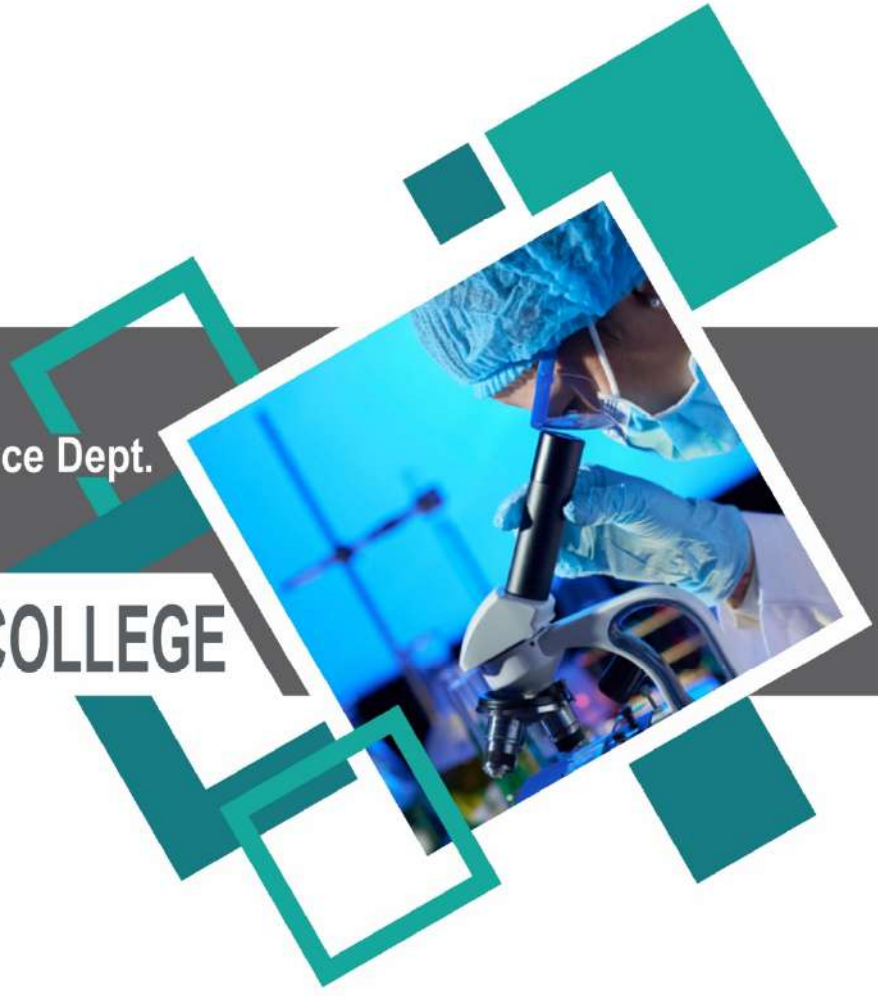
BACHELOR OF MEDICAL LABORATORY TECHNOLOGY LAB MANUAL

1st Year



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Paper 102 (Practical)

EXPERIMENT 1. Microscopy - Light microscopy, Bright & Dark Field microscopy, Fluorescence microscopy, Phase Contrast microscopy, Concept of Electron Microscopy

Microscope is the instrument, the most important characteristic of microbiology laboratories. The magnification, it provides, enables us to see microorganisms and their structures otherwise invisible to the naked eye. The magnitudes attainable by microscopes range 100X- 400000X. A microscope may be defined as an optical instrument, consisting of a lens or a combination of lenses, for making enlarged or magnified images of minute objects. (*Micro*: small; *scope*: to view).

Antony Von Leeuwenhoek is considered to be the first person who has seen a microorganism through a simple microscope made by him with a magnification of 270-480 times. He described the size, shape, movements of bacteria, protozoa and algae. These findings were later confirmed after the development of compound microscope by Robert Hooke. The characteristic morphological studies enabled by the discovery of powerful microscopes, helped the scientists to classify microorganisms. Later improvements in the compound microscopes were made and Amici discovered oil immersion lens, which enabled the scientists to study the characteristics more minutely.

Microscopes are continuously improved to enable us to have higher magnifications and better resolutions. Microscopes are of two categories: Light or optical microscopes and Electron microscopes depending upon the principle on which the magnification is based. Light microscopy, in which the magnification is obtained by a system of optical lenses uses light waves .The light microscopy includes bright field microscopy, dark-field microscopy, fluorescence microscopy and phase contrast microscopy. On the other hand the electron microscopy uses a beam of electrons in place of light waves for visualization of objects .This includes transmission electron microscopy and scanning electron microscopy.

Light microscopy

Light microscopy is the simplest form of microscopy. It has tools that are used to observe the small organisms or object and even macromolecules. It has wide variety of microscopic tools for studying the biomolecules and biological processes. It includes all forms of microscopic methods that use electromagnetic radiation to achieve magnification.

Instrumentation of a typical light microscope- The typical diagram of a light microscope is given below. The light is produced by a lamp (with tungeston filament) as source and light rays are focused on the specimen by the condenser. The specimen is kept on the stage and firmed by clipped present on the side. The light diffracted by the sample is then collected by the objective lens (objective lens varies from 10x-100x magnification) and additional magnification is achieved by the eyepiece (usually gives additional 10x magnification). Hence, if you observe a sample with 40x objective lens, microscope is actually magnifying the object by 400x (40x from objective and 10x from the eye piece, 40x10=400x).

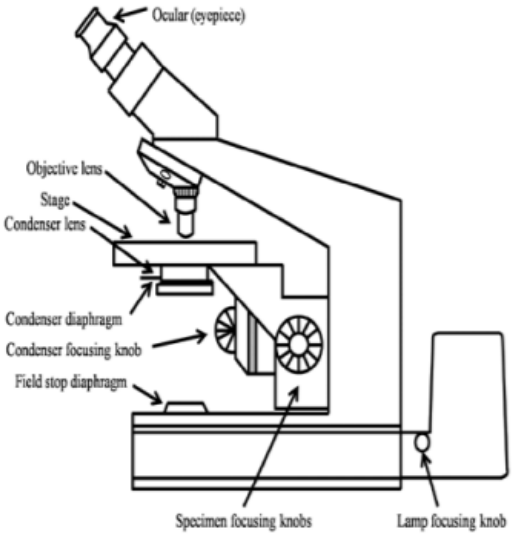


Figure. Instrumentation of a typical light (binocular) microscope with its different components.

Bright & Dark Field microscopy

The brightfield microscope, perhaps the most commonly used type of microscope, is a compound microscope with two or more lenses that produce a dark image on a bright background. Some brightfield microscopes are monocular (having a single eyepiece), though most newer brightfield microscopes are binocular (having two eyepieces), like the one shown in Figure 1; in either case, each eyepiece contains a lens called an ocular lens. The ocular lenses typically magnify images 10 times (10×). At the other end of the body tube are a set of objective lenses on a rotating nosepiece. The magnification of these objective lenses typically ranges from 4× to 100×, with the magnification for each lens designated on the metal casing of the lens. The ocular and objective lenses work together to create a magnified image. The total magnification is the product of the ocular magnification times the objective magnification: ocular magnification × objective magnification ocular magnification × objective magnification ocular magnification × objective magnification ocular magnification × objective magnification

For example, if a 40×40× objective lens is selected and the ocular lens is 10×10×, the total magnification would be

(40×)(10×)=400×(40×)(10×)=400×

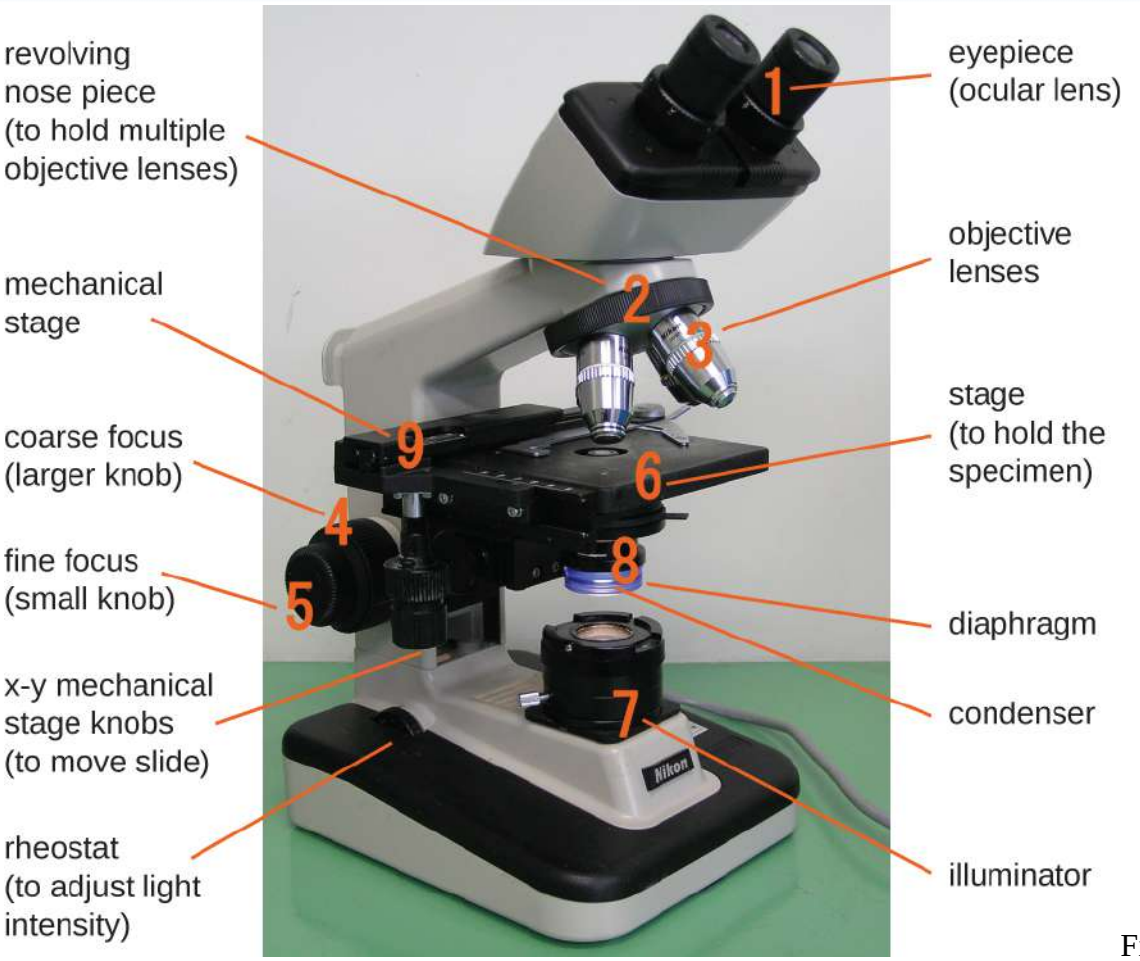


Figure 1: Components of a typical brightfield microscope.

Components of a typical brightfield microscope.

The item being viewed is called a specimen. The specimen is placed on a glass slide, which is then clipped into place on the stage (a platform) of the microscope. Once the slide is secured, the specimen on the slide is positioned over the light using the x-y mechanical stage knobs. These knobs move the slide on the surface of the stage, but do not raise or lower the stage. Once the specimen is centered over the light, the stage position can be raised or lowered to focus the image. The coarse focusing knob is used for large-scale movements with 4× and 10× objective lenses; the fine focusing knob is used for small-scale movements, especially with 40× or 100× objective lenses.

When images are magnified, they become dimmer because there is less light per unit area of image. Highly magnified images produced by microscopes, therefore, require intense lighting. In a brightfield microscope, this light is provided by an illuminator, which is typically a high-intensity bulb below the stage. Light from the illuminator passes up through condenser lens (located below the stage), which focuses all of the light rays on the specimen to maximize illumination. The position of the condenser can be optimized using the attached condenser focus knob; once the optimal distance is established, the condenser should not be moved to adjust the brightness. If less-than-maximal light levels are needed, the amount of light striking the specimen can be easily adjusted by opening or closing a diaphragm between the condenser and the specimen. In some cases, brightness can also be adjusted using the rheostat, a dimmer switch that controls the intensity of the illuminator.

A brightfield microscope creates an image by directing light from the illuminator at the specimen; this light is differentially transmitted, absorbed, reflected, or refracted by different structures. Different colors can behave differently as they interact withchromophores (pigments that absorb and reflect particular wavelengths of light) in parts of the specimen. Often, chromophores are artificially added to the specimen using stains, which serve to increase contrast and resolution. In general, structures in the specimen will appear darker, to various extents, than the bright background, creating maximally sharp images at magnifications up to about 1000×. Further magnification would create a larger image, but without increased resolution. This allows us to see objects as small as bacteria, which are visible at about 400× or so, but not smaller objects such as viruses.

At very high magnifications, resolution may be compromised when light passes through the small amount of air between the specimen and the lens. This is due to the large difference between the refractive indices of air and glass; the air scatters the light rays before they can be focused by the lens. To solve this problem, a drop of oil can be used to fill the space between the specimen and an oil immersion lens, a special lens designed to be used with immersion oils. Since the oil has a refractive index very similar to that of glass, it increases the maximum angle at which light leaving the specimen can strike the lens. This increases the light collected and, thus, the resolution of the image (Figure 2). A variety of oils can be used for different types of light.

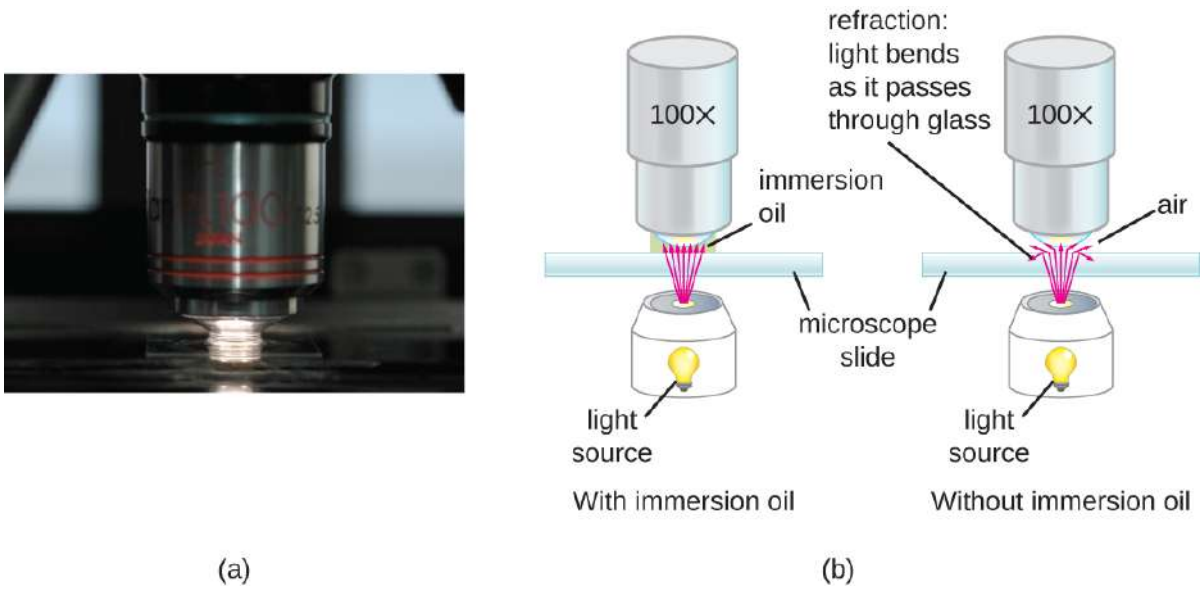


Figure 2. (a) Oil immersion lenses like this one are used to improve resolution. (b) Because immersion oil and glass have very similar refractive indices, there is a minimal amount of refraction before the light reaches the lens. Without immersion oil, light scatters as it passes through the air above the slide, degrading the resolution of the image.

MICROSCOPE MAINTENANCE: BEST PRACTICES

Even a very powerful microscope cannot deliver high-resolution images if it is not properly cleaned and maintained. Since lenses are carefully designed and manufactured to refract light with a high degree of precision, even a slightly dirty or scratched lens will refract light in unintended ways, degrading the image of the specimen. In addition, microscopes are rather delicate instruments, and great care must be taken to avoid damaging parts and surfaces. Among other things, proper care of a microscope includes the following:

- cleaning the lenses with lens paper
- not allowing lenses to contact the slide (e.g., by rapidly changing the focus)
- protecting the bulb (if there is one) from breakage
- not pushing an objective into a slide
- not using the coarse focusing knob when using the 40× or greater objective lenses
- only using immersion oil with a specialized oil objective, usually the 100× objective
- cleaning oil from immersion lenses after using the microscope
- cleaning any oil accidentally transferred from other lenses
- covering the microscope or placing it in a cabinet when not in use

Darkfield Microscopy

A **darkfield microscope** is a brightfield microscope that has a small but significant modification to the condenser. A small, opaque disk (about 1 cm in diameter) is placed between the illuminator and the condenser lens. This opaque light stop, as the disk is called, blocks most of the light from the illuminator as it passes through the condenser on its way to the objective lens, producing a hollow cone of light that is focused on the specimen. The only light that reaches the objective is light that has been refracted or reflected by structures in the specimen. The resulting image typically shows bright objects on a dark background (Figure 3).

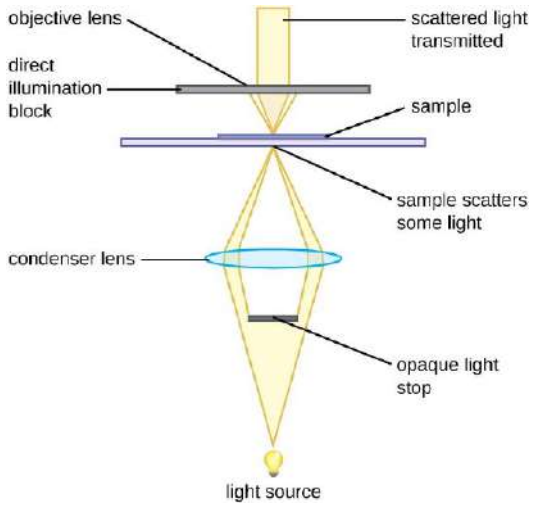


Figure 3. An opaque light stop inserted into a brightfield microscope is used to produce a darkfield image. The light stop blocks light traveling directly from the illuminator to the objective lens, allowing only light reflected or refracted off the specimen to reach the eye.

An opaque light stop inserted into a brightfield microscope is used to produce a darkfield image. The light stop blocks light traveling directly from the illuminator to the objective lens, allowing only light reflected or refracted off the specimen to reach the eye.

Darkfield microscopy can often create high-contrast, high-resolution images of specimens without the use of stains, which is particularly useful for viewing live specimens that might be killed or otherwise compromised by the stains.

Fluorescence microscopy

Principle: To observe the sample through a fluorescence microscope, it should be first labeled with a fluorescent dyes/substance known as a fluorophore. Higher energy light shorter wavelength of lights (UV rays or blue light) generated from mercury vapor arc lamp passes through the excitation filter which allows only the short wavelength of light to pass through and removes all other non-specific wavelengths of light. The filtered light is reflected by the dichroic filter and falls on the sample (i.e. fluorophore-labeled). The fluorochrome absorbs shorter wavelength rays and emits rays of longer wavelength (lower energy) that passes through the emission filter. The emission filter blocks (suppresses) any residual excitation light and passes the desired longer emission wavelengths to the detector. Thus the microscope forms glowing images of the fluorochrome-labeled microorganisms against a dark background. To the observer, the background is dark, as there is no visible light and only the labelled specimen (cells, microorganisms etc.) appear bright (fluoresce)

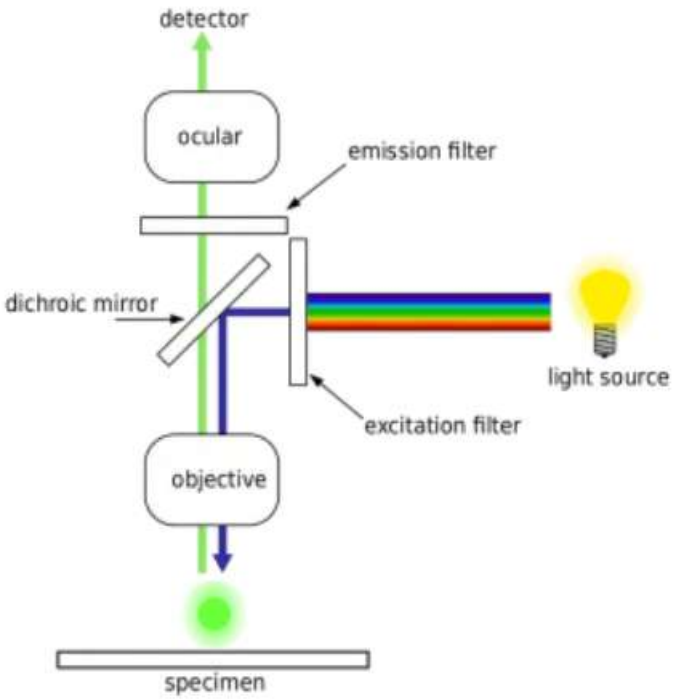


Fig. Working mechanism of fluorescence microscope

Typical components of a fluorescence microscope are:

Fluorescent dyes (Fluorophore)

A fluorophore is a fluorescent chemical compound that can re-emit light upon light excitation.

Fluorophores typically contain several combined aromatic groups, or plane or cyclic molecules with several π bonds.

Many fluorescent stains have been designed for a range of biological molecules.

Some of these are small molecules that are intrinsically fluorescent and bind a biological molecule of interest. Major examples of these are nucleic acid stains like DAPI and Hoechst, phalloidin which is used to stain actin fibers in mammalian cells.

A light source

Four main types of light sources are used, including xenon arc lamps or mercury-vapor lamps with an excitation filter, lasers, and high- power LEDs.

Lasers are mostly used for complex fluorescence microscopy techniques, while xenon lamps, and mercury lamps, and LEDs with a dichroic excitation filter are commonly used for wide-field epifluorescence microscopes.

The excitation filter

The exciter is typically a bandpass filter that passes only the wavelengths absorbed by the fluorophore, thus minimizing the excitation of other sources of fluorescence and blocking excitation light in the fluorescence emission band.

The dichroic mirror

A dichroic filter or thin-film filter is a very accurate color filter used to selectively pass light of a small range of colors while reflecting other colors.

The emission filter.

The emitter is typically a bandpass filter that passes only the wavelengths emitted by the fluorophore and blocks all undesired light outside this band – especially the excitation light.

By blocking unwanted excitation energy (including UV and IR) or sample and system autofluorescence, optical filters ensure the darkest background.

Types of Fluorescence Microscopes

There are various types of fluorescence microscopes. Some of the common types are:

Epifluorescence microscopes: The most common type of fluorescence microscope in which, excitation of the fluorophore and detection of the fluorescence are done through the same light path (i.e. through the objective).
Confocal microscope: In this type of fluorescence microscope, high-resolution imaging of thick specimens (without physical sectioning) can be analyzed using fluorescent-labeled dye.
Multiphoton microscope: In this type of microscope, multiphoton fluorescence excitation results in the capture of high-resolution three-dimensional images of specimen tagged with highly specific fluorophores.
Total internal reflection fluorescence (TIRF) microscope: Total internal reflection fluorescence microscopy (TIRFM) exploits the unique properties of an induced evanescent wave or field in a limited specimen region immediately adjacent to the interface between two media having different refractive indices.

Applications of Fluorescence Microscope

Fluorescence microscopy is widely used in diagnostic microbiology and in microbial ecology (for enumerating bacteria in natural environments).

a. Detection of acid-fast bacilli (AFB) in sputum or CSF when stained with auramine fluorescent dye.
b. Detection of *Trichomonas vaginalis*, intracellular gonococci, and other parasites when stained by acridine orange.
c. In immunodiagnosis of infectious diseases, using both direct and indirect antibody techniques.

Phase contrast microscopy

Principle

The phase contrast microscopy is based on the principle that small phase changes in the light rays, induced by differences in the thickness and refractive index of the different parts of an object, can be transformed into differences in brightness or light intensity. Contrast is defined as the difference in light intensity between the image and the adjacent background relative to the overall background intensity.

When light passes through cells, small phase shifts occur, which are invisible to the human eye. In a phase-contrast microscope, these phase shifts are converted into changes in amplitude, which can be observed as differences in image contrast. The basic principle to making phase changes visible in phase-contrast microscopy is to separate the illuminating (background) light from the specimen-scattered light and to manipulate these differently.

The ring-shaped illuminating light that passes the condenser annulus is focused on the specimen by the condenser. Some of the illuminating light is scattered by the specimen. The remaining light is unaffected by the specimen and forms the background light. When observing an unstained biological specimen, the scattered light is weak and typically phase-shifted by −90° (due to both the typical thickness of specimens and the refractive index difference between biological tissue and the surrounding medium) relative to the background light. This leads to the foreground and background having nearly the same intensity, resulting in low image contrast.

In a phase-contrast microscope, image contrast is increased in two ways, by generating constructive interference between scattered and background light rays in regions of the field of view that contain the specimen, and by reducing the amount of background light that reaches the image plane. First, the background light is phase- shifted by −90° by passing it through a phase-shift ring, which eliminates the phase difference between the background and the scattered light rays.

When the light is focused on the image plane (where a camera or eyepiece is placed), this phase shift causes background and scattered light rays originating from regions of the field of view that contain the sample (i.e., the foreground) to constructively interfere, resulting in an increase in the brightness of these areas compared to regions that do not contain the sample. Finally, the background is dimmed ~70-90% by a gray filter ring. This method maximizes the amount of scattered light generated by the illumination (i.e., background) light, while minimizing the amount of illumination light that reaches the image plane. Some of the scattered light (which illuminates the entire surface of the filter) will be phase-shifted and dimmed by the rings, but to a much lesser extent than the background light (which only illuminates the phase-shift and gray filter rings). This is the feature of negative phase contrast. In its positive form, the background light is instead phase-shifted by +90°. The background light will thus be 180° out of phase relative to the scattered light. The scattered light will then be subtracted from the background light to form an image with a darker foreground and a lighter background. Thus, there are two main types of phase contrast, positive and negative. Since the observed particles are usually thin and transparent, these polar contrasts provide strikingly different images. Positive phase contrast reveals medium to dark gray images on a lighter grey background; these images often have a bright halo along the edge of the sample. Negative phase contrast is the opposite. The specimen appears lighter with a dark background; they also have a dark halo outlining the image.

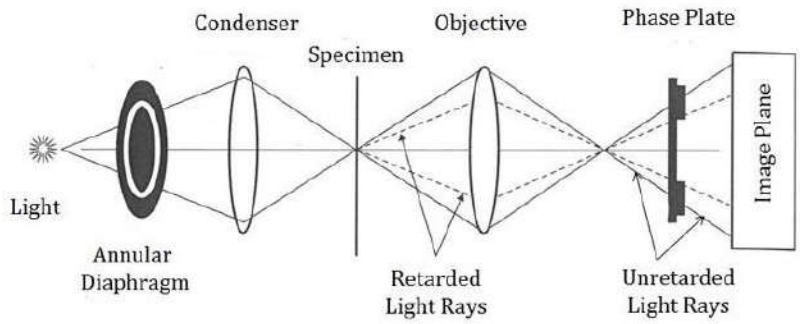


Fig: Phase contrast microscope

Applications of Phase Contrast Microscope

Phase-contrast microscopy is particularly important in biology. It reveals many cellular structures that are not visible with a simpler bright-field microscope. These structures were made visible to earlier microscopists by staining, but this required additional preparation and thus killing the cells. The phase-contrast microscope made it possible for biologists to study living cells and how they proliferate through cell division. It is one of the few methods available to quantify cellular structure and components that does not use fluorescence. Phase Contrast Microscopy is used to produce high-contrast images of transparent specimens, such as -

1. living cells (usually in culture),
2. microorganisms,
3. thin tissue slices,
4. lithographic patterns,
5. fibers,
6. latex dispersions,
7. glass fragments, and
8. subcellular particles (including nuclei and other organelles).

Phase-contrast microscopy is used in numerous biological researches.

Advantages of Phase Contrast Microscope

- Living cells can be observed in their natural state without previous fixation or labeling.
- It makes a highly transparent object more visible.
- No special preparation of fixation or staining etc. is needed to study an object under a phase-contrast microscope which saves a lot of time.
- Examining intracellular components of living cells at relatively high resolution.

  e.g. The dynamic motility of mitochondria, mitotic chromosomes & vacuoles.
- It made it possible for biologists to study living cells and how they proliferate through cell division.

- Phase-contrast optical components can be added to virtually any bright field microscope, provided the specialized phase objectives conform to the tube length.

Limitations of Phase Contrast Microscope

- Phase-contrast condensers and objective lenses add considerable cost to a microscope, and so phase contrast is often not used in teaching labs except perhaps in classes in the health professions.
- To use phase-contrast the light path must be aligned.
- Generally, more light is needed for phase contrast than for corresponding bright- field viewing, since the technique is based on the diminishment of the brightness of most objects.

EXPERIMENT 2. Sterilization: Principles & operations – Autoclave, Hot air oven, Filtration, Laminar Air Flow / BioSafety cabinet.

Sterilization describes a process that destroys or eliminates all forms of microbial life and is carried out in health-care facilities by physical or chemical methods.

The various methods of sterilization are:

1. Physical Method

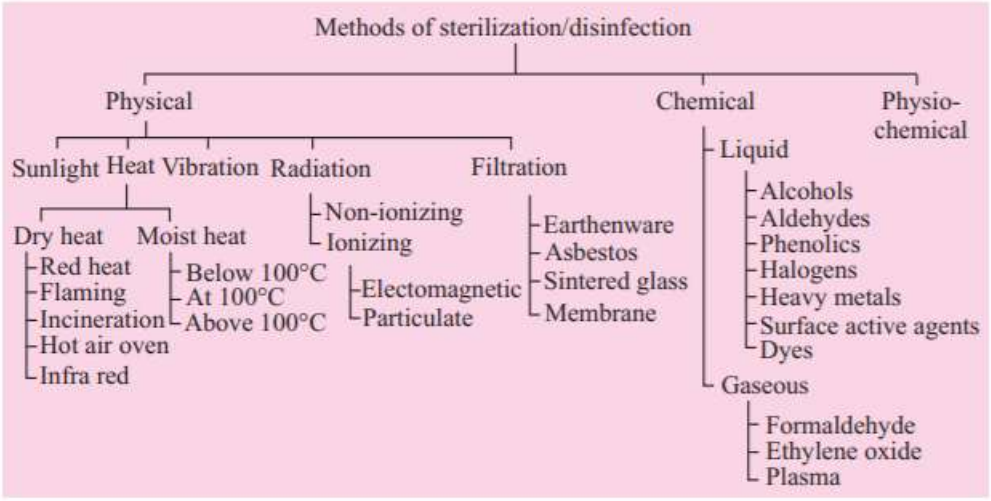
(a) Thermal (Heat) methods

(b) Radiation method

(c) Filtration method

2. Chemical Method

3. Gaseous method



Heat Sterilization

Heat sterilization is the most widely used and reliable method of sterilization, involving destruction of enzymes and other essential cell constituents. The process is more effective in hydrated state where under conditions of high humidity, hydrolysis and denaturation occur, thus

lower heat input is required. Under dry state, oxidative changes take place, and higher heat input is required.

