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Hematoxylin in the 21st century

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ABSTRACT
Hematoxylin continues to be a popular substance in the 21st century. I present here some recent developments including the use and misuse of the terms, hematoxylin and hemalum, in biological staining. The medical use of hematoxylin and its application as a dye for hair or textiles continues to be of interest. Unusual applications including its use as a biosensor and for study of vibrational properties of wood are included.

KEYWORDS
biological staining; biosensors; haemalum; haematein; haematoxylin; hair dyes; hemalum; hematoxylin; medical uses; metal complexes; textile dyeing; wood impregnation

The names

The three major subjects discussed here have two names each with different spellings: hematoxylin/haematoxylin, hematein/haematein and hemalum/haemalum. The Biological Stain Commission (BSC) suggests that the use of the words hematoxylin/haematoxylin is country dependent: "hematoxylin (USA) or haematoxylin (elsewhere)" (Dapson et al. 2019), but my investigation of the 21st century usage of these terms revealed the issue to be rather more complicated. Using Google Scholar to search for words in publications, I found 146,000 results for hematoxylin and 57,700 results for haematoxylin for the period 2000 – 2020. Looking at the country of residence of the main or corresponding author for the top 20 publications each year, I found that 51 countries favored hematoxylin and 60 countries favored haematoxylin. The United States, China, India, Japan and the UK all appeared in the top eight in lists of both words. More observations about the frequency of the spellings used can be found below in the biological staining section.

Hemalum is a staining solution made by dissolving hematoxylin together with an oxidizing agent, an aluminum salt and a weak acid in water. There are many variations of the actual reagents, their ratios and their concentrations. Hemalum was not mentioned by Conn in 1925 who preferred the alternative, haemalum (Conn 1925), but by 1953, hemalum prevailed exclusively in this standard reference work (Conn 1953). In the 21st century, in the published literature, hemalum is more frequent by about 3:1. Further information can be found in the section for hemalum below.

Metal complexes

The literature concerning metal complexes of hematoxylin is extensive. Invariably, the complexes are formed from hematein, the oxidation product of hematoxylin. For example, simultaneous determination of trace amounts of Al, Fe, Cu and Pb in water samples was achieved by adding hematoxylin solution and ammonia buffer to a sample in a test tube with a conical bottom (Alpdogan and Zor 2017). Oxygen dissolved in the water converted the hematoxylin into hematein. A mixture of methanol, the disperser solvent, and carbon tetrachloride, the extraction solvent, was injected and the mixture centrifuged at 4,500 rpm. The heavy carbon tetrachloride layer containing the metal complex was separated, dried using a nitrogen stream, the residue dissolved in methanol and this solution injected into an HPLC system equipped with a reverse phase column and detected at 545 nm. Good separation was achieved with retention times of 7.3 min. (hematoxylin), 9.8 min. (Cu complex), 11.3 min. (Fe complex), 14.4 min. (Pb complex) and 19.0 min (Al complex) (Alpdogan and Zor 2017). An alternative detection technique using flame atomic absorption spectrometry also has been used to detect, in addition, Co(II) and Mn(II). The proposed method was applied successfully to cigarettes, hair and some vegetable species (Zor and Alpdogan 2016).

Even without chromatographic separation, it sometimes is possible to detect two different metals in solution, because different hematein complexes exhibit different absorption maxima. For example, both Al and Bi can be detected in vegetable or water samples using solutions of hematoxylin with cetyltrimethylammonium

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bromide as a surfactant at pH 5.8. The λ_{max} values for the Al complex, 500 nm, and the Bi complex, 550 nm, are sufficiently different to allow each metal to be detected in the concentration ranges of 0.1 – 11.0 and 0.1 – 7.0 µg/ml, respectively (Bagheban-Shahri and Niazi 2016).

