Hematoxylin Formulae

Bryan D Llewellyn

Produced for distribution through

StainsFile

The Internet Resource For Histotechnologists

http://stainsfile.info

This document includes formulae for the alum and iron hematoxylin solutions included on the ***StainsFile*** Internet site. Some of these are no longer in use, and others are variations of other, more common, formulae. I have included a discussion of the relationship between the dye and the mordant, and how the relationship between them affects the staining character of a formula. The formulae have been collected from several sources, including older reference texts and the Internet.

If any reader knows of a hematoxylin formula not listed in this document, I would most appreciate receiving details so that it may be included in the future. I may be contacted by e-mail through the ***StainsFile*** Internet site.

Bryan D. Llewellyn
October, 2013

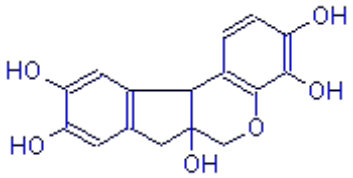
October 2013	1.0	Initial document

October, 2013.

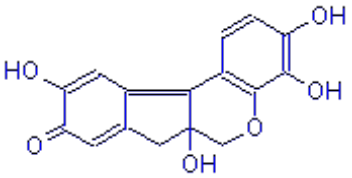
Table of Contents

Hematoxylin and Hematein

Common name (North America)	Hematoxylin	Hematein
Common name (Britain)	Haematoxylin	Haematein, Haematine
Color Index number	75290	75290
Color Index name	Natural black 1	Natural black 1
Ionises	Acid	Acid
Solubility in water	3%	1.5%
Solubility in ethanol	3%	7%
Colour	Yellow brown	Dark brown
Formula weight	302.3	300.3
Empirical formula	$C_{16}H_{14}O_6$	$C_{16}H_{12}O_6$



Hematoxylin



Hematein

Hematoxylin is a natural dye which is extracted from the heartwood of the tree *Haematoxylum campechianum*, although histotechnologists are probably more familiar with the name as *Haematoxylon campechianum*. The genus names *Haematoxylum* and *Haematoxylon* are derived from two Greek words: *haimatos* which means blood, and *xylon* which means wood. The two words together mean "wood of blood" or "bloodwood", a reference to the colour of the tree's heartwood from which hematoxylin is extracted.

The hematoxylin which we buy is extracted from this heartwood of the bloodwood tree. There may be some differences in method, but one is to chip the heartwood of freshly logged trees, then boil the chips in water. An orange-red solution is obtained, which turns yellow, then black on cooling. The water is evaporated leaving crude hamatoxylin. Depending on the genetic line of the tree, hematoxylin content ranges from 0% - 10%. Further purification is undoubtedly done.

This dyestuff may be referred to as haematoxylin or hematoxylin, with spellings of haematein or hematein for the oxidation product. Both spellings are valid, being merely the British and American regional variants respectively. In this document hematoxylin and hematein will be used.

Although it is common practice to use hematoxylin, it is not itself the dye. During the preparation of staining solutions hematoxylin is converted into hematein. This is usually accomplished with chemical oxidising agents, but is sometimes accomplished by atmospheric oxygen over time. Sodium iodate is the most common oxidising agent for this purpose, although there are others, such as potassium permanganate, iodine, bleach and mercuric oxide. It is now strongly recommended that mercuric oxide not be used for this oxidation as it eventually makes its way into the environment as a poisonous pollutant. Hematein itself is rarely used in staining solutions as it continues to oxidise and forms non-staining or poorly staining products. In addition, the quality of hematoxylin is usually higher and more consistent than the quality of hematein, and solutions made with it are more easily standardised.

The dye is usually used in conjunction with a mordant, the two commonest being aluminum (as ammonium or potassium alum), or iron (ferric chloride or iron alum). Other mordants are used much less frequently but include chrome alum and phosphotungstic acid. The tissue component most frequently demonstrated is nuclear chromatin using an aluminum mordant in the Hematoxylin and Eosin general oversight staining method. Using ferric salts as the mordant, it is also used for acid resistant nuclear staining, the demonstration of muscle striations and numerous other elements. With phosphotungstic acid it can demonstrate fibrin, muscle striations and some neuroglia fibres. There are many published formulae.

Due to the widespread use of this dye in medical histology, it is important that a steady supply be available. This has not always been the case. A shortage occurred in the early part of the 1970s. During this period several dyes were tested as substitutes with some success. Unfortunately, none of them have the wide variety of uses that hematoxylin has. Celestine blue B and mordant blue 3 are probably the most successful. Please refer to the StainsFile web site for details.

Hematoxylin has not yet been fully synthesised, but the

compound has been split into some precursors, which have then been successfully re-converted to the original compound.

References

Horobin R W and Kiernan J A, (2002). *Conn's Biological Stains*, 10th ed. BIOS Scientific Publishers, Oxford, UK

Budavari, S., Editor, (1996). *The Merck Index, Ed. 12*. Merck & Co., Inc., Whitehouse Station, NJ, USA

Lemmens, R. H. M. J. and Wulijarna-Soetjipto, N., Editors. (1992). *Plant resources of South East Asia No. 3, Dye and tannin-producing plants.* PROSEA, Bogor, Indonesia.

Morsingh, F., and Robinson, R., (1970). *The synthesis of brazilin and haematoxylin.* Tetrahedron, **v.** 26, **p.** 281, UK

James Cook University, School of Marine and Tropical Biology, Townsville, Queensland, Australia. http://www-public.jcu.edu.au/discovernature/plants_t/JCUDEV_012192

Oxidation

Hematoxylin itself is not a dye, and it has to be oxidised to hematein, which is a dye, before it can be used. This process is called ripening, and can be accomplished in two distinct ways. Simple alcoholic or aqueous solutions made with hematoxylin are usually pale yellow-brown in colour. On oxidation the colour changes to a deep, mahogany brown. When combined with an aluminum salt such as aluminum potassium sulphate, the colours are pale, transparent violet (unripened) and deep opaque purple (ripened). They may also be combined with iron salts. In these the colour is deeper, usually a very dark violet.

Natural ripening
It was common practice in the past to use natural, atmospheric oxidation in the belief that it gave a more reliable and longer lasting solution. Natural ripening is accomplished by putting the solution in an oversize flask, so it can be shaken, with the top plugged loosely with cotton batting, allowing air to enter. This is left in a warm, light and airy place (a window sill) for oxidation to take place. The flask is shaken periodically. Oxidation may take several months, and is determined by testing the solution from time to time. When the solution gives a satisfactory depth of staining, it is transferred to a brown bottle and tightly stoppered for storage in the dark to retard further oxidation.

Chemical ripening
The other way to ripen hematoxylin is to use chemical oxidising agents. The most common is sodium iodate at about 200 mg for each gram of hematoxylin for complete oxidation. Others have also been suggested for particular formulas, but sodium iodate can be substituted for all of them if used at the stated amount.

Mercuric oxide was often recommended as an oxidant in the past. It is now strongly recommended that it ***not*** be used. It is very toxic, and should be avoided whenever possible. If it must be used, then full safety precautions should be taken (refer to an MSDS), and the used solution must be disposed of in compliance with government regulations to avoid contamination of the environment.

Boiling a solution of hematoxylin with an oxidant invariably causes rapid oxidation and such solutions may be used as soon as cooled. Boiling is not always necessary as oxidation can also take place at room temperature over a few days with most oxidising agents, including sodium iodate. When a solution is needed rapidly, boiling does enable it to be made available quickly with no negative effects on the staining.

Oxidant per gram of hematoxylin

Oxidising agent	*Formula*	*Maximum*	*Recommended*
Sodium iodate	$NaIO_3$	200 mg	40-150 mg
Mercuric oxide	HgO	500 mg	100 mg
Potassium permanganate	$KMnO_4$	177 mg	175 mg
Potassium periodate	KIO_4	50 mg	50 mg
Hydrogen peroxide USP	H_2O_2	2.0 mL	

Calcium hypochlorite has also been recommended by Anderson, but the amount suggested for each gram of hematoxylin varies from 1.6 grams to 10 grams. Debiden used 105 milligrams of sodium hypochlorite for five grams hematoxylin in the form of 2 millilitres of commercial Javex bleach. Refer to the formulae for details.

Half oxidation

The term "half oxidation" is occasionally encountered, although it is a misnomer unless 50% of the dye content is oxidised. It refers to partial ripening of the hematoxylin content of a solution to obtain any degree of ripening that gives satisfactory staining. It is more common in stronger, regressive alum hematoxylin solutions than in the progressive formulae and its use goes back many decades. The ripening procedure for Ehrlich's alum hematoxylin from the late 19th century, for instance, required ripening atmospherically until it stained well, then the container was tightly stoppered to inhibit further oxidation and extend the life of the solution. This procedure may be thought of as natural half oxidation, although the percentage of hematoxylin actually converted to hematein could not be known.

Half oxidation is based on the rationale that the dye content is high enough that not all of it is needed at once, and since atmospheric oxidation will take place throughout the solution's life, the presence of some unoxidised hematoxylin will enable the solution to be stable for a longer time than if all of the hematoxylin is converted to

hematein initially. During natural ripening the solution becomes usable when a significant portion of the hematoxylin has been oxidised. These solutions have an extended life because they are usable before full conversion takes place. Throughout their life, oxidation of hematoxylin and further oxidation of already converted hematein continues. They do not become unusable until oxidation proceeds so far that insufficient hematein remains to give acceptable staining.

Chemical half oxidation seeks to emulate this process by adding sufficient oxidant to convert only part of the hematoxylin to hematein. Oxidation will then continue as for a naturally ripened solution. In practice, the limiting factor for use of a solution is more likely to be carry over of alkaline tap water, neutralising the acid and allowing the lake to precipitate. This is why alum hematoxylin solutions can often be rejuvenated by the addition of a small amount of acetic acid.

Many formulae use this practice, often without drawing attention to it. Refer to the chart of oxidising agents above and you will note that sodium iodate is recommended at 40-200 milligrams per gram of hematoxylin. Complete oxidation is achieved with 200 milligrams, so a solution using less than that is “half oxidised”.

Iron hematoxylin solutions are rarely oxidised by adding an oxidising agent for that purpose as the ferric salts present, being oxidising agents themselves, do double duty as both mordant and ripener. Nevertheless, some formulae do call for a ripened alcoholic solution to be used. This is particularly so when the mordant and dye are applied seperately. It is not an uncommon practice when iron hematoxylin solutions are made by combining stock solutions just before use, to wait a short time before using the resulting solution and allow the hematoxylin to be oxidised to some extent by the ferric salt mordant first.

Hematoxylin versus Hematein

The question arises, if hematein is the dye and if hematoxylin needs to be oxidised to hematein before it can stain, why not use hematein in the first place? The answer lies in the difficulty of obtaining satisfactory hematein. It is quite easy to obtain good hematoxylin, and most companies' products are perfectly satisfactory. Only very rarely is a poor quality hematoxylin provided. Hematein is another story, however. It is not uncommon to purchase unsatisfactory dye, even from reputable suppliers. It is recommended that hematein be purchased in relatively small samples as continued oxidation increases the poorly staining components.

The problem is that hematein continues to oxidise to poorly staining substances. It seems to do this as a powder, and it certainly does it in solution. If we start with hematein, oxidation will start to diminish staining capacity right from the start, shortening the working life of the solution. However, if you have a good sample of hematein, alum-hematein solutions made from it do work well and can be used immediately. Please refer to the formulae by: Baker, Launoy, Mann, Martinotti, Masson, and Rawitz.

Keep in mind that all six of these solutions could be made with hematoxylin if an oxidant were added to the solution. Similarly, solutions specifying hematoxylin can be made with hematein if the oxidant is omitted. In practice, the experience of many microscopists is that the most convenient, reliable and reproducible approach is to start with hematoxylin and chemically oxidise it to hematein, either completely or partially. With sodium iodate, this can be done so rapidly and with such ease that hematein is scarcely needed at all.

Stock alcoholic hematoxylin

When hematoxylin first began to be used as a histological dye, microscopists had to extract it themselves from logwood chips or sawdust. One such procedure is given with the formula for Mitchell's Alum Hematoxylin, where hematoxylin is extracted directly into an aqueous alum solution. Other procedures involved extraction of the dye into ethanol. Logwood can contain anywhere from 0% to 10% hematoxylin, depending on the tree’s genetics, so the concentration of dye in the resulting alcoholic solution can be variable. There are ways around this, of course, such as repeated extractions into the same ethanol or evaporation until crystallisation begins.

This ethanolic hematoxylin became something of a standard laboratory fixture and most had a solution labelled as 10% alcoholic hematoxylin, made by adding the equivalent of 10 grams hematoxylin to 100 millilitres of absolute ethanol. This solution was kept in a cupboard as a stock solution. Over a period of time the hematoxylin would slowly oxidise atmospherically to hematein, and the stock solution would become the source of dye for those occasions when a staining solution needed to be made and ripened quickly. Most staining solutions made from this stock solution, ripened or not, were based on it having a 10% dye concentration. However, hematoxylin dissolves in ethanol to about 3% and its oxidation product, hematein, to about 7%. Certainly, a freshly made stock solution would contain only 3 grams dye per 100 mL, so any staining solution made with it would contain less dye than expected, unless the stock solution was shaken before measuring out the volume to be incorporated into the staining solution being made. I suspect this shaking was common, whether deliberately or accidentally.

The error introduced into staining solutions by using this stock solution would be less if it was an old, well ripened solution. In that case the hematein content would be close to 7%. I have no information on whether the hematein content of 7% was in addition to the hematoxylin content of 3%, or instead of it. If in addition, the total dye content would be close to 10% in a fully ripened stock solution, but less than that in one not fully ripened. It should also be noted that hematein continues

to oxidise and forms poorly staining or non staining compounds. There are some practical problems that derive from this. This kind of stock solution is inherently very variable as it is not certain how much hematoxylin has oxidised to hematein, nor how much hematein has overoxidised and is no longer functional as a dye. This introduces a variable into staining procedures. In many cases this may not matter, but in others it may be important.

Due to the ongoing oxidation of both hematoxylin and hematein the dye content of the stock solution may decline over time, and really old solutions may not contain sufficient hematein to provide an adequate amount of dye for a staining solution. This does take a very long time, but can happen. The author has encountered this situation twice during his working life, both times using very old stock solutions. Always keep in mind that poor staining results from staining solutions made from a stock solution of hematoxylin may well be due to the stock solution no longer being viable.

With the availability of today's relatively pure hematoxylin and considering how easily hematoxylin may be oxidised to hematein with sodium iodate, the reality is that a stock 10% hematoxylin solution is no longer needed. More consistency is obtained by weighing a known amount of hematoxylin, dissolving it into a suitable solvent and oxidising chemically with 0.2 grams sodium iodate for each gram of hematoxylin.

References

Lillie, R.D., (1954). *Histopathologic technique and practical histochemistry Ed.2.* Blakiston, New York, USA.

Gray, Peter. (1954). *The Microtomist's Formulary and Guide*. The Blakiston Co.

Lynch M.J. et. al. *Medical Laboratory Technology*. W. B. Saunders Company, Toronto, Canada.

Horobin R W & Kiernan J A, (2002). *Conn's Biological Stains, 10th ed.* BIOS Scientific Publishers, Oxford, UK

Mordant

Mordants are indispensible in histology, and our most common staining method, the H&E, depends on them. Despite their importance there is sometimes some confusion as to what mordants are, or are not. The term is often used as a catch all term to describe any and every procedure which facilitates staining, secondary fixation of sections with Bouin's fluid and trapping methyl violet in a Gram's stain being two common examples. Neither involves a mordant.

A mordant is a metal with a valency of at least two. The commonest metals used in histotechnology are aluminum and ferric iron, both with valencies of three. Their attachment to dyes is by a covalent and a coordinate bond. This is otherwise known as chelation, and is a relatively common phenomenon. The word *chelation* is apparently derived from the name of the large claw, or *chela*, of a lobster. Gripping a metal atom by two different bonds has a fanciful similarity to gripping food with the two parts of this claw.

The term *lake* is derived from *lac*, which is the exudate of an insect from India and other countries. The collected material is washed and separated into its constituents, including a resinous material which is used for various products (shellac varnish) and as a coating for candy (sweets). One of the constituents is a dye, also called lac, which forms bright red compounds with aluminum. Over time the word *lac* has been changed to *lake*, which is used to refer to all such dye-metal complexes.

A mordant may be defined as:

A polyvalent metal ion whch forms coordination complexes with certain dyes.

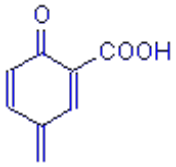
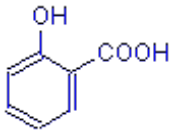
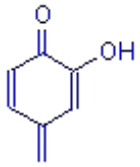
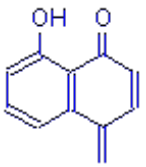
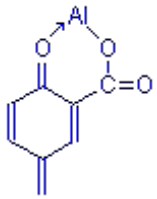
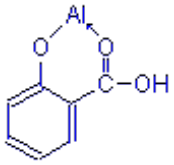
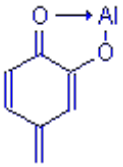
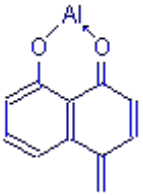
A lake may be defined as:

A coordination complex formed between a polyvalent metal ion and certain dyes.

How lakes form

Two types of bonds are involved in the fundamental reaction between a mordant dye and a mordant. One is a covalent bond with a hydroxyl oxygen. The other is a coordinate bond with another oxygen as the the electron donor. The only difference between them is the source of the shared electrons.

In the table below are the four most common configurations found in mordant dyes. There are others, particularly in the textile industry, but nearly all mordant dyes used in biological staining have one or more of these configurations. Note that in each case a double bonded oxygen and a hydroxyl group (or a carboxyl group) are involved. The covalent bond forms between the hydroxyl oxygen and the metal (straight bond), while the coordinate bond forms between the double bonded oxygen and the metal (arrow).

Structure				
Lake				

Since aluminum and ferric iron both have valencies of three, it is possible that three molecules of dye could attach to each atom of the mordant metal. In practice, it is unlikely that this happens, since attachment to the tissue is also by means of the mordant metal. Varying the amount of mordant present with the dye is a way to exert some control over the staining characteristics of some lakes. This is quite effective with alum hematoxylin solutions. Regressive formulations usually have larger amounts of dye present in comparison to progressive formulations. In other words, the amount of mordant available to each dye molecule is lower.

It is often remarked that the addition of a mordant to an appropriate dye solution results in a very sudden, dramatic change in colour. This is due to the incorporation of the metal atom into the delocalised electron system of the dye. Metals have relatively low energy levels, so their incorporation into a delocalised system results in a lowering of the overall energy. The absorbance of the

lake, and thus its colour, is related to this.

Probably the single most commonly used dye in histotechnology is hematoxylin or hematein, mostly used to stain nuclei. Such solutions usually contain hematoxylin and an alum, and are called hemalums or alum hematoxylin solutions. Many formulae have been suggested. They vary in the amount of hematoxylin, the amount and type of aluminum salts, solvents, oxidizing agents and stabilisers. The variations are, at times, almost contradictory and make the underlying principles on which these solutions are based difficult to make clear.

As a minimum, the three items needed to produce an effective nuclear staining alum hematoxylin are:–

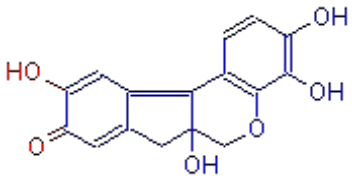
- Dye: hematoxylin or hematein.
- Mordant: an aluminum salt.
- Solvent: water being the simplest.

In addition to these three items, other ingredients may be added to modify or facilitate staining in some fashion:–

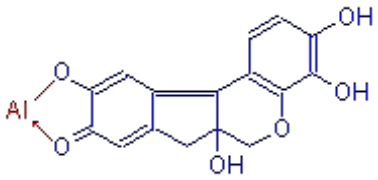
- Oxidizing agents to convert hematoxylin to hematein.
- Acids to adjust pH and extend the life of the solution, and which may affect the selectivity of nuclear staining.
- Stabilisers to inhibit further oxidation once the hematein has been formed.
- Additions to the solvent, often to inhibit evaporation or precipitation.

Co-ordination complex

From the structural formulae above in the section on hematoxylin and hematein it can be seen that they differ by only one hydrogen atom. Removal of this hydrogen from hematoxylin is accomplished either naturally by atmospheric oxygen or by using mild oxidizing agents, and results in a compound with a hydroxyl group adjacent to a carbonyl group. This configuration is one that facilitates the formation of co-ordination complexes with metals including, but not limited to, aluminum. Thus the aluminum may be perceived as a link, or bridge, between the anionic dye hematein and a negatively charged nuclear phosphate group.



Adjacent hydroxyl and carbonyl groups of hematein



Co-ordination complex between hematein and aluminum

It is the lake which is the staining component of hemalum solutions. The simplest view is that it reacts as a cation and attaches to tissue anions, such as phophate groups of DNA and carboxyl groups of proteins. A more complete view is that the attachment, at least to DNA, is also by means of covalent and co-ordinate bonding.

The amount of hematoxylin in the various solutions varies widely from about 1 gram per litre in Mayer's solution, to twenty times that in Masson's. Obviously, the actual dye content must have an affect on the staining properties, and it can be observed that the more concentrated the dye content the more likely it is that the solution stains regressively. Most progressive solutions have about 1 gram per litre or a little more, and the regressive solutions have 4 or more grams per litre. However, the dye content alone does not determine this characteristic. Both the amount of mordant present and the pH also have an influence. In general, however, if the concentration of mordant remains constant, a solution with a lower dye content will likely be more nuclear selective than that with a higher content. This can be shown easily enough by adding increasing amounts of a ripened 10% alcoholic hematoxylin solution to 5 mL of a 10% solution of alum, then adjusting the volume to 10 mL with water.

