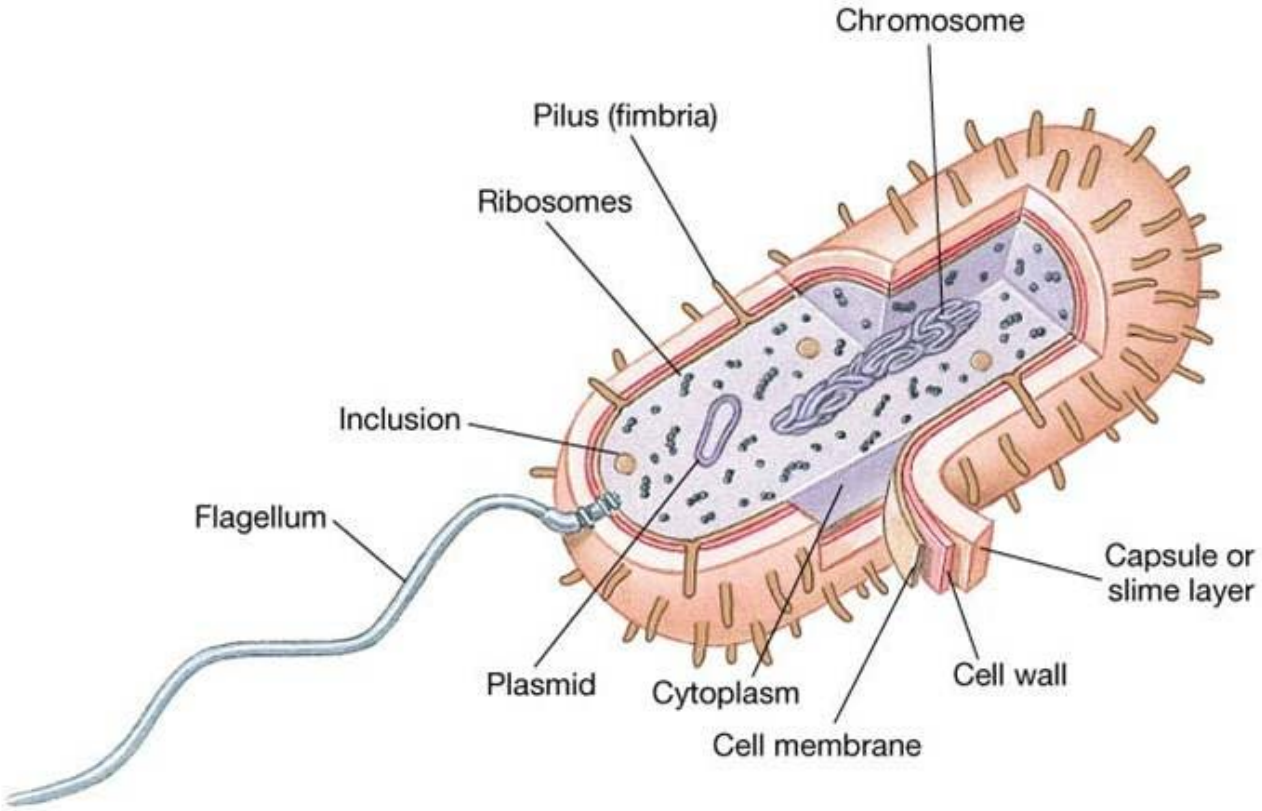
STAINS & STAINING

A DISCUSSION ABOUT
OBSERVING BACTERIA

PROKARYOTIC CELL STRUCTURE



A COMPARISON OF PROKARYOTES & EUKARYOTES

CHARACTERISTIC	PROKARYOTES	EUKARYOTES
Nucleus	Absent	Present
DNA structure	Single, circular chromosome	Multiple linear chromosome
Membranes	Cell membrane only	Cell & organelle membranes
Organelles	Absent	Present in a variety of forms
Ribosome	(70S), free in cytoplasm	(80S), free/ bound to ER surface
Cytoskeleton	Absent	Present
Cell walls	Generally present	Present in fungi, algae & plants
Flagella	Rotating movement	Whipping movement
Cilia	Absent	Present

Why is staining needed ?

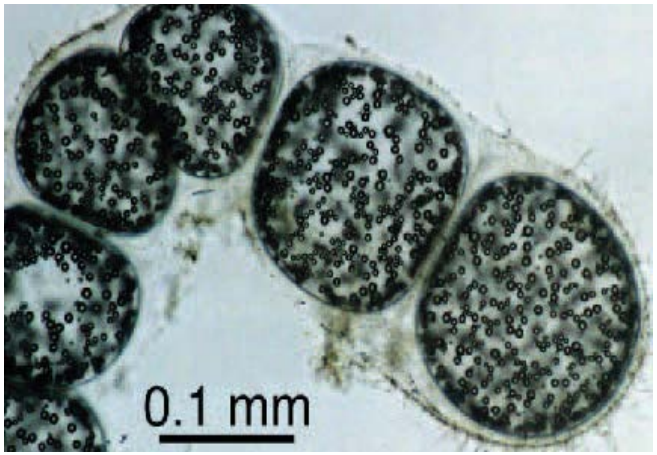
- Bacteria are minute in size (~ 0.5 micro meter in length).
- Bacteria are transparent and colorless when suspended in an aqueous medium.
- To reveal diverse types of bacterial shapes and sizes.
- To show the presence of various internal and external structures.
- To study morphological properties and to divide microorganisms into specific groups for diagnostic purposes.

Some facts about bacterial sizes

- Viruses ~ 0.01 micro meter (poliovirus) – 0. 25 micro meter (small pox virus)
- Bacteria ~ 0.25 micro meter (chlamydiae & mycoplasmas) – 5 micro meter

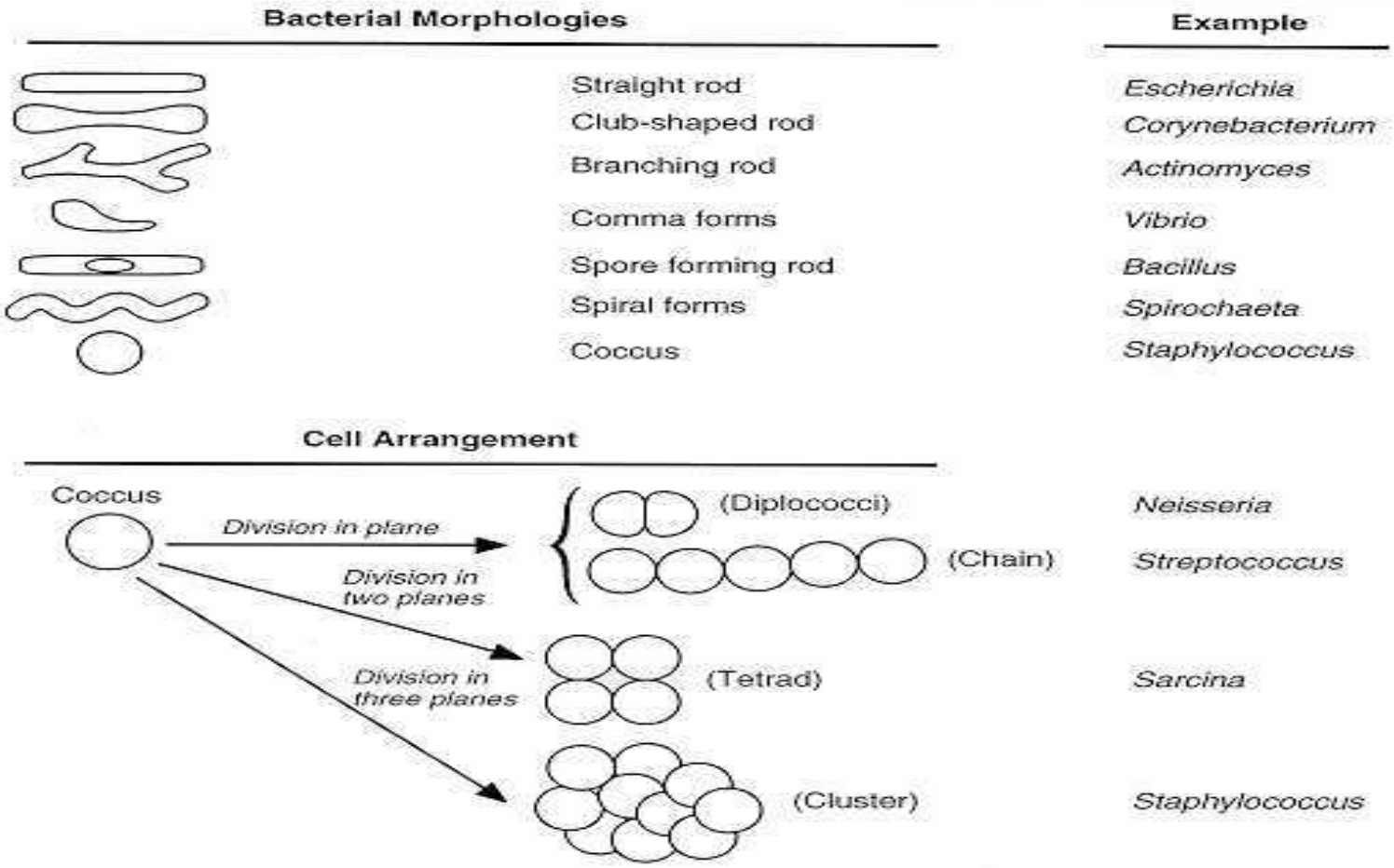
THE LARGEST BACTERIUM – *Thiomargarita namibiensis* (0.1 – 0.3 mm)