The long history of the use of aluminum and hematein is discussed in the section on biological staining below. The structure of aluminum-hematein complexes has been of particular interest. From extensive investigations using absorption spectrometry, four different structures, seven if tautomers are included, have been described that exist in solution at different pH or depending on the aluminum:hematein ratio (Bettinger and Zimmermann 1991). The structures have been illustrated earlier in this journal (Cooksey 2010) and in a summary by Kiernan (2018), and for completeness are reproduced here with the original Bettinger and Zimmermann numbers in Roman numerals (Figures 1–4). In summary, in weakly acidic media the violet 1:1 and 1:2 complexes (VII) and (VIII) were observed, and in strongly acidic solution the red 1:1 complex (IX) occurs, whereas in weakly alkaline solution the blue 1:1 complex (X) was detected.

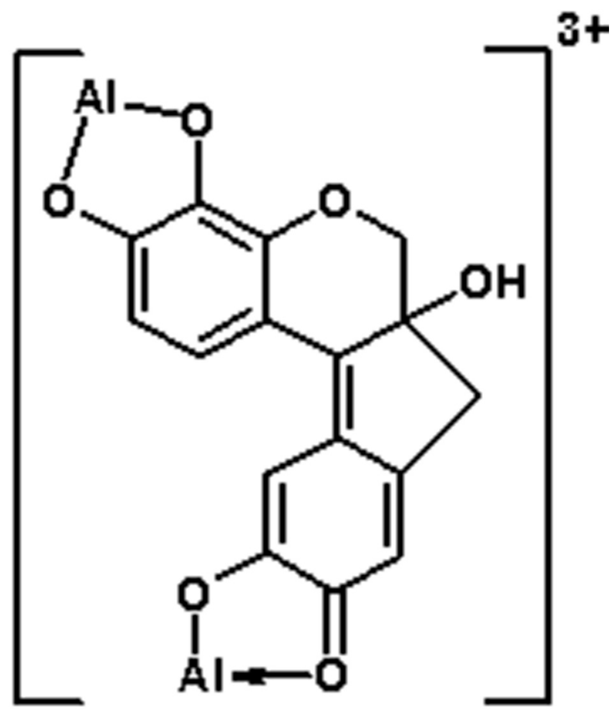

Figure 1. Tautomers of the violet 1:1, pH 4, aluminum-hematein complex compound VII (Bettinger and Zimmermann 1991).

Biological staining

John Randal Baker FRS (1900–1984) was professor of cytology at Oxford University in the UK. He suggested that "aluminium-haematein has probably been employed more extensively in the study of living organisms than any other dye. It was introduced into microtechnique by Bohmer in 1865" (Baker 1962). The emphasis on hematein is to be appreciated, because most authors suggest that hematoxylin is responsible. Sometimes the reference to hematoxylin as a dye is quite explicit, e.g., "Hematoxylin is a natural dye which is extracted from the heartwood of the logwood tree" (Beitollahi et al. 2018a), "Hematoxylin, a natural dye obtained from logwood, is the traditional and most widely used dye in histology, histochemistry, and cytology analyses … " (Kim et al. 2020), "Tissue sections were counterstained with the nuclear dye hematoxylin (blue)" (Ueha et al. 2020). Newcomers to the subject may wonder how a colorless substance,

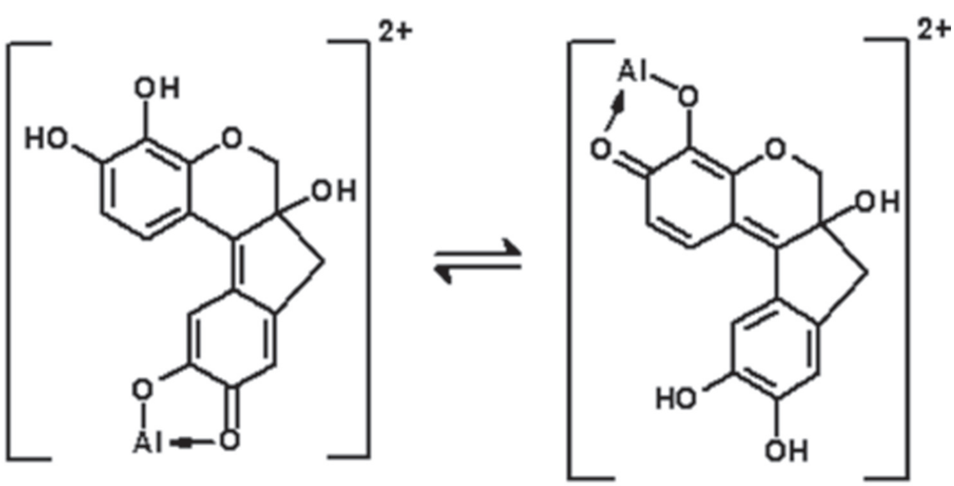

Figure 2. Violet 1:2 aluminum-hematein complex, compound VIII at pH 4.7 (Bettinger and Zimmermann 1991).

