B.Sc. MEDICAL MICROBIOLOGY LAB MANUAL

1st Semester

Prepared By

Paramedical & Allied Science Dept.

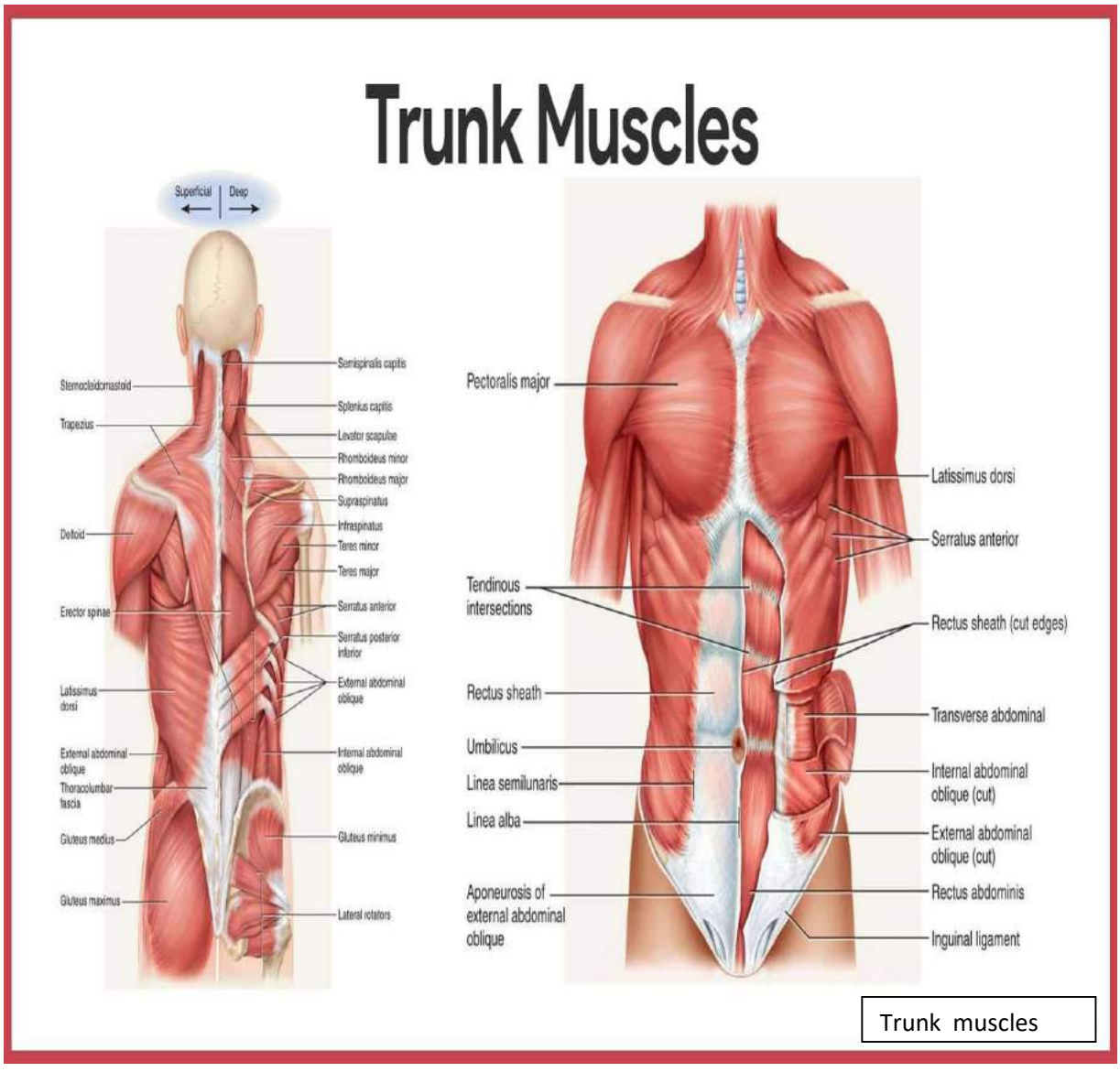
BMLT

MIDNAPORE CITY COLLEGE

LABORATORY MANUAL

BMM-195, B.SC MEDICAL MICROBIOLOGY

DEMONSTRATION ON MUSCLES OF TRUNK



Trunk muscles

DEMONSTRATION ON MUSCLES OF FACE

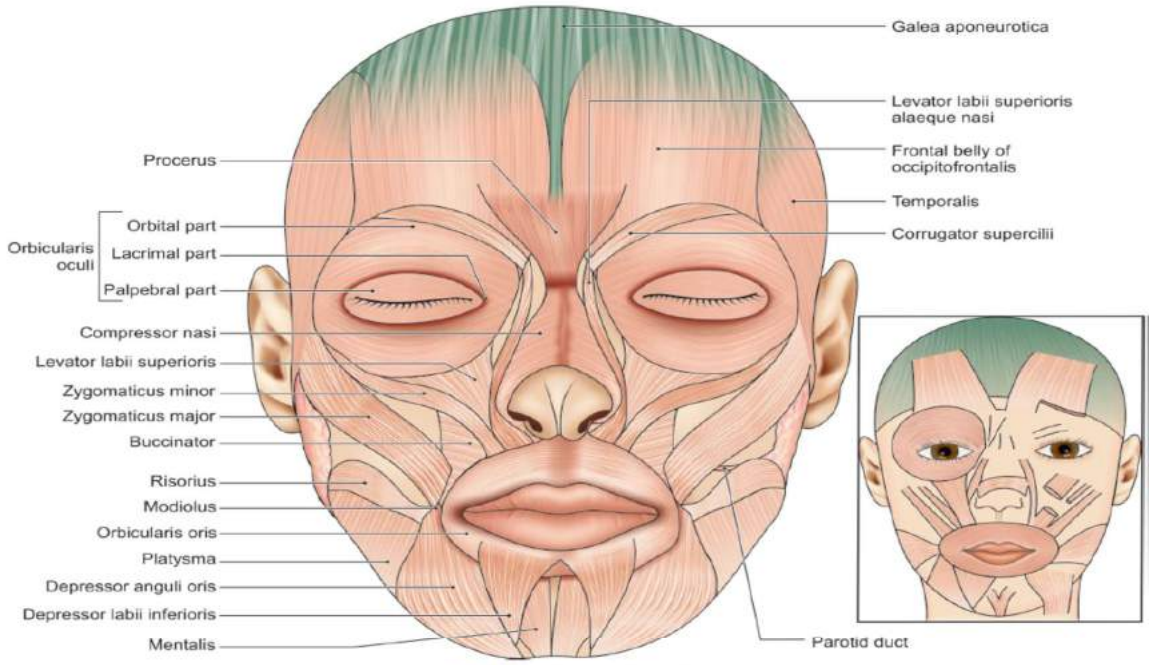


Fig. 2.9: The facial muscles

Muscles around the Mouth

1 Orbicularis oris (Fig. 2.9)
2 *Buccinator* (Latin cheek) (Fig. 2.10)
3 Levator labii superioris alaeque nasi (Fig. 2.10)
4 Zygomaticus major (Fig. 2.9)
5 Levator labii superioris (Fig. 2.9)

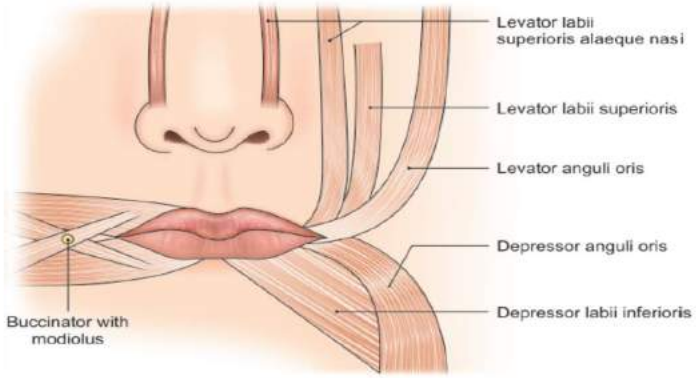
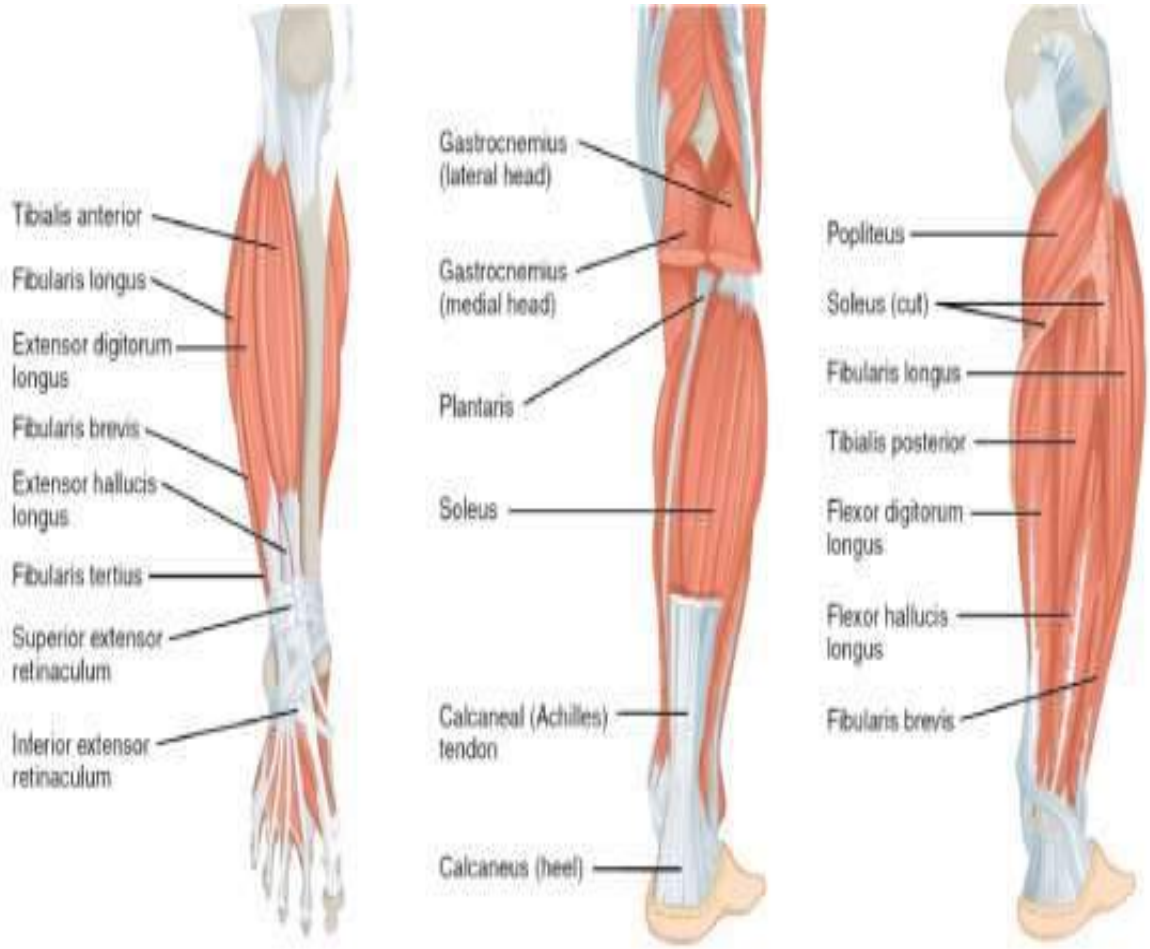
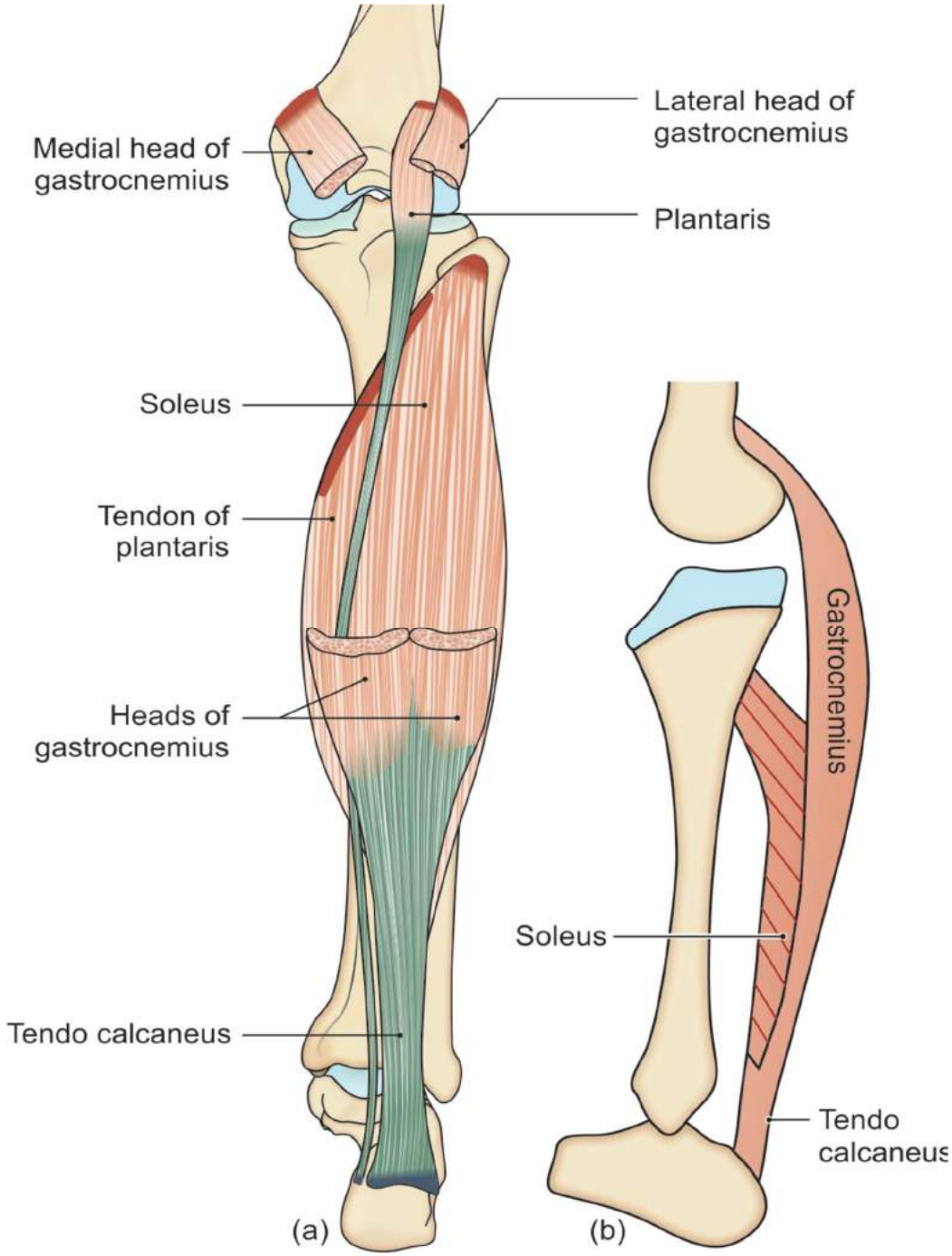