A chain of *Thiomargarita namibiensis* cells (as viewed with the light microscope)

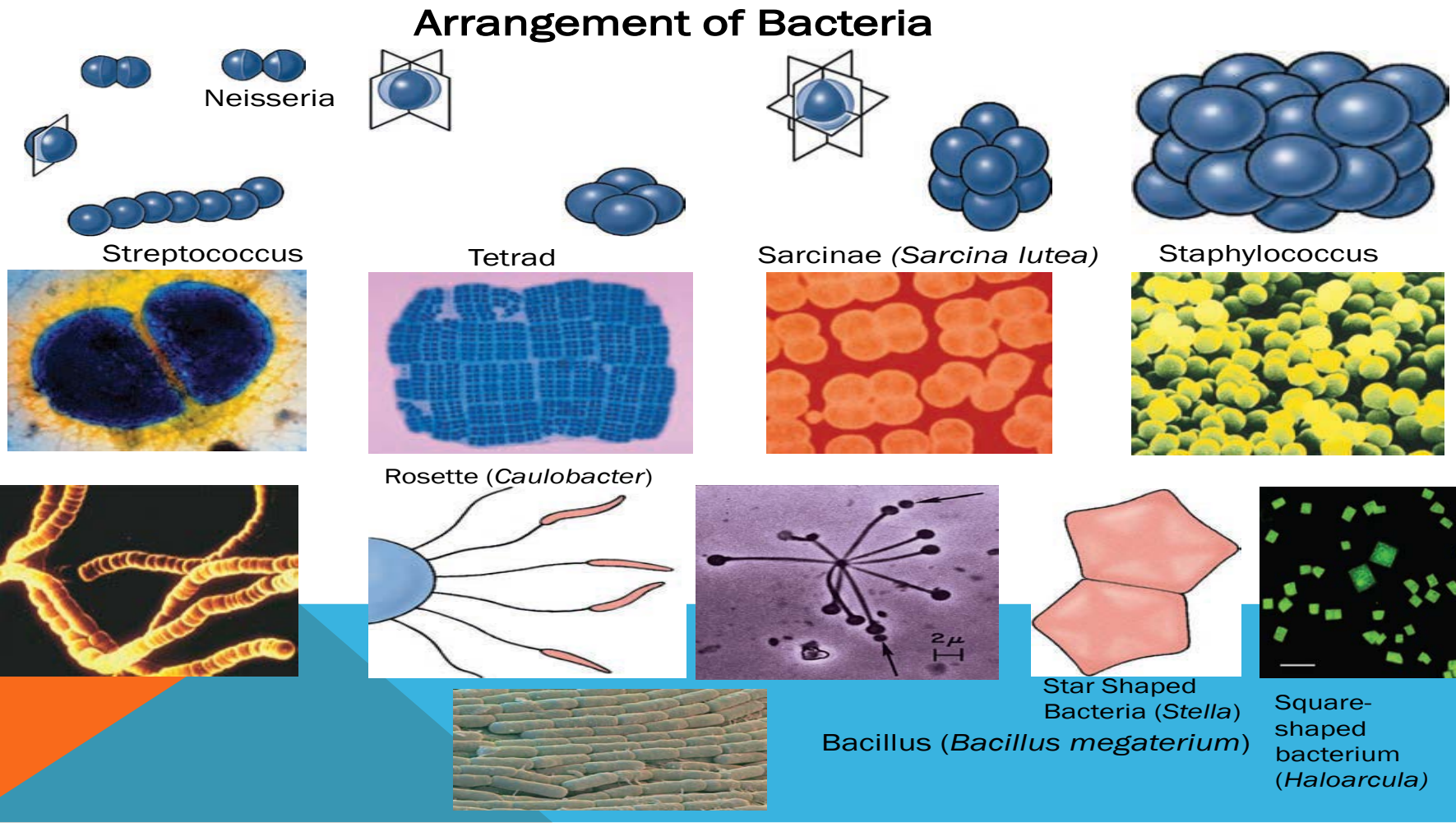


- Yeasts ~ 8 micro meter
- Molds ~ 40 micro meter
- Protozoa ~ 100 micro meter
- Diameter of a carbon atom ~ 0.1 nm (0.0001 micro meter)
- Size of structures observable by unaided eye > 500 micro meter (0.5 mm).
- Observation range of electron microscope ~ 2 nm – 100 micro meter
- Observation range of light microscope ~ 200 nm – 0.5 mm

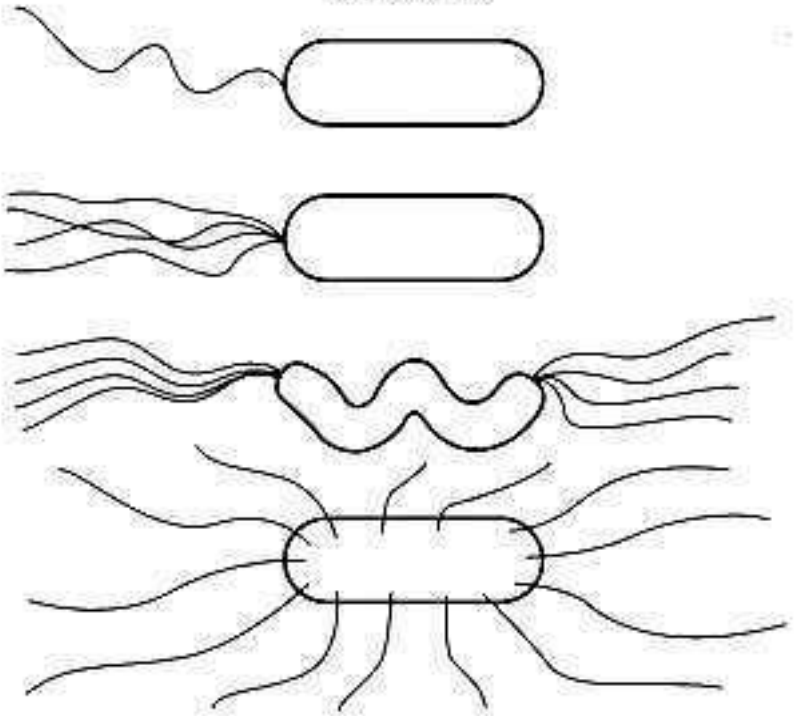
Diversity of bacterial shapes



Diversity of Bacterial Shapes



Diversity of bacterial external structures



Structure	Flagella Type	Example
	Monotrichous	*Vibrio cholerae*
	Lophotrichous	*Bartonella bacilliformis*
	Amphitrichous	*Spirillum serpens*
	Peritrichous	*Escherichia coli*

Dyes & Stains

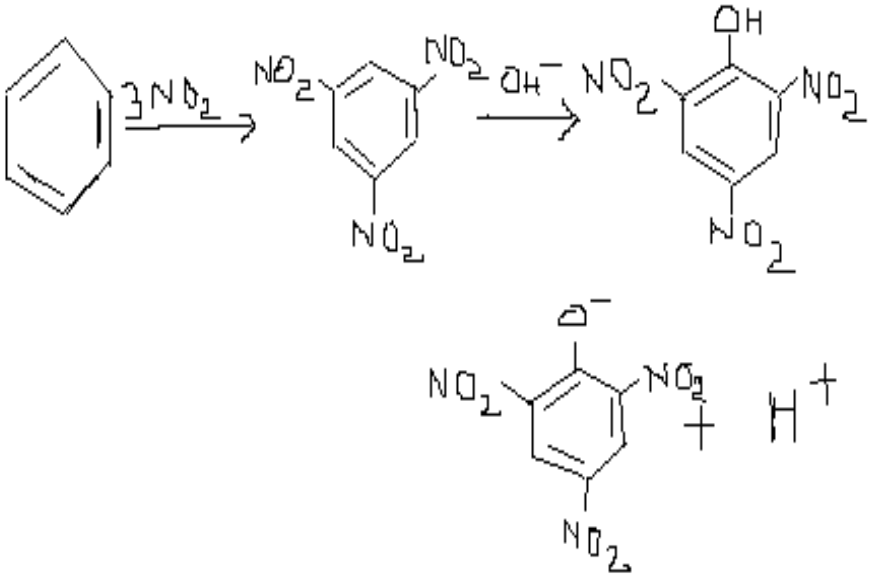
- On the basis of usage, a coloring agent that is used for general purposes is called dye and a coloring agent that is used for biological purposes is called a stain.
  For ex.- aniline compounds are dyes while crystal violet is a stain.
- On the basis of chemical composition, stains are organic compounds 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

STAINS

- However, electron dense substances used as negative stains in electron microscopy, like osmium tetroxide, uranyl acetate, phosphotungstic acid etc. are not dyes but are used as stains.

