Stains and staining techniques:

- **Dye:** Coloring agent that is used for general purposes
- **Stain:** coloring agent that is used for biological purposes
  Ex: Aniline compounds are dyes while Crystal violet is a stain.

- stains are organic compounds containing both chromophore and auxochrome groups linked to benzene rings.

Benzene = Organic colorless solvent

+

= Chromogen (colored not stain)

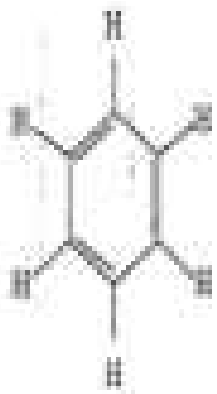
Chromophore = imparts color to benzene

+

Auxochrome = conveys the property of ionization to the chromogen enabling it to form salts and bind to fibers or tissues

Stain

may be defined as an organic compound containing a benzene ring plus a chromophore and auxochrome group (Figure III.1).



Benzene

Organic colorless solvent

+ Chromophore: Chemical group that imparts color to benzene

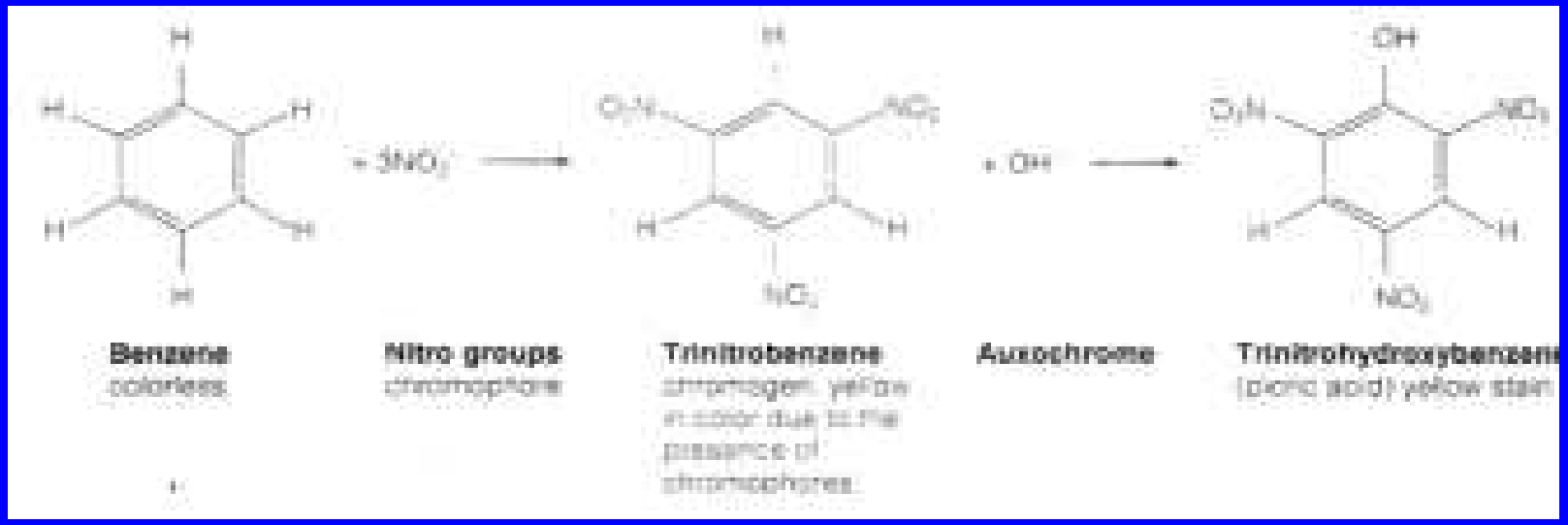
= Chromogen: Colored compound, not a stain

Auxochrome: Chemical group that conveys the property of ionization to the chromogen, enabling it to form salts and bind to fibers or tissues

Stain

Picric acid is used to explain chemical nature of acidic stain.

1. Benzene is colorless.
2. Addition of the chromophore – nitro group yields pale yellow colored trinitrobenzene, which is a chromogen , but not a stain.
3. Trinitrohydroxybenzene (picric acid) is a bright yellow colored stain, as addition of the auxochrome – OH group renders the property of ionization to this compound.
4. The negatively charged ion has a strong affinity for the positively charged components of the cell.

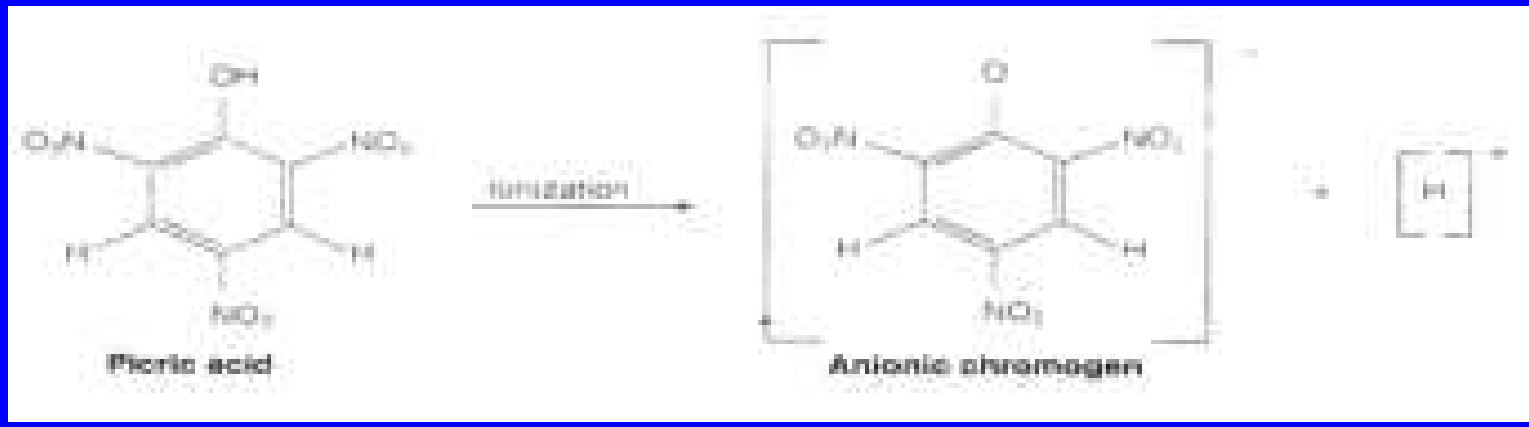


CHEMICAL CLASSIFICATION OF STAINS

- The ability of a stain to bind to macromolecular cellular components such as proteins or nucleic acids depends on the electrical charge found on the stain as well as on the cellular components to be stained.
- On the basis of the electrical charge properties of stains, there are two types of stains- ACIDIC and BASIC.

- **ACIDIC STAINS** are anionic, i.e. on ionization, the stains exhibit a negative charge and has a strong affinity for the positively charged cellular components.

  For ex. Picric Acid.



- **BASIC STAINS** are cationic, i.e. on ionization, the stains exhibit a positive charge and has a strong affinity for the negatively charged cellular components. For ex. Methylene Blue.