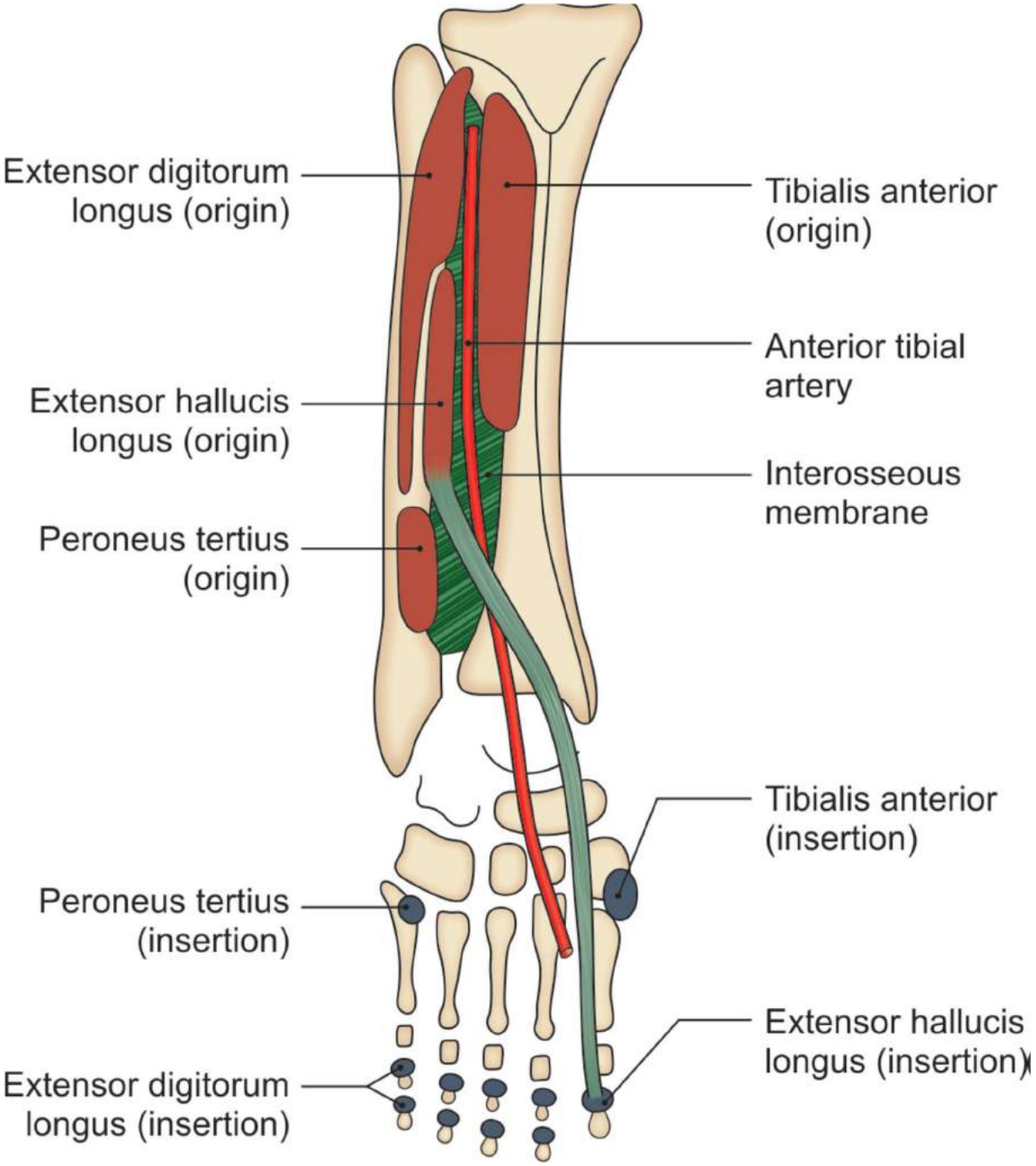
Fig. 2.10: Some facial muscles

DEMONSTRATION ON MUSCLES OF LOWER EXTREMITIES

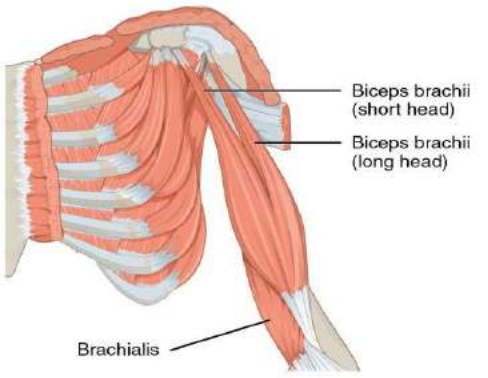




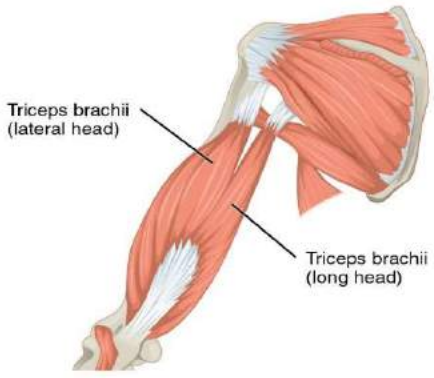
lowerextrimities muscles



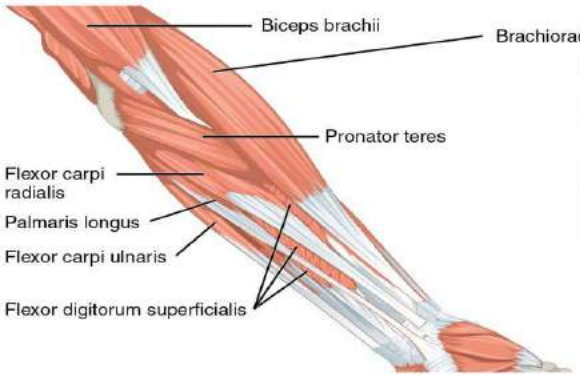
upper extrimities muscles



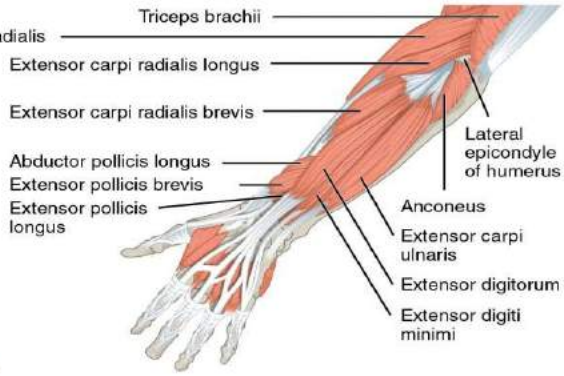
Left upper arm muscles (anterior lateral view)



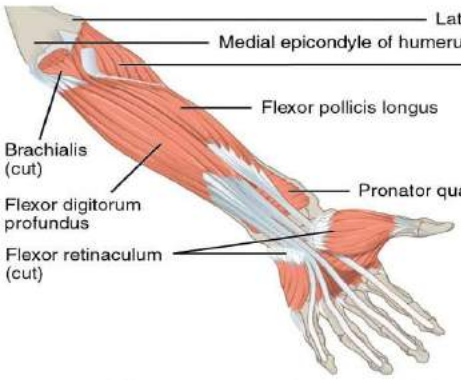
Left upper arm muscles (posterior view)



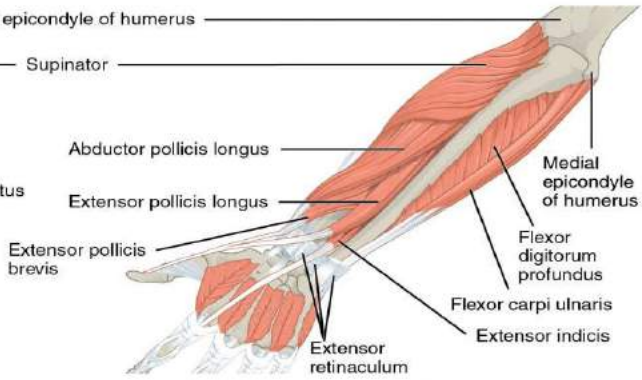
Left forearm superficial muscles (palmar view)



Left forearm superficial muscles (dorsal view)



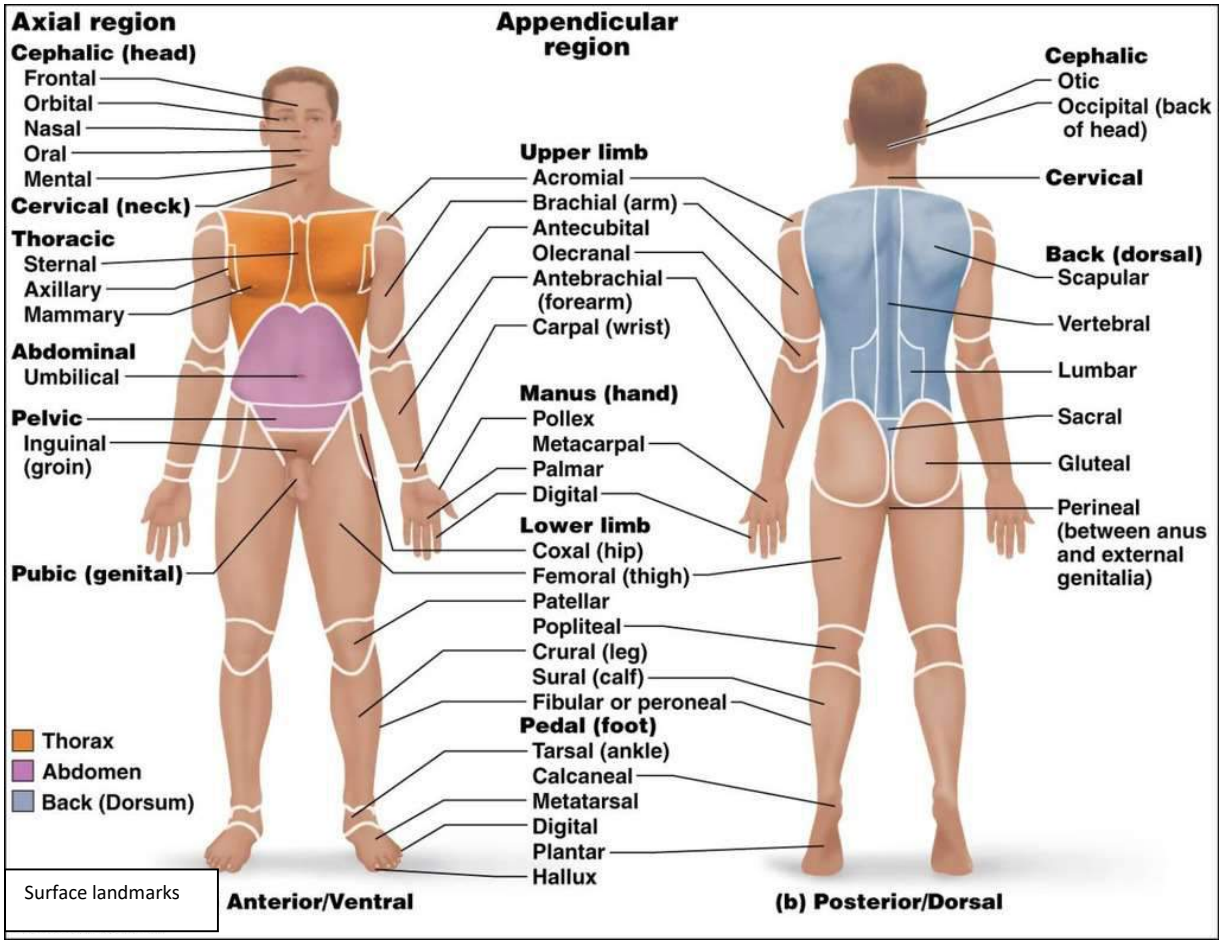
Left forearm deep muscles (palmar view)



Left forearm deep muscles (dorsal view)

DEMONSTRATION ON MUSCLES OF UPPER EXTREMITIES

Identification of surface land marks of a human body



Surface landmarks

The anatomical position - This is a standard position used in anatomy and clinical medicine to allow accurate and consistent description of one body part in relation to another • The head is directed forwards with eyes looking into the distance. • The body is upright, legs together, and directed forwards. • The palms are turned forward, with the thumbs laterally.

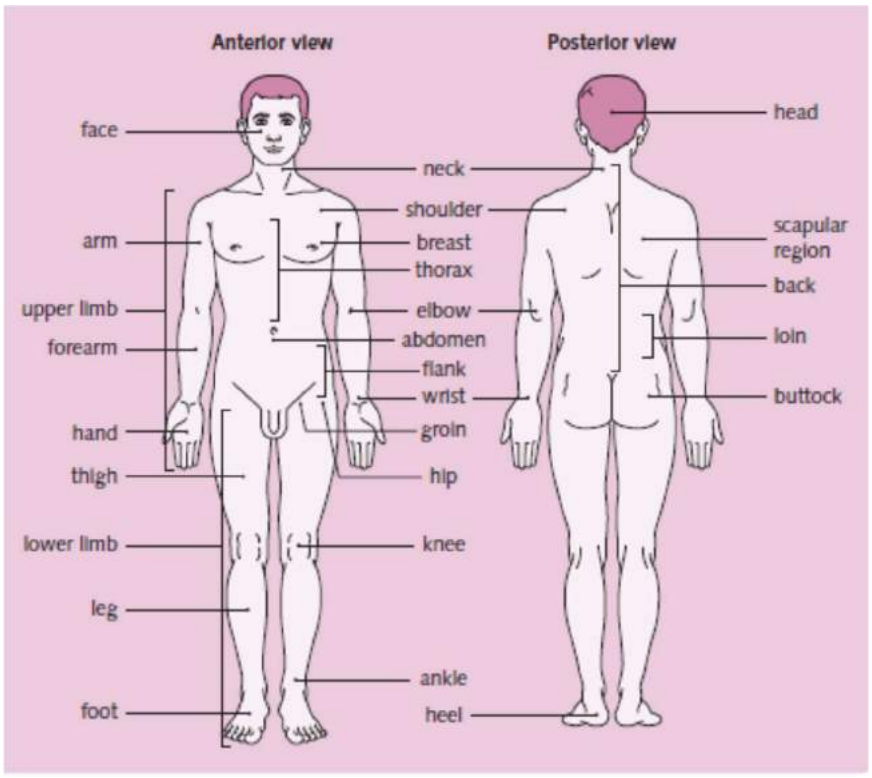
Anatomical planes these comprise the following • **The median sagittal plane** is the vertical plane passing through the midline of the body from the front to the back. Any plane parallel to this is termed **paramedian or sagittal**. • **Coronal (or frontal)** *planes* are vertical planes perpendicular to the sagittal planes. • **Horizontal or transverse planes** lie at right angles to both the sagittal and coronal planes.

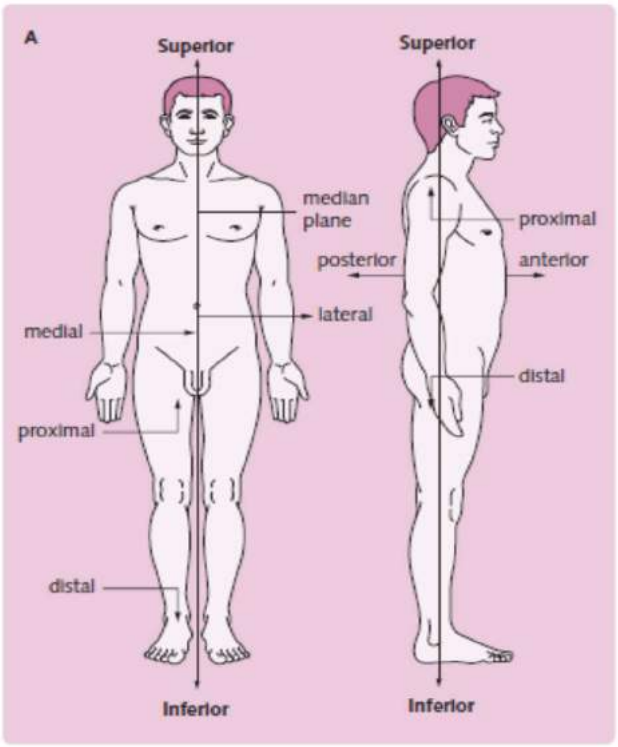
Such anatomical planes are frequently used in computer tomography (CT) scans and magnetic resonance imaging (MRI), to visualize muscle, bone, lung and other soft tissues as well as pathologies, for example pancreatic cancer or a brain abscess.

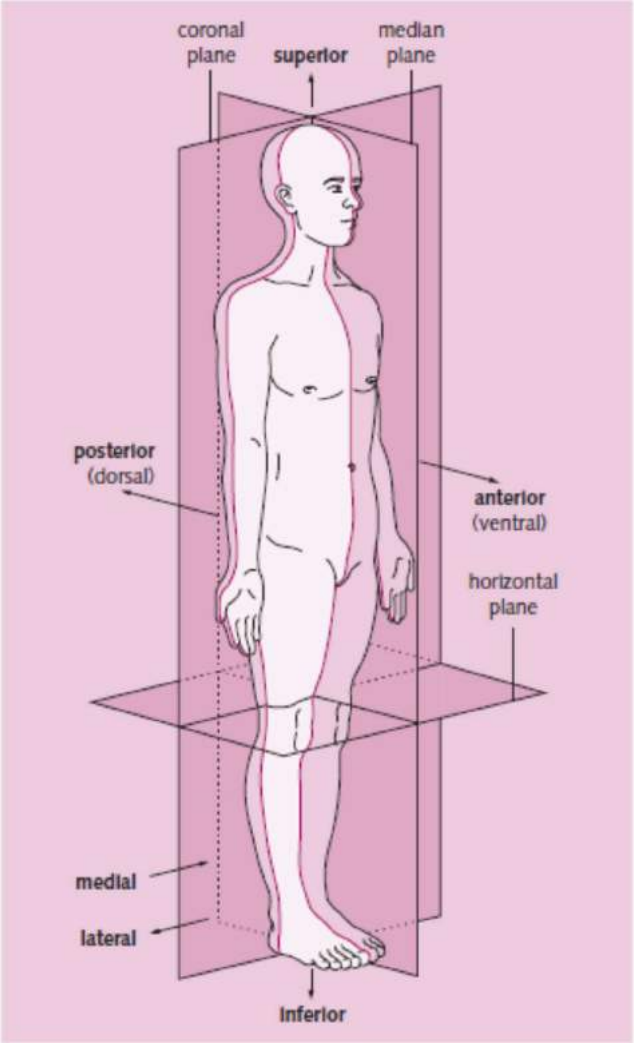
Terms of position The terms of position commonly used in clinical practice and anatomy are illustrated in

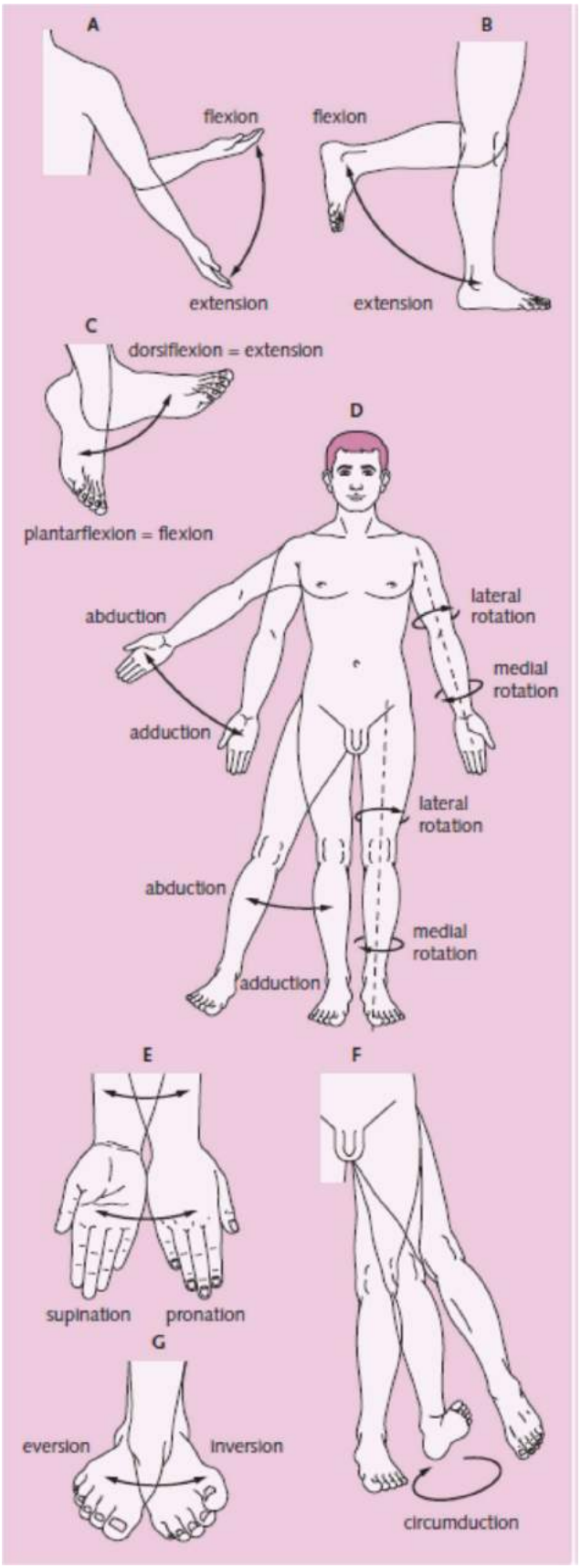
Terms of movement various terms are used to describe movements of the body • **Flexion**—forward movement in a sagittal plane which in general reduces the angle at thejoint, e.g. bending the elbow. Exceptions areat the ankle joint (when the angle is increased) and the shoulder joint (when the angle between the upper limb and trunk is increased). • **Extension**—backward movement in a sagittal plane which in general increases the angle atjoints except at the ankle joint (when theangleis decreased) and the knee joint due to lower limb rotation during embryonic development. **Abduction**—movement away from the median plane. • **Adduction**—movement towards the median plane. • **Supination**—lateral rotation of the forearm, causing the palm to face anteriorly. • **Pronation**—medial rotation of the forearm, causing the palm to face posteriorly. • **Eversion**—turning the sole of the foot outwards. • Inversion—turning the sole of the foot inwards. • **Rotation**—movement of part of the body around its long axis. • **Circumduction**—a combination of flexion, extension, abduction, and adduction. The terms used to describe movements of the thumb are perpendicular to the movements of the body, e.g. flexion of the thumb is at 90°to that of flexion of the fingers

