. The main bone and tooth proteins.

80. Collagen. Processing of collagen.
81. The biochemical character of pulp. Mineralization of bone and tooth tissue.
82. The influence of a diet on the state of teeth. The role of dietary carbohydrates, proteins, microelements, and vitamins in the maintenance of a normal physiological state of teeth.
83. Microelements: fluorine and strontium, their role in mineralization of bone and tooth tissue.
84. The physiological role of saliva. Gingival liquid. The peculiarities of its chemical composition.
85. Proteins of saliva. Salivary enzymes
86. Inorganic components of saliva: cation and anion composition of saliva.
87. Calcium and phosphorus. Their distribution in stimulated and unstimulated saliva.

LITERATURE

1. Joy P P, Surya S. and Aswathy C.- KERALA AGRICULTURAL UNIVERSITY, Laboratory manual of Biochemistry, 2015
2. Rodney Boyer-Biochemistry Laboratory: Modern theory and techniques, 2nd edition, 2012
3. DM Vasudevan, Subir Kumar Das-Practical textbook of biochemistry for medical students, 2nd edition, 2013
4. Cheremisinoff, N. P., & Davletshina, T. A. (2013). *Fire and explosion hazards handbook of industrial chemicals*. Elsevier
5. Richmond, J. Y., & McKinney, R. W. (2009). *Biosafety in microbiological and biomedical laboratories*. US Government Printing Office
6. Chemical Hazards of the Workplace. Proctor, N. and J. Hughes. J.B. Lippincott Co., Philadelphia, PA (latest edition).
7. Prudent Practices for Handling Hazardous Chemicals in Laboratories. National Research Council Committee on Hazardous Substances in the Laboratory. National Academy Press, Washington, D.C. (latest edition).
8. Dangerous Properties of Industrial Materials. Sax, N. Irving. Van Nostrand Reinhold, NewmYork, NY (latest edition).
9. CRC Handbook of Laboratory Safety, Steere, N. ed. CRC Press, Inc., Boca Raton, FLAn(latest edition).
10. The Merck Index. Windholz, M., ed. Merck and Co. Inc., Rahway, N.J. (latest edition).
11. Nelson, D. L., Lehninger, A. L., & Cox, M. M. (2008). *Lehninger principles of biochemistry*. Macmillan.
12. Moran, L. A., Horton, R. A., Scrimgeour, K. G., & Perry, M. D. (2014). *Principles of biochemistry*.
13. Vasudevan, D. M., Sreekumari, S., & Vaidyanathan, K. (2016). *Textbook of biochemistry for medical students*. JP Medical Ltd.
14. Shivananda Nayak B, MSc PhD, Essentials of Biochemistry (for medical students), 2nd edition, 2013

Չափսը՝ 70x100, 1/16:
Ծավալը 10.25 տպագրական մամուլ:
Տպաքանակը՝ 100 օրինակ:
Տպագրվել է «Ոսկան Երևանցի» տպագրատանը:

ACKNOWLEDGEMENTS

We acknowledge with gratitude the generous help we have received from the University of Traditional Medicine in Armenia, Campus International LLC and the editors of this manual. Further suggestions towards the improvement of this manual are welcomed.