A Taipiece: Hematoxylin & Eosin (H & E)
A special case, though not a special stain. H & E is so widely used that no account of staining mechanisms can fail to mention it — the more so as H & E still puzzles us in various ways.

What is H & E? — The easy bit is to say what “eosin” is: Eosin Y is a normal acid (anionic) dye of moderate size and well studied chemistry. “Hematoxylin” however is a misnomer, hematoxylin being a colorless natural product whose oxidation can produce hematein. And “hematoxylin” in H & E is the product of the reaction of hematein with aluminium ions, so is better termed Al-hematein. But to ask “what is this?” is to encounter puzzle number one. The nature of the Al-hematein complex or complexes in staining solutions remains uncertain. Both cationic and anionic complexes could be present; for experimental data and summary of prior work see Bettinger & Zimmermann (1991) and Puchtler et al (1986). Moreover the Al-hematein complex present in tissue sections following “blueing” is probably different again, perhaps a poorly soluble cation polymer (Puchtler et al 1986).

How does H & E work? — Again starting with the easy bit: eosin stains sections by acid dying. The Al-hematein staining mechanism however is puzzle number two. Its staining behavior differs from nuclear and Nissl staining basic dyes such as cresyl violet or toluidine blue. Extraction of DNA from cell nuclei inhibits nuclear staining by such basic dyes, but does not eliminate Al-hematein staining. Routine blue. Extraction of DNA from cell nuclei inhibits nuclear staining by basic dyes readily wash out of sections if over-enthusiastically

Further Reading

Chapter 19 | On Chemical Reactions and Staining Mechanisms

What is the difference between argentaffin and argyrophilic reactions?
Both terms relate to the formation of colloidal metallic silver (Latin argentum, Greek argyros) (1), which may range in color from yellow through brown to black depending on particle size and density, in specific structural components of tissues. The metal is formed at sites of reduction of silver ions derived from a staining solution. These ions may be simple Ag+, as in a solution of silver nitrate, or they may be complex ions such as silver diammine, [Ag(NH3)2]⁺ or silver methenamine, [Ag(C6H12N4)3]3+, which are formed respectively by adding ammonium hydroxide or hexamethylenetramine to solutions of silver nitrate. (Hexamethylenetramine, the reaction product of formaldehyde and ammonia, is also used as a urinary antiseptic; its pharmaceutical name is methenamine in the USA and hexamine in the UK.) These complex silver ions are more easily reduced than Ag+. By convention, argentaffin is used when the reducing agent is present in the tissue. Structures are said to be argentophilic when bright light or a solution of a reducing agent is applied to the object after exposure to a silver solution.

An example of an argentaffin reaction with silver nitrate as the reagent is the histochemical demonstration of ascorbic acid in such sites as the adrenal cortex and the embryonic eye. Tiny fragments of fresh tissue are immersed in an acidified solution of silver nitrate and then in a solution of sodium thiosulfate (2, 3, 4). The latter reagent removes unreduced silver, which otherwise would be slowly reduced by the action of light, discoloring all parts of the tissue. This slower reduction, which forms the basis of several traditional methods for showing intercellular clefts and spaces (5), is an argentophilic, not an argentaffin reaction, because the light serves as an external reducer, acting probably on silver chloride precipitated in extracellular fluid. The same holds for von Kossa’s stain for calcified material. In this, silver ions from aqueous AgNO₃ displace calcium from deposits of calcium carbonate or phosphate, forming insoluble silver carbonate or phosphate, which is quickly decomposed by the action of light to form black colloidal silver.

The only argentaffin reaction of importance in histopathology is the reduction of an alkaline solution containing silver diammine in the Masson-Fontana stain, which detects small quantities of melanin and, in suitably fixed tissues, the amines derived from tyrosine and tryptophan: dopamine, noradrenaline, adrenaline and serotonin (6).

