

Theories and Principles of Staining

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Abstract: *Special staining is considered to be a chemical reaction which demonstrates the presence of a particular type of tissue, cell, organelle or substance. The main objective of staining tissue sections is to impart colour to specific tissue constituents. It is used to highlight important features of the tissue as well as to enhance the tissue contrast. Therefore it is necessary to understand how a dye molecule exhibit a certain color and how it becomes attached to a specific site. Stained materials absorb certain components of white light illuminating the specimen and therefore appear colored.*

Keywords: Special staining, Dye molecule, Tissue contrast.

1. Introduction

The theory of staining is very complex subject and is not fully understood. Therefore many theories have been put forth to explain the facts. Histological staining is based on some chemical and physical properties. Numerous special staining techniques rely on the fundamental chemical processes of oxidation and reduction. In special staining techniques, oxidizing or reducing agents act on tissue sections, so that subsequently applied dyes or reagents can bind to or react with particular tissue elements, resulting in visualization.

1) Electrostatic reactions

Dyeing substances are composed of chromophores (ex- azo groups) and auxochrome groups (ex- hydroxyl, carboxyl or nitro groups), and the latter properties define the dye as an acid dye or a basic dye.

Any group that makes an organic compound coloured is called a chromophore. To turn a coloured compound into a dye requires the addition of an ionizable group that will allow binding to the tissues. Such binding groups are called auxochromes. These dyeing substances are required for electrostatic reactions.

2) Metal impregnation

Metal impregnation is the method of increasing contrast to the tissue section. The commonest metal that is used in light microscopy is silver, which produces a dense, black, fine deposit of silver and silver oxide where the silver ions have been reduced. Silver impregnation is also called silver staining, but the mechanism is quite different to the effects of dyes and the structures are actually plated with the silver rather than the silver being reversibly bound to the section. It has the advantage of being stable and permanent.

There are three different ways of producing silver deposits. These are the argentaffin reaction, the argyrophil reaction and ion-exchange reactions.

The argentaffin reaction

In the argentaffin reaction, the tissue contains reducing groups that are sufficiently strong and present in sufficient quantity to give a visible deposit without added reducing agents. These groups are often aldehyde groups and silver solutions can be used to replace the Schiff's reagent in the periodic acid-Schiff technique to give periodic acid-silver.

The argyrophil reaction

Many tissue groups are able to absorb silver, possibly by ionic mechanisms as for dyeing. The silver is mainly adsorbed as silver ions but small amounts are reduced to silver atoms. These silver atoms are deposited at the site of reduction. These silver atoms then act as catalytic sites where more silver can be deposited by the reducing action of a developer, e.g. formaldehyde. In this case the developer does the main reduction and the tissue simply provides places where there are silver atoms to catalyse the reduction. This type of reaction where an external reducer or developer is added is called an argyrophilic reaction.

Ion-exchange reactions

Ion exchange can also deposit silver and this is used to detect mineralization of bone using the von kossa technique. The section is treated with silver solution (silver nitrate) and the phosphates and carbonates in the mineralized bone form insoluble silver salts. The silver salts are then blackened by UV light or hydroquinone solutions. This method is used to demonstrate calcification in bone.

3) Selective staining

Selective staining method depends on the solubility of the dyes, for example fat staining with Sudan, Oil red and other fat dyes which rely on differences in solubility of the dye within two media, i.e. diffusion from alcoholic dye solution into the fat of specimens.

4) Cytochemical reactions

The Cytochemical reactions involves application of defined chemical reactions, for example chemical complex formation for Fe³⁺ staining, enzyme substrate reactions for peroxidases, phosphatases, or selective staining of tissue molecules after their chemical modification, for example - introducing reactive groups such as aldehydes for the Feulgen and PAS reaction)

5) Ligand specific probes

The selectivity of cellular staining is defined by the applied molecular probe and their specificity for cellular molecules with which they react, for example, antibodies, lectins, nucleotides.