This method of sterilization can be applied only to the thermostable products, but it can be used for moisture-sensitive materials for which dry heat (160-180°C) sterilization, and for moisture-resistant materials for which moist heat(121-134°C) sterilization is used.

The efficiency with which heat is able to inactivate microorganisms is dependent upon the degree of heat, the exposure time and the presence of water. The action of heat will be due to induction of lethal chemical events mediated through the action of water and oxygen. In the presence of water much lower temperature time exposures are required to kill microbe than in the absence of water. In this processes both dry and moist heat are used for sterilization.

Dry Heat Sterilization: Examples of Dry heat sterilization are:

1. Incineration

2. Red heat

3. Flaming

4. Hot air oven

It employs higher temperatures in the range of 160-180°C and requires exposures time up to 2 hours, depending upon the temperature employed. The benefit of dry heat includes good penetrability and non-corrosive nature which makes it applicable for sterilizing glass-wares and metal surgical instruments.

It is also used for sterilizing non-aqueous thermo-stable liquids and thermostable powders. Dry heat destroys bacterial endotoxins (or pyrogens) which are difficult to eliminate by other means and this property makes it applicable for sterilizing glass bottles which are to be filled aseptically.

Hot-air oven

Dry heat sterilization is usually carried out in a hot air oven, which consists of the following:

(i) An insulated chamber surrounded by an outer case containing electric heaters.

(ii) A fan

(iii) Shelves

(iv) Thermocouples

(v) Temperature sensor

(vi) Door locking controls.

Operation

(i) Articles to be sterilized are first wrapped or enclosed in containers of cardboard, paper or aluminium.

(ii) Then, the materials are arranged to ensure uninterrupted air flow.

(iii) Oven may be pre-heated for materials with poor heat conductivity.

(iv) The temperature is allowed to fall to 40°C, prior to removal of sterilized material.

Autoclave

The autoclave is a steam-pressure sterilizer. Steam is the vapour given off by water when it boils at 100°C. If steam is trapped and compressed, its temperature rises as the pressure on it increases. As pressure is exerted on a vapour or gas to keep it enclosed within a certain area, the energy of the gaseous molecules is concentrated and exerts equal pressure against the opposing force. The energy of pressurized gas generates heat as well as force. Thus, the temperature of steam produced at 100°C rises sharply above this level if the steam is trapped within a chamber that permits it to accumulate but not to escape. A kitchen pressure cooker illustrates this principle because it is, indeed, an "autoclave." When a pressure cooker containing a little water is placed over a hot burner, the water soon comes to a boil. If the lid of the cooker is then clamped down tightly while heating continues, steam continues to be generated but, having nowhere to go, creates pressure as its temperature climbs steeply. This device may be used in the kitchen to speed cooking of food, because pressurized steam and its high temperature (120 to 125°C) penetrates raw meats and vegetables much more quickly than does boiling water or its dissipating steam. In the process, any microorganisms that may also be present are similarly penetrated by the hot pressurized steam and destroyed.

Essentially, an autoclave is a large, heavy-walled chamber with a steam inlet and an air outlet. It can be sealed to force steam accumulation. Steam (being lighter but hotter than air) is admitted through an inlet pipe in the upper part of the rear wall. As it rushes in, it pushes the cool air in the chamber forward and down through an air discharge line in the floor of the chamber at its front. When all the cool air has been pushed down the line, it is followed by hot steam, the temperature of which triggers a thermostatic valve placed in the discharge pipe. The valve closes off the line and then, as steam continues to enter the sealed chamber, pressure and temperature begin to build up quickly. The barometric pressure of normal atmosphere is about 15 lb to the square inch. Within an autoclave, steam pressure can build to 15 to 30 lb per square inch above atmospheric pressure, bringing the temperature up with it to 121 to 123°C. Steam is wet and penetrative to begin with, even at 100°C (the boiling point of water). When raised to a high temperature and driven by pressure, it penetrates thick substances that would be only superficially bathed by steam at atmospheric pressure. Under autoclave conditions, pressurized steam kills bacterial endospores, vegetative bacilli, and other microbial forms quickly and effectively at temperatures much lower and less destructive to materials than are required in a dry-heat oven (160 to 170°C).

Temperature and time are the two essential factors in heat sterilization. In the autoclave (steam-pressure sterilizer), it is the intensity of steam temperature that sterilizes (pressure only provides the means of creating this intensity), when it is given time measured according to the nature of the load in the chamber. In the dry-heat oven, the temperature of the hot air (which is not very penetrative) also sterilizes, but only after enough time has been allowed to heat the oven load and oxidize vital components of microorganisms without damaging materials.

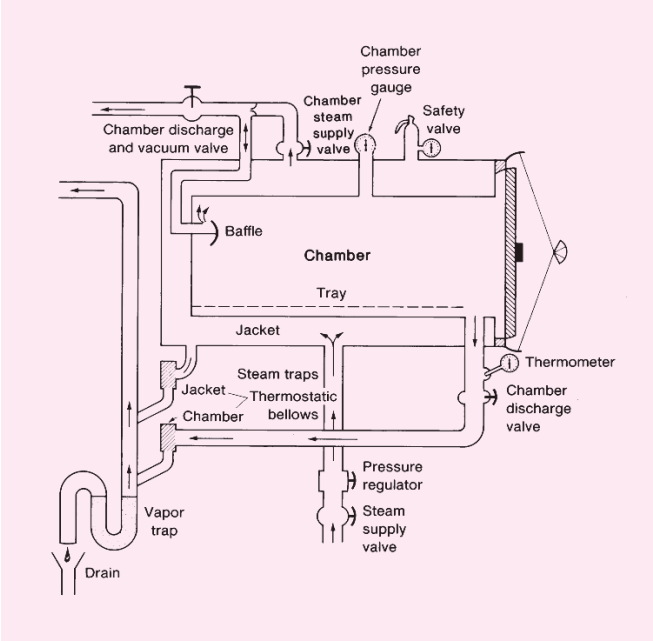


Fig. Autoclave

Table. Pressure-Temperature-Time Relationships in Steam-Pressure Sterilization.

Steam Pressure, Pounds per Square Inch (Above Atmospheric Pressure)	*Temperature*		*Time (Minutes Required to Kill Exposed Heat-Resistant Endospores)*
	Centigrade	*Fahrenheit*	
0	100°	212°	—
10	115.5°	240°	15–60
15	121.5°	250°	12–15
20	126.5°	260°	5–12
30	134°	270°	3–5

Filtration Sterilization

Filtration process does not destroy but removes the microorganisms. It is used for both the clarification and sterilization of liquids and gases as it is capable of preventing the passage of both viable and non viable particles. The major mechanisms of filtration are sieving, adsorption and trapping within the matrix of the filter material. Sterilizing grade filters are used in the treatment of heat sensitive injections and ophthalmic solutions, biological products and air and other gases for supply to aseptic areas. They are also used in industry as part of the venting systems on fermentors, centrifuges, autoclaves and freeze driers.

Membrane filters are used for sterility testing.

Application of filtration for sterilization of gases: HEPA (High efficiency particulate air) filters can remove up to 99.97% of particles >0.3 micrometer in diameter. Air is first passed through prefilters to remove larger particles and then passed through HEPA filters. The performance of HEPA filter is monitored by pressure differential and airflow rate measurements.

There are two types of filters used in filtration sterilization

(a) Depth filters: Consist of fibrous or granular materials so packed as to form twisted channels of minute dimensions. They are made of diatomaceous earth, unglazed porcelain filter, sintered glass

or asbestos.
(b) Membrane filters: These are porous membrane about 0.1 mm thick, made of cellulose acetate, cellulose nitrate, polycarbonate, and polyvinylidene fluoride, or some other synthetic material. The membranes are supported on a frame and held in special holders. Fluids are made to transverse membranes by positive or negative pressure or by centrifugation.

Application of filtration for sterilization of liquids: Membrane filters of 0.22 micrometer nominal pore diameter are generally used, but sintered filters are used for corrosive liquids, viscous fluids and organic solvents. The factors which affects the performance of filter is the titre reduction value, which is the ratio of the number of organism challenging the filter under defined conditions to the number of organism penetrating it. The other factors are the depth of the membrane, its charge and the tortuosity of the channels.

Laminar Air Flow

A Laminar flow hood/cabinet is an enclosed workstation that is used to create a contamination-free work environment through filters to capture all the particles entering the cabinet.
These cabinets are designed to protect the work from the environment and are most useful for the aseptic distribution of specific media and plate pouring. Laminar flow cabinets are similar to biosafety cabinets with the only difference being that in laminar flow cabinets the effluent air is drawn into the face of the user. In a biosafety cabinet, both the sample and user are protected while in the laminar flow cabinet, only the sample is protected and not the user.

Components/ Parts of Laminar flow hood
A laminar flow cabinet consists of the following parts:

1. Cabinet: The cabinet is made up of stainless steel with less or no gaps or joints preventing the collection of spores. The cabinet provides insulation to the inner environment created inside the laminar flow and protects it from the outside environment. The front of the cabinet is provided with a glass shield which in some laminar cabinets opens entirely or in some has two openings for the user's hands to enter the cabinet.
2. Working station: A flat working station is present inside the cabinet for all the processes to be taken place. Culture plates, burner and loops are all placed on the working station where the operation takes place. The worktop is also made up of stainless steel to prevent rusting.
3. Filter pad/ Pre-filter: A filter pad is present on the top of the cabinet through which the air passes into the cabinet. The filter pad traps dust particles and some microbes from entering the working environment within the cabinet.
4. Fan/ Blower: A fan is present below the filter pad that sucks in the air and moves it around in the cabinet. The fan also allows the movement of air towards the HEPA filter sp that the remaining microbes become trapped while passing through the filter.
5. UV lamp: Some laminar flow hoods might have a UV germicidal lamp that sterilizes the interior of the cabinet and contents before the operation. The UV lamp is to be turned on 15 minutes before the operation to prevent the exposure of UV to the body surface of the user.
6. Fluorescent lamp: Florescent light is placed inside the cabinet to provide proper light during the operation.
7. HEPA filter: The High-efficiency particulate air filter is present within the cabinet that makes the

environment more sterile for the operation. The pre-filtered air passes through the filter which traps fungi, bacteria and other dust particles. The filter ensures a sterile condition inside the cabinet, thus reducing the chances of contamination.

Principle/ Working of Laminar flow hood

The principle of laminar flow cabinet is based on the laminar flow of air through the cabinet.
The device works by the use of inwards flow of air through one or more HEPA filters to create a particulate-free environment. The air is taken through a filtration system and then exhausted across the work surface as a part of the laminar flow of the air. The air first passes through the filter pad or pre-filter that allows a streamline flow of air into the cabinet. Next, the blower or fan directs the air towards the HEPA filters. The HEPA filters then trap the bacteria, fungi and other particulate materials so that the air moving out of it is particulate-free air. Some of the effluent air then passes through perforation present at the bottom rear end of the cabinet, but most of it passes over the working bench while coming out of the cabinet towards the face of the operator. The laminar flow hood is enclosed on the sides, and constant positive air pressure is maintained to prevent the intrusion of contaminated external air into the cabinet.

Procedure for running the laminar flow cabinet

The procedure to be followed while operating a laminar flow cabinet is given below:
Before running the laminar flow cabinet, the cabinet should be checked to ensure that nothing susceptible to UV rays is present inside the cabinet.
The glass shield of the hood is then closed, and the UV light is switched on. The UV light should be kept on for about 15 minutes to ensure the surface sterilization of the working bench.
The UV light is then switched off, and a time period of around 10 minutes is spared before the airflow is switched on.
About 5 minutes before the operation begins, the airflow is switched on.
The glass shield is then opened, and the fluorescent light is also switched on during the operation.
To ensure more protection, the working bench of the cabinet can be sterilized with other disinfectants like 70% alcohol.
Once the work is completed, the airflow and florescent lamp both are closed and the glass shield is also closed.

Types of laminar flow cabinet
Depending on the direction of movement of air, laminar flow cabinets are divided into two types:
1. Vertical laminar flow cabinet
In the vertical flow cabinets, the air moves from the top of the cabinet directly towards the bottom of the cabinet.
A vertical airflow working bench does not require as much depth and floor space as a horizontal airflow hood which makes it more manageable and decreases the chances of airflow obstruction or movement of contaminated air downstream.
The vertical laminar flow cabinet is also considered safer as it doesn't blow the air directly towards the person carrying out the experiments.
2. Horizontal laminar flow cabinet
In the horizontal laminar flow cabinets, the surrounding air comes from behind the working bench,

which is then projected by the blower towards the HEPA filters.
The filtered air is then exhausted in a horizontal direction to the workplace environment.
One advantage of this cabinet is that airflow parallel to the workplace cleanses the environment with a constant velocity.
The eluent air directly hits the operator, which might reduce the security level of this type of laminar flow cabinets.

Uses of Laminar flow hood

The following are some common uses of a laminar flow cabinet in the laboratory:
Laminar flow cabinets are used in laboratories for contamination sensitive processes like plant tissue culture.
Other laboratories processes like media plate preparation and culture of organisms can be performed inside the cabinet.
Operations of particle sensitive electronic devices are performed inside the cabinet.
In the pharmaceutical industries, drug preparation techniques are also performed inside the cabinet to ensure a particulate-free environment during the operations.
Laminar flow cabinets can be made tailor-made for some specialized works and can also be used for general lab techniques in the microbiological as well as the industrial sectors.

Precautions

While operating the laminar airflow, the following things should be considered:
The laminar flow cabinet should be sterilized with the UV light before and after the operation.
The UV light and airflow should not be used at the same time.
No operations should be carried out when the UV light is switched on.
The operator should be dressed in lab coats and long gloves.
The working bench, glass shield, and other components present inside the cabinet should be sterilized before and after the completion of work.

Biosafety Cabinets Definition

Biosafety Cabinets (BSCs) are enclosed workspaces with a ventilated hood that is designed to contain pathogenic microorganisms during microbiological processes.

The primary purpose of biosafety cabinets is to protect the laboratory personnel and the environment from the pathogenic microorganism as aerosols might be formed during the processing of such microorganisms.
Biosafety cabinets are only used for certain risk group organisms and for processes that might result in aerosol formation.
These cabinets are provided with HEPA-filters that decontaminate the air moving out of the cabinet.
Biosafety cabinets might be confused with the laminar hood as both of these pieces of equipment work as enclosed workspaces. But, laminar hood only provides protection to the sample and not to the personnel and the environment, whereas biosafety cabinets protect all three.
The use of biosafety cabinets or other such physical containment is not required in the biosafety level 1, but depending on the risk assessment, some processes might require such containment.
BSCs are an essential part of biosafety as they minimize the formation of aerosol, protecting the environment, the pathogen, and the laboratory personnel.
Besides, most BSCs also function to sterilize biological materials that are kept inside the cabinets.

Biosafety Cabinet Classes

Biosafety cabinets are classified into three classes by the U.S. Centers for Disease Control and Prevention (CDC), each with specific performance characteristics and applications.
Class I and II Biosafety cabinets are used for Biosafety levels I and II but, when used correctly in conjunction with useful microbiological techniques, these provide an effective containment system for safe manipulation of moderate and high-risk microorganisms.
Class III BSCs are most suitable for work with hazardous agents that require Biosafety Level 3 or 4.

1. Biosafety Cabinet Class I
Class I is the most basic biosafety cabinet that provides protection to the environment and the laboratory personnel.
It doesn't, however, provide protection to the product as the unsterilized room air is drawn over the work surface.
Class I biosafety cabinets are typically used to either enclose specific equipment like centrifuges or for procedures like aerating cultures that might potentially generate aerosols.
Biosafety cabinets of this class are either ducted (connected to the building exhaust system) or unducted (recirculating filtered exhaust back into the laboratory).
In the Class I BSC, the room air is drawn in through the opening that also allows the entry of the operator's arm during work.
The air inside the cabinet then takes in the aerosol particles that may have been generated and moves it away from the operator towards the HEPA filter.
The air moving out of the cabinet is thus, sterilized via the HEPA filters before its discharge to the environment.
In this way, the cabinets protect the operator and the environment from the aerosol but not the sample.

2. Biosafety Cabinet Class II
BSC-Class II cabinets provide both kinds of protection (of the samples and the environment) since makeup air are also HEPA-filtered.
The principle of operation of Class II cabinets involves a fan mounted in the top of the cabinet that draws a curtain of sterile air over the workstation where the biological products are being handled.
The air then moves underneath the work station and back up to the top of the cabinet before passing through the HEPA filters.
The exhaust that moves out of the facility consists of air being drawn into the front of the cabinet underneath the work surface.
The air drawn in acts as a barrier against the potentially contaminated air coming back out to the operator.
Class II BSCs are further divided into five types depending on the exhaust system and the mechanism of work (recirculation of the exhaust air); Type A1, Type A2, Type B1, Type B2, and Type C1.

a. Type A1
The type A1 cabinets have a minimum inflow velocity of 75ft/min where the contaminated divided just above the work station and mixes with the inflow air.
The mixed air is then drawn through a duct network so that it reaches the back of the cabinet.

After this, air might be either recirculated after passing through the HEPA filters or exhausted out of the cabinet, also through a HEPA filter.
This type of cabinet is not as widely used as it is not safe to work with hazardous chemical substances.

b. Type A2
The type A2 cabinets have a minimum inflow velocity of 100 ft/min.
In Class II, Type A2 BSC air enters the chamber through the front aperture, which provides operator protection.
The inflow air mixes with the downflow air (from the top of the cabinet)and enters the front intake grille and then passes over the workstation where the air splits.
Approximately 60% to 70% of the contaminated air is recycled and pushed back into the workstation in the chamber through the downflow HEPA filter, while the remaining 30% to 40% is exhausted through the exhaust HEPA filter.
However, if hazardous, volatile chemicals are to be used within the cabinet, along with the microbiological work, exhaust must be released into the atmosphere through the direct duct system.
Because of the chances of the release of hazardous chemicals into the environment, A2 type cabinets are also not extensively used.

c. Type B1
Type B cabinets are different from Type A cabinets as they use single-pass airflow to control the flow of hazardous vapors.
Type B1 cabinets divide the airflow so that the contaminated air is directed towards the exhaust system while the air between the operator and the workstation mixes with the inflow and is recirculated.
The exhaust air dispersed out of the facility should be passed through the HEPA filters to provide protection to the environment.
These cabinets have a dedicated duct system which allows the release of the contaminated air out of the facility.
In the case of Type B1 cabinets, 40% of the air is recirculated, whereas the remaining 60% is exhausted out of the facility.

d. Type B2
For a Type B2 BSC, like in Type A cabinets, the air is drawn in from the front opening creating an air barrier that protects the operator.
Air is also drawn in from an opening at the top of the cabinet that supplies the downflow of air in the cabinet.
The air then passes through a HEPA filter, where 100% of the air is exhausted through a dedicated duct system with an exhaust fan motor. The air moving out of the facility is thus sterilized before its release into the atmosphere.
The advantage of this system is the removal of toxic vapors that are generated in the cabinet with no recirculation within the BSC.
All of the contaminated airflow (100%) in a Type B2 cabinet is externally exhausted which means the air drawn into the cabinet is 100% exhausted into the atmosphere.
As a result, none of the air drawn into the B2 from either inflow or downflow is recycled within the airflow system.

Because none of the air is recirculated, these cabinets are the best to be used for tasks involving the release of chemical vapors.
Type B2 cabinets, however, are expensive, and their use is limited to toxicology laboratories where protection against hazardous chemicals is imperative.

e. Type C1
Type C1 cabinets are similar to Type B cabinets in their working mechanism, but these are designed to reduce operating costs add flexibility to the laboratories.
These cabinets work by using the single-pass airflow system where the cabinets move the air by mixing it with the downflow air separated into columns for recirculation.
The air above the workstation is drawn with a second fan which moves the contaminated air out through the exhaust system with a HEPA filter.
In this way, the cabinets provide protection to the environment, the operator, and the workstation or the biological material.
Type C cabinets are different from Type A cabinets as they use a single-pass airflow mechanism where the air is not circulated.
These differ from Type B cabinets in that they don't require a dedicated ducted exhaust system, can work for an extended duration to increase operator protection in the case of exhaust failure, and can even run without the exhaust at all.

3. Biosafety Cabinet Class III
Class III cabinets are leak-tight, totally enclosed but ventilated cabinets, where all air that either enters or leaves through the facility pass through a HEPA filter.
The cabinets are provided with rubber gloves that are attached to the system to be used during operations in the cabinet. This is why these cabinets are also termed 'glove boxes'.
The cabinet even has a transfer chamber that facilitates the sterilization of materials before they leave the glove box.
Even though the gloves restrict the hand movement of the operator inside the cabinet, it prevents direct contact between the operator and the samples.
The exhaust air is treated with double HEPA filters or HEPA filters in combination with incineration.
These cabinets can be used for all four Biosafety levels (1, 2, 3, and 4). But these are the most important for the manipulation of biological materials in the Biosafety level 4.
These cabinets are mostly custom-built for specific laboratories with lab equipment built inside the chamber.
All of these structural and design features provide maximum protection to the operator, the environment, and the sample against the high-risk group 4 pathogenic organisms.

EXPERIMENT 3. Microbial media: Preparation of media for bacteria

Principle:

A number of media have been formulated for growing bacteria. Media generally contain a carbon source, nitrogen source and some essential minerals and salts. Some media may contain additional nutritional supplements. In addition solid media contain agar as a solidifying agent. Meat extract and peptone are the commonest sources of carbohydrates and amino acids.

Media are of different types. These are:

1 Basal media: These contain nutrients that support the growth of non-fastidious bacteria. They do not confer any selective advantage, e.g. nutrient agar.

2 Enriched medium: These are solid selective media. These media, in addition to basal nutrients also contain nutritional supplements like blood, serum, etc., which favour the growth of fastidious bacteria. e.g. blood agar, chocolate agar, Löwenstein- Jensen medium, etc.

3 Enrichment media: These are liquid selective media. They favor the growth of some bacteria by extending the lag phase of others eg. Selenite F broth.

4 Selective media: These media contain ingredients that selectively enable the growth of some species, while inhibiting others eg. Deoxycholate citrate agar (DCA) medium. This medium is a selective medium for growth of *Salmonella* spp. present in stool which contains a mixed bacteria flora. This medium inhibits Escherichia coli and other Gram negative bacteria.

5 Differential media: These media differentiates between species of bacteria depending on a specific property.

Example: MacConkey agar is a differential medium. This medium is used to demonstrate lactose fermenting properties, and differentiate between lactose and non-lactose fermenting bacteria.

REQUIREMENTS

I Equipments: Bacteriological incubator.

II Reagents and media: Different kinds of media such as nutrient agar, blood agar, MacConkey agar and Selenite F broth.

III Specimen: 24 hour broth cultures of *Staphylococcus aureus*, *E. coli*, *Proteus mirabilis* and *Salmonella spp*.

PROCEDURE

1 Inoculate a loopful of the test organism, using a sterile inoculating loop, into appropriately labeled plates and tubes.

2 Incubate the plates and tubes for 18 hours at 37°C.

3 Examine the plate and tubes for growth and record observations.

QUALITY CONTROL

1 One un-inoculated set of media as sterility control

2 Nutrient agar: Colonies of non-fastidious bacteria such as *S. aureus*.

3 Blood agar: Haemolytic strain of *S. aureus* streaked on the plate surrounded by a zone of hemolysis.

4 MacConkey agar: Pink, lactose fermenting colonies of *E. coli* and colorless colonies of *Proteus* spp.

5 Selenite F broth: Growth positive *Salmonella* spp, and growth negative *Proteus* spp.

OBSERVATIONS

All the inoculated bacteria (e.g. *S. aureus, E. coli, P. mirabilis* and *Salmonella* spp) produce colonies on the nutrient agar (basal medium) and blood agar (enriched medium). In addition *S. aureus* may or may not produce haemolysis on the blood agar.

EXPERIMENT 4. Collection of samples and processing in microbiology laboratory

PRINCIPLE

It is critical that the laboratory provide complete guidelines for the proper collection and transport of specimens to ensure quality patient care. All diagnostic information from the microbiology laboratory is contingent on the quality of specimen received. Consequences of a poorly collected and/or poorly transported specimen include failure to isolate the causative microorganism and recovery of contaminants or normal microbiota, which can lead to improper treatment of the patient. Often, direct specimen smears are utilized to determine the quality of the specimen, to provide rapid information for diagnosis and therapy, and to allow the physician to determine if additional, better quality specimens should be collected. This procedure addresses instructions for physicians, nurses, and laboratory assistants on collecting and transporting samples.

Specimen Safety considerations

a. Follow universal precaution guidelines. Treat all specimens as potentially biohazardous.

b. Laboratory workers should use appropriate barrier protection (such as gloves and laboratory coat or gown) when collecting or handling specimens. If splashing may occur, protective eyewear, face masks, and aprons may be necessary.

c. Do not contaminate the external surface of the collection container and/or its accompanying paperwork.

d. Minimize direct handling of specimens in transit from the patient to the laboratory. Use plastic sealable bags with a separate pouch for the laboratory requisition orders or transport carriers (for example, small buckets with rigid handles).

NOTE: Specimens obtained by a physician using needle aspiration should be transferred to a sterile tube or anaerobic transport vial prior to transport of the specimen to the laboratory. If there is little

material in the syringe, the physician should draw a small amount of sterile nonbacteriostatic 0.85% NaCl or sterile broth through the syringe and then transfer the specimen to a sterile tube. Alternatively, and only if the specimen will be compromised by transferring it from the syringe, a small amount of sterile 0.85% NaCl or broth may be drawn into a syringe prior to removal of the needle. The physician should use a protective device while removing the needle to avoid injury and should cap the syringe with a sterile cap prior to transporting it to the laboratory.

General guidelines for proper specimen collection

a. Collect specimen before administering antimicrobial agents when possible.

b. Collect specimen with as little contamination from indigenous microbiota as possible to ensure that the sample will be representative of the infected site.

c. Utilize appropriate collection devices. Use sterile equipment and aseptic technique to collect specimens to prevent introduction of microorganisms during invasive procedures.

d. Clearly label the specimen container with the patient's name and identification number or date of birth (DOB). Always include date and time of collection and collector's initials.

e. Collect an adequate amount of specimen. Inadequate amounts of specimen may yield false-negative results.

f. Develop an understanding of the microbiology laboratory's source identification schemes. Know when to include "rule-out" request. For example, the laboratory may routinely screen for *Shigella*, *Salmonella*, and *Campylobacter* species in stool cultures but not for *Yersinia* or *Vibrio* species.

g. Identify the specimen source and/or specific site correctly so that proper culture media will be selected during processing the laboratory.

Procedure for Specific Specimen Collection

Anaerobic Culture. Specimens are to be collected from a prepared site using a sterile technique. Contamination with normal flora must be avoided. Some anaerobes will be killed by contact with oxygen for only a few seconds. Ideally, pus obtained by needle aspiration through intact surface, which has been aseptically prepared, is put directly into anaerobic transport media. Sampling of open lesions is enhanced by deep aspiration using a sterile plastic catheter or needle. Curettings of the base of an open lesion may also provide a good yield. If irrigation is necessary, nonbacteriostatic sterile normal saline may be used. Pulmonary samples may be obtained by transtracheal percutaneous needle aspiration or by physicians trained in this procedure. Superficial collection (ie, a swab of the lesion) is not the best specimen for anaerobic culture. If swabs must be used, two should be collected; one for culture and one for Gram stain. Swabs of the throat or genital tract are not appropriate specimens for anaerobic culture.
The following are clinical symptoms suggestive of anaerobic infection:

- Foul-smelling discharge
- Location of infection in proximity to a mucosal surface
- Necrotic tissue, gangrene, pseudomembrane formation

- Gas in tissues or discharges
- Endocarditis with negative routine blood cultures
- Infection associated with malignancy or other process producing tissue destruction
- Infection related to the use of aminoglycosides (oral, parenteral, or topical)
- Septic thrombophlebitis
- Bacteremic picture with jaundice
- Infection resulting from human or other bites
- Black discoloration of blood-containing exudates (may fluoresce red under ultraviolet light in *B melaninogenicus* infections)
- Presence of "sulfur granules" in discharges (actinomycosis)
- Classical clinical features of gas gangrene
- Clinical setting suggestive for anaerobic infection (septic abortion, infection after gastrointestinal surgery, genitourinary surgery, etc)

Upper Respiratory Tract. This section describes procedures for obtaining culture specimens from the nasopharyngeal area and the throat.

1. A nasopharyngeal culture is obtained by inserting a thin sterile swab gently through the nose to touch the pharynx; gently rotate and remove.
2. A throat culture is obtained by introducing a sterile swab into the mouth. Use a tongue blade to avoid contaminating the specimen with oral secretions. Firmly swab both tonsillar fossae, posterior pharynx, and any inflamed or ulcerated areas.

Lower Respiratory Tract: Sputum. This section discusses sputum cultures, including such alternatives as induced sputum, tracheal aspiration, and bronchial washings.

1. Rinsing the mouth with saline or water (but not mouthwash) may reduce contamination with normal oropharyngeal flora.
2. Encourage deep cough with expectoration of the sputum into a sterile specimen collection cup that is labeled with the patient's name.
3. Do not send saliva (spit) for culture.
4. When the patient is unable to cough productively, notify the physician. An alternative method may be ordered, such as:

a. Induced sputum. This is done by a respiratory therapist on the orders of the physician. Involuntary deep coughing is induced by irritation.

b. Tracheal aspiration. The trachea is gently irritated with a small lumen suction catheter, which causes deep, productive coughing. Also, the specimen may be aspirated with a syringe.

c. Bronchial washings. These are done by the physician in the operating room at the time of bronchoscopic examination. Sputum following bronchoscopy can be very productive for the recovery of mycobacteria.