Chemical nature of stain- an example



- Benzene is colorless.
- Addition of the chromophore – nitro group yields pale yellow colored trinitrobenzene, which is a chromogen , but not a stain.
- Trinitrohydroxybenzene (picric acid) is a bright yellow colored stain, as addition of the auxochrome – OH group renders the property of ionozation to this compound.
- 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?

- Acidic stains are used mainly to stain cytoplasm whereas the basic stains color acidic cellular components like nucei and cell wall or plasma membrane.

- 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.

- Bacterial cell surface is negatively charged due to –

1. presence of outer membrane lipopolysaccharides outside of the cell wall in Gram negative bacteria which are often composed of negatively charged phospholipids.
2. 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.

Sample fixation methods

- The purpose of sample fixation is to immobilize the sample cells without considerable structural distortions in cell shape. So that the original cell structure can be properly seen under microscope after staining.
- Sample fixation methods are of two types- heat fixation and chemical fixation.
- Heat fixation is a easier and crude method of fixation which often causes structural distortions in cells due to overheating.
- Chemical fixation is a much more satisfactory methods as chemical fixatives usually immobilize target cellular structures without causing significant structural distortions.

The two most commonly used chemical fixatives are-

1. Glutaraldehyde ($HOC\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{—}CHO$), which cross-links proteins by forming covalent bonds using its two reactive aldehyde groups.
2. Osmium tetroxide (OsO_4), which binds to and stabilizes lipid bilayers of cell membrane and proteins by reacting with the C=C bonds present in these cellular components.

WHY DO WE SEE COLOR?

- Visible light consists of electromagnetic wavelengths of 400- 750 nm.
- Each wavelength is associated with a definite energy and produces a particular color sensation as it strikes the retina.
- When a beam of visible light strikes a colored substance, certain wavelengths are absorbed and others are reflected. Those reflected wavelengths compose the color of the substance.
- Relationship of color absorbed and complementary color observed-

Wavelength absorbed (nm)	Color absorbed	Complementary color
400- 435	Violet	Yellow- green
435- 480	Blue	Yellow
480- 490	Blue- green	Orange
490- 500	Green- blue	Red
500- 560	Green	Purple
560- 580	Yellow- green	Violet
580- 595	Yellow	Blue
595- 605	Orange	Green- blue
605- 750	Red	Blue- green

Modern theory of color

- When a molecule absorbs light, its bonding electrons are excited by absorbing energy and are promoted to orbitals of higher energy.
- The energy absorbed by such a transition is equal to the difference of the energy of molecular orbitals in the ground state and the energy of molecular orbital to which the electron is promoted.
- The relative energies of the molecular orbitals in the ground state are-

  π delocalised > π > σ
- To promote an electron to the same higher energy orbital, minimum energy will be required for delocalized electrons.
- For example, methane having σ bonds only, on excitation, absorbs large energy in the far UV region. Hence it is colorless.
- In a compound having a highly conjugated system, the p orbitals overlap, causing extensive delocalization of the π electrons, which being in a higher energy orbital, require less energy for excitation that fall in the range of the visible spectrum. The compound, therefore, shows color.

Colored properties of benzene derivatives- a case study

- Benzene has a ring structure made of three conjugated double bonds where 6 delocalised π electrons are involved. It absorbs in the UV region and is colorless.

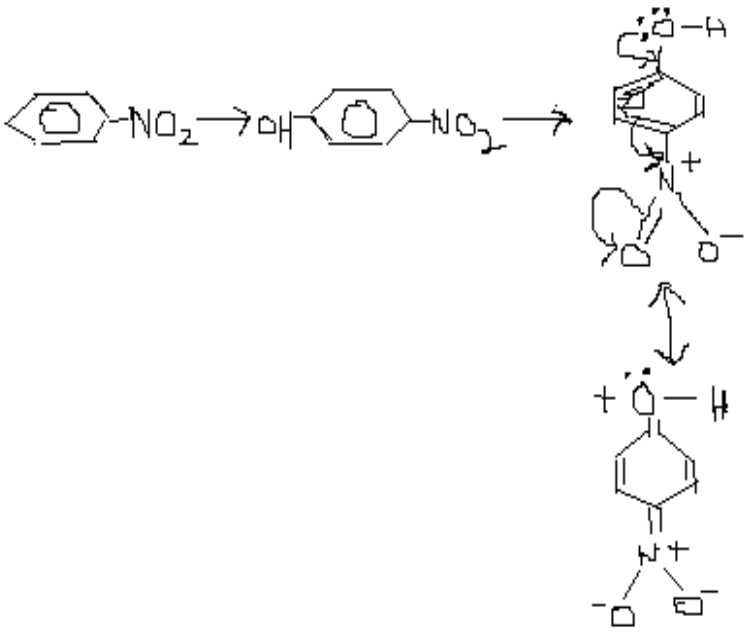
- When it is conjugated with chromophoric groups which contain double bonds, electron delocalization increases and the compound is colored.

- Electron delocalization is further enhanced by groups which can donate unshared electron pair for greater extension of the conjugated system. As a result, the absorption is shifted to a longer wavelength, making the color more intense.

Chemical effect of an auxochrome

- The auxochrome –OH gr. deepens the color of p-nitophenol by extending the conjugated system between the chromophore and the auxochrome due to resonance.

- So, nitrobenzene is a pale yellow substance, but when the auxochrome –OH gr. is present in ortho or para position, the product become deep yellow.



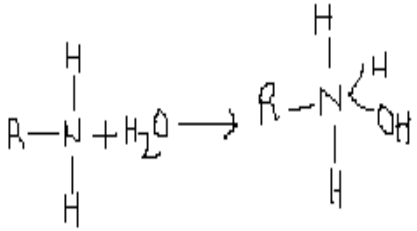
Bathochromic and hypsochromic shift

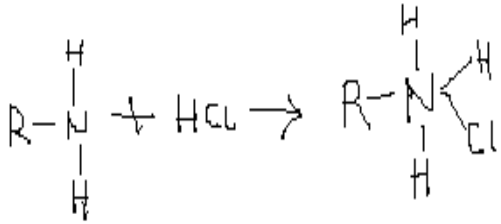
- Chromophore is now defined as any isolated functional group which shows absorption in UV or visible region.

- Any chemical groups that cause deepening of the color, i.e. absorption shifted toward red wavelength are known as bathochromic groups and the effect as bathochromic shift.

- On the other hand, the chemical groups that cause the opposite effect, i.e. absorption shifted toward violet wavelength, are termed hypsochromic groups and the effect is referred to as hypsochromic shift.

Acidic and basic auxochromes





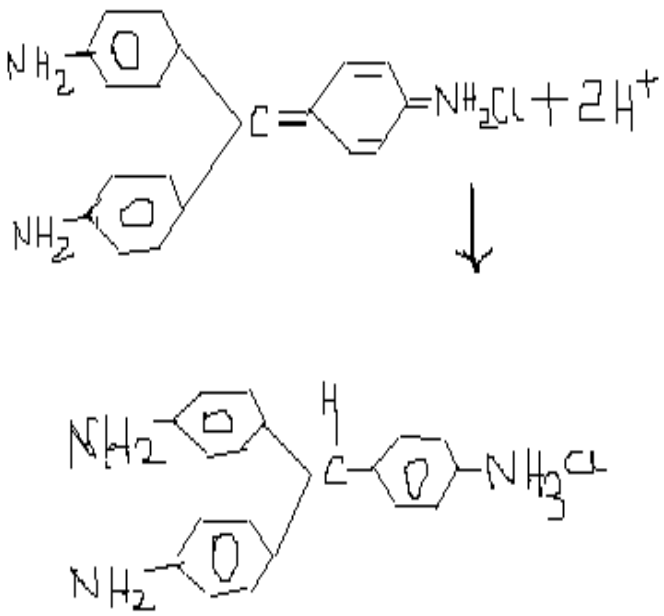
- -OH^-, sulfonic gr. (SO_2OH^-) are acidic auxochrome while amino gr. is a basic auxochrome.
- Amino gr. has the ability of its N atom to become pentavalent on addition of water or acid.
- On the other hand, OH^- gr. has the ability to yield H^+ ions by dissociation.
- The more of either of these two groups are present in a compound, the more strongly acidic or basic it becomes.
- The amino gr. is more strongly basic than the OH^- gr. is acidic. If one of each of these two radicals is present, the basic nature of the amino gr. predomonates.

