Review Article

Metachromasia and Metachromatic Dyes: A review

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ABSTRACT

Metachromasia is a phenomenon of characteristic alteration in the color of staining process that is carried out in biological tissues shown by certain stains after their binding to particular substances, which are present in tissues, called chromotropes. This concept of metachromasia requires the presence of polyanions within the tissue. When a concentrated basic dye solution such as toluidine blue is used for staining such tissues, the bound dye molecules result in the formation of dimeric and polymeric aggregates. The spectrum of light absorption of these dye aggregates varies from the individual monomeric dye molecules. Cell and tissue structures, which contain high concentrations of ionized sulfate and phosphate groups such as the ground substance of cartilage, mast cell granules, and rough endoplasmic reticulum of plasma cells, show the phenomenon of metachromasia. This article reviews about the concept of metachromasia and various metachromatic dyes used for histopathological studies.

Keywords: Amyloid, Glycosaminoglycans, Metachromasia, Metachromatic dyes, Toluidine blue

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INTRODUCTION

The phenomenon of metachromasia was first reported, in 1875, by Jurgens, who noted metachromatic staining of amyloid by dahlia. In the same year, Ranvier observed metachromatic staining of cartilage with cyanine and Cornil described the phenomenon in amyloid and cartilage stained with methyl violet. The term "metachromatic" was coined by Ackroyd, in 1876, to describe color changes undergone by certain substances when heated. The first use of the term in a biological context was, in 1879, by Ehrlich, who observed that connective tissue mast cells were colored differently from the dye with which they were stained.[1,2] The current understanding of metachromasia was described by a Belgian histologist. Lison described who its value in the quantitative estimation of sulfate esters of higher molecular weight and nucleic acids metachromasia. In the year 1970, Toepfer published spectral shifts with increase in the concentration of the thiazine dyes which correctly matched the spectrum of dye-heparin composites, which exhibited that metachromasia can be reproduced by increase in the concentration of the stain alone in solution.[1,3,4]

DEFINITION

"Metachromasia may be defined as the staining of tissue or tissue components such that the color of the tissue-bound dye complex differs significantly from the color of the original dye complex to give a marked contrast in color."[5]

METACHROMASIA

Metachromasia is a phenomenon whereby a dye may absorb light at different wavelengths depending on its concentration and surroundings. The dye, which exhibits this phenomenon without changing its chemical structure, is said to be metachromatic. When the tissues are stained with a concentrated basic dye solution, such as toluidine blue, the bound dye molecules are close

enough to form dimeric and polymeric aggregates. The phenomenon of chromatic shift is explained on the basis of an effect on dye color because of interference between the numerous dye molecules that are bound by strong acid molecules. In a dilute solution, dyes such as toluidine blue are monomeric and it exhibits blue. In a concentrated or a hypertonic solution, dye molecules polymerize and therefore exhibit the metachromatic color shift.[6] Hence, the effect of polyanions, because of their high-density negative charge, is to functionally polymerize toluidine blue molecules in tissues and the metachromatic staining reaction is a strong predictor of tissue polyanions.[4,7,8]

Structures, which have greater concentrations of sulfate ion and phosphate ionic groups, namely, ground substance of cartilage, granules of mast cells, and the rough endoplasmic reticulum of plasma cells show the phenomenon metachromasia. This depends on the charge density of the negative sulfate and carboxylate positive ions in the glycosaminoglycan (GAG).[9] The GAG polyanion stabilizes the positively-charged dye molecules which results in a spectral shift. The higher the degree of stuffing, the greater the metachromatic shift. Hence, hyaluronic acid, lacking sulfate groups and with only moderate charge density, causes slight metachromasia; chondroitin sulfate, with an additional sulfate residue per GAG saccharide dimer, is an effective metachromatic substrate while heparin is strongly metachromatic. Hence, toluidine blue appears purple to red when it stains.[4,10,11]

TYPES OF METACHROMASIA

Three types of metachromasia are usually described (Table 1).[4,8]

Two forms of metachromasia are recognized.

Positive metachromasia, in which chromotropes induce a hypsochromic shift in basic dye, a shift in the peak of the absorption curve of dye in aqueous solution toward shorter wavelengths. The term "hypsochromic" (hypso, high) refers to the heightening of color (the transmitted color is at a longer wavelength than that of the original dye).