5. A small amount of sputum is all that is required, but it must be sputum and not oral secretions.
6. Three sputa collected on consecutive days is recommended for the recovery of mycobacteria.

Specimens of Wound Exudate. Follow these steps for using a sterile transport swab in collecting wound exudate specimens.

1. Gently cleanse the area, using dry, sterile gauze to remove any contaminants.
2. Using a sterile bacterial culture collection system, introduce deeply enough to obtain a moist specimen; replace the swab in the container. Do **not** break the container.
3. Store at room temperature.

Urine for Culture. When a urine culture is ordered, follow these steps for collecting a clean-catch specimen.

1. Explain carefully to patients the mechanics of midstream collection and the importance of collecting an uncontaminated specimen. Teach them how to handle the specimen container to keep it sterile.
2. A clean-catch specimen is necessary to confirm the presence or absence of infecting organisms in urine. The specimen must be free of any contaminating matter that might be present on the genital organs; therefore, patients should be urged to follow the steps outlined below.

a. Instructions for the Female Patient.

- If you are menstruating, first insert a fresh tampon or use cotton to stop the flow.
- Separate the skin folds around the urinary opening.
- Wash the urinary opening and its surroundings from front to back with a sterile antiseptic pad.
- Begin urinating into the toilet, making sure you keep the skin fold apart with the fingers of one hand.
- Wait until the urine stream is well established before moving the container into the path of the stream to catch the rest of the urine. Do **not** touch the container to the genital area.

b. Instructions for the Male Patient.

- Wash the end of the penis well with soapy water. Let it dry.
- Begin urinating into the toilet. Wait until the urine stream is well-established before moving the container into the path of the stream to catch the rest of the urine. Do **not** touch the container to the genital area.

3. Cleansing agents, such as soap or detergent, must be rinsed away from the urethral area before the specimen is collected.
4. A urine specimen from a catheterized patient is obtained by using a sterile 21- to 23-gauge needle and a 3-mL syringe. Prepare an area on the distal end of the rubber catheter with an antiseptic sponge. Insert the needle at a 45° angle, pointed toward the drainage tubing. If urine is not obtained, try lifting the catheter tubing carefully. If necessary, kink the tubing three inches from the catheter and hold in place with a rubber band until urine is visible.
5. Urine for culture must be transferred to a urine transport tube that contains preservative immediately after collection.

Note: Do **not** collect urine specimens from a drainage bag.

Stool for Culture. When collecting stool specimens, follow these guidelines.

1. A small amount is all that is required, about the size of a walnut. If several different types of cultures are requested, submit a walnut-sized sample for each. Place the specimen in stool culture transport medium (C&S vial).
2. When stool specimens are not readily obtainable, rectal swabs are acceptable; however, it must be indicated whether the specimen is a stool or a rectal swab. Place the swab in stool culture transport medium (C&S vial).

Use of Sterile Swab Bacterial Collection Kit

The swab system is guaranteed sterile until the seal is broken. Directions for use:

1. Peel open and remove the swab from the package.
2. Remove the cap/swab stick from the tube.

3. Collect the appropriate specimen and put the cap/swab into the tube. Push the cap to bring the swab into contact with the transport medium.
4. Print the patient's name and the culture site on the specimen tube.
5. Place the specimen in a specimen bag and put the completed test request form in the side pouch.
6. Store it at room temperature.
7. Send specimen to the laboratory.

Normal Flora. The common practice in microbiology is to identify "significant" organisms from cultures. Significance is determined in part by the quantitation of an organism relative to other organisms present, the "pathogenicity" of isolates, and the site from which the specimen was obtained. When the organisms present are known to be part of the expected flora from a particular body site, the result reported is often "routine (site) flora". The following list representative flora from various body sites.

Skin Flora

- α-Hemolytic (Alpha-hemolytic) *Streptococcus* species
- *Bacillus* species
- Coagulase-negative *Staphylococcus* species
- *Corynebacterium* species

Respiratory Flora

- α-Hemolytic (Alpha-hemolytic) *Streptococcus* species not *Enterococcus*
- *Corynebacterium* species
- *Neisseria* species
- Nonhemolytic *Streptococcus* species

The following potential pathogens may be part of the routine flora if not predominating:

- Coagulase-negative *Staphylococcus* species
- *Haemophilus influenzae*
- *Haemophilus parainfluenzae*
- *Moraxella catarrhalis*
- *Neisseria meningitidis*
- *Streptococcus pneumoniae*

Genitourinary Tract Flora

- α-Hemolytic (Alpha-hemolytic) *Streptococcus* species not *Enterococcus*
- Coagulase-negative *Staphylococcus* species (if not predominating)
- *Corynebacterium* species
- *Lactobacillus*
- Nonhemolytic *Streptococcus* species

EXPERIMENT 5. Methods of inoculation of different microbes in common media : Streak plate, spread plate, pour plate, serial dilution

Purpose

A. To isolate pure cultures from a specimen containing mixed flora
B. To culture and study the normal flora of the mouth

Principle

The skin and many mucosal surfaces of the human body support large numbers of microorganisms that comprise the normal, or indigenous, flora. When clinical specimens are collected from these surfaces and cultured, any pathogenic microorganisms being sought must be recognized among, and isolated from, other harmless organisms. Colonies of the pathogenic species must be picked out of the mixed culture and grown in isolated pure culture. The microbiologist can then proceed to identify the isolated organism by examining its biochemical and immunological properties. Pure culture technique is critical to successful, accurate identification of microorganisms

Materials

Nutrient agar plates
Blood agar plates
Sterile swabs
A mixed broth culture containing Serratia marcescens (pigmented), Escherichia coli, and Staphylococcus epidermidis
A demonstration plate culture made from this broth, showing colonies isolated by good streaking technique
Glass slides
Gram-stain reagents

Procedures

A. Streaking a Mixed Broth Culture for Colony Isolation

1. Make certain the contents of the broth culture tube are evenly mixed.
2. Place a loopful of broth culture on the surface of a nutrient agar plate, near but not touching the edge. With the loop flat against the agar surface, lightly streak the inoculum back and forth over approximately one-eighth the area of the plate; do not dig up the agar.
3. Sterilize the loop and let it cool in air.
4. Rotate the open plate in your left hand so that you can streak a series of four lines back and forth, each passing through the inoculum and extending across one side of the plate.
5. Sterilize the loop again and let it cool in air.
6. Rotate the plate and streak another series of four lines, each crossing the end of the last four streaks and extending across the adjacent side of the plate.
7. Rotate the plate and repeat this parallel streaking once more.
8. Finally, make a few streaks in the untouched center of the plate. Do not touch the original inoculum.
9. Incubate the plate (inverted) at 35°C.

B. Taking a Culture from the Mouth
1. Rotate a sterile swab over the surface of your tongue and gums.
2. Roll the swab over a small -cm square of surface of a blood agar plate, near but not touching one edge. Rotate the swab fully in this area.
3. Discard the swab in a container of disinfectant.
4. Using an inoculating loop, streak the plate.
5. Incubate the plate (inverted) at 35°C.

Results
A. Examination of Plate Streaked from Mixed Broth Culture
1. Examine the incubated nutrient agar plate carefully.
Make a drawing showing the intensity of growth in each streaked area.

Pour-Plate Techniques

Purpose
A. To learn the pour-plate technique for obtaining isolated colonies

Principle
An alternative method for using agar plates to obtain isolated colonies, other than streaking their surfaces, is to prepare a "pour plate." In this case, an aliquot of the specimen to be cultured is placed in the bottom of an empty, sterile petri dish, and then melted, cooled agar is poured over it. Quickly, before the agar cools, the plate is gently rocked to disperse the inoculum. When the agar has solidified and the plate is incubated, any bacteria present in the specimen will grow wherever they have been embedded within the agar layer or localized on its surface. Their colonies will be isolated and can be removed from subsurface positions by inserting the inoculating loop or a straight wire into the agar. To prepare pour plates, the inoculum must be a liquid specimen or culture. If it is not, it must be suspended in sterile fluid before being placed in the petri dish.
Another method for preparing a pour plate is to inoculate the specimen or culture directly into the tube of melted, cooled, but not yet solidified agar. Mix it by rolling it back and forth between the outstretched fingers of both hands, and pour the inoculated agar into a sterile petri dish. These steps must be performed quickly before the agar cools enough to harden.
When primary isolation plates have been properly poured or streaked, individual colonies can be picked up on an inoculating loop or straight wire and inoculated to fresh agar or broth media. These new pure cultures of isolated organisms are called subcultures. If they are indeed pure and do not contain mixtures of different species, they can be identified in stepwise procedures as you will see in later exercises.

Materials
Tubed nutrient agar (10 ml per tube)
Sterile petri dishes
Sterile 1-ml pipettes (cotton plugged)
Mixed broth culture containing Escherichia coli and Staphylococcus epidermidis
Nutrient agar plates (prepared in Exercise 8)
Nutrient agar broth (prepared in Exercise 8)
Nutrient agar plate cultures (streaked), containing isolated colonies of three bacterial species

Procedures

A. Pour-Plate Technique

1. Place a tube of sterile nutrient agar in a boiling water bath. (A simple water bath can be set up by placing a glass beaker or tin can half filled with water on a tripod over a Bunsen flame. An asbestos mat must be used under glass vessels. The water should be kept at a steady but not rapid boil. Keep the water level at the halfway mark. An electric burner may be used instead.)
2. When the agar is liquefied, remove the tube and allow it to cool to about 50°C.
3. Place an empty sterile petri dish before you, top side up.
4. Remove a sterile 1-ml pipette from its container, keeping your fingers on the plugged mouth end.
5. Pick up the mixed broth culture in the other hand, remove its closure with the little finger of the hand holding the pipette (do not touch the pipette to anything), and insert the pipette into the broth.
6. Holding the tube and pipette vertically, poise your index finger over the pipette mouth. Allow the pipette to fill to the level of the broth in the tube and then close off its mouth with your finger (there should be about 0.3 to 0.4 ml of culture in the pipette).
7. Keeping your finger pressed on its top, raise the pipette until the tip is free of the broth and then slowly allow the material in the pipette to run back into the tube until only the last 0.1 ml remains. Now press your finger tightly to close the pipette's mouth and prevent further dripping. Never use your mouth to draw fluid into a pipette.
8. Before you withdraw the pipette from the tube, touch its tip against the dry inner wall to remove any drop hanging from it.
9. Withdraw the closed pipette, replace the tube closure, and put the tube down in the rack.
10. Now, with your free hand, remove the top of the petri dish (do not put it down), place the tip of the pipette against the bottom of the dish, release your finger from the mouth, and let 0.1 ml of broth culture run into the plate bottom.
11. Replace the dish top and discard the pipette into a container of disinfectant.
12. Pick up the tube of melted, cooled agar, remove its closure, and put it down on the bench top.
13. With your free hand, remove the top of the petri dish (again, do not put it down). Quickly pour the agar into the dish.
14. Replace the petri dish cover (the tube may be set aside for washing). Gently rock the closed dish, or rotate it in circular fashion on the bench top, being careful not to allow the still melted agar to wave up over the edge of the bottom half or onto the cover.
15. Let the agar solidify without further disturbance. When it is quite firm (about 30 minutes), invert the plate and place it in the 35°C incubator.

EXPERIMENT 6. Staining techniques : Differential Staining : Gram staining, Albert staining, Acid fast staining, capsule staining, spore staining

Gram Staining

Principle: Differential staining requires the use of at least three chemical reagents that are applied sequentially to a heat fixed smear. The first reagent is called the primary stain. Its function into impart its colour to all cells. In order to establish a colour contrast the second reagent is the discolouring agent. Based on the chemical composition of cellular components the decolourising agent may or may not remove the primary stain from the entire cells or only from certain cell structures. The final reagent, the counter stain has a contrasting than that of the primary stain.

Following decolourisation, if the primary stain is not washed out, the counter stain can't be observed and the cells or their components will retain the colour of the primary stain. If the primary

stain is removed, it accepts the contrasting colour of counter-stain. In this way cell type or their structures can be distinguished from each other on the basis of the stain that cells retained.

Purposes: To become familiar with-

i) The chemical and theoritical basis for differential staining procedures.
ii) The chemical basis of gram-stain.
iii) Performance of the procedure for differentiating between the two principle group of bacteria-
   a. Gram positive bacteria.
   b. Gram negative bacteria.

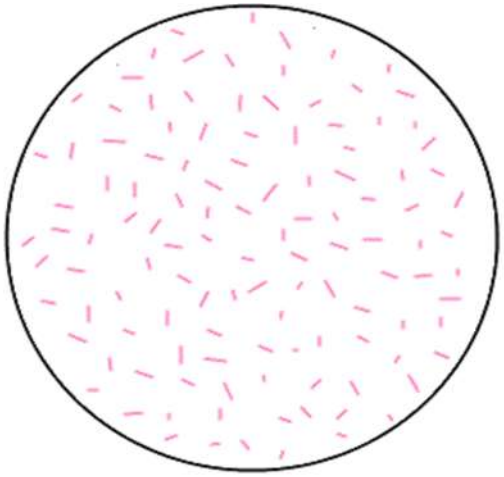
Materials:

- **Culture:** Twenty four hours old culture
- **Reagents:** Crystal violet- Primary stain
  Gram Iodine- Mordant
  Decolourising agent- 70% ethyl alcohol
  Counter Stain- Safranine
- **Equipment:** Bunsen burner, inoculating loop, staining tray, glass slide, lens paper and microscope.

Procedure:

- A clean glass slide was obtained.
- The smear was prepared by placing a drop of culture by using sterile inoculating loop.
- The smear was allowed to air dry and then heat fixed by using Bunsen-burner.
- The smear was covered with several drops of crystal violet and incubated for 30 seconds to 1 minute.
- The slide was gently washed with drops of tap water.
- The smear was then flooded with the Gram's iodine and incubated for one minute.
- The slide was gently washed with drops of tap water.
- The slide was then decolourized with 90% ethyl alcohol.
- The slide was air dried followed by counter staining with safranine for 45 seconds.
- The slide was gently washed with drops of tap water.
- The slide was air dried and observed under oil immerson microscope (100x).

Observation and Result:

Draw a Representative field:	
Cell Morphology: **Shape:** **Arrangement:** **Cell Colour:** **Gram characteristic:**	**Rod Shape** **Single** **Violet** **Gram Positive**

Comment: Hence the supplied bacterial sample was Gram Positive rod shaped bacteria.

Albert staining

PRINCIPLE

The diphtheria bacillus, *Corynebacterium diphtheriae* has well developed granules within their bacterial cytoplasm. These granules are known as Volutin granules, Babes Ernst granules, polar bodies or metachromatic granules. These granules are made up of polymetaphosphate and are seen in unstained wet preparations as round, refractile bodies within the bacterial cytoplasm. With basic dyes, granules tend to stain more strongly than the rest of the bacterium. With toluidine blue or methylene blue, they stain metachromatically, and appear reddish purple in colour. These granules are clearly demonstrated best by special stains such as Albert's, Neisser's or Puch's stain. With Albert's staining, the bacilli appear green with bluish-black metachromatic granules.

REQUIREMENTS

I Equipments: Compound light microscope

II Reagents and glass wares: Bunsen flame, loop wire, glass slides, Albert's stain I and II.

Preparation of Albert's stain I: This stain is composed of 1.5 grams toluidine blue, 2 grams malachite green, 10 ml glacial acetic acid, 10 ml alcohol (95% ethanol) and 1 litre distilled water. Toluidine

blue and malachite green are dissolved in the alcohol and then added to the water and acetic acid. The stain is then allowed to stand for one day and then filtered.

Note: Toluidine blue stains the granules bluish black due to metachromatic effect and malachite green stains the bacilli green.

Preparation of Albert's stain II: Albert's II (also known as Albert's iodine) is composed of 6 gram iodine, 9 gram potassium iodide and 900 ml distilled water. The solution is made by first dissolving 2 gram potassium iodide in 10 ml distilled water and then 1 gram of iodine is further added to it with dissolution. Then 290 ml of distilled water is added and final volume is made up to 300 ml.

III Specimen: Exudate smear collected directly from pseudomembrane obtained using a throat swab / culture smear of *C. diphtheriae*.

PROCEDURE

1 Heat fix the smears by passing the slide 2–3 times gently over the flame with the smear side up. Allow the smears to be air dried.

2 Put the smears on a slide rack and cover the smears with Albert's stain I. Allow it to stain for 3-5 minutes.

3 Rinse the smears gently under tap water and blot those dry.

4 Then cover the smear with Albert's stain II. Allow it to act for 1 minute.

5 Rinse the smears again under tap water and blot those dry.

6 Observe the smear first under low power (10x) objective, and then under oil immersion (100x) objective.

7 Record the observations in the note book. Findings are recorded, together with grading of the positive smear.

OBSERVATION

1–2 µm sized green coloured bacilli showing Chinese letter arrangement at angles to each other, containing bluish-black metachromatic granules, are seen.

RESULTS AND INTERPRETATION

The stained smear contains malachite green stained bacilli showing bluish-black metachromatic granules.

The smear is positive for bacilli showing bluish-black metachromatic granules.

Probably, the smear contains *Corynebacterium diphtheriae*.

Acid fast staining

PRINCIPLE

Acid fastness of acid-fast bacilli is attributed to the presence of large quantities of unsaponifiable wax fraction called mycolic acid in their cell wall and also the intactness of the cell wall. The degree of acid fastness varies in different bacteria.

In this staining method, application of heat helps the dye (a powerful staining solution containing carbol fuchsin and phenol) to penetrate the tubercle bacillus. Once stained, the stain cannot be easily removed. The tubercle bacilli resist the decolourizing action of acid-alcohol which confers acid fastness to the bacteria. The other microorganisms, which are easily decolourised by acid-alcohol, are considered non-acid fast. The non-acid fast bacilli readily absorb the colour of the counter stain (methylene blue) appearing blue, while the acid-fast cells retain the red colour of primary stain (carbol fuchsin).

REQUIREMENTS

Equipments: Compound light microscope.

Reagents and glass wares Bunsen flame/ torch soaked in methylated spirit, loop wire, glass slides, slide rack, strong carbol fuchsin, acid-alcohol (3 ml HCl + 97 ml ethanol) (decolourising agent), and Loeffler's methylene blue (counter stain).

Preparation of strong carbol fuchsin: This solution is prepared by dissolving 5 grams basic fuchsin powder in 25 grams crystalline phenol by placing them in a 1 litre flask. The flask containing solution is kept over a boiling water-bath for about 5 minutes, shaking the contents from time to time. When the solution is complete, 50 ml of 95% alcohol or 100% ethanol is added to the solution and mixed thoroughly. Then 500ml of distilled water is added to it and the mixture is filtered before use.

Preparation of 20% sulphuric acid: 800ml of water is collected in a large flask. The 200ml concentrated sulphuric acid (about 98% or 1.835g / ml)) is poured slowly down the side of the flask into the water, about 50 ml at a time. The mixture becomes hot. Remainder of acid is added in same manner. Note: The acid must be added to the water. It is dangerous to add the water to the acid. Great care must be taken to avoid spilling the acid on skin, clothing or elsewhere.

Preparation of 95% alcohol: This is prepared by adding 95 ml of ethanol and adding water to it to make 100ml.

Preparation of acid-alcohol decolouriser: This solution contains 75 ml concentrated hydrochloric acid (HCl) and 25 ml of industrial methylated spirit. Methylated spirit is poured into a large flask. The flask is placed in 5–8 cm of cold water in the sink. Then hydrochloric acid is added slowly and the top of the flask is covered to stop the fumes from escaping. It is left for 10 minutes. It is then decanted into a labelled bottle for use. The final concentration of HCl is 3%.

Specimen: Sputum smear positive for tubercle bacilli / culture smear of Mycobacterium species.

PROCEDURE

1) Heat fixes the smears by passing the slide 2–3 times gently over the flame with the smear side up. Allow the smear to be air dried.

2) Put the smears on a slide rack and cover the smears with strong carbol fuchsin. Allow it to stain for 5 minutes.
3) During this period, heat the slides from below intermittently by Bunsen flame or torch soaked in methylated spirit without boiling the solution, until the steam rises. Do not allow the stain to dry on the slide, and if necessary add more carbol fuchsin to cover the smear.
4) Rinse the smears gently under tap water.
5) Cover the smear with 20% sulphuric acid for at least 10 minutes for decolourisation.
6) Wash the slides thoroughly with water to remove all traces of acid. Note: Decolourisation with 95% alcohol for 2 minutes is only optional and may be omitted.
7) Cover the smear with Loeffler's methylene blue for 15–20 seconds.
8) Rinse the smears again under tap water and air dry it.
9) Observe the smear first under low power (10x) objective, and then under oil immersion (100x) objective.
   Note: The smear should be examined following a zig-zag pattern for at least 10 minutes or 300 fields, before declaring the smear negative.
10) Record the observations in the note book.

OBSERVATION

Presences of pink coloured slender rod shaped structures were seen with curved ends, and are scattered amidst blue coloured round cells with darkly stained multilobed nucleus.

RESULTS AND INTERPRETATION

The stained smear contains pink coloured acid fast bacilli seen among the blue coloured multilobed pus cells. The smear is positive for acid fast bacilli. Probably, the smear contains *Mycobacterium tuberculosis.*

Capsule staining

PRINCIPLE

Negative staining procedure is so called because the background gets stained and the organism remains colourless. It is also known as 'Indirect staining. The procedure requires the use of acidic stains such as India ink or Nigrosin. Negative staining finds its utility for the demonstration of capsule and bacteria difficult to stain such as *Treponema palladium*. Wet film India-ink method is the best method for staining capsules of bacteria from cultures in either liquid or solid media.
The acidic dyes such as India ink, nigrosin or eosin have negatively charged chromogen, and will not readily combine with the negatively charged bacterial cytoplasm. Instead it forms a deposit around the organism, leaving the organism itself colourless. Therefore, the unstained cells are easily discernible against the coloured background.

REQUIREMENTS

Equipment: Compound light microscope.
Reagents and glass wares: These include Bunsen flame, staining tray, glass slides and coverslips nigrosin stains. Nigrosin staining solution is prepared by adding 0.03 gram of nigrosin in 100 ml of distilled water.
Specimen: 24 hour broth culture of *Klebsiella pneumoniae* (A capsulated bacterium).

PROCEDURE

1) Take a clean grease free glass slide.
2) Put a small drop of nigrosin close to one end of a clean slide.
3) Using a sterile loop, a loopful of broth culture of the capsulated organism is mixed with the nigrosin drop.
4) With the edge of a second slide, held at 30° angle and held in front of the bacterial suspension mixture, spread the drop along the edge of the applied slide. The slide is then pushed away from the previously spread drop of suspended organism, forming a thin smear.
5) Air dry the preparation without any heat fixation.
6) Observe the stained smear under oil immersion (100x) objective.
7) Record the observations in the note book.

OBSERVATION

The bacterial organism is seen as a clear halo against a black or dark background in the wet film or dry film preparation.

RESULTS AND INTERPRETATION

Hence the supplied bacterial sample contained rod / coccus shaped bacteria.

Spore staining

PRINCIPLE

Malachite green stain, also known as Schaeffer-Fulton Method for bacterial endospores uses two different reagents: primary stain (malachite green) and counter stain (0.5% safranine or 0.05% basic fuchsin).Ordinary tap water acts as decolourising agent. Unlike most of the vegetative cells that are stained by common procedures, the spore, because of its impervious coats, are not stained by the primary stain easily. The application of heat facilitates penetration of the primary stain, malachite green. After the primary stain is applied and the smear is heated, both the vegetative cell and spore appear green. Once the spore is stained with the malachite green, it cannot be decolourised by tap water, which removes only the excess primary stain. The spore remains green. On the other hand, water removes the stain from the vegetable cells, because the stain does not demonstrate a strong affinity for the vegetative cell components and these vegetable cells therefore become colourless.
Red coloured-safranine as counterstain is used as the second reagent to colour the decolourised vegetative cells, which will absorb the counterstain and appear red. The spores retain the green of the primary stain.

REQUIREMENTS
I Equipments: Compound light microscope.

II Reagents and lab wares: Bunsen burner, beaker of boiling water, staining tray, glass slides, inoculating loop, malachite green and safranine.
Preparation of malachite green stain: This stain is prepared by dissolving 5 gram of malachite green in 100 ml of distilled water.

Preparation of safranine stain: This stain is prepared by dissolving 0.5 gram of safranine in 100 ml distilled water.

III Specimen: Smear collected from 48 hours to 72 hours nutrient agar slant culture of *Bacillus cereus*/ thioglycollate culture of *Clostridium butyricum.* On a clean glass slide, a smear from the culture is made in saline, then air dried and fixed with heat.

PROCEDURE

1. Heat fix the smears by passing the slide 2–3 times gently over the flame with the smear side up. Allow the smear to be air dried.
2. Put the slide with the smear over a beaker of boiling water, resting it on the run with the bacterial film upper most. When, within several seconds, large droplets have condensed on the underside of the slide, flood the smear with 5% acqueous solution of malachite green and allow acting for 1 minute, while the water continues to boil.
3. Wash the smears with cold water.
4. Then cover the smear with 0.5% safranine or 0.05% basic fuchsin. Allow it to act for 30 seconds.
5. Rinse the smears again under tap water and blot those dry.
6. Observe the smear first under low power (10x) objective, and then under oil immersion (100x) objective.
7. Record the observations in the note book.

OBSERVATION

Bacterial endospores stain green, and vegetative bacilli stain red.

RESULTS AND INTERPRETATION

A 2 – 3 µm red coloured rod-shaped structure seen along with an intracellular 0.5 µm sized spherical green coloured structure.

It represents red coloured vegetative bacilli with green coloured spores by the malachite green staining method. The sample may be sporebearing bacilli (eg. *Bacillus* species or *Clostridium* species).

EXPERIMENT 7. Observation of morphology of bacteria – shape and arrangement

Principle: Nutrient agar is used as a general purpose medium for the growth of a wide variety of non-fastidious microorganisms. It consists of peptone, beef extract and agar. This relatively simple formulation provides the nutrients necessary for the replication of a large number of non-fastidious microorganisms. The characteristics of the components used in nutrient agar:

Beef extract is an aqueous extract of lean beef tissues. It contains water-soluble substances of animal tissue, which include carbohydrates, organic nitrogen compounds, water soluble vitamins, and salts.

Peptone is made by digesting proteinaceous materials e.g., meat, casein, gelatin, using acids or enzymes. Peptone is the principal source of organic nitrogen and may contain carbohydrates or vitamins. Depending up on the nature of protein and method of digestion, peptones differ in their constituents, differing in their ability to support the growth of bacteria.

Agar is a complex carbohydrate obtained from certain marine algae. It is used as a solidifying agent for media and does not have any nutritive value. Agar gels when the temperature of media reaches 45°C and melts when the temperature reaches 95 °C.

Materials:

Glass petri plates, conical flasks (250 ml), pipettes (1 ml and 10 ml), spirit lamp, 70% alcohol, fuel alcohol, non-absorbent cotton, tissue paper, distilled water
Media Composition: Nutrient broth 13.0 gm
Agar 20.0 gm
Distilled Water 1000 ml
Final pH 6.8 ± 0.2.

Procedure:

Media preparation:

Required amount (1.3 gm) of nutrient broth was dissolved into 100 ml of distilled water kept in a conical flask.
pH was adjusted to 6.8 ± 0.2.
Then 2.0 gm of agar was added to 100 ml of nutrient broth.
The media was sterilized at 15 lb pressure and 121 ^{o}C for 15 min by using autoclave.
After autoclaving, the media was cooled down to 50 ^{o}C and poured in to the autoclaved petri plates.

Dilution preparation:

Nine milliliter of autoclaved distilled water was added to 6 test tubes.
One milliliter of water sample (Tap water) was mixed with 9 ml of distilled water and thus 10^{-1} dilution was prepared.
Then 1 ml of water from10^{-1} dilution was poured in another test tube containing 9 ml of autoclaved distilled water and thus 10^{-2} dilution was prepared.
Like this, upto 10^{-6} dilution was prepared.

Plating:

One hundred microliter of 10^{-4}, 10^{-5}, 10^{-6} dilutions were spread on the solidified nutrient agar plates.
The plates were incubated at 37 ^{o}C for 24 h.

Result:

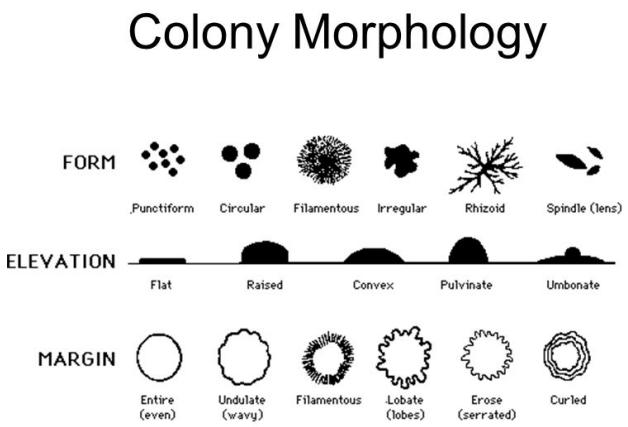
After 24 h, the colony appeared on the petri plates were enumerated and the colony characteristics were observed.

Enumeration of bacteria from water sample:

Sample number	Dilution no.	Sample added (ml)	CFU numbers/ 0.1 ml	CFU numbers/ 1 ml	CFU No./ml in original sample	Average number of CFU No./ml in original sample

Colony characteristics:

Colony number	Colour	Form	Elevation	Margin	Figure



Comment: Hence, the supplied sample contained X CFU/ g of cultivable bacteria.

EXPERIMENT 8. Hanging drop preparation

Principle: Hanging drop preparation is a special type of wet mount (in which a drop of medium containing the organisms is placed on a microscope slide), often is used in dark illumination to observe the motility of bacteria.

In this method a drop of culture is placed on a coverslip that is encircled with petroleum jelly (or any other sticky material). The coverslip and drop are then inverted over the well of a depression slide. The drop hangs from the coverslip and the petroleum jelly forms a seal that prevents evaporation. This preparation gives good views of microbial motility.

Materials required:

1. Parafin wax
2. Loop
3. Cover slip

4. Microscope
5. Bunsen burner
6. Young broth culture of motile bacteria
7. Glass slides (glass slide with depression) or normal glass slide with adhesive or parasfin ring

Procedure: 1. Take a clean glass slide and apply paraffin ring, adhesive tape ring to make circular concavity (this step is not needed if a glass slide with depression is available).

2. Hold a clean coverslip by its edges and carefully apply vaselin on its corners using a tooth pick.

3. Place a loopfull of the broth culture to be tasted in the centre of the prepared coverslip.

4. Turn the prepared glass slide or concavity slide upside down (concavity down) over the drop on the coverslip to the slide around the cavity.