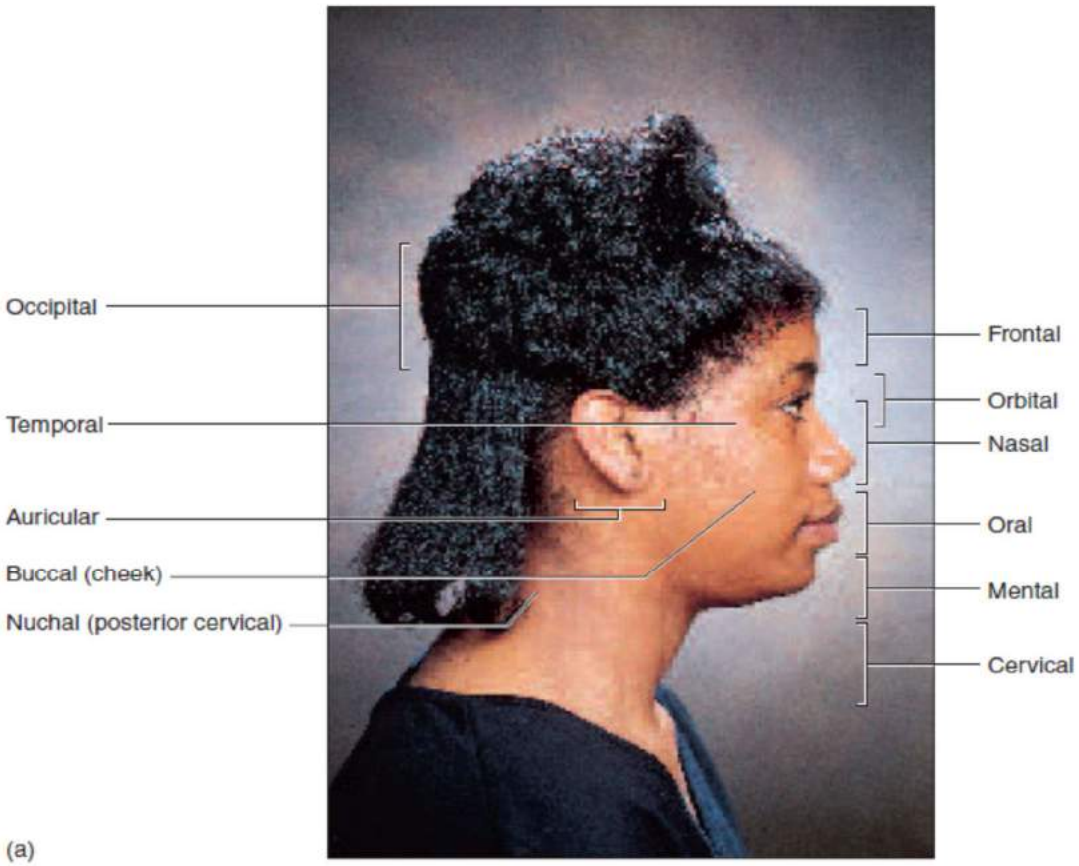
Anatomical landmarks



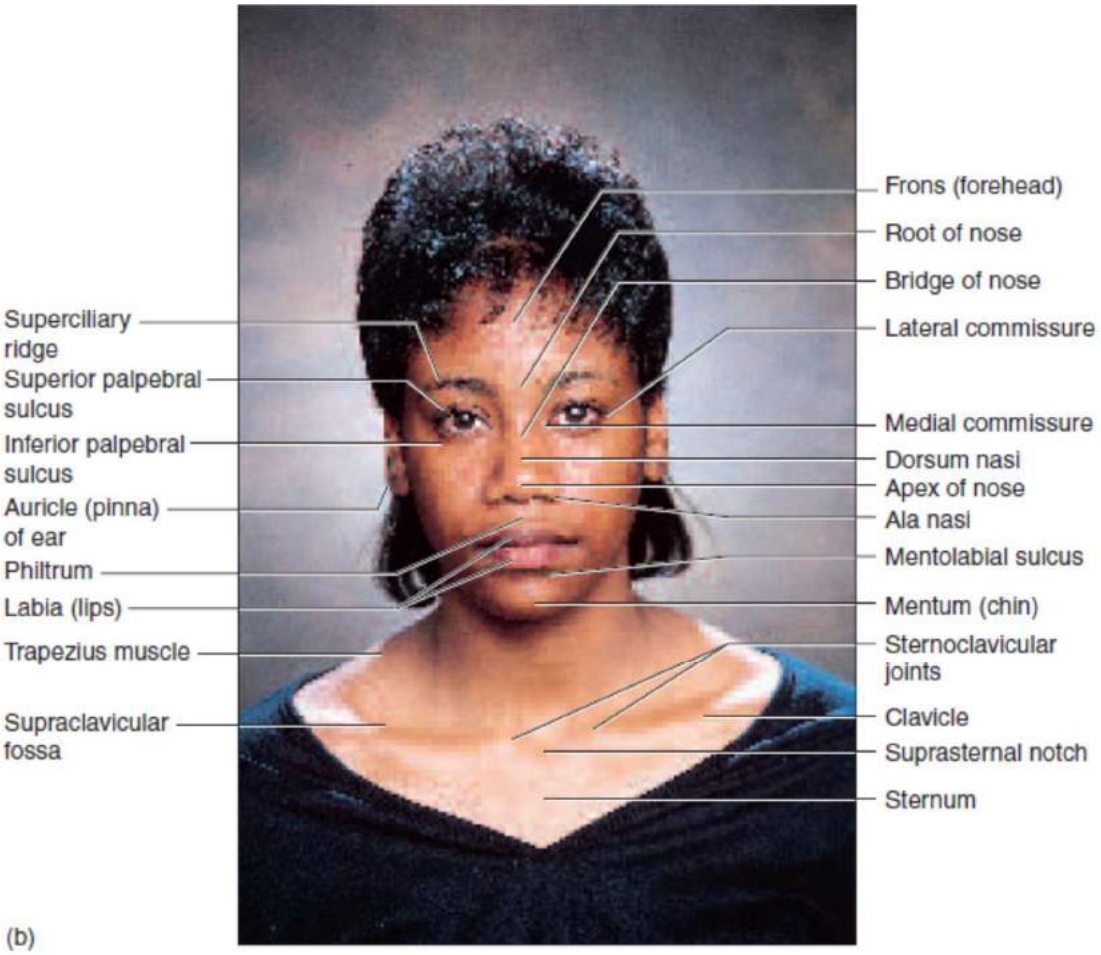



coronal plane
superior
median plane
posterior (dorsal)
anterior (ventral)
horizontal plane
medial
lateral
inferior

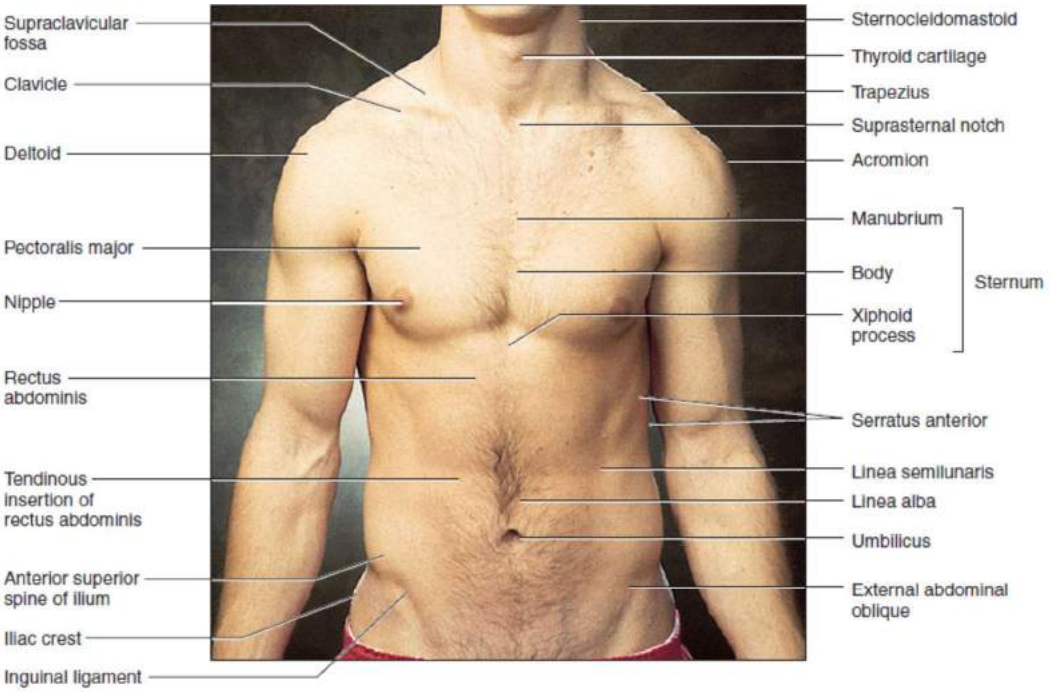

A
flexion
extension
B
flexion
extension
C
dorsiflexion = extension
plantarflexion = flexion
D
abduction
adduction
lateral rotation
medial rotation
lateral rotation
abduction
medial rotation
adduction
E
supination
pronation
F
circumduction
G
eversion
inversion



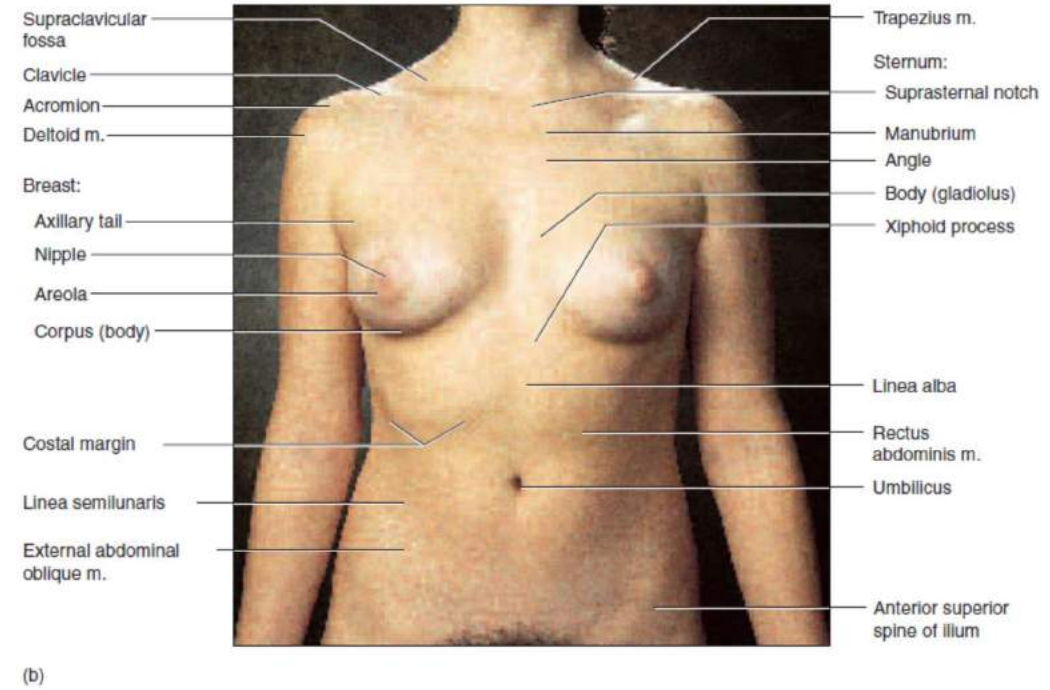
ANATOMICAL SURFACES OF FACE


Frons (forehead)
Root of nose
Bridge of nose
Lateral commissure
Medial commissure
Dorsum nasi
Apex of nose
Ala nasi
Mentolabial sulcus
Mentum (chin)
Sternoclavicular joints
Clavicle
Suprasternal notch
Sternum
Superciliary ridge
Superior palpebral sulcus
Inferior palpebral sulcus
Auricle (pinna) of ear
Philtrum
Labia (lips)
Trapezius muscle
Supraclavicular fossa

(b)

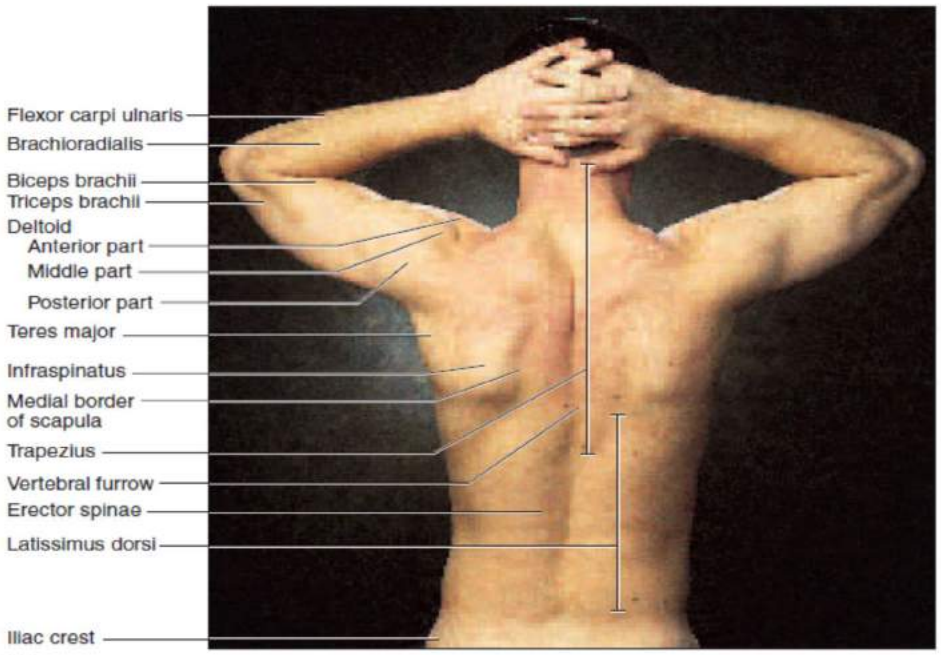


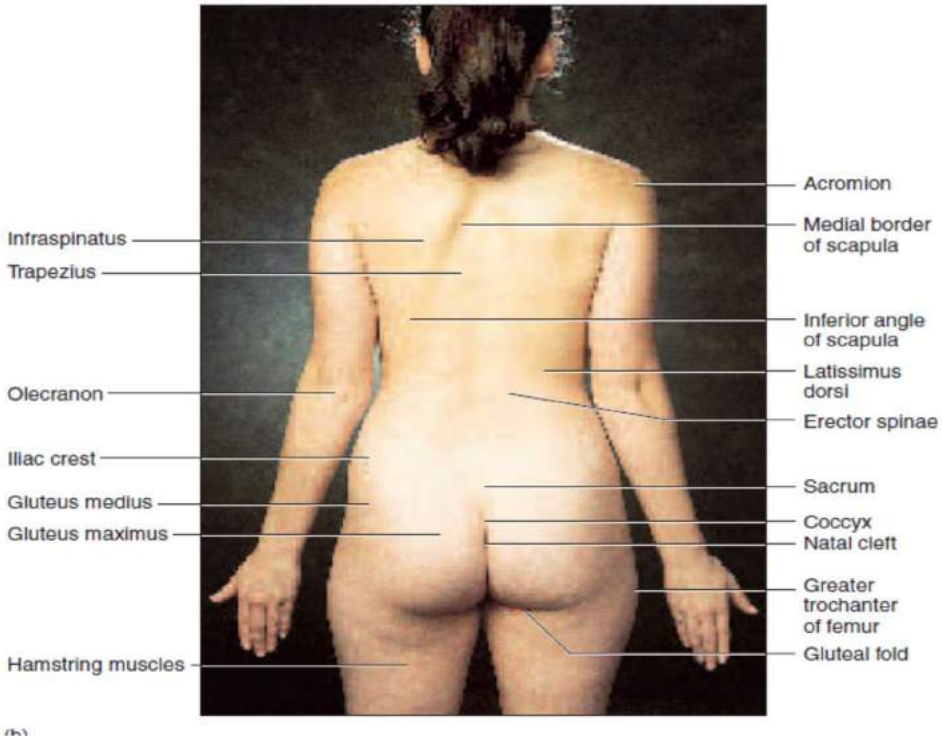
(a)