Most types of melanoma and some tumors of intestinal endocrine cells give positive Masson-Fontana argentaffin reactions (7, 8). Nearly all the cell-types of the diffuse endocrine system (DES) are argentophilic (9, 10), as are the axons of neurons and reticulin, a term that applies to thin collagen fibers and basement membranes. The technical details of argentophil silver methods vary with the structures to be demonstrated, but in all cases there are at least two steps. In the first, which is often called impregnation, the tissue (whole pieces or sections) is exposed to a silver-containing solution. Silver ions bind non-specifically to the tissue and at certain significant sites some of the ions are reduced to silver “nuclei”, each consisting of several atoms of the metal and far too small to be visible. In the second step, known as development, a reducing agent changes dissolved silver ions (which may or may not be derived from those non-specifically bound by the tissue) into deposits of colloidal metal. This development reaction is catalyzed by the silver nuclei, which are thereby enlarged enough to form visible deposits. Thus, the second (development) step of an argentophil method is a chemical amplification of the invisible signal generated in the first (impregnation) step (11). Two examples of argentophilic methods will serve to illustrate different determinants of specificity and types of catalytic amplification.
In the Grimelius technique, for showing cells of the diffuse endocrine system (10), sections are impregnated with 0.004 M AgNO₃ in a 0.02 M acetate buffer at pH 5.6 for three hours at 60 °C. This solution has been found empirically to provide for nucleation of silver in and around the secretory granules of cells of the DES. The slides are then transferred to a 0.09 M solution of hydroquinone in 0.4 M sodium sulfite, in which they remain for one minute. Sulfite ions react with silver ions (partly derived from residual impregnating solution and partly from silver non-specifically bound to the tissue) to form a complex anion, [Ag(SO₃)₂]⁻. This is reduced by hydroquinone and the resulting black colloidal silver accumulates around the initially formed nuclei in the cytoplasms of cells of the DES. A positive result with this simple, inexpensive technique may avoid multiple expensive immunohistochemical tests for the various peptides and proteins that characterize different DES cell-types and tumors derived from them (8).

There are several silver methods for reticulin that have in common three major steps (12). Gomori's technique (13) is typical. The initially formed nuclei in the cytoplasms of cells of the DES have been articles of commerce for many years. Consequently, pararosaniline and new fuchsine are available as individual dyes, whereas products sold as basic fuchsin typically are mixtures (2, 3). All four components of basic fuchsin are suitable for making Schiff's reagent, but pararosaniline has been the one used in chemical studies. Triphenylmethane dyes are commonly formulated as shown in Figure 1 (5, 6, 7). However, it is now generally agreed to be an alkylsulfonic acid as shown (for Schiff's reagent made from pararosaniline) in Figure 1 (5, 6, 7).

References

How does pH influence the staining mechanism of Schiff’s reagent in biological tissue samples?

Schiff’s reagent is a solution made by reaction of basic fuchsin, a red dye, with sulfurous acid. The reagent ideally is colorless, but it may be light yellow on account of impurities in the dye. It was introduced by Hugo Schiff in 1866 as a chromogenic test for aldehydes. Traditionally, basic fuchsin was a mixture of four cationic red triphenylmethane dyes (pararosaniline, roseaniline, magenta II and new fuchsine) made by oxidizing a crude mixture of aniline and mixed ortho- and para-isomers of toluidine (1). Pure aniline, p-toluidine and o-toluidine have been articles of commerce for many years. Consequently, pararosaniline and new fuchsine are available as individual dyes, whereas products sold as basic fuchsin typically are mixtures (2, 3). All four components of basic fuchsin are suitable for making Schiff’s reagent, but pararosaniline has been the one used in chemical studies. Triphenylmethane dyes are commonly formulated as shown in Figure 1 (5, 6, 7). However, it is now generally agreed to be an alkylsulfonic acid as shown (for Schiff’s reagent made from pararosaniline) in Figure 1 (5, 6, 7).

Figure 1. The components of basic fuchsin (I - IV) and the formation of Schiff’s reagent by reaction of pararosaniline with sulfurous acid (V, VI).
Studies with NMR spectroscopy indicate that the major component is the zwiterionic form shown (VI) in which one of the amino groups is protonated (6). The colorless solution is stable only at low pH and in the presence of an excess of sulfurous acid. Raising the pH or reducing the concentration of sulfurous acid increases the sensitivity of the reagent for detecting aldehydes but also leads to regeneration of the original dye. Some of the equilibria that account for this instability (6, 8) are summarized in Figure 2 (VII, V-VI). For histochemical purposes high sensitivity is not usually needed and the reagent must be stable while exposed to the air and losing sulfur dioxide for 15 to 20 minutes. Accordingly, Schiff’s reagent for use as a biological stain must be acidic (pH about 2 is best for most applications) and it must contain more sulfurous acid than was needed to decolorize the basic fuchsin. If the pH approaches 3 a white precipitate may form after a few months of storage (4, 9). In equation VII it is seen that a solution at pH 2 contains predominantly bisulfite ions and sulfurous acid molecules. Reversal of the sulfonation, VI → V, occurs if there is loss of SO2 into the atmosphere (see VII) or if the pH is raised towards neutrality. SO2 can escape from a loosely capped bottle. The Schiff’s reagent then becomes pink and is unfit for use because it now contains some regenerated basic fuchsin, which could bind to polyanions in the tissue and give false-positive staining in cartilage matrix, mast cell granules and other materials that are stainable by cationic dyes at low pH. Reaction V → V of Figure 2 is reversible, so Schiff’s reagent that has become pink from losing SO2 can be regenerated by adding a source of sulfurous acid, such as thionyl chloride or sodium metabisulfite. Excessive acidification causes additional protonation, leading to the formation of IX, which does not react with aldehydes (6, 8).