5. Turn the slide over so the coverslip is an top and the drop can be observed hanging from the coverslip over the concavity.

6. Place the preparation in the microscope slide holder and align it using the naked eye so on edge of the drops is under the low power objectives.

7. Turn the objective to its lowest position using the coarse adjustment and close the diaphragm.

8. Look through the eyepiece and raise the objectives slowly using the coarse adjustment knob until the edge of the drop is observed as an irregular line coursing the field.

9. Move the slide to make that line (the edge of the drop) passes through the centre of the field.

10. Without raising or lowering the tube, swing high dry objective into position (be sure the high dry objective is clean).

11. Observe the slide through the eyepiece and adjust the fine adjustment until the edge of the drop can be seen as a thick usually dark fine.

12. Focus the edge of the drop carefully and look at each slide of that line for very small objects that are the bacteria. The cells will look either like dark or slightly grey. Very small rods are spheres. Remember the high dry objective magnifies a little loss than half as much as the oil immersion objective.

13. Adjust the slide using the diaphragm lever to maximize the visibility of the cells.

14. Observe the cells nothing there morphology and grouping and determine whether true motility can be observe.

15. Brownian movement should be visible on slides of all the organism, but there should also show the true motility.

16. Wash the depressive slide and after soaking in Lysol buckets or discard the prepared glass slide.

Fig: Organisms are motile in nature

Observation: Observe the slide under the microscope. First focus it under low power objectives and then place a drop of oil immersion on the coverslip. The motility is observed under oil immersion lense of the compound microscope.

Result: From the result it was the supplied organisms are motile in nature.

EXPERIMENT 8. Identification of common Pathogenic bacteria:- by staining & their biochemical tests *(Catalase, Coagulase, Oxidase, IMVIC, Urease , etc.)* and Serological tests

Catalase test:

PRINCIPLE

Chemically, catalase is a haemoprotein, similar in structure to haemoglobin, except that the four iron atoms in the molecule are in the oxidized (Fe^{3+}) rather than the reduced (Fe^{2+}) state.

The enzyme converts hydrogen peroxide into water and oxygen.

$2H_2O_2$ —— $2H_2O + O_2$ (gas bubbles)

Production of the enzyme catalase can be demonstrated by adding hydrogen peroxide to colonies of the bacteria. If catalase is present it is indicated by the presence of free gas bubbles. If catalase is absent, no bubbles will be seen.

The catalase test is most commonly used to differentiate members of the family *Micrococcaceae* from members of the family *Streptococcaceae*.

Catalase test is also carried out for Mycobacteria to differentiate tubercle bacilli from atypical mycobacteria.

REQUIREMENTS

I Reagents and glass wares: 3% hydrogen peroxide, glass slides, test tubes, glass rod / platinum loop / plastic loop and other standard lab wares.

II Specimen: Pure growth of bacteria from solid media preferably from nonblood agar plates (Examples: nutrient agar, Muller-Hinton agar) is tested.

PROCEDURE

Test can be done by 2 methods as follows:

1 Slide method

2 Tube method

Slide method

1 Transfer pure growth of the organism from the agar to a clean slide with a loop or glass rod.
2 Immediately add a drop of 3% hydrogen peroxide to the growth.
3 Observe for bubble formation.

Tube method

1 Take 1 ml of 3% hydrogen peroxide in 12 x 100 mm test tube.
2 Introduce small quantity of bacterial growth into the fluid with the help of a glass rod or plastic loop and touch the side of the tube.
3 Observe the release of bubbles.

OBSERVATIONS

Slide method

Gas bubbles are formed immediately when 3% H_2O_2 is added to the colony.

Tube method

Gas bubbles are released when colonies are introduced into the hydrogen peroxide in the test tube.

RESULTS AND INTERPRETATION

1 The rapid and sustained appearance of bubbles or effervescence constitutes a positive test. It means bacteria possesses the enzyme catalase, hence is catalase positive.
2 Some bacteria possess enzymes other than catalase that can decompose hydrogen peroxide. Hence, forming a few tiny bubbles after 20-30 seconds is not considered a positive test.

List of catalase positive and negative bacteria.

Catalase positive bacteria	Catalase negative bacteria
Staphylococci	*Streptococcus pyogenes*
Micrococci	*Gardnerella vaginalis*
Corynebacterium diphtheriae	*Fusobacterium* species
Enterobacteriaceae	*Eikenella corrodens*
	Kingella kinge
	Shigella dysenteriae type 1
	Fatumella ptysees

Coagulase Test

PRINCIPLE

Staphylococcus aureus produces the enzyme coagulase in 2 forms: a. bound coagulase and b. free coagulase.

Bound coagulase

Bound coagulase is also known as clumping factor. It is bound to the bacterial cell wall and is not present in culture filtrates. Presence of this enzyme is tested by slide coagulase test. Fibrin strands are formed between the bacterial cells when suspended in plasma (fibrinogen), causing them to clump into visible aggregates.

Free coagulase

Free coagulase is a thrombin-like substance present in *S. aureus* culture filtrates. Presence of free coagulase is tested by tube coagulase test. In this method, a suspension of coagulase producing staphylococci is prepared in plasma in a test tube, and incubated at 37°C for 3-6 hours. In a positive test, the enzyme coagulase secreted by *S. aureus* is liberated to the medium, which reacts with fibrinogen to produce a visible fibrin clot.

REQUIREMENTS

I Reagents and lab wares

Rabbit plasma with EDTA anticoagulant, saline, glass slides, test tubes, glass rod/platinum loop/plastic loop and other standard lab wares.

II Specimen

Pure growth of *S. aureus* from solid media preferably from nonblood agar plates (Examples: nutrient agar, Muller-Hinton agar).

PROCEDURE

Slide test

1 Take a clean glass slide.
2 Mark it into two halves by a glass marking pencil.
3 Add two drops of sterile saline on two halves of the glass slides.
4 Pick up the colonies of *S. aureus* to be tested from agar culture and gently emulsify with drops of saline.
5 Add a drop of undiluted plasma to the bacterial suspension and mix with a wooden applicator sticks.
6 Place another drop of saline in other half of the slide as a control.
7 Rock the slide, back and froth, and observing for the prompt clumping of the bacterial suspension within 10-15 seconds.

Tube test

1 Take 0.5 ml of rabbit plasma (diluted 1 in 5 with saline) in a test tube.
2 Add approximately 5 drops (250 µl) of overnight broth culture or small amount of the colony growth of *S. aureus* to the diluted plasma in the test tube.
3 Incubate the tube at 37°C for 4 hours.
4 Observe for clot formation by gently tilting the tube.
5 If no clot is observed at that time, reincubate the tube at room temperature and read again after 18 hours.

QUALITY CONTROL

Positive control: *S. aureus* (Coagulase positive bacteria).
Negative control: *S. epidermidis* (Coagulase negative bacteria).
Coagulability of plasma may be tested by adding one drop of 5% calcium chloride to 0.5 ml of the reconstituted plasma. A clot should form within 10 to 15 seconds.

OBSERVATION

In a positive slide test, prompt clumping of the organism shows the presence of the bound coagulase.
In a positive tube test, the plasma in the tube clots and does not flow when the tube is inverted.
Note: On continued incubation, the clot may be lysed by fibrinolysin secreted by some strains.

RESULTS AND INTERPRETATION

In slide test, Positive reaction will be detected within 10–15 seconds of mixing the plasma with the suspension by the formation of a white precipitate and agglutination of the organisms. The test is considered negative if no agglutination is observed after 2 minutes. All strains that are coagulase positive can be reported as *S. aureus.* All strains producing negative slide tests must be tested with the tube coagulase test.
The tube coagulase test is considered positive if any degree of clotting is noted.

List of coagulase positive bacteria

1 *Staphylococcus aureus.*
2 *Staphylococcus schleiferi*
4 *Staphylococcus felis*
5 *Staphylococcus lutrae*
6 *Staphylococcus intermedius*
7 Staphylococcus hyicus
8 *Peptostreptococcus hydrogenalis*

Oxidase test

PRINCIPLE

The cytochromes are iron containing haemoproteins that act as the last link in the chains of aerobic respiration by transferring electrons (hydrogen) to oxygen, with the formation of water. The cytochrome oxidase test uses certain reagent dyes such as p-phenylene diamine dihydrochloride which acts as a substitute for oxygen as artificial electron acceptors. This enzyme oxidises the reagent N-N tetramethyl para-phenylene diamine hydrochloride (a colour less reagent in reduced form) to indophenol blue, a purplish blue coloured product.

REQUIREMENTS

I Reagents and glass wares

Fresh reagent: Tetramethyl – p-phenylene diamine dihydrochloride (1%), and dimethyl – p – phenylene diamine dihydrochloride (1%).
Wood stick/platinum loop/glass rod, and filter paper.

II Specimen

Young culture of bacteria to be tested, preferably less than 24 hours old, growing on an agar plate or agar slant.

PROCEDURE

The test is performed by following two methods:

1 Direct plate technique, and

2 Indirect paper strip procedure.

Direct plate technique

1 Take a nutrient agar plate with colonies of bacteria to be tested.

2 Add 2 to 3 drops of reagent (tetramethyl p-phenylene diamine hydrochloride or dimethyl-p-phenylene diamine dihydrochloride) directly to the bacterial colonies growing on medium in the plate.

3 Note the change of colour of the colonies.

Indirect filter paper strip procedure

1 Take a filter paper strip.

2 Moisten the filter paper strip with freshly prepared 1% oxidase reagent.

Note: Oxidase reagent is freshly prepared in distilled water every day.

3 Pick up the colonies to be tested with the help of a glass rod or plastic loop or platinum wire.

4 Smear the colonies into the reagent zone of the filter paper.

5 Note the change in colour if any within 10 seconds.

QUALITY CONTROL

Positive control: *Pseudomonas aeruginosa* (oxidase positive bacteria).

Negative control: *Escherichia coli* (oxidase negative bacteria).

OBSERVATIONS

Direct plate technique

In a positive test, bacterial colonies on the plate develop adeep blue colour at the site of inoculation within 10 seconds.

In a negative test the colour of the colonies remain unchanged.

Indirect filter paper strip procedure

In a positive test, a deep blue colour develops at the site of smear in the filter paper, within 10 seconds.

In a negative test the colour of the smear in the zone of the filter paper remain unchanged.

RESULTS AND INTERPRETATION

Bacterial colonies having cytochrome oxidase activity develop a deep blue colour at the inoculation site within 10 seconds. In filter paper test, deep blue colour develops at the site of smear within 10 seconds. It means bacteria possesses the enzyme oxidase, hence is oxidase positive.

List of oxidase positive and negative bacteria

Oxidase positive bacteria
Gram negative rods
1. *Pseudomonas* spp(except *Ps. cepacia*).
2. *Vibrio* spp.
3. *Aeromonas* spp.
4. *Camphylobacter* spp.
5. *Plesiomonas* spp.
6. *Flavobacterium* spp.
7. *Alcaligenes* spp.
8. *Haemophilus* spp.
9. *Moraxella* spp.
10. *Chromobacterium* spp.
11. *Bordetella* spp(except *B.parapertusis*)
12. *Brucella* spp (except *B.canis*)
13. *Eikinella* spp.
14. *Cardiobacterium* spp.
15. *Achromobacter* spp.
16. *Pasteurella multocida*

Gram negative cocci
1. *Neisseria* spp.
2. *Branhamella* spp.

Oxidative negative bacteria
1. All genera in family Enterobacteriaceae
2.*Acinetobacter calcoaceticus*
3. *Bordetella parapertusis*
4.*Brucella canis*
5.*Francisella tularensis*
6.*Gardnerella vagimalis*

IMVIC test

Indole Test

OBJECTIVES:

1) Determine the ability of bacteria to degrade the amino acid tryptophan.
2) Distinguish the bacteria based on the indole activity.

PRINCIPLE:

Tryptophan is an essential amino acid that can undergo oxidation by enzymatic activities of some bacteria. Conversion of tryptophan into metabolic products is mediated by the enzyme tryptophanase. The metabolic end products are indole, skatole and indole acetic acid. The ability to hydrolyse tryptophan with the production of indole is not a characteristic of all bacteria. Only some bacteria produce indole.

REQUIREMENTS:

Equipments: Incubator.

IReagents and lab wares: Peptone water / tryptone broth, Kovac's reagent, or Ehrlich's reagent, glass tubes and inoculating wire.

Kovac's reagent consists of para-dimethyl amino benzaldehyde, 5.0 gm; isoamyl alcohol, 75.0 ml; and concentrated hydrochloric acid, 25.0 ml. Ehrlich's reagent consists of p-dimethyl amino benzaldehyde, 4.0 gm; absolute ethyl alcohol, 380.0 ml; and concentrated hydrochloric acid, 80.0 ml.

Specimen: 24 hours to 48 hours peptone water culture of *Escherichia coli* incubated at 37°C.

PROCEDURE

1) Take 0.5 ml of 24 hours to 48 hours peptone water cultures of *E. coli* in a small test tube.
2) Add 0.2 ml of Kovac's reagent to the peptone water and shake.
3) Allow it to stand for few minutes and read the result.

OBSERVATION

In a positive test, a red-violet ring develops within minutes on addition of Kovac's reagent. In a negative test a yellow ring appears.

RESULTS AND INTERPRETATION

Positive indole test is indicated by the appearance of red-violet ring on adding the reagent. Negative reaction is indicated by developing a yellow ring. *E. coli* colonies tested are an indole producing bacteria. *K. pneumoniae* does not produce the indole.

List of Indole positive and negative bacteria

Indole positive bacteria	Indole negative bacteria
1. *Escherichia coli*	*1. Escherichia vulnaris*
2. *Klebsiella oxytoca*	2. *Klebsiella pneumoniae*
3. *Proteus vulgaris*	3. *Proteus mirabilis*
4. *Morganella morganii*	4. Salmonella Typhi
5. *Providencia rettgeri*	5. *Shigella sonnei*
6. *Aeromonas hydrophila*	
7. *Pasteurella multocida*	
8. *Vibrio cholerae*	
9. *Falvobacterium*	
10. *Plesiomonas shigelloides*	

Methyl Red Test

OBJECTIVES

1) Determine the ability of bacteria to oxidise glucose with the production of high concentrations of acidic end products by methyl red test.
2) Differentiate between all glucose oxidizing enteric bacteria particularly *Escherichia coli* and *Enterobacter aerogenes*.

PRINCIPLE

Methyl red is a pH indicator with a range between 6.0 (yellow) and 4.4 (red). The pH at which methyl red detects acid is considerably lower than the pH of other indicators used in bacteriologic culture media. Thus to produce a colour change, the test organism must produce large quantities of acid from the carbohydrate substrate being used.

The methyl red test is a quantitative test for acid production, requiring positive organisms to produce strong acids (lactic acid, acetic acid, formic acid) from glucose through the mixed acid fermentation pathway. Because many species of the Enterobacteriaceae may produce sufficient quantities of strong acids that can be detected by methyl red indicator during the initial phase of incubation, only organisms that can maintain this low pH after prolonged incubation (48–72 hours) overcoming the pH buffering system of the medium can be called methyl red positive.

REQUIREMENTS

Equipments: Incubator.

Reagents and lab wares: Inoculating wire. Methyl red test broth. It consists of poly peptone, 7 gm; glucose, 5 gm; dipotassium phosphate, 5 gm; and distilled water, 1l at a pH of 6.9. Methyl red indicator. It consists of methyl red, 0.1 g in 300 ml of 95% ethyl alcohol.

Specimen: Culture of *E. coli*, *E. aerogenes* and *Klebsiella pneumoniae* in glucose phosphate medium incubated at 30°C for five days.

PROCEDURE

1 Take 0.5 ml of broth cultures of *E. coli* in a small test tube.

2 Add five drops of 0.04% solution of methyl red directly to the broth culture and mix well.

3 Note any change in the colour of medium at once.

OBSERVATION

Look for the development of stable red colour on adding methyl red indicator.

RESULTS AND INTERPRETATION

The development of a stable red colour in the surface of the medium indicates sufficient acid production to lower the pH to 4.4 and constitutes a positive test. Because other organisms may produce smaller quantities of acid from the test substrate, an intermediate orange colour between yellow and red may develop. This does not indicate a positive test. Yellow colour indicates a negative test.

List of MR positive and negative bacteria

MR positive bacteria	MR negative bacteria
1. *E. coli*	1. *K. pneumoniae*
2. *K. ozaenae*	2. *Enterobacter* spp
3. *K. rhinoscleromatis*	
4. *K. ornitholytica*	
5. Edwardsielleae	
6. Salmonellae	
7. Citrobacter	
8. Proteae	
9. Yersinia	

Voges-Proskauer Test

PRINCIPLE

The Voges-Proskauer test determines the capability of some bacteria to produce non-acidic or neutral end products such as acetyl methyl carbinol from the organic acids produced as a result of glucose metabolism. Pyruvic acid, the pivotal compound formed in the fermentative degradation of glucose is further metabolised through various metabolic pathways, depending on the enzyme systems possessed by different bacteria.

One such pathway results in the production of acetoin (acetyl methyl carbinol), a neutral-reacting end product. Enteric bacteria such as members of the Klebsiella-EnterobacterHafnia-Serratia group produce acetoin as the chief end products of glucose metabolism and form smaller quantities of mixed acids.

The test depends on the production of acetyl methyl carbinol from pyruvic acid, as an intermediate product in its conversion to 2: 3 butylene glycol. In the presence of atmospheric oxygen and alkali (40% potassium hydroxide), the small amount of acetyl methyl carbinol present in the medium is converted to diacetyl, which reacts with the peptone of the broth to produce a red colour.

REQUIREMENTS

Equipments: Incubator.

Reagents and lab wares: Inoculating loop. VP broth. It consists of polypeptone, 7 gm; glucose,5 gm; dipotassium phosphate, 5 gm and distilled water, 1 litre at a pH of 6.9. 5% a naphthol. It consists of a naphthol, 5 gm; and absolute ethyl alcohol, 100 ml. It serves as the colour intensifier. 40% potassium hydroxide. It consists of 40 gm potassium hydroxide in 100 ml distilled water. It serves as the oxidising agent.

Specimen: Culture of *Escherichia coli*, *Enterobacter aerogenes* and *Klebsiella pneumoniae* in glucose phosphate medium incubated at 30°C for five days or 37°C for 48 hours.

PROCEDURE

1) Take 1 ml of broth cultures of *E. coli* in a small test tube.

2) First add 40% KOH and then add 0.6 ml of a 5% solution of α-naphthol in ethanol to the broth culture and shake gently. It is essential that the reagents are added in this order.
3) Note any change in the colour of medium within 2-5 minutes.

OBSERVATIONS

Look for the development of pink colour 15 minutes or more after addition of the reagents.

RESULTS AND INTERPRETATION

A positive test is represented by the development of a pink colour 15 minutes or more after addition of the reagents, deepening to magenta or crimson in half an hour. This indicates the presence of diacetyl, the oxidation product of acetoin. A negative test is indicated by colour less reaction for half an hour. The test should not be read after standing for over 1 hour because negative VP test may produce a copper-like colour, leading to a false positive interpretation.

VP positive and negative bacteria

VP positive bacteria	VP negative bacteria
1. *Klebsiella pneumoniae*	1. *Escherichia coli*
2. *Enterobacter cloacae*	2. *Edwardsiella tarda*
3. *Cedicia netri*	3. Salmonellae
4. *Ewingella americana*	4. Proteae
5. *Serratia marcescens*	5. Yersinieae
6. *Aeromonas sobria*	
7. *Vibrio cholerae*	
8. *Chryseomonas luteola*	
9. *Flavimonas oryzihabitans*	
10. *Sphingomonas paucinobilix*	

Citrate Utilisation Test

OBJECTIVES

Differentiate certain enteric organisms on the basis of their ability to utilize citrate as a sole source of carbon.

PRINCIPLE

In the absence of fermentable glucose or lactose, some bacteria are capable of using citrate as a sole source of carbon for their energy. This ability depends on the presence of the enzyme, a citrate permease that facilitates the transport of citrate in the cell.

Citrate is the first major intermediate in the Krebs cycle and is produced by the condensation of active acetyl with oxaloacetic acid. Citrate is acted on by the enzyme citrase which produces oxaloacetic acid and acetate. These products are then enzymatically converted to pyruvic acid and carbon dioxide (CO_2). During this reaction the medium becomes alkaline because the CO_2 that is generated combines with sodium and water to form sodium carbonate, an alkaline product. The presence of sodium carbonate changes the indicator, bromo thymol blue present in the medium from green at pH 6.9 to deep Prussian blue at pH 7.6. Simmon's citrate and Koser's citrate are two examples of different types of citrate media used in the test.

REQUIREMENTS

Equipments: Incubator.

Reagents and lab wares: Inoculating loop, Simmon's citrate medium (It consists of ammonium dihydrogen phosphate, 1 gm; dipotassium phosphate, 1 gm; sodium chloride, 5 gm; sodium citrate, 2 gm; magnesium sulfate, 0.20 gm; agar, 15 g; bromo thymol blue, 0.08 gm and distilled water 1 litre) pH adjusted to 6.9. The medium is poured into a tube on a slant.

Specimen: Culture of *Escherichia coli*, *Enterobacter aerogenes* and *Klebsiella pneumoniae* in glucose phosphate medium incubated at 37°C for 48 hours.

PROCEDURE

1) Using sterile technique, inoculate each bacteria into its appropriately labeled tube by means of a stab and streak inoculation.
2) Incubate all cultures for 24 hours to 48 hours at 37°C.

OBSERVATIONS

Look for the development of deep blue colour within 24-48 hours of incubation of the inoculated tube.

RESULTS AND INTERPRETATION

A positive test is represented by the development of a deep blue colour within 24 hours to 48 hours, indicating that the test organism has been able to utilize the citrate contained in the medium, with the production of alkaline products. A negative test is indicated by no change of colour of the citrate medium.

List of citrate positive and negative bacteria

Citrate positive bacteria	Citrate negative bacteria
1. *Klebsiella pneumoniae*	*1. Escherichia coli*
2. *Citrobacter diversus*	2. Salmonella Typhi
3. *Enterobacter cloacae*	3. Salmonella Paratyphi A
4. *Serratia marcescens*	4. *Shigella* species
5. *Providencia alcalifaecians*	5. *Yersinia enterocolitica*
6. *Euringella americana*	6. *Edwardsiella tarda*
7. *Acroncobacter oxylosoxidans*	7. *Vibrio holisae*
8. *Vibrio vulnificus*	

Urease test

PRINCIPLE

Urea is a diamide of carbonic acid. Urease, the enzyme produced by the bacteria and fungi, hydrolyses urea and releases ammonia and carbon dioxide. Ammonia reacts in solution to form ammonium carbonate, which is alkaline leading to an increase in pH of the medium. Phenol red that is incorporated in the medium changes its color from yellow to red in alkaline pH, thus indicating the presence of urease activity.

REQUIREMENTS

I Equipments: Incubator.

II Reagents and glass wares: Inoculating wire, Christensen's urea agar, and 12 × 100 mm test tubes.

III Specimen

Pure growth of *Proteus mirabilis* from solid media preferably from non-blood agar plates (Examples: nutrient agar, Muller- Hinton agar) is tested.

PROCEDURE

1 Pick up the colonies of *P. mirabilis* from the culture on nutrient agar.
2 Inoculate Christensen's urea agar slope with these bacterial colonies.
3 Incubate the tube at 37°C for 18 hours.
4 Observe any change of colour in the inoculated medium.

QUALITY CONTROL

Positive control: *P. mirabilis* (urease positive bacteria).
Negative control: *Escherichia coli* (urease negative bacteria).
An uninoculated medium is incubated along with the test to compare the colour change.

OBSERVATION

Examine the medium after four hours and after overnight incubation**.** The test should not be considered negative till after four days of incubation.
The uninoculated medium is colour less. In a positive test, after incubation, the colour of the medium changes to purple pink.

RESULTS AND INTERPRETATION

Positive reaction is detected after 18 hours of incubation. When positive, the color of the medium changes to purple pink (*P. mirabilis)*.The test is considered negative if no colour change of the medium is observed (*E. coli)*.
P. mirabilis tested is a urease producing bacteria. *E. coli* does not produce the enzyme urease.

Urease producing bacteria and fungi

Urease producing bacteria

Strong (or) most rapid urease producers

Brucella species
Helicobacter pylori

Rapid urease producers

Proteus species
Morganella species

Slow urease producers

Klebsiella species
Enterobacter species

Urease producing fungi

Cryptococcus neoformans
Trichophyton mentagrophytes

Serological tests

As per the manufacturer's protocol

1st Year BMLT Biochemistry practical (Paper – 103)

Phlebotomy and collection of blood samples

Phlebotomy is the process of making a puncture in a vein, usually in a vein, usually in the arm, with a cannula for the purpose of drawing blood. The procedure itself is known as a venipuncture, which is also used for intravenous therapy. A person who performs phlebotomy is called a phlebotomist, although most doctors, nurses, and other technicians can also carry out a phlebotomy. In contrast, phlebectomy is the removal of a vein.

Phlebotomies that are carried out in the treatment of some blood disorders are known as therapeutic phlebotomies.

Phlebotomy – Drawing of blood

Venipuncture is one of the most routinely performed invasive procedures and is carried out for any of five reasons :

1. To obtain blood for diagnostic purposes;

2. To monitor levels of blood components;[3]

3. To administer therapeutic treatments including medications, nutrition, or chemotherapy;

4. To remove blood due to excess levels of iron or erythrocytes (red blood cells); or

5. To collect blood for later uses, mainly transfusion either in the donor or in another person.

Blood is most commonly obtained from the superficial veins of the upper limb. The median cubital vein, which lies within the cubital fossa anterior to the elbow, is close to the surface of the skin without many large nerves positioned nearby. Other veins that can be used in the cubital fossa for venipuncture include the cephalic, basilic, and median antebrachial veins.

Minute quantities of blood may be taken by fingerstick sampling and collected from infants by means of a heelprick or from scalp veins with a winged infusion needle.

Phlebotomy (incision into a vein) is also the treatment of certain diseases such as hemochromatosis and primary and secondary polycythemia.

Complications :

A 1996 study of blood donors (a larger needle is used in blood donation than in routine venipuncture) found that 1 in 6,300 donors suffered a nerve injury.

Equipment:

There are many ways in which blood can be drawn from a vein, and the method used depends on the person's age, the equipment available, and the type of tests required.

Most blood collection in the US, UK, Canada and Hong Kong is done with an evacuated tube system. Two common systems are Vacutainer (Becton, Dickinson and company) and Vacuette (Greiner Bio-One). The equipment consists of a plastic adapter, also known as a tube or needle holder/hub, a hypodermic needle and a vacuum tube. Under certain circumstances, a syringe may be used, often with a butterfly needle, which is a plastic catheter attached to a short needle. In the developing world, the evacuated tube system is the preferred method of drawing blood.

With evacuated or vacuum tubes

Greiner Bio-One manufactured the first ever plastic evacuated blood collection tube in 1985 under the VACUETTE brand name. Today, many companies sell vacuum tubes as the patent for this device is now in the public domain. These tubes are manufactured with a specific volume of gas removed from the sealed tube. When a needle from a hub or transfer device is inserted into the stopper, the tube's vacuum automatically pulls in the required volume of blood.

The basic Evacuated Tube System (ETS) consists of a needle, a tube holder, and the evacuated tubes. The needle is attached to the tube holder by the phlebotomist prior to collection, or may come from the manufacturer as one unit. The needle protrudes through the end of the tube holder, and has a needle on each end. After first cleaning the venipuncture site and applying a tourniquet, the phlebotomist uncaps the needle attached to the tube holder, inserts the needle into the vein, then slides evacuated tubes into the tube holder, where the tube's stopper is pierced by the back end of the needle. The vacuum in the tube then automatically draws the needed blood directly from the vein. Multiple vacuum tubes can be attached to and removed in turn from a single needle, allowing multiple samples to be obtained from a single procedure. This is possible due to the multiple sample sleeve, which is a flexible rubber fitting over the posterior end of the needle cannula which seals the needle until it is pushed out of the way. This keeps blood from freely draining out of the back of the needle inserted in the vein, as each test tube is removed and the next impaled. OSHA

safety regulations require that needles or tube holders come equipped with a safety device to cover the needle after the procedure to prevent accidental needle stick injury.

Fittings and adapters used to fill evacuated tubes from butterfly needle kits and syringes are also available.

There are several needle gauges for a phlebotomist to choose from. The most commonly used are as follows: a 21g (green top) needle, a 22g (black top) needle, a 21g (green label) butterfly needle, a 23g (light blue label) butterfly needle, and a 25g (orange or dark blue label) butterfly needle (however this needle is only used in pediatrics or extreme cases as it is so small that it can often result in hemolyzing the blood sample). There are also a variety of tube and bottle sizes and volumes for different test requirements.

Additives and order of draw

The test tubes in which blood is collected may contain one or more of several additives. In general, tests requiring whole blood call for blood samples collected in test tubes containing some form of the anticoagulant EDTA. EDTA chelates calcium to prevent clotting. EDTA is preferred for hematology tests because it does minimum damage to cell morphology. Sodium citrate is the anticoagulant used in specimens collected for coagulation tests. The majority of chemistry and immunology tests are performed on serum, which is produced by clotting and then separating the blood specimen via centrifuge. These specimens are collected in either a non-additive tube or one containing a clotting activator. This clotting activator can interfere with some assays, and so a plain tube is recommended in these cases, but will delay testing. Tubes containing lithium heparin or sodium heparin are also commonly used for a variety of chemistry tests, as they do not require clotting and can be centrifuged immediately after collection. A combination of sodium fluoride and potassium oxalate is used for glucose tests, as these additives both prevent clotting and stop glycolosis, so that blood glucose levels are preserved after collection.[8] Another specialty tube is an opaque amber colored tube used to collect blood for light sensitive analytes, such as bilirubin.

Test tubes are labeled with the additive they contain, but the stopper on each tube is color coded according to additive as well. While colors vary between manufacturers, stopper colors generally are associated with each additive as listed below. Because the additives from each tube can be left on the needle used to fill the tubes, they must be drawn in a specific order to ensure that cross contamination will not negatively affect testing of the samples if multiple tubes are to be drawn at once. The "order of draw" varies by collection method. Below in the order of draw generally required for the Evacuated Tube System (ETS) collection method are the most common tubes, listing additive and color.