Usually stains are taken into tissue due to dye-tissue or reagent-tissue affinities. The word affinity has two distinct phases. A tissue component has high affinity means that the component becomes intensely stained. Affinity means there is a tendency of a stain to transfer from solution onto a section. It is also used to describe attractive force binding

dye to tissue which depends upon various factors which includes stain-tissue, solvent-solvent, stain-solvent and stain-stain interactions.

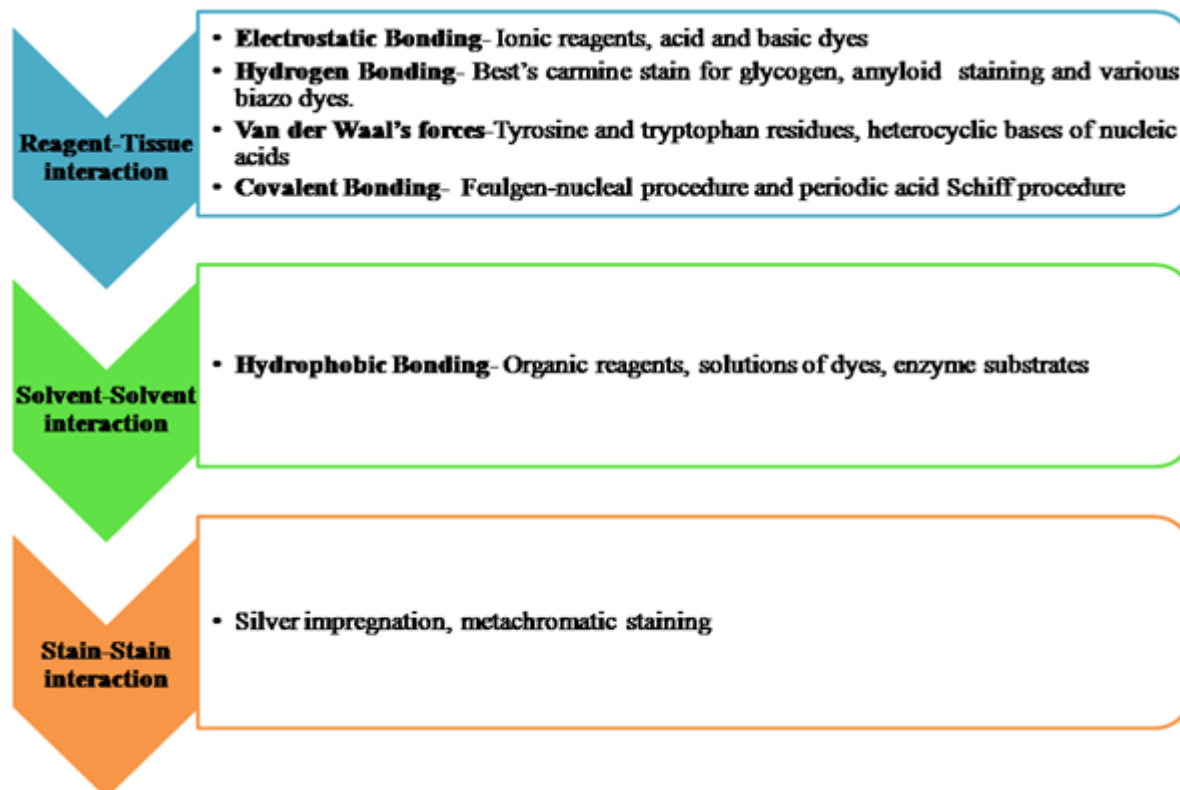


Table 1: Dye-tissue interactions

The binding of dyes to tissues mainly occurs by chemical bonding and the mechanisms rely on the same binding forces. The dye must form some type of bond or link to the tissue or they will be rinsed out of the tissue when the section is washed in another reagent. The usual forms of bonding can be involved. Each type has its own characteristics and bond strengths.

