STAINS AND STRAINING REACTIONS

The microscopical examination of stained preparation enables the morphology, relative, size and arrangement of microorganism to be seem clearly. Unstained microorganisms cannot be seen under the ordinary microscope because their refractive index is nearly similar to that of the suspending medium. So, stains are used to create contrast between cell and medium.

Five reasons why cells are stained in microbiology

Any five of the following

1. To reveal the size and shape of m/os
2. To enhance visualization of cells or certain cellular components under a microscope
3. Cells may also be stained to highlight metabolic processes
4. To differentiate b/w live and dead cells in a sample
5. Cells may also be enumerated by staining to determine the biomass in an environment of interest
6. Cells may also be stained to determine the presence or absence of internal structures
7. Cells are also stained to diferentiate b/w different types of organism

The commonly used stains are chemically salts of organic (aromatic) compounds possessing colour. They can be basic, acidic or neutral, depending on whether the colouring component is contained in the basic or acidic part of the stain.

Basic Stains consist of a coloured base (a cation) and a colourless acid radical (an anion) e.g methylene blue chloride, conventionally called

methylene blue. Other examples of basic stains include crystal (gentian) violet and basic safranin. Most of the stains that are used to stain bacteria are of the basic types because bacterial cells are rich in nucleic acids (bearing negative charges as phosphate groups) which combine with the positively charged basic dyes.

Acidic stains contain the colouring substance in the acidic radical (anion) while the base (cation) part is colourless, e.g Na^+ eosinate conventionally called eosin dye. Other examples of acidic stains include nigrosin, Indian ink, picric, acid, congo red and acid fuschin. Acidic stains are not very often used in microbiology except to provide background staining e.g in negative staining.

Neutral stains consist of coloured acidic and basic components. They are usually formed from the precipitate which is produced when aqueous solution of certain acidic and basic stains combine. Examples include Leishman and the haematoxylin-eosin stain used in the staining of animal tissues. Neutral dyes stain nucleic acid and cytoplasma.

Synthetic and Natural Stains

Synthetic stains are those prepared from aromatic compounds.

The majority of the stains used in microbiology (specifically in bacteriology) is synthetic and are mainly derivates of aniline dye. Examples include congo red, malachite green, crystal violet, methlene blue, acid and basic fuschin, safranin and eosin etc.

Natural stains are those obtained from animal or vegetable sources.

An example is haematoxylin which is a basic dye prepared from logwood.

Staining Techniques

Staining techniques employed in microbiology are varied and are specifically adapted to reveal certain types or parts of microorganisms. Thus the Gram staining technique differentiates microorganisms into either Gram positive or Gram negatives while the Ziehl-Neelsen technique differentiates acid fast from non-acid fast bacteria. Similarly, the Schaeff fulton technique and the Feulgen reaction respectively reveal bacteria endospores and nuclear material.

General Terminologies associated with Staining Techniques

There general terminologies associated with staining techniques include:

Indirect staining – This involves the staining of an organism or tissue only when a chemical is added before, during or after the application of a stain. The chemical which is required to bring about the staining reaction is called a **mordan**t. Mordants could be acidic, basic or simple metals. Basic mordants react with acidic stain and acidic mordants react with basic stains. Examples of mordants include phenol contained in the carbol fuchsin stain used in the Ziehl-Neelsen technique and iodine, which is added after application of crystal violet in the Gram technique. Metallic mordants include salts of chromium, aluminum, iron and tin which are used with acidic dyes too.

The simple staining of an organism or tissue without the use of a mordant is called **direct staining** e.g the Methylene blue technique.

Occasionally, a chemical may be added to a stain to speed up its reaction or to make its reaction more intense or selective. Such a chemical is called an **accelerator**. Unlike a mordant, an accelerator does not combine with a stain. An example of accelerator is Potassium hydroxide, which is used in Loeffler's methylene blue. In what is termed **regressive staining**, all the constituents of a preparation are stained. This is then followed by the

selective removal, or washing out of the excess stain. This process is referred to as **differentiation** or **decolourization**. For basic stains, the decolourizer is usually an acid solution and for acid stains, it is usually an alkaline solution. An example of a decolourizer is the acid-alcohol used in the Ziehl-Neelsen technique and the alcohol or acetone-alcohol used in Gram staining technique.

The term **progressive staining** is used to describe a staining technique in which several stains are applied in sequence for specific times without the use of decolourizer.

Differential staining is one in which two or more reagents are applied to impart colour to a cell or its parts, whereas **simple staining** involves the application of a single stain or reagent. **Negative staining** technique incorporates materials such as Indian ink or at most Nigrosin which is composed of particles too large to enter a cell. It involves staining the background with an acidic dye, leaving the cells contrastingly colourless.