The simplest (theoretically) and oldest way to make Schiff’s reagent is to bubble gaseous sulfur dioxide through a 0.3-1.0% (0.01-0.03M) aqueous solution of basic fuchsin. This is the method implied in Figure 2. The acidity of the solution is then due only to ionization of sulfurous acid (pK1 = 1.9) but it is almost impossible to adjust the amount of added SO2Cl to the range between the minimum needed to decolorize the dye and the maximum dictated by the solubility of SO2Cl2 gas in water. Another technique is to add some thionyl chloride, SO2Cl2, to the dye solution. This liquid reacts instantly with water: SO2Cl2 + 2H2O → 2HCl + SO2 + 2H2O providing both sulfurous and hydrochloric acids in a 1:2 molar ratio. Schiff reagents made with thionyl chloride can be stored for a few years in securely capped bottles (4, 9). Probably the most popular recipes for Schiff’s reagent (4, 7, 10) are those in which the sulfurous acid is formed by dissolving sodium metabisulfite in the dye solution and then adding hydrochloric acid, allowing control of both the pH and sulfurous acid content as well as avoiding the use of more hazardous chemicals. Many vendors sell Schiff’s reagent as a ready-made solution. A survey of 20 material safety data sheets (MSDS) shows that all were made with sodium metabisulfite, bisulfite or sulfite (0.1-2.0%), and hydrochloric acid, with concentrations of basic fuchsin ranging from 0.01 to 1.0%. The pH was reported in only seven of the MSDS, usually as a range such as 1.1-5.5 or 1.5-2.0.

The two most frequent histochemical applications of Schiff’s reagent are the periodic acid-Schiff (PAS) method for neutral mucosubstances and the Feulgen reaction, a specific stain for DNA. Investigations in the 1950s indicated that maximum staining intensity was achieved at pH 2.4 for the PAS method, whereas pH optima ranging from 2.3 to 4.3 were reported for the Feulgen technique (4). Current practice favors more acidic and therefore more stable solutions. The ambient pH changes when slides are washed after immersion in Schiff’s reagent. Rinses in 0.03 M sulfurous acid to remove unbound reagent were formerly commonplace (10, 11) but they weaken the final color. Rapid washing in water does not result in false-positive staining (12) and is currently recommended (9, 13). The rise in pH associated with washing in water for 10 minutes reduces the protonation of the stable dye-tissue complex (X in Figure 2), with resultant intensification of the color. The same effect can be achieved in 15 seconds with 0.3% sodium tetraborate, an alkaline solution (14).

References
What is Giemsa’s stain and how does it color blood cells, bacteria and chromosomes?

Stains for Blood

Probably the first use of a staining solution containing methylene blue (a cationic thiazine dye) and eosin (a red anionic xanthene dye) was in 1888 by C. Chenzinsky, to stain malaria parasites in blood films. F. Plehn in 1890 and E. Malachowski and D. L. Romanowsky in 1891 all independently described similar staining solutions that imparted a variety of colors to erythrocytes, leukocytes and malaria parasites. Notably, leukocyte nuclei were purple and the parasite nuclei were bright red; these colors could not be obtained with any technique in which the two dyes were used sequentially. Romanowsky’s thesis (in Russian) and publication (in German) noted that the desired colors were obtained only when the methylene blue was derived from a stock solution old enough to have mold growing on its surface. The improved staining properties of “ripened” methylene blue solutions were studied by Unna (1899), who also published in 1891 and found that the aging process could conveniently be accelerated by heat and alkalinity. Artificially ripened or polychrome methylene blue was used by most later inventors of blood stains, including L. Jenner (1899), B. Nouch (1899), W. Leishman (1901), R. May and L. Grunwald (1902), and J. Wright (1902) (1). The products of polychroming, studied by Kehrmann (4) and later by Home and French (5), result from demethylation of methylene blue and also from oxidation of the resulting dyes (Fig. 1).