Vacutainer/sample tube types for venipuncture/phlebotomy

Tube cap color or type	Additive	Usage and comments
Blood culture bottle	Sodium polyanethol sulfonate (anticoagulant) and growth media for microorganisms	Usually drawn first for minimal risk of contamination. Two bottles are typically collected in one blood draw; one for aerobic organisms and one for anaerobic organisms

Light blue	Sodium citrate	Coagulation tests such as prothrombin time (PT) and partial thromboplastin time (PTT) and thrombin time (TT). Tube must be filled 100%
Plain red	No additive	Serum: Total complement activity, cryoglobulins
Gold (sometimes red and grey "tiger top")	Clot activator and serum separating gel	Serum –separating tube: Tube inversions promote clotting. Most chemistry, endocrine and serology tests, including hepatitis and HIV.
Dark green	Sodium heparin (anticoagulant)	Chromosome testing, HLA typing, ammonia, lactate
Mint green	Lithium heparin (anticoagulant)	Plasma. Tube inversions prevent clotting
Lavender ("purple")	EDTA (chelator / anticoagulant)	Whole blood, CBC, ESR, Coombs test, platelet antibodies, flow cytometry, blood levels of tacrolimus and cyclosporine.
Pink	EDTA (chelator / anticoagulant)	Blood typing and cross-matching, direct Coombs test, HIV viral load.
Royal blue	EDTA (chelator / anticoagulant)	Trace elements, heavy metals, most drug levels, toxicology
Tan	EDTA (chelator / anticoagulant)	Lead
Gray	Sodium fluoride (glycolysis inhibitor Potassium oxalate (anticoagulant)	Glucose, lactate
Yellow	Acid-citrate-dextrose A (anticoagulant)	Tissue typing, DNA studies, HIV cultures
Pearl ("white")	Separating gel and (K2) EDTA	PCR for adenovirus, toxoplasma and HHV-6

In children

Use of lidocaine iontophoresis is effective for reducing pain and alleviating distress during venipuncture in children. A needle-free powder lignocaine delivery system has been shown to decrease the pain of venipuncture in children. Rapid dermal anesthesia can be achieved by local anesthetic infiltration, but it may evoke anxiety in children frightened by needles or distort the skin, making vascular access more difficult and increasing the risk of needle exposure to health care workers. Dermal anesthesia can also be achieved without needles by the topical application of local anesthetics or by lidocaine iontophoresis. By contrast, noninvasive dermal anesthesia can be established in 5–15 min without distorting underlying tissues by lidocaine iontophoresis, where a direct electric current facilitates dermal penetration of positively charged lidocaine molecules when placed under the positive electrode.

One study concluded that the iontophoretic administration of lidocaine was safe and effective in providing dermal anesthesia for venipuncture in children 6–17 years old. This technique may not be applicable to all children. Future studies may provide information on the minimum effective iontophoretic dose for dermal anesthesia in children and the comparison of the anesthetic efficacy and satisfaction of lidocaine iontophoresis with topical anesthetic creams and subcutaneous infiltration.

Non-pharmacological treatments for pain associated with venipuncture in children includes hypnosis and distraction. These treatments reduced self reported pain and when combined with cognitive-behavioural therapy (CBT) the reduction of pain was even greater. Other interventions have not been found to be effective and these are suggestion, blowing out air, and distraction with parent coaching did not differ from control for pain and distress.

With needle and syringe

Some health care workers prefer to use a syringe-needle technique for venipuncture. Sarstedt manufactures a blood-drawing system (S-Monovette) that uses this principle. This method can be preferred on the elderly, those with cancer, severe burns, obesity, or where the veins are unreliable or fragile. Because syringes are manually operated, the amount of suction applied may be easily controlled. This is particularly helpful when veins are small which may collapse under the suction of an evacuated tube. In children or other circumstances where the quantity of blood gained may be limited it can be helpful to know how much blood can be obtained before distributing it amongst the various additives that the laboratory will require. Another alternative is drawing blood from indwelling cannulae.

Blood cultures

There are times when a blood culture collection is required. The culture will determine if there are pathogens in the blood. Normally blood is sterile. When drawing blood from cultures use a sterile solution such as Betadine rather than alcohol. This is done using sterile gloves, while not wiping away the surgical solution, touching the puncture site, or in any way compromising the sterile process. It is vital that the procedure is performed in as sterile a manner as possible as the persistent presence of skin commensals in blood cultures could indicate endocarditis but they are most often found as contaminants. It is encouraged to use an abrasive method of skin preparation. This removes the upper layers of dead skin cells along with their contaminating bacteria.[22] Povidone-iodine has traditionally been used but in the UK a 2% chlorhexidine in 70% ethanol or isopropyl alcohol solution is preferred and time must be allowed for it to dry. The tops of any containers used when drawing a blood culture should also be disinfected using a similar solution. Some labs will actively discourage iodine use where iodine is thought to degrade the rubber stopper through which blood enters the bottle, thus allowing contaminates to enter the container.

The blood is collected into special transport bottles, which are like vacuum tubes but shaped differently. The blood culture bottle contains transport media to preserve any microorganisms present while they are being transported to the laboratory for cultures. Because it is unknown whether the pathogens are anaerobic (living without oxygen) or aerobic (living with oxygen), blood is collected to test for both. The aerobic bottle is filled first, and then the anaerobic bottle is filled. However, if the collection is performed using a syringe, the anaerobic bottle is filled first. If a butterfly collection kit is used, the aerobic bottle is filled first, so that any air in the tubing is released into the oxygen-containing bottle.

Specially designed blood culture collection bottles eliminate the need for either the syringe or butterfly collection method. These specially designed bottles have long necks that fit into the evacuated tubes holders that are use for regular venipuncture collection. These bottles also allow for collection of other blood specimens via evacuated tubes, to be collected without additional venepuncture.

The amount of blood that is collected is critical for the optimal recovery of microorganisms. Up to 10mL of blood is typical, but can vary according to the recommends of the manufacturer of the collection bottle. Collection from infants and children are 1 to 5 mL. If too little blood is collected, the ratio of blood-to-nutrient broth will inhibit the growth of microorganisms. If too much blood is collected, there is the risk of a hospital-induced anemia and the ratio of blood-to-nutrient broth will tilt in the opposite direction, which also is not conductive to optimal growth.

The bottles are then incubated in specialized units for 24 hours before a lab technician studies and/or tests it. This step allows the very small numbers of bacteria (potentially 1 or 2 organisms) to multiply to a level which is sufficient for identification +/-antibiotic resistance testing. Modern blood culture bottles have an indicator in the base which changes color in the presence of bacterial growth and can be read automatically by machine. (For this reason the barcoded stickers found on these bottles should not be removed as they are used by the laboratory's automated systems.)

Taking blood samples from animals

Blood samples from living laboratory animals may be collected using following methods.

Blood collection not requiring anesthesia:

Saphenous vein (rat, mice, guinea pig)

Dorsal pedal vein (rat, mice)

Blood collection requiring anesthesia (local/general anesthesia):

Tail vein (rat, mice)

Tail snip (mice)

Orbital sinus (rat, mice)[25]

Jugular vein (rat, mice)

Temporary cannula (rat, mice)

Blood vessel cannulation (guinea pig, ferret)

Tarsal vein (guinea pig)

Marginal ear vein or artery (rabbit)

Terminal procedure:

Cardiac puncture (rat, mice, guinea pig, rabbit, ferret)

Orbital sinus (rat, mice)

Posterior vena cava (rat, mice)

The volume of the blood sample collection is very important in experimental animals. All nonterminal blood collection without replacement of fluids is limited up to 10% of total circulating blood volume in healthy, normal, adult animals on a single occasion and collection may be repeated after three to four weeks. In case repeated blood samples are required at short intervals, a maximum of 0.6 ml/kg/day or 1.0% of an animal's total blood volume can be removed every 24 hours. The estimated blood volume in adult animals is 55 to 70 ml/kg body weight. Care should be taken for older and obese animals. If blood collection volume exceeds more than 10% of total blood volume, fluid replacement may be required. Lactated Ringer's solution (LRS) is recommended as the best fluid replacement by National Institutes of Health (NIH). If the volume of blood collection exceeds more than 30% of the total circulatory blood volume, adequate care should be taken so that the animal does not suffer from hypovolemia.

Preparation of normal, molar and percent solutions

INTRODUCTION :

Solutions of chemical reagents are a big part of biochemistry, biological and chemical based work. For a beginner of experimental procedure making solutions can also be the most frustrating part. Preparation and handling solutions are essential part of experimental biochemistry. Thus any of new science graduates should be clear in preparing reagents, buffers, and accuracy in pipetting.

The concentration of a dissolved salt in water refers to the amount of salt (solute) that is dissolved in water (solvent). Solutes are the substance of interest to be dissolved and the term solvent denotes the material in which the solute is dissolved.

Solution is a mixture that contains solute and a solvent. Solute can be denoted as the component of a solution present in the lesser amount and the solvent is the component of a solution present in the greater amount. Concentration can be written as the amount of a solute present in a solution per amount of solvent.

UNITS OF CONCENTRATION

- There are many ways to express concentrations. Concentration may be expressed several different ways and some of the more common concentration units are:

1. Equivalent weight
2. Molarity
3. Molality
4. Normality
5. Percent solution (weight/weight)
6. Percent solution (weight/volume)
7. Percent solution (volume/volume)

Equivalent Weight

The equivalent weight is determined by dividing the atomic or molecular weight by the valence. A major use of the concept of equivalents is that one equivalent of an ion or molecule is chemically equivalent to one equivalent of a different ion or molecule. The mass of a substance especially in grams is chemically equivalent to eight grams of oxygen or one gram of hydrogen : the atomic or molecular weight divided by the valence.

Valance could be determined as

1. The absolute value of ion charge

2. The number of H+ or OH– that a species can react with

3. The absolute value of change in charge on a species when undergoing a

chemical reaction.

Preparation of NaOH

Solutions of NaOH can be prepared by either dissolving solid NaOH pellets in water or by diluting a concentrated solution of NaOH. However, the exact concentration of the solution prepared by these methods cannot be calculated from the weighed mass or using the dilution equation for two reasons:

1. Solid sodium hydroxide is hygroscopic ("water-loving"). Pellets of NaOH exposed to air will increase in mass as they become hydrated so the actual mass of pure NaOH is not accurately known.

2. Sodium hydroxide in solution reacts with carbonic acid and its concentration decreases over time. The acid is formed when small amounts of CO2 gas (which is always present in air) dissolves in solution.

$$H_2CO3(aq) + NaOH(aq) = H_2O + Na+(aq) + HCO3-(aq)$$

The water used to make the NaOH solution can be boiled to expel the dissolvedCO2 gas but this time-consuming procedure is often not possible in a short laboratory period. A stock solution of NaOH can be made in advance with boiled water but will re-absorb CO2 over a period of time unless stored in airtight containers. Therefore, if we want to know the exact concentration of a freshly made NaOH solution, we need to "standardize" it. That is, determine its exact concentration by titrating it with a known mass of a primary standard acid.

A "primary standard" is a substance that is used to determine the concentration of a solution. A primary standard should have the following properties: It should be available in very pure form at reasonable cost and should have a high equivalent weight to minimize weighing errors. It should be stable at room temperature, easy to dry, and should not easily absorb water when exposed to air (hygrophobic).

Potassium hydrogen phthalate ("KHP") is the primary standard reagent commonly used to standardize NaOH. It is a monoprotic acid whose formula is KHC8H4O4 and molecular weight is 204.22 g/mol.

$$KHC8H4O4(aq) + NaOH(aq) \longrightarrow H2O + Na+(aq) + K+(aq) + C8H4O4(aq)$$

The white powdery acid is normally heated at 110°C for one hour to remove any loosely bound waters of hydration and then cooled in a desiccator before use.The exact mass (and number of moles of acid) is determined by weighing the dried acid on an analytical balance. The acid is then dissolved in water and NaOH is added until an endpoint (the point at which an indicator changes color) is reached. The phenophthalein indicator used in this experiment is colorless in acid and pink in base. Therefore, the solution containing KHP will remain colorless as long as some KHP is still present. Once the last of the KHP has reacted, the solution will turn pink with one excess drop of base. The exact concentration of NaOH is calculated by using the stoichiometry fromreaction to convert the number of moles of KHP used to moles of NaOH and then dividing by the volume of NaOH used to reach the endpoint of the reaction.

Equivalent Weight of an Acid

In an acid base titration, the equivalence point is the volume of added base where the moles of –OH added (from the base) equal the moles of H+ initially present (from the acid). moles of H+ initially present = moles –OH added (at equivalence point).

To approximate the equivalence point, an indicator with an endpoint close to the equivalence point is added to the analyte solution. A balanced equation can be written describing the chemical reaction occurring between the titrant (the base in this experiment) and analyte (the acid in this experiment) if the identity of both is known.

For example, the titration of hydrochloric acid with potassium hydroxide can be

written:

$$HCl(aq) + KOH(aq) \longrightarrow H2O + KCl(aq)$$

In the case if the identity of the acid is unknown, but the number of acidic hydrogens (H+) carried by the acid is known, a balanced equation can still be written.

For example, the titration of a triprotic acid (an acid with 3 H+) with sodium

hydroxide can be written:

$$H3X(aq) + 3KOH(aq) \longrightarrow 3H2O(l) + K3X(aq)$$

(X: the unknown anion of the acid)

The formula weight of this unknown acid can be calculated by using dimensional analysis. First, the base's concentration is used to convert the base's volume at the endpoint to moles. Then, multiplying by the mole ratio between acid and base from the balanced chemical equation allows for the calculation of the moles of the acid. Now the mass of acid titrated must be divided by the moles of acid calculated giving a result with the units of g/mol.

Equivalent Weight of an Oxidizing Agent

The concept of equivalents and equivalent mass is not restricted to acid-base reactions alone. Unlike acid-base reactions in redox reactions, the electrons are the active units (the equivalents) and the equivalent weights are the masses of oxidizing or reducing agent that deliver or accept 1 mole of electrons. But in case of acid and base the hydrogen or hydroxide ions plays key role in determination of equivalent weight.

Molarity

Molarity is based on the volume of solution containing the solute. Since density is a temperature dependent property a solution's volume, and thus its molar concentration, changes with temperature. By using the solvent's mass in place of the solution's volume, the resulting concentration becomes independent of temperature.

Molarity is the common way of referring to concentrations of solutions. The goal of most basic molarity problems shall be to get the moles from grams by dividing the molecular weight and then dividing by the total number of liters or by given the molarity find the number of grams of the solution by multiplying the volume then the molecular weight. Molarity might give you the density of the solution, from which you can obtain the mass by multiplying the density by the volume.

Although there are several ways in which the concentration of a solution can be quantified, molarity is one of the most basic and widely used. Molarity (M) is defined as the number of moles of solute dissolved in one liter of solution. The higher the molarity, the more concentrated or strong the solution is. For example, a 12 M (which is said "twelve molar") solution of HCl (ie. hydrochloric acid) is much more concentrated than a 0.10 M solution! The basic formula for calculating molarity is:

Molarity (M) = moles of solute (mol) per liters of solution (L)

To solve for moles of solvent, we can use algebra to manipulate the above equation producing the following derived formulas:

Moles of solute (mol) = Molarity (M) × liters of solution (L)

In simple terms, the following formula could be used for preparation of molar

Solutions for lab solutions preparation

For preparation of molar solution

[Molecular weight of the compound (A)/ 1000] × Required morality (B) × Required volume of solution (C) = D gram

In the above equation for preparation of solution of 'B' molarity, 'D' grams of the solute could be dissolved in 'C' ml of solvent.

Molality

The molal unit is not used nearly as frequently as the molar unit and is used in thermodynamic calculations where a temperature independent unit of concentration is needed. A molality is the number of moles of solute dissolved in one kilogram of solvent. The term molality and molarity

should not be confused. While expressing the Molality it is represented by a small “m,” whereas molarity is represented by an upper case “M”

In case of preparation of molar solution except water all other solvent must be weighed. The water is exempted from weighing because; one liter of water has a specific gravity of 1.0 and weighs one kilogram. So one can measure out one liter of water and the solute could be directly added to it. But other solvents might have a specific gravity greater than or less than one. Therefore, one liter of any solvent other than water is not likely to occupy a liter of space.

For example to make a one molal aqueous (water) solution of sodium chloride (NaCl), measure out one kilogram of water and add one mole of the solute, NaCl to it. The atomic weight of sodium is 23 and the atomic weight of chlorine is 35. Therefore the formula weight for NaCl is 58. So 58 grams of NaCl could be dissolved in 1kg water for preparation of 1 molal solution of NaCl.

Normality

The concentration of a solution could also be expressed in terms of Normality. It is based on an alternate chemical unit of mass called the equivalent weight. The normality of a solution is the concentration expressed as the number of equivalent weights (equivalents) of solute per liter of solution. In a chemical mixture 1 normal (1 N) solution contains 1 equivalent weight of solute per liter of solution. Since normality simplifies the calculations required for chemical concentration, it is widely used in analytical chemistry.

Every substance may be assigned an equivalent weight. The equivalent weight may be equal to the formula weight (molecular weight, mole weight) of the substance or equal to an integral fraction of the formula weight (i.e., molecular weight divided by 2, 3, 4, and so on).

The above phenomenon could be better explained with the following example to gain an understanding of the meaning of equivalent weight:

$$\mathbf{HCl(aq) + NaOH(aq) \longrightarrow NaCl(aq) + H_2O}$$

$$\mathbf{HCl(aq) + NaOH(aq) \longrightarrow NaCl(aq) + H_2O}$$

1 mole 1 mole

(36.5 grams) (40.0 grams)

$$\mathbf{H_2SO_4(aq) + 2NaOH(aq) \longrightarrow Na_2SO_4(aq) + 2H_2O}$$

1 mole 1 mole

(98.1 grams) (80.0 grams)

In the above chemical reaction 1 mole of hydrochloric acid (HCl) reacts with 1 mole of sodium hydroxide (NaOH) and 1 mole of sulfuric acid (H2SO4) reacts with 2 moles of NaOH. If you made 1 molar solutions of these substances, 1 liter of 1 M HCl will react with 1 liter of 1 M NaOH and 1 liter of 1 M H_2SO_4 will react with 2 liters of 1 M NaOH. Therefore, H2SO4 has twice the chemical capacity of HCl when reacting with NaOH. The equivalent weight of HCl is equal to its molecular weight, but that of H2SO4 is 1⁄2 its molecular weight.

Expressions for normality are shown below. Notice the similarity to molar solution definition.

Normality (N) = Number of equivalents of solute Equivalents/1 liter of solution = Equivalents/liter

where Number of equivalents of solute = (grams of solute/equivalent weight of solute)

finally N = grams of solute/eq wt solute × L solution = grams/eq wt × L

So, 1 liter of solution containing 36.5 grams of HCl would be 1 N, and 1 liter of solution containing 49.0 grams of H2SO4 would also be 1 N. A solution containing 98.1 grams of H2SO4 (1 mole) per liter would be 2 N.

Percentage Solutions

The percentage solution could be expressed in terms of weight percent (% w/w), volume percent (% v/v) and weight-to-volume percent (% w/v) units ofsolute present in 100 units of solution. For example a solution of 1.5 gram of NH_4NO_3, contains 1.5 gram of NH4NO3 in 100 mL of solution.

Percent by weight (% w/w)

In case of preparing a solution based on percentage by weight, one would simply determine what percentage was required (for example, a 20% by weight aqueous solution of sodium chloride) and the total quantity to be prepared.

If the total quantity needed is 1 kg, then it would simply be a matter of calculating 20% of 1 kg which, of course is:

20 /100 * 1000 g/kg = 200 g NaCl/kg.

Thus finally to bring the total quantity to 1 kg, it would be necessary to add 800g water.

- **Percent by volume (% w/v)**

  Preparation of solutions based on percent by volume it requires the calculationsame as for percent by weight, except that calculations are based on volume. In simple terms one should plan that what percentage was desired (for example, a 20% by volume aqueous solution of sodium chloride) and the total quantity to be prepared in terms of volume.

  For example if 20 % is to be prepared for a total quantity of 1 liter, then it would simply be a matter of calculating 20% of NaCl in 1 liter, the formula can be written as:

  20/100 * 1000 ml/l = 200 g NaCl/l.

- **Percent by volume (% v/v)**

  Volume percent or volume/volume percent most often is used when preparing solutions of liquids. This is typically only used for mixtures of liquids. Volume percent is relative to volume of solution, not volume of solvent. The advantage of volume/volume units is that gaseous concentrations reported in these units do not change as a gas is compressed or expanded.

  For example, 70% v/v rubbing alcohol may be prepared by taking 700 ml of isopropyl alcohol and adding sufficient water to obtain 1000 ml of solution (which will not be 300 ml).

Calculation of molecular weight and Equivalent weight

Molecular weight

Molecular weight is a measure of the sum of the atomic weight values of the atoms in a molecule. Molecular weight is commonly abbreviated by M.W. or MW. Molecular weight is either unitless or expressed in terms of atomic mass units (amu) or Daltons (Da).

Both atomic weight and molecular weight are defined relative to the mass of the isotope carbon-12, which is assigned a value of 12 amu.

Sample Molecular Weight Calculation :

The calculation for molecular weight is based on the molecular formula of a compound (i.e., not the simplest formula, which only includes the ratio of types of atoms and not the number). The number of each type of atom is multiplied by its atomic weight and then added to the weights of the other atoms.

For example, the molecular formula of hexane is C_6H_{14}. The subscripts indicate the number of each type of atom, so there are 6 carbon atoms and 14 hydrogen atoms in each hexane molecule. The atomic weight of carbon and hydrogen may be found on a periodic table.

Atomic weight of carbon: 12.01

Atomic weight of hydrogen: 1.01

molecular weight = (number of carbon atoms)(C atomic weight) + (number of H atoms)(H atomic weight) so we calculate as follows:

molecular weight = (6 x 12.01) + (14 x 1.01)

molecular weight of hexane = 72.06 + 14.14

molecular weight of hexane = 86.20 amu

Equivalent weight

Equivalent weight (also known as gram equivalent) is the mass of one equivalent, that is the mass of a given substance which will combine with or displace a fixed quantity of another substance. The equivalent weight of an element is the mass which combines with or displaces 1.008 gram of hydrogen or 8.0 grams of oxygen or 35.5 grams of chlorine. These values correspond to the atomic weight divided by the usual valence; for oxygen as example that is 16.0 g / 2 = 8.0 g.

For acid–base reactions, the equivalent weight of an acid or base is the mass which supplies or reacts with one mole of hydrogen cations (H^+). For redox reactions, the equivalent weight of each reactant supplies or reacts with one mole of electrons (e^-) in a redox reaction.

Equivalent weight has the units of mass, unlike atomic weight, which is now used as a synonym for relative atomic mass and is dimensionless. Equivalent weights were originally determined by experiment, but (insofar as they are still used) are now derived from molar masses. Additionally, the equivalent weight of a compound can be calculated by dividing the molecular mass by the number of positive or negative electrical charges that result from the dissolution of the compound.

Equivalents of Acids and Bases

$H_2SO_4 + 2OH^- \rightarrow 2H_2O + SO_4^{2-}$

MW of each element and adding 2(1) + (32) + 4(16) = 98.0.

Note that this acid can donate two protons, as the sulfate ion is left with a charge of −2. This the equivalent weight is 98.0/2 = 49.0

For a base, the reasoning is the same. Ammonium hydroxide can accept a proton in solution to become an ammonium ion:

For a base, the reasoning is the same. Ammonium hydroxide can accept a proton in solution to become an ammonium ion:

$NH_4OH + H^+ = H_2O + NH_4^+$

The MW of ammonium hydroxide is (14) + (4)(1) + (16) + 1 = 35.0. Since only once proton is consumed, E for this compound is 35.0/1 = 35.0.

A gram equivalent (geq) is the number of grams of substance present divided by its equivalent weight. It can also be expressed as the number of charge elements contained times the number of moles n.

Measurement of hydrogen ion concentration using PH meter

PREPARATION OF BUFFER

AIM:

To prepare the buffer at required pH.

PRINCIPLE:

The pH meter measures at electrical potential developed by pair of electrode pins in a solution. For measurement of pH, an electrode system sensitive to change in H+ ion concentration of solution is taken. The electrode system consists of sequence of electrode whose potential raise with pH (H+ concentration of the solution).

PROCEDURE:

1. ACETIC ACID- SODIUM ACETATE BUFFER:

REAGENTS REQUIRED:

Acetic Acid 0.2M: 1.5 ml of glacial acetic acid is made upto 100ml with distilled water.

Sodium Acetate Solution: 0.64 gm of sodium acetate or 2.72gm of sodium acetate trihydrate is dissolved in 100ml Distilled water.

PROCEDURE:

Pipette out exactly 36.2ml of sodium acetate solution into 100ml of standard flask and add 14.8ml of glacial acetic acid, make the volume 100ml using distilled water using distilled water. This gives 0.2 M of acetic acid and sodium acetate buffer. The pH is measured with pH meter. The pH meter is first standararised with pH buffer. Wash electrode with distilled water and introduced into 0.2M acetic acid-sodium acetate buffer prepared, the pH of solution is 4.6.

RESULT:

36.2ml Sodium acetate and 14.8 ml glacial acetic acid were mixed and buffer was prepared. pH was measured initial reading observed was 4 which made upto 4.6 with 5N NaOH.

2. BARBITONE BUFFER:

REAGENTS REQUIRED:

- Diethyl barbituric acid.
- Sodium diethyl barbititrate.

PROCEDURE:

Dissolve 2.85gm of diethyl barbituric acid and 14.2gm of sodium diethyl barbititrate in distilled water and upto 1 liter. This gives the barbitone buffer. The pH meter is first standararised with pH buffer. Wash electrode with distilled water and introduced into barbitone buffer prepared, the pH of solution is 6.8.

3. CITRATE BUFFER:

REAGENT S REQUIRED:

- Citric acid: Dissolve 2.101 gm of citric acid in 100ml distilled water.
- Sodium citrate solution 0.1 M: Dissolved 2.941gm of sodium citrate in 100ml distilled water.

PROCEDURE:

46.5ml of citric acid with 3.5ml of sodium citrate solution and upto 100ml with distilled water. It corresponds to 0.1 M citrate buffer and standardised with pH meter and measures the pH of the prepared solution. This gives citrate buffer at pH 2.5.

RESULT:

Citrate buffer was prepared and the pH observed was 4.8 which was adjusted to 2.5 using 1N Hcl and 5N NaoH.

CARBONATE- BICARBONATE BUFFER:

REAGENTS REQUIRED:

- Sodium carbonate solution 0.2M: Dissolve 2.12gm of anhydrous sodium

carbonate in 100ml Distilled water.

- Sodium bicarbonate solution: Dissolve 1.68gm of sodium bicarbonate in 100ml of distilled water.

  PROCEDURE:

  Pipette out exactly 27.5ml of sodium carbonate (Na2Co3) solution. To this add 22.5ml of sodium bicarbonate solution and made upto 100ml with distilled water which corresponds to 0.2 M sodium carbonate and bicarbonate buffer.

  Standardise pH meter and measure the pH of required buffer. This gives the Carbonate bicarbonate buffer pH 10.2.

  RESULT:

  Carbonate bicarbonate buffer was prepared and pH observed was 7.5 which was adjusted to 10.2 using 1N Hcl and 5N NaoH.

4. PHOSPHATE BUFFER:

REAGENTS REQUIRED

- Monobasic: Dissolve 2.78gm of sodium dihydrogen phosphate in 100 ml of distilled water.
- Dibasic sodium phosphate (0.2M): Dissolve 5.3 gm of disodium hydrogen phosphate or 7.17 gm sodium hydrogen phosphate in 100ml distilled water.

PROCEDURE:

39 ml of dihydrogen sodium phosphate is mixed with 61 ml of disodium hydrogen phosphate This made up to 200ml with distilled water. This gives phosphate (Po4)2 buffer of 0.2M.Standardized pH meter with standard buffer. Washed electrode with distilled water and introduced it into phosphate buffer prepared. The pH of the solution is 6.8.

RESULT:

Phosphate buffer was prepared and pH was observed 8.5 which was made upto 6.8 using 1N Hcl and 5N NaoH.

5. POTASSIUM PHOSPHATE BUFFER:

REAGENTS REQUIRED:

- Dipotassium hydrogen phosphate
- Potassium dihydrogen phosphate

PROCEDURE:

174.18 g/mol dipotassium hydrogen phosphate and 136.09 g/mol potassium dihydrogen phosphate was taken and made up to 200ml using distilled water. This gives the potassium buffer.
Standardised pH meter with standard buffer. Washed electrode with distilled water and introduced it into potassium buffer prepared. The pH of the solution is 6.5.

Result:

Dipotassium hydrogen phosphate (K2HPO4) and potassium dihydrogen phosphate (KH2PO4) solution were prepared and the pH was measured to be 9.87 and 4.23 respectively, the solution were made using 1N Hcl and 5N NaoH respectively and the pH was found to be 6.5.

Qualitaive analysis : Identification of carbohydrates

Potato consists of different carbohydrates like starch, reducing sugars etc. Difficulties are encountered in the qualitative and quantitative analysis of samples containing mixtures of carbohydrates, particularly the sugars, because of their structural and chemical similarity and also with respect to their stereoisomers. During biochemical investigations it may because necessary to establish whether a given sample, particularly of a purified preparation, consist carbohydrates or not. Several rapid tests are available the presence or absence of a sugar or a carbohydrate in a sample. These tests are based on specific colour reactions typical for their group and are described below. For laboratory practical, it may be advised to perform these tests with the individual rather than mixture of sugars. Use of sugar solutions of different concentrations (0.1-1%) during these experiments would also provide valuable information about the sensitivity of these tests. The types of carbohydrates detected by these tests are:

Name of the test Application

1. Molisch's Test General test for carbohydrates

2. Anthone Test General test for carbohydrates

3. Iodine Test For glycans (starch, glycogen)

4. Barfoed's Test To distinguish between mono-saccharides from reducing

diasaccharides

5. Seliwanoff's Test For Ketones

6. Fehling's Test For reducing sugars

7. Bendict's Test For reducing sugars

8. Picric acid Test For reducing sugars

9. Bial's Test For pentoses

MOLISCH'S TEST

Principle

This is a general test for all carbohydrates. Conc. H_2SO_4 hydrates glycosidic bonds to yield monosaccharides which in the presence of an acid get dehydrated to form furfural and its derivatives. These products react with sulphonated α-naphthol to give a purple complex. Polysaccharides and glycoproteins also give a positive reaction.

Reagents

1. Conc. H_2SO_4

2. α-naphthol: 5% (w/v) in ethanol (prepare Procedure and

observations)

Add 2-3 drops of α-naphthol solution to 2 ml of the test solution. Very gently pipette 1ml conc.H_2SO_4 along the side if the test tube so that the two distinct layers are formed. Carefully observe any color change at the junction two layers. Appearance of purpose color indicates the presence of carbohydrates in the sample preparation or the test solution.