Why basic stains are commonly used for bacterial staining?

1. Basic stains are commonly used for bacterial staining as the presence of a negative charge on the bacterial surface acts to repel most acidic stains and thus prevent their penetration into the cell.
2. Acidic stains are used mainly to stain cytoplasm whereas the basic stains color acidic cellular components like nucei and cell wall or plasma membrane

Bacterial cell surface is negatively charged due to –

1. **Gram negative**: Presence of outer membrane lipopolysaccharide outside of the cell wall in Gram negative bacteria which are often composed of negatively charged phospholipids.
2. **Gram positive**: Presence of negatively charged teichoic acid molecules attached at the outer surface of Gram positive cell wall peptidoglycan.

MORDANTS

Some stains have no natural affinity for the cells, cellular parts and tissues. These stains are mixed with salts like oxides of aluminium or chromium to form an insoluble co-ordination complex which binds with target cellular component with greater affinity. These salts are called mordants. Mordants are not stains, but facilitates the firm binding of the stain compound with cells.

For example, Grams iodine (40% KI soln.) is a mordant in Gram staining. It forms insoluble crystal violet- iodine complex (CV-I) with the primary stain, thereby increasing the affinity of crystal violet for binding with the bacterial cell wall peptidoglycan.

STAINING TECHNIQUES

- Types of staining techniques-

1. Simple staining- use of single stain for visualization of morphological shape (cocci, bacilli or spiralli) and arrangement (chains, clusters, tetrads etc.)

2. Differential staining- use of two contrasting stains for separation of microorganisms into groups (Gram staining and acid fast staining) and visualization of structures (flagella stain, capsule stain, endospore stain etc.).

   The first stain is called primary stain. Its function is to impart its color to all cells.

   The second stain, sometimes called counter stain, imparts contrasting color to that of the primary stain.

SIMPLE STAINING

PRINCIPLE:

The bacterial smear is stained with a single, positively charged staining reagent as negatively charged bacterial cell surface strongly attracts and binds to the cationic stains.

PURPOSE:

To elucidate the morphology and arrangement of bacterial cells.

PROCEDURE:

1. A heat fixed smear of a overnight grown bacterial suspension is made upon a grease free slide.
2. The smear is flooded with an appropriate stain for about 1 minute and then washed with tap water to remove excess stain.
3. The slide is blot dried and examined under microscope.

Function of decolorizing agent

- In order to establish a color contrast, decolorizing agent is used.
- Based on the chemical composition of cellular components, the decolorizing agent may or may not remove the primary stain from entire cell or only from certain cell structures.
- Following decolorization, if the primary stain is not washed out, the counter stain can not be absorbed and the cell or its components will retain the color of the primary stain.
- If the primary stain is removed, the decolorized cellular components will accept the contrasting color of the counter stain.