Negative or bathochromic metachromasia occurs when different groups of chromotropes cause the peak of the absorption curve of basic dye solutions to shift to longer wavelengths. This bathochromic effect (bathys, deep) is characterized by a lowering or shift of the metachromatic color of the dye to a wavelength shorter than the orthochromatic color. Negative metachromasia has its main application in invertebrate and plant histology.[2]

Table 1: Various types of metachromasia

Type	Color	Structure
α (alpha) (orthochromatic)	Blue	Monomeric (single molecules)
β (beta)	Purple	Di and trimeric (aggregates 2 and 3 molecules or a mixture of α and β
γ (gamma)	Red	Polymeric (long chain aggregates of molecules)

METACHROMATIC DYES

Metachromatic dyes share important characteristics: The dye molecules are unicationic or unianionic (low charge), small and planar.

Cationic Dyes

The most commonly used metachromatic dyes are the basic or cationic dyes toluidine blue O, azures A and B, thionin and methylene blue. Less commonly used dyes include crystal violet, methyl violet and pinacyanol, safranin O, brilliant cresyl blue, cupric, ferric and aluminum lakes of celestion blue, Bismarck brown Y, cresyl violet acetate, gallocyanin, acridine orange, and Janus green.

Anionic Dyes

These are used less frequently than cationic dyes. Biebrich scarlet, for example, is bound metachromatically by polyamino-proteins, especially histones under certain conditions. The metachromatic effects of bromophenol blue with anionic chromotropes are considered to be the examples of dichromatism. However, compared to the large metachromatic shifts of basic dyes, spectral shifts exhibited by acid dyes are much smaller, possibly limiting wider applications.[2]

THEORIES OF METACHROMASIA

Various authors have proposed different theories regarding metachromasia.

Michaelis (1902) – Chromotropic tissue elements bind one of the two tautomeric equilibrium forms of a metachromatic dye, and this dye has a distinct absorption spectrum.

Hansen (1908) – Metachromatic dyes are hydrolytically dissociated in solution, and the dye base becomes fixed to the chromotropic tissue elements.

Pappenheim (1908) – The dye base is absorbed to the chromotrope in the carbinol form.

Lison (1935) – Metachromatic color is due to a polymerization of a tautomeric form of the dye that, in aqueous solution, could exist as an imino base.

Michaelis and Granick (1945) – Metachromasia is due to polymerization of the dye molecules.

Sciechter and Campani (1948) – Metachromasia is due to a combination of the chromotrope with the resonance form of the dye.

Sylven (1954 and 1959) – Metachromasia is a special type of orderly dye aggregation, involving water molecules and characterized by the formation of new intermolecular bond between adjacent dye molecules.[2,12]

FACTORS AFFECTING METACHROMASIA

1. Concentration of the dye: Increase in the concentration favors metachromasia
2. Temperature*:* Decreasing the temperature favors metachromasia
3. pH*:* Less pH (acidic) favors metachromasia
4. Aqueous solvent: Water, a polar solvent contributes to the efficiency of Van der Waal's forces by which the molecules are held together
5. The orderly alignment of dye molecules is favored if the molecules present hydrophobic and hydrophilic parts.[1,8,12]

It has been estimated by Sylven (1954) that the intercharge distance necessary for metachromasia (i.e. distance between anionic tissue groups) is 0.5 nm or less. It is, therefore, possible for water molecules dimensions approximately 0.4 nm to be intercalated between dye molecules. Dehydration abolishes most metachromasia. Strongly hydrated tissues such as cartilage (chondroitin sulfate) and mast cells (heparin) retain metachromasia better than less hydrated tissues such as non-sulfated acid mucopolysaccharides.[8,12]

MECHANISM OF METACHROMASIA

Certain tissue structures or substances known as "chromotropes" carry acidic groups with a minimum surface density of not more than 0.5 nm between adjacent negatively-charged groups. These chromotypes react with metachromatic dyes to produce a color which is different to that normally exhibited by the dye. The dye exists in normal monomeric (orthochromatic) form. The negative charges on the chromotrope attract significantly positively-charged polar groups on the dye, bringing about dye aggregation in a specialized orderly form (dye-dye interaction or intermolecular attraction), forming a polymeric (metachromatic) form. Principally, Vander wall's forces hold dye molecules together to form dimmers, trimers, or polymers. Other forces implicated are hydrogen bonding and hydrophobic bonding. Hence, any factor favoring dye-dye interaction, such as high charge density substrates or salty dye baths, will tend to give rise to metachromatic staining.[7,8,10]

Examples such as thiazine dyes as they undergo a color shift from blue (monomeric, orthochromatic, 620-630 nm) through purple (dimeric, trimeric, 590 nm) to red, (polymeric, 550 nm).[5,7,8]

Formation of purple (partial metachromasia) can be due to:

