Nuclear staining with alum hematoxylin

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Abstract

The hematoxylin and eosin stain is the most common method used in anatomic pathology, yet it is a method about which technologists ask numerous questions. Hematoxylin is a natural dye obtained from a tree originally found in Central America, and is easily converted into the dye hematein. This dye forms coordination compounds with mordant metals, such as aluminum, and the resulting lake attaches to cell nuclei. Regressive formulations contain a higher concentration of dye than progressive formulations and may also contain a lower concentration of mordant. The presence of an acid increases the life of the solution and in progressive solutions may also affect selectivity of staining. An appendix lists more than 60 hemalum formulations and the ratio of dye to mordant for each.

Key words: H & E, hematoxylin, hemalum, mordant, nuclei

Of all the staining methods used in histology laboratories, hematoxylin and eosin (H & E) must surely hold pride of place. It would be difficult to imagine a histology laboratory without it. It is often said that, “familiarity breeds contempt,” so perhaps it is not surprising that of all the stains we use, H & E still generates many questions.

H & E staining is an old method dating back to the later part of the 19th century. Conn’s Biological Stains (Horobin and Kiernan 2002) gives a date of 1865 for Bohmer’s use of hematoxylin with alum and 1891 for Mayer’s comments about ripening hematoxylin. It has been a standard staining method since those times and histotechnologists are quite familiar with the technique, yet questions are asked by experienced and inexperienced technologists alike regarding the most appropriate formulation to use in a given circumstance.

Technologists also need to know what to do when the staining does not meet expectations. As an example, comments are sometimes made about unexpected mucin staining with Gill III and other strong hemalum solutions. Within the context of the comments is the inference that this is an undesirable thing. Is it undesirable? If so, how do we stop it? Integral to this issue are the technologist’s expectations and control of the staining process to optimize the appearance of the stained section. Surely, if a histotechnologist is to understand any staining technique it should be this one. It is so fundamental to the functioning of a modern histology laboratory and likely to remain so for a long time to come, that lacking understanding constitutes a distinct drawback.

Nuclear material

There are two components to nuclear staining. They are the mordanted hematein and the nuclear material stained. Many years ago, when nobody really knew what was being colored in the cell nucleus, the word “chromatin” was introduced as a convenience to describe the stained material. According to Baker (1958a), this word was introduced by Flemming with the definition: “that substance in the cell nucleus which takes up the dye in the treatments with dyes known as nuclear coloring.” Since the early days, the material in the nucleus that reacts with dyes has been identified as mostly DNA. The word “chromatin” came to be used as a synonym for fixed and preserved DNA, and later fell into disuse. It is well worth emphasizing, however, that
“chromatin” and “DNA” are not synonyms. Stainable material in the nucleus includes DNA, but is not confined to it. It is possible to stain the nuclear structure with hemalum despite having removed the DNA, indicating that some other material, probably protein, also can be stained. Baker investigated this in 1962 (Baker 1962), so it is not something new. He had this to say: “Although the dark blue of chromatin in a differentiated preparation is almost entirely due to its nucleic acid content, yet the proteins of chromatin are capable of giving a closely similar appearance after the nucleic acids have been removed.”

**Fixation and processing**

We tend to think of fixation, dehydration, clearing and infiltration as separate processes. Actually, they are all part of a single sequence and they affect each other. There is no doubt that the quality of an H & E stain depends on thorough performance of this sequence from start to finish.

Regrettably, owing to a desire for a speedy diagnosis, inadequate fixation is common in service laboratories. It requires 24–48 h for a 3 mm thick piece of tissue to be fixed with 4% neutral buffered formaldehyde (NBF); even a 1 mm core biopsy should be treated with NBF for that length of time for optimal fixation. It is, after all, a chemical process that requires a certain period of time to be completed. In practice, it is not uncommon for tissue to be in NBF for only 6–8 h, then processed. Larger specimens may be trimmed and unfixed pieces put into NBF for only a few hours before processing. From a chemical point of view, this is completely inadequate, even with the application of heat, and it is a significant cause of sub-optimal staining with H & E. The best results always are obtained with well fixed, dehydrated and infiltrated material that is sectioned reasonably thinly.

**Hematoxylin**

The major component for H & E nuclear staining is a hematoxylin derivative. For convenience, we usually refer to this as hematoxylin, although the actual dyestuff is hematein, an oxidation product of hematoxylin. Oxidation takes place by the removal of one hydrogen atom from a hydroxyl group leaving an oxygen with double bonds. This forms a para-quinonoid ring as shown below. It should be noted that there are other hydrogen atoms that could be replaced, i.e., further oxidation can take place. When this happens the dye usually is rendered unsuitable for use, so we usually refer to it as over-oxidation, a process we try to prevent.

Hematoxylin is a natural product extracted from a small tree, *Haematoxylum campechianum* L., also known as *Hematoxylon campechianum*. Both names are commonly used. A Google search on the Internet located more than 54,000 references for *Haematoxylum* and almost 50,000 for *Haematoxylon*. The genus name comes from the Greek “haimatos” meaning blood and “xylon” meaning wood. In North America it is usual to drop the letter “i” from the name. The wood goes by many names; most are translations into various languages of “blood wood” or “Campeche wood” or something similar.

There is a comprehensive article on the history of logwood, as hematoxylin wood is known, on Wayne’s Word (Armstrong 1992). It is interesting to note that Belize, originally named British Honduras, developed as a British possession primarily because of the logwood industry and the importation of logwood chips to Britain. The Belize flag refers to this, displaying two logwood cutters. The Wikipedia article about Belize (Wikipedia 2008a) says: “Taking advantage of Spain’s inability to establish control over present-day Belize, Englishmen began to cut logwood (*Haematoxylum campechianum* L.), a dyewood greatly valued in Europe as the principal dyestuff for the expanding wool industry.”

Logwood still has commercial importance. PROSEA (Plant Resources of South-East Asia) is a Dutch-based foundation devoted to economic development in areas formerly under Dutch control. Their third publication is titled “Dye and tannin producing plants” (Lemmens et al. 1992). One such plant is *Haematoxylum campechianum*, and the book gives a full description of the tree. It is a leguminous tree, i.e., a member of the pea family. It is bushy and fairly slow growing, but can reach 15 m tall. The trunk is not straight enough for use as lumber, but small articles can be made. There are many photographs of the tree on the Internet. Wayne’s Word has several and the Tropical Plant Database has a photograph of the leaves (Raintree 1996), as does “The Banana Tree” (Banana Tree). There are some beautiful photographs of the

![Hematoxylin and Hematein](image)
flowers and leaves (Carr 2004) on an Internet site at the University of Hawaii.

The method of dye extraction that PROSEA describes is to make small chips of the heartwood of the tree, then extract the dye with boiling water. The extract then is cooled and the water removed by evaporation. The resulting powder is crude hematoxylin. This undoubtedly is further purified before sale to laboratories.

Oxidation

It used to be a common practice for histology laboratories to keep a stock alcoholic solution of hematoxylin, often several years old and said to be a 10% solution. Because hematoxylin originally was oxidized atmospherically, it was necessary to keep such a solution for occasions when hematein was required rather than hematoxylin. Originally, this solution was made by direct extraction of the dye from wood chips into ethanol. Because logwood is a biological product, wood from a tree, material other than hematoxylin was extracted and the extract was quite variable in concentration. Today if we need an ethanolic solution of hematoxylin, it is a very simple matter to dissolve the dye in the ethanol.