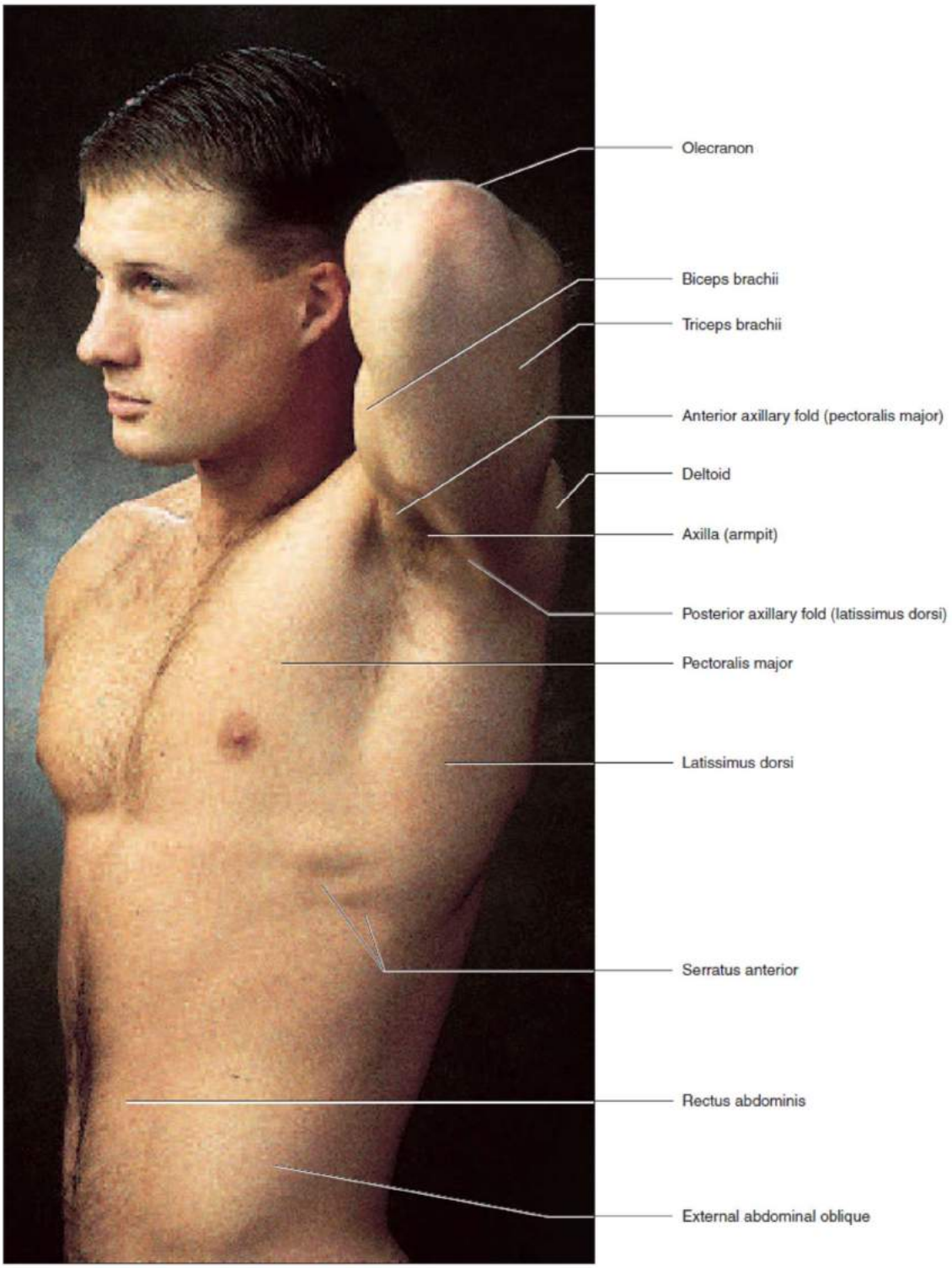
(b)

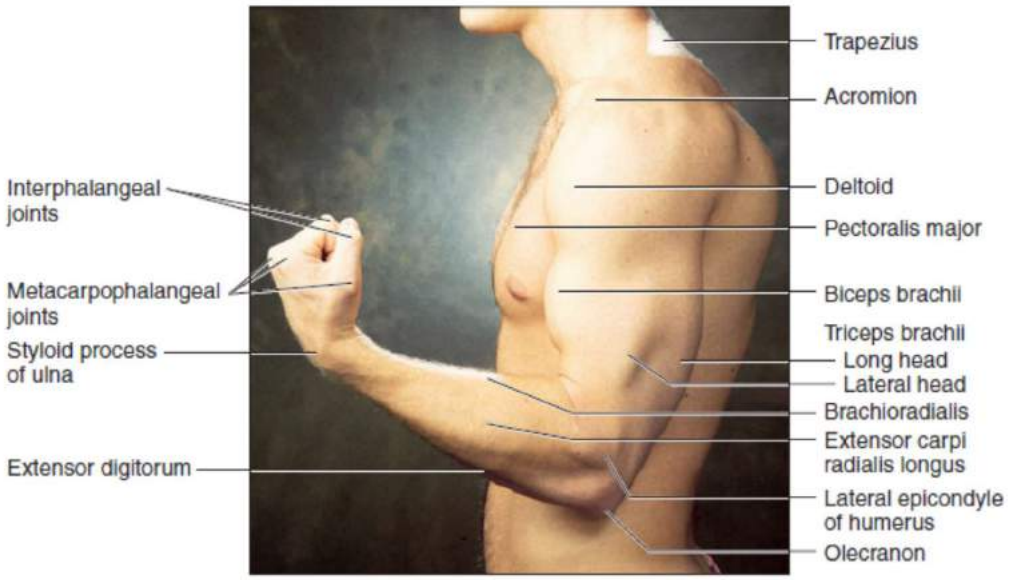
ANATOMICAL SURFACES OF CHEST AND ABDOMEN



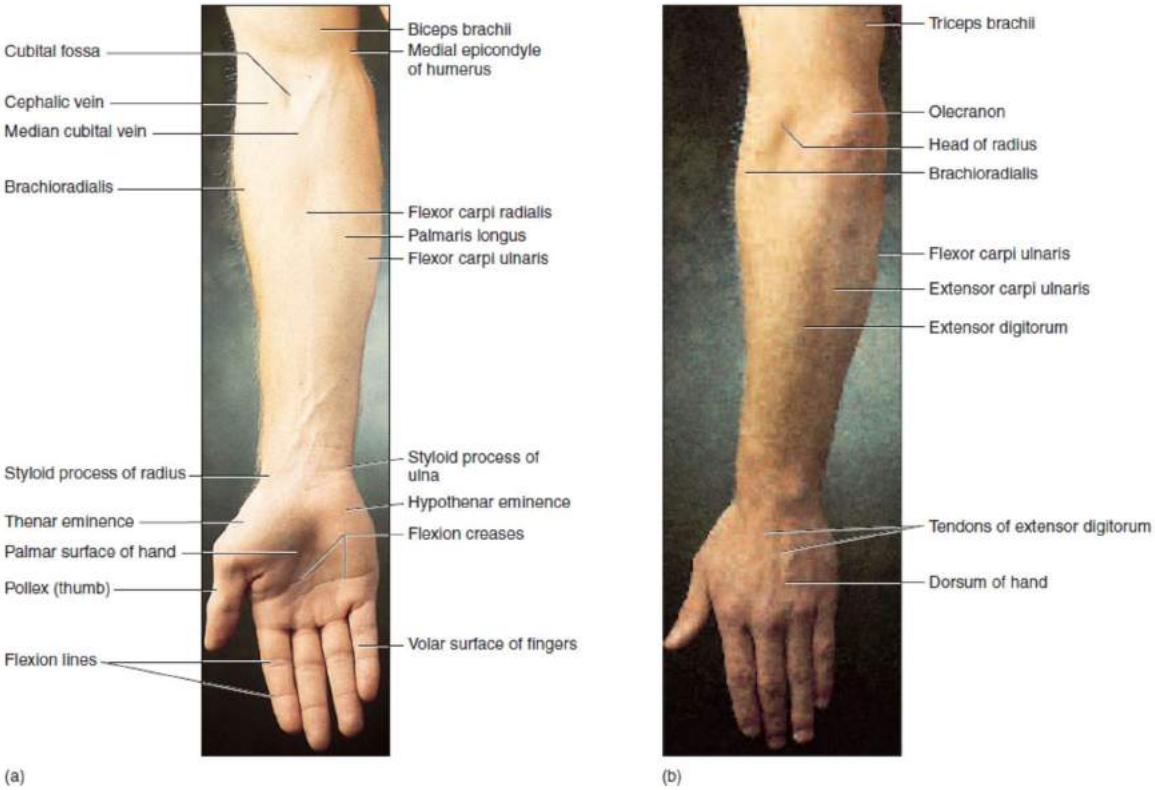


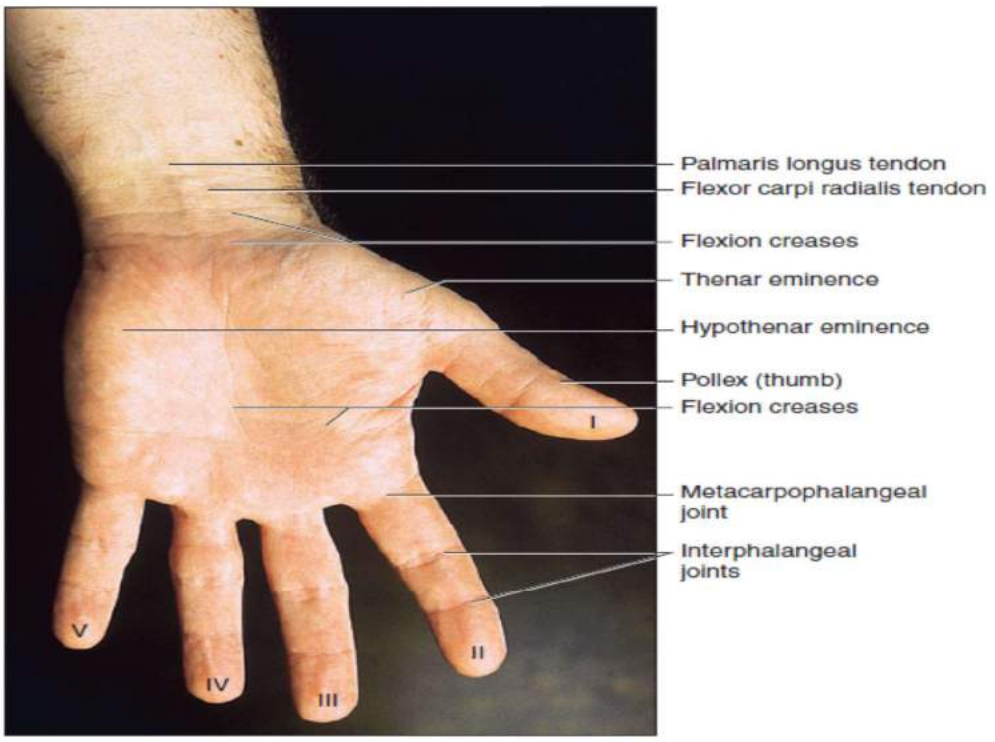
ANATOMICAL SURFACES OF BACK


Olecranon
Biceps brachii
Triceps brachii
Anterior axillary fold (pectoralis major)
Deltoid
Axilla (armpit)
Posterior axillary fold (latissimus dorsi)
Pectoralis major
Latissimus dorsi
Serratus anterior
Rectus abdominis
External abdominal oblique

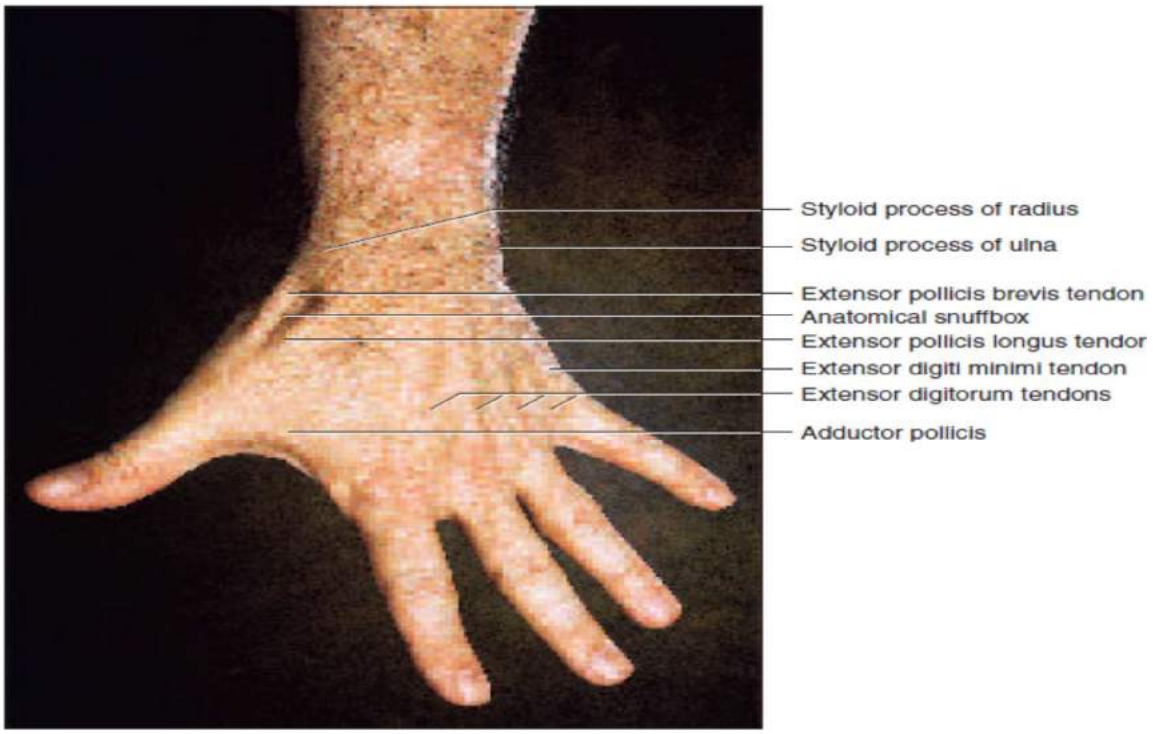


The Upper Limb, Lateral Aspect.



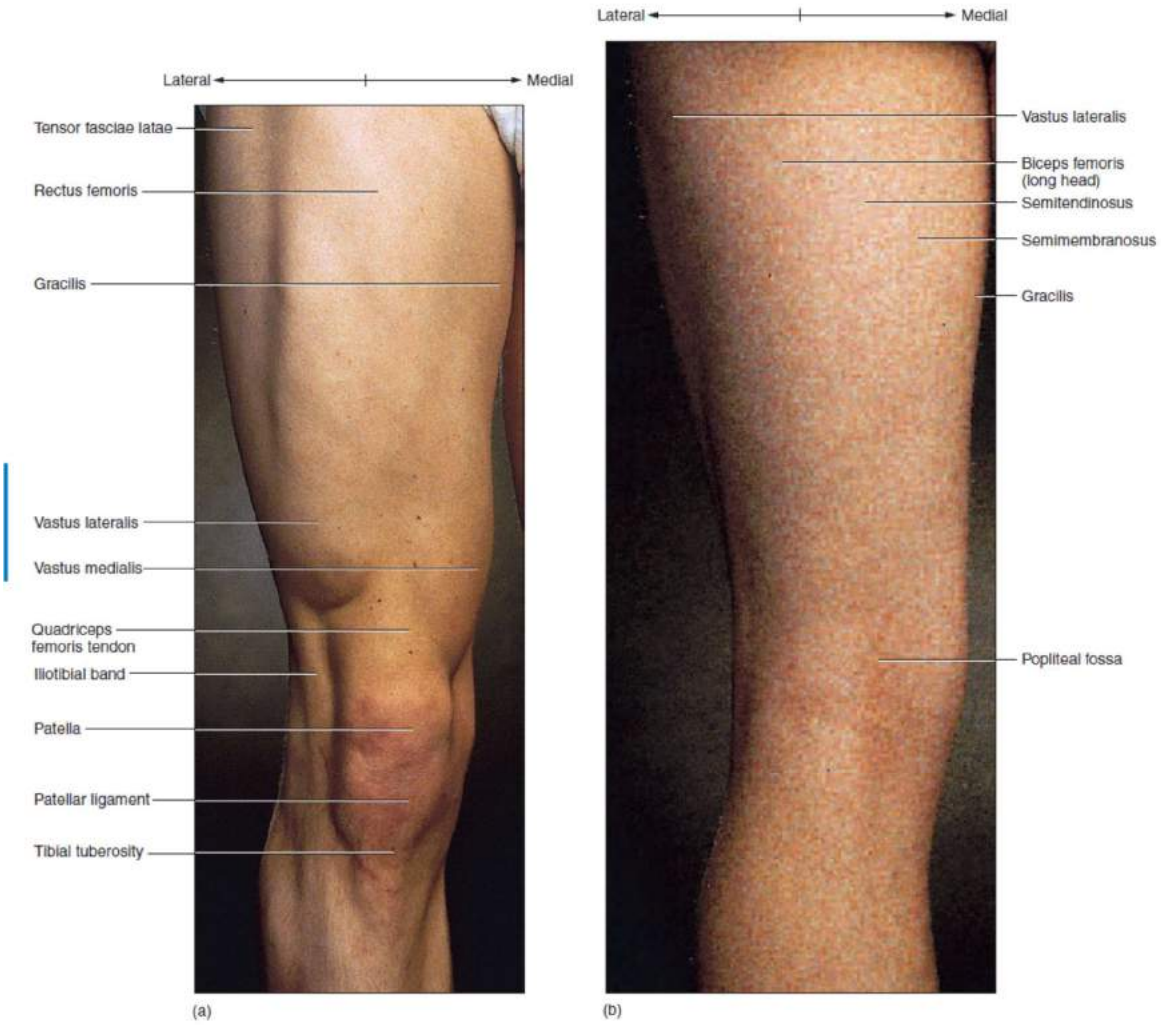


(a)