1. A mixture of blue monomeric dye and red polymerized dye are present giving a purple coloration
2. The cationic groups are not large enough to permit full polymerization, only dimmers, and trimers of the dye being formed.[8]

The dye polymers exhibit usually a "hypsochromic" effect, which means that there is a shift of emission toward the longer wavelengths of light. Examples include toluidine blue (blue – purple – red), thionin, (blue – purple – red), azure A (blue – purple – red), methylene blue (blue – purple – red), and acridine orange (yellow – red). Occasionally, dyes show "bathochromic" effect where there is a shift of emission toward the shorter wavelengths of light. Examples such as safranin (red – yellow) and Bismarck brown (brown – yellow).[8]

APPLICATIONS OF METACHROMASIA

Metachromasia is used in demonstration of:

- Mucins
- Amyloid
- Mast cell granules
- Neuroendocrinal cells
- Glycogen (induced metachromasia)
- Sulfatides
- Metachromatic granules of *Corynebacterium diphtheriae*
- Nucleus
- Decalcification.

DEMONSTRATION OF MUCINS

Mucins can be acidic and neutral sulfated and carboxylated mucins, which are acidic, are metachromatic while neutral mucin is not. Acidic mucins are produced by fibroblasts, endothelial cells, osteocytes, chondrocytes, and mast cells. These include chondroitin sulfate (A, B, C), heparin/heparin sulfate, keratin sulfate, and hyaluronic acid.[13] Acidic mucins are stained metachromatically by toluidine blue, thionin, azure A, acridine orange by fluorescence.

Feurter's Technique

About 1% thionin or toluidine blue in 0.5% aqueous solution of tartaric acid is used. Thionin and toluidine blue are basic dyes of thiazine group, which has a blue (orthochromatic). When stained for the mucin, they stain red metachromatically.[7,10]

Azure A

Azure A is a basic dye of thiazine group formed by oxidation of methylene blue. It has a blue (orthochromatic). In staining sections with azure A, an alcohol-fast metachromasia is used. A mordent type differentiation, uranyl nitrate is used to get alcohol fastness and increased brightness. The acid mucins are stained purple to red (metachromatic) and the background blue (orthochromatic). By varying the pH and electrolyte content of dye solution sulfated and carboxylated mucins can be differentiated. Sulfated mucins show metachromasia below first 3.0 but not carboxylated mucins.[7,10]

Acridine Orange

It is a cationic fluorochrome and has an orthochromatic yellow. If a section is stained by iron hematoxylin and subsequently stained by acridine orange, mucins fluoresce-orange/red.[7,10]

AMYLOID

Amyloid is metachromatically stained by methyl violet, a triphenylmethane dye introduced by Cornil, in 1875, and orthochromatically by standard toluidine blue method.

Methyl Violet

About 1% aqueous methyl violet is used for staining for 5 min followed by differentiation with 1% acetic acid sections are mounted in aqueous media of high sugar or salt content to prevent diffusion of the stain. Amyloid stains pink to red and other elements stain violet. Its staining metachromatically with methyl violet is controversial since methyl violet is a mixture of tetra, penta, and hexamethyl pararosaniline. The coloration of amyloid is probably by selective absorption of one of these colored fractions, hence polychromasia would be a more likely explanation of this staining.[7,10]

Toluidine Blue

Stains amyloid blue (orthochromatic) but when examined under polarized light amyloid gives a dark red birefringence.[7,10]

Crystal Violet

This method uses ammonium oxalate, which accentuates the polychromatic effect. Formic acid can also be used as an accentuator of the polychromic effect.

Methyl Green

This method is slightly more selective, in that tissue sections are stained with methyl violet and then differentiated and counterstained simultaneously with methyl green, which replaces the former dye in tissue components other than amyloid.[7,10]

MAST CELL GRANULES

Mast cells were first recognized by virtue of metachromasia, in 1877, by Paul Ehrlich. Mast cells contain heparin and histamine usually in the form of granules which are difficult to see in H and E stained sections. The compound being a primary cause for metachromasia was recognized as heparin. Toluidine blue is a tiny weak cationic dye which is hydrophilic in nature. Adherent to nucleic acids such as DNA or RNA, in chromatin or Nissl substance, the stain gives blue. Adherent to GAG, in mast cell granules or cartilaginous matrix, the stain exhibits metachromatic purple. Toluidine blue is characteristically applied from weakly acidic aqueous solutions. DNA, RNA, and GAG are polyanionic, but most of the proteins are protonated and are polycationic. They are stained metachromatically and appear different for various stains. The examples include toluidine blue – purple, azure A – red, and thionin – purple. Other basic dyes, which stain the mast granules metachromatically are methylene blue, methylene violet, cresyl – violet, brilliant cresyl blue, acridine red, neutral red, pyronin, and safranine.[4,13]