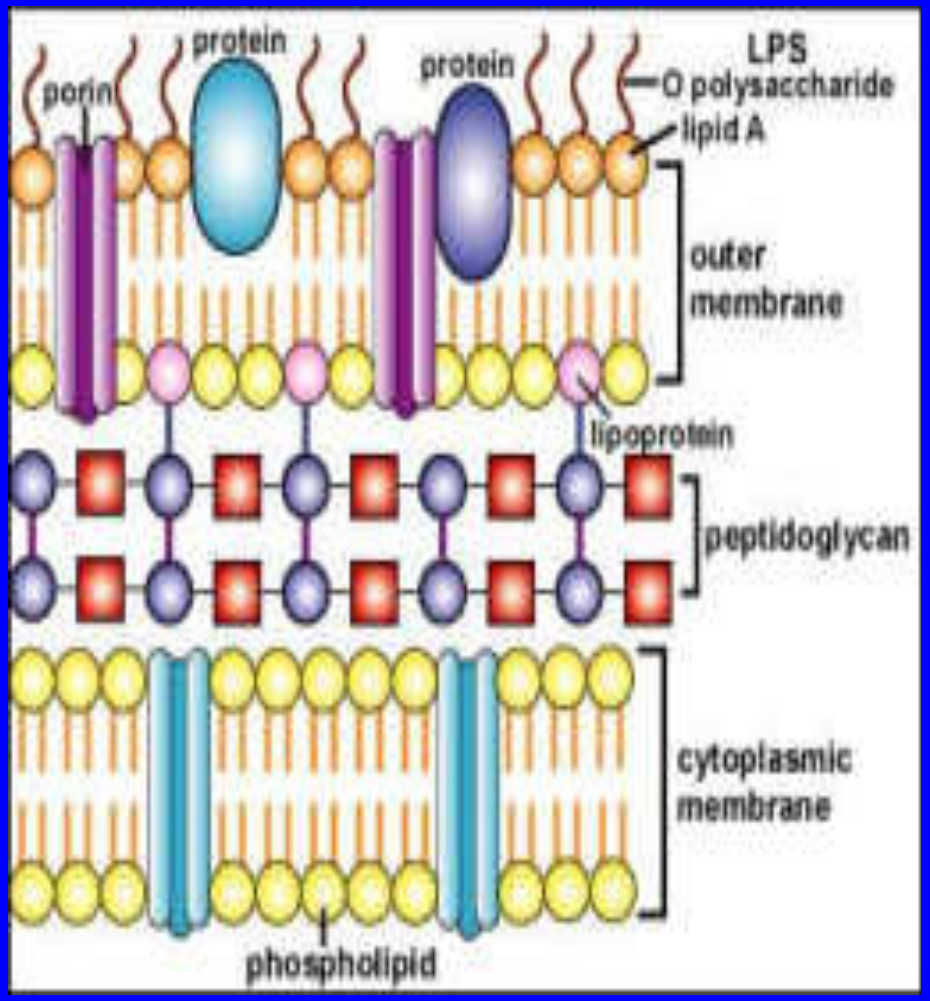
Principle of Gram staining

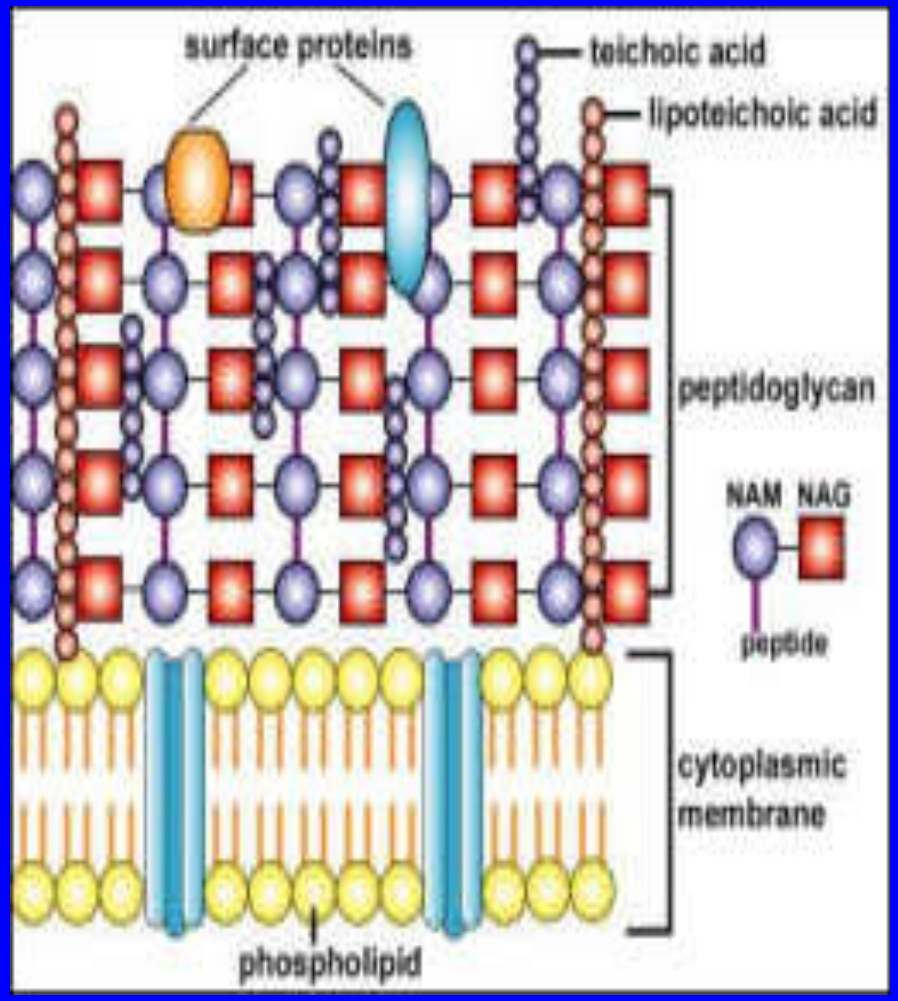
- This staining technique divides bacterial cells into two major groups – Gram positive and Gram negative, which makes it an essential tool for classification and differentiation of microorganisms.

- The Gram stain reaction is based on the difference in the chemical composition of bacterial cell walls.

- Gram positive cells have a thick peptidoglycan layer, whereas the peptidoglycan layer in Gram negative cells is much thinner and surrounded by an outer lipopolysaccharide containing layers.