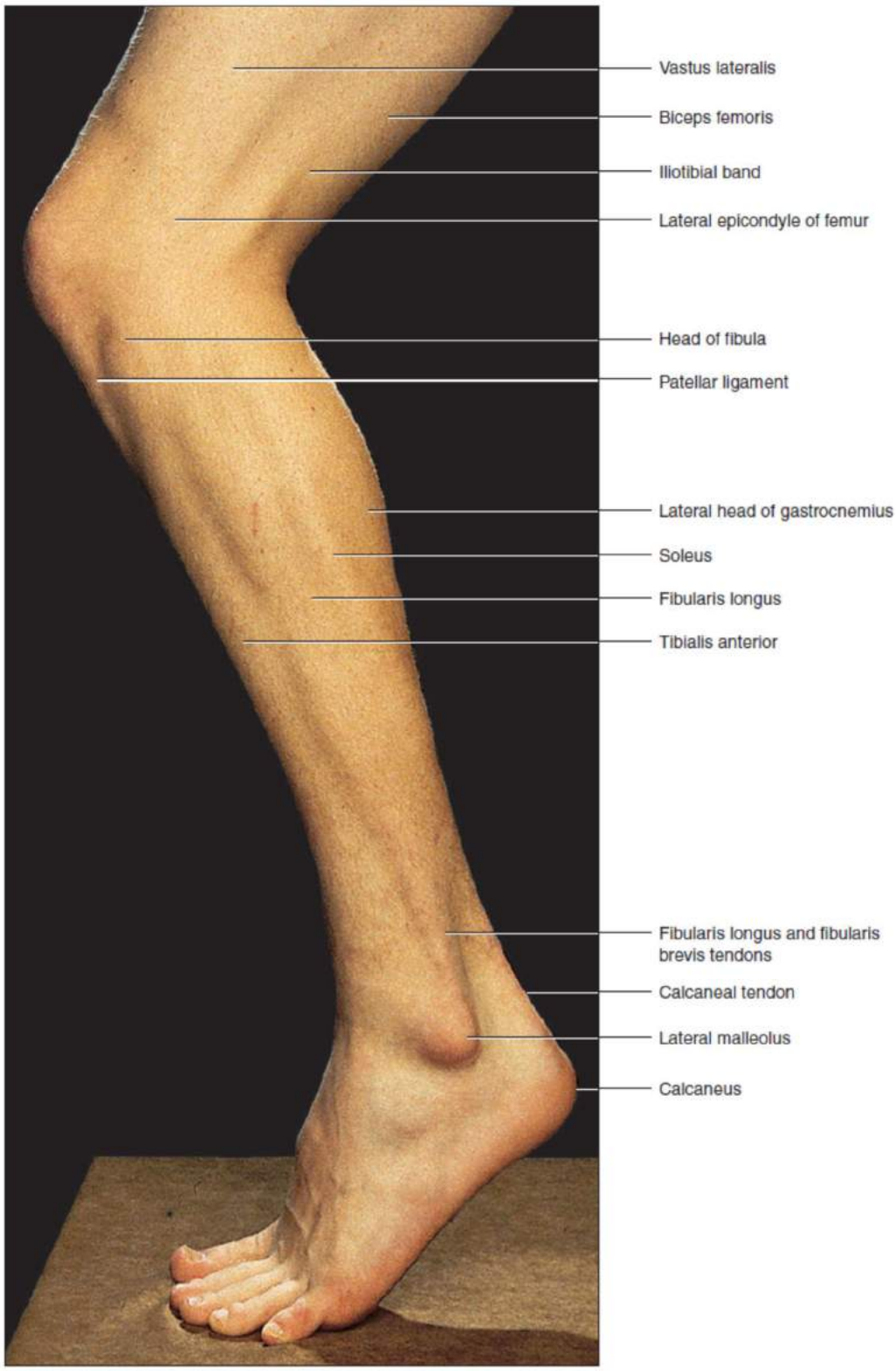


(b)

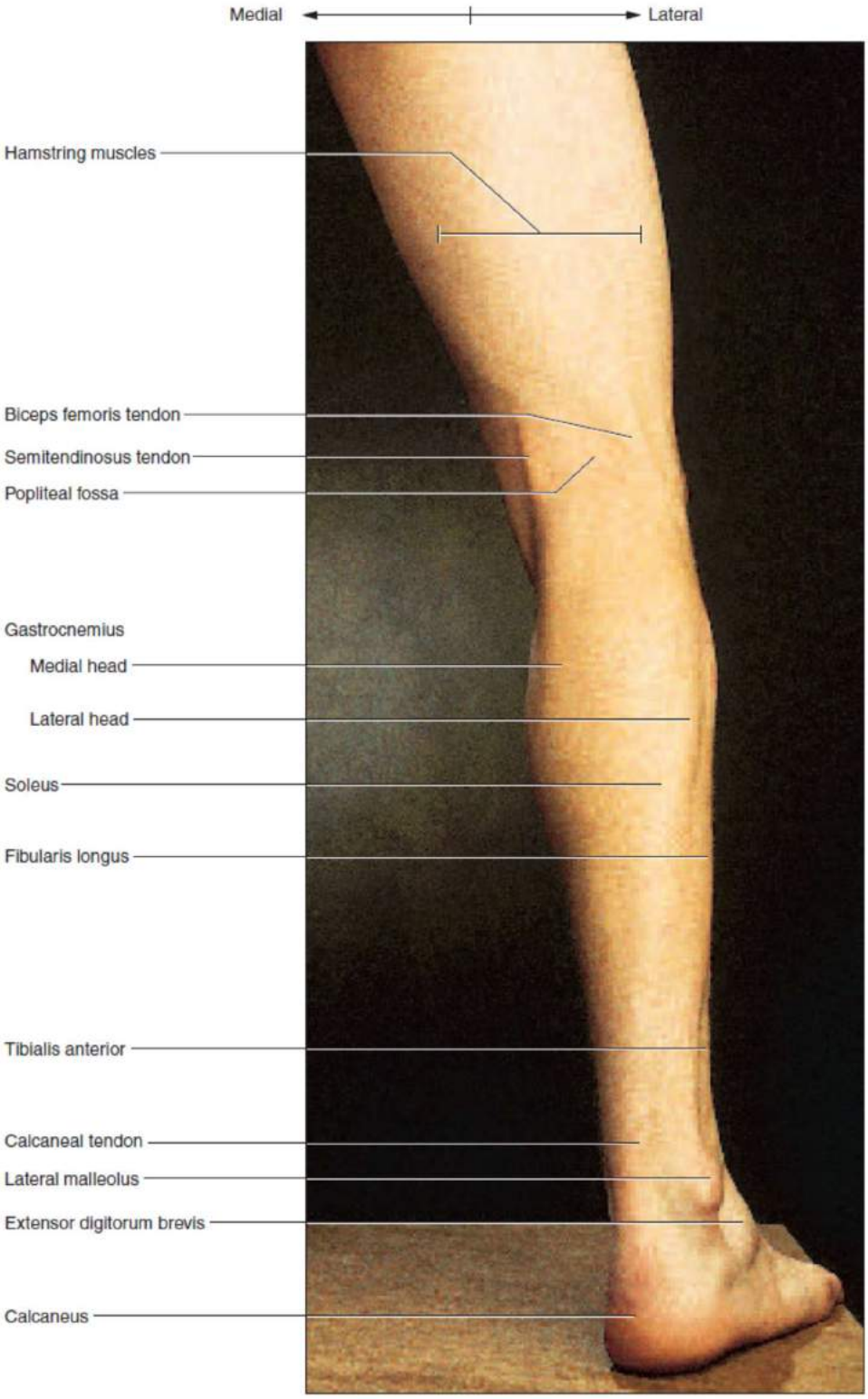
ANATOMICAL SURFACES OF THE FINGER



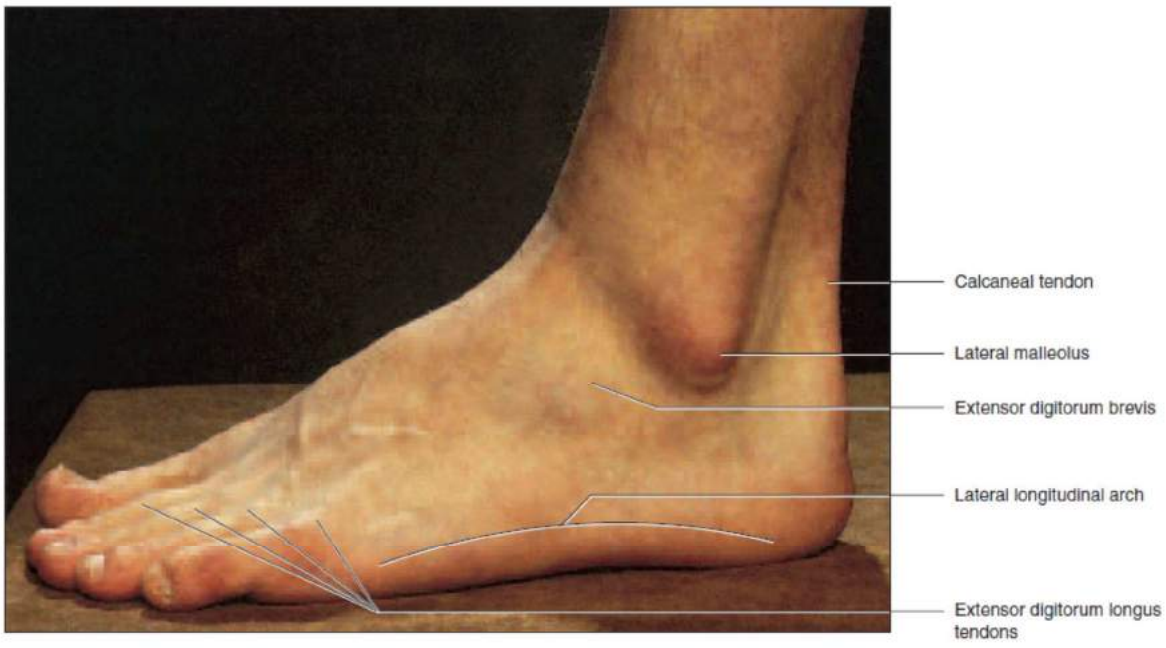
ANATOMICAL SURFACES OF LEG



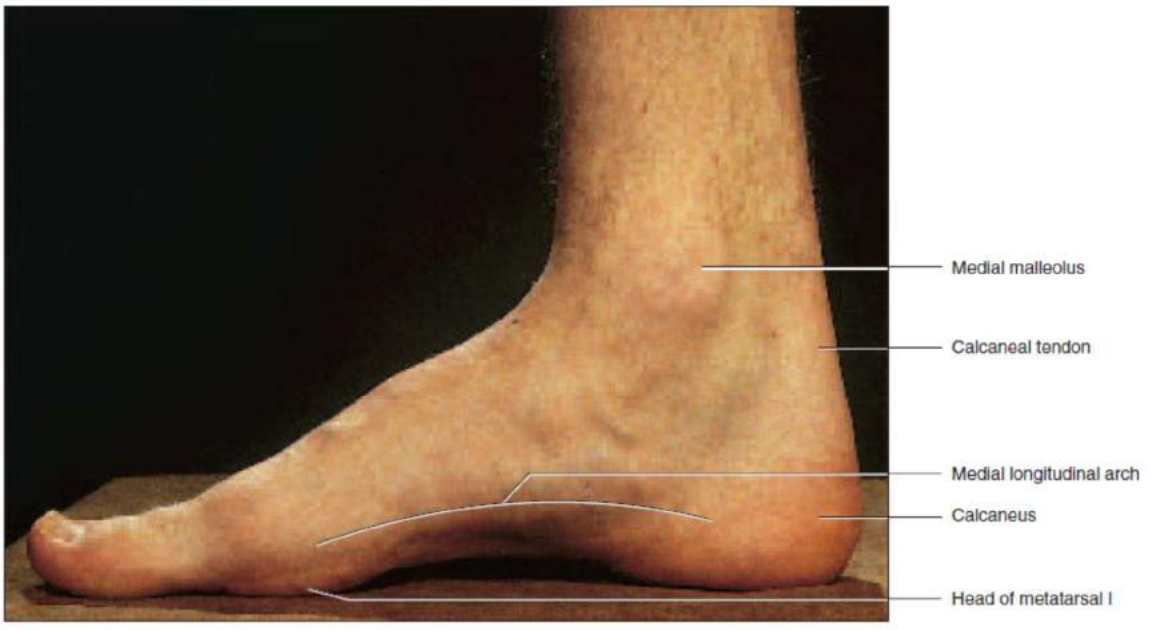
The Leg and Foot, Lateral Aspect.



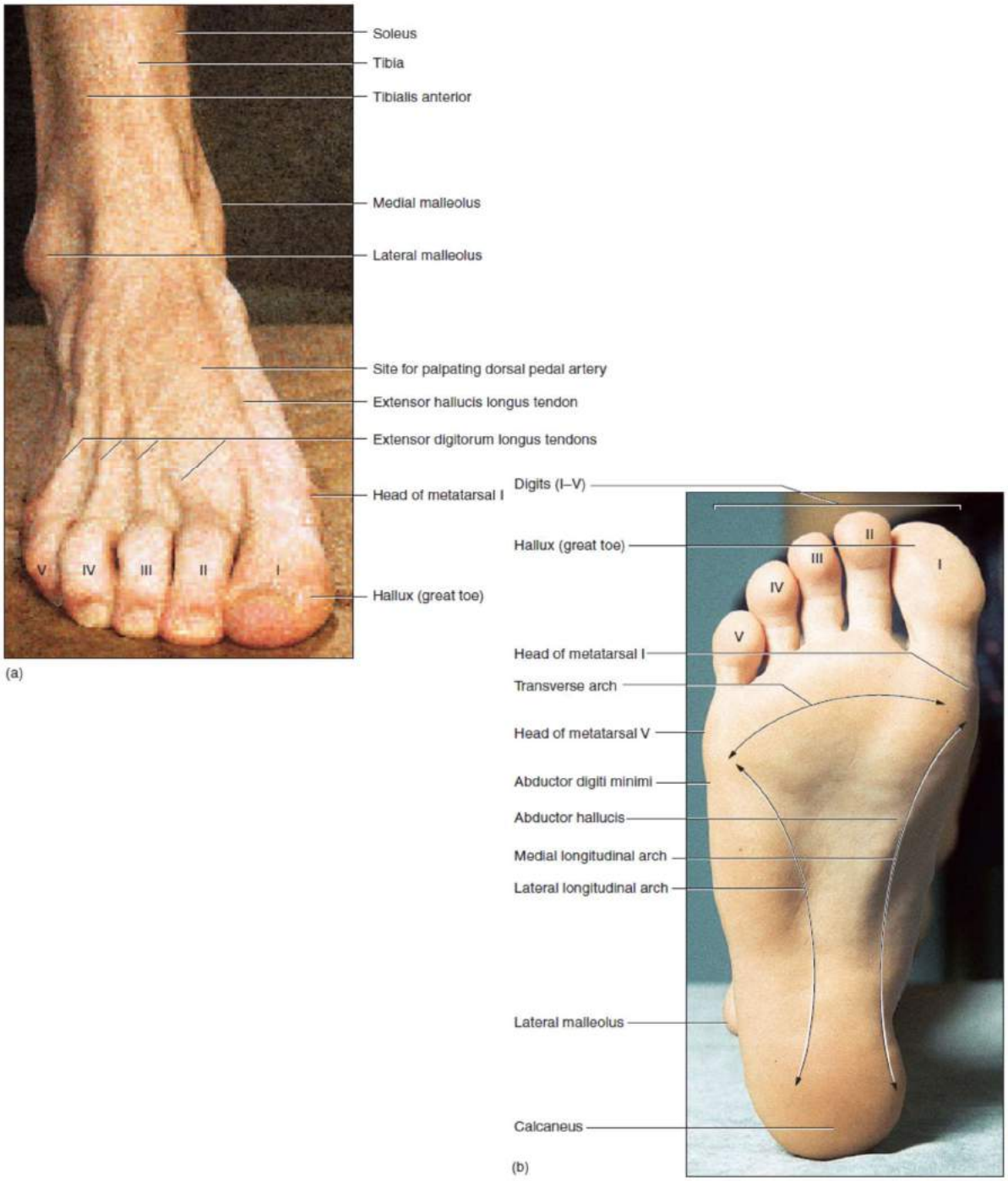
The Leg and Foot, Dorsal Aspect.


Calcaneal tendon
Lateral malleolus
Extensor digitorum brevis
Lateral longitudinal arch
Extensor digitorum longus tendons

(a)


Medial malleolus
Calcaneal tendon
Medial longitudinal arch
Calcaneus
Head of metatarsal I

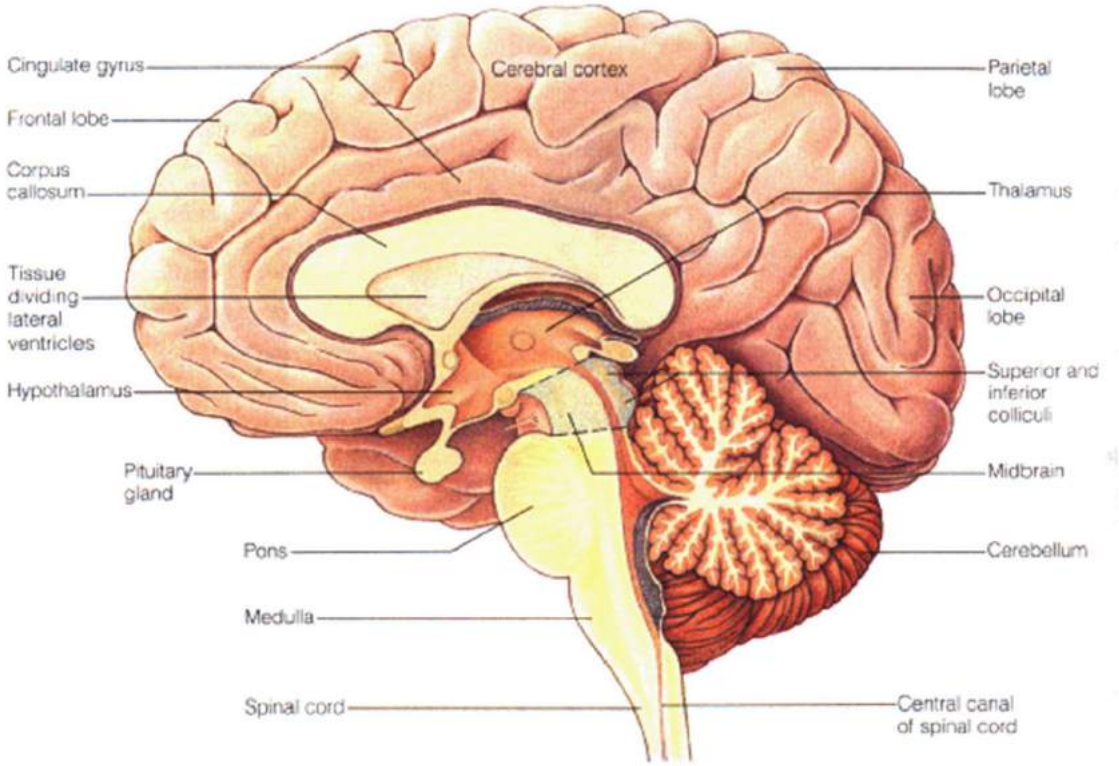
(b)