Acidic and basic chromophores

- Acidic - Nitro gr., the quinonoid gr. etc. found in crystal violet, methyl green.

- Basic – Azo gr. (-N=N-) in methyl red, Indamine gr. (-N=) in methylene blue.

Leuco compounds



- Chromophores are easily reduced by combining with H^+ ion at the double bonds which results in a loss of color. These decolorized stains are known as leuco compounds.
- For example, the decolorization of pararosaniline by reduction yields leucopararosaniline.

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 counterstain, 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.

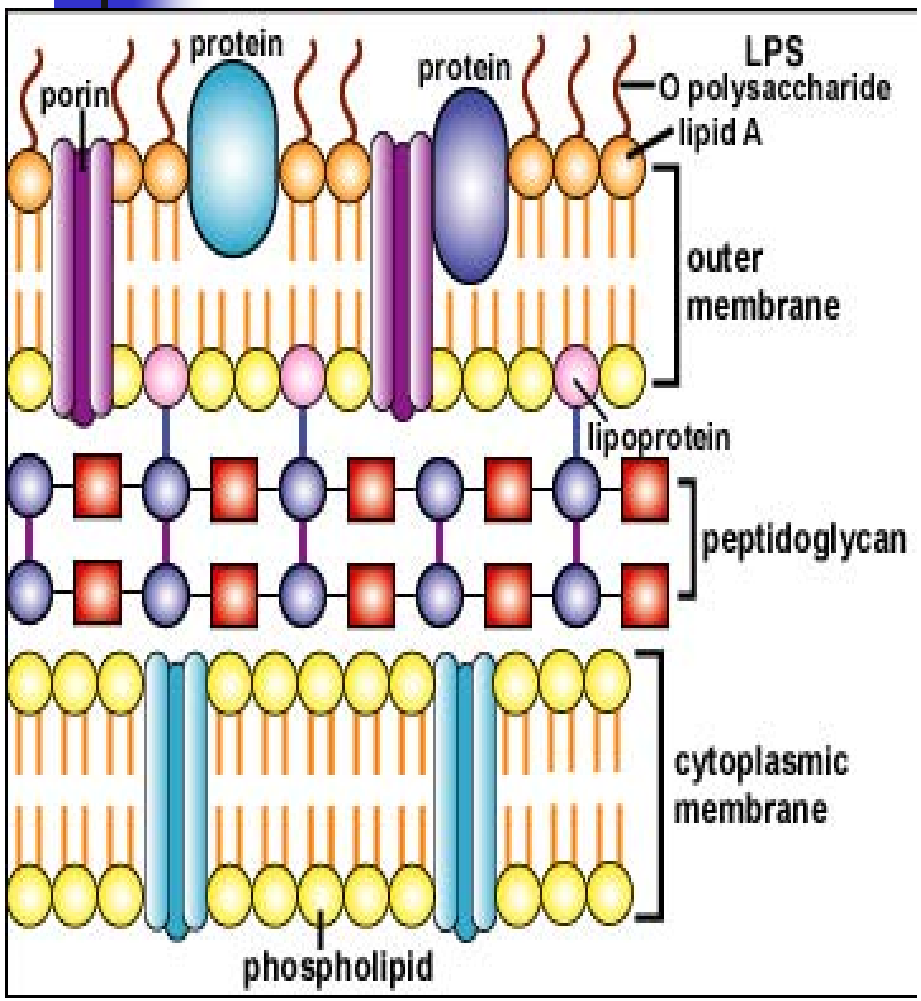
Function of decolorizing agent

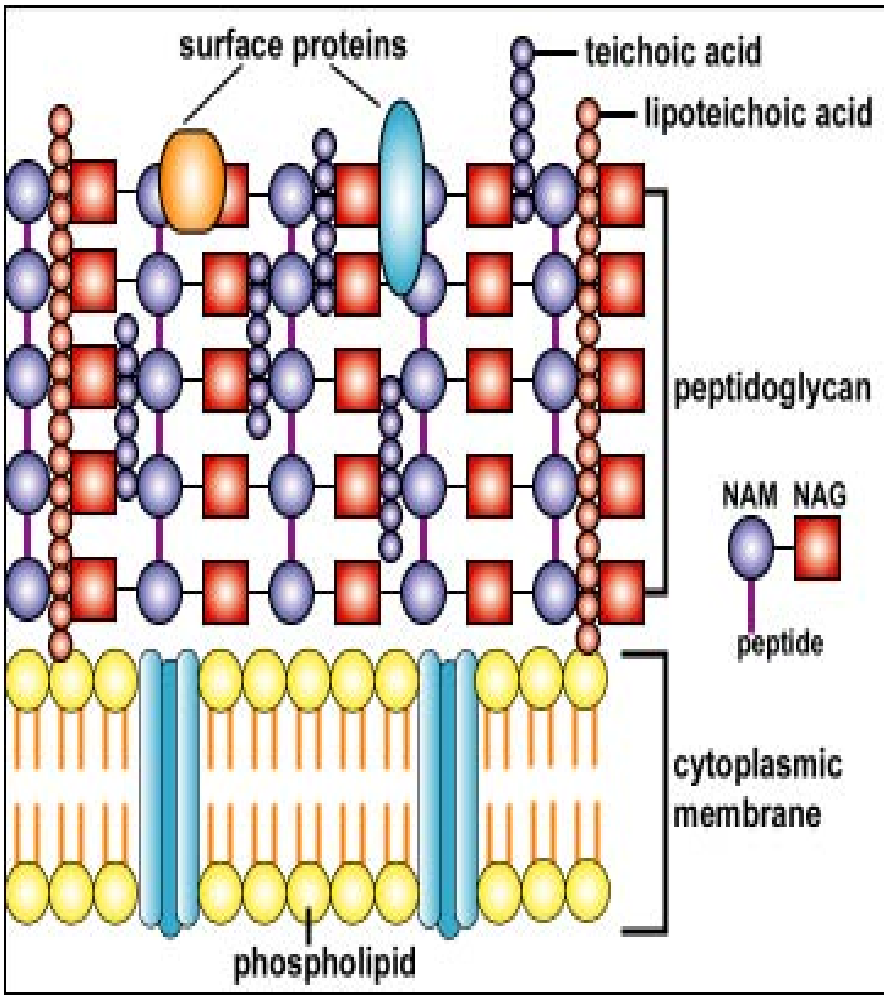
- In order to establish a color contrast, decolorizing agent is used.
- Based on the chemical composition of cellular components, the decolorizing agent may or may not remove the primary stain from entire cell or only from certain cell srtuctures.
- Following decolorization, if the primary stain is not 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 counterstain.

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.

Contd.

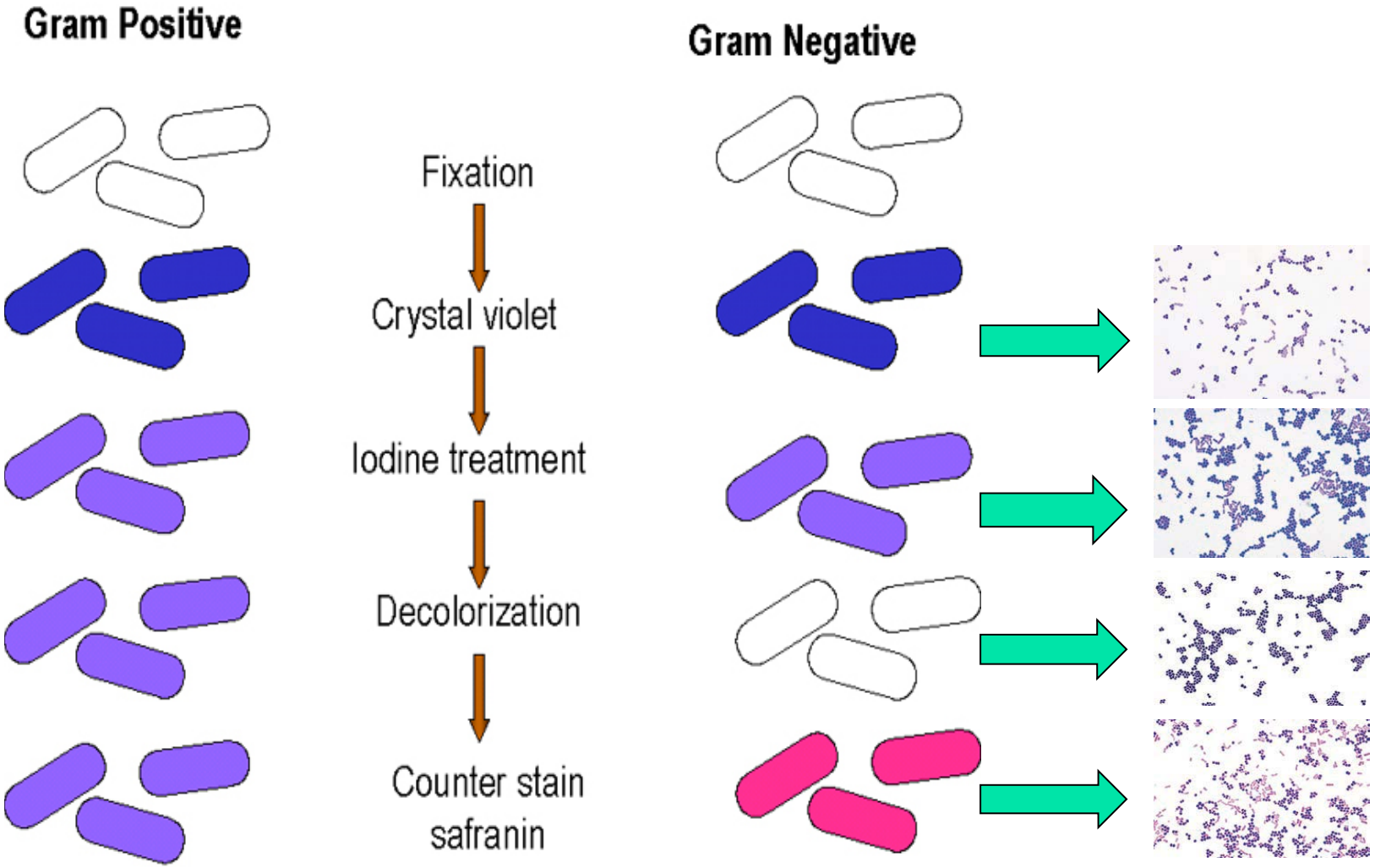
- **Decolorizing agent**: 95% ethyl alcohol, which serves as a protein dehydrating agent and as a lipid solvent.