How much hematoxylin would such a solution contain? The Sigma Aldrich Handbook of Stains, Dyes and Indicators (Green 1990) says that hematoxylin dissolves in both ethanol and water to 3%. Conn’s Biological Stains (Horobin and Kiernan 2002) also gives 3% for water and ethanol, but for hematein, gives 1.5% in water and 7.5% in ethanol. Clearly, a stock ethanolic solution cannot be 10%, so everything prepared from it must contain less dye than expected. In a solution containing excess undissolved dye, the actual concentration depends on how much has oxidized to hematein, because the solubilities of hematein and hematoxylin differ. The concentration increases until full oxidation has taken place, but will not exceed the saturation point of about 7.5%. Such solutions should not be filtered to remove any sediment until they are used, because the sediment is the source of the increase in concentration. These solutions have a very long life. In fact, in the past it was often stated that they never over-oxidized. That is not true, although it may take some years for it to happen if the solution is used only occasionally and is stored in a tightly capped container. Such solutions are not needed today, because an oxidized hematoxylin solution may be prepared quickly by dusting a small aliquot of a freshly made solution with sodium iodate.

Many of the methods requiring ethanolic hematoxylin are iron-mordanted techniques. The mordants we use for iron hematoxylins, ferric salts, are oxidizing agents anyway, so well ripened solutions may not be the most desirable in the first place.

Oxidation of hematoxylin to hematein originally was atmospheric, but that is not the most common practice today. Most laboratories either purchase solutions already made and oxidized chemically or they add chemical oxidants to their own solutions. Some technologists believe the naturally oxidized solutions to be superior to those chemically oxidized, but others see little difference between solutions oxidized either way. There certainly are no published experimental data to support one way over the other, other than the obvious convenience of chemical oxidation.

The most common oxidant in use is sodium iodate at the rate of 0.2 g for each gram of hematoxylin for full oxidation. At room temperature, oxidation usually is complete after 24 h. Boiling the solution brings full oxidation immediately. Some technologists add somewhat less than 0.2 g per gram dye to strong alum hematoxylin solutions, i.e., those containing 4 g or more of dye per liter. Half oxidation often is used with strong alum hematoxylins to extend their useful lives. Half oxidation, as the term implies, requires 0.1 g of sodium iodate for each gram of hematoxylin in a solution. The unoxidized hematoxylin slowly oxidizes atmospherically and this increases the useful life of the solution.

Sodium iodate is so convenient and fast that we scarcely need other oxidants. Others have been used in the past, but rarely are used now. Mercuric oxide was used for Harris’ hemalum, which is quite popular in North America, and required boiling of the solution. Today, Harris’ is usually made with sodium iodate as the oxidant owing to concerns about worker safety and contamination of the environment with mercury, and this often is called “mercury-free Harris’ hematoxylin.” Common oxidants are listed in Table 1.

Hematoxylin or hematein

Because we know that the actual dye is hematein, not hematoxylin, the obvious question is why do we not use hematein to make the solutions and avoid all this fuss about oxidation? The answer is that, despite manufacturing advances, hematoxylin powders are more reliable than hematein powders. A good, reliable sample of hematoxylin can be purchased from almost any supplier with
confidence, but hematein varies considerably in quality. The most likely cause of this is that hematein can be oxidized atmospherically to form products that do not stain satisfactorily. Hematoxylin also is oxidized, but the product is hematein, so this oxidation does not affect staining. Notwithstanding that, a good sample of hematein can be used to formulate quite satisfactory hemalum solutions. Some formulae that specify hematein are listed in Table 2. Hematein can be used to make any hemalum by the simple expedient of omitting the oxidant. Similarly, any hemalum specifying hematein can be made with hematoxylin if an appropriate amount of an oxidant is added.

Mordants

In the late 1800s it was noted that when mixed with alum, hematoxylin could be used to stain nuclei. The original formula was by Böhmer (Gatenby and Beams 1950, Gray 1954). This required an ethanolic extract of logwood chips that had been allowed to stand and oxidize until deep brown. For use, a small amount of a 0.3% solution of ammonium alum, AlNH₄(SO₄)₂ 12H₂O, was placed in a watch glass and a few drops of the tincture of hematoxylin added. If a watch glass holds 5 ml and a “few drops” is 0.3 ml (three drops of a solution containing 7% hematein), then this amounts to about 4 g hematoxylin and 30 g alum per liter. The aluminum in the alum plus the dye form a strongly staining compound that preferentially colors nuclei. Böhmer’s technique would still work, of course, but our knowledge of what is needed to produce intense staining is more complete than it was back then and the method is obsolete.

Böhmer’s procedure does make clear the minimum requirements for a hemalum: (1) a solvent such as water; (2) ripened hematoxylin; and (3) an aluminum source as a mordant. Other ingredients may facilitate the staining in some fashion, but these three are essential.

The terms “mordant” and “mordanting” often are used in histology to describe a variety of unrelated processes. For example, the terms are often used to describe postfixation treatment of sections from formalin fixed tissue with Bouin’s picro-formalin-acetic mixture prior to staining with Masson’s trichrome. The terms also are used to describe iodine treatment of crystal violet stained sections in a Gram’s stain. In fact, almost any treatment of histological material that has the end result of improved staining in some fashion is referred to as mordanting and it has become something of a catch-all term. Speaking precisely, however, none of these is true mordanting. Bouin’s fluid is used as a secondary fixative for sections and iodine in Gram’s stain functions as a trapping agent. Other procedures may involve pH control to promote or inhibit staining, or may indeed involve some treatment that does function as a true mordant.

For the purposes of this discussion the term mordant will be used correctly, i.e., a polyvalent metal ion that forms coordination complexes with certain dyes. The “certain dyes” are those given the Color Index the functional name, “mordant” dyes and include also some natural dyes. Hematein is one of those “certain dyes.” As a natural source dye, it has been given the functional name of natural black 1 (Horobin and Kiernan 2002).

The three metals most commonly used with hematein are aluminum, usually from ammonium or potassium alum; iron, usually as ferric chloride or ferric ammonium sulfate (iron alum); and tungsten from phosphotungstic (tungstophosphoric) acid. Several other metals have been used, but the following discussion will concentrate on aluminum-mordanted hematein. Aluminum is the usual mordant for general

| Table 1. Oxidant per gram of dye (Gatenby and Beams 1950, Gray 1954, Lillie 1954, Culling et al. 1976, Debidin 1987, 1991) |
|-----------------|-----------------|-----------------|-----------------|
| Oxidant         | Formula         | Maximum         | Recommended     |
| Sodium iodate   | NaIO₃           | 200 mg          | 40-150 mg       |
| Mercuric oxide  | HgO             | 500 mg          | 100 mg          |
| Potassium permanganate | KMnO₄ | 177 mg          | 175 mg          |
| Potassium periodate | KIO₄ | 50 mg           | 50 mg           |
| Hydrogen peroxide 3% aqueous | H₂O₂ | 2.0 ml          |                 |
| Sodium hypochlorite (5.25% Javex® bleach) | NaOCl₂ | 2.0 ml          |                 |
| Calcium hypochlorite |               | Anderson used 1.6 g per g hematoxylin in one variant and 8 g per g hematoxylin in another. |
nuclear staining with H & E, evidenced by the use of the term “hemalum” as a short way to refer to these solutions. Originally applied to Mayer’s progressive solutions, the term now generally is used for all aluminum-mordanted hematein solutions.

For histology, a limited number of “certain dyes” are used. Hematein and carminic acid are used commonly as are some oxazone dyes, such as celestine blue and galloycyanine, and the triaryl methane dye, eriochrome cyanine R. Less common is brazilein, a close relative of hematein, and some natural dyes related to carminic acid and also derived from insects. Of these, the oldest known may be kermes or kermesic acid, mentioned in the Bible in the book of Exodus (Darby 1890), and laccaic acids (Llewellyn 2005) used in India and other Eastern countries since ancient times to color lips and nails, and during weddings to color the soles of the feet (Food and Agricultural Organization of the United Nations 1995). It may also be used to color food in modern times (Canadian Patents Database 1996).

Laccaic acids are important from a historical perspective even though the dyes rarely are used histologically. Like kermesic and carminic acids, laccaic acids, or lac dye as they are collectively known in producing countries, are extracted from the bodies of insects that feed on tree sap. In the case of lac, the insects also exude a gum that is cleaned, dissolved in an alcohol and used as wood finish shellac or as an edible coating for foods such as fruit or candy. The Hindi word, laksha, from which the English word lac is derived through Latin refers to the insect, the mordanted dye and the gum (Platts 1884). This term was anglicized to lac, which was translated as lake and is still used to describe mordant-dye complexes used in histological procedures.