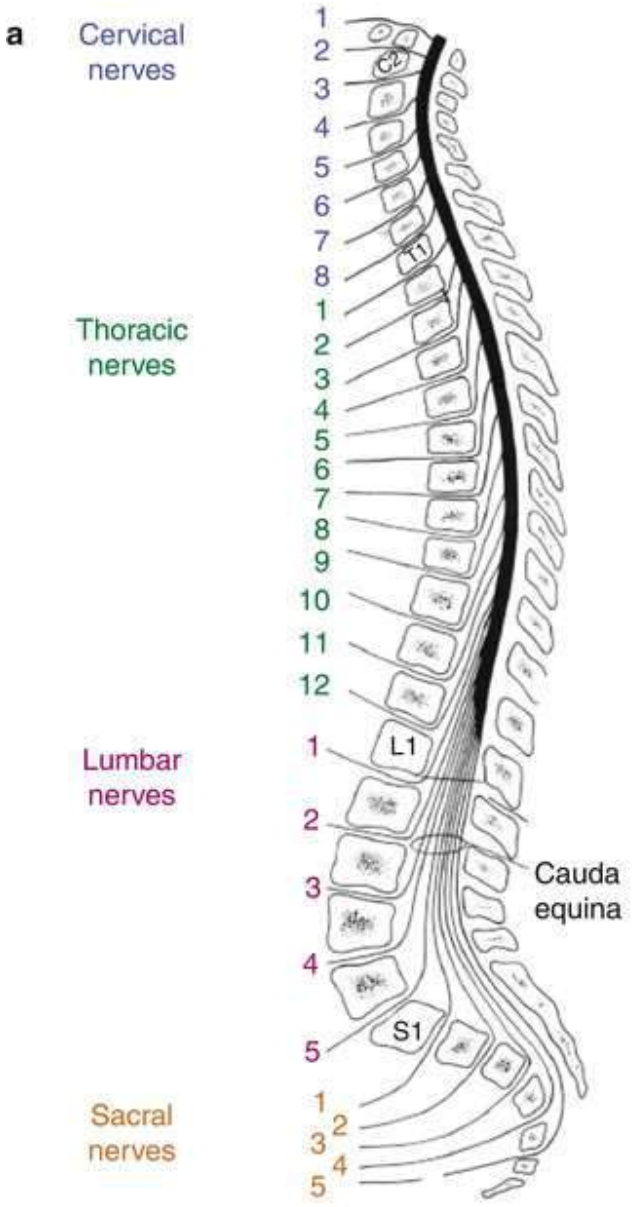


ANATOMICAL SURFACES OF FOOT

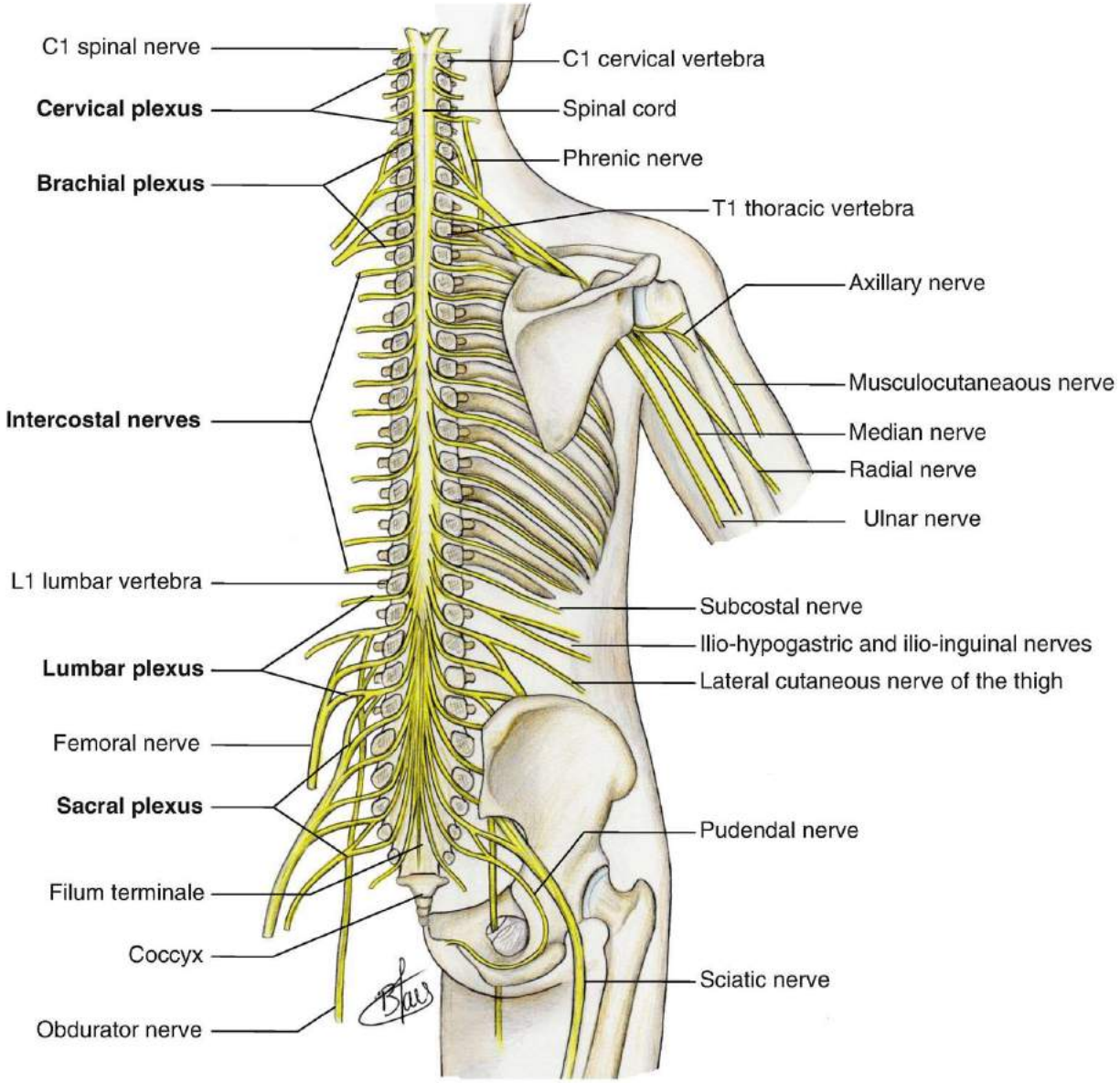
ANATOMY OF CNS

A. Anatomy of BRAIN



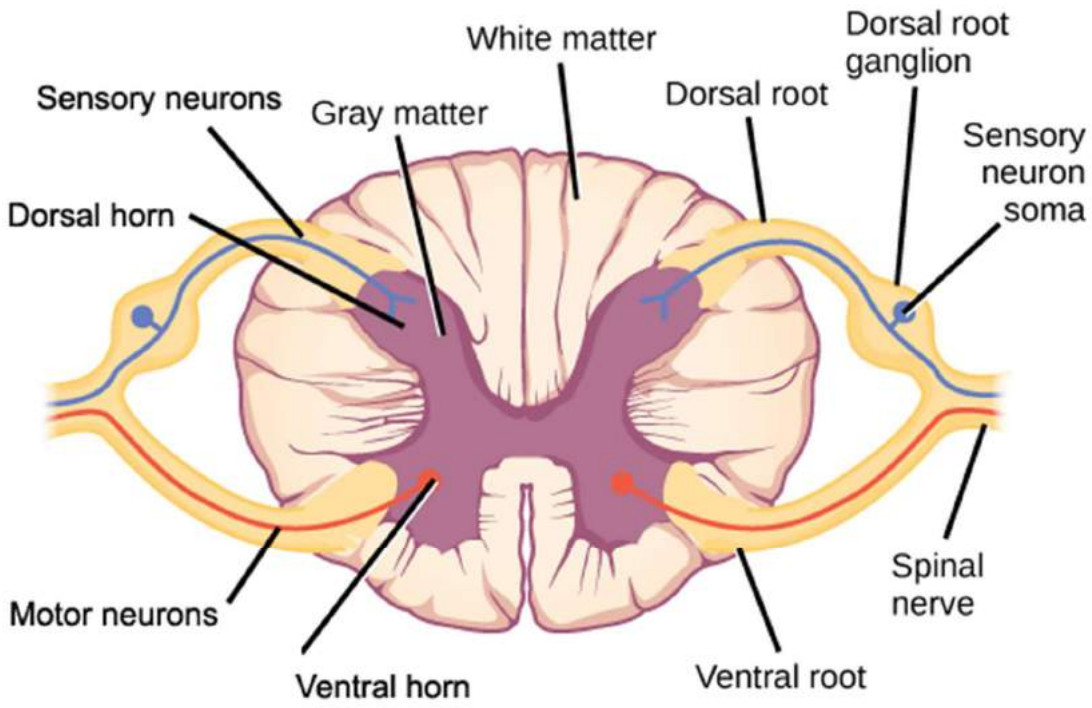


B. **ANATOMY OF SPINAL CORD**



ANATOMY OF SPINAL NERVES

CROSS SECTION OF SPINAL CORD



Cross Section of Spinal Cord

The spinal nerves are peripheral nerves that transmit messages between the spinal cord and the rest of the body, including muscles, skin, and internal organs. Each spinal nerve is dedicated to certain regions of the body.

Structure

The spinal nerves are relatively large nerves that are formed by the merging of a sensory nerve root and a motor nerve root. These nerve roots emerge directly from the spinal cord—sensory nerve roots from the back of the spinal cord and the motor nerve roots from the front of the spinal cord. As they join, they form the spinal nerves on the sides of the spinal cord.

The spinal cord is composed of nerve cells that serve to relay messages between the brain and the peripheral nerves.

The spinal nerves receive sensory messages from tiny nerves located in areas such as the skin, internal organs, and bones. The spinal nerves send sensory messages to the sensory roots, then to sensory fibers in the posterior (back or dorsal) part of the spinal cord.

The motor roots receive nerve messages from the anterior (front or ventral) part of the spinal cord and send the nerve messages to the spinal nerves, and eventually to small nerve branches that activate muscles in the arms, legs, and other areas of the body.

There are 31 pairs of spinal nerves including:

- Eight cervical spinal nerves on each side of the spine called C1 through C8
- Twelve thoracic spinal nerves in each side of the body called T1 through T12
- Five lumbar spinal nerves on each side called L1 through L5
- Five sacral spinal nerves in each side called S1 through S5
- One coccygeal nerve on each side, Co1

Location

Spinal nerves are distributed approximately evenly along the spinal cord and spine. The spine is a column of vertebral bones that protects and surrounds the spinal cord. Each spinal nerve exits the spine by traveling through the foramen, which are openings at the right and left sides of the vertebral bones of the spine.

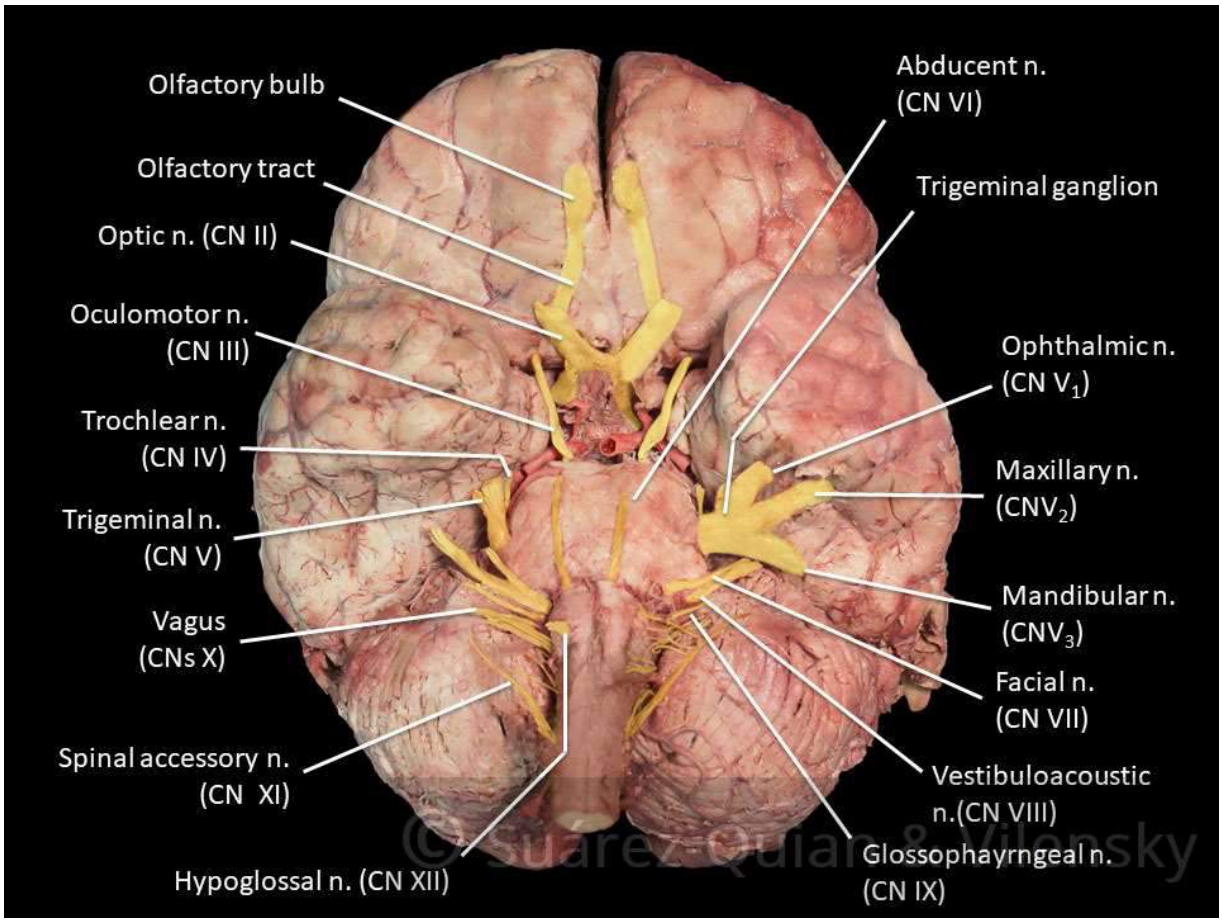
The spinal nerves are formed within a few centimeters of the spine on each side. Some groups of spinal nerves merge with each other to form a large plexus. Some spinal nerves divide into smaller branches, without forming a plexus.

A plexus is a group of nerves that combine with each other. There are five main plexi formed by the spinal nerves:

- **Cervical Plexus**: Composed of the merging of spinal nerves C1 through 5, these divide into smaller nerves that carry sensory messages and provide motor control to the muscles of the neck and shoulders.
- **Brachial Plexus**: Formed by the merging of spinal nerves C5 through T1, this plexus branches into nerves that carry sensory messages and provide motor control to the muscles of the arm and upper back.
- **Lumbar Plexus**: Spinal nerves L1 through L4 converge to form the lumbar plexus. This plexus splits into nerves that carry sensory messages and provide motor control to the muscles of the abdomen and leg.
- **Sacral Plexus**: Spinal nerves L4 through S4 join together, and then branch out into nerves that carry sensory messages and provide motor control to the muscles of the legs.

- **Coccygeal Plexus**: Composed of the merging of nerves S4 through Co1, this plexus supplies motor and sensory control of the genitalia and the muscles that control defecation.

ANATOMY OF CRANIAL NERVES



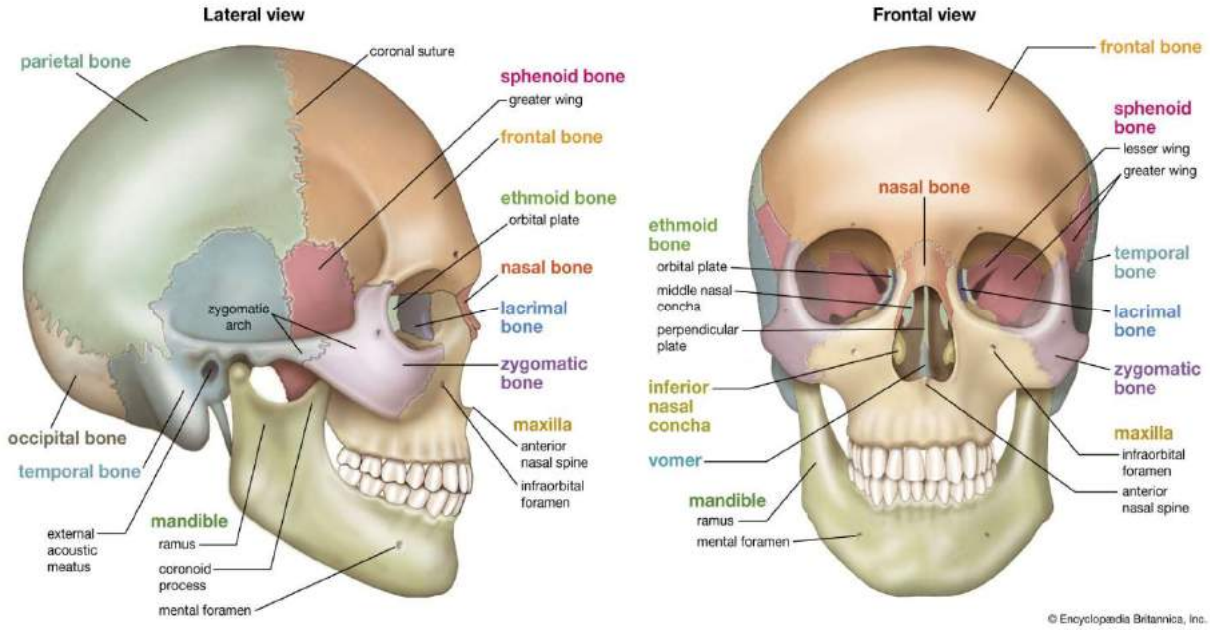
Twelve pairs of nerves (the cranial nerves) lead directly from the brain to various parts of the head, neck, and trunk. Some of the cranial nerves are involved in the special senses (such as seeing, hearing, and taste), and others control muscles in the face or regulate glands. The nerves are named and numbered (according to their location, from the front of the brain to the back).