  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 cross-linked peptidoglycan layer. The washing-out effect of alcohol releases the unbound CV-I complex, leaving the cells colorless.

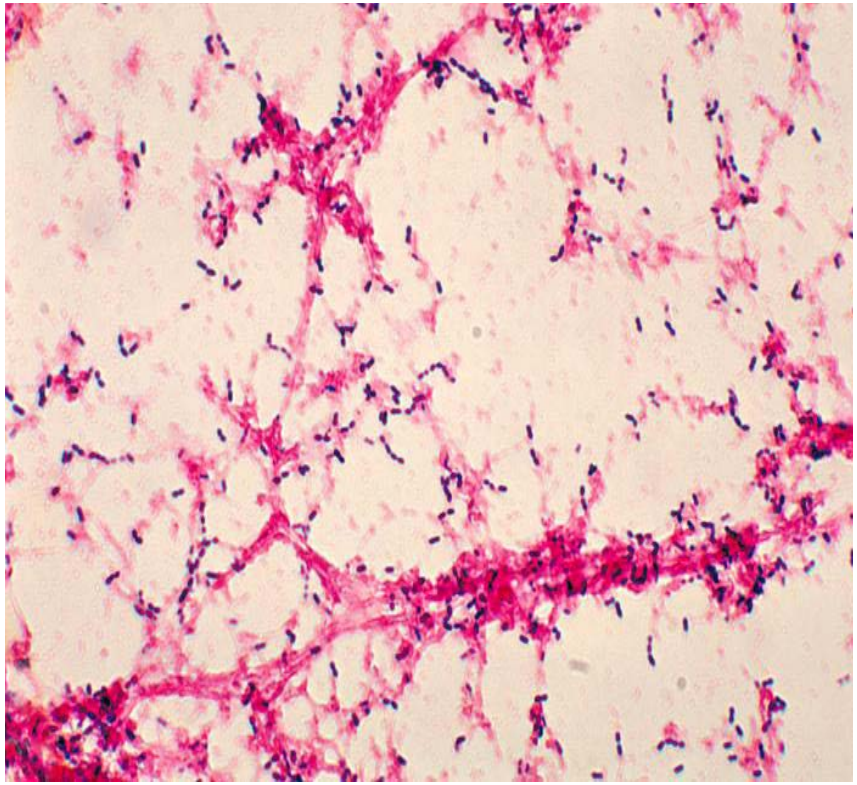
  The much thicker peptidoglycan layer in Gram positive cells will retain the CV-I complex, as the pores are made smaller due to the dehydrating effect of the alcohol. Thus the tightly bound primary stain is difficult to remove and the cells remain purple.

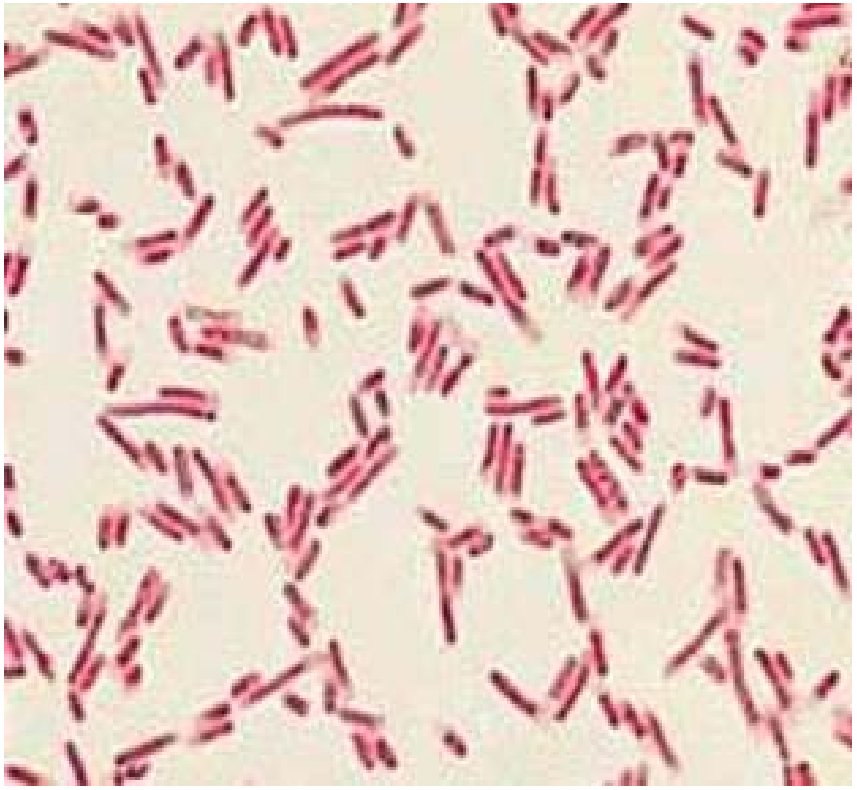
- **Counterstain**: Safranin, which used to stain Gram negative cells red, as these cells are previously decolorized and can absorb the counter stain.

Procedure of Gram staining



STAINED GRAM POSITIVE COCCI & GRAM NEGATIVE RODS





Gram variable organisms:

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

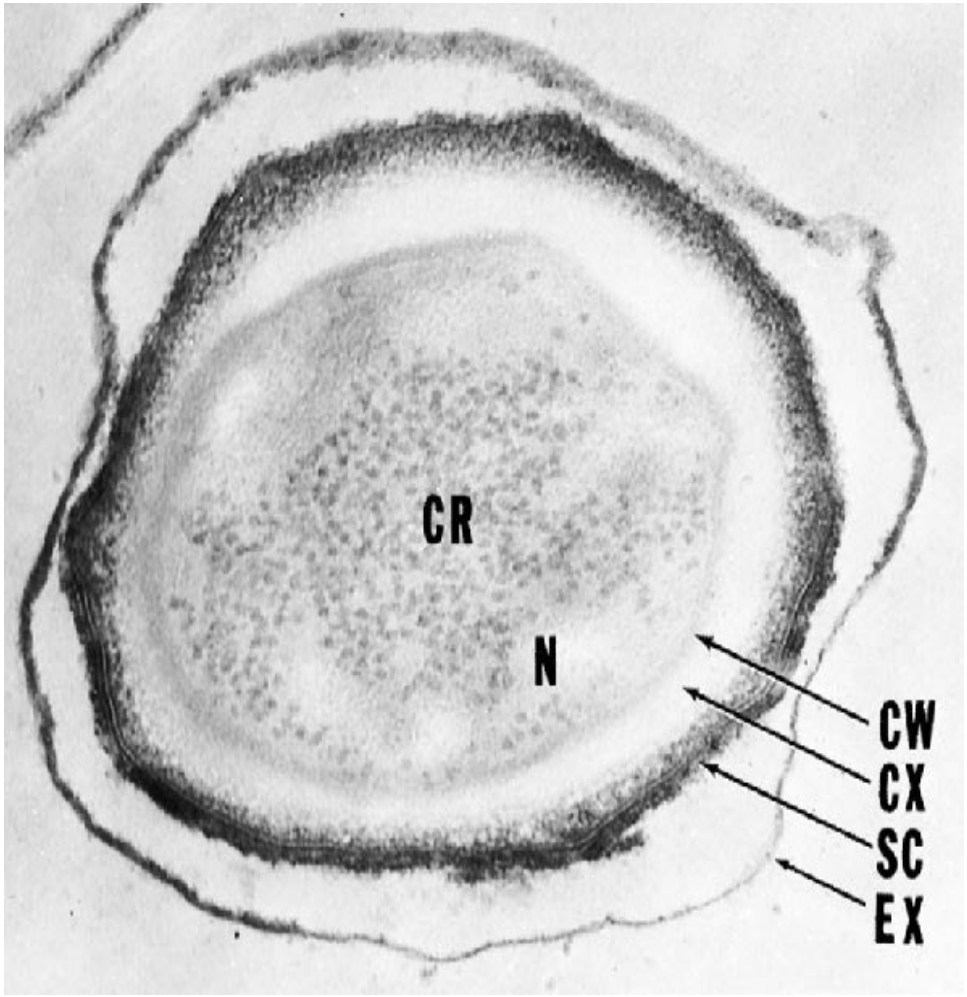
Endospore staining (Schaeffer-Fulton method)

- 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 to penetrate the impervious spore coat by application of heat. After applying this stain, both the vegetative cell and endospore will appear green.