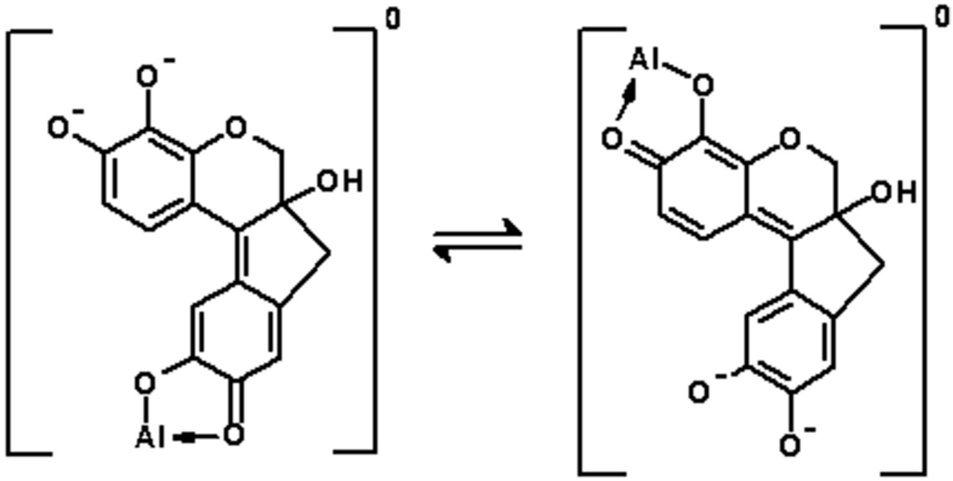

Figure 3. Tautomers of the red 1:1 aluminum-hematein complex, compound IX at pH 2.6. (Bettinger and Zimmermann 1991).

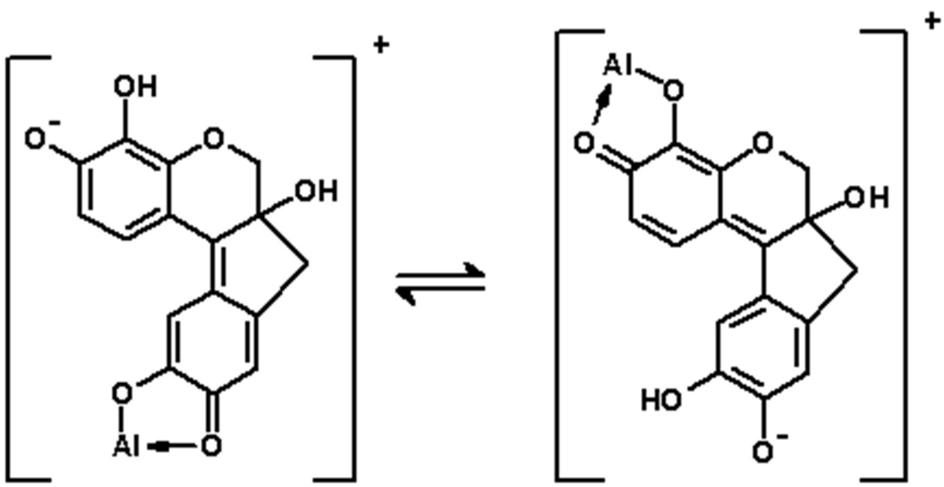

Figure 4. Tautomers of the blue 1:1 aluminum-hematein complex, compound X at pH 8.7 (Bettinger and Zimmermann 1991).

hematoxylin, can act as a dye, but it has long been known that the actual dye is its oxidation product, hematein. “Hematein is formed by oxidation of hematoxylin, which is colorless when pure and protected from oxygen (Lalor and Martin 1959; Bettinger and Zimmermann 1991); it is not a dye” (Kiernan 2018).