Ionic bonding

Ionic bonding is also called as salt links or electrostatic bonds which involve electrostatic attraction between oppositely charged ions. Anionic (negatively charged) dyes

will bind to cations (positively charged) in the tissue, and cationic dyes will bind to tissue anions. Ionic bonding is the single most important form of bonding in most histological staining. Negatively charged ionic substances like eosin will stain positively charged tissue ions. Eosin is an example of an anionic dye and is attracted to protein groups that are positively charged (cations) such as amino groups. First, the aminogroups in the protein become ionized by binding to a hydrogen ion and this charged group then attracts the eosin ion.

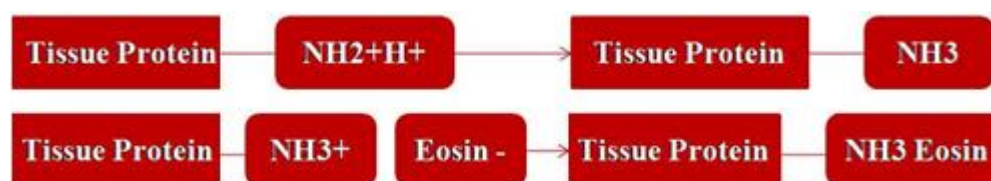


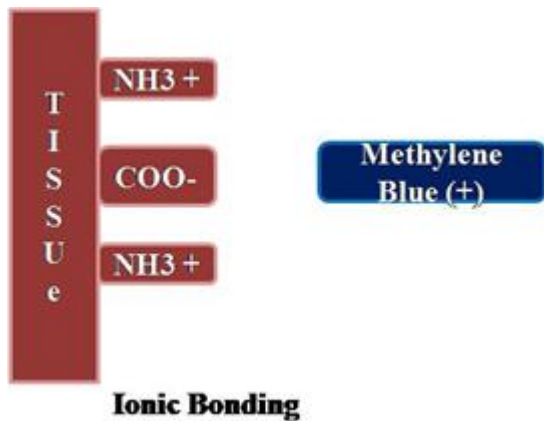
Figure 1: Ionization of tissue amines and subsequent binding of eosin

Anionic dyes are also called acid dyes because they are derived from coloured acids. Anything that will stain with an acid dye is called acidophilic. In the case of materials staining with eosin, they could also be termed eosinophilic. Tissue components that are acidophilic include collagen, red blood cells and the cytoplasm of many cells.

Positively charged methylene blue ions will stain negatively charged tissue ions. Methylene blue is an example of a cationic dye and will bind to tissue anions such as carboxylic acid, sulphuric acid and phosphoric acid groups. These groups need to be ionized to bind the dyes. Cationic dyes are

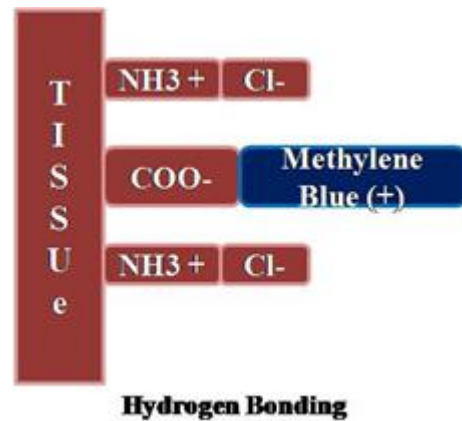
commonly called basic dyes and so substances staining with such dyes are called basophilic. The components that bind basic dyes include nucleic acids and acid mucins.

Electrostatic attraction between opposite charges depicts although there is a negatively charged carboxyl group in the tissue that could bind the dye, the dye will not be attracted to it because of the two nearby positive charges. The net charge on the surface of the protein is positive, so the methylene blue ion is repelled.