Smear Preparations and Fixation

In the preparation of cells for microscopic examination, one pertinent problems that must be solved is the deposition of sufficiently thin layer of cells on the microscope slide so that light can pass through, making them visible through the microscope. In plant and animal tissue, the usual procedure is to cut thin layers (usually 5 to 10 micrometer thick) using a microtone, staining and than observing with a microscope. When dealing with microorganisms, this problem is considerably simpler. Here one needs only to perform smear preparations for most stains. When a smear preparation is made from a **broth culture** of microorganism, the broth suspension is applied directly to the slide without prior dilution. It is often helpful to use 2 to 3 loopfuls on a small area of the slide and the suspension

should not be spread out too thinly. When making a smear from **solid media** only a very small portion of growth (about the size of a pin head) should be removed in order to avoid the problem of getting too many cells. This portion of growth should be emulsified with a drop of distilled water on the slide and then spread out over a fairly large area.

After making smears, the slides should be left in a safe place to air dry, protected from flies and dust. Thereafter, and before staining; the smear is usually **fixed** using heat, alcohol or occasionally, other chemicals. The purpose of fixation is to: (1) preserve microorganisms and (2) to prevent smears from being washed from slides during staining. Fixation preserves microorganisms in that it (3) stops all enzymatic activity in the cell before observation. Most, if not all cells contain enzymes that are capable of destroying much of the material within the cell after it dies. Therefore, if a cell is not properly fixed before staining, much detail of cell structure will often be destroyed by these enzymes.

As mentioned above, fixation could be accomplished by any of the methods below:

A. Heat treatment. B. Alcohol C. Treatment with other chemicals e.g Formaldehyde and Potassium permanganate.

A. Heat fixation:

This is widely used but can damage organisms and alter their staining reaction especially if excessive heat is used. Heat fixation also damages leucocytes and is therefore unsuitable for fixing smears which may contain intracellular organisms such as *Neisseria gonorrhoeae* and *Neisseria meningitidis.* Also, organisms such as *Mycobacterium tuberculosis* are not killed by the usual heat techniques used to fix sputum smears. Heat fixation

generally results in cell shrinkage so that cells appear smaller than their actual size under the microscope. When used, heat fixation must be carried out with great care and the following technique is usually recommended:

a. Allow the smear to air-dry completely.

b. Rapidly pass the slide, smear upper most, three times through a Bunsen burner flame. Note that after passing the slide three times through the flame, it should be possible to lay the slide on the back of the hand without the hand feeling uncomfortably hot. If this cannot be done, too much heat has been used.

c. Allow the smear to cool before staining.

B. Alcohol Fixation

This form of fixation is far less damaging to microorganisms than heat. Cells especially pus cells are well preserved. Alcohol fixation is therefore, recommended for fixing smears when looking for Gram negative intracellular diplococci e.g *N. gonorrhoeae* or *N. meningitidis*. Alcohol fixation is more bactericidal than heat, for example, *M. tuberculosis* is not killed by the usual heat treatment but is rapidly killed in sputum smears after applying 70%v/v alcohol.

Alcohol fixing of smears may be accomplished thus:

1. Allow the smear to air-dry completely

2. Depending on the type of smear, alcohol-fix as follows:

For detection of intracellular Gram negative, fix with one or two drops of absolute methanol or ethanol.

For the detection of other organisms including *M. tuberculosis* fix with one or two drops of 70% v/v methanol or ethanol (absolute methanol can also be used but a 70% v/v solution is adequate).

3. Leave the alcohol on the smear for a minimum of 2 minutes or until the alcohol air dries on the smear.

C. Other Chemical Fixative

The use of other chemical is sometimes necessary to fix smears which contain particularly dangerous organisms to ensure that all the organisms are killed, e.g 40g/1 Postassim permanganate is recommended for fixing smears which may contain *Mycobacterium* species. Formaldehyde-fixed smears, however, tend to stain poorly and the chemical itself is toxic with injurious vapour.

Staining Techniques

1. Gram Staining Technique

An important taxonomic characteristic of bacteria is their response to Gram stain. Most bacteria can be differentiated by Gram reaction due to differences in their cell wall structure. The Gram staining procedure begins with the application of basic dye, crystal violet. A solution of iodine (which acts as a mordant) is then applied; all bacteria will be stained purple at this point in the procedure. The cells are then treated with alcohol. Gram-positive cells retain the crystal violet-iodine complex, remaining purple while Gram-negative cells are completely decolourized by alcohol, acetone or a mixture of alcohol and acetone. As a last step, a counter stain (such as neutral red, safranin or other red counter stain) is applied so that the decolourized Gram-negative cells will take on a contrasting colour while the Gram-positive cells now appear purple.

It is worthwhile to mention here that the Gram-staining reaction of many species is variable. Occasionally, Gram positive organisms lose their ability to retain crystal violet and stain Gram negatively due to one or more of the following reasons.