The reluctance of authors to mention hematein can be illustrated by counting the number of publications that use this word, as mentioned in the names section above, compared to the number that mention hematoxylin. The results are noteworthy in two respects. First, the large number of references to hematoxylin compared to references to hematein and second, the steady increase of references to hematoxylin from 2000 to 2012 followed by a steady decrease to half the number by 2019. The results of counting references to haematoxylin exhibit only a steady increase over time from 2000 to 2019. These findings are illustrated in Figure 5. To show the results on one graph, it was necessary to plot the number of references for hematoxylin/day, assuming that publication proceeds for 365 days each year, and the number of references for hematein are plotted/year.

Biosensors

Biosensors are multi-component devices that use living organisms or biological molecules to detect the presence of chemicals. Hematoxylin has been employed as a component of a variety of biosensors.

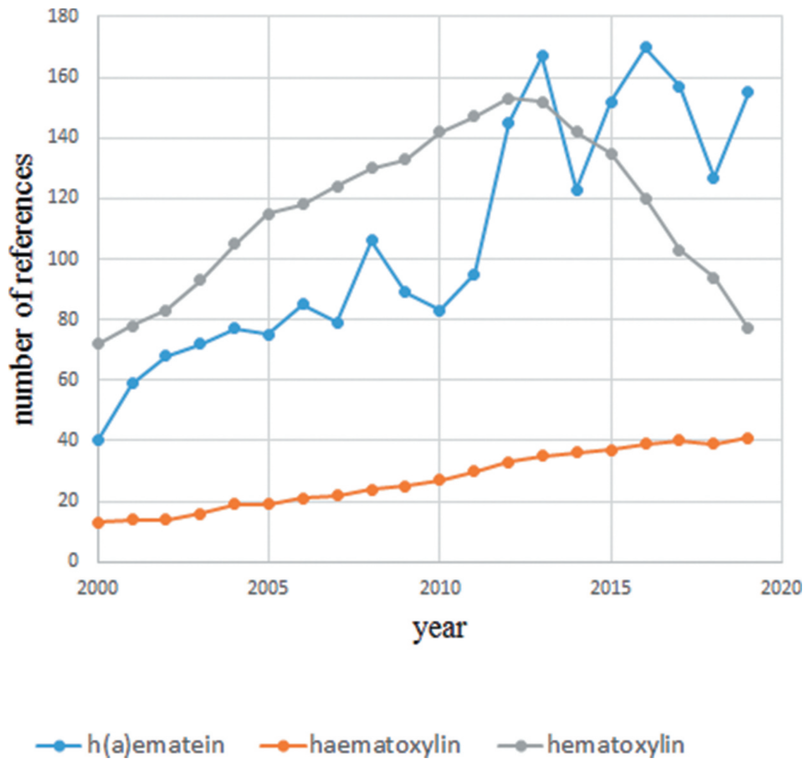

Figure 5. References per year for hematein + haematein (blue), per day for haematoxylin (orange), and per day for hematoxylin (gray).

Such is the interest in these devices that the publishers, Elsevier and MDPI, both devote journals specifically to them.

An application that uses hematoxylin as the electrochemical signal generator was first described by Nodoushan et al. (2019a). It employed reduced graphene oxide and gold nanourchins. Staphylococcal enterotoxin B competes with hematoxylin and consequently, when present, the signal observed decreases. A wide linear range from 5.0 to 500.0 fmol/l was achieved and the limit of detection was calculated as 0.21 fmol/l. The construction of this sensor for staphylococcal enterotoxin B and a description of how it works are provided by Li et al. (2020).