Ionic Bonding

Figure 2: Ionic bonding



Hydrogen Bonding

Figure 3: Hydrogen bonding

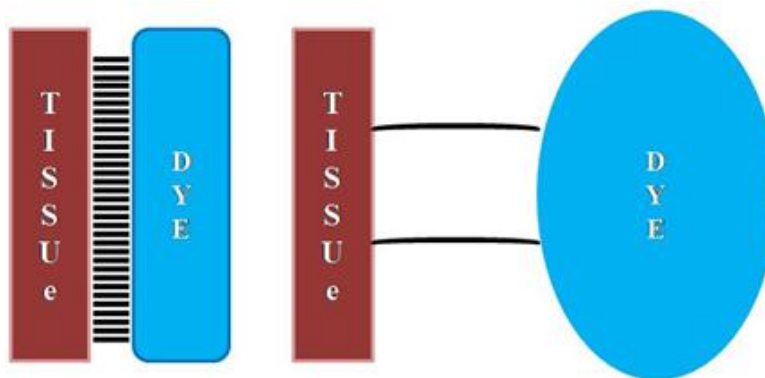
Hydrogen bonding

It is a dye-tissue attraction arising when a hydrogen atom lies between two electronegative atoms. It is an intermolecular force. Hydrogen bonds are highly selective as they can occur only between certain groups. In the staining of elastin fibres hydrogen bonds are probably more important. In whole or partial non-aqueous solutions hydrogen bonding can play a more significant role. Examples include Best's carmine stain for glycogen, staining for amyloid and various biazo dyes.

The methylene blue dye is able to bind to the carboxyl group, since the repelling charges on the amino groups are temporarily neutralized with chloride ions. The net charge on the surface of the protein is negative.

Van der Waals forces

These are short-range forces and will only have an effect if the two atoms are between about 0.12 and 0.2 nm apart. If they are further apart, then there is no effective bonding force. Van der Waals forces can occur between any two atoms and are not specific for any atom or group. If the surface shapes of the tissue protein and the shape of the dye matches, then many van der Waals bonds can be formed. Thus, although they are individually very weak, they may add up to a significant binding force if the dye and protein have complementary molecular surfaces. Groupings such as tyrosine and tryptophan residues, heterocyclic bases of nucleic acids favor van der Waals forces.

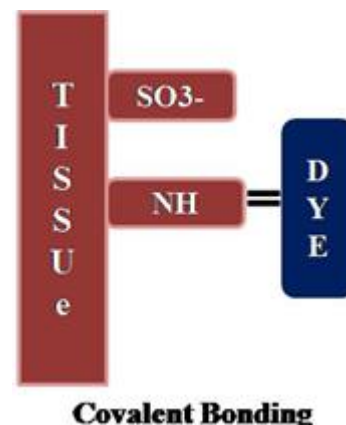


Van der Waals forces

Figure 4: Van der Waals forces

Covalent bonds

These are very strong bonds and are not easily broken once formed. They are important in some histochemical techniques e.g. periodic acid-Schiff, and in the attachment of dyes to antibodies in immunofluorescence. The so-called reactive methods use covalent bonds to bind but are not used much in histology. The Feulgen-nuclear procedure and periodic acid Schiff procedure is an example for reactive methods.



Covalent Bonding

Figure 5: Covalent bonds

Hydrophobic interactions

These are called as hydrophobic bonds, the forces are not chemical bonds since they hold dyes in tissues by the exclusion of water from the regions of hydrophobic groups. The exclusion of water stabilizes the two groups. Hydrophobic interactions are short range and are unaffected by hydrogen-bonding agents or salts. Altering the pH may change a particular group from a hydrophilic to a hydrophobic form by altering its ionization and this will alter the staining with hydrophobic dyes. Hydrophobic interactions are important in selectivity and play a major role in the staining of fats by Sudan dyes.

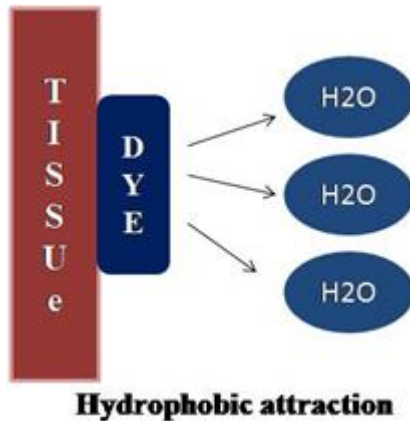


Figure 6: Hydrophobic attraction

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