Image 1: Twelve pairs of cranial nerves emerge from the underside of the brain, pass through openings in the skull, and lead to parts of the head, neck, and trunk. The nerves are named and numbered, based on their location, from the front of the brain to the back. Thus, the olfactory nerve is the 1st cranial nerve, and the hypoglossal nerve is the 12th cranial nerve

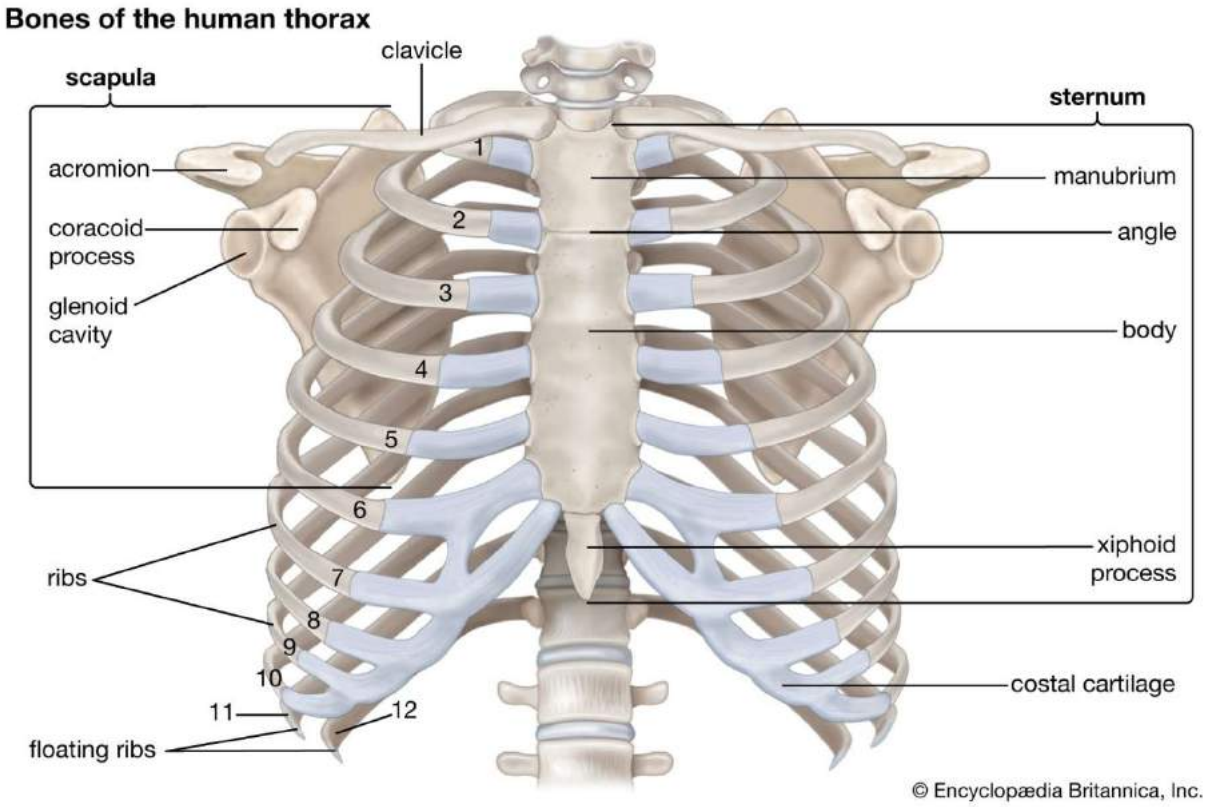
Unlike spinal nerves whose roots are neural fibers from the spinal grey matter, cranial nerves are composed of the neural processes associated with distinct brainstem nuclei and cortical structures.[1]

The names of the cranial nerves (CN) are: CN I - Olfactory, CN II - Optic, CN III - Oculomotor, CN IV - Trochlear, CN V - Trigeminal, CN VI - Abducens, CN VII - Facial, CN VIII - Vestibulocochlear, CN IX - Glossopharyngeal, CN X - Vagus, CN XI - Accessory, and CN XII - Hypoglossal.

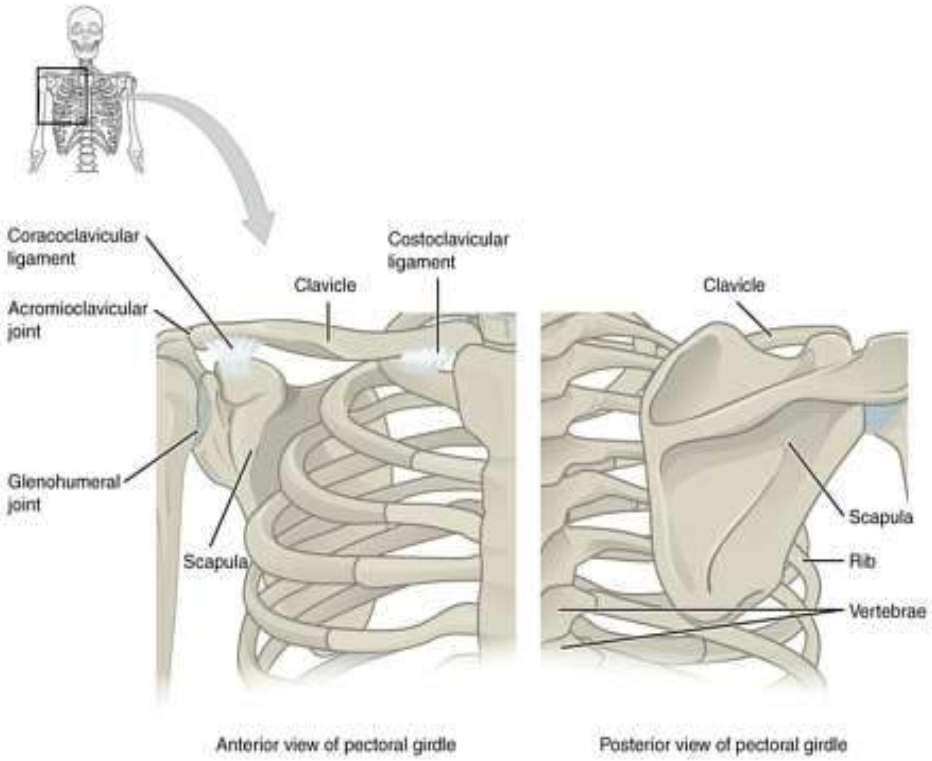
BONES OF THE SKULL



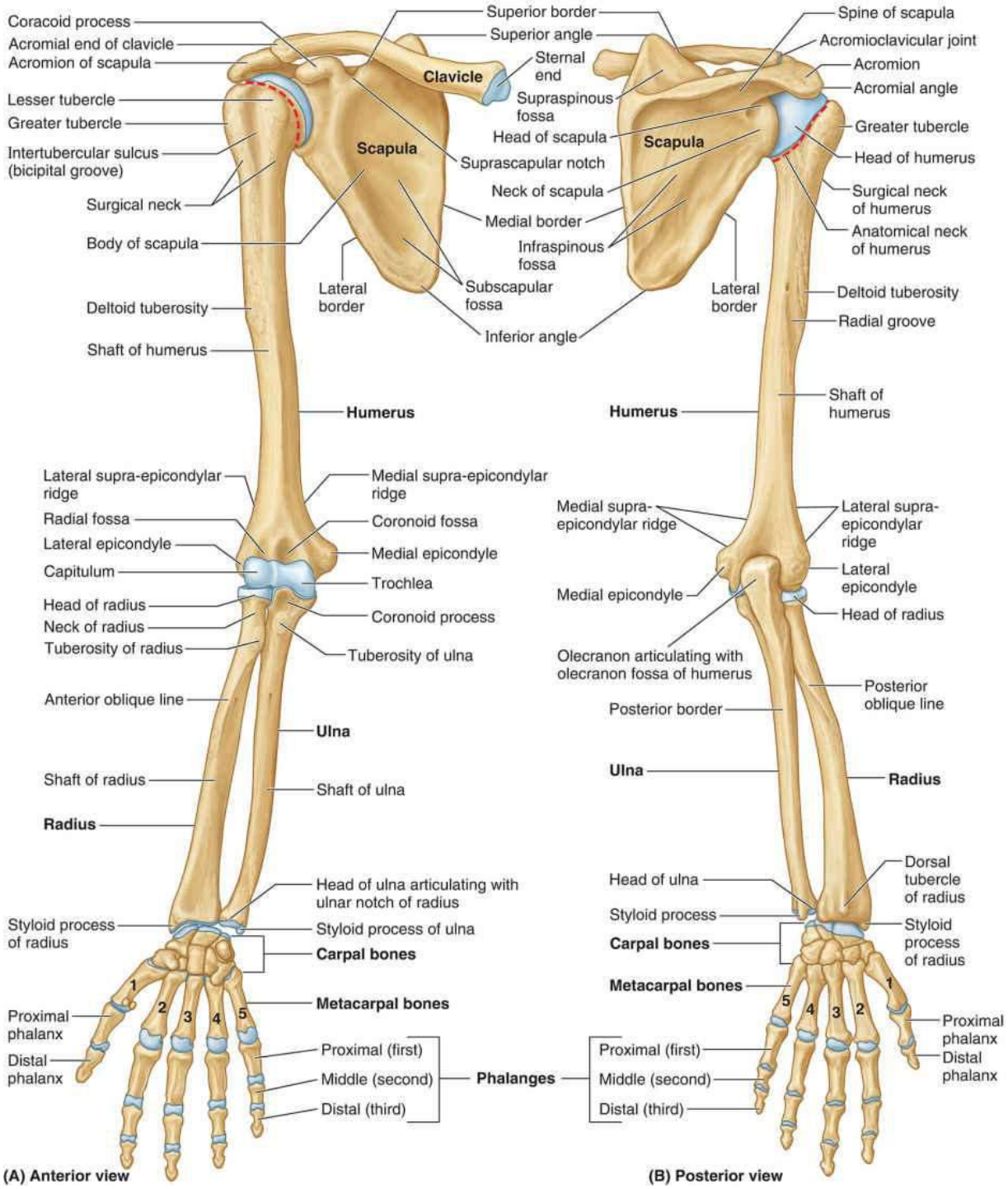
BONES OF THE THORAX



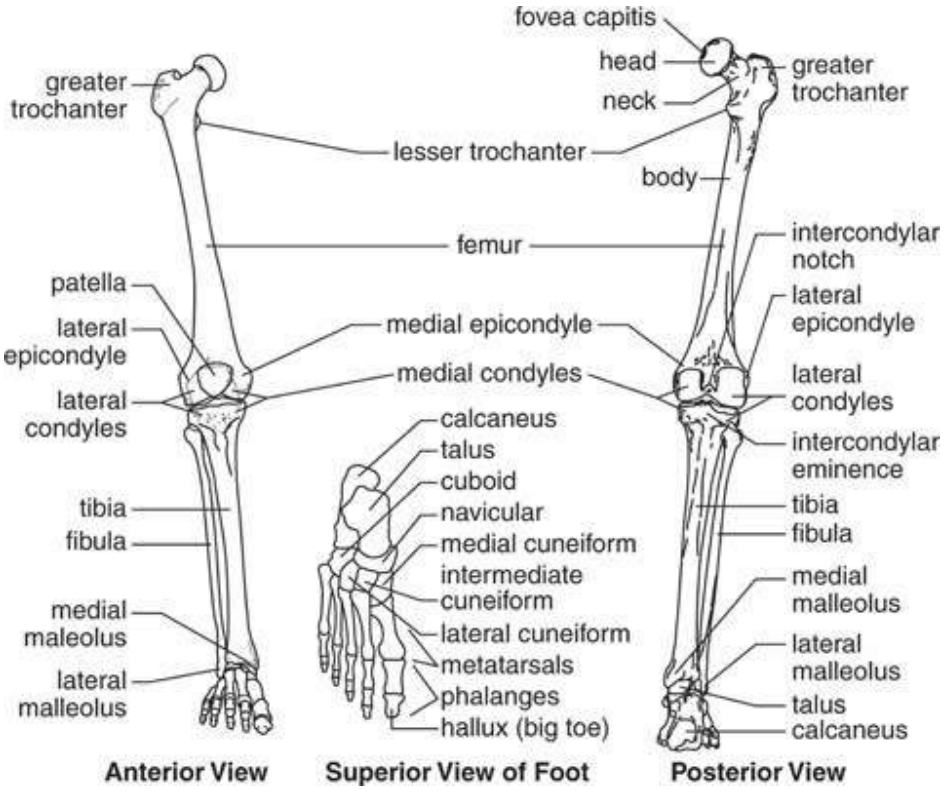
BONES OF PECTORAL GIRDLE



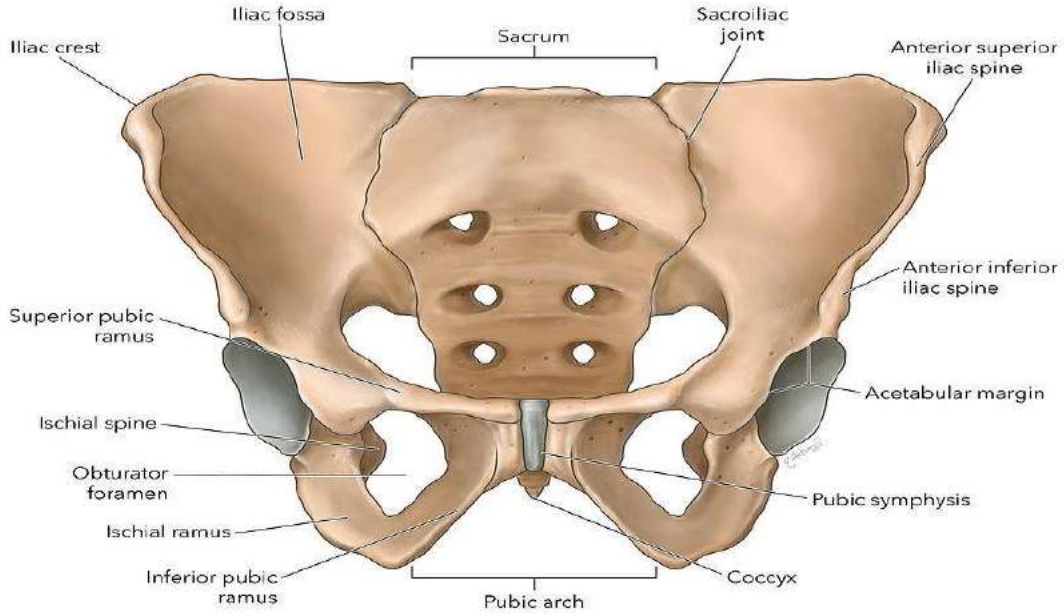
BONES OF UPPER LIMBS



BONES OF LOWER LIMB



BONES OF PELVIC GIRDLE



BMM 196; BACTERIOLOGY

1. Staining methods- simple staining, grams staining, capsule staining, spore staining, spirochete staining, Methods of motility testing: hanging drop preparation

Simple staining

Principle: Simple staining involved applying a single basic dye to impart colour to the bacterial cell. Basic dyes are positively charged and work well with bacteria because the bacterial cells bear a slight negative charge. Further basic dyes are attached to the acidic part of the cell such as techoic acid. In general there are two main stain types. Positively charged stains have a positive chromophore. The second type, negatively charged stains, has a chromophore that carries a negative charge. Positively charged stains are excellent in binding negatively charged structures such as bacterial cell walls and, if they can enter the cell, many macromolecular structures such as DNA and proteins.

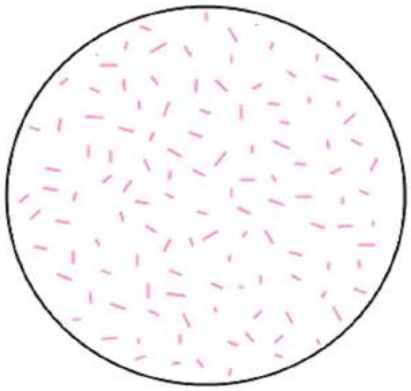
Example of the basic dye includes methylene blue, crystal violet and safranin.

Materials: Glass slide, bacterial culture, cotton, crystal violet, microscope, tray of disinfectant, inoculating loop bunsen- burner

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 sec-1 min.
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:**	 **Rod Shape** **Single** **Violet**

Comment: According to the above result, the supplied bacterial sample was rod shaped and arranged in single. (Please change accordingly with your result).

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:

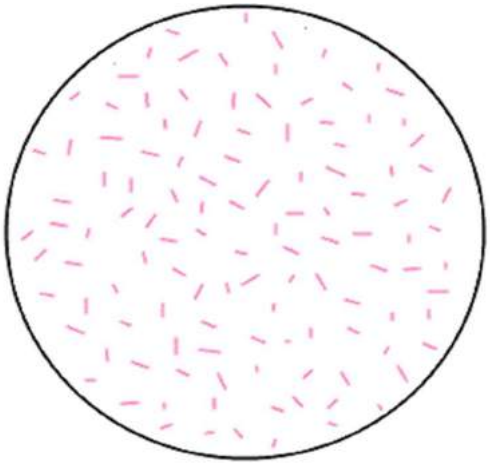
- **Culture:** Twenty four hours old culture
- **Reagents:** Crystal violet- Primary stain
  Gram Iodine- Mordant
  Decolourising agent- 70% ethyl alcohol
  Counter Stain- Safranine
- **Equipment:** Bunsen burner, inoculating loop, staining tray, glass slide, lens paper and microscope.

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.

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).

METHODS OF MOTILITY TESTING: HANGING DROP PREPARATION

INTRODUCTION: Hanging drop preparation is one of the easiest method to observe motility in a clinical microbiological laboratory. This is carried out by putting a loop full of bacterial suspension on the cover slip and placing it over a cavity slide and observing it under a microscope. Advantage of this method is that by this method *live bacteria* can be observed. Examination of living organisms is useful to observe cell activities, viz. motility, binary fission, and also to observe natural sizes and shapes of the cells.

Motility of bacteria can also be demonstrated by i. Craigie 's tube method , ii. swarming of the bacteria on a non inhibitory medium (e.g, blood agar) and iii. by dark ground microscopy.

Capillary tube method is a useful method for demonstrating the motility of anaerobic bacteria.

PRINCIPLE: Microorganisms such as bacteria, because of their small size and a refractive index that closely approximates that of water, do not lend them readily to microscopic examination in a living, unstained state. Bacteria are motile due to the presence of flagella. Depending on the location of the attachment of the flagella, bacteria can be classified as i. monotrichous (single polar flagellum at one end) e.g, *Vibrio cholerae*, ii. amphitrichous (single flagellum at both the ends) e.g *Pseudomonas aeruginosa*, iii. lophotrichous (tufts of flagella at one or both ends) e.g, Spirilla, and iv.peritrichous (fagella arranged all around the cell) e.g, *Escherichia coli, Salmonella* sp , etc.