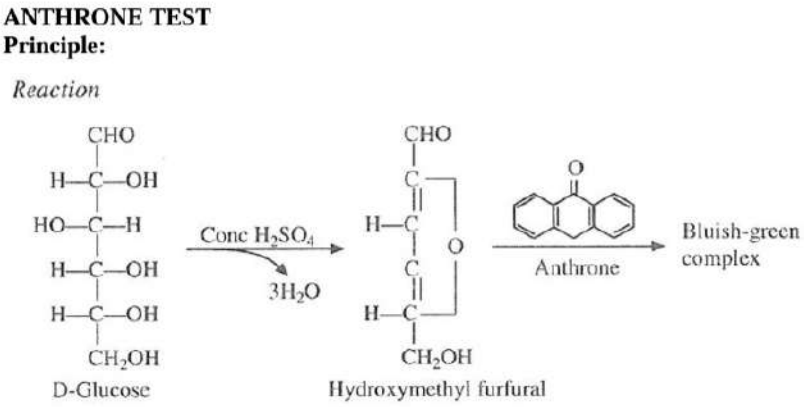
Precautions

1. α-naphthol solution is unstable and should be prepared fresh.

2. Conc. H_2SO_4 should be along the sides of the test tubes causing minimal disturbance to the contents in the tube.

ANTHRONE TEST

Principle:



Anthrone reaction is another general test for carbohydrates. In this the furfural producereacts with anthrone to give bluish green colored complex.

Materials and Reagents

1. Boiling water bath.

2. Conc. H2SO4

3. 0.2% (w/v) anthrone solution Procedure and observations

Add 0.5 - 1 ml of the test solution to about 2 ml of anthrone reagent and mix thoroughly. Observe whether the color changes to bluish green. If not, examine the tubes again keeping them in boiling water bath for 10 min.

IODINE TEST

Principle

Iodine forms colored adsorption complexes with polysacchaides. Starch gives blue color with iodine, while glycogen reacts to form reddish brown complex. Hence it is useful, convenient and rapid test for detection of amylase, amylopectin and glycogen.

Reagents

Iodine solution: Prepare 0.005N iodine solution in 3% (w/v) potassium iodine solution.

1% Test solutions of glucose, sucrose, starch, glycogen, cellulose etc.

Procedure and observations

Take 1 ml of the sample extract or test solution in a test tube. Add 4 - 5 drops of iodine solution to it and mix the contents gently. Observe if any coloured product is formed.

Barfoed's Test

Principle:

This test is used for distinguishing monosaccharides from reducing disaccharides.

Monosaccharides usually react in about 1 - 2 min while the reducing disaccharides take

much longer time between 7 - 12 min to get hydrolysed and then react with the reagent.

Brick red color is obtained in this test which is due to the formation of cuprous oxide.

Reaction

(CH3COO)2Cu2 + H2O ® 2CH3COOH + Cu(OH)2

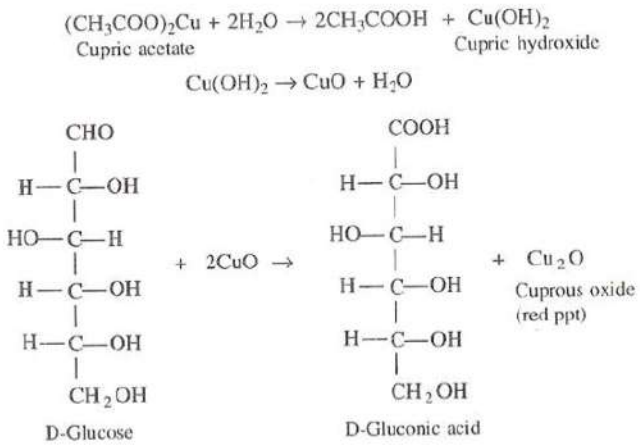
Cupric acetate Cupric hydroxide Cu(OH)2

® CuO+H2O

$(CH_3COO)_2Cu_2 + H_2O$ ® $2CH_3COOH + Cu(OH)_2$

Cupric acetate Cupric hydroxide $Cu(OH)_2$

® $CuO+H_2O$



Materials and Reagents

1. Boiling water bath

2. Barfoed's reagents: Dissolve 13.3 g of copper acetate in 200 ml water and add 1.8 ml of

glacial acetic acid to it.

Procedure and observations

Take 2 ml of Barfoed's solution in a test tube and add 1ml of sample solution to it. Keep the test tubes in a boiling water bath. A briskly boiling water bath should be used for obtaining reliable results. Look for the formation of brick red color and also note the time taken for its appearance.

SELIWANOFF'S TEST

Principle

This test is used to distinguish aldoses from ketoses. Ketoses undergo dehydration to give furfural derivatives, which then condense with resorcinol to form a red complex. Prolonged heating will hydrolyze disaccharides and other monosaccharides will also eventually give color.

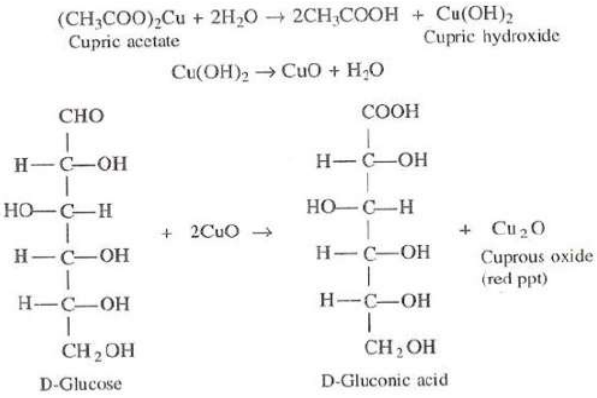
Materials and Reagents

Reaction

$(CH_3COO)_2Cu_2 + H_2O$ ® $2CH_3COOH + Cu(OH)_2$

Cupric acetate Cupric hydroxide $Cu(OH)_2$

® $CuO+H_2O$



3. Boiling water bath

4. Seliwanoff's reagent: 0.05% (w/v) resorcinol in 3 HCl Procedure and

Observations

Add 1ml of the test solution to 2 ml of Seliwanoff's reagent and warm in a boiling water bath for 1min. Note for the appearance of a deep red color. This would indicate that the sample solution contains a keto sugar.

Fehling's

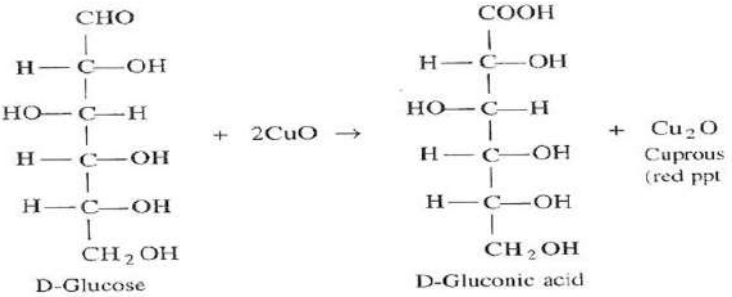
Principle

Fehling's test is a specific and highly sensitive for detection of reducing sugars.

Formation of yellow or red cuprous oxide denotes the presence of reducing

sugars. Rochelle salt acts as the chelating agent in this reaction.

Reaction



Materials and Reagents :

5. Boiling water bath.

6. Fehling's solution A: Dissolve 35 g of CuSO4.5H2O in water and make the volume to 500 ml.

7. Fehling's solution B: Dissolve 120 g of KOH and 173 g Na-K tartrate (Rochelle salt) in water and make the volume to 500 ml.

8. Fehling's reagent: Mix equal volumes of Fehling's solution A and B. These solutions must be mixed immediately prior to use.

Procedure and observations

Add 1 ml of Fehling's reagent (Reagent No. 4) to 1 ml of aliquot of the test solution. Mix thoroughly and place the test tubes in vigorously boiling water bath. Look out for the formation of red ppt of cuprous oxide which would indicate the presence of reducing sugars in the solution.

Benedict's Test

Benedict's test is more convenient and this reagent in more stable. In this method sodium citrate functions as a chelating agent. Presence of reducing sugars results in the formation of red ppt of cuprous oxide.

Reaction

$$Na_2CO_3 + 2H_2O \rightarrow 2NaOH + H_2CO_3$$
$$2NaOH + CuSO_4 \rightarrow Cu(OH)_2 + Na_2SO_4$$
$$Cu(OH)_2 \rightarrow CuO + H_2O$$
$$\text{D-Glucose} + 2\ CuO \rightarrow \text{D-gluconic acid} + Cu_2O \quad (\text{Red ppt})$$

Materials and Reagents

- Boiling water bath.
- Benedict's reagents: Dissolve 173 g of sodium citrate and 100 g of anhydrous Na2CO3 in 600 ml of hot H2O. Dilute to 800 ml with water.
- Dissolve 17.3 g of CuSO4.H2O in 100 ml hot water. Cool and dilute to 100 ml.
- Add Reagent No.2 to Reagent No.3 slowly with constant stirring. Make the

final volume to 1 L.

Procedure and observations

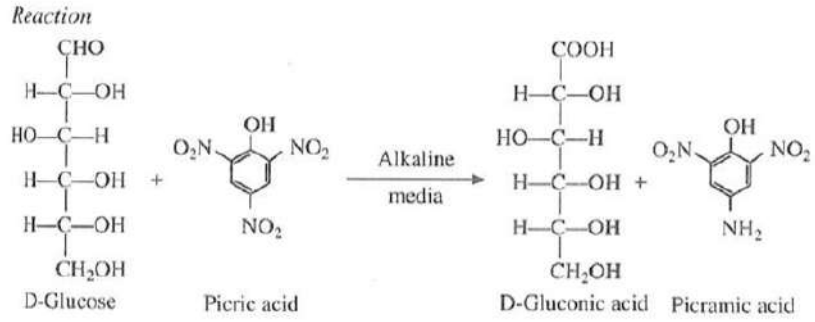
Add 0.5 - 1 ml of the test solution or sample extract to 2 ml of Benedict's reagent (Reagent No. 4). Keep the test tubes in a vigorously boiling water bath. Observe for the formation of red precipitates whose appearance would suggest the presence of reducing sugars in the given or sample extract.

Picric acid Test

Principle:

It is another test for detection of reducing sugars. The reducing sugars react with picric acid to form a red colored picramic acid.

Reaction



Materials and Reagents

- Boiling water bath.
- Saturated picric acid: Dissolve 13 g picric acid in distilled water, boil and

cool.

- 10% Na2CO3

Procedure and observations

Add 1 ml saturated picric acid to 1 ml of sample solution followed by 0.5 ml 10% Na2CO3.

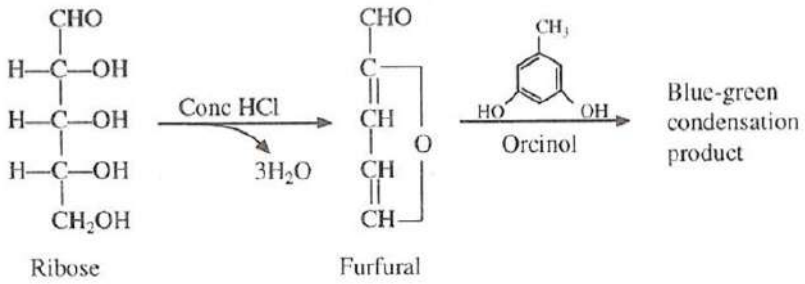
Heat the test tubes in a boiling water bath. Appearance of red color would indicate the presence of reducing sugars in the sample solution.

Bial's test

Principle

This test is useful in the determination of pentose sugars. Reaction is due to formation of furfural in the acid medium which condenses with orcinol in presence of ferric ions to give a blue-green colored complex which is soluble in butyl alcohol.

Reaction



Materials and Reagents

- Boiling water bath
- Dissolve 1.5 g of orcinol in 100 ml of conc. HCl and add 20-30 drops of 10 % ferric chloride solution to it.

Procedure and observations

To 2 ml of Bial's reagent add 4-5 drops of test solution and heat in a boiling water bath. Observe for the formation of blue-green colored complex.

Known Test for Glucose

Sl.No	Experiment	Observation	Inference
1.	**Molisch's Test**: A carbohydrate solution of 2-3 ml is taken in a test tube. Molish's reagent (2-3 drops) is added to carbohydreate solution and mix it well. Then 1-2 ml of concentrated H2SO4 solution is added slowly in the test tube without stirring or mixing.	Development of a purple-coloured ring between the junction of two liquids in the test tube.	This is general test for all Carbohydrate due to the formation of hydroxymethyl furfural. Glucose being a monosaccharide it reacts with the solution.
2.	**Benedict's Test:** Given carbohydrate solution (2-3ml) is taken in a dry and clean test tube. Half Benedict reagent is added to an amount of half of the carbohydrate solution and mixed. Then boil the solution on the reducing flame of a Bunsen burner.	Greenish yellow colour is developed initially after that it turns to brick red colour after vigorous boiling**.**	This is the positive test for all reducing sugar. Glucose being a reducing monosaccharide so, it gives red colour due to formation of Cu2O (Cuprous oxide**)**
3.	**Barfoed's Test:** About 2-3 ml of supplied carbohydrate solution is taken in a dry test tube. Then just 2-3 drops of Barfoed's reagent is added to it and mix well. Then heat vigorously and finally cooled at room temperature	Red precipitate is developed at the bottom of the test tube	This is a positive test for only reducing monosaccharide it reacts with the solution due to the formation of Cu2O.
4.	**Fehling's Test**: About 2-3 ml of supplied	Initially yellow colour developed and then red precipitate is	For reducing sugar due to formation of Cu2O.

	carbohydrate solution is taken in a dry test tube Then just 2-3 drops of Fehling's reagent are added to it and mix well. Then heat vigorously.	formed.	Glucose being a reducing monosaccharide, it reacts with the solution
5.	**Iodine Test:** Given carbohydrate solution is taken in a dry and clean test tube. Then 2-3 drops of Iodine solution is added to it and mix well and wait for 3 mins	No change was observed	This is the positive test for the polysaccharide. As glucose is a reducing monosaccharide so, it does not react with iodine.

Known test for Fructose

Sl.No.	Experiment	Observation	Inference
1.	**Molisch's Test:** Molisch's Test: A carbohydrate solution of 2-3 ml is taken in a test tube. Molish's reagent (2-3 drops) is added to carbohydreate solution and mix it well. Then 1-2 ml of concentrated H_2SO_4 solution is added slowly in the test tube without stirring or mixing	Development of a purple coloured ring between the junction of two liquids in the test tube.	This is general test for all Carbohydrate due to the formation of hydroxymethyl furfural. As fructose is a monosaccharide it reacts with the solution.
2.	Barfoed's Test: About 2-3 ml of supplied carbohydrate solution is taken in a dry test tube. Then just 2-3 drops of Barfoed's reagent is added to it and mix well. Then heat vigorously and	Red precipitate is developed at the bottom of the test tube	This is a positive test for only reducing monosaccharide. As fructose is a reducing monosaccharide so it gives positive result. It reacts with the solution due to the formation of Cu_2O.

	finally cooled at room temperature		
3.	Fehling's Test: About 2-3 ml of supplied carbohydrate solution is taken in a dry test tube Then just 2-3 drops of Fehling's reagent is added to it and mix well. Then heat vigorously.	At first yellow colour developed and then red precipitate is formed.	This is the positive test for reducing sugar due to formation of Cu2O. As fructose is a Reducing monosaccharide, it reacts with the solution.
4.	**Iodine Test:** Given carbohydrate solution is taken in a dry and clean test tube. Then 2-3 drops of Iodine solution is added to it and mix well and wait for 3 mins.	No change was observed	Positive test for the polysaccharide. Fructose is a reducing monosaccharide so, it does not react with iodine.
5.	Benedict's Test: Given carbohydrate solution (2-3ml) is taken in a dry and clean test tube. Half Benedict reagent is added to an amount of half of the carbohydrate solution and mixed. Then boil the solution on the reducing flame of a Bunsen burner.	Greenish yellow colour is developed nitially after that it turns to brick red olour after vigorous boiling	Positive test for all reducing sugar. Fructose is a reducing monosaccharide so, it gives red colour due to formation of Cu2O (Cuprous oxide).

Known Test for Lactose

SL. No.	Experiment	Observation	Inference
1.	**Molisch's Test:** A carbohydrate solution of 2-3 ml is taken in a test tube. Molish's reagent (2-3 drops) is added to carbohydreate solution and mix it well. Then 1-2 ml of concentrated	Development of a purple-coloured ring between the junction of two liquids in the test tube.	This is general test for all Carbohydrate so, lactose (reducing disaccharide) react with molisch's test due to the formation of hydroximethayl furfural.

	H_2SO_4 solution is added slowly in the test tube without stirring or mixing		
2.	**Benedict's Test:** Given carbohydrate solution (2-3ml) is taken in a dry and clean test tube. Half Benedict reagent is added to a amount of half of the carbohydrate solution and mixed. Then boil the solution on the reducing flame of a Bunsen burner.	Greenish yellow colour is developed initially after that it turns to brick red colour after vigorous boiling.	Positive test for all reducing sugar. As lactose is a reducing disaccharide so, it gives red colour due to formation of Cu_2O (Cuprous oxide).
3.	**Iodine Test:** Given carbohydrate solution is taken in a dry and clean test tube. Then 2-3 drops of Iodine solution are added to it and mix well and wait for 3 mins.	No change was observed	**positive test for the polysaccharide**

5.	**Barfoed's Test:** About 2-3 ml of supplied carbohydrate solution is taken in a dry test tube. Then just 2-3 drops of Barfoed's reagent is added to it and mix well. Then heat vigorously and finally cooled at room temperature	No change was observed	Due to slow reaction with disaccharide no change was observed (negative test)

Known test for Maltose

Sl. No.	Experiment	Observation	Inference
1.	Molisch's Test: A carbohydrate solution of 2-3 ml is taken in a test tube. Molish's reagent (2-3 drops) is added to carbohydreate solution and mix it well. Then 1-2 ml of concentrated H2SO4 solution is added slowly in the test tube without stirring or mixing.	Development of a purple-coloured ring between the junction of two liquids in the test tube.	It is general test for all Carbohydrate so, maltose (reducing disaccharide) react with molisch's test due to the formation of hydroximethayl furfural.
2.	**Fehling's Test:** Given carbohydrate solution is taken in a dry and clean test tube. 2-3 drops of Fehling's reagent is added to it and mix well. After that solution was heated vigorously.	Yellow colour Developed initially and then red precipitate is observed.	This is the positive test for reducing sugar due to formation of Cu2O. As maltose is a reducing monosaccharide, it reacts with the solution.
3.	**Iodine Test:** Given carbohydrate solution is taken in a dry and clean test tube. Then 2-3 drops of Iodine solution are added to it and mix well and wait for 3 mins	No change was observed.	This is the positive test for the polysaccharide. Maltose is a disaccharide so; it does not react with iodine reagent
4.	**Barfoed's Test:** Given carbohydrate solution is taken in a dry and clean test tube. 2-3 drops of Barfoed's reagent is added to it and mix well. Then heat the solution vigorously and finally cooled at room temperature.	No change was observed.	Due to slow reaction with disaccharide no change was observed (negative test)
5.	**Benedict's Test:** Given carbohydrate solution (2-3ml) is taken in a dry and clean test tube. Half Benedict reagent is added to an amount of half of the carbohydrate solution and mixed. Then boil the solution on the reducing flame of a Bunsen burner	At first greenish yellow colour is developed just after heating then it turns to brick red colour after vigorous boiling.	This is the positive test for all reducing sugar. Maltose is a reducing monosaccharide so, it gives red colour due to formation of Cu2O(Cuprous oxide).

Known test for Sucrose

Sl. No.	Experiment	Observation	Inference
1.	**Molisch's Test:** A carbohydrate solution of 2-3 ml is taken in a test tube. Molish's reagent (2-3 drops) is added to carbohydreate solution and mix it well. Then 1-2 ml of concentrated H_2SO_4 solution is added slowly in the test tube without stirring or mixing**.**	Development of a purple-coloured ring between the junction of two liquids in the test tube.	Sucrose is present in the sample. Hydroximethyl furfural is formed in the molisch's test due to reaction of carbohydrate with concentrated H_2SO_4.
2.	**Benedict's Test:** Given carbohydrate solution (2-3ml) is taken in a dry and clean test tube. Half Benedict reagent is added to a amount of half of the carbohydrate solution and mixed. Then boil the solution on the reducing flame of a bunsen burner**.**	No change was observed	Sucrose is a nonreducing sugar so, it does not react with this solution
3.	**Barfoed's Test:** Given carbohydrate solution is taken in a dry and clean test tube. 2-3 drops of Barfoed's reagent is added to it and mix well. Then heat the solution vigorously and finally cooled at room temperature.	No change was observed.	Sucrose is a nonreducing sugar so, it does not react with this solution.
4.	**Hydrolysis Test:** 2-3ml of given carbohydrate solution is taken in a dry and clean test tube. Then few drops of conc. H_2SO_4, con. HCl is added to it and then boiled. After boiling cooled the solution under tap water and then neutralize the solution by adding	Developed greenish yellow colour after heating then it turns to brick red colour after vigorous boiling.	This is the positive test for all carbohydrate. Sucrose is a nonreducing sugar and converted to glucose after acid hydrolysis.Reducing sugar test is perform by using Benedict's Test and it gives the brick red colour due to formation of Cu_2O

	Na2CO3 until no further bubbles occur. Then perform Benedict's Test		
5.	Iodine Test: Given carbohydrate solution is taken in a dry and clean test tube. Then 2-3 drops of Iodine solution is added to it and mix well and wait for 3 mins**.**	No change was observed	This is the positive test for the polysaccharide. as sucrose is a disaccharide so, it does not react with iodine reagent.
6.	**Fehling's Test:** Given carbohydrate solution is taken in a dry and clean test tube. 2-3 drops of Fehling's reagent are added to it and mix well. After that solution was heated vigorously.	No change was observed	This is the positive test for all reducing sugar. Sucrose is a nonreducing sugar so, it does not react with Fehling's solution.

Known test for Starch

Sl. No.	Experiment	Observation	Inference
1.	**Molisch's Test:** A carbohydrate solution of 2-3 ml is taken in a test tube. Molish's reagent (2-3 drops) is added to carbohydreate solution and mix it well. Then 1-2 ml of concentrated H2SO4 solution is added slowly in the test tube without stirring or mixing..	Development of a purple coloured ring between the junction of two liquids in the test tube.	Sucrose is present in the sample. hydroxymethyl furfural is formed in the molisch's test due to reaction of carbohydrate with concentrated H2SO4
2.	**Benedict's Test:** Given carbohydrate solution (2-3ml) is taken in a dry and clean test tube. Half Benedict reagent is added to a amount of half of the carbohydrate solution and mixed. Then boil the solution on the reducing flame of a Bunsen burner.	No change was observed	Starch is a nonreducing sugar so, it does not react with this solution.

3.	**Barfoed's Test:** Given carbohydrate solution is taken in a dry and clean test tube. 2-3 drops of Barfoed's reagent are added to it and mix well. Then heat the solution vigorously and finally cooled at room temperature.	No change was observed	Starch is a nonreducing sugar so, it does not react with this solution.
4.	**Iodine Test:** Given carbohydrate solution is taken in a dry and clean test tube. Then 2-3 drops of Iodine solution is added to it and mix well and wait for 3 mins.	Blue color was observed	This is the positive test for the polysaccharide. Starch is a polysaccharide; it reacts with iodine reagent..
5.	**Fehling's Test:** Given carbohydrate solution is taken in a dry and clean test tube. 2-3 drops of Fehling's reagent are added to it and mix well. After that solution was heated vigorously.	No change was observed	This is the positive test for all reducing sugar. Starch is a nonreducing sugar so, it does not react with Fehling's solution.
6.	**Hydrolysis Test:** 2-3ml of given carbohydrate solution is taken in a dry and clean test tube. Then few drops of conc. H_2SO_4, con. HCl is added to it and then boiled. After boiling solution was cooled under tap water and then neutralize the solution by adding Na_2CO_3 until no further bubbles occur. Then perform Benedict's Test.	Developed greenish yellow colour after heating then it turns to brick red colour after vigorous boiling	This is the positive test for all carbohydrate. Starch is a nonreducing sugar and converted to glucose after acid hydrolysis.Reducing sugar test is perform by using Benedict's Test and it gives the brick red colour due to formation of Cu_2O.
7.	**Barfoed's Test:** Given carbohydrate solution is taken in a dry and clean test tube. 2-3 drops of Barfoed's reagent is added to it and mix well. Then heat the solution vigorously and finally cooled at room temperature.	No change was observed.	Sucrose is a nonreducing sugar so, it does not react with this solution.
8.	**Hydrolysis Test:** 2-3ml of given carbohydrate solution is taken in a dry and clean test tube. Then few drops of conc. H_2SO_4, con. HCl is added to it and then boiled. After boiling cooled the solution under tap water and then neutralize the solution by adding Na_2CO_3 until no further bubbles occur. Then perform Benedict's Test	Developed greenish yellow colour after heating then it turns to brick red colour after vigorous boiling.	This is the positive test for all carbohydrate. Sucrose is a nonreducing sugar and converted to glucose after acid hydrolysis.Reducing sugar test is perform by using Benedict's Test and it gives the

			brick red colour due to formation of Cu2O
9.	Iodine Test: Given carbohydrate solution is taken in a dry and clean test tube. Then 2-3 drops of Iodine solution is added to it and mix well and wait for 3 mins**.**	No change was observed	This is the positive test for the polysaccharide. as sucrose is a disaccharide so, it does not react with iodine reagent.
10.	**Fehling's Test:** Given carbohydrate solution is taken in a dry and clean test tube. 2-3 drops of Fehling's reagent are added to it and mix well. After that solution was heated vigorously.	No change was observed	This is the positive test for all reducing sugar. Sucrose is a nonreducing sugar so, it does not react with Fehling's solution.

Known test for Peptone

Sl. No.	Experiment	Observation	Inference
1	**Biuret test:** 1% copper sulphate solution, one drop taken in a dry clean test tube. Then 3ml of 10% NaOH solution added to it. Then equal volume of supplied protein solution is taken and mixed well.	rose pink (Violet) colour observed.	Positive test for peptone
2	**Million's Test:** Given protein solution (2-3 ml) taken in a dry and clean test tube. Then 3-4 drops of Million's reagent added to it and heated slightly heated with stirring.	At first very little precipitate was observed and after heating it was dissolve	Positive test for peptone
3.	**Xanthoprotein Test:** About 2-3ml of supplied protein solution is taken in a dry test tube then HNO3 is added to it. Then heated and boiled and cooled.	Yellow colour is developed without precipitate.	positive test for peptone
4.	**Adamkiewicz Test:** Abo Given protein solution (2-3 ml) is taken in a dry test tube. 2ml of glacial CH3COOH added to it and mixed well. Then con. H2SO4 mixed into the test tube.	A purple colour is developed at the junction of two liquid	positive test for peptone
5.	**Esbach test:** Few ml of supplied protein sample is taken in a dry test tube. Then few ml of Esbach solution is added to it.	No precipitate is formed	Presence of peptone
6.	**Heat coagulation:** Little amount of given protein solution is taken in a dry and clean test tube. Then few drops of glacial CH3COOH is added to it and heated.	No coagulation Observed after heating	It is the negative test for peptone.

Beer-Lambert's Law

The Beer–Lambert law, also known as Beer's law, the Lambert–Beer law, or the Beer–Lambert–Bouguer law relates the attenuation of light to the properties of the material through which the light is travelling. The law is commonly applied to chemical analysis measurements and used in understanding attenuation in physical optics, for photons, neutrons, or rarefied gases. In mathematical physics, this law arises as a solution of the BGK equation.

Mathematical formulation :

A common and practical expression of the Beer–Lambert law relates the optical attenuation of a physical material containing a single attenuating species of uniform concentration to the optical path length through the sample and absorptivity of the species. This expression is:

$$A = \varepsilon l c$$

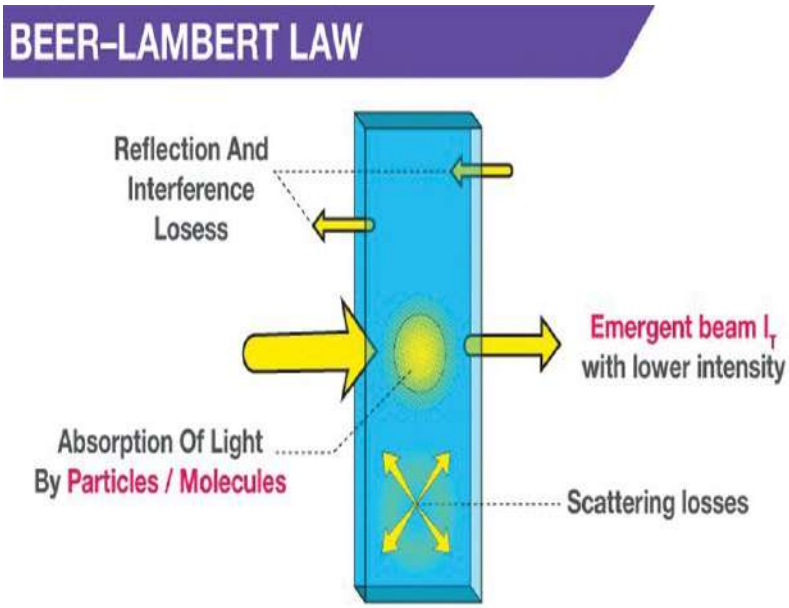
Where

***A* is the absorbance**

ε is the molar attenuation coefficient or absorptivity of the attenuating species

l is the optical path length in cm

c is the concentration of the attenuating species



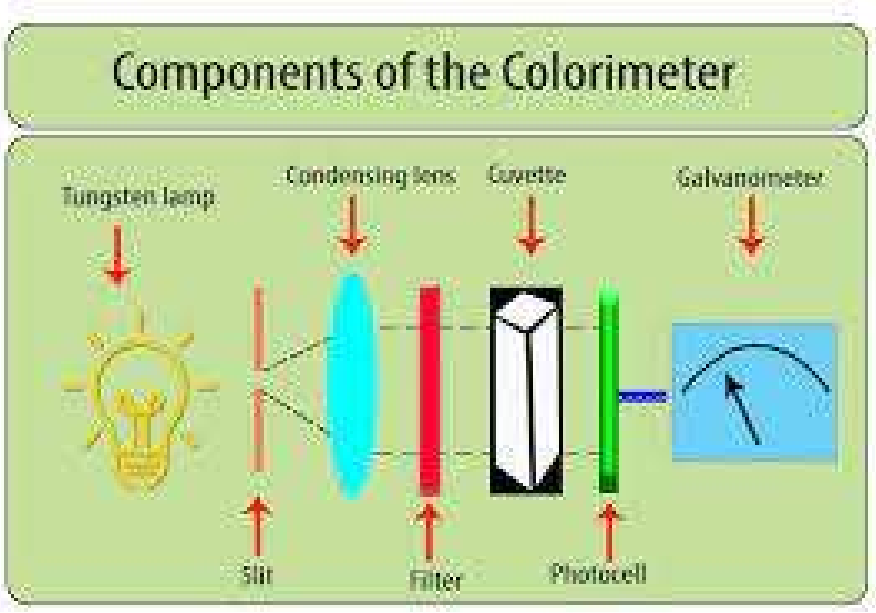
Colorimeter: A colorimeter is an instrument that compares the amount of light getting through a solution with the amount that can get through a sample of pure solvent. A colorimeter contains a photocell which is able to detect the amount of light passing through the solution under investigation.