1. Cell wall damage due to antibiotic therapy.
2. Cell wall damage due to excessive heat fixation of smear.
3. Over-decolourization of the smear.
4. Use of an iodine solution which is too old (yellow instead of brown in colour) and therefore not acting as an effective mordant.
5. Preparation of smear from an old culture.
6. Occasionally, Gram negative organisms may not be fully decolourized and appear as Gram positive (false positive result) if a smear is too thick. Some other cells besides bacteria may respond to the Gram stain e.g. yeast cells stain Gram-positive while animal cells are always Gram-negative.

Technically, one cannot give such organisms a Gram stain classification since their cell walls have a different molecular composition from Gram-positive or Gram-negative bacteria.

2. Ziehl-Neelsen Staining Technique

This is also known as acid fast or non acid fast staining method. The Ziehl-Neelsen staining technique reveals acid-fast bacteria. Acid-fast bacteria are those that retain carbol fuchsin (basic fuchsin dissolved in a phenol-alcohol-water mixture) even when decolourized with hydrochloric acid in alcohol.

A smear of cells on a slide is flooded with carbol fuchsin and heated on a steam bath. Following this, the decolourization with acid-alcohol is carried out, and finally a contrasting (blue or green) counter stain is applied. Acid-fast bacteria (Mycobacteria and some of the related Actinomycetes) appear red while others take on the colour of the counter stain.

The Ziehl-Neelsen technique could further be categorized into methods I and II based on the degree of acid fastness of the microorganism

and consequently the concentrations of acid-alcohol required for decolourization. Method 1 is used to stain strongly acid-fast organisms such as *Mycobacterium tuberculosis* and *Mycobacterium ulcerans* and requires 30% v/v acid solution to decolourize the smear. Method II is used to stain *Mycobacterium leprae* which is only weakly acid-fast and thus requires only 1% v/v acid solution for decolourization of the smear.

3. Spore Staining Technique

Spores are most simply observed as intracellular refractive bodies in unstained cell suspension or as colourless areas in cells stained by conventional methods. An excellent laboratory technique for the differentiation of spores and vegetative cells is the Schaeff-Fulton technique. This involves heating the smear after the application of an aqueous primary dye (e.g. malachite green or carbol fuchsin) in order to enhance penetration of the dye into the relatively impermeable spore coat. This is followed by a period of alcohol treatment and then finally application of a counter stain (usually safranin red). The same impermeability of the spores serves to prevent decolourization by alcohol treatment sufficient to decolourize vegetative cells. Vegetative cells thus take up the colour of the counter stain (red for safranin) while the spores retain the colour of the primary dye.

4. Capsule Staining Technique

Capsules are usually demonstrated by the negative staining procedure or a modification of it. One such capsule staining technique called the **Welch method** involves treatment with hot crystal violet solution followed by a rinsing with copper sulphate solution. The latter is used to remove excess stain because the conventional washing with water would dissolve the capsule. The copper salt also gives colour to the background, with the

result that the cell and the background appear dark blue and the capsule a much paler blue.

The conventional method of capsule staining still used routinely in our laboratories involves making a smear of the culture with Indian ink or nigrosin, air –drying and then finally applying a basic dye e.g. Safranin, which penetrates bacterial cell wall. Upon examination under a microscope, capsule will appear as a clear zone surrounding the cell wall.

5. The Flagella Stain

Flagella are too fine (12-30nm) to be visible in the light microscope. However, their presence and arrangement can be demonstrated by treating the cells with an unstable colloidal suspension of tannic acid salts, causing a heavy precipitate to form on the cell walls and flagella. In this manner, the apparent diameter of the flagella is increased to such an extent that subsequent staining with basic fuchsin makes the flagella visible in the light microscope. Another variation of the flagella staining is usually carried out on motile organisms. In this case, young cultures grown on fresh agar slants are gently washed with 2-3ml of sterile distilled water and the bacterial suspension incubated for a few minutes. Droplets are transferred by capillary pipette from the top of the suspension, where motile cells are most numerous to one end of a tilted, scrupulously clean microscope slide and allowed to run down the slide and dry in air without mechanical agitation that would break off the flagella. The slide is flooded with a solution of the Leifson stain and allowed to stand for 10 minutes, washed, dried and examined under oil objective lens. Flagella are stained pink by this technique.

6. Cell Wall Stain

The Bisset and Hale's procedure conssits of immersing thick smear in 1% phosphomolybdic acid for 3-5 minutes and then in 1% methyl green for the same interval. After washing and drying the smear is examined under oil objective lens. Cell walls stain dark green or purple; cytoplasm is unstained.

7. Nuclear Stain

The Feulgen reaction and the various modifications of it are used to demonstrate the nuclear materials in bacteria. This reaction is for aldehydes, but is considered to indicate the presence of DNA. Smear fixed with osmic vapour are subjected to a mild hydrolysis with 1N HCL (for 5-7min. at 58^{o}C) and treated with Schiff's reagent. A light pink colour indicates DNA since RNA is not stained after acid hydrolysis. The colour produced by DNA is often not strong, but other stains have been used with success namely Giemsa stain, Azure A or thionin, following the hydrolysis step.