A lake may be defined as a coordination complex formed between a polyvalent metal ion and certain dyes. Lakes are formed between the metal and dye by chelation. It is stated in Wikipedia (2008b) that the term chelate was first used in 1920 by Morgan and Drew, who said, “The adjective chelate, derived from the great claw or chele of the lobster or other crustaceans, is suggested for the caliper-like groups which function as two associating units and fasten to the central atom so as to produce heterocyclic rings.” With dyes, the metal atom is “gripped” firmly by two covalent bonds, one of which is polar with both its electrons derived from a double bonded oxygen atom of the dye. The non-polar covalent bond is between a hydroxyl or a carboxyl group and the metal (Baker 1958b).

Although it is possible for more than one dye molecule to bond to an aluminum atom, it is unlikely, because the mordant usually is present in considerable excess. It is more likely that an aluminum atom would be associated with only one molecule of dye and that there also would be many free aluminum ions unattached to dye molecule.

**Attachment to tissue**

It still is not clear how a lake attaches to chromatin. Baker explained it by stating that “aluminum-haematein is a typical cationic dye” and he emphasized that the most common material involved is DNA, although he noted also that chromatin could be stained with hemalums after removing the DNA. He explained this as owing to staining of acidic proteins. Lillie et al. (1974) proposed that basic amino acids were involved, including arginine, and Horobin has suggested that the attachment may involve both ionic and non-ionic forces (Kiernan 1999).

One well-known characteristic of strong hemalums is that they often stain acid mucopolysaccharides. This can be so distinct that Mayer recommended an aluminum hematein formula for
selective mucin staining that is similar to his mucicarmine (see Appendix 3).

**Using mordants**

There are four ways in which dyes and mordants may interact with the tissues. The terms come from the textile dyeing industry and include the word, “chrome.” In this case, chrome refers to chromium, which is or used to be a common mordant for textile dyeing.

**Onchrome**

The mordant is applied first and attaches to some tissue components. The dye then is applied and reacts with the mordant. This is fairly common in histology; Heidenhain’s iron hematoxylin is a typical example.

**Metachrome**

The mordant and dye are combined in a single solution, then applied together. The soluble lake then attaches to tissue components. This is the common way that mordant dyes are applied in histology including iron, aluminum and tungsten-mordanted hematein.

**Afterchrome**

The dye is applied to the tissue, then washed off and the mordant applied. Lake formation takes place after the dye has stained the tissue. This procedure is rare in histology. Lillie et al. (1975b), however, used the dye phenocyanin TC to stain nuclei as a hematoxylin substitute in this manner using a ferrous salt as the mordant.

**Mordant substitution**

Displacement involves staining sections with a lake made with one mordanted dye, then applying a second mordanted dye to replace the original. The celestine blue–hemalum sequence is of this type. Some technologists use an acidified iron alum solution rather than celestine blue–iron alum as the primary stain in this procedure with equally satisfactory results, indicating that the iron alum is the active ingredient.

**Mass action**

Often, when we talk about how dyes, mordants and tissue components react with each other, we do so by reference to individual atoms and molecules. In the real world of histological staining, however, there is no procedure that uses a single atom or molecule. When we apply a solution of a mordanted dye to a section we are attempting to manipulate millions, perhaps billions of molecules. What may happen at the discrete level is only part of the story; the rest of the story is what happens at the mass level. By looking at chemical reactions from a gross perspective, we can see how factors other than the different types of chemical bonds can influence the end result. We may take into account that many staining reactions take some time to reach a certain level of intensity, that temperature differences may increase or decrease intensity for any given time of application, or that the relative amounts of the chemicals in a solution may have an effect on how strongly a tissue component is stained.

**Hemalum formulae**

For H & E staining, metachrome solutions invariably are used in modern practice. There are numerous formulae for making these hemalum solutions; more than 60 are on the StainsFile web site. A list of these formulae is appended to this document (Llewellyn 2009). See Appendix 4 for a list of formulae organized by the amount of hematoxylin and their dye:aluminum ratios. The formulae show considerable variability, ranging from 1 to 20 g hematoxylin and 6 to 140 g alum per liter of solvent, with or without some acid. The solvent varies from plain tap water to various mixtures of water, ethanol, methanol, and isopropanol and may include glycerol, ethylene glycol or propylene glycol.

Hemalum solutions are described as either regressive or progressive depending on whether they are used with or without differentiation. Although the extremes are quite far apart, many progressive hemalums have about 1–1.5 g of dye per liter, while those usually thought of as regressive have 4–6 g of dye per liter. The amount of alum is more consistent, with most containing about 40–60 g per liter, occasionally up to 100 g. The most obvious difference is the amount of hematoxylin present. The most significant factors are the amount of dye present and the ratio between the dye and the mordant. These ratios are presented in a chart at the end of this document (Appendix 2). In some solutions, the amount and type of acid also may be a factor, but the presence of acid is not as decisive as often is assumed. Comparing Lillie’s (1954) regressive formula (Lillie
Comparison of Mayer (1891) var-b formula shows the difference clearly. Mayer’s hemalum has 1 g of hematoxylin with 50 g of ammonium alum, while the Lillie solution has 5 g of hematoxylin with 50 g of ammonium alum, and both contain a little acetic acid (Table 3). That is, the Lillie formulation has a five-fold more concentrated dye solution than Mayer’s. The difference in staining, however, is quite striking. With a 10 min application, Mayer’s gives classic progressive staining, with sharp nuclei and little cytoplasmic color. Lillie’s formulation gives a dense overstained section that must be differentiated. Even then, there is significant coloration in cytoplasm and there may be some mucin staining.

A point must be stressed here. The depth of staining does not just increase in intensity five-fold. The increased concentration of dye causes material to stain that was left unstained in weaker solutions. The increased concentration of dye has an effect not only on intensity, but also on what stains. Mucin is an example. It is rare for progressive formulations to demonstrate mucins, but it is quite common in strong, well ripened formulations, especially if they are acid-deficient.

There are a few formulations that do not fall clearly into either of the two usual groups. I have referred to these as “intermediate” formulations. They may be thought of as either strongly staining progressive solutions or as lightly staining regressive formulations. Their importance is in the way they validate the role of dye concentration in the staining patterns obtained. They show that depth of staining is a continuum ranging from the highly selective nuclear stain to the completely non-selective over-staining obtained with strong formulae. We may obtain any degree of staining on that continuum simply by modifying the amount of hematoxylin in the solution. This approach is not common, but may be more rational than changing the hemalum used because it is too dark or too light. The addition of a solution containing all the ingredients except the dye would reduce the intensity of background staining, whereas adding a concentrated dye solution would increase it. That the amount of dye present has the most significant effect on whether a hemalum is best used progressively or regressively can be demonstrated in an exercise comparing the staining obtained with a fixed concentration of alum and varying amounts of dye. Differences in staining observed with these solutions are attributable to differences in dye concentration. See Appendix 5 for details.

Dye concentration, however, is not the only factor. For a given concentration of dye, increasing the concentration of mordant usually causes greater selectivity of staining. Compare Bennett’s and Langeron’s hemalums, for example. Both contain the same ingredients at the same concentrations except for the alum. Bennett’s formula contains 90 g per liter, whereas Langeron’s has 50 g. Owing to this, it is expected that Bennett’s hemalum would be more selective for nuclei than would Langeron’s.

A second point is that for a given concentration of hematoxylin, a hemalum with a higher concentration of alum is more selective for nuclei. This can be shown also in a staining exercise, this time using a fixed concentration of dye and varying amounts of alum. Differences in staining are attributed to differences in mordant concentration. See Appendix 5 for details.