Principle of colorimeter:

- Beer-lambert's law
- When a monochromatic light passes through a colored solution, that monochromatic light is absorbed by the colored solution, which is depend on –
- Type of colour
- Colour density
- Distance travelled by light.

Components of colorimeter:

- Light source
- Slit
- Monochromator(Filter)
- Cuvette
- Photocell
- Galvanometer



Application Of Colorimetry :

- It is used by hospitals as well as laboratories for analysing biochemical samples such as urine, cerebrospinal fluids, plasma, biochemical samples, and serum.
- It is widely used to generate a quantitative estimation of the serum components, proteins, glucose, and various biochemical compounds.

- It is also used in food industries and by manufacturing industries to make textiles and paints. A colorimeter has immense significance in the ever-expanding world of science. Further research is going to enhance its features and functionalities.

Spectrophotometry

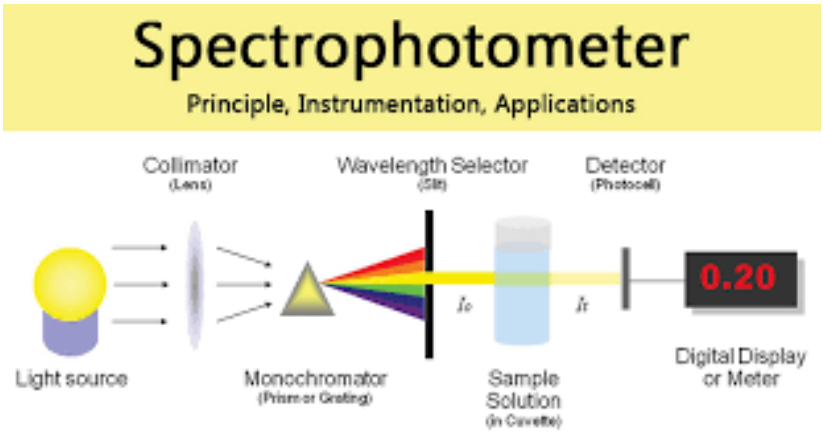
Spectrophotometry is a branch of electromagnetic spectroscopy concerned with the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength. Spectrophotometry uses photometers, known as spectrophotometers that can measure the intensity of a light beam at different wavelengths. Although spectrophotometry is most commonly applied to ultraviolet, visible, and infrared radiation, modern spectrophotometers can interrogate wide swaths of the electromagnetic spectrum, including x-ray, ultraviolet, visible, infrared, and/or microwave wavelengths.

Principle of Spectrophotometer :

The spectrophotometer technique is to measure light intensity as a function of wavelength. It does this by diffracting the light beam into a spectrum of wavelengths, detecting the intensities with a charge-coupled device, and displaying the results as a graph on the detector and then on the display device. In the spectrophotometer, a prism (or) grating is used to split the incident beam into different wavelengths by suitable mechanisms, waves of specific wavelengths can be manipulated to fall on the test solution. The range of the wavelengths of the incident light can be as low as 1 to 2nm.

The spectrophotometer is useful for measuring the absorption spectrum of a compound, that is, the absorption of light by a solution at each wavelength.

Instrumentation of Spectrophotometer



The essential components of spectrophotometer instrumentation include:

1. A table and cheap radiant energy source

- Materials that can be excited to high energy states by a high voltage electric discharge (or) by electrical heating serve as excellent radiant energy sources.

2. A monochromator, to break the polychromatic radiation into component wavelength (or) bands of wavelengths.

- A monochromator resolves polychromatic radiation into its individual wavelengths and isolates these wavelengths into very narrow bands.

Prisms:

A prism disperses polychromatic light from the source into its constituent wavelengths by virtue of its ability to reflect different wavelengths to a different extent

Two types of Prisms are usually employed in commercial instruments. Namely, 600 cornu quartz prism and 300 Littrow Prism.

Grating:

- Gratings are often used in the monochromators of spectrophotometers operating ultraviolet, visible and infrared regions.

3. **Transport vessels** (cuvettes), to hold the sample

- Samples to be studied in the ultraviolet (or) visible region are usually glasses (or) solutions and are put in cells known as “CUVETTES”.
- Cuvettes meant for the visible region are made up of either ordinary glass (or) sometimes Quartz.

4. A Photosensitive **detector** and an associated **readout system**

- Most detectors depend on the photoelectric effect. The current is then proportional to the light intensity and therefore a measure of it.
- Radiation detectors generate electronic signals which are proportional to the transmitter light.
- These signals need to be translated into a form that is easy to interpret.

This is accomplished by using amplifiers, Ammeters, Potentiometers and Potentiometric recorders.

Applications:

- Detection of concentration of substances
- Detection of impurities
- Structure elucidation of organic compounds
- Monitoring dissolved oxygen content in freshwater and marine ecosystems
- Characterization of proteins
- Detection of functional groups
- Respiratory gas analysis in hospitals
- Molecular weight determination of compounds
- The visible and UV spectrophotometer may be used to identify classes of compounds in both the pure state and in biological preparations.

Physiology and Anatomy Lab Manual for BMLT 1st Year (WBUHS)

BMLT WBUHS (paper – 104)

1. Measurement of Pulse rate

Principle:

Pulse/heart rate is the wave of blood in the artery created by contraction of the left ventricle during a cardiac cycle. The strength or amplitude of the pulse reflects the amount of blood ejected with myocardial contraction (stroke volume). Normal pulse rate range for an adult is between **60-100 beats per minute**. A well-trained athlete may have a resting heart rate of 40 to 60 beats per minute, according to the American Heart Association.

Procedure:

Ask whether the patient has walked, climbed stairs, or otherwise exerted themself in the last 20 minutes. If not, you can proceed. If the answer is yes, wait 20 minutes before taking the reading. This will help to prevent false readings.

Make sure the patient is relaxed and comfortable.

Place the tips of your first and second finger on the inside of the patient's wrist.

Press gently against the pulse. Take your time to note any irregularities in strength or rhythm.

If the pulse is regular and strong, measure the pulse for 30 seconds. Double the number to give the beats per minute (e.g.: 32 beats in 30 seconds means the pulse is 64 beats per minute). If you noticed changes in rhythm or strength, you must measure the pulse for a full minute.

Record the pulse rate (the number of beats per minute) in the patient's notes and describe its strength and rhythm. Compare the pulse rate with the values in the Table land record whether the pulse is normal, slow or fast. Any abnormalities should be recorded and reported to the senior nurse and doctor.

Strength of the pulse is a very subjective measurement, but an experienced nurse will compare it with what has been felt previously in other patients. Describe the puse as ‘weak’, ‘faint’, ‘strong' or' bounding’.

Think about the rhythm of the pulse. Is it regular? If irregular, in what way? Cardiac problems may present as a regular missed beat, for example, so is the irregularity regular (described as regularly irregular) or is there no pattern (described as irregularly irregular)?

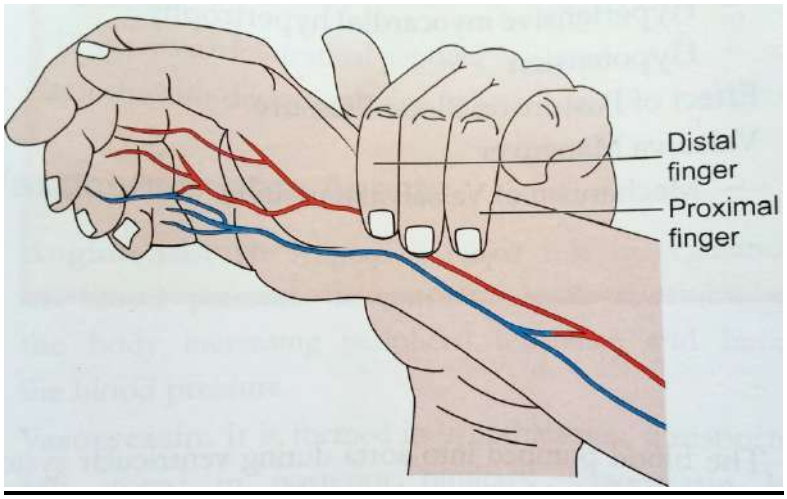
Discuss with your patient the result of the pulse measurement and if any further investigations are required.

Wash and dry your hands.

Pulse point	Area of palpation
Temporal pulse	Over the temple, in front of ear on superficial temporal artery
Carotid pulse	In the neck along anterior border of sternocleidomastoid muscle on common carotid artery
Brachial pulse	In cubital fossa along medial border of biceps muscle on brachial artery
Radial pulse	Over the thumbside of wrist between tendons of brachioradialis and flexor carpi radialis muscles on radial artery
Ulnar pulse	Over the little fingerside of wrist on ulnar artery
Femoral pulse	In the groin on femoral artery
Popliteal pulse	Behind knee, in the popliteal fossa on popliteal artery

Measurement of redial artery pulse –

1. With the palm up, look at the area between wrist bone and the tendon on the thumb side of wrist. Radial pulse can be taken on either wrist.
2. Use the tip of the index and third fingers of other hand to feel the pulse in radial artery between wrist bone and the tendon on the thumb side of wrist.
3. Apply just enough pressure so can feel each beat. Do not push too hard, it will obstruct the blood flow.
4. Watch the second hand on watch or a clock to count how many times.
5. Record your pulse rate.



Measurement of carotid artery pulse –

Ask the client to sit upright. Locate the carotid artery medial to the sternomastoid muscle (between the muscle and the trachea at the level of the cricoid cartilage, which is in the middle third of the neck). With the pads of your three fingers, gently palpate the carotid artery, one at a time.

Evaluation Of Pulse

1. Rate :-

   Count the pulse for 1 min / at least 30 sec

   Normal : 60 – 100 /min

   Tachycardia : >100 /min

   Bradycardia : <60 /min

- ❖ Increased pulse rate due to- exercise, fever, anxiety, hyperthyroidiam and atrial and ventricular trachycardias.

  Decreased pulse rate in bradycardia, hypothyroidism and incomplete heart blocks.

2. Rhythms –

Assessed by palpating radial artery

It is noted regular or irregular.

Under normal sinus bradycardia or sinus trachycardia pulse appears at regular intervals.

Irregular pulse rhythm is a feature of extra systole, Atrial Tachyarrhythmia, atrial fibrillation.

Observation-

Regularly irregular : Atrial Tachyarrhythmia with fixed AV block .

Irregularly irregular : Atrial / ventricular ectopic, Atrial Fibrillation.

3. Volume

Assessed by palpating – carotid artery

Correlates with stroke volume.

High volume – elderly person, anxiety, Aortic regurgitation, fever, thyrotoxicosis.

Observation: Normal / High/ Low volume.

4. Character of blood vessel-

- Thickened and palpable / soft and not palpable.
- It is usually evaluated at right carotid artery.
- It becomes more prominent in some abnormal conditions such as anacrotic pulse, water hammer pulse, pulsus paradoxus.

5. Character of pulse :

Catacrotic / Aanacrotic

Palpitation / measurement format for the arterial pulse rate (beats/min).

❖ Measurement No….

- Name of subject-
- Age –
- Sex-

Pulse Rate	Carotid artery pulse (Beats/min)	Radial artery pulse (Beats/min)
Resting (Before Exercise) (15 sec x 4)		
Just After step test for 5 minutes		
After 10 minutes of resting		
After 20 minutes of resting		
After 30 minutes of resting		

- Evaluation of resting pulse (before exercise) - -

1. Rate-
2. Rhythm –
3. Volume-
4. Character of arterial wall-
5. Character of pulse-

Interpretation of resting results: -

……
……

Measurement of blood pressure

The term blood pressure refers to the force exerted by the blood as it presses against and attempts to stretch the walls of blood vessels. Although blood exerts this outward force throughout the CVS, the term blood pressure, used unqualified, refers to systemic arterial blood pressure (others are: venous, capillary pressure, etc). The blood pressure (BP) is not steady (unchanging) throughout the cardiac cycle but fluctuating, i.e. it is pulsatile. It rises and falls; reaching its maximum during systole of the heart, when it is called the systolic blood pressure (SBP); and falling to its minimum during diastole of the heart when it is called diastolic blood pressure (DBP) Measurement of blood pressure is an important clinical procedure as it provides valuable information about the cardiovascular system (CVS) under normal and disease conditions.

PRINCIPLE:

A sufficient length of a single artery is selected in the arm (brachial artery), or in the thigh (femoral artery). The artery is first compressed by inflating a rubber bag (connected to a manometer) placed around the arm (or thigh) to stop the blood flow through the occluded section of the artery. The pressure is then slowly released and the flow of blood through the obstructed segment of the artery is studied by:
i. Feeling the pulse—the palpatory method.
ii. Observing the oscillations of the mercury column—the oscillometric method, and
iii. Listening to the sounds produced in the part of the artery just below the obstructed segment—the auscultatory method.

PROCEDURES:

The subject may be lying down (supine) or sitting, but should be mentally and physically relaxed and free from excitation and anticipation.

Lay the arm bare up to the shoulder and record the blood pressure first with the palpatory method, followed by auscultatory method. The upper arm on which the BP cuff is to be tied must be at the level of the heart. (In the supine position, the arm resting on the bed will be nearly at the heart level. In the sitting position the arm resting on the table of a suitable height will be at the correct level).

In obese subjects, the cuff may be applied on the forearm with the stethoscope placed over the radial artery for auscultatory method. (If no sounds are heard a reasonably reliable determination can be obtained by palpation at the wrist).

Palpatory Method (Riva Rocci 1896)

1. Make the subject sit or lie supine and allow 5 minutes for mental and physical relaxation.
2. Open the lid of the apparatus until you hear the "click". Release the lock on the mercury reservoir and check that the mercury is at the zero level. If it is above zero, subtract the difference from the final reading. If it is below zero, add the required amount of mercury to bring it to zero level.
3. Place the cuff around the upper arm, with the centre of the bag lying over the brachial artery, keeping its lower edge about 3 cm above the elbow. Wrap the cloth covering around the arm so as to cover the rubber bag completely, and to prevent it bulging out from under the wrapping on inflation. The cuff should neither be too tight nor very loose.
4. Palpate the radial artery at the wrist and feel its pulsations with the tips of your fingers. Keeping your fingers on the pulse, hold the air bulb in the palm of your other hand and tighten the leak valve screw with your thumb and fingers.
5. Inflate the cuff slowly until the pulsations disappear; note the reading then raise the pressure another 30–40 mm Hg.
6. Open the leak valve and control it so that the pressure gradually falls in steps of 2–3 mm. Note the reading when the pulse just reappears. **The pressure at which the pulse is first felt is the systolic pressure**. (It corresponds to the time when, at the peak of each systole, small amounts of blood start to flow through the compressed segment of the brachial artery). Deflate the bag quickly to bring the mercury to the zero level.

7. Record the pressure in the other arm. Take 3 readings in each arm, deflating the cuff for a few minutes between each determination.

Advantages of palpatory method.

This method avoids the pitfall of the auscultatory method in missing the auscultatory gap.

Disadvantages of palpatory method:
This method measures only the systolic pressure, the diastolic pressure cannot be measured.
This method lacks accuracy because the systolic pressure measured by it is lower than the actual by 4–6 mm Hg. It assumes that the first escape of blood under the cuff will cause pulsations in the peripheral artery (radial in this case). However, there is no evidence that the amount of blood that escapes when the artery first opens is enough to produce a pulse wave detectable by the fingers. Thus, definite pulsation may not occur until the cuff pressure has been reduced by 6–8 mm Hg.

Auscultatory Method (Korotkoff, 1905)

1. Place the cuff over the upper arm as described above, and record the BP by the palpatory method.

2. Locate the bifurcation of brachial artery (it divides into radial and ulnar branches) in the cubital space just medial to the tendon of the biceps which can be easily palpated in a semi-flexed elbow as a thick, hard, elongated structure. Mark the point of arterial pulsation with a sketch pen.
3. Place the chest-piece of the stethoscope on this point and keep it in position with your fingers and thumb of the left hand (if you are right-handed).
4. Inflate the cuff rapidly, by compressing and releasing the air pump alternately (sounds may be heard as the mercury column goes up). Raise the pressure to 40 to 50 mm Hg above the systolic level as determined by the palpatory method.
5. Lower the pressure gradually until a clear, sharp, tapping sound is heard. Continue to lower the pressure and try to note a change in the character of the sounds.

These sounds are called Korotkoff sounds and show the following phases:

Phase I This phase starts with a clear, sharp tap when a jet of blood is able to cross the previously obstructed artery. (Sometimes this phase may start with a faint tap, especially when the systolic pressure is very high). As the pressure is lowered, the sounds continue as sharp and clear taps. This phase lasts for 10–12 mm Hg fall in pressure.

Phase II The sounds become murmurish and remain so during the next 10–15 mm Hg fall in pressure when they again become clear and banging.

Phase III It starts with clear, knocking, or banging sounds that continue for the next 12 to 14 mm Hg pressure, when they suddenly become muffled.

Phase IV The transition from phase III to phase IV is usually very sudden. The sounds remain muffled, dull, faint and indistinct (as if coming from a distance) until they disappear. The muffling of sounds and their disappearance occurs nearly at the same time, there being a difference of 4–5 mm Hg (i.e. phase IV lasts for 4–5 mm Hg).

Phase V This phase begins when the Korotkoff sounds disappear completely. If you reduce the pressure slowly, you will note that total silence continues right up to the zero level.
6. Take 3 readings with the auscultatory method and repeat 3 readings on the other arm.
7. Effects of posture, gravity, and muscular exercise on blood pressure are discussed in the next experiment.

Effect of postural changes in Blood Pressure

The effect of changes in posture depends on whether these are recorded immediately after standing from supine position, or after prolonged standing. They also depend on whether a person stands against a support (e.g. a wall), or is standing 'free' and still.

Immediate Effect. As the person assumes erect position, blood tends to pool in the lower parts of the body (especially in the veins) due to gravity. This decreases the venous return, and hence CO and BP. A pooling of 250–300 ml of blood can decrease the systolic pressure by 10–15 mm Hg. However, within some 8–10 seconds the sino-aortic baroreceptor mechanism restores BP to normal level.

Effect of Prolonged Standing. If a person stands still, especially against a support, more than 500 ml of blood may pool in the lower body. Also, increased capillary hydrostatic pressure causes fluid to be filtered out into the tissues, which further reduces venous return. The CO and BP fall, resulting in cerebral ischemia that causes the person to fall down unconscious. The fainting is actually a homeostatic mechanism, as it restores venous return, CO and BP, thus relieving cerebral ischemia.

PROCEDURES

1. Allow the subject to rest and relax for a few minutes in the supine position. Record the heart rate (pulse rate) and BP by the palpatory method and auscultatory method (later on by auscultatory method alone). Disconnect the cuff from the BP apparatus.

2. Ask the subject to sit up and immediately record the BP and heart rate (HR). Repeat the determinations after 1 minute, 2 and 5 minutes.

3. Make the subject lie down again and rest for a few minutes. Then record the BP and HR. Now ask him to suddenly stand up, and record the BP and HR.

4. Record your observations in your workbook.

Interpretation of Results

Measurement of arterial blood pressure by palpatory method-

- Measurement No….
  - Name of subject-
  - Age –
  - Sex-

	Pulse Dis-appears (mmHg)	Pulse Re-appears / Systolic blood pressure (mmHg)
Resting (Before Exercise)		
Just After step test for five minutes		
After 10 minutes of resting.		
After 20 minutes of resting.		
After 30 minutes of resting.		

Interpretation of your results: -

……

Measurement of arterial blood pressure by auscultatory method-

- Measurement No….
  - Name of subject-
  - Age –
  - Sex-

	Systolic BP (mmHg)	Diastolic BP (mmHg)	Pulse pressure (mmHg)	Mean arterial pressure (mmHg)
Resting (Before Exercise)				
Just After step test for five minutes				
After 10 minutes of resting.				
After 20 minutes of resting.				
After 30 minutes of resting.				

Interpretation of your results: -

……
……

Postural changes and Blood Pressure

Measurement No….

- Name of subject-

Three posture	Systolic BP (mmHg)	Diastolic BP (mmHg)	Pulse pressure (mmHg)	Mean arterial pressure (mmHg)
In supine				
In sitting				
In standing				

Interpretation of your results: -

……

Measurement of respiratory rate

Principle:

Respiratory rate (RR) is an essential vital sign and a fundamental element of patient assessment. Generally, changes in RR occur automatically in response to a physiological demand but it is possible to consciously increase or decrease RR for short periods. Changes from as little as three to five breaths per minute (bpm) may indicate a change in the patient's condition and are often the first sign of deterioration. RR therefore provides a baseline for future comparisons and helps determine the patient's acuity.

Other key indications for measuring RR include:

- Post-operative monitoring;
- Detecting complications such as pulmonary oedema associated with blood transfusion and administration of intravenous fluids;
- Identifying patients who are deteriorating and are critically ill;
- Evaluating response to treatment – for example use of opiates, which can cause respiratory depression;
- Monitoring chronic lung disease such as chronic obstructive pulmonary disease, and response to treatment;
- Monitoring patients receiving oxygen therapy

Procedure

1. Wash hands with soap and water to reduce infection risk.

2. Position the patient in a comfortable position. Maintain a constant temperature to prevent shivering, which can increase RR. If possible, remove bulky clothing or bed covers from the upper part of the patient's body to facilitate counting RR and observing depth, symmetry and pattern of breathing. If the patient is sitting, their feet must be flat on the floor; sitting with legs suspended can reduce venous return, which may increase heart rate and subsequently RR.

3. If a patient has been prescribed oxygen, ensure the oxygen mask or nasal cannula is correctly positioned and recorded on the observation chart before recording RR.

4. Allow the patient to rest, if possible, for 20 minutes before taking the measurement. RR may increase after activity, giving an abnormal baseline. Some medication can affect RR so this should also be taken into consideration.

5. While you are preparing the patient, observe their respiratory function, for example, whether they can talk in full sentences. Taking a breath mid-sentence or one-word answers may be a sign of respiratory distress.

6. Note whether the patient is alert and orientated to time and place. Changes in cognitive status, such as confusion, may be due to hypoxia, cerebral injury or side-effects of medication, such as opiates.

7. It may be useful to assess RR at the same time as pulse rate or oxygen saturation. This will give a more accurate rate and minimise any subconscious influence, as patients may alter their breathing if they know they are being observed.

8. Using a watch with a second hand, count breaths (number of times the chest moves up and down) for a full minute. This length of time is needed as changes can occur in the respiratory pattern and rate.

9. While observing the RR, note the rhythm, which may indicate signs of underlying illness. Respirations should be regular with equal pause between each breath.

10. Observe the patient's lips for signs of cyanosis (blue tinge), which may indicate hypoxia (low oxygen saturation [SpO2]). Pulse oximetry is a valuable tool to measure SpO2 but it has limitations when a patient's peripheral circulation or condition is compromised – for example, through tremor, shivering, hypovolaemia, hypothermia, heart failure or vaso-constriction.

11. Record the RR on the observation chart and report any abnormalities.

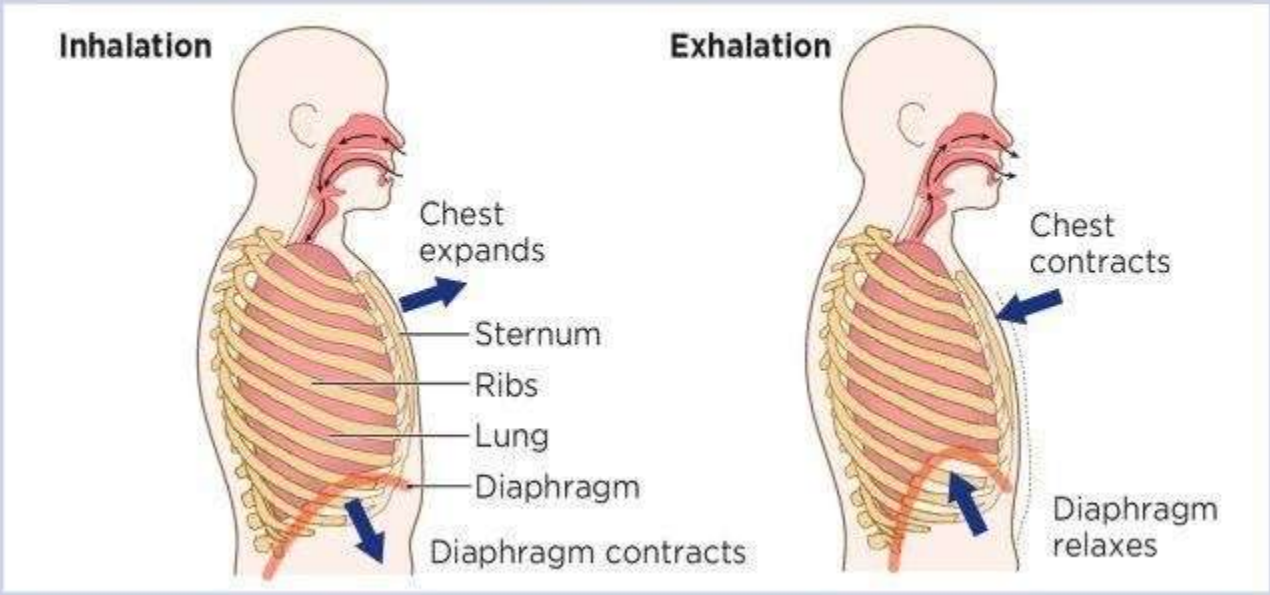
Normal and abnormal RR

Table 1. **Respiratory rate classification in adult patients**

RR	Range
Eupnoea (normal relaxed breathing)	12-20bpm (Royal College of Physicians, 2017)
Normal range >65 years	12-25bpm (Rodrigues-Molinero et al, 2013)
Normal range >80 years	10-30bpm (Rodrigues-Molinero et al, 2013)
Bradypnoea (slow RR)	<12bpm (RCP, 2017)
Tachypnoea (fast RR)	>20bpm (RCP, 2017)

bpm = breaths per minute; RR = respiratory rate

Fig 1. **Chest movements with inhalation and exhalation**



Measurement of respiratory rate (breaths/min).

- Measurement No….
  - Name of subject-
  - Age –
  - Sex-

Pulse Rate	Breathing rate
Resting (Before Exercise)	
Just After step test for 5 minutes	
After 10 minutes of resting	

Interpretation of resting results: -

……

Clinical significances:

Tachypnoea

- Anxiety, Emotional distress, Pain, Fever, Exercise, Asthma, Pulmonary embolism
- Pneumonia
- Acute respiratory distress syndrome
- Anaphylaxis
- Heart failure
- Shock
- Diabetic ketoacidosis
- Neuromuscular disorders
- Chronic obstructive pulmonary disease

Bradypnoea

- Depression of the respiratory centre
- Opioid overdose
- Increased intracranial pressure
- Diabetic coma
- Exhaustion caused by severe airway obstruction
- Sleep apnoea
- Obesity hypoventilation syndrome

Estimation of body composition

Body composition is the percentage of fat and non-fat mass present in your body. Body that has a desirable lower percentage of body fat is referred as healthy body composition and a higher percentage of non-fat mass includes organs, muscles and bones. There are many approaches to assess body composition like densitometry, ultrasound, bioelectric impedance, anthropometry, CAT scanning, etc. As the name suggests some of the methods are invasive, expensive and time consuming, whereas anthropometry is non-invasive, affordable and subject friendly. Anthropometric measurements and indices are also used such as weight, height, skinfold thickness, diameter, length and circumference that involves mathematical components. All these indices are the main components in estimating body segments.

Fat Percentage and Muscle Mass with Skinfold Thickness

Body composition and growth are the key aspects of health in populations. These factors can be assessed by measuring the fat percentage and the muscle mass. Skin fold thickness methods have been used to determine the subcutaneous fat. As discussed above, our body is mainly composed of two types of fat: Body fat, and Non-fat mass.
Body fat is found in muscle tissue, under the subcutaneous fat deposit or skin, and are also present around the visceral fat i.e., organs. These are the essential fats that helps protect body's internal organs, stores energy that act as fuel and also normalizes body hormones which are important for our body. Whereas, Non-fat mass areas (also called as lean tissues) include muscle, organs, tissues, water and bone. These tissues are metabolically active as they help burn calories while body fat cannot do that. Body fat percentage is used to estimate the total body fat on the basis of specific measurements and there are several ways to estimate it such as bio-electric impedance and Skinfold measurements. A skinfold caliper is used for the determination of skinfold thickness through which a prediction can be made of total body fat mass.
Skinfolds are generally used for determining total body fat:

Biceps Skinfold- It measures the skinfold thickness at the front of the upper arm at the level marked for taking the upper arm circumference. The landmark is when the skinfold is picked up with your thumb and index finger directly above the centre of cubical fossa.

Instrument: Skinfold caliper

Method: The subject stands with the arms hanging freely on the sides of the body. The vertical fold with your thumb and index finger one centimeter above the upper arm circumference is lifted. The jaws of the caliber are placed on the folds and the reading in mm is recorded. The reading is noted when needle is at standstill approximately seconds of applying caliper.

Precautions
 Take care not to prolong the time of the application of the caliper to the skin because prolonging causes the displacement of the fat, hence erroneous reading.
 The hold of the pinch above the skinfold should not be loosened while taking the measurements.
 With the caliper, if the subject feels pain, this happens when muscle is also pinched along with subcutaneous fat.
Triceps Skinfold: It is measured in the midline of the posterior surface of the arm, over the triceps muscle in vertical direction at a point half way on the line connecting the lateral

projection of acromion process of the scapula and interior margin of the olecranon process of the ulna.