The usual source of the mordant for nuclear staining with hemalum is an aluminum double sulphate, or alum. Ammonium or potassium aluminum sulphates are the most common, although there is no reason at all why sodium aluminum sulphate should not be used. The usual explanation for the use of alums is that they were available in good purity in the late 1800s when these solutions were being introduced. It was therefore a simple convenience to use them in preference to other sources of aluminum. Of course, the second metal in the double sulphate should not be capable of acting as a mordant.

Other aluminum salts have been used. Examples are aluminum acetate in Haug's formula, or aluminum nitrate in Rawitz' 1909 variant. The more modern Gill's hema-

lums use aluminum sulphate. From these examples, it is clear that the source of aluminum is not critical, and purity is no longer a concern.

In modern practice alum solutions of about 50 grams to a litre of solvent, mostly water, are fairly typical. The saturation points of ammonium alum and potassium alum are 142 grams and 139 grams per litre respectively in water. Formulae have been published with amounts of alum ranging from 6 grams per litre up to 142 grams per litre.

When alum and hematein are combined a lake is formed. This is a specific chemical compound and is what does the staining of nuclei. Sometimes heat is used to speed up the formation of the lake, but it eventually happens regardless of that. In strong solutions it, or products derived from it, may precipitate as a dark sludge, particularly as the hematein continues to oxidise to other compounds. In practical terms this means that hemalum solutions should be filtered frequently, and that doing so is more important for the stronger formulations.

There are four ways that the dye and mordant may be combined for effective staining, afterchrome, metachrome, onchrome and displacement. The first three of those terms were taken from the textile dyeing industry, and the *chrome* in them refers to chromium which was quite common as a textile mordant.

Onchrome

This refers to procedures in which the mordant is applied first, then a dye is applied and forms a lake with the mordant in the tissue. It is common with iron hematoxylin methods of the Heidenhain type.

Metachrome

Mordant and dye are combined in solution to form a soluble lake. This lake is then used to stain the tissue. The vast majority of alum hematoxylin solutions are of this type, as are some iron hematoxylin solutions, such as Weigert's solution frequently used as an acid resistant nuclear stain.

Afterchrome

These are rare in histotechnology. It refers to those methods in which the dye is applied first, followed by the mordant. Staining nuclei with Phenocyanin TC, followed by ferrous iron mordant is an example.

Displacement

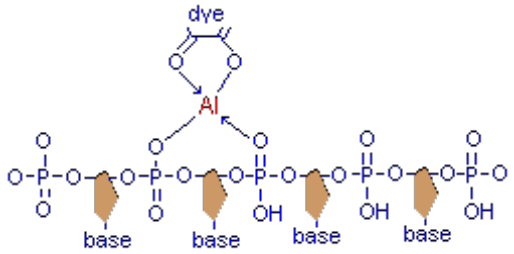
One reagent can replace another in chemical processes, provided both have similar reactions. It is the basis for trichrome staining, in which an acid dye stains the tissue, then a polyacid replaces it in some areas, followed by another acid dye which replaces the polyacid. In the hematoxylin context, it is the basis for the celestine blue-hemalum sequence which converts an iron mordanted celestine blue B nuclear stain to an iron mordanted hematoxylin nuclear stain to obtain better acid resistance, *i.e.* the hematoxylin displaces celestine blue.

Reactions With Tissue

Obviously, there is a reaction between the lake and tissue components, although it is not as straightforward as it may appear. There does appear to be more than one mechanism at work, including covalent bonds, hydrogen bonds and van der Waal's forces.

One early explanation was that the mordant attaches to DNA in nuclei by much the same mechanism as it attaches to the dye, *i.e.* by chelation - covalent and coordinate bond formation, carrying the hematoxylin along with it.

Phosphate hydroxyl groups of the nucleic acids provide means for covalent bonding, and other atoms in the vicinity can donate electrons for the coordinate bond. The DNA strand has a repeating sequence of phosphate and deoxyribose with a base attached. The four bases pair up in a complementary manner forming the double helix. For histological staining the bases are not significant, but the phosphate groups are fundamentally important. Attachment of mordanted dye is due to the mordant forming a chelate with a phosphate hydroxyl and another atom in a manner very similar to that between the mordant and the dye. Although the diagram below shows the source of electrons for the co-ordinate bond to also be a phosphate oxygen, this is for illustration purposes only and the actual source of electrons is not clear, if there is a single source



Attachment of an aluminum lake to DNA phosphate

Nuclear chromatin contains protein components in addition to DNA, so it is quite possible for two seperate staining events to be taking place at the same time. Proteins have many reactive groups available, including hydroxyl, carboxyl and amino. That is why some staining methods. including hemalums, may still demonstrate nuclei even after all DNA has been removed. This can happen with both acid and basic dyes, including lakes.

Even a cursory glance at a section stained with a regressive hemalum clearly shows that more than nuclei have been stained. With some the tissue is deep blue with little difference between the structures, and requires removal of some of the staining with acid alcohol. This removal, or differentiation, is not even, some structures such as chromatin resisting removal better than the overall background staining. That is, of course, the whole rationale behind the procedure. However, it does emphasise that there must be a difference in how the lake is attached to the different components, otherwise we would expect that dye from all components would be removed with equal ease.

Hematoxylin is a dye that is known to form hydrogen bonds, and it is likely that these are responsible for a significant part of the overall staining. Staining from a solution containing urea, a know preferential hydrogen bonding compound, diminishes the degree of background staining, but it does not eliminate it altogether. The final component is likely van der Waal's forces. Both hydrogen bonds and van der Waals forces are relatively weak and dye is easily removed with acid alcohol. They may also be involved in the staining of chromatin.

Low acid, well ripened hemalums also stain mucins *(See next section)*.

In summary, staining with hemalums may be due to:–

1. Covalent bonding to phosphate groups in DNA.
2. Bonding to nuclear associated protein, whether covalent or ionic.
3. Ionic bonding to acid mucopolysaccharides.
4. Hydrogen bonding.
5. Van der Waal's forces.

Reference

Baker, J. R., (1958). *Principles of biological microtechnique,* p. 207-227. Methuen, London, UK.

Staining Mucin

Some hemalums, particularly those that have a high hematein content and are low in acid, stain acid mucin. This is not an anomaly, it is a standard part of the characteristics of hemalums. Mayer even used this ability as a selective staining technique for acid mucin, coining the term ***mucihematein*** for his solution. The method is now obsolete, but was designed to accentuate the blue colour of mucins seen with many strong, regressive hemalum formulations. The same staining is seen with well oxidised, strong, regressive formulae such as Ehrlich's.

Mayer's Mucihematein for Mucin

Ingredient	*Var I*	*Var II*	*Var III*	*Var IV*	*Function*
Hematein	0.2 g	0.2 g	0.2 g	1 g	Dye
Aluminum	0.1 g	0.1 g	0.1 g	0.5 g	Mordant
Distilled	60 mL				Solvent
Glycerol	40 mL				Stabiliser
Ethanol, 70%		100 mL	100 mL	100 mL	Solvent
Nitric acid		2 drops (opt)	0.05 mL	drops	pH control

Compounding procedures

Var I, II & III

Grind the hematein with a few drops of the solvent. Add the aluminum chloride, and mix. Add the rest of the solvent, and mix. Add nitric acid if it is being used. Test the solution on a positive control (*e.g.* stomach).

Var IV

Make the 70% ethanol from absolute ethanol and tap water. Dissolve the hematein and aluminum chloride in it, and leave for one week. Test the solution on a positive control (*e.g.* stomach). If too pale add 1 drop of fresh 1% nitric acid and retest. Repeat if necessary, but no more than about 3 drops should be added.

Method

1. Bring sections to water
2. Place in the staining solution for 10-60 minutes
3. Wash well with tap water to blue.
4. Optionally, counterstain with eosin.
5. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Expected results

- Nuclei — blue
- Mucins — blue

Notes

1. Var I is from Mallory & Wright.
2. Var II is from Gatenby & Beams.
3. Var III is from Gray.
4. Var IV is from Bensley, who recommended preparing the hematein in house and staining for 5 minutes.
   This variant is much stronger than the others.

Reference

Bensley R. R. and Bensley, S. H., (1938). *Handbook of Histological and Cytological Technique.* U. Chicago Press, Chicago, USA

Gatenby, J.B. and Beams, H.W., (1950). *The Microtomist's Vade-Mecum. 11 ed.*, Churchill, London, UK.

Gray, Peter. (1954). *The Microtomist's Formulary and Guide.* The Blakiston Co.

Mallory, F. B. & Wright, J.H., (1904). *Pathological technique, Ed.3*. W. B. Saunders, Philadelphia, USA.

Dye:Mordant Ratio

One gram of ammonium alum contains 0.0595 grams of aluminum, and one gram of potassium alum contains 0.0569 grams. 50 grams would therefore contain 2.975 grams and 2.845 grams of aluminum respectively. Hematoxylin requires 0.0899 grams of aluminum to allow one aluminum atom for each molecule in one gram of dye. That is the aluminum content of 1.51 grams ammonium alum or 1.58 grams potassium alum. In addition, aluminum is trivalent and could, presumably, combine with three molecules of dye, although that is unlikely in practice as one valency would be required for attachment to appropriate tissue groups and an excess of mordant would tend to distribute the aluminum to as many dye molecules as possible. Even taking all this into account, it is plain that most hemalums have a considerable excess of aluminum available. It is this excess that can be used to control the staining characteristics of the various formulae, usually by altering the dye content.

The ratio of dye to mordant has a significant affect on the staining characteristics of the various formulae. Just as altering the dye content while keeping the alum concentration constant can have an influence on the nuclear selectivity of the solutions, so can increasing the alum concentration while keeping the dye concentration constant. In general, the higher the alum content, the more nuclear selective the solution is likely to be. Once again, this is easily shown by making a series of alum solutions from 0.1% to 10% and adding 0.1 mL of ripened 10% hematoxylin to 10 mL of each of them.

The usual explanation for this phenomenon is that in mass action systems there is a "competition" for the dye between mordant attached to the tissue and mordant still in solution. There is a tendency for the dye to equilibriate between the two, so that when there is a higher dye content, *i.e.* the ratio of mordant to dye is lower, the equilibrium favours the tissue. When the mordant to dye ratio is greater due to a lower dye concentration, then the equilibrium favours the solution and less dye attaches to the tissue.

The charts below compare the ratio of mordant to dye in aluminum hematoxylin formulae. The figure in the columns marked ***Dye*** is the hematoxylin or hematein content in grams. The column marked ***Mordant*** is the amount of source aluminum compound in grams. In most, but not all, cases this is ammonium or potassium alum. Please check the individual salts in the data chart for specifics. The column marked ***D:M*** is the ratio between them with the dye content normalised to 1. The figures in the column marked ***Needed*** is the amount of aluminum, in grams, required to give a 1:1 molecular ratio with the dye. The column marked ***Available*** is the actual amount of aluminum available in grams. The column marked ***N:A*** is the multiple by which the amount of aluminum available exceeds that needed. A number less then 1 in this column indicates there is less than a 1:1 molar ratio and that the dye is in excess. The two ratios are therefore the ***D:M*** column, based on weight, and the ***N:A*** column, based on molecules. The latter permits comparison between formulae using any source of aluminum.

Column headings in comparison lists

Formula	Name of the solution
Dye	Amount of hematoxylin or hematein in grams.
Mordant	Amount of aluminum source compound in grams.
D:M	Amount of aluminum source compound for each gram of dye.
Needed	Amount of aluminum needed, in grams, to give one atom of aluminum for each molecule of dye.
Available	The amount of aluminum, in grams, actually available.
N:A	The multiple by which the aluminum available exceeds that needed as defined above.

Aluminum hematoxylins sorted alphabetically

Formula	Dye	Mordant	D:M	Needed	Available	N:A
Anderson 1923a	2.5	20	8.0	0.22	1.19	5.3
Anderson 1923b	2.5	125	50	0.22	7.44	33.1
Anderson 1929	5	30	6	0.45	1.79	4
Apathy	3	30	10	0.27	1.79	6.6
Baker Hematal-8	0.94	8	8.4	0.08	1.24	14.7
Baker Hematal-16	0.47	8	16.8	0.04	1.24	29.4
Bencosme	2	133	67.5	0.18	7.57	42.1
Bennett	1	90	90	0.09	5.12	57
Bosma	2.5	25	10	0.22	1.49	6.6
Bullard	8	60	2.5	0.72	3.57	4.95
Carazzi	1	50	50	0.09	2.84	31.6
Cole 1903	6	6	1	0.54	0.36	0.7
Cole 1943	1.5	100	66.7	0.13	5.95	44.2
Debiden	5	100	20	0.45	5.69	12.7
de Groot	2	22	11	0.18	1.31	7.3
Delafield	6	90	15	0.54	5.36	9.9
Ehrlich	6	50	8.3	0.54	2.84	5.3
Friedlander	6	6	1	0.54	0.34	0.6
Gadsdon	5.5	60	10.9	0.49	3.41	6.9
Gage	1	40	40	0.09	2.28	25.3
Galigher	5	3	0.6	0.45	0.18	0.4
Garvey	2.5	45	18	0.22	2.56	11.4
Gill I	2	18	8.8	0.18	2.78	15.4
Gill III	4	70	17.6	0.36	11.10	30.9
Gill III	6	158	26.4	0.54	24.98	46.3
Graham	2	25	12.5	0.18	3.94	21.9
Hamilton	2	75	37.5	0.18	4.46	24.8
Harris	5	100	20	0.45	5.69	12.6
Harris & Power	20	60	3	1.8	3.41	1.9
Haug	5.5	5	0.9	0.49	0.96	1.9
Hine	1	4	3.8	0.09	0.59	6.6
Horneyold	8.4	4	0.5	0.75	0.25	0.3
Krutsay	1	50	50	0.09	2.84	31.6
Langeron 1924	4	50	12.5	0.36	2.84	7.9
Langeron 1942	1	50	50	0.09	2.84	31.6
Launoy	10	5	0.5	0.9	0.79	0.9
Lee	1	50	50	0.09	2.98	33.1
Lillie	5	50	10	0.45	2.98	6.6
McLachlan var I	2	17.5	8.8	0.18	2.76	15.4
McLachlan var II	2	25	12.5	0.18	1.49	8.3
Mallory	2.5	50	20	0.22	2.84	12.7
Mallory & Wright	1	140	140	0.09	8.33	92.7
Mann	6	35	5.8	0.54	1.99	3.7
Martinotti	2	15	7.5	0.18	0.89	5
Masson	20	60	3	1.80	3.41	1.9
Mayer 1891, 1903	1	50	50	0.09	2.98	33.1
Mayer 1901	1	50	50	0.09	2.84	31.6
Mayer 1896	4	50	12.5	0.36	2.98	8.3
Molnar I	10	50	5	0.90	2.98	3.3
Molnar II	4	50	12.5	0.36	2.98	8.3
Papamiltiades	4	10	2.5	0.36	1.58	4.4
Pusey	1.6	60	37.5	0.14	3.57	24.8
Rawitz 1895a	10	10	1	0.90	0.57	0.6
Rawitz 1895b	2.5	15	6	0.22	0.89	4
Rawitz 1909	2	20	10	0.18	1.44	8
Reddy	6.4	60	9.4	0.58	3.57	6.2
Sass var I	10	140	14	0.90	8.33	9.3
Sass var II	1	50	50	0.09	2.98	33.1
Schmorl	5	100	20	0.45	5.95	13.2
Scott	3	21	7	0.27	1.19	4.4
Slidders	1	100	100	0.09	5.69	63.3
Unna	3	140	46.7	0.27	8.33	30.9
Watson	6	6	1	0.54	0.36	0.7

Aluminum hematoxylins sorted by dye content

Formula	Dye	Mordant	D:M	Needed	Available	N:A
Baker Hematal-16	0.47	8	16.8	0.04	1.24	29.4
Baker Hematal-8	0.94	8	8.4	0.08	1.24	14.7
Hine	1	4	3.8	0.09	0.59	6.6
Gage	1	40	40	0.09	2.28	25.3
Carazzi	1	50	50	0.09	2.84	31.6
Krutsay	1	50	50	0.09	2.84	31.6
Langeron 1942	1	50	50	0.09	2.84	31.6
Mayer 1901	1	50	50	0.09	2.84	31.6
Lee	1	50	50	0.09	2.98	33.1
Mayer 1891, 1903	1	50	50	0.09	2.98	33.1
Sass var II	1	50	50	0.09	2.98	33.1
Bennett	1	90	90	0.09	5.12	57
Slidders	1	100	100	0.09	5.69	63.3
Mallory & Wright	1	140	140	0.09	8.33	92.7
Cole 1943	1.5	100	66.7	0.13	5.95	44.2
Pusey	1.6	60	37.5	0.14	3.57	24.8
Martinotti	2	15	7.5	0.18	0.89	5
de Groot	2	22	11	0.18	1.31	7.3
Rawitz 1909	2	20	10	0.18	1.44	8
McLachlan var II	2	25	12.5	0.18	1.49	8.3
Gill I	2	18	8.8	0.18	2.78	15.4
McLachlan var I	2	17.5	8.8	0.18	2.76	15.4
Graham	2	25	12.5	0.18	3.94	21.9
Hamilton	2	75	37.5	0.18	4.46	24.8
Bencosme	2	133	67.5	0.18	7.57	42.1
Rawitz 1895b	2.5	15	6	0.22	0.89	4
Anderson 1923a	2.5	20	8.0	0.22	1.19	5.3
Bosma	2.5	25	10	0.22	1.49	6.6
Garvey	2.5	45	18	0.22	2.56	11.4
Mallory	2.5	50	20	0.22	2.84	12.7
Anderson 1923b	2.5	125	50	0.22	7.44	33.1
Scott	3	21	7	0.27	1.19	4.4
Apathy	3	30	10	0.27	1.79	6.6
Unna	3	140	46.7	0.27	8.33	30.9
Gill III	4	70	17.6	0.36	11.10	30.9
Papamiltiades	4	10	2.5	0.36	1.58	4.4
Langeron 1924	4	50	12.5	0.36	2.84	7.9
Mayer 1896	4	50	12.5	0.36	2.98	8.3
Molnar II	4	50	12.5	0.36	2.98	8.3
Galigher	5	3	0.6	0.45	0.18	0.4
Anderson 1929	5	30	6	0.45	1.79	4
Lillie	5	50	10	0.45	2.98	6.6
Harris	5	100	20	0.45	5.69	12.6
Debiden	5	100	20	0.45	5.69	12.7
Schmorl	5	100	20	0.45	5.95	13.2
Haug	5.5	5	0.9	0.49	0.96	1.9
Gadsdon	5.5	60	10.9	0.49	3.41	6.9
Friedlander	6	6	1	0.54	0.34	0.6
Cole 1903	6	6	1	0.54	0.36	0.7
Watson	6	6	1	0.54	0.36	0.7
Mann	6	35	5.8	0.54	1.99	3.7
Ehrlich	6	50	8.3	0.54	2.84	5.3
Delafield	6	90	15	0.54	5.36	9.9
Gill III	6	158	26.4	0.54	24.98	46.3
Reddy	6.4	60	9.4	0.58	3.57	6.2
Bullard	8	60	2.5	0.72	3.57	4.95
Horneyold	8.4	4	0.5	0.75	0.25	0.3
Rawitz 1895a	10	10	1	0.90	0.57	0.6
Launoy	10	5	0.5	0.9	0.79	0.9
Molnar I	10	50	5	0.90	2.98	3.3
Sass var I	10	140	14	0.90	8.33	9.3
Harris & Power	20	60	3	1.8	3.41	1.9
Masson	20	60	3	1.80	3.41	1.9

Aluminum hematoxylins sorted by weight ratio

Formula	Dye	D:M
Horneyold	8.4	0.5
Launoy	10	0.5
Galigher	5	0.6
Haug	5.5	0.9
Rawitz 1895a	10	1
Friedlander	6	1
Cole 1903	6	1
Watson	6	1
Papamiltiades	4	2.5
Bullard	8	2.5
Harris & Power	20	3
Masson	20	3
Hine	1	3.8
Molnar I	10	5
Mann	6	5.8
Rawitz 1895b	2.5	6
Anderson 1929	5	6
Scott	3	7
Martinotti	2	7.5
Anderson 1923a	2.5	8.0
Ehrlich	6	8.3
Baker Hematal-8	0.94	8.4
Gill I	2	8.8
McLachlan var I	2	8.8
Reddy	6.4	9.4
Bosma	2.5	10
Apathy	3	10
Lillie	5	10
Rawitz 1909	2	10
Gadsdon	5.5	10.9
de Groot	2	11
Langeron 1924	4	12.5
McLachlan var II	2	12.5
Mayer 1896	4	12.5
Molnar II	4	12.5
Graham	2	12.5
Sass var I	10	14
Delafield	6	15
Baker Hematal-16	0.47	16.8
Gill III	4	17.6
Garvey	2.5	18
Harris	5	20
Mallory	2.5	20
Debiden	5	20
Schmorl	5	20
Gill III	6	26.4
Pusey	1.6	37.5
Hamilton	2	37.5
Gage	1	40
Unna	3	46.7
Carazzi	1	50
Krutsay	1	50
Langeron 1942	1	50
Mayer 1901	1	50
Lee	1	50
Mayer 1891, 1903	1	50
Sass var II	1	50
Anderson 1923b	2.5	50
Cole 1943	1.5	66.7
Bencosme	2	67.5
Bennett	1	90
Slidders	1	100
Mallory & Wright	1	140

Aluminum hematoxylins sorted by molecular ratio

Formula	Dye	N:A
Horneyold	8.4	0.3
Galigher	5	0.4
Rawitz 1895a	10	0.6
Friedlander	6	0.6
Cole 1903	6	0.7
Watson	6	0.7
Launoy	10	0.9
Harris & Power	20	1.9
Masson	20	1.9
Haug	5.5	1.9
Molnar I	10	3.3
Mann	6	3.7
Rawitz 1895b	2.5	4
Anderson 1929	5	4
Scott	3	4.4
Papamiltiades	4	4.4
Bullard	8	4.95
Martinotti	2	5
Anderson 1923a	2.5	5.3
Ehrlich	6	5.3
Bennett	1	57
Reddy	6.4	6.2
Hine	1	6.6
Bosma	2.5	6.6
Apathy	3	6.6
Lillie	5	6.6
Gadsdon	5.5	6.9
de Groot	2	7.3
Langeron 1924	4	7.9
Rawitz 1909	2	8
McLachlan var II	2	8.3
Mayer 1896	4	8.3
Molnar II	4	8.3
Sass var I	10	9.3
Delafield	6	9.9
Garvey	2.5	11.4
Harris	5	12.6
Mallory	2.5	12.7
Debiden	5	12.7
Schmorl	5	13.2
Baker Hematal-8	0.94	14.7
Gill I	2	15.4
McLachlan var I	2	15.4
Graham	2	21.9
Pusey	1.6	24.8
Hamilton	2	24.8
Gage	1	25.3
Baker Hematal-16	0.47	29.4
Unna	3	30.9
Gill III	4	30.9
Carazzi	1	31.6
Krutsay	1	31.6
Langeron 1942	1	31.6
Mayer 1901	1	31.6
Lee	1	33.1
Mayer 1891, 1903	1	33.1
Sass var II	1	33.1
Anderson 1923b	2.5	33.1
Bencosme	2	42.1
Cole 1943	1.5	44.2
Gill III	6	46.3
Slidders	1	63.3
Mallory & Wright	1	92.7

Data for calculations

Item	***Dye***	***AlM***	***AlK***	***AlS***	***AlA***	***AlN***	***AlC***	***Al***
FW	300.272	453.33	474.39	342.15	140.65	375.14	133.34	26.9815
Al atoms	—	1	1	2	1	1	1	—
Amount of Aluminum*	0.0899	0.0595	0.0569	0.1577	0.1918	0.0719	0.2023	—

****** *For the dye column this is the amount of aluminum in grams* ***required*** *to provide one atom for each molecule in one gram hematoxylin. For the other columns it is the amount of aluminum in grams* ***contained*** *in each gram of mordant.*

Column headings in Data chart

Item	*Parameter specified.*
Dye	Hematoxylin or hematin as specified by each formula.
AlM	Ammonium aluminum sulphate, ammonium alum.
AlK	Potassium aluminum sulphate, potassium alum
AlS	Aluminum sulphate
AlA	Aluminum acetate
AlN	Aluminum nitrate
AlC	Aluminum chloride
Al	Data for aluminum

Acids

Alum solutions are acidic, a 0.2 molar (5.16%) solution of potassium alum has a pH of 3.3, for instance. This is very close to the 50 grams per litre commonly used in hemalum formulae. At this pH the lake is soluble. As the solution is used and alkaline tap water is introduced into the solution, the pH rises until the lake begins to precipitate. This is shown by a colour change from cherry red to purple red, eventually becoming blue. It does not take too much contamination from tap water for this to happen, and non-acidified hemalums do not have a long useful life. Where acid is not used, it is advantageous to rinse the sections with distilled water before putting them into the hemalum so as to reduce any alkaline carry over.