Difference between Gram positive & Gram negative cell wall

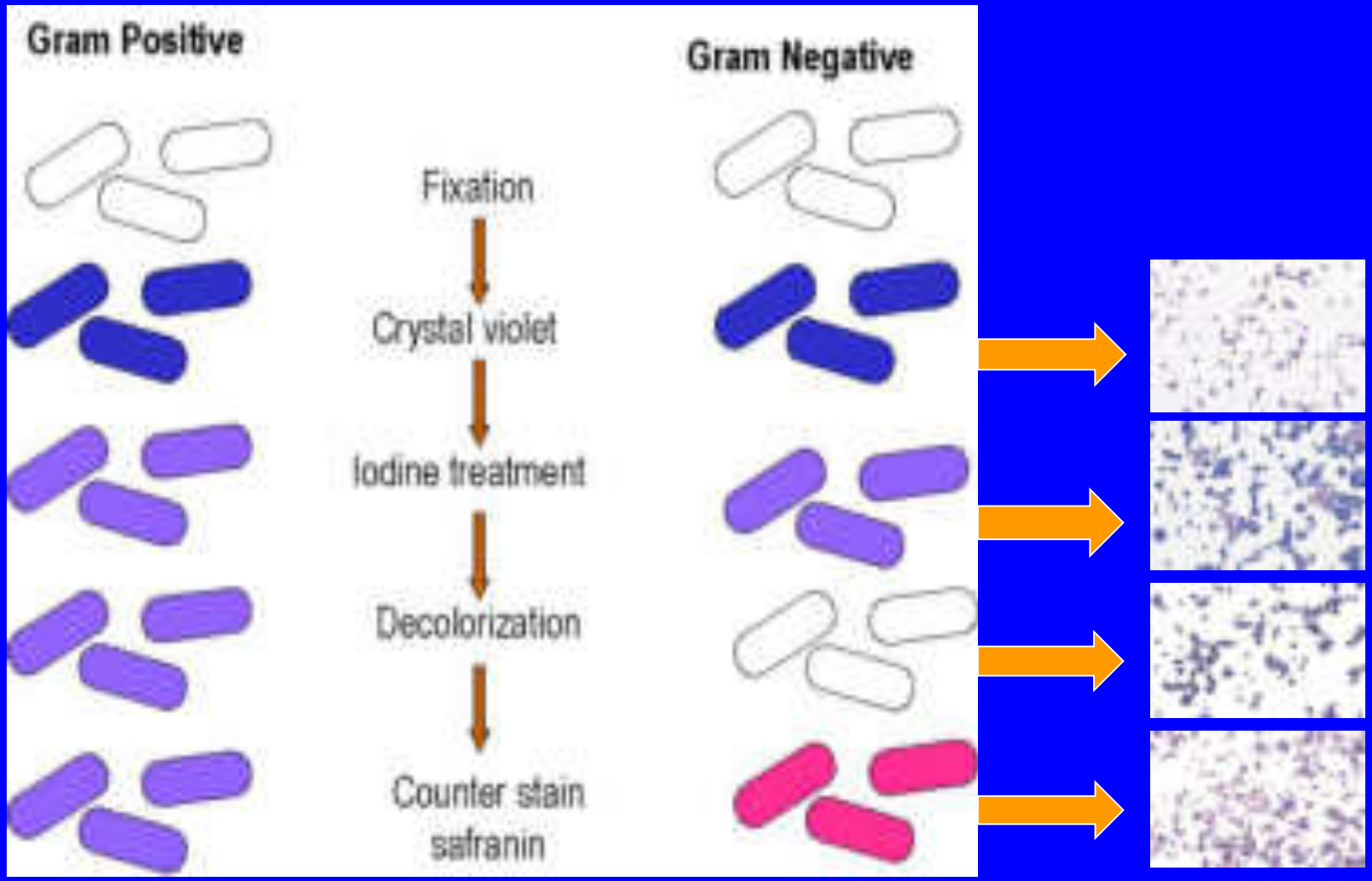




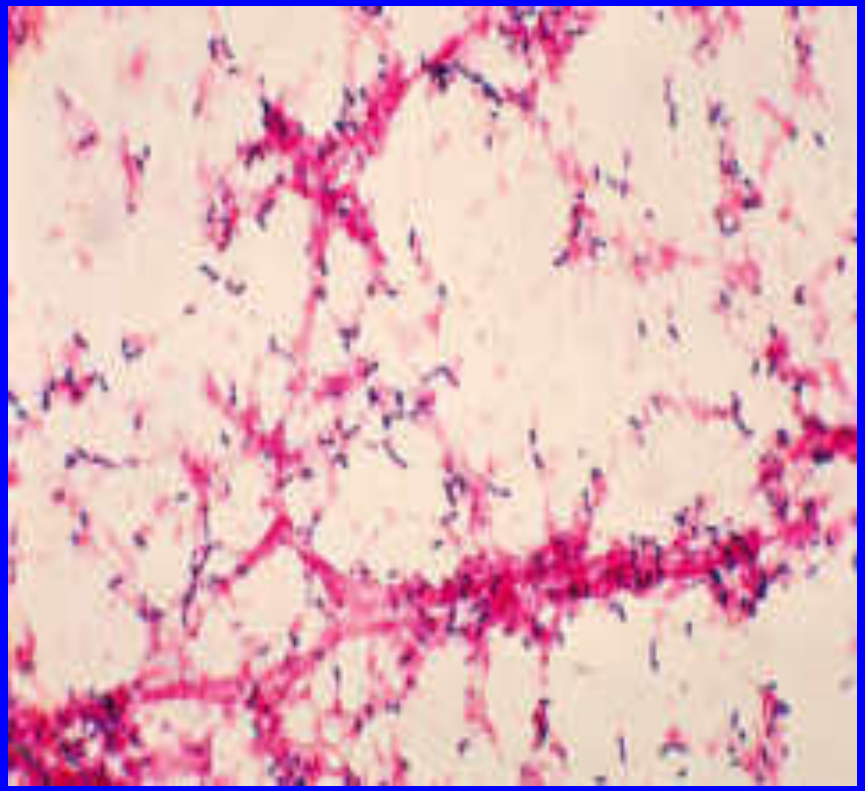
Functions of Gram staining reagents

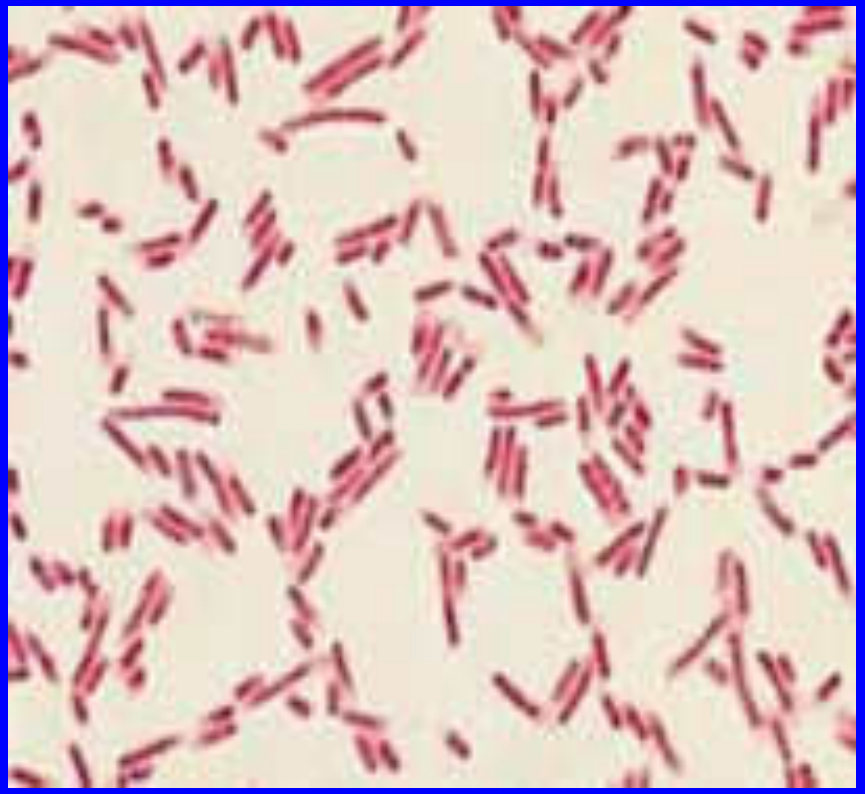
- **Primary stain: Crystal violet**, which stains all cells purple.
- **Mordant**: **Gram's iodine**, which increases the affinity of cells for crystal violet by binding to it, thus forming an insoluble crystal violet – iodine complex (CV-I), that serves to intensify the color of the stain, thereby making all cells purple – black.
- **Decolorizing agent**: 95% ethyl alcohol, which serves as a protein dehydrating agent and as a lipid solvent. In Gram negative cells, alcohol increases the porosity of the cell wall by dissolving the lipids in the outer membrane. Thus, the CV- I complex can be more easily removed from the thinner and less highly cross-linked peptidoglycan layer. The washing-out effect of alcohol releases the unbound CV-I complex, leaving the cells colorless. The much thicker peptidoglycan layer in Gram positive cells will retain the CV-I complex, as the pores are made smaller due to the dehydrating effect of the alcohol. Thus the tightly bound primary stain is difficult to remove and the cells remain purple.
- **Counterstain**: Safranin, which used to stain Gram negative cells red, as these cells are previously decolorized and can absorb the counter stain.

Procedure of Gram staining



STAINED GRAM POSITIVE COCCI & GRAM NEGATIVE RODS





Gram variable organisms:

- Over-decolorization will result in loss of the primary stain, causing Gram positive organisms to appear Gram negative.

- Under-decolorization will not completely remove the CV-I complex, causing Gram negative organisms to appear Gram positive.

- As cultures age, especially in the case of Gram positive cells, the organisms tend to lose their ability to retain the primary stain and appear Gram negative.

- If Gram positive cells are treated with the enzyme lysozyme or the antibiotic penicillin, then cell wall will be partially removed and the cells will stain Gram negative.

  These cells, which show wrong Gram staining properties, are called Gram variable organisms.

Endospore staining (Schaeffer-Fulton method)

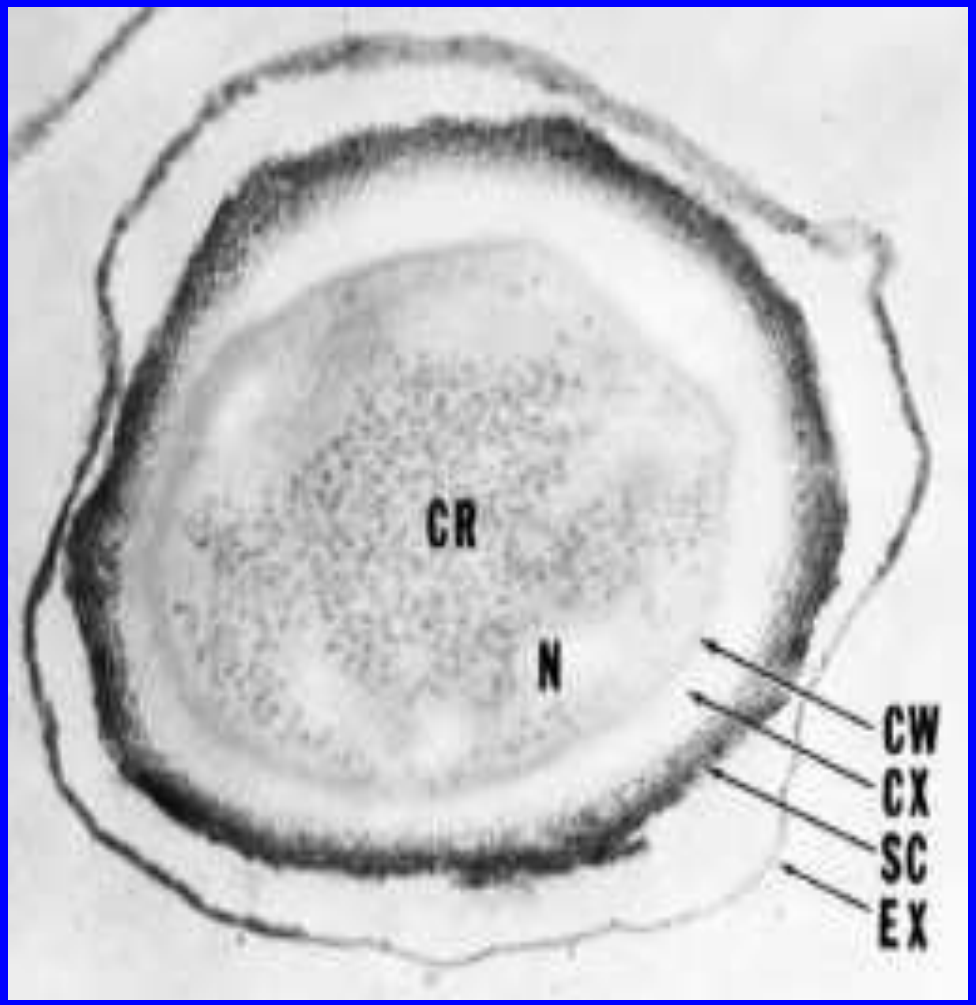
- Anaerobic genus of Clostridium and aerobic genus of Bacillus can exist either as metabolically active vegetative cells or as highly resistant and metabolically inactive endospores.