A third factor is whether there is any acid present and if so, what kind. This often is said to be the deciding factor, but its effect often is overstated. Acids appear to have been added originally to extend the useful life of the solutions. As hemalums are used, small amounts of tap water may be carried over and this contaminates them. In solutions that do not incorporate acid, the low pH necessary for staining is obtained from the alum. The buffering capacity is low, however, and contamination with water quickly raises the pH of such solutions to a level that does not permit staining to take place and the solution develops a murky blue appearance. The addition of some acid, in the range of 20–50 ml glacial acetic acid per liter, increases the buffering capacity and extends the usable life of the solution from days to months.

Table 3. Comparison of Mayer’s and Lillie’s hemalums

| Formula         | Dye | Mordant | Source            | Water | Acid  | Other          |
|-----------------|-----|---------|-------------------|-------|-------|----------------|
| Mayer 1891      | 1.0 | 50 g    | Ammonium alum     | 1000  | Acetic | E95 = 50       |
| var-b (Gray 1950) |     |         |                    |       |        |                |
| Lillie (1954)   | 5.0 | 50 g    | Ammonium alum     | 700   | Acetic | GI = 300, NI = 0.5 |

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Whether acid is added or not, it is good practice to rinse sections in distilled water prior to placing them in the hemalum; this significantly reduces problems. Adding a small amount of acetic acid often rejuvenates old solutions, although there is a limit to the number of times this can be done.

The addition of some acid has a greater effect on causing weaker dye solutions to become nucleus-selective than stronger ones. An acidified Lillie formula (Lillie 1954), for example, stains much the same whether the acid is present or not. On the other hand, the progressive Cole’s hemalum (Drury and Wallington 1980), usually used without acid and giving staining comparable to that obtained with a differentiated regressive hemalum, becomes a very nucleus-selective progressive stain when 20 ml per liter acetic acid is incorporated. In this case, the presence of acid improves nuclear staining selectivity distinctly.

The type of acid also can be a factor. There is little to choose between 20 ml glacial acetic acid per liter and 1 g citric acid; they bring about much the same effect. The addition of concentrated hydrochloric acid, however, is a different matter. Hydrochloric acid lowers the pH enough that only nuclear structures stain. For that reason, Krutsay’s hemalum (Humason 1967), which contains 5 ml hydrochloric acid per liter, probably is the most specifically nuclear stain of all hemalums; nothing stains but nuclei. In addition, the hydrochloric acid removes calcium deposits so they are not detected. Depending on the goal, this may or may not be desirable. The effects of added acid can be demonstrated by repeating the previous two exercises, but with the addition of acetic, citric or hydrochloric acids.

Apart from the oxidizing agent, the other chemicals that may be added to hemalums are either anti-evaporants or antioxidants. These are added to extend the useful life of the solution, or to produce a more even staining pattern by making the solution more viscous. In addition, “thicker” solutions may trap some of the precipitate that strong formulations often produce. These other ingredients have little effect on the staining patterns and are added primarily for convenience. In practice, regular filtering is advised for all formulations, strong or weak, but particularly if a metallic scum forms on the surface.

The usual explanation for why some formulae are progressive and some regressive is that in a solution with a lower dye concentration there is more aluminum available to combine with each molecule of dye. There is, in effect, a “competition” for the dye by each aluminum atom. With a higher dye:aluminum ratio, more dye is retained by the aluminum in solution and less is available to combine with nucleic acids. The dynamics are biased in favor of the solution over the dye. The greater the aluminum content, i.e., the higher the dye:alum ratio, the greater this bias and the more selective the staining. When there is more dye or less aluminum present, the number of aluminum atoms available is less for each dye molecule, so the bias is favor of the nucleic acids and other stainable material. Stronger solutions also are more prone to non-ionic staining, i.e., hydrogen bonding and van der Waal’s forces. The end result is that non-selective staining generally increases as the dye concentration increases.

**Application time**

There is some correlation between the length of time that a hemalum is applied to a section and the depth of staining obtained, but it is not absolute. Progressive solutions, for example, stain rapidly at first, but once a certain level of intensity is reached, they stain more slowly; they exhibit a degree of self-limitation. Indeed, that is what makes them progressive in the first place. This holds true within reasonable times of application. If applied for unreasonable periods, the intensity darkens and less selective staining is obtained, which requires differentiation. Reasonable times are within the range of 5–30 min, and unreasonable times are measured in hours.

Regressive hemalums are more prone to produce densely stained sections no matter how long the solution is applied. Certainly, many of them produce dense nuclear and cytoplasmic staining after 5–10 min and require differentiation. A few, however, were designed to be applied for longer periods. It often is recommended that Ehrlich’s hematoxylin be applied for a minimum of 20–30 min, for example. In general, if hemalums are applied for equal periods, a regressive solution stains more deeply with more cytoplasmic coloration than a progressive formula.

Harris’ formula sometimes is considered suitable as a regressive stain if used without acid and as a progressive stain if acetic acid is added. This is greatly exaggerated. Even with 50 ml acetic acid per liter, Harris’ hemalum must be applied for less than 40 sec to obtain any degree of nuclear selectivity; longer application causes deep cytoplasmic staining, although a little less than with no acid added. Unfortunately, very short application times can result in uneven staining unless agitation is used immediately. I suspect
that many regressive formulae could be used in this fashion.

**Differentiation**

After staining with a regressive formula, 0.5 or 1% hydrochloric acid in 70% ethanol is applied. A few seconds of this treatment removes much of the background staining and some of the nuclear staining, the actual amounts depending on the desired end result. Experience is a large factor in determining how long the acid ethanol should be applied. Those who prefer a more darkly stained preparation may prefer to avoid differentiation, but even in these cases some detail can be obscured and may require a brief treatment, so a more dilute hydrochloric acid solution sometimes is preferred (perhaps 0.1 or 0.01%) depending on how little background staining removal is desirable. We sometimes refer to this as “cleaning up the background” rather than differentiation, although fundamentally it is the same. Acetic acid may be more useful for this than hydrochloric acid, because it extracts the dye more slowly.

There is no reason why simple aqueous solutions could not be used for differentiation. The effect of using ethanol is to cause currents between the water in and on the section and the ethanol that is applied. This, it sometimes is claimed, ensures a degree of evenness in differentiation. Simple agitation of the sections while applying the acid would have much the same effect. It is possible that the less polar ethanol reduces the effects of the acid. If this is the case, a more dilute aqueous solution would accomplish much the same result.

There are other methods of differentiation including treatment with a mordant solution or application of oxidizing agents, but these other methods do not give as sharply defined results as acid extraction nor are they as rapid; they are not recommended.

**Bluing**

After staining with a hemalum, the color of the tissue is red. It is necessary to change it to blue for both visual effect and to stabilize the stain. This usually is accomplished with a tap water wash. In cases where speed is essential or tap water is too acid, it may be done by applying an alkaline medium such dilute ammonia water, sodium acetate or lithium carbonate solution, or a complex mixture like Scott’s tap water substitute. These media do not need to be strongly alkaline and a pH near 8 is more than adequate. Treatment with these solutions should not extend beyond what is needed for bluing as it may remove some staining. The excess should be removed with a brief rinse with water to avoid carryover to the eosin.

In a few localities the tap water is the source of staining problems. For example, this may be due to the chemical treatment drinking water undergoes or from periodic increases in humic acid content during the snow melt period in mountainous areas. There also are places where the tap water is quite soft and may have a lower pH than that required. In these instances, the water can affect the bluing, or the chemical treatments may bring about fading. Rather than using distilled or deionized water in these cases, percolating or forcing the water through marble chips or crushed oyster shells, both readily available sources of calcium carbonate, should increase the pH sufficiently to resolve the problem.

**A good hematoxylin and eosin (H & E)**

What makes a good nuclear hematoxylin stain? That question should elicit a very subjective answer. The fact is that there are no objective criteria for determining what a “good” nuclear stain should look like. The nuclei should be clear and distinct, but the depth of staining, the amount of background cytoplasmic or mucin staining, etc. can vary tremendously and still be what a pathologist prefers to see. Perhaps that is the answer; a “good” H & E is what the person making the diagnosis prefers to see.