It has become common practice to add small amounts of acids to hemalum solutions, initially to extend their useful life. Acetic acid at 2-5% is probably the most common, although others have been used, 0.1% citric acid in Langeron's 1942 formula, for instance, a solution usually erroneously referred to as "Mayer's" hemalum.

Solutions containing added acid have a much longer useful life, and when they eventually do begin to change colour, may be rejuvenated by the addition of a further small amount of acid. There is a limit, of course, to the number of times this can be done. It is a truism, however, that the useful life of hemalums is more closely linked to the depletion of the acid content than to depletion of the dye content. This is especially so with the stronger, regressive formulae where the dye content is so high that it would take months of continual, intensive use for it to be significantly lowered by the small amounts removed by attaching to DNA. Again, rinsing with water or with dilute acid of the kind used to acidify the hemalum, is advantageous in reducing alkaline carry over and extends the useful life of the solution.

An observed effect from the addition of acid is that nuclear staining is sometimes more selective. This shows up more obviously with those hemalums having a lower dye content. In fact, some of the stronger type, such as Ehrlich's or Lillies', do not show any significant difference. Coles's 1943 formula, however, has a noticeably different appearance, with much greater nuclear selectivity. Unacidified, and applied in a progressive stain for about ten minutes, this solution resembles a differentiated regressive stain. If acidified and similarly applied, it is selectively nuclear with noticeably less cytoplasmic staining. This is one of the best examples of how a small amount of acid can improve nuclear selectivity.

The improved nuclear selectivity is probably due to a slight lowering of the pH when extra acid is added. This may be sufficient to eliminate some of the reactions with acidic groups of cytoplasmic proteins. As to why it does not do so with stronger formulae, it may be that the high dye content just makes it more likely that cytoplasmic groups would react. It should also be mentioned that hematein can participate in non-ionic attachment of dye to tissues, and the presence of salts can promote these dipole-dipole interactions. Hematein can, in fact, be used in a Best's carmine type procedure to demonstrate glycogen, a method that is believed to be dependant on hydrogen bonding.

Since lowering the pH can make nuclear staining more selective, lowering it enough to completely inhibit staining from carboxyl groups, leaving only nuclear phosphate to stain, would seem to make sense. Krutsay’s hemalum is such a solution. It contains hydrochloric acid and is very highly selective for nuclei with a very clean, unstained background. It may be the most nuclear selective, progressive hemalum of them all. However, give usually involves take, and the disadvantage is that the very low pH results in removal of calcium deposits with no indication of their location, and the presence of small calcium deposits may be a useful indicator for some conditions.

The effect of adding acid to hemalum solutions can be seen by repeating the two previously described exercises with the addition of 0.2 mL glacial acetic acid or one drop of concentrated hydrochloric acid to each solution.

Differentiation

One very common way of staining with hematoxylin solutions is to overstain and then remove, or differentiate, the excess. This is the essence of ***regressive*** staining. With ***progressive*** staining, the hematoxylin solution is applied long enough to stain the target elements, then removed. It is timed so that no excess staining takes place, and no differentiation is needed.

The amount of staining to be removed depends on the particular characteristics of the formula used, but also on the personal preference of the microscopist who will be viewing the slides. This latter can vary significantly, ranging from almost none to quite significant degrees of differentiation. There is no "correct" degree. Some microscopists prefer a darkly stained background so they can detect ground substance and mucins, which they find useful in reaching a conclusion. Others prefer a clean background with clear and sharp nuclear staining.

Mordanted hematoxylin can be extracted from tissues in several ways. The two commonest means are extraction by acids and by mordants, although the latter is usually confined to iron hematoxylin staining of the Heidenhain type and may use the mordant at reduced strength. It is generally considered that acid alcohol produces the sharpest nuclear delineation, particularly with alum mordants. Mordant differentiation of hemalums often produces poorly defined structures and should be avoided.

Removal of excess background staining with aluminum mordanted hematoxylin is usually done with acid alcohol. That is, a solution of 0.5% or 1% hydrochloric acid in 70% ethanol. A greater degree of control can be exerted by using weaker concentrations of acid than that as it takes longer to accomplish. It is unusual to use stronger concentrations as they remove dye so quickly that it becomes difficult to control. Ethanol is not absolutely necessary, but it facilitates even differentiation compared to water. If water is used then increased agitation is necessary. Methylated spirits or iso-propanol may be used to replace ethanol.

The acid alcohol should be flooded onto the slide or the slide completely immersed in it for a few seconds, then removed and washed with water to eliminate all traces of hydrochloric acid. The time of differentiation is variable, depending on the degree of background staining and the depth of nuclear staining wanted. Over differentiation can be corrected by putting slides back into the alum hematoxylin solution and restaining.

Acids could attack the attachment of dye to nuclei at two points. The first is at the dye-mordant bond and the second is at the tissue-mordant bond. Both attachments are thought to be similar and depend on coordinate bonds between the mordant and the dye or the mordant and components of the DNA. Baker studied this very thoroughly using staining-destaining-restaining sequences and concluded that hydrochloric acid in acid alcohol broke the tissue-mordant bonds. In other words, the mordant is removed from the tissue and goes back into solution. It is likely that other forms of staining, hydrogen bonding for instance, are simply overwhelmed by the acid and the whole complex removed from the tissue component concerned.

Blueing

When a section of tissue is first stained and the hemalum solution rinsed off, the tissue is usually coloured red. ***Bluing*** refers to the procedure which converts this to a blue colour.

Blueing is accomplished by washing the stained sections with alkaline water. Anything with a pH of 8 or above is suitable and blues quite rapidly. Even water at pH 7 will do, particularly if warm. The usually recommended practice is to wash for ten minutes in running tap water, provided the tap water has a pH higher than 7. In many parts of the world this is quite satisfactory.

It is not universal, however, either because of an acidic local water supply or due to seasonal runoff making the local water temporarily acidic for a period of time. In those cases, tap water should not be used. Instead, a solution of a mildly alkaline salt will accomplish the same thing, and quite rapidly. The simplest, but not necessarily the best, is to add 1 or 2 drops of strong ammonia to each 100 millitres tap water in a staining jar, sufficient to render it mildly alkaline. Too much ammonia will detach the sections from the slide because of the softening effect it has on proteins, including any adhesive used.

Lithium carbonate, sodium carbonate, sodium bicarbonate or sodium acetate are used similarly. Usually a 0.5% aqueous solution is suitable, the concentration being adjusted to give conveniently fast blueing, *i.e.* the lowest concentration that will blue within about ten seconds and will treat several batches of slides, but which is not so strong that it detaches sections from slides. Many laboratories keep a saturated solution of one of these chemicals and dilute appropriately each day.

Many others may be suitable as well, but it is recommended that strong alkalis, such as hydroxides, not be used as they are likely to cause detachment of the sections from slides. Carbonates are usually satisfactory.

Some microscopists prefer to use Scott's tap water substitute, a solution designed to mimic hard, alkaline tap water. The presence of calcium and magnesium ions may also improve eosin counterstaining with this solution. It blues quite rapidly

Scott's tap water substitute:

Potassium bicarbonate ($KHCO_3$)	2 g.
Calcium chloride ($CaCl_2.2H_2O$)	0.5 g.
Magnesium sulphate ($MgSO_4.7H_2O$)	20 g.
Distilled water	1 L.

Blueing should be applied for the minimum time necessary to blue the sections completely. Once blued, the sections should be removed from the blueing solution. The higher the concentration, the more important this is. Alkaline solutions will slowly remove alum hematoxylin from nuclei. This does take some time, and with normal practice and times it may not be seen, but leaving sections in an alkaline medium for an extended time, overnight for instance, will cause nuclei to be less sharply stained. Bear in mind, also, that alkaline solutions soften protein adhesives, so the longer the sections remain in these solutions the more likely it is that sections will detach.

After blueing, residual blueing agent must be removed from the tissues as it may affect subsequent eosin staining if allowed to remain. This is done by washing, usually in tap water, although if the tap water is acidic, several changes of distilled water should be used. If the time required to wash out residual blueing agent is longer than the time required to blue the sections with tap water in the first place, then the chemical blueing is redundant and blueing with tap water should be used unless there is some other reason it is necessary.

For those places where acidic tap water is a significant problem, a water supply in which the acid has been neutralised can be set up fairly easily. It requires a large, perhaps 200 litre, plastic container with a suitable stand which has an opening at the bottom. The bottom of the container should be drilled to fit a hose connection to a tap. The bottom of the container is lined with a fine screen, then clean marble chips are placed on the bottom to a depth of about 10 centimetres. This is the neutralising medium. The container is filled with tap water, and drawn off for use through the tap connected to the hose at the bottom. As water is removed it flows over the neutralising medium and should become slightly alkaline. If the container is fitted with a ballcock mechanism connected to a water supply, the level of water in the container should remain fairly constant. A container with a suitably large volume ensures that the water comes in contact with calcium carbonate for long enough to be neutralised.

Choosing a hemalum

The four most influential factors determining the type of staining to be expected from individual hemalums are:

1. the amount of dye in the solution
2. the amount of mordant
3. the ratio between the dye and mordant
4. the pH

To these we should also add, perhaps, the time for which the solution is applied.

In practical application, the formulae that are most popular seem to fall into two groups. The first is those which have 1 gram dye in conjunction with 50 grams alum, or thereabouts, and which stain ***progressively***. The second is those which have 5 grams dye with 50 grams alum, or thereabouts, and which stain ***regressively***. The D:M ratios are 1:50 and 1:10 respectively, and are as suitable to use as reference points for comparison as any.

Having said that, one very popular formula falls outside it. Harris' hemalum has 5 grams dye with 100 grams alum (D:M ratio = 1:20). Interestingly, this solution is often said to be regressive without acid, and progressive with acid. This may be overstated as it also requires a short staining time of less than one minute and gives a noticeably blue background, but it does serve to illustrate the point. Despite the exceptions, the staining characteristics of an untested hemalum formula can be fairly accurately estimated by looking at these amounts and ratios.

Although the majority of formulae are clearly intended to be used either progressively (1 or 2 grams dye per litre) or regressively (5-6 grams per litre) a few are not clearly one or the other. These ***intermediate*** hemalum formulae have between 2 and 4 grams per litre with much the same concentration of alum as the other types. Their staining also falls between, giving darkly stained nuclei with more background staining than given by an obviously progressive formula. Each of these may be able to be used both progressively and regressively, depending on the time for which they are applied and how darkly the background staining should be.

Perhaps, rather than think of hemalum solutions as falling into one of two defined groups, we should consider the various formulae to represent a continuum of staining characteristics ranging from the sharp and highly selective staining obtained with Krutsay's hemalum to the dense overstaining of a formula such as Lillie's or Ehrlich's, with a continuous darkening of the staining as the dye content increases, modified only by the amounts of alum and acid.

It should also be possible for a custom hemalum to be formulated based on these principles. Adjusting the amounts of dye and mordant to increase or decrease nuclear selectivity should enable a progressive hemalum to be made that gives the desired degree of nuclear and background staining that a microscopist would find most useful. Apart from tailoring the appearance for this reason, it is also a useful exercise to do simply for itself as it teaches control of the H&E staining process. This method is of such importance that all technologists should have a thorough understanding of the principles involved and be capable of manipulating the end appearance as required.

These principles also apply with other mordants, such as metachrome iron hematoxylins, although the application is less striking. The ratio between dye and mordant is also a factor with other dyes, including at least one hemalum substitute. Mordant blue 3, in particular, responds similarly. In practical use, sharper progressive staining with mordant blue 3 is obtained as the amount of mordant decreases, whereas with hematoxylin selectivity improves as the amount of mordant increases.

Reference

Baker, John R., (1958). *Principles of biological microtechnique*. Methuen, London, UK.

Baker, J. R., (1962), *Experiments on the action of mordants: 2. Aluminium-haematein*. Quarterly Journal of Microscopical Science, **v**. 103, pt. 4, pp. 493-517.

Budavari, S., Editor, (1996). *The Merck Index, Ed. 12*. Merck & Co., Inc., Whitehouse Station, NJ, USA

Staining With Hemalum

Aluminum mordanted hematein
There are two ways to stain with aluminum mordanted hematein, progressively and regressively, however, the difference between the two methods is minor.

Progressive Staining Method for paraffin sections
1. Bring sections to water with xylene and ethanol.
2. Place into the staining solution for 10 minutes.
3. Rinse with water.
4. Blue.
5. Wash well with water.
6. Counterstain if desired.
7. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Regressive Staining Method for paraffin sections
1. Bring sections to water with xylene and ethanol.
2. Place into the staining solution for 10 minutes.
3. Rinse well with water.
4. Differentiate with acid alcohol.
5. Rinse with water.
6. Blue.
7. Wash well with water.
8. Counterstain if desired.
9. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Expected results
- Nuclei — blue
- Background — as counterstain or unstained

Even a cursory examination shows that the only difference between these two procedures is that the regressive method includes a treatment with acid alcohol to remove excess staining. Some microscopists believe that sharper nuclear staining is obtained with regressive staining. Others consider it to be necessary to obtain the amount of non nuclear staining they prefer. Both may be overcome if the dye and acid contents are adjusted properly, since just about any degree of background staining can be obtained progressively by modifying the amounts of dye and alum, and increasing the acid content and type of acid can sharpen nuclear staining The advantage of doing it that way is greater consistency, as a variable in the procedure is removed, *i.e.* the length of time acid alcohol is applied.

The time of application of the hemalum is given as 10 minutes. This can be adjusted to produce the depth of staining required. Progressive staining tends towards being self limiting, the nuclei staining fairly rapidly initially, then increasing in depth of colouration more slowly. With regressive staining, the excess is removed with acid alcohol, so as long as a degree of overstaining is initially obtained, the time of differentiation can be altered to bring about the desired results.

Iron Hematoxylin

Types of iron hematoxylin
There are two ways that iron hematoxylins are used. Metachrome procedures, in which the mordant and hematoxylin are combined in a single solution and applied together, and onchrome procedures, in which the sections are treated with the mordant first, then the dye is applied and subsequently differentiated either with a solution of the mordant or some other means.

Onchrome staining
Onchrome staining in the context of iron hematoxylins refers to the Heidenhain type, so called as Heidenhain introduced the first such technique. These methods are three step staining procedures as follows:—

1. Treat sections with the mordant solution.
2. Place in hematoxylin solution. The sections should be black when staining is complete.
3. Differentiate. This is often done with the mordant, full strength or diluted, but acids may also be used.

These methods are not designed for rapid staining. The first two steps may take a minimum of a few hours each, and are often applied overnight. Differentiation time is variable depending on what is being demonstrated, and often required visual microscopic control. There are modifications to these methods requiring higher temperatures (60°C) for an hour or two each, so they can be done within one day, but they are often considered to be inferior in the quality of results. This is, perhaps, overstated.

Iron hematoxylins produce black staining rather than the blue or purple-blue of alum hematoxylin. This is ideal for monochrome photography and made them popular in the past, but this is less of a concern today with the excellent colour recording obtained with modern digital photography.

Metachrome staining
Metachrome staining refers to those iron hematoxylin solutions where the mordant and dye are combined before they are applied to the tissue. Weigert's iron hematoxylin solution is a typical example.

These solutions have much in common with hemalums, which are also metachrome solutions, except that aluminum salts are not involved and the mordant is an iron salt, usually ferric chloride or ferric ammonium sulphate (iron alum). The principles are much the same, however.

There are a group of metachrome iron hematoxylin solutions that are used for purposes other than nuclear staining. These are the myelin stains, often incorporating lithium carbonate into the formula, making them less selective for nuclei and permitting other structures to be stained.

Verhoeff's iron hematoxylin is a metachrome solution which is used most often to demonstrate elastic, but which may also be used to stain myelin. Unlike most metachrome hematoxylin procedures, Verhoeff's stain is differentiated with a solution of the mordant.

Metachrome iron hematoxylin solutions tend to be stable for relatively short periods, ranging from a few hours to a few weeks. This is primarily due to the use of ferric (Fe^{+++}) salts as the mordant, usually ferric ammonium sulphate (iron alum) or ferric chloride. Both of these are oxidising agents which eventually cause the formation of poorly staining compounds, often giving an ill defined, muddy brown appearance to the tissue. It is usually advantageous to make up these solutions with a fresh, unoxidised hematoxylin solution, rather than an old, ripened one. The mordant will oxidise the hematoxylin when combined with it, but it is important to allow sufficient time for this to occur before using the solution for staining. Usually 15-30 minutes after combining the hematoxylin and mordant is adequate, depending on the specific solution being made.

Counterstaining Hemalum

Probably the most common counterstain to alum hematoxylin is eosin. As an acid dye, eosin staining has much in common with other acid dyes, such as those used for single solution and multi-step trichrome methods and the various quad stains. The basic underlying principles are very similar, merely the application being different.

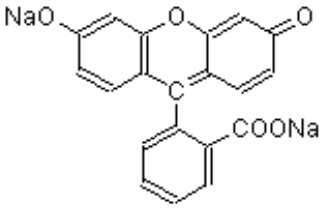
Acid dyes as a group are quite diverse, ranging from picric acid, which has a molecular weight of 229, to the large molecular weight dyes such as fast green FCF at 809. In fact, this difference in molecular weights is one of the features of these dyes that enables us to manipulate them so successfully.

So what is an acid dye? Simply put, it is an organic salt in which the anion is intensely coloured, and which will attach to cations while retaining its colour. That definition gives a clear indication as to the chemical basis for this staining, that of ionic interaction between dye anions and tissue cations. The former are carboxyl, hydroxyl and sulphonic groups predominantly, while the latter are nearly always amino groups. In most cases it involves plain ionic bonds.

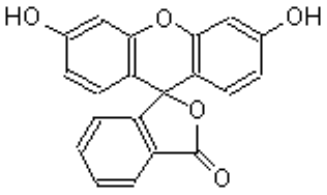
The Eosins

Eosin belongs to a group of dyes called fluorones, which are derived from fluorescien. The sodium salt of fluorescien is called uranin. It is not used histologically, but its relationship to eosin is quite apparent when the formulae are compared. These dyes are fluorescien homologues halogenated with bromine, iodine or chlorine. Eosin Y ws itself is tetrabromofluorescien, for instance, *i.e.* fluorescien with four bromine atoms attached.

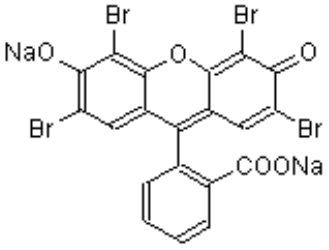
The free anion of eosin Y is called eosinol Y, and its relationship to fluorescien is also apparent. Eosinol, and similar compounds from the other dyes related to eosin such as phloxine and erythrosine, can be used to stain tissues.



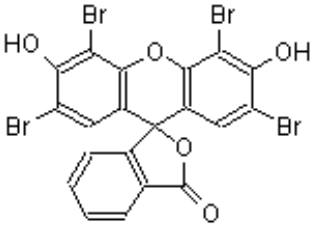
Uranin



Fluorescien



Eosin Y ws



Eosinol Y

Eosin is commonly applied as a solution in hard tap water, from 0.5% - 2%. It is applied for a couple of minutes, the excess is then washed out until it is stained the way we want, then dehydrated, cleared and mounted. This works well in London, England. It happens that the water there is rich in calcium and magnesium ions and is slightly alkaline. It turns out that water with those characteristics is just about ideal for eosin staining, and for gaining the maximum variation in staining intensity with that.