Spirochaetes are the examples of bacteria which are motile but without presence of any external flagella.

REQUIREMENTS

I Equipments

Compound light microscope.

II Reagents and glass wares

Normal saline, inoculating loop wire, staining tray, cavity slides and cover slips.

III Specimen

Log phase broth culture of *E. coli*

PROCEDURE

1 Take a clean grease free cavity slide.

2 Take a clean coverslip, apply paraffin to four corners of coverslip.
3 Place a drop of broth culture on the coverslip with the help of inoculating loop.
4 Place the cavity slide (cavity down) over the coverslip so that the drop is placed in center.
5 Invert the slide, and observe under microscope.
6 First observe under low power (10x), locate the edge of the drop, shift the focus to high power (40x) and observe.
7 Record the observation in a notebook.

OBSERVATIONS
The bacteria showing motility are demonstrated in the hanging drop preparation.
Note: It is important to differentiate active motility from brownian movement. Brownian movement is not true motility, instead it is exibited due to movement of organism as a result of their collision with water molecules. This movement is usually seen around the axis of bacteria.

RESULTS AND INTERPRETATION
The wet mount preparation shows motile bacteria.

2. Preparation of common culture media

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.

3. Sterilization methods

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

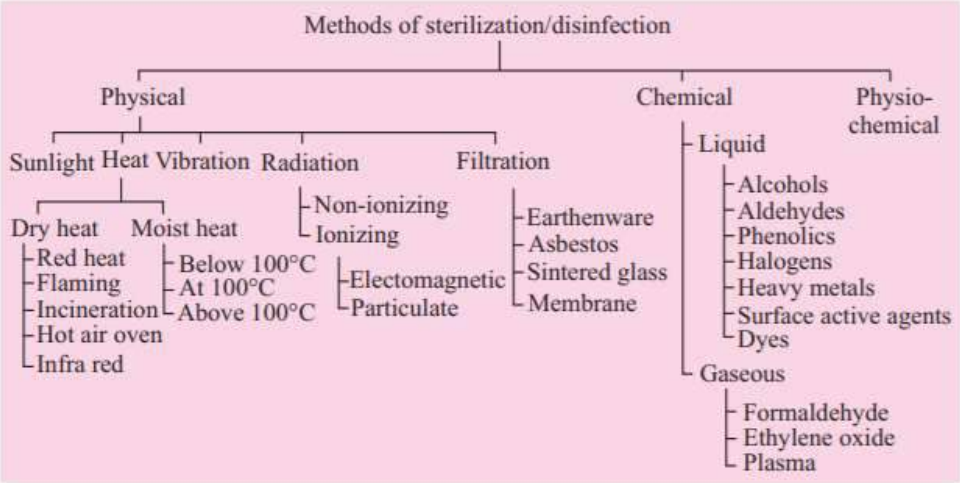
(a) Thermal (Heat) methods

(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.

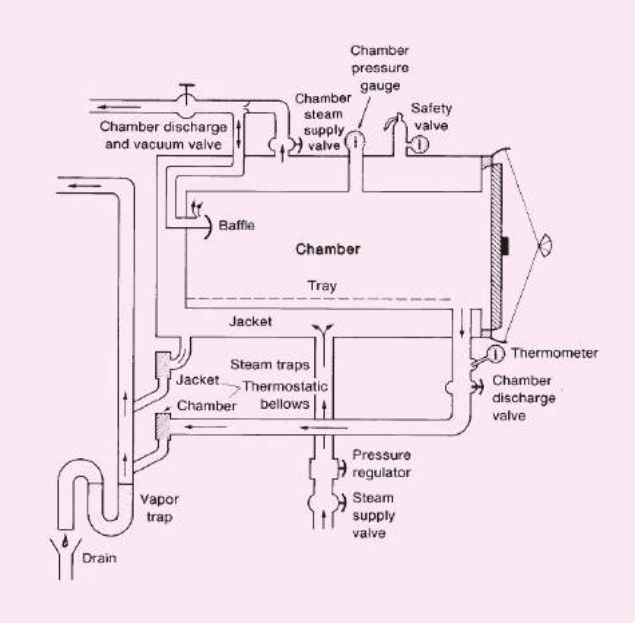


Fig. Autoclave

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.

4. Culture methods
4A. Culture of Aerobic Bacteria
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:

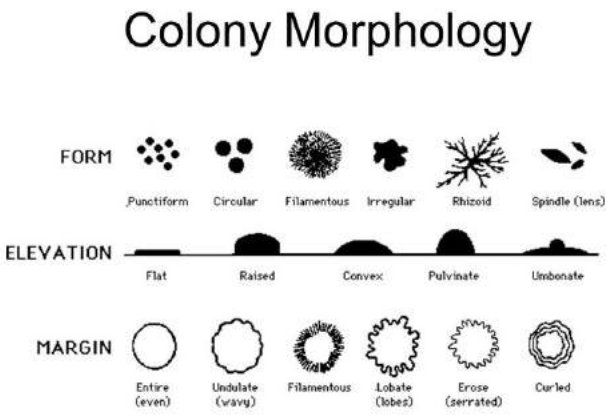
After 24 h, the colony appeared on the petri plates were enumerated and the colony characteristics were observed.

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.

4B. Culture of Anaerobic Bacteria

PRINCIPLE

There are different ways of creating anaerobic conditions suitable for the growth of obligate anaerobes. Deep nutrient agar tubes are the simplest method. The tubes are inoculated while still molten, cooled rapidly and incubated. Anaerobes grow in the depths of the medium, and the number of colonies becomes fewer towards the surface. Strict anaerobes will not grow within a centimeter of the surface.

Alternatively, reducing agents like 0.5-1% glucose, 0.1% ascorbic acid, 0.1% cysteine, 0.1% thioglycollate can be added.

Cooked meat particles also act as a good reducing agent Example. Robertson Cooked meat medium.

For culture of anaerobes, oxygen must be excluded either by combustion or by replacing it with an inert gas.

In many laboratories, combustion involves the combining of oxygen with hydrogen to form water in the presence of a catalyst like palladium or palladinized asbestos. Anaerobic jars are a constant feature of anaerobic culture. They include the McIntosh and Fildes jar, which has inlets to admit hydrogen and carbon dioxide, a vacuum pump for evacuating oxygen, and a catalyst fitted into the lid.

A simpler but more expensive technique is the Gaspak system. This utilizes a transparent polycarbonate jar with a lid bearing a screened catalyst chamber. The catalyst, consisting of pellets of sodium borohydride, cobalt chloride, citric acid and sodium bicarbonate is contained in sachets. Water is added to the sachet and it is immediately placed in the jar, which is then sealed tightly. The resulting reaction liberates hydrogen and carbon dioxide. An indicator is also added to demonstrate anaerobiosis.

REQUIREMENTS

I Equipments

Anaerobic culture systems: McIntosh and Fildes jar and Gaspak
system.

II Reagents and media

Blood agar plates and thioglycollate broth culture.

III Specimen

A 48 hour thioglycollate broth culture of anaerobic bacteria (*Clostridium sporogenes, Bacteroides* spp), and 24 hour
cultures of facultative anaerobes (*Escherichia coli*), and obligate aerobes (*Pseudomonas aeruginosa*).

PROCEDURE

1 Divide each plate into 4 quadrants.
2 Inoculate a loopful of each organism into a quadrant.
3 Stack the plates into the anaerobic jars, introduce the catalyst and quickly seal the lid.
Note: Anaerobic condition should be checked by alkaline methylene blue indicator.
4 Incubate the plates at 37ºC for 48 hours.
5 Incubate one plate aerobically.
6 Remove the plates from the jars and examine for growth.

QUALITY CONTROL

1 Blood agar inoculated with *P. aeruginosa,* the bacteria that does not grow anaerobically

2 Thioglycollate broth culture inoculated with *Cl. sporogenes*, the bacteria that does not grow aerobically

OBSERVATIONS

If anaerobiosis is complete, obligate anaerobes like *Cl. sporogenes* will grow, while obligate aerobes like *P. aeruginosa* will not grow.

RESULTS AND INTERPRETATION

P. aeruginosa will not show growth on the plates incubated anaerobically, while *Cl. sporogenes* and *Bacteroides* spp will grow on the blood gar plates. *P. aeruginosa* is a strict aerobe that cannot grow in the absence of oxygen.

P. aeruginosa will show growth on the aerobically incubated plate while *Cl. sporogenes* or *Bacteroides* spp will not grow.

Cl. sporogenes and *Bacteroides* are strict anaerobes that cannot grow if oxygen is present.

E. coli will grow on all the plates, either incubated aerobically or anaerobically. *E. coli* is a facultative anaerobe and can grow either in the presence or absence of oxygen.

LIST OF ANAEROBIC BACILLI AND COCCI

Anaerobic bacilli

Gram positive bacilli	*Gram negative Bacilli*
Bifidobacterium	*Bacteroides*
Propionobacterium	*Fusobacterium*
Eubacterium	
Lactobacillus	
Actonomyces	

Anaerobic cocci

Gram positive cocci	*Gram Negative cocci*
Peptosteptococcus	*Veillonella*
Coprococcus	*Acidaminococcus*
Ruminococcus	*Megasphaera*

5. Cultivation of bacteria and in laboratory

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:

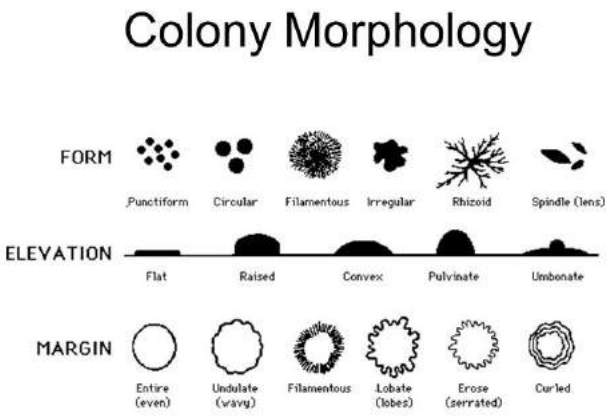
After 24 h, the colony appeared on the petri plates were enumerated and the colony characteristics were observed.

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.

6. Biochemical test used for identification of bacteria

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 vaginalis*

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
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

7. Anaerobic culture methods
PRINCIPLE
There are different ways of creating anaerobic conditions suitable for the growth of obligate anaerobes. Deep nutrient agar tubes are the simplest method. The tubes are inoculated while still molten, cooled rapidly and incubated. Anaerobes grow in the depths of the medium, and the number of colonies becomes fewer towards the surface. Strict anaerobes will not grow within a centimeter of the surface.
Alternatively, reducing agents like 0.5-1% glucose, 0.1% ascorbic acid, 0.1% cysteine, 0.1% thioglycollate can be added.
Cooked meat particles also act as a good reducing agent Example. Robertson Cooked meat medium.
For culture of anaerobes, oxygen must be excluded either by combustion or by replacing it with an inert gas.
In many laboratories, combustion involves the combining of oxygen with hydrogen to form water in the presence of a catalyst like palladium or palladinized asbestos. Anaerobic jars are a constant feature of anaerobic culture. They include the McIntosh and Fildes jar, which has inlets to admit hydrogen and carbon dioxide, a vacuum pump for evacuating oxygen, and a catalyst fitted into the lid.
A simpler but more expensive technique is the Gaspak system. This utilizes a transparent polycarbonate jar with a lid bearing a screened catalyst chamber. The catalyst, consisting of pellets of sodium borohydride, cobalt chloride, citric acid and sodium bicarbonate is contained in sachets. Water is added to the sachet and it is immediately placed in the jar, which is then sealed tightly. The resulting reaction liberates hydrogen and carbon dioxide. An indicator is also added to demonstrate anaerobiosis.
REQUIREMENTS
I Equipments
Anaerobic culture systems: McIntosh and Fildes jar and Gaspak
system.
II Reagents and media
Blood agar plates and thioglycollate broth culture.
III Specimen
A 48 hour thioglycollate broth culture of anaerobic bacteria (*Clostridium sporogenes, Bacteroides* spp), and 24 hour
cultures of facultative anaerobes (*Escherichia coli*), and obligate aerobes (*Pseudomonas aeruginosa*).
PROCEDURE

1 Divide each plate into 4 quadrants.
2 Inoculate a loopful of each organism into a quadrant.
3 Stack the plates into the anaerobic jars, introduce the catalyst and quickly seal the lid.
Note: Anaerobic condition should be checked by alkaline methylene blue indicator.
4 Incubate the plates at 37ºC for 48 hours.
5 Incubate one plate aerobically.
6 Remove the plates from the jars and examine for growth.

QUALITY CONTROL
1 Blood agar inoculated with *P. aeruginosa,* the bacteria that does not grow anaerobically
2 Thioglycollate broth culture inoculated with *Cl. sporogenes*, the bacteria that does not grow aerobically

OBSERVATIONS
If anaerobiosis is complete, obligate anaerobes like *Cl. sporogenes* will grow, while obligate aerobes like *P. aeruginosa* will not grow.
RESULTS AND INTERPRETATION
P. aeruginosa will not show growth on the plates incubated anaerobically, while *Cl. sporogenes* and *Bacteroides* spp will grow on the blood gar plates. *P. aeruginosa* is a strict aerobe that cannot grow in the absence of oxygen.
P. aeruginosa will show growth on the aerobically incubated plate while *Cl. sporogenes* or *Bacteroides* spp will not grow.
Cl. sporogenes and *Bacteroides* are strict anaerobes that cannot grow if oxygen is present.
E. coli will grow on all the plates, either incubated aerobically or anaerobically. *E. coli* is a facultative anaerobe and can grow either in the presence or absence of oxygen.

LIST OF ANAEROBIC BACILLI AND COCCI

Anaerobic bacilli

Gram positive bacilli	*Gram negative Bacilli*
Bifidobacterium	*Bacteroides*
Propionobacterium	*Fusobacterium*
Eubacterium	
Lactobacillus	
Actonomyces	

Anaerobic cocci

Gram positive cocci	*Gram Negative cocci*
Peptosteptococcus	*Veillonella*
Coprococcus	*Acidaminococcus*
Ruminococcus	*Megasphaera*

8. Maintenance & preservation of bacterial cultures

Once a microorganism has been isolated and grown in pure culture, it becomes necessary to maintain the viability and purity of the microorganism by keeping the pure culture free from contamination. Normally in laboratories, the pure cultures are transferred periodically onto or into a fresh medium (subculturing) to allow continuous growth and viability of microorganisms. The transfer is always subject to aseptic conditions to avoid contamination.

Since repeated subculturing is time-consuming, it becomes difficult to maintain a large number of pure cultures successfully for a long time. In addition, there is a risk of genetic changes as well as contamination. Therefore, it is now being replaced by some modern methods that do not need frequent subculturing. These methods include refrigeration, paraffin method, cryopreservation, and lyophilization (freeze-drying).

Periodic Transfer to Fresh Media

Strains can be maintained by periodically preparing a fresh culture from the previous stock culture. The **culture medium**, the storage temperature, and the time interval at which the transfers are made vary with the species and must be ascertained beforehand. The **temperature** and the type of medium chosen should support a slow rather than a rapid rate of growth so that the time interval between transfers can be as long as possible. Many of the more common heterotrophs remain viable for several weeks or months on a medium like **nutrient agar**.

The transfer method has the disadvantage of failing to prevent changes in the characteristics of a strain due to the development of variants and mutants.

Refrigeration

Pure cultures can be successfully stored at 0-4°C either in refrigerators or in cold-rooms. This method is applied for short duration (2-3 weeks for bacteria and 3-4 months for fungi) because the metabolic activities of the microorganisms are greatly slowed down but not stopped. Thus their growth continue slowly, nutrients are utilized and waste products released in medium. This results in, finally, the death of the microbes after sometime.

Paraffin Method/ preservation by overlaying cultures with mineral oil

This is a simple and most economical method of maintaining pure cultures of bacteria and fungi. In this method, sterile liquid paraffin is poured over the slant (slope) of culture and stored upright at room temperature. The layer of paraffin ensures anaerobic conditions and prevents dehydration of the medium. This condition helps microorganisms or pure culture to remain in a dormant state and, therefore, the culture can be preserved from months to years (varies with species).

The advantage of this method is that we can remove some of the growth under the oil with a transfer needle, inoculate a fresh medium, and still preserve the original culture. The simplicity of the method makes it attractive, but changes in the characteristics of a strain can still occur.

Cryopreservation

Cryopreservation (i.e., freezing in liquid nitrogen at -196°C or in the gas phase above the liquid nitrogen at -150°C) helps the survival of pure cultures for long storage times.

In this method, the microorganisms of culture are rapidly frozen in liquid nitrogen at -196°C in the presence of stabilizing agents such as glycerol or dimethyl sulfoxide (DMSO) that prevent cell damage due to the formation of ice crystals and promote cell survival.

This liquid nitrogen method has been successful with many species that cannot be preserved by lyophilization and most species can remain viable under these conditions for 10 to 30 years without undergoing a change in their characteristics, however, this method is expensive.

Lyophilization (Freeze-Drying)

Freeze-drying is a process where water and other solvents are removed from a frozen product **via sublimation.** Sublimation occurs when a frozen liquid goes directly to a gaseous state without entering a liquid phase.

It is recommended to use slow rates of cooling, as this will result in the formation of vertical ice crystal structures, thus allowing for more efficient water sublimation from the frozen product. Freeze-dried products are hygroscopic and must be protected from moisture during storage. Under these conditions, the microbial cells are dehydrated and their metabolic activities are stopped; as a result, the microbes go into a dormant state and retain viability for years. Lyophilized or freeze-dried pure cultures are then sealed and stored in the dark at 4°C in refrigerators.

Freeze-drying method is the most frequently used technique by culture collection centers. Many species of bacteria preserved by this method have remained viable and unchanged in their characteristics for more than 30 years.

Advantage of Lyophilization

1. Only minimal storage space is required; hundreds of lyophilized cultures can be stored in a small area.
2. Small vials can be sent conveniently through the mail to other microbiology laboratories when packaged in a special sealed mailing container.
3. Lyophilized cultures can be revived by opening the vials, adding the liquid medium, and transferring the rehydrated culture to a suitable growth medium.