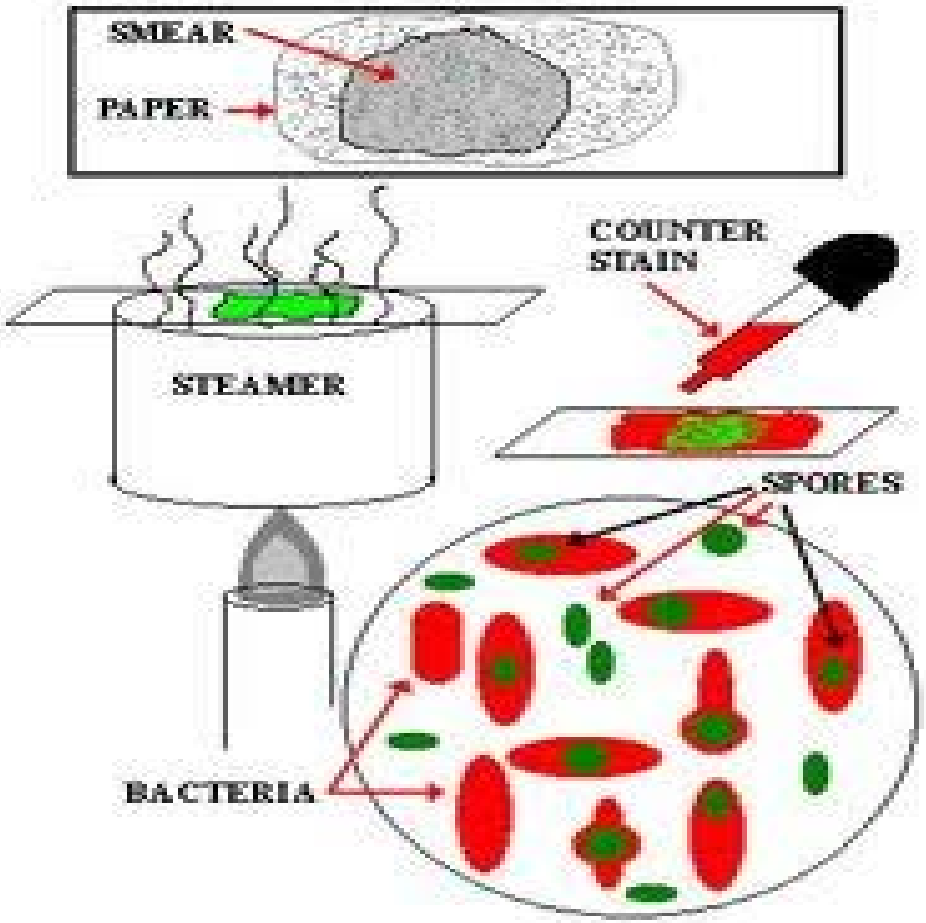
- **Decolorizing agent**:

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.

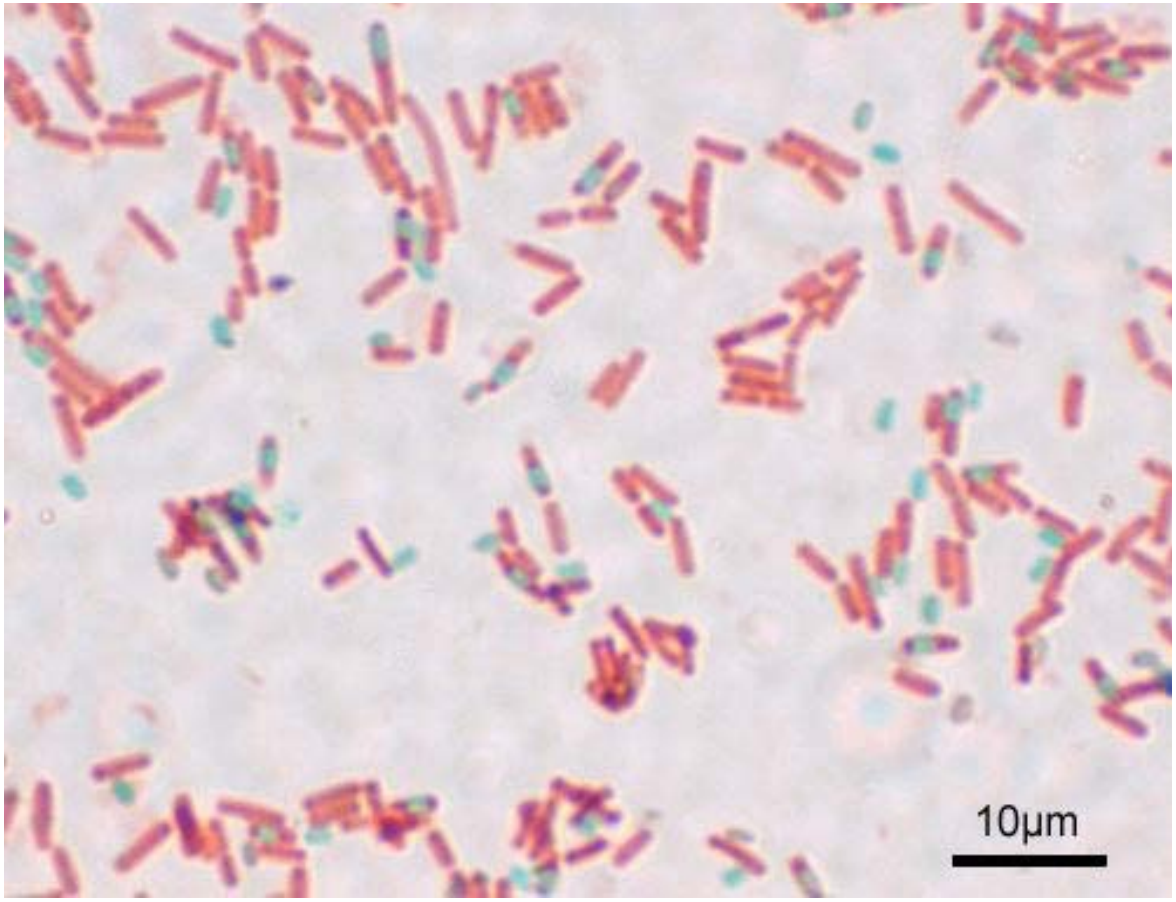
- **Counter stain**:

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

PROCEDURE OF ENDOSPORE STAINING

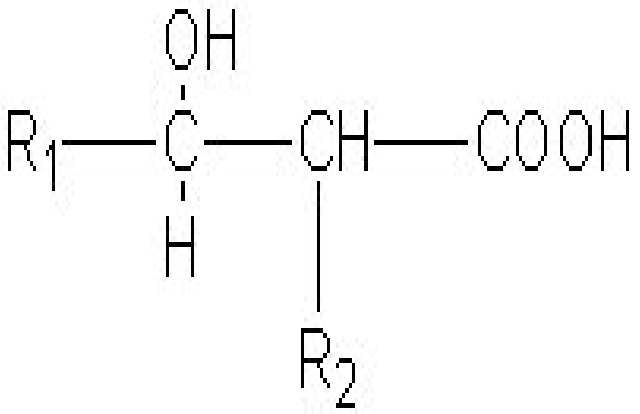


ENDOSPORE OF *Bacillus subtilis*



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.



Mycolic acid structure proposed by Asselineau (1950)

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.