For London it is a very fortuitous circumstance as, due to the local water quality, an H&E stained section can show considerable variation in intensity between the various types of tissue, with deep pink erythrocytes, paler muscle and even paler collagen.

Unfortunately for eosin staining elsewhere, not everyone in the world has access to London tap wate.

Fortunately eosin Y can also be used in alcoholic solution. I became familiar with this very early in my

career as the hospital in which I worked as a student, in London as it happened, used a 0.1% solution in 80% industrial methylated spirits. It was applied for 15 seconds, followed by a slow rinse with absolute methylated spirits and clearing with xylene. The results very closely approximated those obtained with aqueous staining.

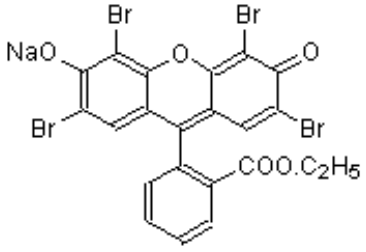
I have used this method extensively in Canada with ethanol rather than methylated spirits and have always obtained quite satisfactory results for about 40 years.

To use eosinol instead of eosin it must first be made since it is rarely, if ever, listed by dye vendors. Make a strong solution of eosin Y ws in water (10%). Slowly add concentrated hydrochloric acid little by little. A precipitate will form. Keep adding hydrochloric acid until no more precipitate forms. Filter out the precipitate using a filter paper. The precipitate is the eosinol. It is insoluble in water, so it can be washed with distilled water to remove any residual acid, then dried at room temperature. When dry it may be ground into a powder and stored in a jar like any other dye. It is quite stable at room temperature.

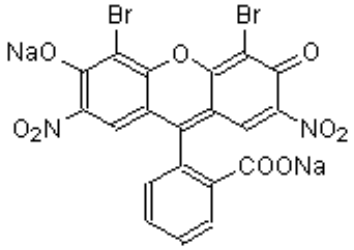
For use, a small amount is dissolved into the final dehydrating ethanol or into the first clearing xylene. The sections are allowed to remain in it until the staining is at the required depth, then clearing is completed and the section coverslipped. The staining is somewhat homogenous, but this procedure enables material to be stained that otherwise does not take up any eosin. It may be useful for some necrotic, decayed or fetal tissues which are causing problems. It is not recommended as a regular procedure.

There is a variant of eosin, ethyl eosin, which is sold as alcohol soluble eosin, but this is quite redundant. It has the same formula as eosin Y but with an ethyl group replacing the sodium. Ordinary eosin Y is ethanol soluble at sufficient concentration to make it satisfactory as a stain from ethanol solution.

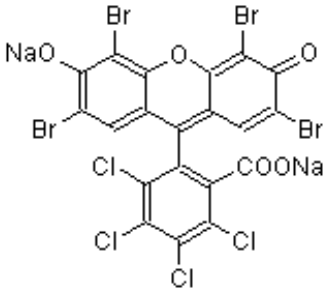
There is also an eosin with a blue cast, eosin B, which is rarely used but stains essentially the same as eosin Y but has a cold cast. Neither of these dyes is particularly popular.



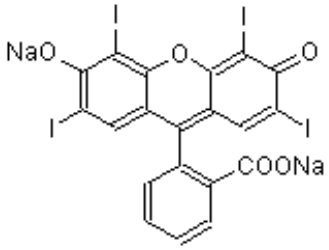
Ethyl eosin



Eosin B



Phloxine B



Erythrosine B

There are other dyes related to eosin Y which are sometimes added to the simple ethanolic solution in order to increase differential staining. These are phloxine B and erythrosine B. Of these two, most commonly phloxine B is used.

Mixed dyes

Adding small amounts of other dyes to eosin Y is quite prevalent in the United States. It comes about in an attempt to increase the intensity of staining in a part of the world where water quality varies greatly in different places. In some parts of the country the water may be soft and acidic periodically, during the spring snow melt, for instance. In other places acetic acid is added to the eosin in order to increase staining intensity deliberately. Applying an acid dye from an acid medium does increase staining intensity, but usually also results in a "flattening" of staining, that is, the differential effect obtained with eosin Y and

London tap water is lost and the staining becomes homogenous.

Due to this, some laboratories add a small amount of phloxine B to the eosin. This usually results in more intensely stained erythrocytes, in effect putting back some differential effect. It is quite surprising how little phloxine needs to be added to accomplish this.

The first two formulae below are quite typical.

Acidified Eosin		***Acidified Eosin & Phloxine***		***Eosin Trichrome***
Ethanol	800 mL	Ethanol	800 mL	Ethanol
Distilled water	200 mL	Distilled water	200 mL	Isopropanol
Glacial acetic acid	5 mL	Glacial acetic acid	5 mL	Methanol
Eosin Y ws	2 g	Eosin Y ws	2 g	Acetic acid
		Phloxine	1 g	Eosin Y ws
				Phloxine B
				Orange G

The solution would be applied for a minute or so, then washed well with 80% ethanol, then absolute ethanol and cleared. Sometimes mixtures of alcohols may be used, such as iso-propanol, methanol, ethanol, but the effect is the same.

In an attempt to get even more differentiation into the counterstain, sometimes the small molecular weight dye, Orange G is added. This gives an orange cast to some structures and the picture is not unpleasing. Companies may provide solutions like this under various names. Ingredients may include those in the third column above in varying proportions.

Perhaps the most unusual mixture of dyes used as a hemalum counterstain is Meter's Eosin, which is quite popular in some laboratories: The biebrich scarlet has much the same function as the orange G, but with a darker and redder colour.

Meter's Eosin	
Distilled water	800 mL
95% ethanol	200 mL
Biebrich scarlet	0.4 g
Eosin Y ws	5.0 g
Phloxine B	2.1 g

HPS

Yet another approach to counterstaining hemalum was taken by others. In the past this method was quite popular in one very large hospital in Ontario, Canada. In North America it is known as the HPS or Hematoxylin phloxine saffron. Other places may know it better as the HES, or hematoxylin erythrosine saffron. The technique requires an aqueous solution of phloxine B or erythrosine B and a completely water free alcoholic solution of saffron. As the world's supply of saffron is now much less than formerly, its role as a dye having been replaced by synthetic alternatives, the price has increased and the cost of using it in this method makes it prohibitive. I should note that cheaper saffrons do not work well in this technique, although Indian saffron as used in Indian cooking is usually satisfactory and is not too expensive.

The procedure is to stain nuclei with hemalum and blue, apply the phloxine or erythosine, dehydrate thoroughly, then soak the section in water-free alcoholic saffron until collagen has stained yellow but muscle is still pink. The end point is determined visually.

The saffron is extracted into the absolute ethanol in an incubator over a few days, and even trace amounts of water cause the staining to fail. Needless to say, the procedure never became very popular, but it does give a nice looking section with differential staining. Alternatives using synthetic dyes have been suggested, but the extra attention required in busy laboratories make using them unattractive as a routine method, although they retain their usefulness as special stains.

All of these procedures are interesting from another point of view, quite apart from their value as counterstains for hemalum. They all attempt to bring about some form of differential staining whether it be by preferentially removing some of the dye by washing it out, mixing together different dyes and letting them preferentially stain different components from a single solution, or applying dyes sequentially so that the second can replace the first as with phloxine and saffron.

The point is that in other staining methods these same practices have been used to bring about the one-step and multi-step trichrome methods as well as the methods based on the phloxine tartrazine technique, which itself depends on the same principles as the HPS.

References

Horobin, R. W., and Kiernan, J.A., (2002). *Conn's Biological Stains*, 10 ed., Biological Stain Commission. BIOS, Scientific Publishers, Oxford, UK

Lee F. W., (1969). *Eosinol counterstain for routine paraffin embedded tissue.* Journal of Medical Laboratory Technology, **v. 26** (Jan), pp. 36-7

Alum Hematoxylin Formulae

(Hemalum)

Anderson`s Alum Hematoxylin

There are three variations listed for Anderson's alum hematoxylin. 1923a is likely an intermediate type. 1923b is likely a darkly staining progressive or intermediate type. 1929 is a typical regressive formula.

Ingredient	***1923a***	***1923b***	***1929***	***Function***
Hematoxylin	2.5 g	2.5 g	5 g	Dye
Ammonium alum	20 g	100 g	30 g	Mordant
Distilled water	900 mL	900 mL	700 mL	Solvent
95% ethanol	50 mL	50 mL	50 mL	Solvent
Calcium hypochlorite	4 g	—	40 g	Oxidant
Chloramine T *or* lime chloride		4 g		Oxidant
Glacial acetic acid	50 mL	50 mL	50 mL	Acidifier

Compounding procedure

1923a and 1929

- Dissolve the hypochlorite in 200 mL water.
- Dissolve the hematoxylin in 200 mL water.
- After 4 hours, combine the solutions.
- Dissolve other ingredients in 500 mL water.
- Combine with the hematoxylin solution.

1923b

- Dissolve the alum in 700 mL water.
- Dissolve the chloramine T in 200 mL water.
- Leave four hours. Shake occasionally.
- Dissolve the hematoxylin in the ethanol.
- Add the oxidant solution to the hematoxylin.
- Mix for a few seconds.
- Slowly add to the alum solution, mixing well.
- Add acetic acid.

Notes

Anderson's formulae specify chloramine T or calcium hypochlorite as the oxidising agent. It is recommended that sodium iodate be used instead at the ***maximum*** amounts given below.

1923a 0.5 g

1923b 0.5 g

1929 1 g

References

Gray, Peter. (1954). *The Microtomist's Formulary and Guide.* Blakiston, New York, USA.
Gatenby, J.B. and Beams, H.W., (1950). *The Microtomist's Vade-Mecum. 11 ed..* Churchill, London, UK.
Susan Budavari, (1996). *The Merck Index, Ed. 12.* Merck & Co., Inc., Whitehouse Station, NJ, USA

Apathy's Alum Hematoxylin

This is another intermediate hemalum.

Ingredient	*Amount*	*Function*
Hematoxylin	3 g	Dye
Ammonium alum	30 g	Mordant
Distilled water	450 mL	Solvent
95% ethanol	250 mL	Dye solvent
Glycerol	350 mL	Stabiliser
Glacial acetic acid	10 mL	Acidifier
Salicylic acid	0.3 g	Acidifier

Compounding
Dissolve the dye in 100 mL water and 250 mL ethanol.
Oxidise atmospherically at room temperature (or chemically for immediate use).
Dissolve the alum and acids in 350 mL water.
Combine both solutions.
Add glycerol.

Notes

Lillie leaves out the salicylic acid.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide.* Blakiston, New York, USA.
Lillie, R.D., (1954). *Histopathologic technique and practical histochemistry Ed.2*, Blakiston, New York, USA.

Baker's Hematal

Hematal Variants

Ingredient	*Stock A*	*Stock B*	*Function*
Aluminum sulphate	15.76 g	—	Mordant
Distilled water	1 L	—	Solvent
Haematein	—	1.876 g	Dye
Ethylene glycol, 50%, aqu.	—	1 L	Solvent

Ingredient	*Hematal-8*	*Hematal-16*
Stock A	1 vol	2 vol
Stock B	1 vol	1 vol
Ethylene glycol, 50%, aqu.	—	1 vol

Compounding procedure

Prepare each stock solution seperately.
Combine as specified.
The solution may be used immediately.

Staining time
Progressive

Hematal-8 — 2-5 minutes.

Hematal-16 — 10 minutes.

Regressive

Hematal-8 — 30 minutes.

Notes

1. Both are suitable for routine staining.
2. 0.5% aqueous sulphuric acid was recommended for differentiation, but hydrochloric acid is suitable.
3. Hematal-8 has 8 atoms of aluminum for each molecule of hematein, Hematal-16 has 16, hence the names.

Reference

Baker, J. R., (1962), *Experiments on the action of mordants: 2. Aluminium-haematein.* Quarterly Journal of Microscopical Science, **v**. 103, pt. 4, pp. 493-517.

Baker's Standard Alum Hematoxylin

Ingredient	*Solution A*	*Solution B*	*Function*
Aluminum sulphate	3.94 g	—	Mordant
Distilled water	1 L	—	Solvent
Haematoxylin	—	5 g	Dye
Sodium iodate	—	0.5 g	Dye
Distilled water	—	1 L	Solvent

Compounding procedure
Prepare each solution separately.
Solution B should be brought to a boil, then immediately cooled.

Method
1. Place dewaxed sections into solution A for 1 hour.
2. Rinse with water.
3. Place into solution B for 20 minutes.
4. Rinse with water.
5. Differentiate with 0.5% sulphuric acid for 40 seconds.
6. Rinse with water.
7. Blue with 0.5% ammonia water for 5 seconds.
8. Wash well with water.
9. Optionally, counterstain with eosin.
10. Dehydrate, clear and mount with a resinous medium.

Notes
1. This formula was not intended for routine use. It was designed for experimental purposes to facilitate varying each step.

Reference
Baker, J. R., (1962), *Experiments on the action of mordants: 2. Aluminium-haematein.* Quarterly Journal of Microscopical Science, **v**. 103, pt. 4, pp. 493-517.

Bencosme's Alum Hematein

	Original	Variant	
Ingredient	***Amount***	***Amount***	***Function***
Hematein	2 g	2.5 g	Dye
Potassium alum	133 g	120 g	Mordant
Distilled water	920 mL	1 L	Solvent
Glacial acetic acid	20 mL	10 mL	Acidifier

Compounding procedure
Add the alum to the water in a 2 L flask and bring to the boil for 1 minute.
Add the hematein and mix by swirling.
Place an inverted funnel over the flask and simmer for 10 minutes, shaking frequently.
Cool to room temperature and add the acetic acid.
Filter before use and twice weekly.
It may be used immediately.

Notes
1. This solution uses hematein rather than hematoxylin. Hematoxylin could be used, but the original formula would require a maximum of 0.4 grams sodium iodate and the variant formula 0.5 g sodium iodate for complete oxidation.
2. When freshly made this solution stains in 2 minutes. Staining time slowly increases to 10 minutes as the solution ages. Life is about a month.

Reference
Lynch M.J., Raphael S.S., Mellor L.D., Spare P.D. and Inwood M.J.H., (1969). *Medical Laboratory Technology, ed. 2.* W. B. Saunders Co., Toronto, On., Canada

Bennett's Alum Hematoxylin

Ingredient	*Amount*	*Function*
Hematoxylin	1 g	Dye
Potassium alum	90 g	Mordant
Distilled water	1 L	Solvent
Sodium iodate	0.2 g	Oxidant
Chloral hydrate	50 g	Stabiliser
Citric acid	1 g	Acidifier

Compounding procedure
Heat the water. Then add in order:-
Hematoxylin, sodium iodate, alum, citric acid and chloral hydrate.
Dissolve each ingredient before adding the next.
Cool to room temperature and filter.
It may be used immediately.

Notes
1. Putt gives no information regarding staining time and characteristics, but the similarity of the formula to Langer-on's (Mayer's) hemalum suggests it is progressive and selectively nuclear. A staining time of 10-20 minutes should be adequate.

Reference
Putt, F. A., *Manual of Histopathological Staining Methods*. John Wiley & Sons, New York, NY., USA

Böhmer's Alum Hematoxylin

Böhmer's formula is the original alum hematoxylin for nuclear staining. Although not used anymore it is of educational and historical interest.

Solution A	***Formula 1***	***Formula 2***	***Function***
Hematoxylin	3.5 g	8 g	Dye
100% ethanol	100 mL	100 mL	Solvent

Solution B	***Formula 1***	***Formula 2***	***Function***
Ammonium alum	0.3 g	0.3 g	Mordant
Distilled water	100 mL	100 mL	Solvent

Compounding procedure
Formula 1 is taken from the Microtomist's Formulary and Guide, and Formula 2 from the Microtomist's Vade-Mecum. The difference in concentration of the hematoxylin may be due to converting an alcoholic logwood extract to grams of dye. In any case, the way it is used makes the differences irrelevant.

Originally solution A would have been made by soaking logwood chips in ethanol until a suitable concentration of dye was obtained. The solution would then have been allowed to ripen for a long time until it was distinctly deep brown, and filtered before it was used. In a modern variation, simply dissolve the dye in ethanol and leave to ripen, or add a small amount of sodium iodate.

The original called for a few drops of solution A to be added to a small quantity of solution B in a watch glass until the depth of colour was judged to be correct. For today's use, perhaps 5 mL solution A added to 45 mL solution B, more or less, would be satisfactory.

Method
1. Place a small amount of staining solution into a watch glass.
2. Place frozen sections into the staining solution for an appropriate time.
3. Transfer sections through at least two changes of clean water.
4. Mount onto slides.
5. Dehydrate in ethanol, clear in xylene and mount with a resinous medium

or

1. Bring sections to water via xylene and ethanol.
2. Place into the staining solution for an appropriate time.
3. Rinse with water and blue.
4. Rinse well with water.
5. Counterstain if desired.
6. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Notes
1. The appropriate time should be determined by trial, but 10-20 minutes should be long enough. The time will depend on the amount of solution A added to solution B. Smaller amounts are likely to take longer to stain with a lighter final intensity.

References
Gray, Peter. (1954). *The Microtomist's Formulary and Guide.* The Blakiston Co.
Bolles Lee, A., Gatenby, J.B. and Beams, H.W., (1950). *The Microtomist's Vade-Mecum. 11 ed.*, Churchill, London

Bosma's Alum Hematoxylin

Ingredient	*Amount*	*Function*
Hematoxylin, 10% alc.	25 mL	Dye
Ammonium alum	25 g	Mordant
Tap water	865 mL	Solvent
Diethylene glycol	100 mL	Stabiliser
Sodium iodate	0.2 g	Oxidant
Glacial acetic acid	10 mL	Acidifier

Compounding procedure

Place 815 mL hot tap water in a large flask.
Add the ammonium alum, mix well to dissolve and cool to room temperature.
Add the alcoholic hematoxylin solution.
Add the sodium iodate dissolved in 50 mL cold tap water and mix well.
Add the diethylene glycol and mix well.
Add the acetic acid and mix well.
The pH should be 3.1 to 3.3 and the solution may be used immediately.

Notes

1. Hematoxylin dissolves in ethanol to about 3%. When an undissolved excess of hematoxylin is present, the dye content in solution increases as hematoxylin oxidises to hematein, which dissolves to about 7.5%, and 25 mL of a fully oxidised solution would have just less than 2 grams hematein in it. Since it is hematein, *i.e.* already oxidised, adding further oxidant would likely overoxidise the solution. Since extra chemical oxidation is done, it would seem that partially oxidised alcoholic hematoxylin is meant to be used. However, that introduces a variable, since it cannot be known how much hematoxylin has been converted to hematein in the alcoholic solution, and how much dye is actually incorporated into the final hemalum.
2. Due to the concerns in *Note 1*, this formula would likely best be made with a freshly prepared, unripened solution of 2.5 grams hematoxylin in 25 mL ethanol. This should be added to the other ingredients without being filtered. The amount of sodium iodate should then be adjusted to give the degree of oxidation desired. Complete oxidation would be accomplished with 0.5 grams sodium iodate.

Reference

Bosma, Rob., (1988*). A useful hematoxylin without toxic chemicals.* Histologic, **v**. 18, No. 1.
Green, F. J., (1990). *Sigma Aldrich Handbook of Stains, Dyes and Indicators*. Aldrich Chemical Company, Milwaukee, Wisconsin, USA.
Horobin, R. W. and Kiernan, J. A., (2002). *Conn's Biological Stains ed. 10*. Biological Stain Commission, BIOS Scientific Publishers, Oxford, UK.

Bullard's Alum Hematoxylin

Ingredient	*Amount*	*Function*
Hematoxylin	8 g	Dye
Ammonium alum	20 g	Mordant
Distilled water	250 mL	Solvent
50% ethanol	144 mL	Solvent
Glacial acetic acid	16 mL	Acidifier
Red mercuric oxide	8 g	Oxidant
95% ethanol	275 mL	Solvent
Glycerol	330 mL	Stabiliser
Glacial acetic acid	18 mL	Acidifier
Ammonium alum	40 g	Mordant

Compounding procedure
Combine the first five ingredients and bring to the boil.
Add the mercuric oxide with caution.
Cool and filter.
Add the last four ingredients.
The solution may be used immediately, and is stable for more than a year.

Notes
1. This solution calls for mercuric oxide as the oxidant. It is now recommended that mercuric oxide ***not*** be used due to its eventual contamination of the environment. Substitute 1.6 grams sodium iodate for complete oxidation of the hematoxylin.

Reference
Putt, A., *Manual of Histopathological Staining Methods*. John Wiley & Sons, New York, NY., USA

Carazzi's Alum Hematoxylin

Ingredient	*Amount*	*Function*
Hematoxylin	1 g	Dye
Potassium alum	50 g	Mordant
Distilled water	800 mL	Solvent
Glycerol	200 mL	Stabiliser
Sodium iodate	0.2 g	Oxidant

Compounding procedure
Dissolve the hematoxylin in glycerol.
Dissolve the alum in 750 mL of water.
Dissolve the sodium iodate in the remaining 50 mL water.
Add the alum solution to the hematoxylin solution slowly, while mixing well.
Add the sodium iodate solution. Mix well and filter.
The solution may be used immediately, and is stable for about six months.

Notes
1. This is a progressive solution giving litttle background staining.
2. The amount of hematoxylin may be doubled for a darker nuclear stain.
3. Doubling the amount of hematoxylin may necessitate doubling the oxidant depending on the staining desired.
4. The double strength solution is recommended for frozen sections with about 1 minute staining time.

Reference
Bancroft, J.D. and Stevens A. (1982). *Theory and practice of histological techniques, Ed. 2*. Churchill Livingstone, Edinburgh & London, UK.