**Progressive or regressive**

Is it preferable to use a progressive hemalum and not differentiate, or to use a regressive formula and apply acid ethanol? Which gives optimal staining? This again is a subjective question. Satisfactory H & E staining can be obtained using both methods. Individual technologists and pathologists have their preferences. I suggest that whatever method is used presently should be optimized to bring about greater consistency in staining. If the staining is too dark, it is a simple matter to decrease slightly the hematoxylin content in whatever hemalum solution is used. Even with no other changes, this should result in a slight decrease in staining intensity as well as a slight increase in contrast between the nuclei and the cytoplasm, even without differentiation. Remember to dilute with a solution containing the same alum and acid concentrations as the hemalum being diluted. Simply diluting with water usually does not give
the same effect. The opposite effect also could be obtained by increasing the hematoxylin content in whatever hemalum is being used. This would increase staining intensity and likely cause an increase in cytoplasmic coloration.

There is no magical hemalum formula. Over periods of time, some have become popular, then lost popularity, then the latest and greatest comes to the fore, but they all do the same job. Rather than rely on newly published formulations to solve staining problems you might have, simply adjust the hemalum already used to bring about whatever degree of staining is wanted. Increase or decrease the amount of hematoxylin, increase or decrease the amount of mordant source compound, adjust the acid content with regard to concentration or type, or add or remove solvent constituents. There is no reason why this cannot be done to improve results. Careful small changes to optimize the staining in each laboratory can be beneficial.

**Hematoxylin substitutes**

Approximately 30–35 years ago, hematoxylin powder became difficult to find for a couple of years, and this happened again recently. This situation caused a great deal of consternation and alternative dyes were sought. Several were found and are well documented in the literature for future reference if it becomes necessary. It turned out that Proescher and Arkush (1928) had recommended some dyes as alternatives to hematoxylin in 1928.

After the shortage, other suitable dyes were found and a comprehensive list was given by Lillie et al. (1975a). Most of these dyes use an iron mordant and are progressive. Earlier, other dyes had been recommended including an acid triarylmethane dye with the functional name of mordant blue 3, otherwise known as solochrome cyanine R, eriochrome cyanine R or chromoxane cyanine R (Llewellyn 1974, 1978). This dye also is used with an iron mordant and is stable in solution. It may be used progressively or reggressively. Sections stained with a progressive variant in a solution in excess of 10 years old stained as well as when the solution was fresh.

Most of the substitute dyes, while suitable as nuclear stains, give different tones than hemalum, so they resemble H & E rather than duplicating it. The dye that most closely matches hemalum in tone is the Proescher and Arkush (1928) iron alum galliccyanin. Unfortunately, the solution has a limited life of about a month.

I strongly recommend that all laboratories purchase adequate supplies of one of these substitutes and become familiar with its use; dye powders are stable for many years.

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### Appendix 1. Hemalum formulae, alphabetical

Some formulae are identified as “Anderson 1923 var-a,” etc. This is an arbitrary designation for different formulae that otherwise may be confused.

**Formula:** The name of the formula.

Those marked § have a comment at the end of the list.

**Dye:** Grams of dye required for 1 liter solution.

Those marked ∗ are made with hematein.

Those marked # are made with both hematein and hematoxylin.

Those unmarked are made with hematoxylin.

**Mordant:** Grams of the aluminum salt required for 1 l of the solution.

**Source:** The chemical source of the aluminum.

**Water:** Amount of water for the solution.

**Acid:** The type and amount of acid, milliliters if a fluid, grams if a solid.

**Other:** Other ingredients, milliliters if fluids, grams if solids.

Ac = Acetic acid, glacial

BH = Barium hydroxide

CaH = Calcium hypochlorite

CC = Calcium chloride

Cit = Citric acid

CH = Chloral hydrate

CT = Chloramine T

DG = Diethylene glycol

E95 = 95% Ethanol

E100 = Absolute ethanol

EG = Ethylene glycol

Gl = Glycerol

HCl = Hydrochloric acid

HP = Hydrogen peroxide

Jvx = 5.25% sodium hypochlorite (Javex bleach)

KI = Potassium iodide

LC = Lime chloride

Me = Methanol

MO = Red mercuric oxide

Ni = Sodium iodate

PF = Potassium ferricyanide

PG = Propylene glycol

PP = Potassium permanganate

Sal = Salicylic acid

SB = Sodium bromide

SS = Sublimed sulfur

Th = Thymol

TI = Tincture of iodine

ZS = Zinc sulphate

| Formula | Dye | Mordant | Source | Water | Acid | Other |
|---------|-----|---------|--------|-------|------|-------|
| Anderson 1923 formula var-a (Gray 1954) | 2.5 | 20 | Ammonium alum | 900 | Ac = 50 | E95 = 50, CaH = 4 |
| Anderson 1923 formula var-b (Gatenby and Beams 1950) | 2.5 | 125 | Ammonium alum | 900 | Ac = 50 | E95 = 50 and CT = 4 or LC = 4 |
| Anderson 1929 formula (Gray 1954) | 5.0 | 30 | Ammonium alum | 700 | Ac = 50 | E95 = 50, CaH = 40 |
| Apathy (Gray 1954) | 3.0 | 30 | Ammonium alum | 450 | Ac = 10, Sal = 0.3 | E95 = 250, Gl = 350 |
| Baker hematal-8 (1962) | 0.94# | 8 | Al sulfate | 750 | | EG = 250 |
| Baker hematal-16 (1962) | 0.47# | 8 | Al sulfate | 750 | | EG = 250 |
| Bennett (Putt 1972) | 1.0 | 90 | K alum | 1000 | Cit = 1 | Ni = 0.2, CH = 50 |
| Bosma (1988) | 2.5 | 25 | Ammonium alum | 865 | Ac = 10 | E100 = 25, DG = 100, Ni = 0.2 |
| Bullard (Putt 1972) | 8.0 | 60 | Ammonium alum | 320 | Ac = 35 | E95 = 325, Gl = 330, MO = 8 |