- When environmental conditions become unfavorable for survival of vegetative cells, these cells produce a new intracellular structure, called endospore, which is surrounded by impervious layers called spore coats.

- Due to the presence of tough layers, the spore is resistant to commonly employed microbiological stains and can only be stained with malachite green.

Diversity of bacterial internal structure-the endospore

Bacillus anthracis endospore (151,000).
Note the following structures:



1. Exosporium, EX;

2. Spore coat, SC;

3. Cortex, CX;

4. Core wall, CW;

5. Protoplast or core with its nucleoid, N, and

6. Ribosomes, CR.

The spore staining reagents

Primary stain:

Malachite Green: being a strong stain, malachite green is able penetrate the impervious spore coat by application of heat. After applying this stain, both the vegetative cell and endospore will appear green.

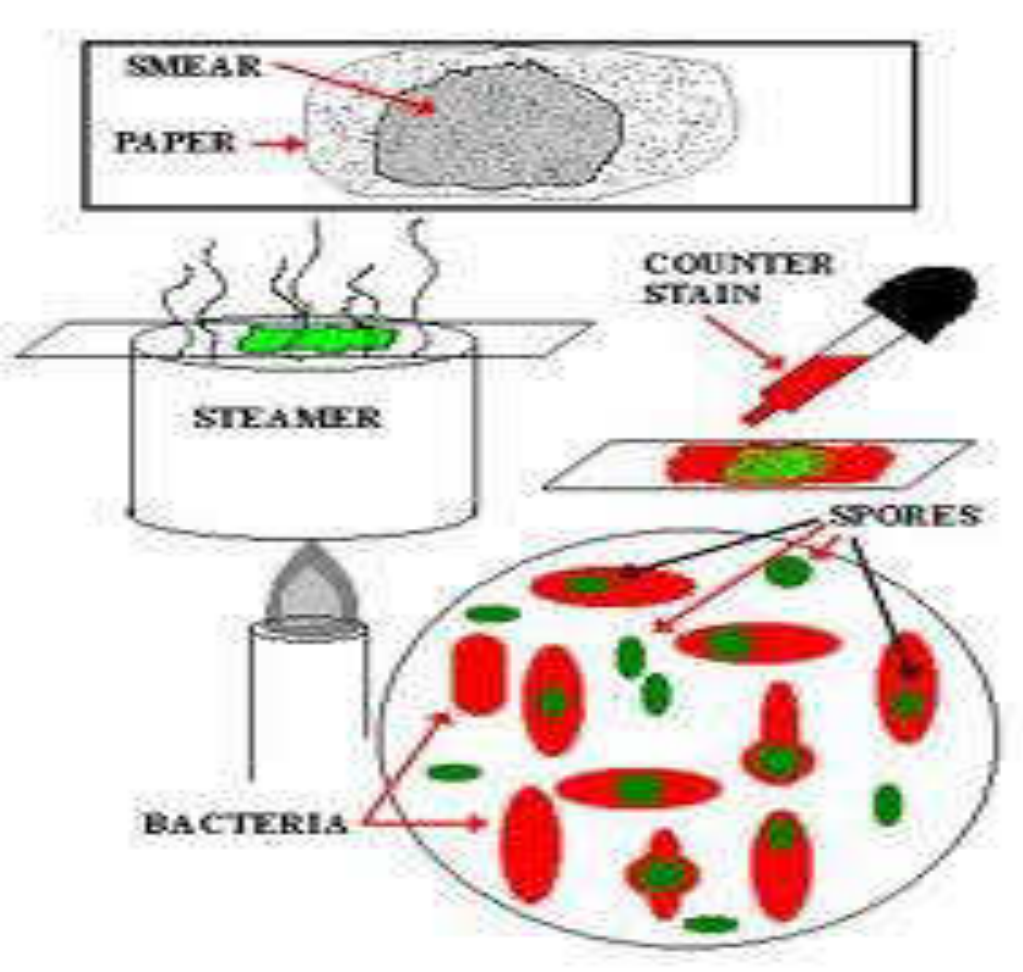
Decolorizing agent:

Water: malachite green does not have a strong affinity for vegetative cells, but once the spore accepts the primary stain, it cannot be decolorized. Only the excess primary stain is removed. The spore remains green and the vegetative cell become colorless.