Cole's Alum Hematoxylin

References to Cole's hemalum that do not specify which of the two formulae is meant, usually refer to the 1943 formula.

Ingredient	***1903***	***1943***	***Function***
Hematoxylin	6 g	1.5 g	Dye
Ammonium alum	6 g	100 g	Mordant
Distilled water	320 mL	950 mL	Solvent
100% ethanol	320 mL	50 mL	Solvent
Glycerol	290 mL	—	Stabiliser
Iodine	—	0.5 g	Oxidant
Glacial acetic acid	75 mL	See Notes 2, 3	Acidifier

Compounding procedure

1903
Dissolve the alum in water.
Dissolve the hematoxylin in ethanol.
Combine, then add the other ingredients and mix well.
The solution must ripen before use.

1943
Dissolve the hematoxylin in 250 mL water with heat.
Dissolve the alum in 700 mL water.
Dissolve the iodine in ethanol.
Combine the dye and iodine solutions.
Add the alum solution.
Bring to the boil.
Cool and filter.
The solution may be used immediately.

Notes

1. The 1903 formula was meant to be oxidised atmospherically. If oxidised chemically, complete oxidation would require 1.2 grams sodium iodate.
2. The original 1943 formula contained no added acid. Used progressively, and applied for 5-10 minutes, it gives results similar to those obtained with a differentiated regressive formula.
3. The addition of 20 mL glacial acetic acid to the 1943 formula increases nuclear selectivity and extends the working life of the solution. This is often used for progressive nuclear counterstaining and in the celestine blue-hemalum sequence.
4. Instead of iodine, 0.3 grams sodium iodate could be used as oxidant in the 1943 formula.

Reference

Culling, C.F.A., Allison, R.T. and Barr, W.T., (1985). *Cellular Pathology Technique, Ed.4.* Butterworth, London, UK.

Drury, R.A.B. and Wallington, E.A., (1980). *Carleton's histological technique Ed. 5*. Oxford University Press, Oxford, UK.

Bancroft, J.D. and Stevens A. (1982). *Theory and practice of histological techniques Ed. 2*. Churchill Livingstone, Edinburgh & London, UK.

Gray, Peter. (1954). *The Microtomist's Formulary and Guide*. The Blakiston Co.

Debiden's Alum Hematoxylin

Ingredient	*1987*	*1991*	*Function*
Hematoxylin	5 g	5 g	Dye
Potassium alum	100 g	100 g	Mordant
Distilled water	1 L	1 L	Solvent
Glacial acetic acid	20 mL	20 mL	Acidifier
Mercuric oxide, red	2.5 g	—	Oxidant
Javex (5.25% sodium hypochlorite)	—	2 mL	Oxidant

Compounding procedure
Dissolve the alum in water in a large flask.
Add the hematoxylin and mix well.
Place in a 60-65°C oven overnight.
Remove from the oven and add the oxidising agent.
Stir until cold. Filter.

1980
Acetic acid may be added as soon as it is cooled following preparation.
It is usable immediately.
It is preferable to prepare small volumes monthly.

1991
Staining improves if allowed to ripen for a week or two.
If acetic acid is used it should be added just before use.

Notes

1. It is recommended that mercuric oxide ***not*** be used for oxidation of hematoxylin due to the eventual contamination of the environement. Substitute a maximum of 1 gram sodium iodate in the 1987 variant, or use the 1991 variant.
2. Acetic acid is optional in Harris' type hemalums. If added, solution life is extended and staining of the background is a little less.

References
Debiden, D., (1987). *Improved preparation of Harris' hematoxylin.* Histologic, **v.**18, **No.**8
Debiden, D., (1991). *A new oxidant for Harris' hematoxylin*. Histologic, **v.**21, **No.**2

De Groot's Alum Hematoxylin

Ingredient	*Amount*	*Function*
Hematoxylin	2 g	Dye
Ammonium alum	22 g	Mordant
Distilled water	270 mL	Solvent
95% ethanol	650 mL	Solvent
Glycerol	80 mL	Stabiliser
Hydrogen peroxide	7.5 mL	Oxidant
Potassium ferricyanide	0.8 g	See Note 2
Calcium chloride	15 g	See Note 3
Sodium bromide	7.5 g	See Note 4

Compounding procedure
Mix the ethanol, water and glycerol to make the solvent.
Add the peroxide to 15 mL of the solvent.
Add the hematoxylin, and dissolve.
Dissolve the calcium chloride and sodium bromide in 250 mL of the solvent.
Mix with the hematoxylin solution.
Add half the alum, and dissolve.
Dissolve the potassium ferricyanide in 400 mL of the solvent.
Add to the hematoxylin solution.
Dissolve the remaining alum in the remaining solvent.
Add to the hematoxylin solution.

Notes
1. The strength of hydrogen peroxide is not specified, but the commonest laboratory strength is 30 vols.
2. Both hydrogen peroxide and potassium ferricyanide are oxidising agents. However, it is not clear if potassium ferricyanide is present for that reason.
3. Although calcium can mordant hematoxylin, it is not clear if it is present for that reason.
4. The purpose of the sodium bromide is not clear. It may be present as a preservative similar to chloral hydrate in some other formulas.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide.* The Blakiston Co.

Delafield's Alum Hematoxylin

Ingredient	*Amount*	*Function*
Hematoxylin	6 g	Dye
Ammonium alum	90 g	Mordant
Distilled water	600 mL	Solvent
95% ethanol	200 mL	Solvent
Glycerol	150 mL	Stabiliser

Compounding procedure
Dissolve the hematoxylin in 50 mL ethanol.
Dissolve the alum in the water.
Add the two solutions.
Leave, loosely stoppered, in a warm, sunlit place for one week.
Filter, then add the glycerol and the rest of the ethanol.
Leave, loosely stoppered, in a warm, sunlit place for 3 months.
Filter and store in a tightly stoppered container in the dark.
This solution is stable for years.

Notes
1. The solution may be partially ripened by adding 0.5 g sodium iodate, but such solutions are considered to be inferior in longevity than those ripened atmospherically.
2. Like many strong regressive formulations, cartilage, cement lines and mucins may be blue.

Reference
Culling, C.F.A., Allison, R.T. and Barr, W.T., (1985). *Cellular Pathology Technique, Ed.4.* Butterworth, London, UK.

Bancroft, J.D. and Stevens A. (1982). *Theory and practice of histological techniques, Ed.2*. Churchill Livingstone, Edinburgh & London, UK.

Duval's Alum Hematoxylin

Ingredient	*Original*	*Modern*	*Function*
Hematoxylin, concentrated ethanolic	8 mL	8 mL	Dye
Ammonium or potassium alum	a little	25 g	Mordant
Distilled water	800 mL	800 mL	Solvent

Compounding procedure
Dissolve the alum in the water.
Add the hematoxylin.
The solution is likely progressive, although this is not stated to be so.

Notes
1. This fomula is from the late 1800's and is now obsolete, although the modern formula should stain satisfactorily.
2. The alcoholic hematoxylin would have been made by soaking logwood chips in ethanol. When fresh this would have contained a maximum of about 3% hematoxylin. After the solution had ripened, further extractions could have increase the dye content, but only to a maximum of about 7% hematein. Eight mL of concentrated alcoholic hematoxylin or hematein would therefore have contained between 0.24 - 0.56 grams of dye.
3. The type of alum was not specified, the most likely being either potassium or ammonium.
4. The formula calls for adding a "little alum" to 800 mL water. Potassium alum saturates at about 14% in water, so 800 mL would contain about 112 g. I have taken just less than 25% of the maximum (*i.e.* 25 grams) as being a "little". Of course, it could be any amount between 1 and 112 grams.
5. The appropriate time should be determined by trial. The instructions are to use full strength for a few minutes.

Reference
Bolles-Lee, A. (1885). *The Microtomist's Vade-Mecum*. J & A Churchill, London, England.
Susan Budavari, Editor, (1996). *The Merck Index, Ed.12*. Merck & Co., Inc., Whitehouse Station, NJ, USA

Ehrlich's Alum Hematoxylin

Ingredient	*Amount*	*Function*
Hematoxylin	6 g	Dye
95% ethanol	300 mL	Solvent
Potassium alum	excess	Mordant
Distilled water	300 mL	Solvent
Glycerol	300 mL	Stabiliser
Glacial acetic acid	30 mL	Acidifier

Compounding procedure
Add acetic acid to ethanol and dissolve the hematoxylin in it.
Mix water and glycerol in an oversized container and dissolve the alum in it.
Add the hematoxylin solution to the alum solution and mix.
Plug the container loosely with cotton wool.
Ripen by leaving in a warm, sunlit place for several weeks.
When sufficiently ripened, tightly stopper and store in a cool, dark place.
The solution is stable for years.

Notes
1. Gray gives 7 grams hematoxylin, and specifies ammonium alum.
2. The alum should be added to excess. This should be about 50 grams, but enough should be added to ensure undissolved alum is present.
3. This is a strongly staining, regressive formula. The staining time should be determined by trial. Usually, 20 minutes is adequate.
4. As with many strong alum hematoxylin solutions, cartilage, cement lines and mucin may stain blue.
5. The solution may be partially ripened chemically by adding 0.5g sodium iodate, but chemically ripened solutions are considered to have inferior longevity.

Reference
Culling, C.F.A., Allison, R.T. and Barr, W.T. (1982). *Cellular Pathology Technique, Ed.4.* Butterworth, London, UK.

Drury, R.A.B. and Wallington, E.A., (1980). *Carleton's histological technique Ed.5*. Oxford University Press, Oxford, UK.

Bancroft, J.D. and Stevens A. (1982). *Theory and practice of histological techniques Ed.2*. Churchill Livingstone, Edinburgh & London, UK.

Gray, Peter. (1954). *The Microtomist's Formulary and Guide.* The Blakiston Co.

Friedlander's Alum Hematoxylin

Ingredient	*Amount*	*Function*
Hematoxylin	6 g	Dye
Potassium alum	6 g	Mordant
Distilled water	300 mL	Solvent
95% ethanol	300 mL	Solvent
Glycerol	300 mL	Stabiliser

Compounding procedure
No directions are given, but try:—
Dissolve the alum in water.
Dissolve the hematoxylin in ethanol.
Combine, and add glycerol.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide*. The Blakiston Co.

Gadsdon's Alum Hematoxylin

This formula is by Prof. Derek Gadsdon at the Sheffield Children's Hospital, UK.

Ingredient	*Amount*	*Function*
Hematoxylin	5.5 g	Dye
Potassium alum	60 g	Mordant
Distilled water	610 mL	Solvent
100% ethanol	100 mL	Solvent
Glycerol	300 mL	Stabiliser
Sodium iodate	0.5 g	Oxidant
Glacial acetic acid	20 mL	Acidifier

Compounding procedure
Dissolve the hematoxylin in 100 mL ethanol.
Dissolve the alum in 600 mL water.
Dissolve the sodium iodate in 10 mL water.
Combine the hematoxylin and alum solutions. Mix well.
Add the sodium iodate solution. Mix well.
Add the acetic acid. Mix well.
Leave overnight.
Add the glycerol. Mix well.
Filter before use.
The solution may be used immediately.
It is stable at room temperature for at least 6 months.

Notes
1. This solution may be used regressively or progressively.
   Regressively, staining time is 6 minutes.
   Progressively, staining time is 3 minutes.
   Frozen sections will stain in 1 minute.

Reference
Red Cross Children's Hospital. *Gadsdon's Haematoxylin Method*. Histonet communication, Nov, 1996.
Jim Elsam, HTEQA Services. *Gadsdon's Hx*. Histonet communication, Nov, 1996

Gages' Alum Hematoxylin

Ingredient	*Amount*	*Function*
Hematoxylin	1 g	Dye
Potassium alum	40 g	Mordant
Distilled water	1 L	Solvent
95% ethanol	20 mL	Solvent
Chloral hydrate	20 g	Oxidant

Compounding procedure
Not given, but based on experience:—
Dissolve the hematoxylin in ethanol.
Dissolve the alum in water.
Combine solutions, and add chloral hydrate.

Notes
1. As no oxidising agent is present the solution needs ripenng. Chemical ripening would require a maximum of 0.2 grams sodium iodate.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide*. The Blakiston Co.

Galigher's Alum Hematoxylin

This formula was recommended as a substitute for Harris' alum hematoxylin.

Ingredient	*Amount*	*Function*
Hematoxylin	5 g	Dye
Ammonium alum	3 g	Mordant
Distilled water	500 mL	Solvent
100% ethanol	500 mL	Solvent
Mercuric oxide	6 g	Oxidant

Compounding procedure
Combine the water and ethanol.
Add the alum and dye, and bring to the boil.
Add the mercuric oxide, then simmer for 20 minutes.
Restore the volume to 1 L with 50% ethanol.
Cool, and filter through double thickness filter paper.
Store in a tightly stopper bottle.
The solution may be used immediately, and is stable for about six months.

Notes
1. It is recommended that mercuric oxide not be used as an oxidant because of eventual contamination of the environment. Substitute up to 1 gram sodium iodate.

Reference
Gatenby, J.B. and Beams, H.W., (1950) . *The Microtomist's Vade-Mecum, ed*.11, Churchill, London, UK.

Garvey's Alum Hematoxylin

Ingredient	Amount	Function
Hematoxylin	2.5 g	Dye
Potassium alum	45 g	Mordant
Distilled water	900 mL	Solvent
100% ethanol	100 mL	Solvent
Sodium iodate	0.3 g	oxidant
Citric acid	1 g	Acidifier

Compounding procedure
Dissolve the hematoxylin in ethanol.
Dissolve the alum indistilled water with heat.
Combine the two solutions, then add sodium iodate and citric acid.
Shake well to dissolve.
The solution may be used immediately, and is stable for several months.

Notes
1. Ammonium alum may be used instead of potassium alum.
2. As the solution was recommended as a substitute for Mayer's alum hematoxylin, staining times and results should be comparable. However, the increased hematoxylin content indicates the solution should stain more darkly.
3. Blueing is done with alkaline solutions such as hard tap water, Scott’s tap water substitute, 0.1% ammonia water, 1% aqueous sodium acetate, 0.5% aqueous lithium carbonate etc.

Reference
Garvey, W. *Modification of the Mayer Hematoxylin stain.* Journal of Histotechnology, **v.**14, **No.**3, *p.*163

Gill's Aluminum Hematoxylin

Ingredient	*Single (I)*	*Double (II)*	*Triple (III)*	*Function*
Hematoxylin	2 g	4 g	6 g	Dye
Aluminum sulphate	17.6 g	70.4 g	158.4 g	Mordant
Distilled water	750 mL	750 mL	750 mL	Solvent
Ethylene glycol	250 mL	250 mL	250 mL	Solvent
Sodium iodate	0.2 g	0.4 g	0.6 g	Oxidant
Glacial acetic acid	20 mL	20 mL	20 mL	Acidifier

Compounding procedure
Mix the ethylene glycol and water.
Add hematoxylin, then sodium iodate.
Add aluminum sulphate and acetic acid
Stir for an hour at room temperature.
Filter before use.
The solutions may be used immediately, and are stable for approximately one year.

Notes
1. The three formulae are for progressive staining.
2. The single strength solution is for cytology. Staining time is 2 minutes.
   The double strength solution is for paraffin sections. Staining time is 3 minutes.
   The triple strength solution is for paraffin sections. Staining time is 1.5 minutes.
3. The aluminum sulphate should be $Al_2(SO_4)_3.18\ H_2O$. Adjust the amount if different.
4. The hematoxylin should be anhydrous. If hydrated ($C_{16}H_{14}O_6.3\ H_2O$) use 2.36 g, 4.72 g and 78 g respectively.

Reference
Culling, C.F.A., Allison, R.T. and Barr, W.T. (1982). *Cellular Pathology Technique, Ed.4.* Butterworth, London, UK.

Graham's Aluminum Hematoxylin

Graham's alum hematoxylin is a modification of Gill's alum hematoxylin, and was designed for routine staining of plant tissue. It should also be satisfactory for animal tissues. It has been formulated so that small quantities may be prepared fresh, sufficient for a single Coplin jar.

Solution A	***Amount***	***Function***
Distilled water	750 mL	Solvent
Propylene glycol	210 mL	Solvent
Glacial acetic acid	20 mL	Acidifier
Aluminum sulphate	17.6 g	Mordant
Sodium iodate	0.2 g	Oxidant

Solution B	***Amount***	***Function***
Hematoxylin	10 g	Dye
Propylene glycol	100 mL	Solvent

Compounding procedure

Solution A
Combine all the ingredients and filter when dissolved.

Solution B
Shake vigorously several times during 48 hours until the hematoxylin dissolves.

Working solution

Solution A - 49 mL
Solution B - 1 mL
Mix well.
It is ready for use when an opaque dark purple.

Notes

1. Staining is progressive, application time being determined by trial (try 2 minutes).
2. Propylene glycol replaces toxic ethylene glycol.
3. The aluminum sulphate should be $Al_2(SO_4)_3.18H_2O$. Adjust the amount if different.

Reference

Graham, E. T., (1991). *A quick mix aluminum hematoxylin stain*. Biotechnic and Histochemistry, pp. 279-281.

Hamilton Alum Hematoxylin

This formula is based on Langeron's 1942 formula, which is often erroniously referred to as Mayer's hemalum, and is used in much the same way, giving somewhat darker staining. I have referred to it as Hamilton alum hematoxylin as I received it via the internet from a histotechnologist at the Hamilton Health Sciences Corporation, Hamilton, Ontario, Canada.

Ingredient	*Amount*		*Function*
Hematoxylin	2	g	Dye
Ammonium alum	75	g	Mordant
Distilled water	950	mL	Solvent
100% ethanol	50	mL	Solvent
Sodium iodate	0.3	g	Oxidant
Glacial acetic acid	30	mL	Acidifier

Compounding procedure
Dissolve the alum in water.
Add ethanol and mix well.
Add hematoxylin and dissolve.
Add sodium iodate and mix well.
Leave overnight in the dark to ripen.
Mix well, and add acetic acid.
Filter.
The solution may be used immediately.

Reference
Personal internet communication, source unidentified.

Harris' Alum Hematoxylin

Ingredient	*Amount*	*Function*
Hematoxylin	5 g	Dye
Potassium alum	100 g	Mordant
Distilled water	1 L	Solvent
95% ethanol	50 mL	Solvent
Mercuric oxide	2.5 g	Oxidant
Glacial acetic acid	40 mL	Acidifier

Compounding procedure
Dissolve the hematoxylin in ethanol.
Dissolve the alum in warmed distilled water.
Combine the solutions in an oversized container.
Bring to the boil.
Add the mercuric oxide, remove from heat.
Cool rapidly.
Add glacial acetic acid if being used.
It may be used immediately when cool.

Notes
1. The solution is a regressive formulation, although it is sometimes used progressively with short staining times.
2. When the mercuric oxide is added, the solution may foam.
3. It is recommended that mercuric oxide ***not*** be used as an oxidant due to eventual contamination of the environment. A maximum of 1 gram sodium iodate may be substituted for total oxidation of hematoxylin. In practice, 0.5 gram works quite well.
4. The acetic acid is optional, but nuclear staining is more precise if it is included.
5. A precipitate often forms, so the solution should be filtered before use.
6. As with many strong hematoxylin solutions, mucin may stain blue.

Reference
Culling, C.F.A., Allison, R.T. and Barr, W.T. (1982). *Cellular Pathology Technique*, Ed.4. Butterworth, London, UK.

Drury, R.A.B. and Wallington, E.A., (1980). *Carleton's histological technique Ed.5*. Oxford University Press, Oxford, UK.

Bancroft, J.D. and Stevens A. (1982). *Theory and practice of histological techniques Ed.2*. Churchill Livingstone, Edinburgh & London, UK.

Harris & Power's Alum Hematoxylin

Ingredient	*Amount*	*Function*
Hematoxylin	20 g	Dye
Potassium alum	60 g	Mordant
Distilled water	100 mL	Solvent
100% ethanol	6 mL	Solvent

Compounding procedure
Mix the alum and hematoxylin in a mortar.
Add the water, little by little, while grinding the mixture.
Filter, and add the ethanol.

Notes
1. Although not stated, presumably this very strong mixture should be left to ripen for some time.
2. Its characteristics and recommended use were not given.
3. The staining time should be determined by trial.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide*. The Blakiston Co.

Haug's Alum Hematoxylin

Ingredient	*Amount*	*Function*
Hematoxylin	5.5 g	Dye
Aluminum acetate	5 g	Mordant
Distilled water	100 mL	Solvent
100% ethanol	5 mL	Solvent

Compounding procedure
Dissolve the hematoxylin in ethanol.
Dissolve the aluminum acetate in water.
Combine.

Notes
1. Although not stated, presumably this very strong mixture should be left to ripen for some time.
2. Its characteristics and recommended use were not given.
3. The staining time should be determined by trial.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide*. The Blakiston Co.

Hine's Alum Hematoxylin for Block Staining

Hine's method is designed to stain blocks of tissue with hematoxylin and eosin. The blocks are then sectioned, dried and immediately coverslipped. Numerous identically stained sections may be prepared for demonstration and educational purposes.