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| Formula                                      | Dye | Mordant | Source         | Water | Acid | Other            |
|---------------------------------------------|-----|---------|----------------|-------|------|------------------|
| Carazzi (Bancroft and Stevens 1982)         | 1.0 | 50      | K alum         | 800   |      | GI = 200, NI = 0.2|
| Cole 1903 formula (Drury and Wallington 1980) | 6.0 | 6      | Ammonium alum  | 320   | Ac = 75 | E100 = 320, GI = 290 |
| Cole 1943 formula (Gray 1954)               | 1.5 | 100     | Ammonium alum  | 950   |      | E100 = 50, I = 0.5 |
| Debiden (1987, 1991)                        | 5.0 | 100     | K alum         | 1000  |      | MO = 2.5 or Jvx = 2 |
| De Groot (Gray 1954)                        | 2.0 | 22      | Ammonium alum  | 270   |      | E95 = 350, GI = 330, HP = 7.5, PF = 0.8, CC = 15, SB = 7.5 |
| Delafield (Bancroft and Stevens 1982)       | 6.0 | 90      | Ammonium alum  | 600   |      | E95 = 200, GI = 150 |
| Ehrlich§ (Drury and Wallington 1980)        | 6.0 | 50      | K alum         | 300   | Ac = 30 | E95 = 300, GI = 300 |
| Friedlander (Gray 1954)                     | 6.0 | 6      | K alum         | 300   |      | E95 = 300, GI = 300 |
| Gadsdon (Histonet)                          | 5.5 | 60      | K alum         | 610   | Ac = 20 | E100 = 100, GI = 300, NI = 0.5 |
| Gage (Gray 1954)                            | 1.0 | 40      | K alum         | 1000  |      | E95 = 20, CH = 20 |
| Galigher (Gatenby and Beams 1950)           | 5.0 | 3      | Ammonium alum  | 500   |      | E95 = 500, MO = 6 |
| Garvey (1991)                               | 2.5 | 45     | K alum         | 900   | Cit = 1 | E100 = 100, NI = 0.3 |
| Gill I (Culling et al. 1985)                | 2.0 | 18     | Al sulfate     | 750   | Ac = 20 | EG = 250, NI = 0.2 |
| Gill II (Culling et al. 1985)               | 4.0 | 70     | Al sulfate     | 750   | Ac = 20 | EG = 250, NI = 0.4 |
| Gill III (Culling et al. 1985)              | 6.0 | 158    | Al sulfate     | 750   | Ac = 20 | EG = 250, NI = 0.6 |
| Graham (1991)                               | 2.0 | 25     | Al sulfate     | 750   | Ac = 20 | PG = 310, NI = 0.2 |
| Hamilton (Histonet)                         | 2.0 | 75     | Ammonium alum  | 950   | Ac = 30 | E100 = 50, NI = 0.3 |
| Harris (Drury and Wallington 1980)          | 5.0 | 100    | K alum         | 1000  | Ac = 40 | E95 = 50, MO = 2.5 |
| Harris and Power§ (Gray 1954)               | 20.0 | 60    | K alum         | 100   |      | E100 = 6 |
| Haug (Gray 1954)                            | 5.5 | 5      | Al acetate     | 100   |      | E100 = 5 |
| Horneyold (Gatenby and Beams 1950)          | 8.4 | 4      | Ammonium alum  | 60    |      | E100 = 20, TI = 20 drops |
| Krutsay (Humason 1967)                      | 1.0 | 50     | K alum         | 1000  | HCl = 5 | NI = 0.2 |
| Langeron 1924 formula (Gray 1954)           | 4.0 | 50     | Ammonium alum  | 700   | Cit = 1 | GI = 300 |
| Langeron 1942 formula (Gray 1954)           | 1.0 | 50     | K alum         | 1000  | Ac = 20 | NI = 0.2, CH = 50 |
| Llauyon (Gray 1954)                         | 10.0* | 5     | K alum         | 1000  |      | NI = 0.2, CH = 50 |
| Lee (Gray 1954)                             | 1.0 | 50     | Ammonium alum  | 1000  |      | GI = 300, NI = 0.5 |
| Lillie (1954)                               | 5.0 | 50     | Ammonium alum  | 700   | Ac = 20 |
| Formula                              | Dye | Mordant         | Source     | Water | Acid | Other                     |
|-------------------------------------|-----|-----------------|------------|-------|------|---------------------------|
| McLachlan variant I (Histonet)      | 2.0 | 17.5 Al sulfate | 700        | Ac = 20 | Gl = 300, Ni = 0.2      |
| McLachlan variant II (Histonet)     | 2.0 | 25 Ammonium alum| 700        | Ac = 20 | Gl = 300, Ni = 0.2      |
| Mallory (Gray 1954)                 | 2.5 | 50 K alum       | 1000       |       | Th = 2.5                 |
| Mallory and Wright (1904)           | 1.0 | 140 Ammonium alum| 100       |       | Optional: dilute with 300 water |
| Mann (Gray 1954)                    | 6.0*| 35 K alum       | 350        | Ac = 30 | E95 = 320, Me = 150, Gl = 150 |
| Martinotti (Gray 1954)              | 2.0*| 15 K alum       | 700        |       | Me = 150, Gl = 150      |
| Masson (Gray 1954)                  | 20.0*| 60 K alum       | 1000       | Ac = 20 | E95 = 50                  |
| Mayer 1892 var-a, var-b formulas    | 1.0 | 50 Ammonium alum| 1000       | Ac = 20 | E95 = 50                  |
| formulas (Gray 1954)                |     | (1891b)         |            |       |                              |
| Mayer 1903 formula (Gray 1954)      | 1.0 | 50 Ammonium alum| 1000       |       | Ni = 0.2                  |
| Mayer 1896 formula (Gray 1954)      | 4.0 | 50 Ammonium alum| 700        |       | Gi = 300                  |
| Molnar I (1975)                     | 10.0| 50 Either alum  | 1000       | Ac = 20 | E95 = 50, MO = 5         |
| Molnar II (1976)                    | 4.0 | 50 Either alum  | 1000       | Cit = 1.5 | Ni = 0.3, CH = 75   |
| Papamiltiades (Putt 1972)           | 4.0 | 10 Al sulfate   | 900        | Ac = 32 | Gi = 100, KI = 4, ZS = 5 |
| Pusey (Villaneuva 1976)             | 1.6 | 60 Ammonium alum| 1000       | Cit = 0.35 | Ni = 0.25, CH = 50    |
| Rawitz 1895 var-a formula (Gray 1954)| 10.0| 10 K alum       | 650        |       | Gl = 350                  |
| Rawitz 1895 var-b formula (Gray 1954)| 2.5*| 15 Ammonium alum| 500        |       | Gl = 500                  |
| Rawitz 1909 formula (Gray 1954)      | 2.0 | 20 Al nitrate   | 500        |       | Gl = 500                  |
| Reddy (2001)                        | 6.4 | 60 Ammonium alum| 640        |       | E100 = 200, Gl = 160    |
| Sass variant I (Gray 1954)          | 10.0| 140 Ammonium alum| 1000       | Ac = 30 | Ni = 10                  |
| Sass variant II (Gray 1954)         | 1.0 | 50 Ammonium alum| 1000       |       | E100 = 60                  |
| Schmorl (Lillie 1954)               | 5.0 | 100 Ammonium alum| 1000      |       | E95 = 300, Gl = 300      |
| Scott (1912)                       | 3.0 | 21 K alum       | 300        | Ac = 30 | E95 = 300                  |
| Slidders (1988)                     | 1.0 | 100 K alum       | 1000       |       | Ni = 0.2                  |
| Unna (Gray 1954)                    | 3.0 | 140 Ammonium alum| 600        |       | E100 = 300, SS = 6       |
| Watson (Gatenby and Beams 1950)     | 6.0 | 6 Ammonium alum  | 300        | Ac = 30 | E100 = 300, PP = 0.3 or |
|                                      |     |                 |            |       | BH = 6                     |

**Notes**

Ehrlich’s hemalum specifies an excess of potassium alum; 50 g has been used for convenience. The actual amount that would dissolve in an equal parts mixture of water, ethanol and glycerol is not stated.

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## Appendix 2. Dye:mordant ratios, alphabetical

Ratios of dye to mordant.

**Formula**: The name of the hemalum.

**Dye**: The weight in grams of hematoxylin or hematein required.

**Mordant**: The weight in grams of the aluminum salt required.

**Source**: The chemical source of the aluminum.

**Ratio 1**: The dye:mordant ratio based on the weights used.
- **Al 1**: Weight of aluminum required to allow 1 atom per molecule of dye.
- **Al 2**: Weight of aluminum actually present in the solution.