Staining solutions made by mixing solutions of polychrome methylene blue and eosin are unstable because the oppositely charged dye ions combine to form salts, known as azure eosinates, that are insoluble in water. Azure eosinates are soluble in alcohols – a mixture of methanol and glycerol is commonly used – and they dissolve in water in the presence of an excess of either thiazine dye or eosin. Furthermore, continued oxidation results in the deposition of other water-insoluble products such as methylene violet (Bernthsen) (Fig. 1). For the production of commercial blood stains the precipitated eosinate is collected and dried (6, 7). Blood stains devised after 1902, such as Macneal’s “tetrachrome” (1922) and the formulation of Roe, Lillie & Wilcox (1941), made use of eosinates of azures A and B and methylene blue. Later work showed that all commercially produced thiazine dyes were mixtures of the compounds shown in Figure 1, with the nominal dye sometimes not the most abundant component (6, 8).

Giemsa’s Formulation

Gustav Giemsa (1867-1948) endeavored to produce mixtures of dyes that would reliably provide the Romanowsky color scheme (6). These formulations were published in 1902-1904, with the most advanced version being an aqueous solution containing a large excess of azure II over eosin Y. Azure II was a deliberate mixture of unoxidized methylene blue with azure I. Azure I was the name used at that time for the dye now called azure B. Modern formulations of Giemsa’s stain are made by mixing azure B eosinate with methylene blue and are commercially available as Giemsa powder, which is dissolved in a 50/50 mixture of methanol and glycerol to provide a concentrated stock solution (9). For staining, the stock solution is diluted in water that is buffered to a pH appropriate to the intended application – pH 6.5-7.0 for alcohol-fixed blood films, lower for sections of formaldehyde-fixed tissues (2).

Dyes Needed for the Romanowsky-Giemsa Effect

The compositions of blood stains were critically studied, using modern chromatographic and analytical methods, in the 1970s, notably by Paul Marshall in the UK (7, 8) and Dietrich Wittekind (2, 10) in Germany. These investigations revealed that the nuclear and all other colors constituting the “Romanowsky-Giemsa effect” could be obtained with solutions containing only azure B and eosin Y. Pure azure B, made by direct synthesis rather than from polychromed methylene blue, has been commercially available (as chloride, tetrafluoroborate or thiocyanate) since 1980 (11) and is used in a “Standardized Romanowsky-Giemsa method” (12) in which a stable methanolic solution of the eosinate is diluted with an aqueous buffer to make the working staining solution.

Pure azure B is more expensive than the crude variety, and there is still considerable demand for Giemsa’s, Wright’s, Macneal’s and related blood stains made from polychrome methylene blue and from impure azures A and B. The chemical compositions and staining properties of these traditional mixtures have been shown to vary...
Special Stains and H&E

Among different suppliers and even among different lots from the same supplier (7, 8). In the USA, samples of batches of methylene blue, azures A, B and C, methylene violet (Bermthsen) and powders for Giemsa’s Jenner’s and Wright’s stains are tested by the Biological Stain Commission (BSC) by assaying dye content, spectrophotometry, thin-layer and high performance liquid chromatography and use in standardized staining procedures (13). Satisfactory batches are certified, and each bottle of dye powder carries a small additional label, provided by the BSC, attesting to this fact. Certified dyes should always be used for preparing solutions of Romanowsky-Giemsa stains. Azure B, the single most important ingredient of these stains, does not have to be the ultra-pure directly synthesized dye to meet the BSC’s requirements for certification (Fig. 2).

Staining Mechanisms

Studies of the interaction of DNA with azure B (14, 15) indicate that in solution the dye cations are present as dimers, each with the two planar ring systems held together by van der Waals forces. The dimers are attracted to the negatively charged phosphate groups of DNA and adhere to the macromolecule as a result of hydrophobic interactions with the purine and pyrimidine rings of the DNA bases. Attraction of eosin anions by un-neutralized positive charges of bound azure B dimers changes the color of the stained DNA from blue to purple (16). Eosin is also attracted to proteins with excess protonated amino and guanidino groups (mainly side chains of lysine and arginine) over ionized carboxy groups (mainly glutamic and aspartic acids); these proteins are hemoglobin in erythrocytes and the major basic protein of eosinophil granules (17). The red coloration of malaria parasite nuclei may be a due to a preponderance of basic proteins (histone) over DNA in the nuclei of protozoa (18, 19) and some invertebrates (20).