Hematoxylin	***Amount***	***Function***	**Compounding procedure**
Hematoxylin, 1% aqu.	100 mL	Dye	Add the ingredients in the order listed. Filter before use.
Aluminum sulphate, 5% aqu.	75 mL	Mordant	
Lugol's iodine	25 mL	Oxidant	
Acetic acid, glacial	8 mL	Solvent	
Glycerol	50 mL	Stabiliser	

Eosin	***Amount***	***Function***	Dissolve the eosin in ethanol, and let stand for one month. For use, dilute with an equal volume of 90% ethanol and filter.
Ethyl eosin	1 g	Dye	
90% ethanol	100 mL	Solvent	

Worcester's fluid	***Amount***	***Function***	Dissolve the mercuric chloride in water. Immediately prior to use add formalin and acetic acid.
Distilled water	200 mL	Solvent	
Mercuric chloride	14 g	Fixing agent	
Formalin, concentrated	22.5 mL	Fixing agent	
Acetic acid, glacial	25 mL	Fixing agent	

Method

1. Fix 1.5 mm slices of fresh tissue in Worcester's fluid and formalin fixed tissue in formol sublimate overnight.
2. Remove from fixative and place in 70% ethanol for one hour.
3. Place tissue into 70% ethanol coloured dark brown with Lugols iodine. Change three times over 48 hours.
4. Transfer to fresh 70% ethanol (no iodine) for two hours, then to distilled water for one hour.
5. Place the blocks into hematoxylin for seven days, then wash in running tap water overnight.
6. Begin dehydration with 70% and 80% ethanols.
7. Place in the working eosin solution for five days.
8. Dehydrate with absolute ethanol, two changes over five to eight hours.
9. Blot off excess ethanol and clear in cedarwood oil.
10. Blot off excess cedarwood oil and place into xylene, two changes for ten minutes each.
11. Impregnate with paraffin wax under vacuum, three changes of one hour each.
12. Block out, section. Bake on. Remove wax with xylene and coverslip with a resinous medium

Notes

1. The method gives good results with all tissue except CNS.
2. Staining of sections has not deteriorated after 18 years.
3. Staining of processed tissues has not deteriorated after 18 years.
4. Bouin's fluid can replace Worcester's fluid, but the yellow colouration should be removed by placing in sodium bicarbonate in 50% ethanol (concentration not specified) instead of the iodinated 70% ethanol.
5. If needed, decalcification can be done with Gooding and Stewart's fluid after removal of mercury pigment and before staining with hematoxylin.
6. The hematoxylin and eosin solutions can be re-used.

Reference

Hine, Ian F., (1981). *Block staining of mammalian tissues with hematoxylin and eosin*. Stain technology, **v** 56, p 119

Horneyold's Alum Hematoxylin

Hematoxylin solution	***Amount***	***Function***
Hematoxylin	0.7 g	Dye
100% ethanol	20 mL	Solvent

Alum solution	***Amount***	***Function***
Ammonium alum	0.35 g	Mordant
Distilled water	60 mL	Solvent

Tincture of iodine	***Amount***	***Function***
Iodine	70 g	Oxidant
Potassium iodide	50 g	—
Distilled water	50 mL	Solvent
100% ethanol	950 mL	Solvent

Compounding procedure

Staining solution
Dissolve the hematoxylin in ethanol and the alum in water.
Combine the two solutions and mix well.
Leave in the light by a window for 3-4 days.
Add 20 drops tincture of iodine.

Tincture of iodine
Mix dry iodine and potassium iodide together.
Add the water and mix well.
Add the ethanol and mix well.

Notes
1. Horneyold's formula is a modification of Böhmer's alum hematoxylin and is considered to be obsolete.
2. This solution is regressive, and is recommended for osmium fixed tissue.
3. Differentiate with acetic ethanol (70% ethanol containing glacial acetic acid). The amount of acetic acid was not given, but 0.5%-1% should be enough.

Reference
Gatenby, J.B. and Beams, H.W., (1950) . *The Microtomist's Vade-Mecum. 11ed.*, Churchill, London, UK.
Lange, N.A., (1966). *Lange's Handbook of Chemistry, Revised 10th ed.*, McGraw-Hill.

Kleinenberg's Alum Hematoxylin

There are two alternate formulae for Kleinenberg's alum hematoxylin.

Formula I

Hematoxylin	***Amount***	***Function***
Hematoxylin	saturated	Dye
100% ethanol	100 mL	Solvent
Ammonium alum	saturated	Mordant
70% ethanol	100 mL	Solvent
Calcium chloride	saturated	Mordant ?
70% ethanol	100 mL	Solvent

Working solution formula I
Alum solution — 85 mL
Calcium solution — 15 mL
Hematoxylin solution —1 mL

Formula II

Hematoxylin	***Amount***	***Function***
Hematoxylin	saturated	Dye
100% ethanol	100 mL	Solvent

Alum A	***Amount***	***Function***
Ammonium alum	saturated	Mordant
Calcium chloride	saturated	Mordant ?
70% ethanol	100 mL	Solvent

Alum B	***Amount***	***Function***
Potassium alum	saturated	Mordant
70% ethanol	88 mL	Solvent
Alum A solution	12 mL	—

Working solution formula II
Alum B solution — 100 mL
Hematoxylin solution — 3 mL

Notes
1. Although not stated, the saturated hematoxylin solution should presumably be allowed to ripen.
2. Formula II is three times the strength of formula I. This may affect the staining times.
3. Calcium can mordant hematoxylin, but it is not certain that is its function.
4. Alum and calcium chloride combined give aluminum chloride hematoxylin.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide*. The Blakiston Co.

Krutsay's Alum and Iron Hematoxylin

Krutsay's alum hematoxylin gives very highly selective staining of nuclei. It may also be easily converted into an iron hematoxylin for use as an acid resistant nuclear stain.

Alum hematoxylin	***Amount***	***Function***
Hematoxylin	1 g	Dye
Potassium alum	50 g	Mordant
Distilled water	1 L	Solvent
Hydrochloric acid	5 mL	Acidifier
Sodium iodate	0.2 g	Oxidant

Iron conversion	***Amount***	***Function***
Iron alum	2 g	Mordant
Hydrochloric acid	0.5 mL	Acidifer
Distilled water	100 mL	Solvent

Compounding procedure for the alum hematoxylin
Mix all reagents together.
Bring to the boil, cool and filter.
The solution may be used when cooled to room temperature.

Compounding procedure for the conversion solution
Dissolve iron alum and hydrochloric acid in water.

To convert the alum hematoxylin to iron hematoxylin

Alum hematoxylin solution	25 mL
Iron conversion solution	2 mL

Notes

1. The alum hematoxylin is very similar to Mayer's hemalum, but with hydrochloric acid instead of acetic acid. This makes it highly selective for nuclei, but removes calcium deposits.
2. The iron alum is ($FeNH_4(SO_4)_2.12\ H_2O$)

Reference
Humason, G. L. *Animal Tissue Techniques.* W. H. Freeman and Co., San Francisco, CA, USA

Langeron's Alum Hematoxylin

Langeron’s 1942 formula is the solution usually meant when “Mayer’s” hemalum is specified.

Both of Langeron’s formulae are modifications of Mayer's formulae. Langeron’s 1924 formula is a modification of Mayer’s 1896 formula, and Langeron’s 1942 formula is a modification of Mayer’s 1901 formula.

Ingredient	***1924***	***1942***	***Function***
Hematoxylin	4 g	1 g	Dye
Ammonium or potassium alum	50 g	50 g	Mordant
Distilled water	700 mL	1 L	Solvent
Glycerol	300 mL	—	Stabiliser
Sodium iodate	—	0.2 g	Oxidant
Glacial acetic acid	20 mL	—	Acidifier
Citric acid	—	1 g	Acidifier
Chloral hydrate	—	50 g	Acidifier

Compounding procedure

1924
Grind the hematoxylin with glycerol in a pestle and mortar.
Dissolve the other ingredients in water.
Add the hematoxylin paste.
Wash out the hematoxylin paste with the solution.
Leave months to ripen.

1942
Dissolve the alum and dye in the water.
Add the other ingredients.
Bring to the boil.
Cool to room temperature.
The solution may be used immediately.

Notes

1. The 1924 solution is better used regressively.
2. The 1942 solution may be used progressively.

Reference

Gray, Peter. (1954). *The Microtomist's Formulary and Guide*. The Blakiston Co.

Launoy's Alum Hematoxylin

Ingredient	Amount	Function
Hematein	10 g	Dye
Potassium alum	5 g	Mordant
Distilled water	1 L	Solvent

Compounding procedure
Combine all ingredients, and dissolve.
The solution may be used immediately.

Notes
1. This solution uses hematein instead of hematoxylin.
2. Although the staining characteristics are not given, the high dye and low alum content would indicate it is likely a strong, regressive solution.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide*. The Blakiston Co.

Lee's Alum Hematoxylin

Ingredient	*Amount*	*Function*
Hematoxylin	1 g	Dye
Ammonium alum	50 g	Mordant
Distilled water	1 L	Solvent
Chloral hydrate	50 g	Stabiliser
Sodium iodate	0.2 g	Oxidant

Compounding procedure
Combine all the ingredients.
Bring to a boil.
Cool and filter.
The solution may be used immediately.

Notes
1. The solution is similar to Mayer's hemalum and should have much the same characteristics.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide*. The Blakiston Co.

Lillie's Alum Hematoxylin

Ingredient	*Amount*	*Function*
Hematoxylin	5 g	Dye
Ammonium alum	50 g	Mordant
Distilled water	700 mL	Solvent
Glycerol	300 mL	Stabiliser
Sodium iodate	0.5 g	Oxidant
Glacial acetic acid	20 mL	Acidifier

Compounding procedure
Dissolve the dye and alum in water.
Add sodium iodate, glycerol and acetic acid.
Leave overnight to ripen before using.

Notes
1. This is a strong, regressive solution.

Reference
Lillie, R.D., *Histopathologic Technic and Practical Histochemistry*. McGraw-Hill, New York, USA

Lugol's Alum Hematoxylin

Lugol's Iodine	***Amount***	***Function***
Iodine	2 g	Oxidant
Potassium iodide	4 g	
Distilled water	100 mL	Solvent

Hematoxylin	***Amount***	***Function***
95% ethanol	100 mL	Solvent
Hematoxylin	10 g	Dye

Alum	***Amount***	***Function***
Potassium alum	6 g	Mordant
Distilled water	500 mL	Solvent

Working solution	***Amount***
Hematoxylin	1.5 mL
Lugol's iodine	1.5 mL
Alum	100 mL

Compounding procedure
Make each solution as listed.
Combine as directed above.
Leave 15 minutes before use.

Notes
1. This is a progressive formula. Staining time is 5-10 minutes.
2. The working solution is stable for a minimum of one week, and will stain at least 100 slides.
3. This formula was not published by Lugol, it was named Lugol's hematoxylin by the authors.

Reference
Clarke, G. and Dodds, H. M., (1983). *Lugol's Hematoxylin*. Stain Technology, **v 58**, No. 4, p. 232

Mallory's Alum Hematoxylin

Ingredient	*Amount*	*Function*
Hematoxylin	2.5 g	Dye
Potassium alum	50 g	Mordant
Distilled water	1 L	Solvent
Thymol	2.5 g	Preservative

Compounding procedure
Dissolve hematoxylin and alum in water.
Add the thymol
Allow to ripen before use.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide.* The Blakiston Co.

Mallory & Wright's Alum Hematoxylin

Ingredient	*Standard*	*Strong*	*Function*
Hematoxylin	1 g	1 g	Dye
Ammonium alum, saturated aqueous	100 mL	100 mL	Mordant
Distilled water	300 mL	—	Solvent

Compounding procedure
Dissolve the hematoxylin in the alum solution by warming if necessary.
Add water if the standard solution is being used.
Plug the container with cotton wool.
Ripen at room temperature for approximately ten days.
Stopper tightly.
Filter before use.
A small crystal of thymol may be added.

Notes
1. The solution is stable for 2-3 months.
2. The standard solution is for routine, formalin fixed tissues.
3. The strong solution is recommended for Zenker fixed tissues.

Reference
Mallory, F. B. & Wright, J.H., (1904). *Pathological technique, Ed.3.* W. B. Saunders, Philadelphia, USA.

Mann's Alum Hematoxylin

Ingredient	*Amount*	*Function*
Hematein	6 g	Dye
Potassium alum	35 g	Mordant
Distilled water	350 mL	Solvent
95% ethanol	320 mL	Solvent
Glycerol	250 mL	Stabiliser
Glacial acetic acid	30 mL	Acidifier

Compounding procedure
Dissolve hematein in acetic acid.
Mix the ethanol and glycerol together, and add to the dye in acetic acid.
Dissolve the alum in the water, and add to the dye solution.
The solution may be used immediately.

Notes
1. This solution uses hematein instead of hematoxylin. Hematoxylin could be substituted but would require up to 1.2 grams sodium iodate for complete chemical oxidation. Since the dye content is so high, approximately 0.5 grams would probably be more appropriate.
2. The high dye content indicates this is a strong, regressive solution.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide.* The Blakiston Co.

Martinotti's Alum Hematoxylin

Ingredient	*Amount*	*Function*
Hematein	2 g	Dye
Ammonium alum	15 g	Mordant
Distilled water	700 mL	Solvent
Methanol	150 mL	Solvent
Glycerol	150 mL	Stabiliser

Compounding procedure
Dissolve the alum in 600 mL water.
Dissolve the hematein in 100 mL water.
Combine, and add the other ingredients.
The solution may be used immediately.

Notes
1. This solution uses hematein instead of hematoxylin. Hematoxylin could be substituted but would require a maximum of 0.4 grams sodium iodate for complete oxidation.

Reference
Gray, Peter. (1954). The Microtomist's Formulary and Guide. The Blakiston Co.

Masson's Alum Hematoxylin

Ingredient	*Amount*	*Function*
Hematein	20 g	Dye
Potassium alum	60 g	Mordant
Distilled water	1 L	Solvent
Glacial acetic acid	20 mL	Acidifier

Compounding procedure
Dissolve the alum in boiling water.
Add the dye.
Filter when cool.
Add acetic acid.
The solution may be used immediately.

Notes
1. This solution uses hematein instead of hematoxylin. Hematoxylin could be substituted but would require a maximum of 4 grams sodium iodate for complete oxidation, although less than that would likely be more reasonable, perhaps 1 gram.
2. The high concentration of dye indicates this is most likely a strong, regressive solution.

Reference
Gray, Peter. (1954). The Microtomist's Formulary and Guide. The Blakiston Co.

Mayer's Alum Hematoxylin Variants

The solution usually meant when “Mayer's” hemalum is specified without further qualification is Langeron’s alum hematoxylin, a modification of Mayer's 1901 formula.

Mayer published several alum hematoxylin variants for nuclear staining, both progressive and regressive.

Ingredient	1891	1896	1901	1903	Function
Hematoxylin	1 g	4 g	1 g	1 g	Dye
Ammonium alum	50 g	50 g	—	50 g	Mordant
Potassium alum	—	—	50 g	—	Mordant
Distilled water	1 L	700 mL	1 L	1 L	Solvent
95% ethanol	50 mL	—	—	—	Solvent
Glycerol	—	300 mL	—	—	Stabiliser
Sodium iodate	—	—	0.2 g	0.2 g	Oxidant
Glacial acetic acid	20 mL	—	—	—	Acidifier

Compounding procedures

1891
Dissolve hematoxylin in ethanol.
Dissolve alum in water.
Combine.
Optionally, add acetic acid.
Ripen for months.

1896
Grind hematoxylin in some glycerol with a pestle and mortar.
Mix all the other constituents together.
Add the hematoxylin paste.
Wash out the paste with the solution.
Ripen for months.

1901, 1903
Dissolve alum hematoxylin in the water.
Add sodium iodate.
Bring to the boil. Cool.

Notes

1. The 1896 formula is regressive. The rest are progressive.
2. The 1901 and 1903 formulae can be used immediately.
3. The 1896 formula is also known as Mayer's glychemalum.
4. The 1901 and 1903 formulae differ only in the use of potassium or ammonium alum.
5. Acetic acid may also be added to the 1896, 1901 and 1903 formulae.

Reference

Gray, Peter. (1954). The Microtomist's Formulary and Guide. The Blakiston Co.

McLachlan's Alum Hematoxylin

This formulation has been named in honor of Dr. H.K.I. McLachlan FRCPA at the request of the originator, Mike Rentsch.

Ingredient	*Var I*	*Var II*	*Function*
Hematoxylin	2 g	2 g	Dye
Aluminum sulphate	17.5 g	—	Mordant
Ammonium alum	—	25 g	Mordant
Distilled water	700 mL	700 mL	Solvent
Glycerol	300 mL	300 mL	Stabiliser
Sodium iodate	0.2 g	0.2 g	Oxidant
Glacial acetic acid	20 mL	20 mL	Acidifier

Compounding procedure
Dissolve aluminum sulphate or alum in about 500 mL water.
Add hematoxylin and dissolve.
Add acetic acid and mix well.
Warm glycerol to reduce viscosity, and add to the mixture.
Mix well, then make up to 1 litre with water.
Add sodium iodate and mix for 5-10 minutes.
Store in a tightly capped bottle in the dark.
The solution remains usable for 2-5 years.

Notes
1. This is a progressive solution.

Reference
Rentsch, M. *Personal communication via the internet.* Australian Biostain P/L.

Mitchell's Alum Hematoxylin

Mitchell's formula is from 1883 and is now obsolete. It does, however, show how alum hematoxylin solutions were originally prepared, and the variability inherent in the procedures for doing so. The modern formula should stain satisfactorily.

Original	***Original***	***Modern***	***Function***
Logwood, ground	2 oz	6 g	Dye
Potassium alum	9 oz	75 g	Mordant
Distilled water	as needed	750 mL	Solvent
Glycerol	4 fl oz	250 mL	Stabiliser

Compounding procedure

Original
Moisten the ground logwood with water and pack it into a funnel.
Pour water onto the wood until it comes through barely coloured.
Remove the wood from the funnel, spread out and dry.
Dissolve the alum in 8 fluid oz of water.
Moisten the logwood with some alum water and pack it tightly into the funnel.
Pour the rest of the alum solution onto the logwood.
When the first drops come through, seal up the tip of the funnel.
Leave 48 hours for the dye to be extracted.
Remove the seal and collect the fluid that comes through.
Pour on more water until 12 fluid oz have been collected.
Add glycerol, mix well and filter.

Modern
Dissolve the alum in the water.
Add the hematoxylin and mix until dissolved.
Add the glycerol, mix well and filter.

Notes
1. The reason for the pre-wash of the logwood in water during the original preparation method was stated to be for the removal of tannin.
2. The original formula gives 255 grams of potassium alum to dissolve in 227 mL water. It takes just over 7 mL water to dissolve 1 gram crystalline potassium alum, so the amount of water specified would be able to dissolve only about 32 grams.
3. The amount of hematoxylin in the modern formula is based on the fact that logwood may contain up to 10% of the dye. Two ounces (about 57g) could contain a maximum of about 6 grams hematoxylin. A 50% extraction would give 3 grams hematoxylin, although it could well have been any amount up to 6 grams, or possibly less.
4. The amounts for the modern formula have been adjusted so that comparison may be made easily with other formulae, all of which are based on a final volume of one litre. Keep in mind that the hematoxylin content could be any amount up to 12 grams.

Reference
Bolles-Lee, A. (1885). *The Microtomist's Vade-Mecum*. J & A Churchill, London, England.
Budavari, S., Editor, (1996). *The Merck Index, Ed. 12*. Merck & Co., Inc., Whitehouse Station, NJ, USA.
Lemmens, R. H. M. J. and Wulijarna-Soetjipto, N., Editors. (1992). *Plant resources of South East Asia No. 3, Dye and tannin-producing plants.* PROSEA, Bogor, Indonesia.

Molnar's Alum Hematoxylin Variants

Ingredient	*Var I*	*Var II*	*Function*
Hematoxylin	10 g	4 g	Dye
Ammonium or potassium alum	50 g	50 g	Mordant
Distilled water	1 L	1 L	Solvent
95% ethanol	50 mL	—	Solvent
Mercuric oxide	5 g	—	Oxidant
Sodium iodate	—	0.3 g	Oxidant
Glacial acetic acid	20 mL	—	Acidifier
Citric acid	—	1.5 g	Acidifier
Chloral hydrate	—	75 g	Antioxidant

Compounding procedure

Var I

Dissolve hematoxylin in ethanol, and alum in water.
Combine in an Erlenmeyer flask and slowly add the mercuric oxide.
Heat until the solution changes colour to a dark purple.
Remove from heat and cool rapidly by swirling the flask in cold water.
When cool, add acetic acid.

Var II

Dissolve alum in water.
Add hematoxylin, sodium iodate, citric acid and chloral hydrate in that order.
Filter before use.

Notes

1. Var I was recommended for double embedded tissues.
2. It is recommended that mercuric oxide not be used as an oxidant for hematoxylin due to eventual contamination of the environment. Complete oxidation of Var I would require 2 grams sodium iodate, although 0.75 grams would give a comparable degree of oxidation for Var I as is specified for Var II.

Reference

Molnar, L. N. *Modification of Harris hematoxylin for sections from tissue double embedded with nitrocellulose and paraffin*. Histologic, v 5, No 1, January, 1975

Molnar, L. N. *Modification of Mayer's hematoxylin-eosin method*. Histologic, v 6, No 4, October, 1976

Papamiltiades' Alum Hematoxylin

Ingredient	*Amount*	*Function*
Hematoxylin	4 g	Dye
Aluminum sulphate	10 g	Mordant
Zinc sulphate	5 g	Mordant
Distilled water	900 mL	Solvent
Potassium iodide	4 g	Stabiliser
Glacial acetic acid	32 mL	Acidifier
Glycerol	100 mL	Stabiliser

Compounding procedure
Dissolve hematoxylin in 400 mL water.
Dissolve aluminum sulphate in 200 mL water.
Dissolve zinc sulphate in 100 mL water.
Dissolve potassium iodide in 100 mL water.
Combine the four solutions, then add the acetic acid and glycerol.
The solution may be used immediately, and is stable for about two months.

Notes
1. The purpose of the zinc sulphate is not clear, but it may be a mordant.
2. The purpose of potassium iodide is not clear. It may be a stabiliser based on the mild reducing ability of iodide.

Reference
Putt, F.A. *Manual of Histopathological Staining Methods*. John Wiley & Sons, New York, NY., USA
Wikipedia. *Potassium iodide*. http://en.wikipedia.org/wiki/Potassium_iodide.

Pusey's Alum Hematoxylin

This is described as a modification of "Mayer's" hemalum, but appears to be based on Langeron's modification.