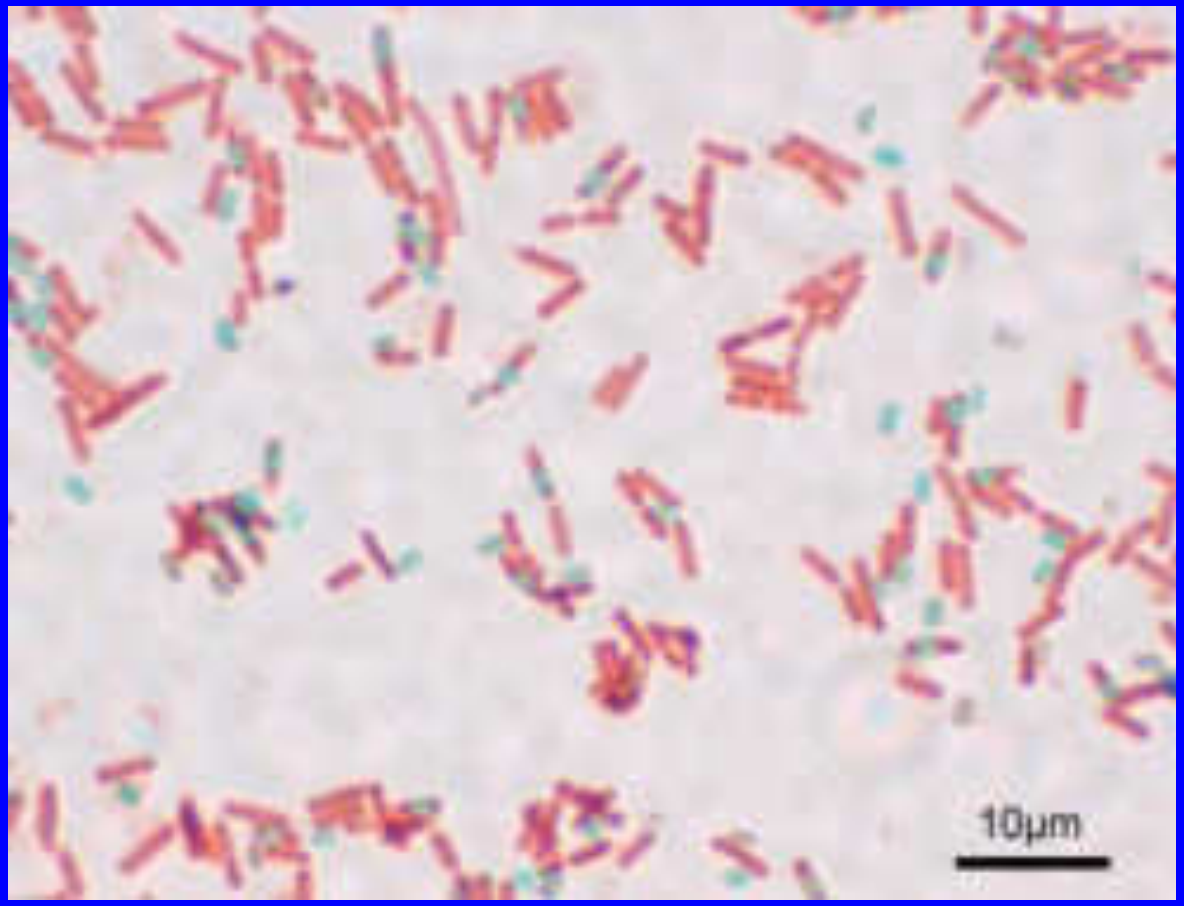
Counter stain:

Safranin: decolorized vegetative cells absorb the counter stain and appear red, while the spore within it is green.

PROCEDURE OF ENDOSPORE STAINING



ENDOSPORE OF *Bacillus subtilis*



Acid-fast staining(Ziehl-Neelsen method)

- The members of the genus *Mycobacterium* are not stainable by Gram staining as these cells posses a thick waxy lipoidal cell wall of mycolic acid that make penetration of stains extremely difficult.

- But, once the stain has penetrated, it cannot be removed even with the use of acid-alcohol as a decolorizing agent. So, these organisms are called acid-fast organisms.

- All other microorganisms, which are easily decolorized by acid-alcohol, are called non acid-fast organisms.

The acid-fast staining reagents

Primary stain:

Carbol Fuchsin: this red phenolic stain is soluble in the lipoidal cell wall of mycobacteria, does penetrate these cells and is retained. Penetration is enhanced by heating.

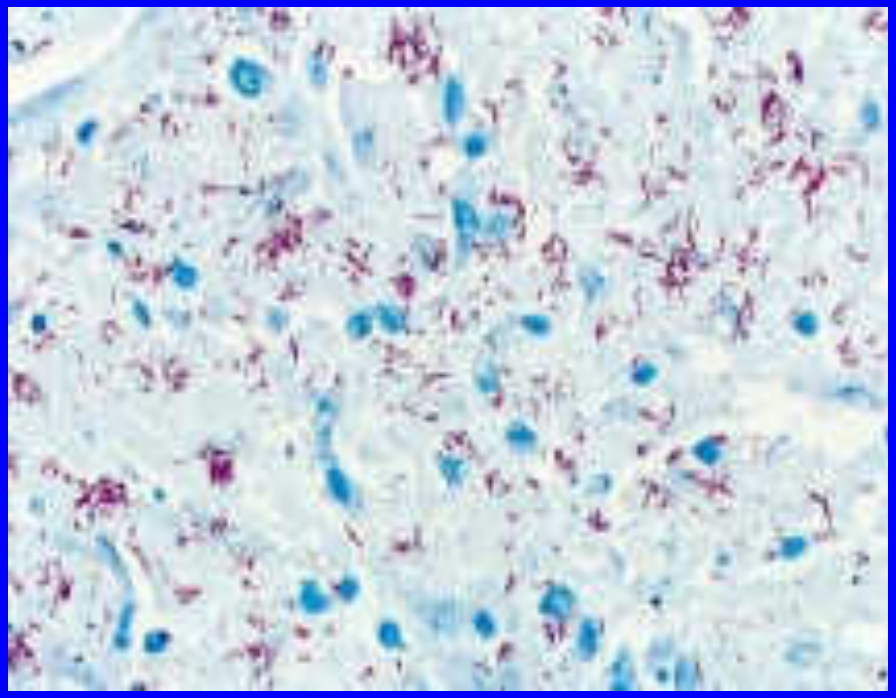
Decolorizing agent:

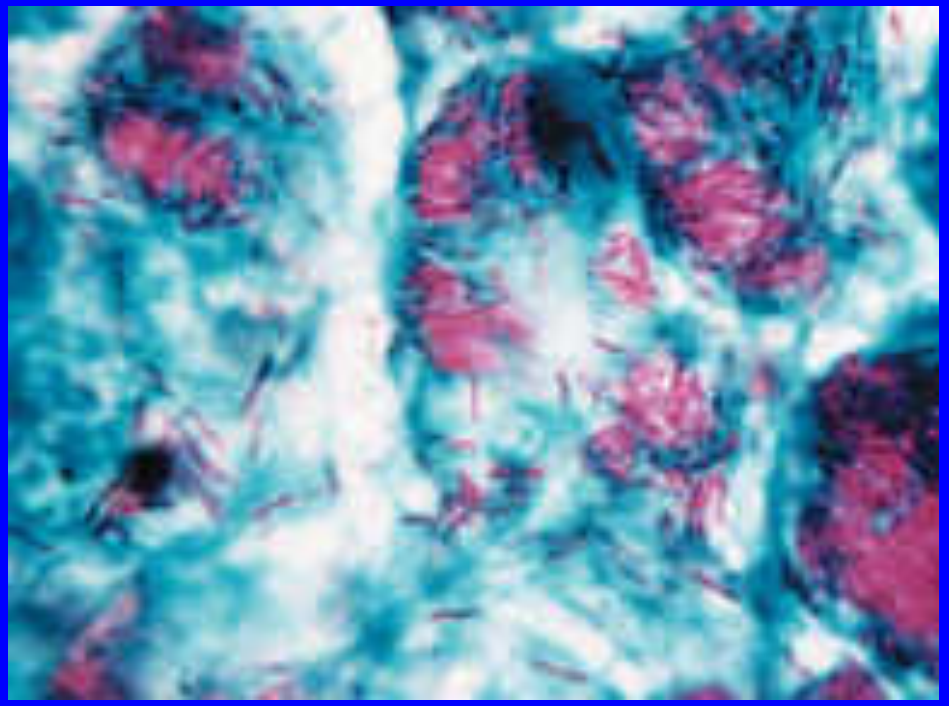
Acid-alcohol (3% HCl + 95% Ethanol): When the cooled smear is treated with acid- alcohol, acid-fast cells will retain the color of the primary stain as the primary stain is more soluble in the mycobacterial cell wall than in the decolorizing agent. Acid-alcohol will easily remove the primary stain from non acid-fast cells as they lack mycolic acid.