**Ratio 2**: The factor by which the aluminum available exceeds that required.

Any discrepancies in the figures below are due to rounding.

| Formula | Dye | Mordant | Ratio 1 | Source      | Al 1 | Al 2 | Ratio 2 |
|---------|-----|---------|---------|-------------|------|------|---------|
| Anderson 1923 formula | 1.9 | 10 | 8.0 | Ammonium alum | 0.22 | 1.19 | 5.3 |
| Anderson 1923 formula var-a (Gray 1954) | 2.5 | 20 | 8.0 | Ammonium alum | 0.22 | 1.49 | 5.3 |
| Apathy (Gray 1954) | 3.0 | 30 | 10.0 | Ammonium alum | 0.27 | 1.79 | 6.6 |
| Baker hematal-8 (1962) | 0.94 | 8 | 8.4 | Al sulfate | 0.08 | 0.65 | 7.7 |
| Baker hematal-16 (1962) | 0.47 | 8 | 16.8 | Al sulfate | 0.04 | 0.65 | 15.4 |
| Bennett (Putt 1972) | 1.0 | 90 | 90.0 | K alum | 0.09 | 5.12 | 57.0 |
| Bosma (1988) | 2.5 | 25 | 10.0 | Ammonium alum | 0.22 | 1.49 | 6.6 |
| Bullard (Putt 1972) | 8.0 | 60 | 2.5 | Ammonium alum | 0.72 | 3.57 | 4.95 |
| Carazzi (Bancroft and Stevens 1982) | 1.0 | 50 | 50.0 | K alum | 0.09 | 2.84 | 31.6 |
| Cole 1903 formula (Drury and Wallington 1980) | 1.5 | 100 | 66.7 | Ammonium alum | 0.13 | 5.95 | 44.2 |
| Cole (1943 formula (Gray 1954) | 5.0 | 100 | 20.0 | K alum | 0.45 | 5.69 | 12.7 |
| de Groot (Gray 1954) | 2.0 | 22 | 11.0 | Ammonium alum | 0.18 | 3.41 | 7.3 |
| Delafield (Bancroft and Stevens 1982) | 6.0 | 90 | 15.0 | Ammonium alum | 0.54 | 5.36 | 9.9 |
| Ehrlich (Drury and Wallington 1980) | 6.0 | 50 | 8.3 | K alum | 0.54 | 2.84 | 5.3 |
| Friedlander (Gray 1954) | 6.0 | 6 | 1.0 | K alum | 0.54 | 0.34 | 0.6 |
| Gadsdon (Histonet) | 5.5 | 60 | 10.9 | K alum | 0.49 | 3.41 | 6.9 |
| Gage (Gray 1954) | 1.0 | 40 | 40.0 | K alum | 0.09 | 2.28 | 25.3 |
| Galilgher (Gatenby and Beams 1950) | 5.0 | 3 | 0.6 | Ammonium alum | 0.45 | 0.18 | 0.4 |
| Garvey (1991) | 2.5 | 45 | 18.0 | K alum | 0.22 | 2.56 | 11.4 |
| Gill I (Culling et al. 1985) | 2.0 | 18 | 8.8 | Al sulfate | 0.18 | 1.46 | 8.1 |
| Gill II (Culling et al. 1985) | 4.0 | 70 | 17.6 | Al sulfate | 0.36 | 5.66 | 15.7 |
| Gill III (Culling et al. 1985) | 6.0 | 158 | 26.4 | Al sulfate | 0.54 | 12.78 | 23.7 |
| Graham (1991) | 2.0 | 25 | 12.5 | Al sulfate | 0.18 | 2.0 | 11.2 |
| Hamilton (Histonet) | 2.0 | 75 | 37.5 | Ammonium alum | 0.18 | 4.46 | 24.8 |
| Harris (Drury and Wallington 1980) | 5.0 | 100 | 20.0 | K alum | 0.45 | 5.69 | 12.6 |

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§2 Harris and Power

Harris and Power’s solution has 20 g dye and 60 g alum in 100 ml water. The reference clearly gives these numbers, but they appear to be unreasonable, because neither would dissolve at those concentrations. Possibly the amount of water should be 1000 ml and the ethanol 60 ml. Compare to Masson’s formula.

§3 Langeron 1942 formula

Langeron’s formula is usually erroneously referred to as “Mayer’s” hemalum. Mayer never recommended the addition of citric acid and chloral hydrate, although he may have originated the use of 1 g of dye and 50 g of alum in 1 liter of water.
### Data used for calculation of ratios

**FW**: The formula weight of each chemical or the atomic weight of aluminum.

**Al atoms**: The number of aluminum atoms present in a molecule of each chemical.

**Amount Al**: The amount of aluminum calculated to be present in 1 g of each chemical. For Hematein it refers to the amount of aluminum required to give one aluminum atom for each molecule in 1 g of hematein.

| Item          | Hematein | Ammonium alum | K alunm | Al sulfate | Al acetate | Al Nitrate | Aluminum |
|---------------|----------|----------------|---------|------------|------------|------------|----------|
| FW            | 300.272  | 453.33         | 474.39  | 666.42     | 140.65     | 375.14     | 26.9815  |
| Al atoms      | –        | 1              | 1       | 2          | 1          | 1          | –        |
| Amount Al     | 0.0899   | 0.0595         | 0.0569  | 0.0809     | 0.1918     | 0.0719     | –        |
Appendix 4. Formulae classified by type

**Progressive formulae**

| Formula | Dye | Alum | Ratio 1 | Source | Al needed | Al present | Ratio 2 |
|---------|-----|------|---------|--------|-----------|------------|--------|
| Mallory and Wright (1904) | 1.0 | 140 | 1:140 | Ammonium alum | 0.09 | 8.33 | 1:93 |
| Slidders (1988) | 1.0 | 100 | 1:100 | K alum | 0.09 | 5.69 | 1:63 |
| Bennett (Putt 1972) | 1.0 | 90 | 1:90 | K alum | 0.09 | 5.12 | 1:57 |
| Cole 1943 formula (Gray 1954) | 1.5 | 100 | 1:67 | Ammonium alum | 0.13 | 5.95 | 1:44 |
| Krutsay (Humason 1967) | 1.0 | 50 | 1:50 | K alum | 0.09 | 2.98 | 1:33 |
| Lee (Gray 1954) | 1.0 | 50 | 1:50 | Ammonium alum | 0.09 | 2.98 | 1:33 |
| Mayer 1891 formula (Gray 1954) | 1.0 | 50 | 1:50 | Ammonium alum | 0.09 | 2.98 | 1:33 |
| Mayer 1901 formula (Gray 1954) | 1.0 | 50 | 1:50 | K alum | 0.09 | 2.84 | 1:31 |
| Mayer 1903 formula (Gray 1954) | 1.0 | 50 | 1:50 | Ammonium alum | 0.09 | 2.98 | 1:33 |
| Sass variant II (Gray 1954) | 1.0 | 50 | 1:50 | Ammonium alum | 0.09 | 2.98 | 1:33 |
| Carazzi (Bancroft and Stevens 1982) | 1.0 | 50 | 1:50 | K alum | 0.09 | 2.84 | 1:32 |
| Langeron 1942 formula (Gray 1954) | 1.0 | 50 | 1:50 | K alum | 0.09 | 2.84 | 1:32 |
| Gage (Gray 1954) | 1.0 | 40 | 1:40 | K alum | 0.09 | 2.28 | 1:25 |
| Pusey (Villaneuva 1976) | 1.6 | 60 | 1:37 | Ammonium alum | 0.14 | 3.57 | 1:25 |
| Hamilton (Histonet) | 2.0 | 75 | 1:37 | Ammonium alum | 0.18 | 4.46 | 1:25 |
| McLachlan variant II (Histonet) | 2.0 | 25 | 1:12 | K alum | 0.18 | 1.49 | 1:8 |
| Martinotti (Gray 1954) | 2.0 | 15 | 1:7 | K alum | 0.18 | 0.89 | 1:5 |