Ingredient	*Amount*	*Function*
Hematoxylin, aged 10% ethanolic	16 mL	Dye
Ammonium alum	60 g	Mordant
Distilled water	1 L	Solvent
Sodium iodate	0.25 g	Oxidant
Citric acid, 5% aqueous	7 mL	Acidifier
Chloral hydrate	50 g	Stabiliser

Compounding procedure
Dissolve alum in water using low heat, but do not boil.
Add hematoxylin solution and mix well.
Add sodium iodate and dissolve.
Leave for 30 minutes.
Add chloral hydrate and dissolve.
Add citric acid and mix well.
Check that the pH is 2.45, adjust with citric acid if not.
It may be used immediately.

Notes
1. The alcoholic hematoxylin solution should be aged (partially oxidised).
2. A saturated (10%) and ripened solution of hematoxylin in ethanol has a hematein content of about 7%. 16 mL of this equates to just over 1.1 grams dye. With 60 grams of mordant and complete oxidation this would indicate a progressive solution.
3. The addition of an oxidising agent (sodium iodate) to an already oxidised hematoxylin solution should cause the solution to become overoxidised. Possibly the aging refers to darkening of the solution to a deep brown with a significant amount of hematoxylin remaining unoxidised. If so, the dye content would be less than expected from the formula as hematoxylin dissolves in ethanol to about 3%. For that reason, any undissolved hematoxylin in the 10% solution should be dispered by shaking before the required amount is removed.
4. The following may be what was intended:

Hematoxylin	1.6 g
Ammonium alum	60 g
Distilled water	1 L
Sodium iodate	0.25 g
Citric acid	0.35 g
Chloral hydrate	50 g

Reference
Villaneuva, A.R., (1976). *Methods of preparing and interpreting mineralized sections of bone*. Proceedings of the First Workshop on Bone Morphometry. Jaworski, Z. F. G., Editor, (1976).

Rawitz' Alum Hematoxylin Variants

Ingredient	*1895a*	*1895b*	*1909*	*Function*
Hematoxylin	10 g	—	—	Dye
Hematein	—	2.5 g	2 g	Dye
Potassium alum	10 g	—	—	Mordant
Ammonium alum	—	15 g	—	Mordant
Aluminum nitrate	—	—	20 g	Mordant
Distilled water	650 mL	500 mL	500 mL	Solvent
Glycerol	350 mL	500 mL	500 mL	Stabiliser

Compounding procedure
Dissolve the aluminum salt and dye in water.
When dissolved, add glycerol.
The 1895a formula should be allowed to ripen.
The 1895b and 1909 formulas may be used immediately.

Notes
1. The 1895b and 1909 formulae use hematein instead of hematoxylin. These could be made with hematoxylin instead. If so, then 0.5 grams sodium iodate would be required to completely oxidise the 1895b formula, and 0.4 grams sodium iodate would be required to completely oxidise the 1909 formula.
2. The Microtomists Formulary and Guide gives the 1895b and 1909 formulae as containing 500 *millilitres* of glycerol. The Microtomists Vade-Mecum gives these two formulae as containing 500 *grams* glycerol. This equates to 400 millilitres based on a specific gravity of 1.25. Since the volume of the 1895a solution totals 1 litre, it is likely that the 1895b solution should also total 1 litre in volume, in which case 500 millilitres glycerol was meant. In either case the difference would not be too significant in practice.

Reference
Gatenby, J.B. and Beams, H.W., (1950). *The Microtomist's Vade-Mecum.* 11 ed., Churchill, London, UK.
Gray, Peter. (1954). *The Microtomist's Formulary and Guide.* The Blakiston Co.

Reddy's Alum Hematoxylin

Ingredient	Amount	Function
Hematoxylin	6.4 g	Dye
Ammonium alum	60 g	Mordant
Ethanol, absolute	200 mL	Solvent
Glycerol	160 mL	Stabiliser
Distilled water	640 mL	Solvent

Compounding procedure
Combine all the ingredients.
Mix for a day.
Leave in the dark for at least a month to ripen.

Notes
1. This formula was recommended for neurological staining.

Reference
Reddy, P. H., (2001). Neurological Sciences Institute. Oregon Health and Science University. *http://www.ohsu.edu/nsi/faculty/reddyh/lab/prothema.html.*

Sass' Alum Hematoxylin

Ingredient	*Var I*	*Var II*	*Function*
Hematoxylin	10 g	1 g	Dye
Ammonium alum	saturated	50 g	Mordant
Distilled water	1 L	1 L	Solvent
Sodium iodate	10 g	1 g	Oxidant
Glacial acetic acid	30 mL	—	Acidifier

Compounding procedure
Dissolve the alum in the water.
Add the hematoxylin.
Add the other ingredients.
Filter.
The solutions may be used immediately.

Notes
1. Both of these formulae specify 1 gram sodium iodate for each gram hematoxylin, but that is far too much. Hematoxylin requires only 0.2 grams sodium iodate for each gram to be fully oxidised. Based on that, Var I would need 2 grams sodium iodate for complete oxidation of the dye and Var II would require 0.2 grams.
2. Due to it's high dye content, the Var I formula is likely regressive.
3. The Var II formula is likely progressive.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide*. The Blakiston Co.

Schmorl's Alum Hematoxylin

Ingredient	*Amount*	*Function*
Hematoxylin	5 g	Dye
Ammonium alum	100 g	Mordant
Distilled water	1 L	Solvent
100% ethanol	60 mL	Solvent

Compounding procedure
Dissolve hematoxylin in ethanol.
Dissolve alum in water.
Combine the solutions and mix well.
Leave at room temperature to ripen.

Notes
1. The hematoxylin content indicates this solution is likely a strong regressive type.

Reference
Lillie, R.D., (1954). *Histopathologic technique and practical histochemistry Ed.2*. Blakiston, New York, USA.

Scott's Alum Hematoxylin

Ingredient	*Amount*	*Function*
Hematoxylin	3 g	Dye
95% ethanol	300 mL	Solvent
Potassium alum	21 g	Mordant
Distilled water	300 mL	Solvent
Glycerol	300 mL	Stabiliser
Glacial acetic acid	30 mL	Acidifier

Compounding procedure
Dissolve hematoxylin in a portion of the water
Add glycerol and mix well.
Dissolve alum in the remaining water with heat.
Combine the two solutions.
Add ethanol and acetic acid, mixing each time
Plug the container loosely with cotton wool.
Ripen by leaving in a warm, sunlit place for several weeks.
When sufficiently ripened, store tightly stoppered in a cool, dark place.
The solution is stable for years.

Section preparation
Scott recommended that tissues be fixed with formalin or one of many common fixative solutions, but that strongly acid fixatives should be avoided since they permitted only nuclei to be stained. He specified that paraffin sections should be fixed to the slide without melting the paraffin and that albumin be used only if necessary.

Notes
1. This is a modification of Ehrlich's formula. It differs by having about half the hematoxylin and a fixed amount of alum. It should therefore stain somewhat more selectively than Ehrlich's formula, but otherwise would be very similar.
2. Scott noted the desirability of chemical oxidation, but made no recommendation. Complete oxidation would require 0.6 to 0.75 grams sodium iodate and half oxidation would require 0.3 to 0.35 grams.
3. Although Scott recommended his tap water substitute for blueing, he noted that distilled water applied for long enough would also be effective, as would any other standard blueing solution.

Scott's tap water substitute:

Potassium bicarbonate ($KHCO_3$)	2 g.
Calcium chloride ($CaCl_2.2H_2O$)	0.5 - 0.75 g.
Magnesium sulphate ($MgSO_4.7H_2O$)	20 g.
Distilled water	1 L.

Reference
Scott, S. G. (1912). *On successive double staining for histological purposes*. Journal of Pathology and Bacteriology, **v.** 16, **p.** 390-398.

Slidders Alum Hematoxylin

This formula differs from others by being made from three stock solutions. It contains no added acid, relying on the high alum:dye ratio for it's progressive staining. Consequently, the carry over of tap water fairly rapidly causes both dilution and raises the pH. Due to these, it has a shorter working life than many other formulations. It was designed to be quickly prepared in laboratories that stain large numbers of slides and prefer to use fresh solution daily, but which would like to avoid the paler background staining obtained with hemalums that include an acid to accentuate nuclei.

Stock mordant	***Amount***	***Function***	
Potassium alum	2 kg	Mordant	Dissolve the alum in 4 litres distilled water, using heat. Add the balance of the water and formalin.
Distilled water	19.8 L	Solvent	
Strong formalin	200 mL	Preservative	

Stock hematoxylin	***Amount***	***Function***
Hematoxylin	20 g	Dye
Strong formalin	200 mL	Solvent

Stock oxidiser	***Amount***	***Function***
Sodium iodate	4 g	Oxidant
Distilled water	200 mL	Solvent

Working solution	***Amount***	
Stock mordant	2 L	Combine 400 mL stock mordant, stock hematoxylin and stock oxidiser in a 2 Litre flask. Bring to the boil (in a fume hood). Remove,
Stock hematoxylin	20 mL	
Stock oxidiser	20 mL	

Notes

1. The formalin in the stock alum and hematoxylin solutions is both a preservative and an anti-oxidant.
2. The working solution is stable for about 2 months at room temperature.
3. The stock solutions are stable at least 6 months at room temperature.
4. The working solution may be made in more traditional manner:—

Ingredient	***Amount***	
Hematoxylin	1 g	Combine the ingredients, then bring to a boil. Immediately remove from heat and cool to room temperature. The solution may be used as soon
Potassium alum	100 g	
Distilled water	1 L	
Sodium iodate	0.2 g	

Reference

Slidders, W. (1988). *Preparation of alum hematoxylin*. Medical Laboratory Sciences, **v** 45, pp. 405.

Unna's Alum Hematoxylin

Ingredient	*Amount*	*Function*
Hematoxylin	3 g	Dye
Ammonium alum	300 g	Mordant
Distilled water	600 mL	Solvent
100% ethanol	300 mL	Solvent
Sublimed sulphur	6 g	Stabiliser

Compounding procedure
Dissolve the hematoxylin in the alcohol.
Dissolve the alum in the water.
Combine the two solutions.
Leave the solution at room temperature to ripen (days).
Add the sulphur and mix well.

Notes
1. The sulphur is added after the solution has been ripened.
2. The sulphur is to stabilise the solution in the oxidised state.
3. Ammonium alum dissolves in water at the rate of about one gram in 7 mL, and it is almost insoluble in ethanol. The formula should therefore dissolve about 90 grams, so the amount specified is in considerable excess.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide*. The Blakiston Co.

Watson's Alum Hematoxylin

Ingredient		*Amount*	*Function*
Hematoxylin		6 g	Dye
Ammonium alum		6 g	Mordant
Distilled water		300 mL	Solvent
100% ethanol		300 mL	Solvent
Glycerol		300 mL	Stabiliser
Glacial acetic acid		30 mL	Acidifier
Potassium permanganate	*or*	0.3 g	Oxidants
Chloramine T	*or*	3.9 g	
Barium hydroxide		6 g	

Compounding procedure
Dissolve alum in water.
If using potassium permanganate add to the alum solution.
Dissolve hematoxylin in ethanol.
Combine the two solutions.
Add glycerol.
Add acetic acid (30 mL, *or* 120 mL if using barium hydroxide).
If using chloramine T or barium hydroxide add to the solution.

Notes
1. Add only one of the oxidants (permanganate *or* chloramine T *or* barium hydroxide). Instead of oxidising with one of those, 0.5 grams sodium iodate could be substituted.
2. This is a modification of Ehrlich's hematoxylin, and is said to be as effective. It was designed to be used immediately after preparation, eliminating the months required for atmospheric oxidation.
3. Gray gives a similar formula for Watson's hemalum with potassium permanganate, but specifies only 0.6 grams of alum. This may be an error.

Reference
Gatenby, J.B. and Beams, H.W., (1950) . *The Microtomist's Vade-Mecum. 11 ed*., Churchill, London, UK.
Gray, Peter. (1954). *The Microtomist's Formulary and Guide.* The Blakiston Co.

Iron Hematoxylin Formulae

Onchrome

Crétin's Iron Hematoxylin

Solution A	***Amount***	***Function***
Ferrous sulphate	4 g	Mordant
Distilled water	100 mL	Solvent

Solution B	***Amount***	***Function***
Potassium ferrocyanide	2 g	Mordant ?
Potassium ferricyanide	1 g	Mordant ?
Distilled water	100 mL	Solvent

Solution C	***Amount***	***Function***
Hematoxylin	0.5 g	Dye
Distilled water	100 mL	Solvent

Solution D	***Amount***	***Function***
Ferric ammonium sulphate	5 g	Mordant
Distilled water	100 mL	Solvent

Method

1. Bring sections to water with xylene and ethanol.
2. Place into solution A for 24 hours.
3. Wash with running tap water overnight.
4. Place into solution B for 3-6 hours.
5. Rinse with distilled water.
6. Place into solution C overnight.
7. Place into solution D until differentiated.
8. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Expected results

- Nuclei —; dense black

Notes

1. The stock solutions should be stable for some time.
2. Both the potassium ferrocyanide and ferricyanide are presumed to function as mordants. However, since solution A, containing ferrous sulphate, is applied before them, it is likely that some of the ferrous salt is converted to Prussian blue (ferric ferrocyanide, $Fe_7(CN)_{18}.14H_2O$) *in situ*, and this may be the mordant.

Reference

Gray, Peter. (1954). *The Microtomist's Formulary and Guide.* The Blakiston Co.

Goldman's Iron Hematoxylin

Solution A	***Amount***	***Function***
Ferric ammonium sulphate	4 g	Mordant
Distilled water	100 mL	Solvent
Acetic acid, glacial	1 mL	Acidifier
Sulphuric acid	0.12 mL	Acidifier

Solution B	***Amount***	***Function***
Picric acid, saturated aqueous	100 mL	Dye and acid
Sulphuric acid	0.1 mL	Acidifier

Solution C	**Amount**	**Function**
Hematoxylin	0.5 g	Dye
Distilled water	100 mL	Solvent

Solution D	***Amount***	***Function***
70% ethanol	100 mL	Solvent
Lithium carbonate, saturated aqueous	5 drops	Base

Compounding procedure
The aqueous hematoxylin should be ripened before use.

Method
1. Bring sections to water, removing mercury pigment if necessary.
2. Place into solution A for ½ - 24 hours.
3. Wash in running tap water for 10 minutes.
4. Place in solution B for 3 hours or longer.
5. Wash in running tap water for 15 minutes.
6. Place in solution C for 1 hour.
7. Wash in running tap water for 15 minutes.
8. Blue with solution D.
9. Counterstain if desired.
10. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Expected results
- Nuclei — black
- Basophil cytoplasm — grey
- Background — as counterstain or unstained

Notes
1. The stock solutions are stable for some time.
2. The technique was originally intended for the demonstration of protozoa.
3. The method was designed for paraffin sections of material fixed with formalin variants, Bouin's or Zenker's fluids.
4. Overstaining occurs only if sections are left in hematoxylin for several hours.
5. Bouin fixed tissue is not as intensely stained as with other fixatives.
6. The time in picric acid (solution B) is necessary, reducing it causes overstaining.

Reference
Lillie, R.D., (1954). *Histopathologic technique and practical histochemistry Ed.2.* Blakiston, New York, USA.
Citing:—
Goldman, (1951). *American Journal of Clinical Pathology*, **v.**21, **p.**198

Heidenhain's Iron Hematoxylin

Heidenhain's iron hematoxylin is the reference type for onchrome iron hematoxylin staining. It is probably the most widely used procedure of the type and is recommended.

Solution A	**Amount**	**Function**
Ferric ammonium sulphate	2.5 g	Mordant
Distilled water	100 mL	Solvent

Solution B	***Amount***	***Function***
Hematoxylin	0.5 g	Dye
Distilled water	100 mL	Solvent

Method

1. Bring sections to water with xylene and ethanol.
2. Place into solution A for 30 minutes to 24 hours.
3. Rinse with distilled water.
4. Place into solution B for 30 minutes to 24 hours.
5. Rinse with tap water.
6. Differentiate in solution A, controlling microscopically.
7. Wash well in running tap water to blue.
8. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Expected results

- Nuclei and other structures — black

Notes

1. The stock solutions are stable for some time.
2. Solution B should be ripened for a minimum of one month.
3. Staining at elevated temperatures (not over 60°C) will shorten the required times.
4. Always differentiate at room temperature.
5. The differentiating solution should not be reused.
6. The other solutions may be reused.
7. This method is usually recommended for monochrome photography.
8. The degree of differentiation will determine which tissue components are prominent. The method can demonstrate many structures, including chromosomes, nuclear components, mitochondria and muscle striations

Reference

Gray, Peter. (1954). *The Microtomist's Formulary and Guide.* The Blakiston Co.

Variants of Heidenhain's Iron Hematoxylin

Heidenhain's iron hematoxylin technique uses two separate solutions for staining. First an iron mordant is applied, usually, but not always, ferric ammonium sulphate or ferric chloride. This is followed by a hematoxylin solution. The overstained preparations are then differentiated in the iron mordant solution, often diluted, or in some similar solution. Numerous variations on this theme have been published, most of them staining very similarly, as they differ largely in the concentration of the reagents and the times for which they are applied.

In the chart below it is to be understood that all solids are in grams and all fluids in millilitres. The amounts given are to be dissolved in sufficient solvent to give a final volume of 100 millilitres for each solution. The solvent is distilled water unless otherwise stated. For example, the formulae for Galiano's variant are:—

Sol A: 3 g. iron alum in 100 mL water.
Sol B: 1 g. hematoxylin and 20 mL acetic acid made up to 100 mL with water.
Sol C: 1.5 g. eosin Y and 25 mL acetic acid made up to 100 mL water

The term "**visual**" in the "**Diff**" column means to control differentiation microscopically.

Variant	*Sol. A*	*Sol. B*	*Sol. C*	*Time A*	*Time B*	*Diff C*	*Comment*
Heidenhain	IA 2.5	HX 0.5	IA 2.5	½ - 24 hrs.	½ - 24 hrs.	visual	
Bütschli	FA 2	HX 0.5	—	24 hrs	3 hrs	—	For protozoa
Diamond	IA 4	HX 0.5 Ter 0.1	PA. sat EA	5 min.	5 min.	3-5 min.	
Dobell	IA 1 E70	HN 1 E70	HCl 0.1 E70	10 min	10 min	visual	For protozoa
Freitas	IA 0.5 E70	HN 1 E70 60 PB 30	PA 0.3 E70	1 hr	1 hr	visual	
French	IA 3.5 E70	HX 1 E95 98 LC 2	IA 1	Overnight	Overnight	visual	
Galiano	IA 3	HX 1 AC 20	EO 1.5 AC 25	15 mins	until dark	visual	Rinse AE
Haggquist	IA 5	HX 1	FC 1	1 hr	1 hr	visual	
Hance	IA 2.5	HS 10 AD 100 SB		½ - 2 hrs	30 min	visual	Add SB until sol B turns plum coloured.

AC	Glacial acetic acid
AD	Distilled water
AE	Ammoniated ethanol
E70	70% ethanol
E95	95% ethanol
EA	Absolute ethanol
EO	Eosin Y ws
FA	ferric acetate
FC	Ferric chloride
HCl	Concentrated hydrochloric acid
HN	Hematein
HS	Saturated alcoholic hematoxylin, well ripened.
HX	Hematoxylin
IA	Iron alum, ferric ammonium sulphate.
LC	Lithium carbonate, saturated aqueous
PA	Picric acid
PB	Phosphate buffer, pH 7.6
SB	Sodium bicarbonate, 2.5% aqueous.
Ter	Tergitol

Masson's Iron Hematoxylin

Solution A	***Amount***	***Function***
Ferric ammonium sulphate	4 g	Mordant
Distilled water	100 mL	Solvent

Solution B	***Amount***	***Function***
Hematoxylin	1 g	Dye
Distilled water	100 mL	Solvent

Compounding procedure
Make each solution seperately.
Solution B should be ripened for a minimum of one month.

Method
1. Bring sections to water with xylene and ethanol.
2. Place into solution A for 5-10 minutes preheated to 50°C.
3. Rinse with distilled water.
4. Place into solution B for 5-10 minutes preheated to 50°C.
5. Rinse with tap water.
6. Differentiate in solution A at room temperature, controlling microscopically.
7. Wash well in running tap water to blue.
8. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Expected results
- Nuclei and other structures — blue to black

Notes
1. This is a modification of Heidenhain's iron hematoxylin, differing in concentration and temperature.
2. The stock solutions are stable for some time.
3. The hematoxylin solution should be ripened.
4. The degree of differentiation will determine which tissue components are prominent. The method can demonstrate many structures, including chromosomes, nuclear components, mitochondria and muscle striations
5. The solutions may be reused, with the exception of the differentiating solution, which should be fresh each time.
6. Counterstaining is not recommended.
7. This method is usually recommended for monochrome photography.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide.* The Blakiston Co.

Murray's Iron Hematoxylin

Solution A	***Amount***	***Function***
Ferric ammonium sulphate	3.5 g	Mordant
Distilled water	100 mL	Solvent

Solution B	***Amount***	***Function***
Hematoxylin	0.5 g	Dye
Distilled water	100 mL	Solvent

Compounding procedure
Make each solution seperately.
Solution B should be ripened for a minimum of one month.

Method
1. Bring sections to water with xylene and ethanol.
2. Place into solution A for 30 minutes to 24 hours.
3. Rinse with distilled water.
4. Place into solution B for 30 minutes to 24 hours.
5. Rinse with tap water.
6. Differentiate in solution A, controlling microscopically.
7. Wash well in running tap water to blue.
8. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Expected results
- Nuclei and other structures — blue to black

Notes
1. This is a modification of Heidenhain's iron hematoxylin, and differs solely in the concentration of the reagents.
2. The stock solutions are stable for some time.
3. The hematoxylin solution needs to be ripened.
4. The degree of differentiation will determine which tissue components are prominent. The method can demonstrate many structures, including chromosomes, nuclear components, mitochondria and muscle striations
5. The solutions may be reused, except for the solution used to differentiate, which should be fresh each time.
6. Counterstaining is not recommended.
7. This method is usually recommended for monochrome photography.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide.* The Blakiston Co.