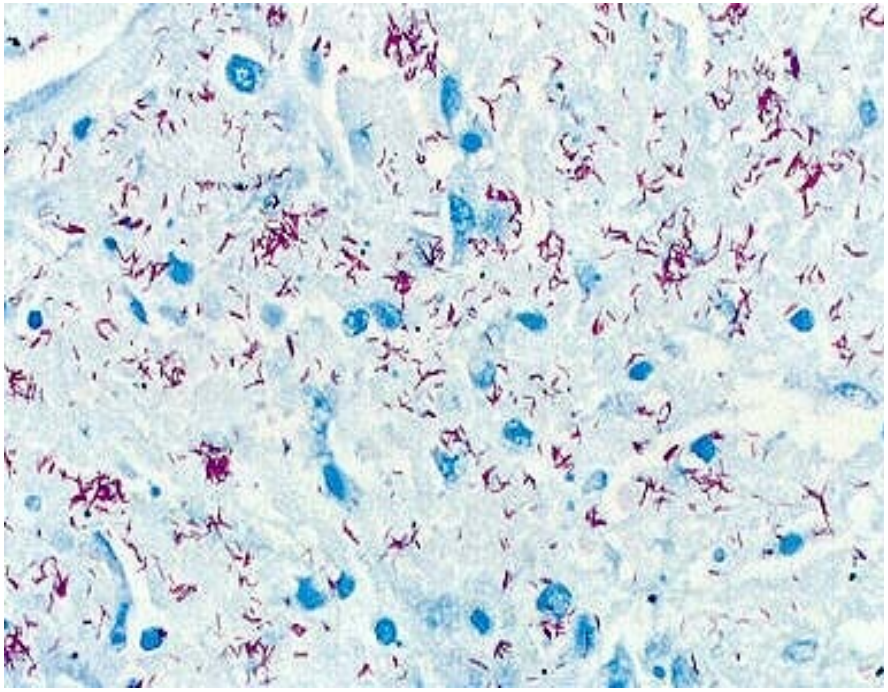
- **Decolorizing agent**:

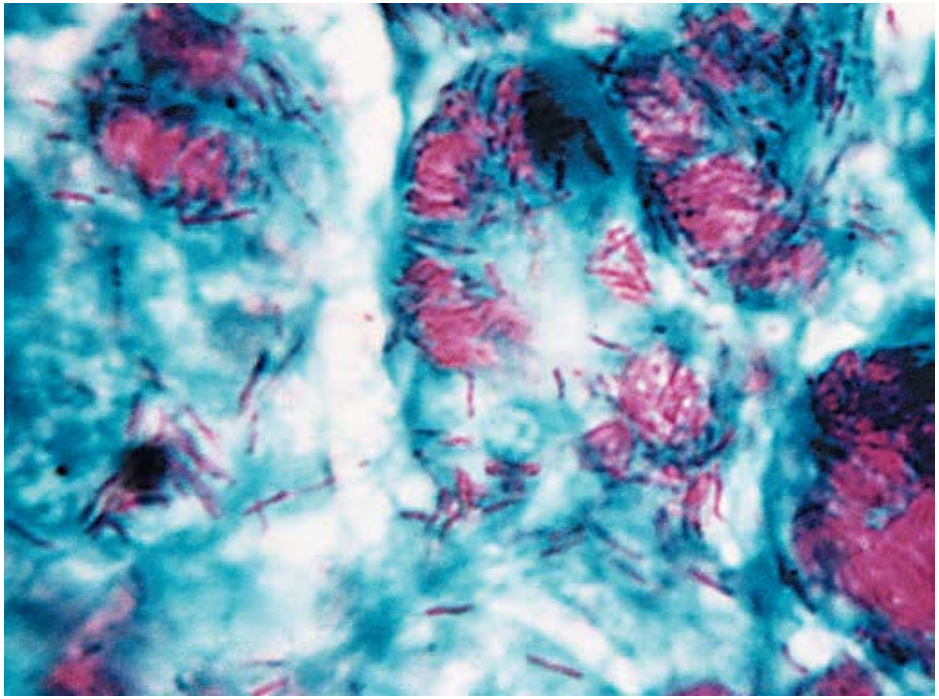
Acid-alcohol (3% HCl + 95% Ethanol): When the cooled smear is treated with acid- alcohol, acid-fast cells will retain the color of the primary stain as the primary stain is more soluble in the mycobacterial cell wall than in the decolorizing agent. Acid-alcohol will easily remove the primary stai n from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

- **Counterstain**:

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 – in a nutshell

- 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:**

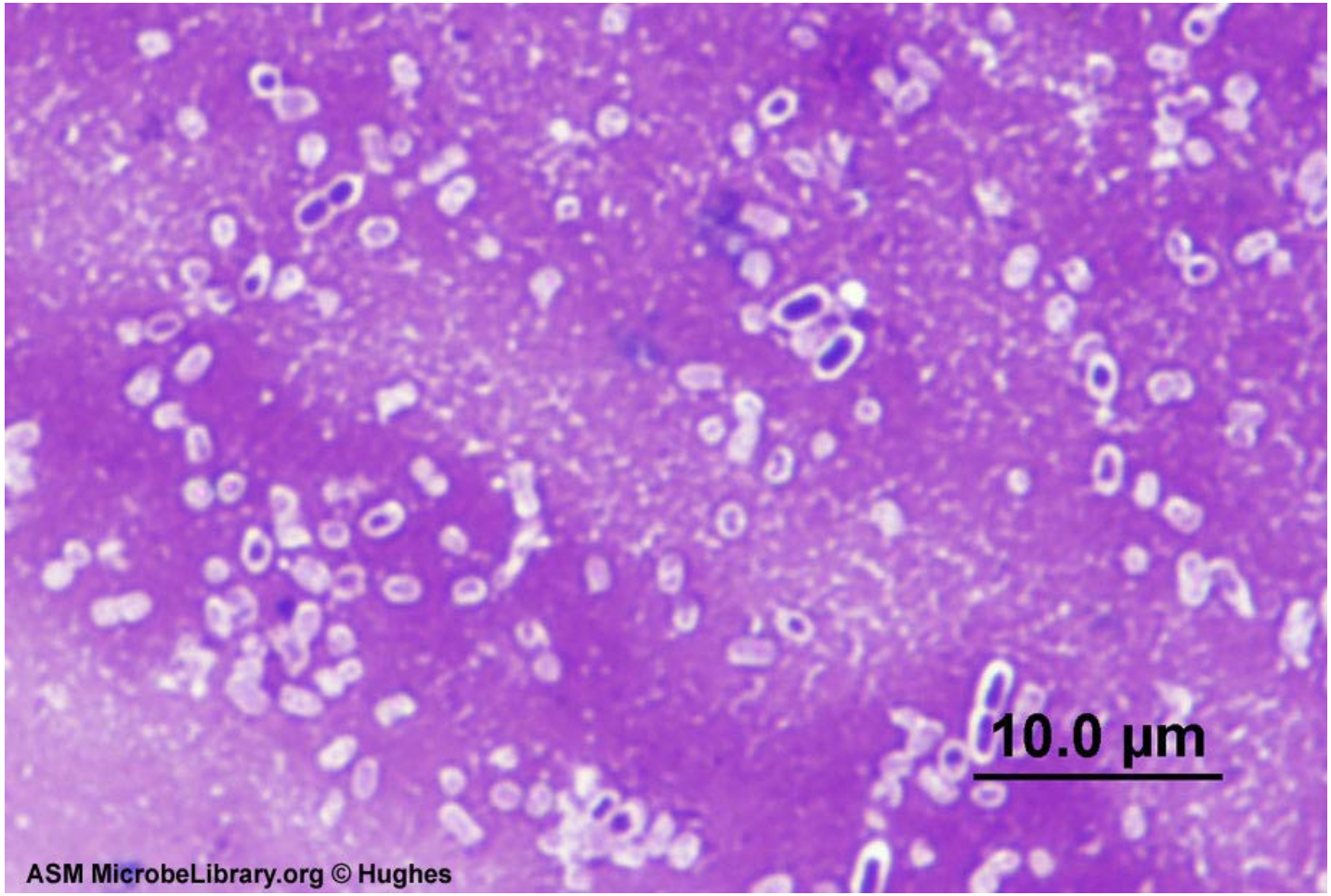
  Crystal violet (1% aqueous solution) : it adheres to the capsule without binding to it (as capsule is non ionic) , when this is applied over a non heat-fixed smear.

- **Decolorizing agent & counterstain :**

  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 counterstain 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.