Instrument: Skinfold' caliper

Method: The subject is asked to stand erect with arms in standard arm hanging position. Remember this is the skinfold thickness measured over the triceps in the middle of the posterior aspect of the arm at the level of the upper arm circumference or the biceps skinfold, in the line with the olecranon process. The skinfold over the triceps muscle of the right arm, one cm above and one cm below the marked point on of the upper arm midway between the acromion process and the superior border of the radius in line with the olecranon process is picked. Keep the jaws of the calipers at the marked level and note the value. The folds should be parallel to the long axis of the arm.

Precautions
- The arms should be hanging loosely and freely at the side of the subject.
- The caliper should be placed parallel to the mid-circumference line.
- While taking the measurement the pressure of the caliper should be released slowly.
- The reading should be taken in 4 seconds and the reading recorded to the nearest 4> millimeter

Subscapular skinfold – It is measured as the fold inferior to the inferior angle of scapula, at natural cleavage. The subscapular skinfold is lifted at 45 degrees to the horizontal plane.

Instrument: Skinfold caliper

Method: The subject stands erect with shoulders relaxed so that the upper extremity is hanging loosely. Standing behind the subject, palpate the vertebral border of the scapula with fingertip running down laterally until the inferior angle is identified. If the subject is obese, the subject is asked to fold hand at the back, this way it it is easier to pick the fold. The subscapular skinfold thickness is measured below the inferior angle of the scapula. Using thumb and index finger, skinfold lightly below the most inferior angle of the right scapula is picked. The skinfold usually is slightly inclined' pointing downward and laterally in the natural cleavage of the skin. The jaws of the calipers are applied at the marked level and note the reading.

Precautions
- The subject should stand comfortably erect with loosely hanging upper extremity.
- The caliper should be placed parallel to the mid-circumference line.
- While taking the measurement the pressure of the caliper should be released slowly.
- The reading should be taken in 4 seconds and the reading recorded to the nearest millimeter
- For all these measurements, subject is required to sit or stand in an upright position.

Supraspinale Skinfold

The supraspinale skinfold site is one of the common locations used for the assessment of body fat using skinfold calipers. It has previously been known as the Suprailiac site.

Location

- The intersection of a line joining the spinale (front part of iliac crest) and the anterior (front) part of the axilla (armpit), and a horizontal line at the level of the iliac crest.
- The pinch is directed medially (towards the centerline) and downward, following the natural fold of the skin (at an approximate angle of 45 degrees).

Table 3 – Percent Fat estimates for Men
(Sum of Biceps, Triceps, Subscapular & Suprailium Skinfolds)

Skin-folds (mm)	Males (Age in years)			
	16-29	30-39	40-49	50+
15	4.8	-	-	-
20	8.1	12.2	12.2	12.6
25	10.5	14.2	15.0	15.6
30	12.9	16.2	17.7	18.6
35	14.7	17.7	19.6	20.8
40	16.4	19.2	21.4	22.9
45	17.7	20.4	23.0	24.7
50	19.0	21.5	24.6	26.5
55	20.1	22.5	25.9	27.9
60	21.2	23.5	27.1	29.2
65	22.2	24.3	28.2	30.4
70	23.1	25.1	29.3	31.6
75	24.0	25.9	30.3	32.7
80	24.8	26.6	31.2	33.8
85	25.5	27.2	32.1	34.8
90	26.2	27.8	33.0	35.8
95	26.9	28.4	33.7	36.6
100	27.6	29.0	34.4	37.4
105	28.2	29.6	35.1	38.2
110	28.8	30.1	35.8	39.0
115	29.4	30.6	36.4	39.7
120	30.0	31.1	37.0	40.4
125	30.5	31.5	37.6	41.1
130	31.0	31.9	38.2	41.8
135	31.5	32.3	38.7	42.2
140	32.0	32.7	39.2	43.0
145	32.5	33.1	39.7	43.6
150	32.9	33.5	40.2	44.1
155	33.3	33.9	40.7	44.6
160	33.7	34.3	41.2	45.1
165	34.1	34.6	41.6	45.6
170	34.5	34.8	42.0	46.1
175	34.9	-	-	-
180	35.3	-	-	-
185	35.6	-	-	-
190	35.9	-	-	-
195	-	-	-	-
200	-	-	-	-
205	-	-	-	-
210	-	-	-	-

Table 3 & 4 provide percent fat estimates for women and men using the sum of four (4) skinfolds (Biceps, Triceps, Subscapular & Suprailium). For example, if the skinfold measurements for a 35 year old male are:

(a) Biceps = 10
(b) Tricep = 11
(c) Subscapular = 17
(d) Suprailium = 22
Total = 60

The percent body fat would be 23.5 %.

(Durnin and Womersley, 1974)

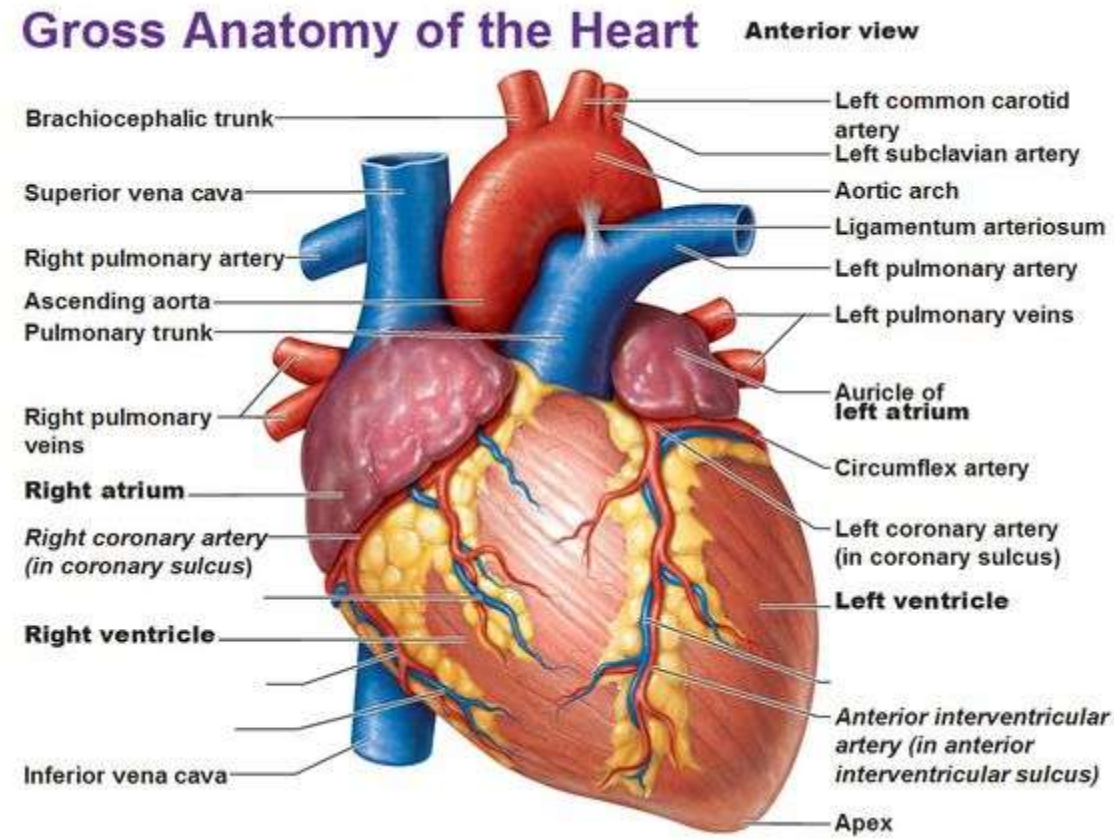
Table 4 – Percent Fat estimates for Women
(Sum of Biceps, Triceps, Subscapular & Suprailium Skinfolds

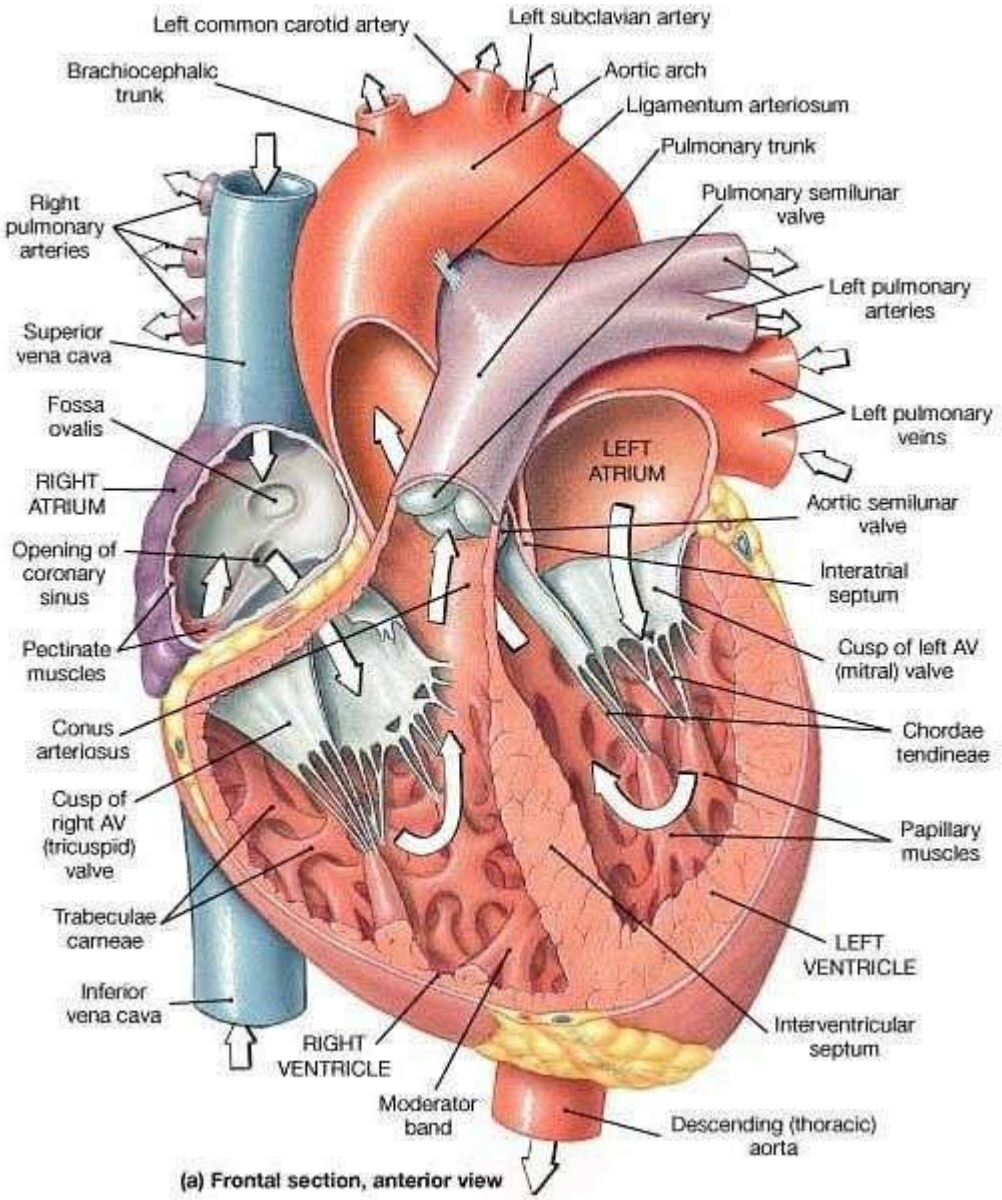
Skin-folds (mm)	Females (Age in years)			
	16-29	30-39	40-49	50+
15	10.5	-	-	-
20	14.1	17.0	19.8	21.4
25	16.8	19.4	22.2	24.0
30	19.5	21.8	24.5	26.6
35	21.5	23.7	26.4	28.5
40	23.4	25.5	28.2	30.3
45	25.0	26.9	29.6	31.9
50	26.5	28.2	31.0	33.4
55	27.8	29.4	32.1	34.6
60	29.1	30.6	33.2	35.7
65	30.2	31.6	34.1	36.7
70	31.2	32.5	35.0	37.7
75	32.2	33.4	35.9	38.7
80	33.1	34.3	36.7	39.6
85	34.0	35.1	37.5	40.4
90	34.8	35.8	38.3	41.2
95	35.6	36.5	39.0	41.9
100	36.4	37.2	39.7	42.6
105	37.1	37.9	40.4	43.3
110	37.8	38.6	41.0	43.9
115	38.4	39.1	41.5	44.5
120	39.0	39.6	42.0	45.1
125	39.6	40.1	42.5	45.7
130	40.2	40.6	43.0	46.2
135	40.8	41.1	43.5	46.7
140	41.3	41.6	44.0	47.2
145	41.8	42.1	44.5	47.7
150	42.3	42.6	45.0	48.2
155	42.8	43.1	45.4	48.7
160	43.3	43.6	45.8	49.2
165	43.7	44.0	46.2	49.6
170	44.1	44.4	46.6	50.0
175	-	44.8	47.0	50.4
180	-	45.2	47.4	50.8
185	-	45.6	47.8	51.2
190	-	45.9	48.2	51.6
195	-	46.2	48.5	52.0
200	-	46.5	48.8	52.4
205	-	-	49.1	52.7
210	-	-	49.4	53.0

Body density = 1.0764 – (0.0008 × suprailiac skinfold) – (0.00088 × triceps skinfold)
% Body fat = (0.41563 × sum of three skinfolds) – (0.00112 × [sum of three skinfolds]2) + (0.03661 × age) + 4.03653

Anatomy Laboratory

Demonstration of heart –

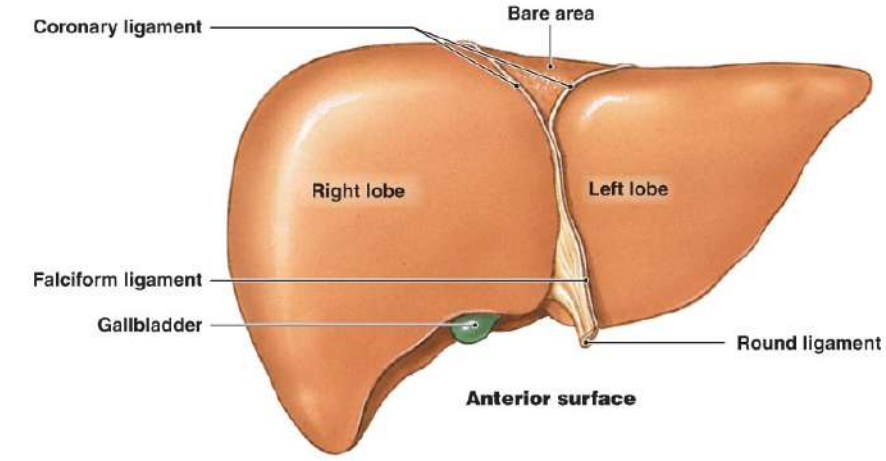



Left common carotid artery
Left subclavian artery
Brachiocephalic trunk
Aortic arch
Ligamentum arteriosum
Pulmonary trunk
Pulmonary semilunar valve
Right pulmonary arteries
Left pulmonary arteries
Superior vena cava
Left pulmonary veins
Fossa ovalis
LEFT ATRIUM
RIGHT ATRIUM
Aortic semilunar valve
Opening of coronary sinus
Interatrial septum
Pectinate muscles
Cusp of left AV (mitral) valve
Conus arteriosus
Chordae tendineae
Cusp of right AV (tricuspid) valve
Papillary muscles
Trabeculae carneae
LEFT VENTRICLE
Inferior vena cava
Interventricular septum
RIGHT VENTRICLE
Moderator band
Descending (thoracic) aorta
(a) Frontal section, anterior view

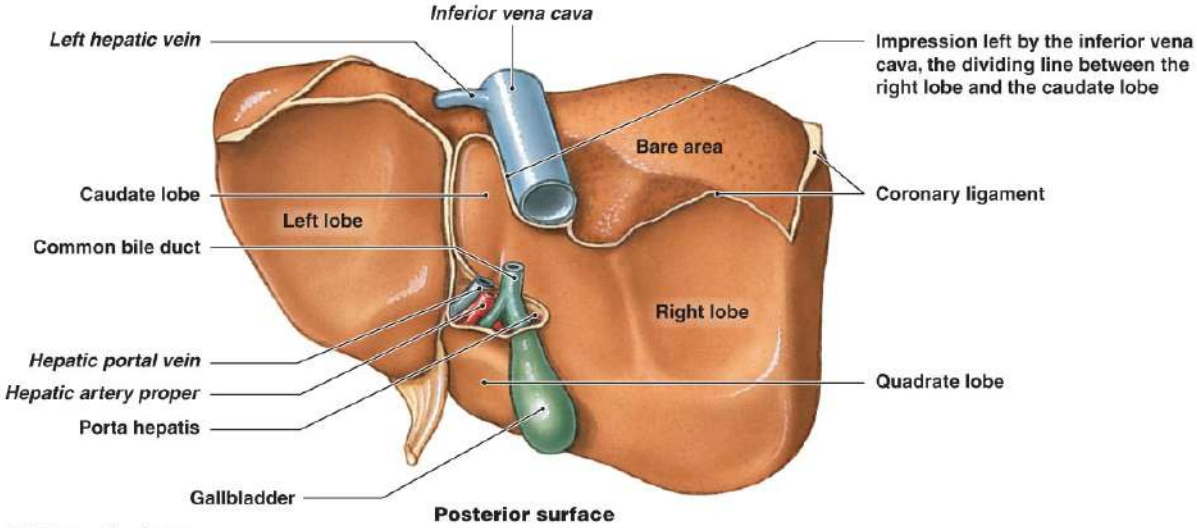
Demonstration of liver –

The major anatomical landmarks and the four lobes of the liver

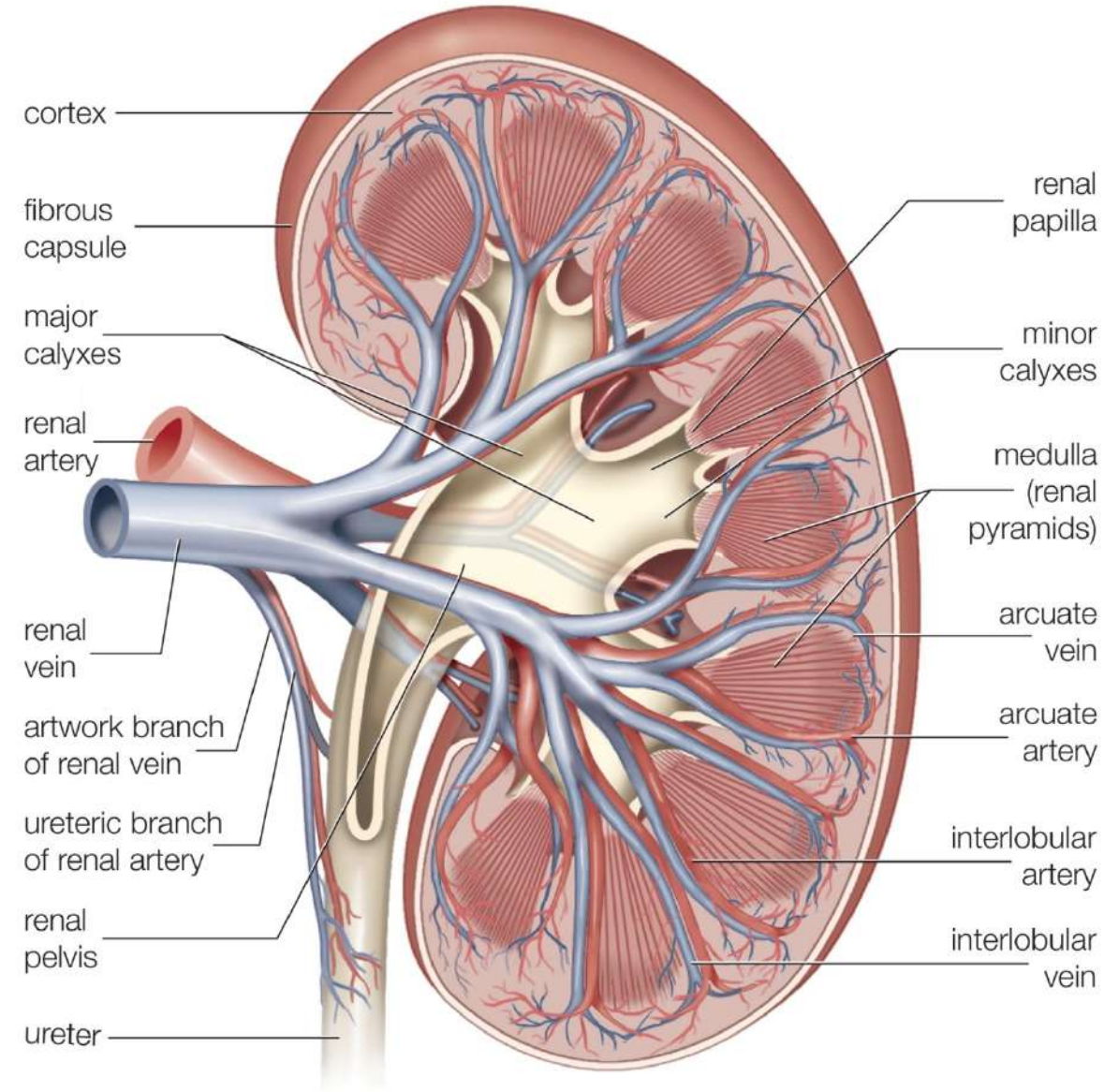
The major anatomical landmarks of the liver as seen in an anterior view



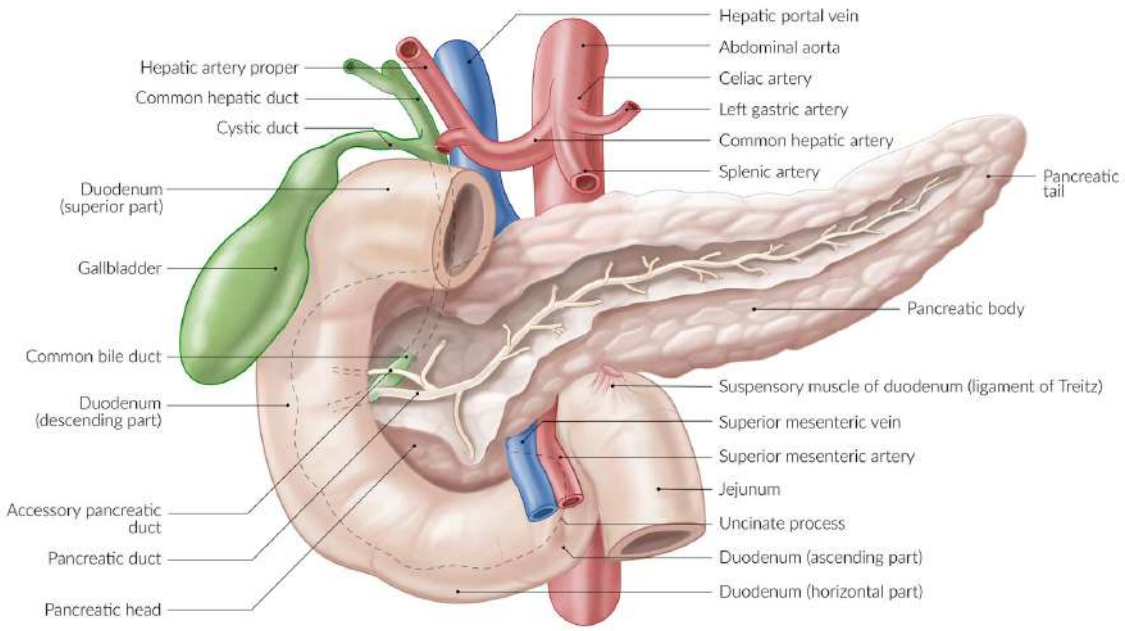
The major anatomical landmarks of the liver as seen in a posterior view



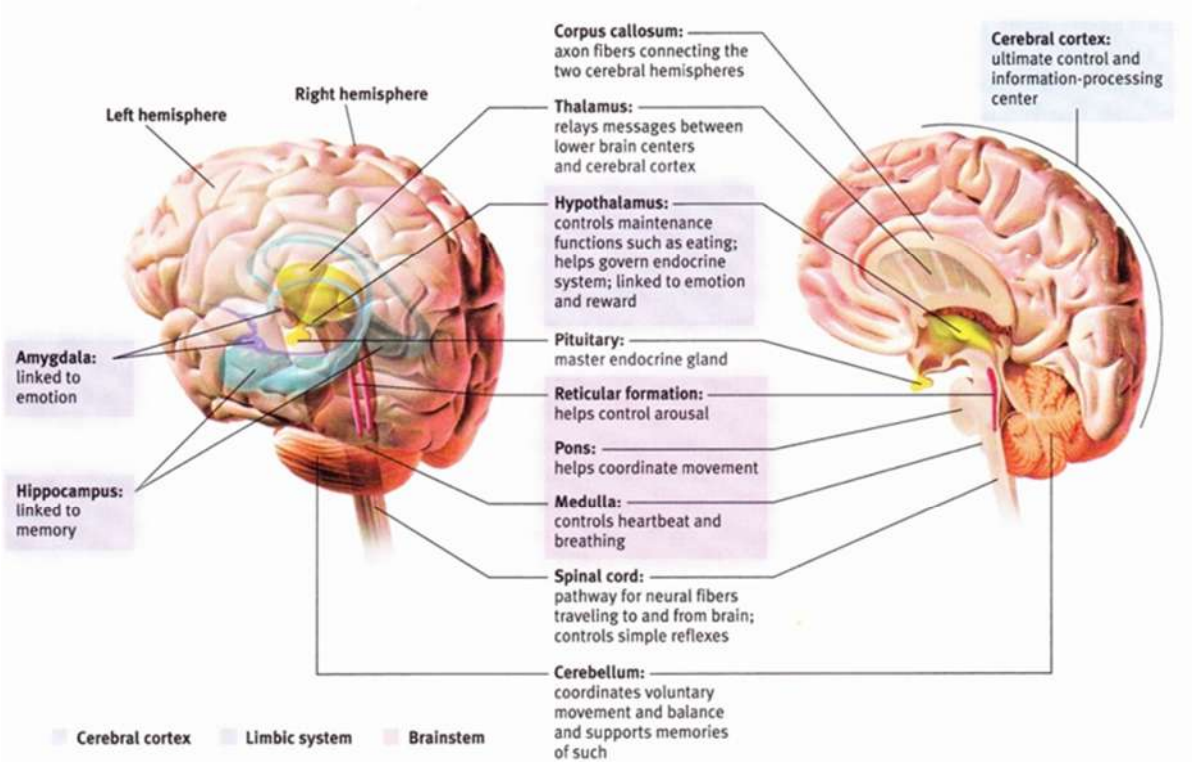
Demonstration of kidney –



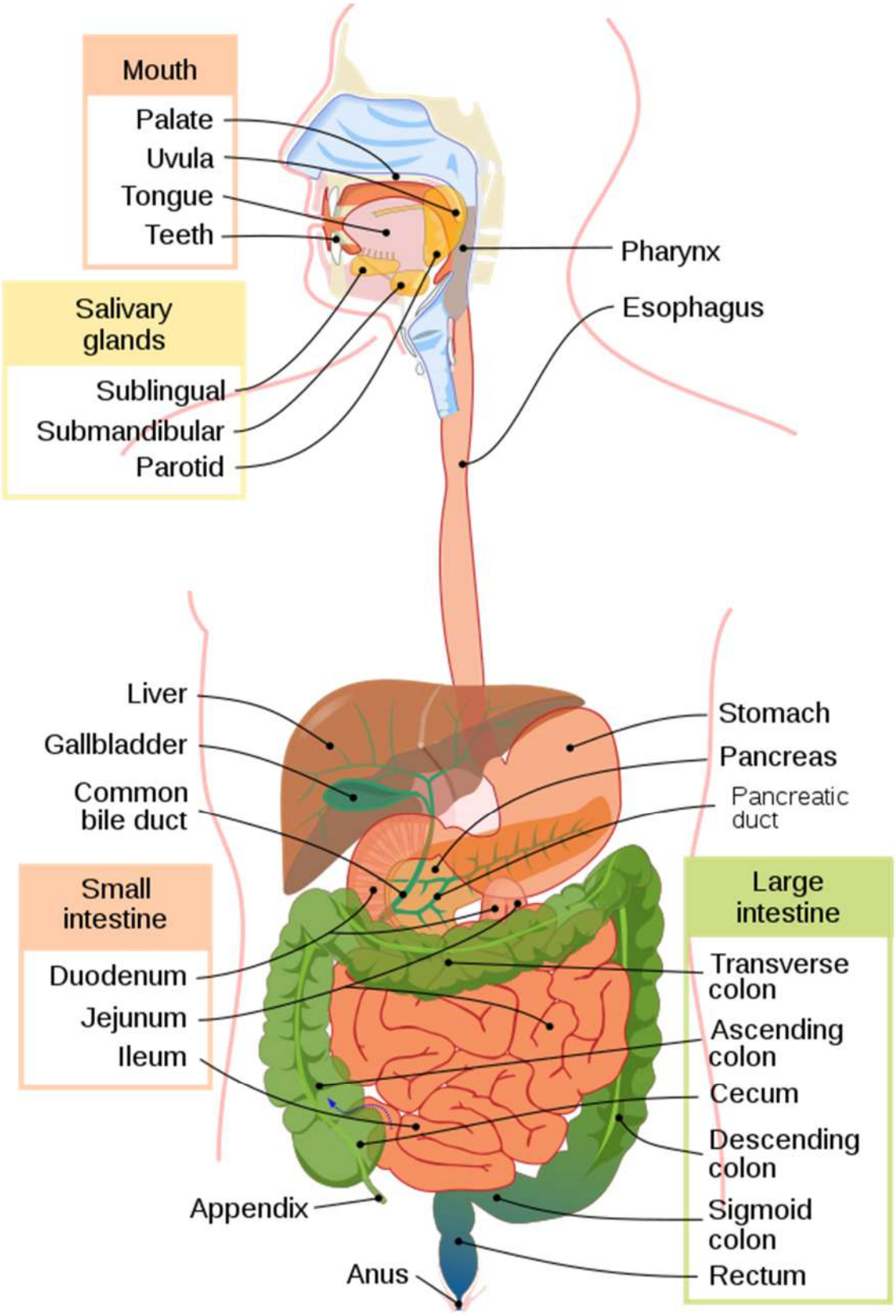
Demonstration of Pancreases-



Demonstration of Brain –



Demonstration of Gastro-intestinal tract-



Demonstration of respiratory tract & lungs –