Régaud's Iron Hematoxylin

Solution A	***Amount***	***Function***
Ferric ammonium sulphate	5 g	Mordant
Distilled water	100 mL	Solvent

Solution B	**Amount**	**Function**
Hematoxylin	1 g	Dye
90% ethanol	10 mL	Solvent
Distilled water	80 mL	Solvent
Glycerol	10 mL	Stabiliser

Solution C	***Amount***	***Function***
Potassium ferricyanide	2.5 g	Bleach
Sodium borate	2 g	pH control
Distilled water	100 mL	Solvent

Compounding procedure
Make each solution seperately.
Solutions A and B may be reused provided they are not contaminated by each other.
Solution C should not be reused, and should be freshly prepared.

Method
1. Bring sections to distilled water with xylene and ethanol.
2. Place into solution A at 50°C for 30 minutes.
3. Rinse well with distilled water.
4. Place into solution B at 50°C for 30 minutes.
5. Rinse well with distilled water.
6. Place into solution C until differentiated.
7. Wash in running tap water until black.
8. Counterstain as required.
9. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Expected results
- Nuclei — black
- Background — as counterstain or unstained

Notes
1. Solutions A and B are stable for some time.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide.* The Blakiston Co.

Iron Hematoxylin Formulae

Metachrome

Anderson's Iron Hematoxylin

Solution A	***Amount***	***Function***
Hematoxylin	0.5 g	Dye
100% ethanol	50 mL	Solvent
Distilled water	50 mL	Solvent
Calcium hypochlorite, 2% aqueous	5 mL	Oxidant

Solution B	**Amount**	**Function**
Ferric ammonium sulphate	3 g	Mordant
Distilled water	100 mL	Solvent
Sulphuric acid	2.5 mL	Acidifier

Compounding procedure
The working solution is made by adding 2 volumes solution A to 1 volume solution B.

Notes
1. The stock solutions are stable for some time.
2. The working solution should be made fresh and may be used immediately, but is not stable for long.
3. The working solution is said to rarely overstain, *i.e.* it is progressive.
4. Solution A oxidises with calcium hypochlorite. Other oxidising agents would also be suitable, sodium iodate (0.1 g or less) being the most common.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide.* The Blakiston Co.

Faure's Iron Hematoxylin

Solution A	***Amount***	***Function***
Hematoxylin	3.2 g	Dye
90% ethanol	100 mL	Solvent

Solution B	***Amount***	***Function***
Ferric chloride	0.2 g	Mordant
Cupric acetate	0.1 g	Mordant
Distilled water	100 mL	Solvent
Hydrochloric acid	2 mL	Acidifier

Working solution	***Amount***
Solution A	1 vol
Solution B	1 vol

Method

1. Bring sections to water with xylene and ethanol.
2. Place into the working solution for 5 seconds.
3. Rinse with tap water.
4. Dip briefly in 1% hydrochloric acid in 70% ethanol.
5. Wash well in running tap water to blue.
6. Rinse with distilled water.
7. Counterstain if desired.
8. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Expected results

- Nuclei — black
- Background — as counterstain or unstained

Notes

1. The stock solutions are stable for some time.
2. The working solution may be used immediately, but is not stable for long
3. The working solution should be made fresh.
4. This method is suitable as an acid resistant nuclear stain.

Reference

Gray, Peter. (1954). The Microtomist's Formulary and Guide. The Blakiston Co.

Hansen's Iron Hematoxylin

Ingredient	*Amount*	*Function*
Hematoxylin	0.75 g	Dye
Ferric ammonium sulphate	4.5 g	Mordant
Distilled water	100 mL	Solvent

Compounding procedure
Dissolve the iron alum in 65 mL of the water.
Dissolve the hematoxylin in the remaining water.
Combine the solutions and bring to the boil.
Cool and filter.

Method
1. Bring sections to water with xylene and ethanol.
2. Place into the staining solution for an appropriate time.
3. Rinse with tap water.
4. Treat briefly in 1% hydrochloric acid in 70% ethanol.
5. Wash well in running tap water to blue.
6. Rinse with distilled water.
7. Counterstain if desired.
8. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Expected results
- Nuclei — black
- Background — as counterstain or unstained

Notes
1. The simplicity of the solution indicates it is not likely stable for a long time.
2. Although a staining time was not given, 30 minutes should be sufficient. Determine by trial.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide.* The Blakiston Co.

Janssen's Iron Hematoxylin

Gray and Lillie give slightly different formulae for this solution.

It is recommended as an acid resistant nuclear stain, and is stable for a month or so.

Ingredient	*Gray*	*Lillie*	*Function*
Hematoxylin	2 g	2 g	Dye
Ferric ammonium sulphate	10 g	20 g	Mordant
Absolute ethanol	10 mL	—	Solvent
Distilled water	140 mL	200 mL	Solvent
Glycerol	30 mL	60 mL	Solvent
Methanol	30 mL	60 mL	Solvent

Compounding procedure

Gray

Dissolve iron alum in water.
Combine ethanol and methanol.
Dissolve hematoxylin in the alcohol mixture.
Combine, and leave for one week at room temperature.
Filter, and add glycerol.

Lillie

Dissolve hematoxylin in methanol.
Dissolve iron alum in water.
Combine and add glycerol.
May be used after about an hour.

Method

1. Bring sections to water with xylene and ethanol.
2. Place into the staining solution for 15-30 minutes.
3. Rinse with tap water.
4. Dip briefly in 1% hydrochloric acid in 70% ethanol.
5. Wash well in running tap water to blue.
6. Rinse with distilled water.
7. Counterstain if desired.
8. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Expected results

- Nuclei — black
- Background — as counterstain or unstained

Notes

1. This solution is recommended as a stable acid resistant nuclear stain to replace Weigert's iron hematoxylin.

Reference

Gray, Peter. (1954). *The Microtomist's Formulary and Guide*. The Blakiston Co.
Lillie, R. D., (1976). *Histopathologic Technic And Practical Histochemistry, ed.4*., p. 200. McGraw-Hill, New York.

Kefalas' Iron Hematoxylin

Ingredient	*Amount*	*Function*
Hematoxylin	1 g	Dye
Ferric chloride	1 g	Mordant
Acetone	100 mL	Solvent
Hydrochloric acid	0.05 mL	Acidifier

Compounding procedure
Dissolve the ferric chloride and hematoxylin in the acetone.
Add the hydrochloric acid.

Method
1. Bring sections to water with xylene and ethanol.
2. Place into the staining solution for 30 minutes.
3. Wash well in running tap water to blue.
4. Rinse with distilled water.
5. Counterstain if desired.
6. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Since this solution contains no water it can be used in a procedure which completely avoids water or ethanol.

1. Remove wax with xylene.
2. Remove xylene with a few changes of acetone.
3. Place into staining solution for 30 minutes.
4. Wash with acetone.
5. Clear with xylene and mount with a resinous medium.

Expected results
- Nuclei — black
- Background — as counterstain or unstained

Notes
1. 0.05 mL hydrochloric acid is about 1-2 drops.
2. The simplicity of the formula indicates it is likely not stable for long.
3. The presence of hydrochloric acid may indicate progressive staining.
4. It is likely suitable as an acid resistant nuclear stain.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide*. The Blakiston Co.

Krajian's Iron Hematoxylin

Ingredient	Amount	Function
Hematoxylin	6 g	Dye
Ferric ammonium sulphate	6 g	Mordant
Ferric chloride	6 g	Mordant
Potassium iodide	6 g	–
95% ethanol	50 mL	Solvent
Distilled water	50 mL	Solvent

Compounding procedure
Dissolve the hematoxylin in the ethanol.
Dissolve the other ingredients in the water.
Combine the solutions.

Method
1. Bring sections to water with xylene and ethanol.
2. Place into the staining solution for 3 minutes.
3. Rinse with tap water.
4. Dip briefly in 1% hydrochloric acid in 70% ethanol.
5. Wash well in running tap water to blue.
6. Rinse with distilled water.
7. Counterstain if desired.
8. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Expected results
- Nuclei — black
- Background — as counterstain or unstained

Notes
1. Gray states that this solution is, "an excellent general purpose hematoxylin".
2. The stability of the solution is not commented on, but it is likely not stable for long.
3. This method is from a technique for micro-organisms, so the staining time may need to be adjusted for sections.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide*. The Blakiston Co.

La Manna's Iron Hematoxylin

Ingredients	*Amount*	*Function*
Hematoxylin	1 g	Dye
Ferric ammonium sulphate	3 g	Mordant
Distilled water	100 mL	Solvent

Compounding procedure
Dissolve the iron alum in half the water.
Dissolve the hematoxylin in the remainder.
Combine.

Method
1. Bring sections to water with xylene and ethanol.
2. Place into the staining solution for an appropriate time.
3. Rinse with tap water.
4. Dip briefly in 1% hydrochloric acid in 70% ethanol.
5. Wash well in running tap water to blue.
6. Rinse with distilled water.
7. Counterstain if desired.
8. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Expected results
- Nuclei — black
- Background — as counterstain or unstained

Notes
1. The simplicity of the formula suggests the solution is not stable for long.
2. No time is given. Determine by trial and error. 30 minutes should be suitable.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide*. The Blakiston Co.

Lillie's Iron Hematoxylin variants

Solution A	***1940***	***1954***	***1967***	***1976***	***Function***
Hematoxylin	1 g	0.5 g	1 g	1 g	Dye
Ferric chloride	1.2 g	1.2 g	0.5 g	2.5 g	Mordant
Ferrous sulphate	—	4.44 g	65 g	4.5 g	Mordant
100% ethanol	—	100 mL	100 mL	100 mL	Solvent
Distilled water	100 mL	292 mL	98 mL	298 mL	Solvent
Hydrochloric acid	1 mL	8 mL	2 mL	2 mL	Solvent

Compounding procedure

1940

Dissolve ferric chloride in half of the water.
Dissolve hematoxylin in the remainder of the water.
Combine and add the hydrochloric acid.

1954, 1967 & 1976

Dissolve hematoxylin in ethanol.
Dissolve ferric chloride, ferrous sulphate and hydrochloric acid in water.
Combine the solutions.

Method

1. Bring sections to water with xylene and ethanol.
2. Place into the staining solution for 5-30 minutes.
3. Wash well in running tap water to blue.
4. Rinse with distilled water.
5. Counterstain if desired.
6. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Expected results

- Nuclei — black
- Background — as counterstain or unstained

Notes

1. The 1940 formula is likely not stable for long. If staining is too dark, dip briefly in 1% hydrochloric acid in 70% ethanol.
2. The 1954 and 1976 formulae are modifications of Wiegert's iron hematoxylin. Lillie states that they are stable for several weeks with occasional use. They are progressive, and do not require differentiation.
3. The 1967 formula may be used after an hour, but is better after 24 hours. It stains nuclei progressively in 2 - 30 minutes, and is usable for several weeks.

Reference

Lillie, R.D., (1954). *Histopathologic technique and practical histochemistry Ed.2*. Blakiston, New York, USA.
Lillie, R.D. and Fullmer, H.M., (1976). *Histopathologic technique and practical histochemistry Ed.4*. Blakiston, New York, USA.
Gray, Peter. (1954). *The Microtomist's Formulary and Guide*. The Blakiston Co.

Lillie & Earle's Iron Hematoxylin

Solution A	***Amount***	***Function***
Hematoxylin	1 g	Dye
95% ethanol	50 mL	Solvent
Glycerol	50 mL	Solvent

Solution B	***Amount***	***Function***
Ferric ammonium sulphate	15 g	Mordant
Ferrous sulphate	15 g	Mordant
Distilled water	100 mL	Solvent

Compounding procedure
Make each solution seperately.
For use, combine equal parts of solutions A and B.
The working solution may be used immediately.

Method
1. Bring sections to water with xylene and ethanol.
2. Place into the staining solution for an appropriate time.
3. Rinse with tap water.
4. If overstained, dip briefly in 1% hydrochloric acid in 70% ethanol.
5. Wash well in running tap water to blue.
6. Rinse with distilled water.
7. Counterstain if desired.
8. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Expected results
- Nuclei — black
- Background — as counterstain or unstained

Notes
1. The stock solutions are stable for some time.
2. The life of the working solution is not given. It is likely not stable for long, although the inclusion of glycerol and ferrous sulphate may improve its stability.
3. The staining time should be determined by trial, but 10-30 minutes should suffice.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide*. The Blakiston Co.

Morel & Bassal's Iron Hematoxylin

Solution A	***Amount***	***Function***
Hematoxylin	1 g	Dye
95% ethanol	100 mL	Solvent

Solution B	***Amount***	***Function***
Ferric chloride	2 g	Mordant
Cupric acetate	0.04 g	Mordant
Distilled water	100 mL	Solvent
Hydrochloric acid	1 mL	Acidifier

Compounding procedure
Make each solution seperately.
For use, combine equal parts of solutions A and B.
The working solution may be used immediately, but is not stable for long.

Method
1. Bring sections to water with xylene and ethanol.
2. Place into the staining solution for an appropriate time.
3. Rinse with tap water.
4. Dip briefly in 1% hydrochloric acid in 70% ethanol.
5. Wash well in running tap water to blue.
6. Rinse with distilled water.
7. Counterstain if desired.
8. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Expected results
- Nuclei — black
- Background — as counterstain or unstained

Notes
1. The stock solutions are stable for some time.
2. The working solution should be made fresh, and is likely not stable for long.
3. The staining time should be determined by trial.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide.* The Blakiston Co.

Paquin and Goddard's Iron Hematoxylin

Hematoxylin solution	***Amount***	***Function***
Hematoxylin	0.8 g	Dye
Ferric ammonium sulphate	5 g	Mordant
Ammonium sulphate	0.7 g	—
95% ethanol	25 mL	Solvent
Glycerol	13 mL	Solvent
Distilled water	75 mL	Solvent

Picric ethanol	***Amount***	***Function***
Picric acid, saturated ethanolic.	6 mL	Acid
95% ethanol	94 mL	Solvent

Compounding procedure
Combine the glycerol and ethanol.
Add the hematoxylin and dissolve using gentle heat.
Dissolve the other ingredients in the water.
Add slowly to the hematoxylin solution with agitation.
Let stand for 24 hours before use.

Method
1. Bring sections to water with xylene and ethanol.
2. Place into the staining solution for 5 minutes.
3. Rinse with tap water.
4. Dip briefly in 1% hydrochloric acid in picric ethanol.
5. Wash well in running tap water to blue.
6. Rinse with distilled water.
7. Counterstain if desired.
8. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Expected results
- Nuclei — black
- Background — as counterstain or unstained

Notes
1. This was specified as an acid resistant nuclear stain prior to Paquin & Goddard's trichrome. It is also suitable for other methods requiring an acid resistant nuclear stain.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide.* The Blakiston Co.

Rozas' Iron Hematoxylin

Rozas' iron hematoxylin is of interest as it contains aluminum and iron salts in one solution. Both are mordants used with hematoxylin, and there will presumably be some kind of competition for available binding sites which could modify the solution's staining characteristics. Since iron can be used to displace aluminum as a mordant for hematoxylin, it is likely that the solution stains more as an iron hematoxylin than as an aluminum hematoxylin.

Solution A	***Amount***	***Function***
Hematoxylin	0.6 g	Dye
Ferric ammonium sulphate	1 g	Mordant
Aluminum chloride	1.2 g	Mordant
Distilled water	74 mL	Solvent
95% ethanol	6 mL	Solvent
Glycerol	20 mL	Stabiliser

Solution B	***Amount***	***Function***
Ferric ammonium sulphate	20 g	Mordant
Distilled water	100 mL	Solvent

Compounding procedure
For solution A, dissolve the hematoxylin in the ethanol.
Dissolve the ferric ammonium sulphate and the aluminum chloride in the water.
Combine, then add the glycerol.

Method
1. Bring sections to water with xylene and ethanol.
2. Place into solution A for 12-24 hours.
3. Place into solution B until differentiated.
4. Wash well in running tap water to blue.
5. Rinse with distilled water.
6. Counterstain if desired.
7. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Expected results
- Nuclei — black
- Background — as counterstain or unstained

Notes
1. The stability of the solutions is not given, but the presence of glycerol indicates they may be stable for some time.
2. The staining procedure indicates that the technique is intended as a primary stain. It is likely a modification of Heidenhain's method and would demonstrate much the same material.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide.* The Blakiston Co.

Seidelin's Iron Hematoxylin

Solution A	***Amount***	***Function***
Ferric chloride	0.6 g	Mordant
Distilled water	95 mL	Solvent
Hydrochloric acid	0.75 mL	Acidifier

Solution B	***Amount***	***Function***
Hematoxylin	1 g	Dye
100% ethanol	100 mL	Solvent

Compounding procedure
Make each solution seperately.
For use, combine 3 parts solution A with 2 parts solution B.
The working solution may be used immediately, but is not stable for long.

Method
1. Bring sections to water with xylene and ethanol.
2. Place into the staining solution for an appropriate time.
3. Wash well in running tap water to blue.
4. Rinse with distilled water.
5. Counterstain if desired.
6. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Expected results
- Nuclei — black
- Background — as counterstain or unstained

Notes
1. The stock solutions are stable for some time.
2. The working solution should be made fresh.
3. This is a modification of Weigert's iron hematoxylin.
4. The staining time should be determined by trial, but is likely about 10-20 minutes.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide.* The Blakiston Co.

Thomas' Iron Hematoxylin

Solution A	***Amount***	***Function***
Hematoxylin	2.5 g	Dye
Dioxane	40 mL	Solvent
Hydrogen peroxide	1 mL	oxidiser

Solution B	***Amount***	***Function***
Ferric ammonium sulphate	3 g	Mordant
Ferric chloride	1.5 g	Mordant
Ferrous chloride	6 g	Mordant
Distilled water	60 mL	Solvent
Acetic acid	6 mL	Acidifier

Compounding procedure
Dissolve the hematoxylin into the dioxane.
Add the peroxide.
Add the acetic acid to the water.
Dissolve the iron salts into the dilute acetic acid.
Filter the solution of iron salts into the hematoxylin solution.

Method
1. Bring sections to water with xylene and ethanol.
2. Place into the staining solution for an appropriate time.
3. Wash well in running tap water to blue.
4. Rinse with distilled water.
5. Counterstain if desired.
6. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Expected results
- Nuclei — black
- Background — as counterstain or unstained

Notes
1. The solution is likely stable for some time.
2. Dioxane is no longer used in histotechnology for safety reasons. The method should be considered obsolete.
3. The solution is progressive, and the staining time should be determined by trial.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide.* The Blakiston Co.

Weigert's Iron Hematoxylin

The modern variant of the 1904 formula is the solution usually meant when Weigert's iron hematoxylin is specified as an acid resistant nuclear stain.

Solution A	***1903***	***1904***	***Function***	***Modern formula for 1904 variant***	
Ferric chloride	0.4 g	0.6 g	Mordant	Ferric chloride 30% aqu.	4 mL
Distilled water	100 mL	100 mL	Solvent	Distilled water	10 mL
Hydrochloric acid	—	0.75 mL	pH control	Hydrochloric acid	1 mL

Solution B	***1903***	***1904***	***Function***		
Hematoxylin	1 g	1 g	Dye	Hematoxylin	1 g
95% ethanol	100 mL	100 mL	Solvent	95% ethanol	100 mL

Solution C	***1903***	—	***Function***
Potassium ferricyanide	2.5 g	—	Bleach
Sodium borate	2 g	—	pH control
Distilled water	100 mL	—	Solvent

Compounding procedure
For both variants:
Make each solution separately.
Immediately before use combine equal parts of solutions A and B.

Method
1. Bring sections to water with xylene and ethanol.
2. Place into the staining solution for:—
   1903 — 1-2 hours
   1904 — 10-30 minutes
3. Rinse with tap water.
4. 1903 — Place in solution C until differentiated.
   1904 — Dip briefly in 1% hydrochloric acid in 70% ethanol.
5. Wash well with running tap water to blue.
6. Rinse with distilled water.
7. Counterstain if desired.
8. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Expected results
- Nuclei — black
- Background — as counterstain or unstained

Notes
1. Solution A of the 1904 variant was originally made from a commercial ferric chloride solution, the amounts in the 1904 column being given by Gray based on its formula. Today, it is usual to use the modern formula.
2. Both variants were originally intended to be used without a counterstain for demonstrating chromatin and other structures usually stained by iron hematoxylin. If the 1904 formula is used for this purpose, rather than as an acid resistant nuclear stain, the staining time should be increased and the excess dye removed with acid ethanol.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide.* The Blakiston Co.
Bancroft, J.D. and Stevens A. (1982) *Theory and practice of histological techniques Ed. 2*. Churchill Livingstone, Edinburgh & London, UK.

Yasvoyn's Iron Hematoxylin

Solution A	***Amount***	***Function***
Hematoxylin	0.1 g	Dye
Distilled water	100 mL	Solvent

Solution B	***Amount***	***Function***
Ferric ammonium sulphate	2.5 g	Mordant
Distilled water	100 mL	Solvent

Compounding procedure
Make each solution seperately.
To 20 drops solution A (about 2 mL), add solution B drop by drop until it just remains blue.
The working solution may be used immediately, but is not stable for long.

Method
1. Bring sections to water with xylene and ethanol.
2. Place into the staining solution for 2-5 minutes.
3. Rinse with water.
4. Remove excess stain with 70% ethanol if necessary.
5. Dehydrate with ethanol, clear with xylene and mount with a resinous medium.

Expected results
- Nuclei — black
- Background — as counterstain or unstained

Notes
1. The stock solutions are stable for some time.
2. The working solution should be made fresh.
3. Although not specified, a tap water wash to blue the hematoxylin may be useful following step 4.
4. Following a wash to blue the nuclei, a counterstain could probably be applied, if wished, before dehydration.

Reference
Gray, Peter. (1954). *The Microtomist's Formulary and Guide.* The Blakiston Co.

Index

Charts