NEUROENDOCRINE CELLS

Many neuroendocrine cells exhibit masked metachromasia. This characteristic can be unmasked by prior treatment of tissue sections by hot acid hydrolysis by hydrochloric acid. This releases carboxyl groups from polypeptides which are then free to react with and change the color of basic dyes such as toluidine blue and azure A. Endocrine cell granules stain purple red.[3]

GLYCOGEN (INDUCED METACHROMASIA)

Sometimes, it is possible to induce metachromasia in substances not normally possessing the requisite polyanionic groups. For example, sodium-bisulfate induced metachromasia of glycogen. In this technique, dialdehyde groups are first formed with periodic acid

and then sections are treated with 50% aqueous sodium bisulfate for 1 h, which forms addition compounds with the aldehyde. As the bisulfate has a polyanion forming capacity, a glycogen chromotype will be constructed and conventional metachromatic dyes used for its demonstration.[2]

SULFATIDES

These sulfate esters of cerebroside are the only lipids sufficiently acidic to induce a metachromatic shift in basic aniline dyes.

Cresyl Violet

Basic aniline dye has an orthochromatic color iliac to violet when stained for sulfatides stains brown metachromatically.[7,10]

Toluidine Blue

It is also called as tolonium chloride, an acidophilic metachromatic dye which specifically stains tissue components that are acidic in nature. It is a dye which is known because of various medical applications after its discovery, in 1856, by William Henry Perkin; later, it was used by the dye industry. It has three isoforms, namely, ortho-toluidine, para-toluidine, and meta-toluidine.[4,11,14]

It has been widely used as a vital stain for many mucosal lesions and has found applications in tissue sections as it specifically stains certain tissue components related to its metachromatic property.[4]

"Metachromatic leukodystrophy" is a rare condition characterized by demyelination of brain and peripheral nerves and in demyelinated areas, sulfatides are deposited as granular bodies.[15,16]

METACHROMATIC GRANULES OF *C. DIPHTHERIAE*

These granules contain polymerized inorganic polyphosphate responsible for metachromasia. With toluidine blue or methylene, blue granules stain red violet contrasting with the blue staining of the bacterial protoplasm. Special stains such as Albert's, Neisser's, and Ponder's have been devised for demonstrating the granules clearly.[16]

NUCLEUS

Thionin and toluidine blue dyes are commonly used for quick staining of the frozen section using their metachromatic property to stain nucleus and cytoplasm differently. Toluidine blue has an affinity for nucleic acids, and therefore, binds to the nuclear material of tissues with a high DNA and RNA content. Nucleic acids stain metachromatically with fluorochrome acridine orange which, because of the low toxicity, is used as a vital dye in diagnostic and research applications. Nucleic acid metachromasia is adversely affected by heat during paraffin wax processing.[2,3,13]

DECALCIFICATION

Metachromasia is adversely affected by strong decalcifying agents such as hydrochloride acid and nitric acid. Suitable decalcifying solutions include 2% formic acid – 20% sodium citrate (pH 5.2), 10% formic acid, formic acid-sodium formate or 15 % EDTA sodium salt, pH 7. Metachromatic staining is conserved in archeological bones decalcified in 2% citric acid - 20% sodium citrate. Prolonged exposure to tissues to buffered citric acid may produce "inversion staining," where cartilage matrix loses its metachromasia while bone and osteoid stain metachromatically.[2]

MOUNTANTS

To overcome loss if metachromasia during dehydration, mounting in water or the use of hydrophilic mountants such as Apathy's medium or glycerol-gelatin are often recommended. Unfortunately, dyes leach into mountants and all but the strongest metachromasia is invariable lost quite rapidly. To conserve metachromasia sections should be air-dried, and mounted with a drop of water beneath coverslip when ready for examinations. Resinous media are also suitable for mounting metachromatically stained sections once the section is adequately air -dried.[2]

CONCLUSION

The generally accepted explanation for the phenomenon of metachromasia is that change in color is due to polymerization. Metachromasia is enhanced when intermolecular distance is reduced. Increasing concentration of dye, decreasing temperature, pH, and water enhance metachromasia. A simple indication of this property is to dissolve such a dye in water at low concentration. If the concentration is increased, a color change occurs. Adding more water reverses the color change. Furthermore, it is the property of various tissues, of staining in a different color to that of the stain. Thus, metachromasia is an important phenomenon where one must be aware of its principles and its applications and about the dyes which exhibit metachromasia subjected to its various applications in histopathology.

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