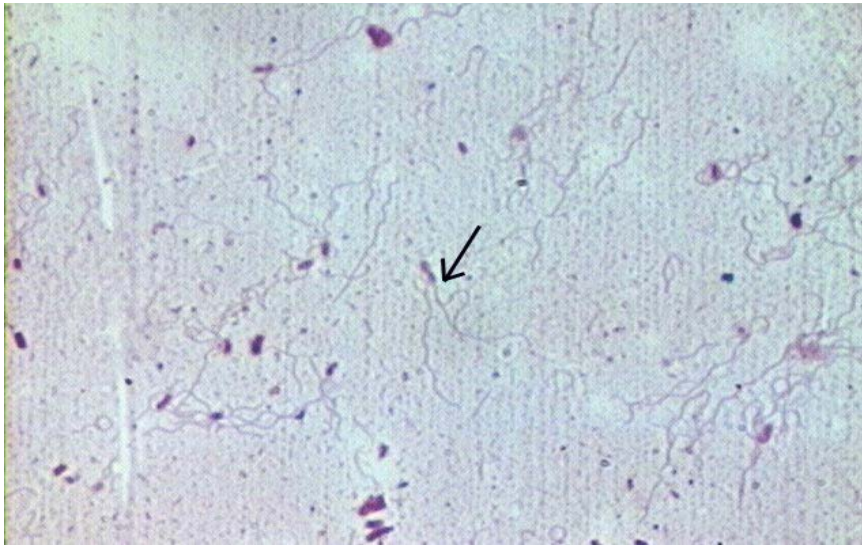
Stained capsule containing cells of *Klebsiella pneumoniae*

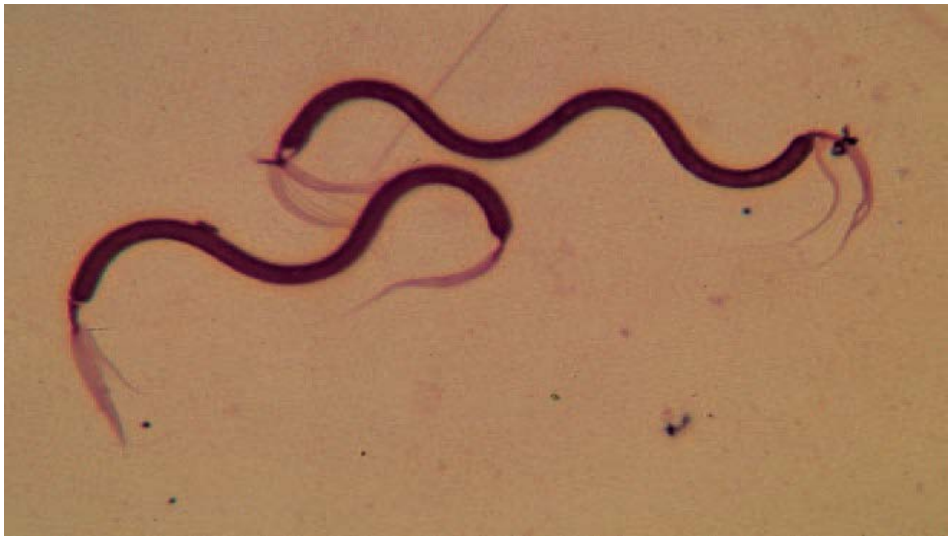


Flagella staining method

- 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 nigrosine or methylene blue is added.
- It is a simple staining method.

Stained flagella preparation





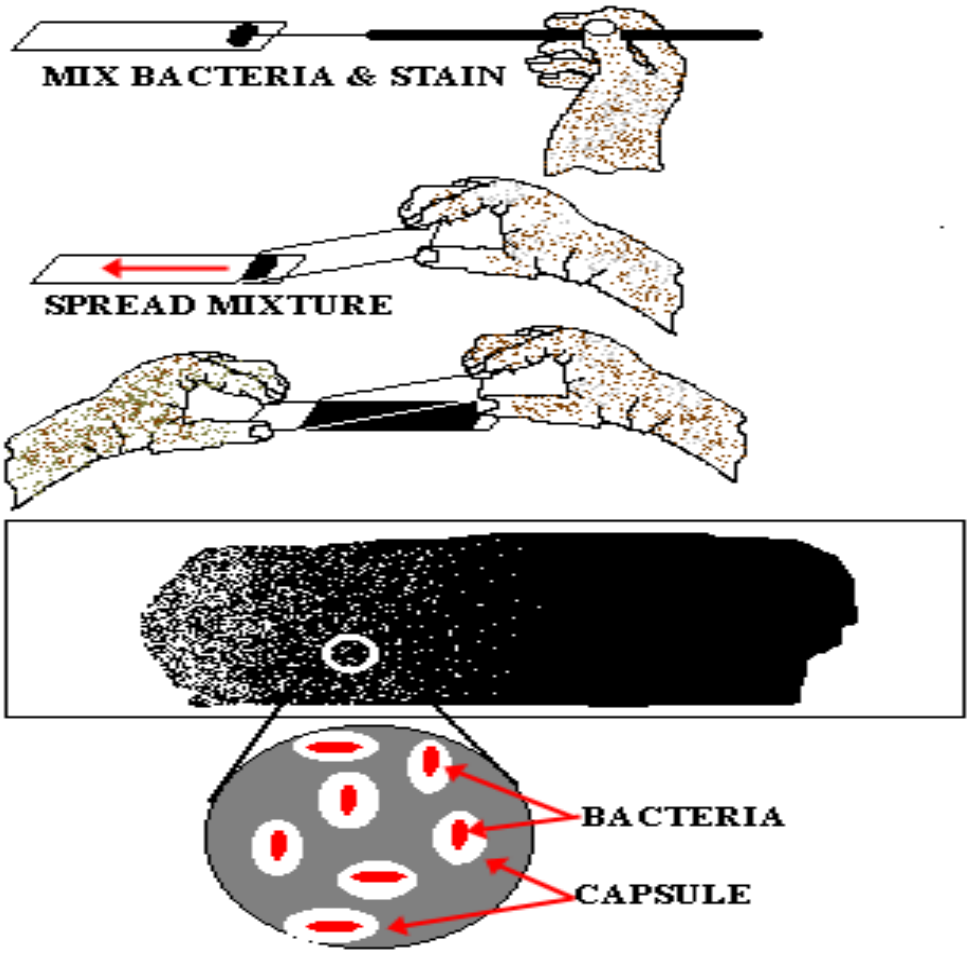
Example of Flagella Staining. *Spirillum volutans* with bipolar tufts of flagella (400).

Negative staining

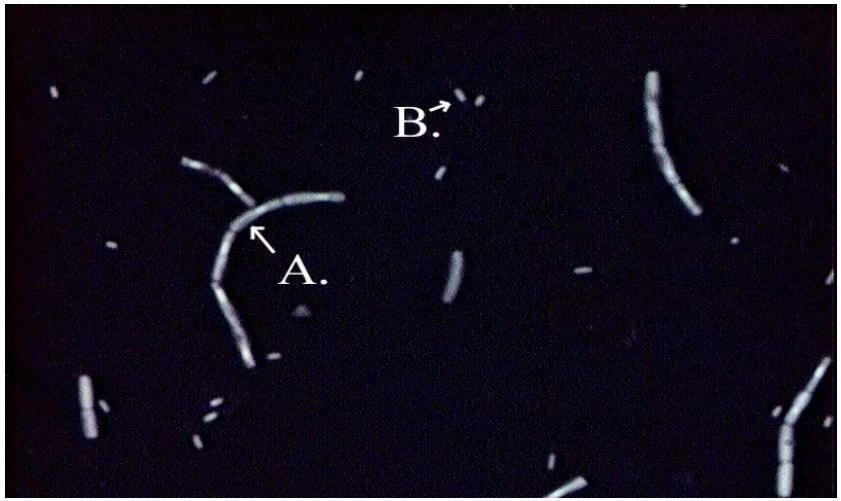
This technique works in a manner opposite to simple technique. Bacteria are mixed on a slide with an acidic dye such as congo red or the black stain, nigrosin. The mixture is smeared across the face of the slide and allowed to air dry.

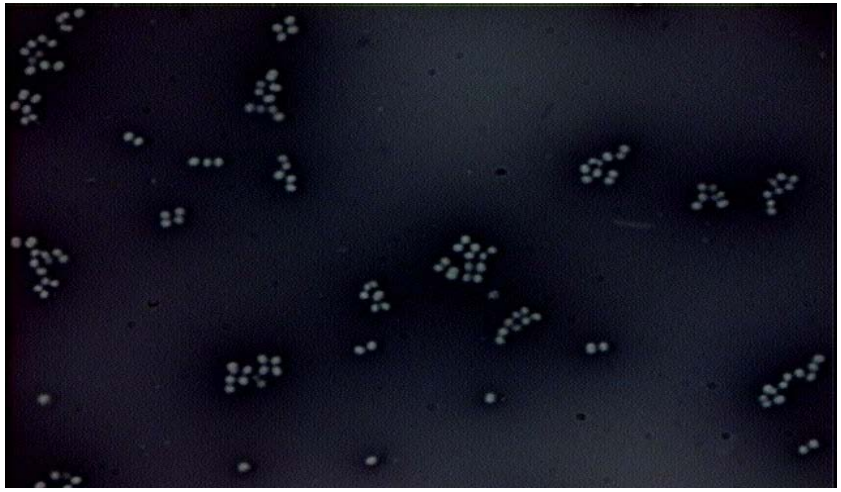
Because the stain carries a negative charge, it is repelled by the bacteria, which also have a negative charge. The stain gathers around the cell. since a chemical reaction has not taken place, and because heat fixing has been avoided, the cells appear less shriveled or distorted. They often appear larger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