Counter stain:

Methylene blue: The decolorized non acid-fast cells now absorb the counter stain and appear blue.

Stained Acid fast organism- *Mycobacterium*





The Ziehl-Neelsen acid-fast stain. This LM stain produces vivid red color in acid-fast organisms such as *Mycobacterium leprae* (magnified 3844X), the cause of leprosy.

CAPSULE STAINING

- Capsule is a gelatinous outer layer secreted by the cell.
- It surrounds and adheres to the cell wall.
- Chemically, capsule is composed of polysaccharide or glycoprotein or polypeptide or a mixture of all these substances.
- Cells that contain a heavy capsule are generally virulent and capable of producing disease, since the structure protects bacteria against the normal phagocytic activities of host cells.

Capsule staining – the principle

- Capsule staining is more difficult than other types of differential staining methods because capsular materials are water soluble and may be dislodged and removed with vigorous washing.

- Smears should not be heated, because the resultant cell shrinkage may destroy the capsule.

Capsule staining – the reagents

- **Primary stain:**

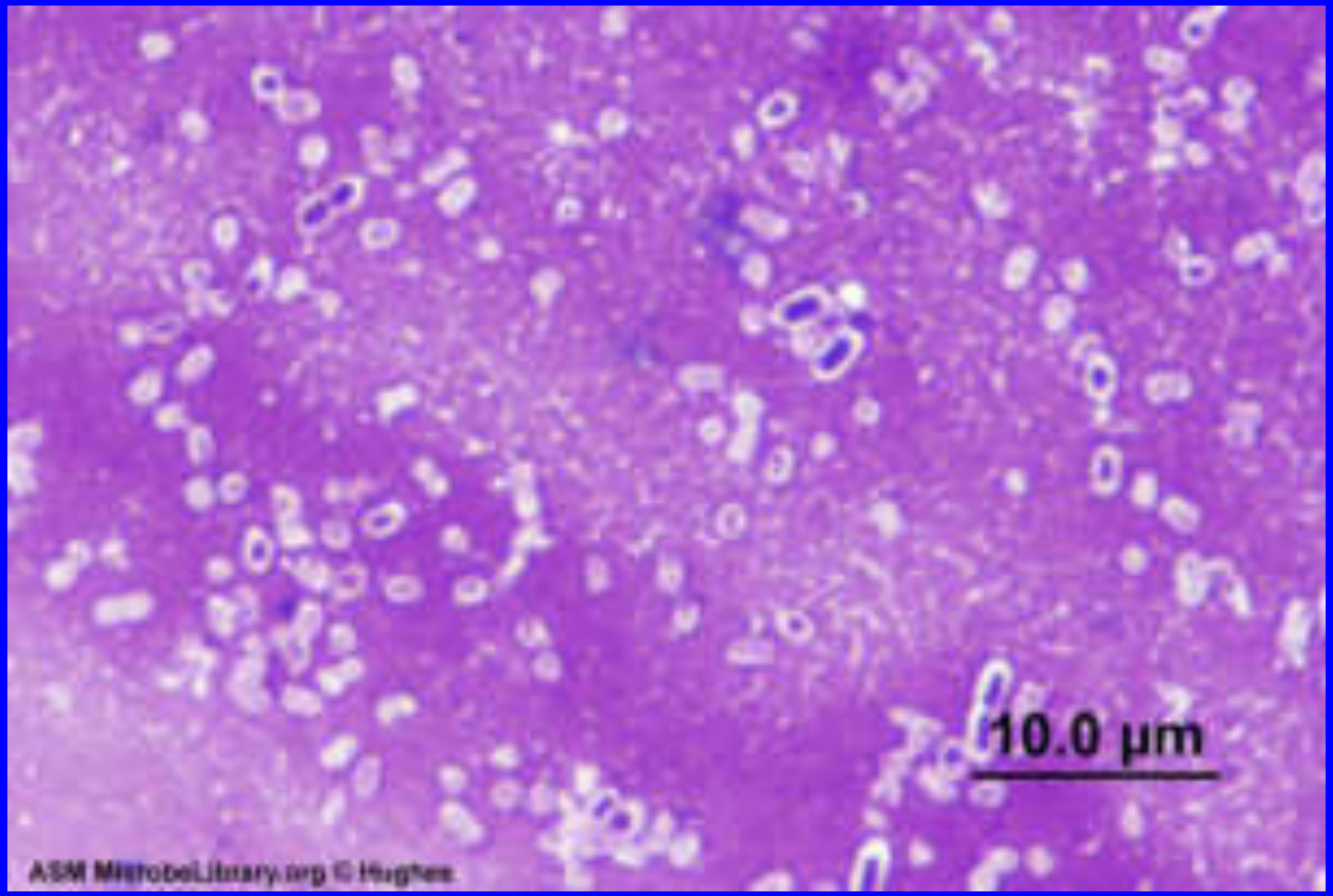
Crystal violet (1% aqueous solution): it adheres to the capsule without binding to it (as capsule is non ionic) , when this is applied over a non heat-fixed smear.

- **Decolorizing agent & counter stain :**

Copper sulfate (20%) : capsule is water soluble. So, copper sulfate, rather than water is used to wash the purple primary stain out of the capsular material without removing the stain bound to the cell wall.

Copper sulfate acts as a counter stain also. It is absorbed into the decolorized capsular material. The capsule will appear light blue in contrast to the deep purple color of the cell.