Giemsa for Tissue Sections

The expected colors in cells of blood or bone marrow are seen only in alcohol-fixed films or smears and when the diluted staining mixture is close to neutrality - usually pH 6.8. Wright’s or Leishman’s stain is usually used, being allowed to act for about 3 minutes. Giemsa’s stain, which is much more stable when diluted, can be allowed to act for 15-45 minutes. After fixation with formaldehyde, which reacts with amino and other basic groups, nearly everything stains blue at pH 6.8. Paraffin sections can be stained with Giemsa at pH 4 to 5. The sections show blue (not purple) nuclei, pink erythrocytes, cytoplasm and collagen, and metachromatic (red-purple) mast cells and cartilage. The colors are not the same as those seen in alcohol-fixed blood films (21-23). Giemsa is often used to stain bacteria in sections of formaldehyde-fixed gastric biopsies. The organisms are dark blue against an unstained or light pink background (23). The substrates of staining are presumably bacterial DNA and RNA, which differ from their animal counterparts in not being associated with basic proteins (24). The teichoic acids of the cell walls of Gram-positive bacteria probably also bind cationic dyes, and the phosphate groups of lipopolysaccharides can be expected to have a similar role in Gram-negative organisms.
Chapter 20 | H&E Staining

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What is the difference between progressive vs. regressive hematoyxlin staining?

Progressive hematoyxlin stains color primarily chromatin and to a much lesser extent cytoplasm to the desired optical density, regardless of the length of staining time. Regressive hematoyxlin stains over-stain chromatin and cytoplasm and require subsequent immersion in dilute acid to pull out the excess color from the chromatin and cytoplasm (Table 1). If differentiation is omitted or incomplete, residual hematoyxlin visually obscures fine chromatin detail and can prevent the uptake of eosin entirely.

Gill hematoyxlin No. 1 and 2 contain 2 and 4 gm hematoyxlin per liter, respectively, and 25% ethyl alcohol. They are progressive stains that can be applied for many minutes without over-staining and without differentiation in a dilute acid bath. Harris hematoyxlin contains 5 gm hematoyxlin per liter of water. It over-stains within minutes and requires differential extraction in dilute HCl to decolorize the cytoplasm (differentiation) and to remove excess hematoyxlin from chromatin. Figure 1 illustrates the difference between the two approaches.

Do you have a preference for progressive or regressive hematoyxlin staining?

I prefer progressive hematoyxlin staining because it does not require differentiation. Under- or over-differentiation can produce over-staining or under-staining. Depending on the degree of timing control exercised in a given laboratory, the results may be satisfactory one day, hyperchromatic another day, and hypocromatic the next. Extreme hyperchromasia can block entirely the uptake of eosin so that H&E becomes simply H.

What is the difference between differentiation and bluing?

Differentiation and bluing (bluing, if you prefer the English spelling) are essential to satisfactory staining by hematoyxlin. Differentiation is used only with regressive hematoyxlin formulations, while bluing is used with both regressive and progressive hematoyxlin formulations. Differentiation effects quantitative changes; bluing, qualitative. See Table 2.

Are there reasons to prefer water or alcohol as the solvent for eosin formulations?

I prefer alcohol-based eosin formulations: 1) they are chemically more stable 2) they minimize, if not eliminate entirely, the unpredictable effects of various impurities such as water-soluble salts that in water may interfere with dye uptake, and 3) they tend to stain more slowly than water-based formulations (promotes a wider range of shades of eosin colors).

Is there a simple way to perform quality assurance (QA) on hematoyxlin and eosin stains before using a batch for the first time?

Yes. Whether buying or making hematoyxlin eosin solutions, you cannot absolutely ensure the product will perform. Apart from unsound methods, limitations in ingredients, incorrect formulations (e.g., precipitated mordant crystals in commercial Harris hematoyxlin formulations), and errors in formulating (e.g., weighing out too much oxidizing agent) can contribute to unsatisfactory results. It doesn’t happen often, but it does happen. Regulatory documentation does not guarantee efficacy.

Formalin-fixed tissue sections or alcohol-fixed buccal smears are immutable probes to: 1) determine the performance of each new lot of stain, 2) select suitable staining times, 3) find out how many slides can be stained satisfactorily by a given volume of each stain, 4) learn when rinses should be changed, and 5) troubleshoot whether a given stain already in use is the cause of an observed staining problem. Once experience imparts confidence to selected staining times, stain and rinse change schedules, the use of control sections or smears is no longer necessary for the remainder of the life of the particular stain that has been validated. However, control preparations should be used when new containers of the same stain with different lot numbers are opened to confirm that the stain does indeed perform as expected. Manufacturers occasionally make bad batches of stain. Simply looking at the first slides stained daily and initialing a stain quality log sheet is of no value if a laboratory has not defined its standards. It is not uncommon to see such sheets dutifully maintained and also to see unsatisfactory staining results.