**Regressive formulae**

| Formula | Dye | Alum | Ratio 1 | Source | Al needed | Al present | Ratio 2 |
|---------|-----|------|---------|--------|-----------|------------|--------|
| Harris (Drury and Wallington 1980) | 5.0 | 100 | 1:20 | K alum | 0.45 | 5.69 | 1:13 |
| Schmorl (Lillie 1954) | 5.0 | 100 | 1:20 | Ammonium alum | 0.45 | 5.95 | 1:13 |
| Debiden (1987, 1991) | 5.0 | 100 | 1:20 | K alum | 0.45 | 5.69 | 1:13 |
| Delafield (Bancroft and Stevens 1982) | 6.0 | 90 | 1:15 | Ammonium alum | 0.54 | 5.36 | 1:10 |
| Sass variant I (Gray 1954) | 10.0 | 140 | 1:14 | Ammonium alum | 0.90 | 8.33 | 1:9 |
| Mayer 1896 formula (Gray 1954) | 4.0 | 50 | 1:12 | Ammonium alum | 0.36 | 2.98 | 1:8 |
| Molnar II (1976) | 4.0 | 50 | 1:12 | Either alum | 0.36 | 2.98 | 1:8 |
| Langeron 1924 formula (Gray 1954) | 4.0 | 50 | 1:12 | Ammonium alum | 0.36 | 2.84 | 1:8 |
| Gadsdon (Histonet) | 5.5 | 60 | 1:11 | K alum | 0.49 | 3.41 | 1:7 |
| Lillie (1954) | 5.0 | 50 | 1:10 | Ammonium alum | 0.45 | 2.98 | 1:7 |
| Reddy (2001) | 6.4 | 60 | 1:9 | Ammonium alum | 0.58 | 3.57 | 1:6 |
| Ehrlich (Drury and Wallington 1980) | 6.0 | 50 | 1:8 | K alum | 0.54 | 2.84 | 1:5 |
| Bullard (Putt 1972) | 8.0 | 60 | 1:3 | Ammonium alum | 0.72 | 3.57 | 1:5 |
| Anderson 1929 formula (Gray 1954) | 5.0 | 30 | 1:6 | Ammonium alum | 0.45 | 1.79 | 1:4 |
| Mann (Gray 1954) | 6.0 | 35 | 1:6 | K alum | 0.54 | 1.99 | 1:4 |
| Molnar I (1975) | 10.0 | 50 | 1:5 | Either alum | 0.90 | 2.98 | 1:3 |
| Harris and Power (Gray 1954) | 20.0 | 60 | 1:3 | K alum | 1.80 | 3.41 | 1:2 |
| Masson (Gray 1954) | 20.0 | 60 | 1:3 | K alum | 1.80 | 3.41 | 1:2 |

Appendix 3. Mucin staining

**Mayer’s mucihematein** (Mallory and Wright 1904, Bensley and Bensley 1938, Gray 1954).

| Variant 1 | Variant 2 | Variant 3 |
|-----------|-----------|-----------|
| Hematein  | 0.2 g     | 1 g       | 0.2 g |
| Aluminum chloride | 0.1 g | 0.5 g | 0.1 g |
| Glycerol 60% | 100 ml | 100 ml | |
| Ethanol 70% | 100 ml | 100 ml | |
| Nitric acid | drops | 0.05 ml | |

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Intermediate formulae

| Formula | Dye | Alum | Ratio 1 | Source | Al needed | Al present | Ratio 2 |
|---------|-----|------|---------|--------|-----------|------------|--------|
| Anderson 1923b formula (Gatenby and Beams 1950) | 2.5 | 125 | 1:50 | Ammonium alum | 0.22 | 7.44 | 1:33 |
| Unna (Gray 1954) | 3.0 | 140 | 1:47 | Ammonium alum | 0.27 | 8.33 | 1:31 |
| Mallory (Gray 1954) | 2.5 | 50 | 1:20 | K alum | 0.22 | 2.84 | 1:13 |
| Garvey (1991) | 2.5 | 45 | 1:18 | K alum | 0.22 | 2.56 | 1:11 |
| Apathy (Gray 1954) | 3.0 | 30 | 1:10 | Ammonium alum | 0.27 | 1.79 | 1:7 |
| Bosma (1988) | 2.5 | 25 | 1:10 | Ammonium alum | 0.22 | 1.49 | 1:7 |
| Anderson 1923a formula (Gray 1954) | 2.5 | 20 | 1:8 | Ammonium alum | 0.22 | 1.19 | 1:5 |
| Scott (1912) | 3.0 | 21 | 1:7 | K alum | 0.27 | 1.19 | 1:4.4 |
| Rawitz 1895b formula (Gray 1954) | 2.5 | 15 | 1:6 | Ammonium alum | 0.22 | 0.89 | 1:4 |

Other formulae

| Formula | Dye | Alum | Ratio 1 | Source | Al needed | Al present | Ratio 2 |
|---------|-----|------|---------|--------|-----------|------------|--------|
| Weak | | | | | | | |
| Baker hematal-16 (1962) | 0.47 | 8 | 1:17 | Al sulfate | 0.04 | 0.65 | 1:15 |
| Baker hematal-8 (1962) | 0.94 | 8 | 1:8 | Al sulfate | 0.08 | 0.65 | 1:8 |
| Intermediate | | | | | | | |
| Graham (1991) | 2.0 | 25 | 1:12 | Al sulfate | 0.18 | 2.0 | 1:11 |
| Gill I (Culling et al. 1985) | 2.0 | 18 | 1:9 | Al sulfate | 0.18 | 1.46 | 1:8 |
| Rawitz 1909 formula (Gray 1954) | 2.0 | 20 | 1:10 | Al nitrate | 0.18 | 1.44 | 1:8 |
| McLachlan variant I (Histome) | 2.0 | 17.5 | 1:9 | Al sulfate | 0.18 | 1.42 | 1:8 |
| Strong | | | | | | | |
| Gill II (Culling et al. 1985) | 4.0 | 70 | 1:18 | Al sulfate | 0.36 | 5.66 | 1:16 |
| Papamiltiades (Putt 1972) | 4.0 | 10 | 2:5 | Al sulfate | 0.36 | 0.81 | 1:2 |
| Haug (Gray 1954) | 5.5 | 5 | 1:1 | Al acetate | 0.49 | 0.96 | 1:2 |
| Gill III (Culling et al. 1985) | 6.0 | 158 | 1:26 | Al sulfate | 0.54 | 12.78 | 1:24 |

Appendix 5. Experiments

The experiments require 0.6 g hematoxylin dissolved in 30 ml distilled water and oxidized with 120 mg sodium iodate, 60 g ammonium or potassium alum dissolved in water and made up to 60 ml, 75 15-ml test tubes and a few pipettes; 102 sections are needed. Placenta is suitable.

Increasing alum

| 100% Alum | 0.01 | 0.05 | 0.1 | 0.2 | 0.5 | 1.0 | 2.0 | 3.0 | 4.0 | 5.0 | 6.0 | 7.0 | 8.0 | 9.0 |
|-----------|------|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 2% Hematein | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Water | 9.49 | 9.45 | 9.4 | 9.3 | 9.0 | 8.5 | 7.5 | 6.5 | 5.5 | 4.5 | 3.5 | 2.5 | 1.5 | 0.5 |
| Ratio | 1:0.1 | 1:0.5 | 1:1 | 1:2 | 1:5 | 1:10 | 1:20 | 1:30 | 1:40 | 1:50 | 1:60 | 1:70 | 1:80 | 1:90 |

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The numbers in 100% alum, 2% hematein and water refer to ml.
Split each into three aliquots of 3.3 ml.
Add 0.2 ml water to one set.
Add 0.2 ml 50% aqueous acetic acid to another.
Add 0.2 ml 2% aqueous hydrochloric acid to the third.
Stain 5 μm sections for 10 min, rinse well and blue.
Do not counterstain.
Dehydrate, clear and mount

### Increasing hematein

| Alum     | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Hematein | 0.05| 0.25| 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 | 4.0 | 5.0 |
| Water    | 9.45| 9.25| 9.0 | 8.5 | 8.0 | 7.5 | 7.0 | 6.5 | 5.5 | 4.5 |
| Hematoxylin per liter equivalent | 0.1 | 0.5 | 1   | 2   | 3   | 4   | 5   | 6   | 8   | 10  |
| Ratio    | 1:500 | 1:100 | 1:50 | 1:25 | 1:16 | 1:12 | 1:10 | 1:8 | 1:6.3 | 1:5 |

The numbers in 100% alum, 2% hematein and water refer to ml.
Split each into three aliquots of 3.3 ml.
Add 0.2 ml water to one set.
Add 0.2 ml 50% aqueous acetic acid to another.
Add 0.2 ml 2% aqueous hydrochloric acid to the third.
Stain two sets of 5 μm sections for 10 min, rinse well.
Treat one set with 0.1% acid alcohol for 5 sec, rinse well.
Blue both sets.
Do not counterstain.
Dehydrate, clear and mount.