Stained capsule containing cells of *Klebsiella pneumoniae*

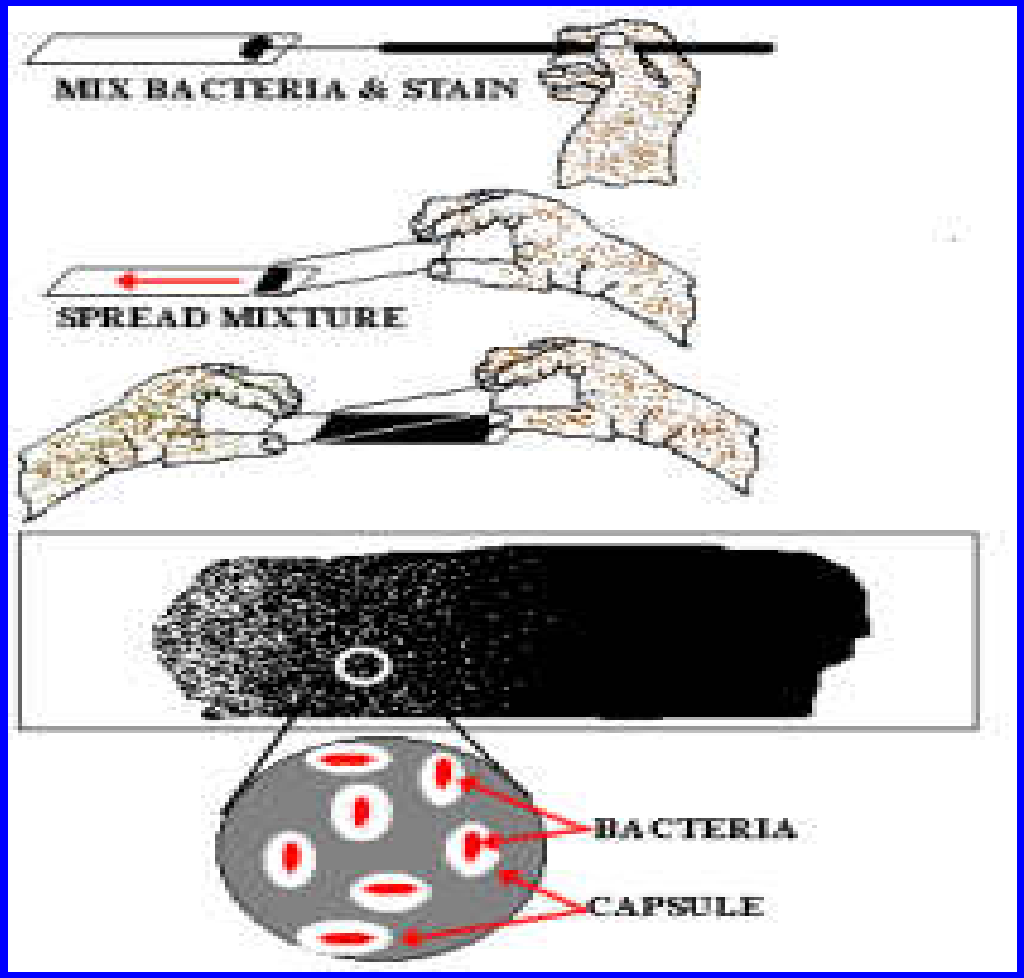


Negative staining

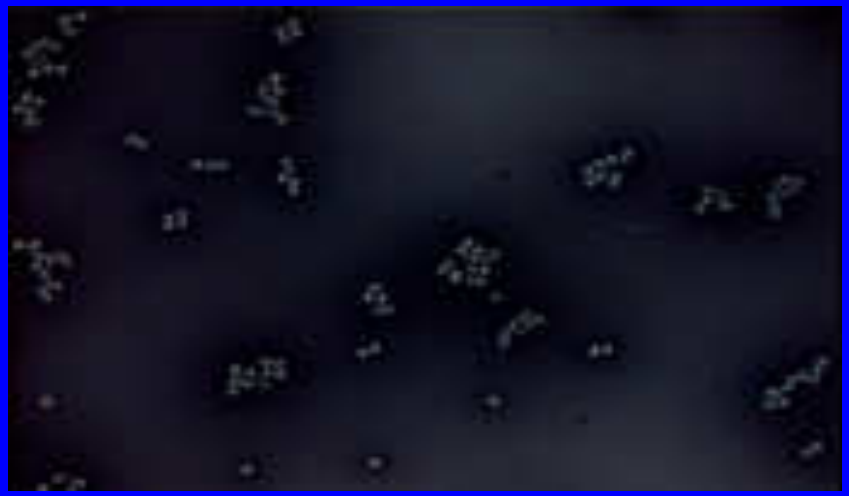
This technique works in a manner opposite to simple technique. Bacteria are mixed on a slide with an acidic dye such as eosin or congo red or the black stain, nigrosin. The mixture is smeared across the face of the slide and allowed to air dry.

Because the stain carries a negative charge, it is repelled by the bacteria, which also have a negative charge. The stain gathers around the cell. since a chemical reaction has not taken place, and because heat fixing has been avoided, the cells appear less shriveled or distorted. They often appear larger than stained cells and more natural.

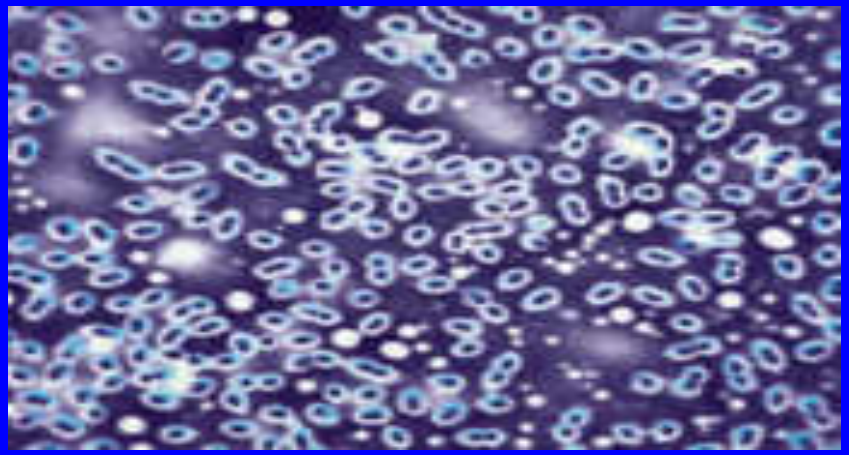
Negative staining technique



Negatively stained rod and cocci cells



Negative staining for capsules reveals a clear area (the capsule, which does not accept stain) in a dark pink background of India ink and crystal violet counterstain. The cells themselves are stained deep purple with the counterstain. The bacteria are *Streptococcus pneumoniae* (3399X), which are arranged in pairs.



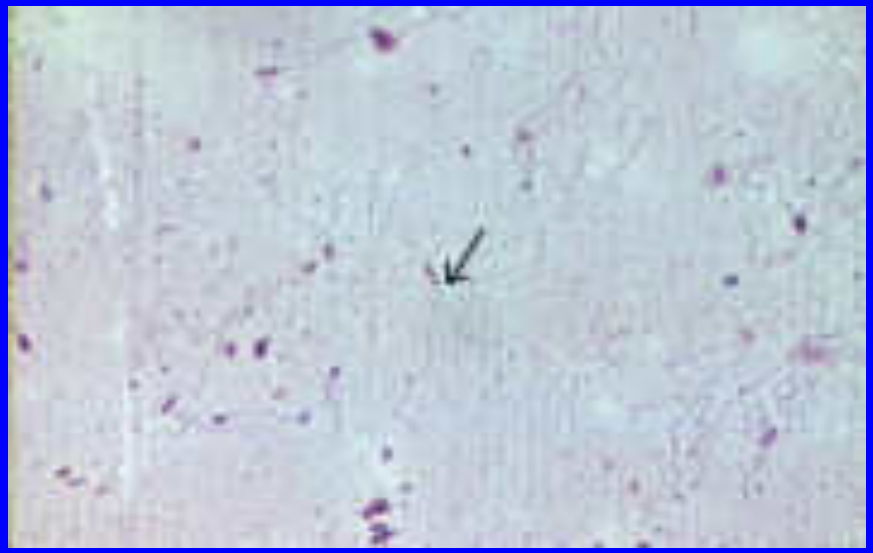
Flagella staining

- Flagella are too slender to be observed under light microscope. It can only be seen under electron microscope.

- For observing flagella under light microscope, it is made thicker by treating the non heat fixed bacterial sample with tannic acid as a mordant at first and then nigrosin or methylene blue is added.

- It is a simple staining method.

Stained flagella preparation



Example of Flagella Staining. *Spirillum volutans* with bipolar tufts of flagella (400).