A nanobiosensor has been designed to detect DNA from genetically modified organisms. It employed exfoliated graphene oxide (EGO) and gold nanourchins, which are used to modify the screen-printed carbon electrode. The linear range of the sensor is 40.0 – 1100.0 fM and the limit of detection is 13.0 fM (Aghili et al. 2017). A similar device for detection of phenylketonuria also has been described (Aghaei et al. 2017). Other applications include detection of aflatoxin B1 (Nodoushan et al. 2019b); simultaneous determination of droxidopa, acetaminophen and tyrosine (Beitollahi et al. 2018a); determination of levodopa (Beitollahi et al. 2018b); simultaneous determination of isoproterenol, acetaminophen and tyrosine (Beitollahi and Salimi 2016); detection of glucose (Dilgin and Gökçel 2015); determination of sulfide (Červenka 2013); determination of hydroquinone (Lu et al. 2012), lead and cadmium (Xu et al. 2011); simultaneous determination of ascorbic acid, adrenaline and uric acid (Zare and Nasirizadeh 2010); and simultaneous detection of noradrenalin and acetaminophen (Nasirizadeh and Zare 2009).

Textile and other fabric dyeing

The dyeing properties of an extract of the logwood tree were discovered by Spanish explorers to the Yucatan in 1502. "A vigorous trade soon developed related to growing and preparing hematoxylin for use in dyeing fabrics in Europe" (Titford 2005). Using HPLC-DAD, logwood was detected in 39 of 52 samples of black dyed wool from five Belgian archives (1650 – 1850) (Ortega Saez and Vanden Berghe 2011; Ortega Saez et al. 2019).

In 1814, Edward Bartholomew Bancroft (1745–1821) declared that "the tingent matter of logwood, is capable of producing, with different mordants or bases, almost all the possible varieties of colour" (Bancroft 1814). The observation that a range of colors can be obtained has been confirmed: "plenty of colors can be obtained by changes and combination of different dye mordants" (Tianzhu et al. 2008). The optimal conditions for using hematoxylin on wool included pre-mordanting for 30 min at pH 4.5 and 50 °C, followed by dyeing for 40 – 50 min at pH of 4–5 and 70 °C (Tianzhu et al. 2008).

Natural dyes were mostly replaced by synthetic ones in the late 19th and early 20th centuries, but logwood survived a little longer as a black dye for silk (Dronsfield and Edmonds 2001). Interest in logwood dyeing persists. The degradation and fading of raw and de-gummed silk fabrics dyed with hematoxylin using aluminum or iron mordants have been reviewed by Yatagai et al. (2001). The pH and temperature that were used while employing an iron mordant with hematoxylin for dyeing silk have been studied and the optimum values were identified for color fastness to washing and to light (Sheng 2013). The use of hematoxylin as a dye for wool, soybean protein fiber, ramie and silk has been promoted as "a promising application due to its merits of non-toxic, harmless, non-pollution and elegant chromaticity" (Ying 2015).

Hair dyes

The effect of different conditions including dyeing temperature, time, pH and mordant concentration have been determined for dyeing white yak hair. Al and Zn mordants produce a brown shade and Fe produces black coloration (Gao et al. 2009). Further kinetic and thermodynamic studies involving yak hair suggest that "[H]aematoxylin could be a good candidate as a natural green dye for hair fibre" (Gao and Gao 2014).

Yaks are not the only beneficiaries. Should you require deep orange hair, hematoxylin with catechinone is recommended and the process is illustrated by Yasunaga et al. (2012). The use of henna mixed with hematoxylin with additional penetration enhancers, e.g., propylene glycol and menthol, was recommended for withstanding repeated washing (Wang and Chen 2011). To avoid the allergenic properties of p-phenylenediamine used in hair dyes, the use of plant colorants has been proposed including henna, curcumin, quercetin and hematoxylin (Gao et al. 2012). Four natural plant dyes, Chinese gallnuts, sapanwood extract, which contains hematoxylin, walnut husks and henna leaves were subjected to a range of tests including dyeing gray human hair collected from 15 70 – 80-year-old Chinese males using an iron(II) mordant (Tang et al. 2019). These plant extracts produced viable hair colors for dyeing unbleached gray human hairs and may prove a less irritating alternative to synthetic hair dyes.

Hemalum

The tradition of naming staining solutions after their inventors began in the 19th century. Consequently, we have Ehrlich's hematoxylin, Delafield's hematoxylin, Mayer's hematoxylin, Harris' hematoxylin etc. Paul Ehrlich (1854 – 1915) developed a recipe using hematoxylin with potassium alum (Ehrlich 1886). Francis Delafield (1841 – 1915) used ammonium alum. Neither of these investigators specified an oxidant to convert the hematoxylin into hematein. Their procedures used oxygen from the air or in water. The first stain to specify an added oxidant, sodium iodate, was that of Paul Mayer (1848 – 1923) a German zoologist who studied Caprellidae, skeleton shrimp, while working at the Zoological Station in Naples, Italy. He is better known for inventing and naming hemalum, the staining solution described in the names section above (Mayer 1891). A recent recipe for Mayer's hematoxylin seems straightforward: "One gram of hematoxylin was dissolved in 1,000 ml distilled water and 200 mg sodium iodate, 3 g potassium alum, 50 g chloral hydrate and 1 g citric acid were added to the solution" (Akita et al. 1985). It also can be purchased from Sigma-Aldrich as "hematoxylin solution according to Mayer" and costs £115/l (Sigma-Aldrich 2020). By 2020, regrettably, the literature referred to "Mayer's hematoxylin" 16 times more often than the more accurate "Mayer's hemalum." One venerable investigator has pointed out the distinction: "The components of the hematoxylin and eosin (H & E) stain (i.e., hemalum and eosin Y)" (Wittekind 2003).

Owing to the variety of components and their possible ratios, the number of variations for the recipes for hemalum is large. Over the years, many authors have suggested variations and some have given their own names to them. Sixty different recipes for hemalum have been reported (Llewellyn 2009).

Medical uses

Like many other natural products with long histories, e.g., turmeric, logwood has been recommended for treating many medical conditions. Dr. Duke's *Phytochemical and Ethnobotanical Databases* contains 42 entries for logwood and lists 19 medical uses (USDA 2020). These uses range from treatment for diagnosed diseases, e.g., cancer or dysentery, to relief from symptoms, e.g., bedsore or diarrhea.

Both hematoxylin and resveratrol have been reported to exhibit anti-cancer potential. Hematoxylin can target cullin-5 (CUL5) to control its expression and inhibit the proliferation and invasion of cancer cells (Gao et al. 2020). CUL5 is a scaffold protein in active cullin-ring ubiquitin ligase (CRL) complexes and is a member of the cullin family of proteins. CUL5, as a target of drug action, will play an invaluable role in future cancer treatment (Gao et al. 2020).

Among anti-cancer studies, four compounds, nalidixic acid, indoprofen, hematoxylin and polydatin were found to inhibit PKM2 kinase at micromolar levels. The tumor pyruvate kinase M2 (PKM2) participates in the glycolytic pathway of lung cancer (Ye et al. 2016). It has been reported that hematoxylin suppresses viral protein R–importin α interaction and consequently could be employed for anti-HIV-1 therapy (Suzuki et al. 2009). Hematoxylin can inhibit bladder cancer cells (T24) by two routes: at a dose > 100 µg/ml, it is cytocidal, and at lower concentrations, it induces apoptosis (Ren et al. 2008).

Wood impregnation

The vibrational properties of wood used as sounding boards in musical instruments, vary with the amount of hematoxylin absorbed by the wood (Obataya et al. 2001). The specific dynamic Young's modulus (E/γ) and loss tangent (tanδ) of hematoxylin impregnated wood were determined in the relative humidity range, 0 – 97%, and compared to other wood treatments by formaldehyde vapor and acetic anhydride, and impregnation with polyethylene glycol. The swelling levels are measured as the ratio of the volume of untreated wood (V_u) to treated wood (V_t). At low swelling levels, $V_t/V_u < 1.03$, the hematoxylin impregnated wood behaved like formalin treated wood and like acetylized wood at mid-levels, $1.07 < V_t/V_u < 1.08$, but at high levels, $V_t/V_u < 1.10$ like unimpregnated wood. It appears that hematoxylin acts as a plasticizer, i.e., due to expanded molecular spacing, the molecules can move around more easily, but only when the hematoxylin molecule is surrounded by approximately 120 water molecules for 24 hr under catalysis of SO_2.

Disclosure statement

The author declares no conflict of interest

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