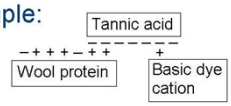
HAEMALUM – Substrates and mechanisms of staining

John A. Kiernan
London, Canada

MORDANTS. From French ***mordre*** - to bite.
Serving to fix colouring matter or gold leaf (1825)

An early example:



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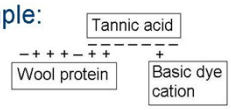

Current usage:

(= Last 100 years)

Metal ion used with a dye.

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A copper ion can complex 4 ammonia molecules: $[Cu(NH_3)_4]^{2+}$

$Cu—NH_3$ Simple covalent

$Cu←NH_3$ Shows e^- donation

$Cu^{\ominus}—^{\oplus}NH_3$ Shows polarity

$Cu\cdots NH_3$ Deliberately vague

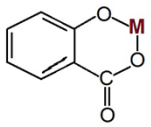
Some mordant dyes can selectively stain cell nuclei (chromatin).

Cationic (basic) dyes stain DNA in chromatin, RNA in cytoplasm, and also polyanions in cartilage, mucus etc.

CHELATION

From Greek *chela* - a claw, as of a crab or scorpion

One metal atom has bonds to 2 atoms of the same complexing molecule (*eg* a dye), making a stable ring.



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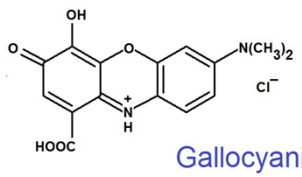
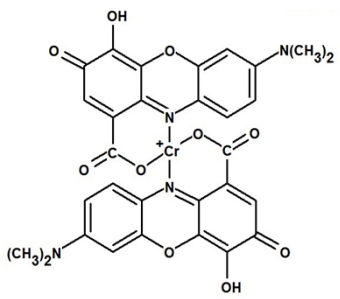


Gallocyanine

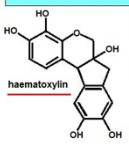
Cationic wt = 300



Chromium chelate Cationic wt = 651

[Marshall PN & Horobin RW (1972) *Stain Technology* 47: 155-161.]

HAEMATOXYLIN AND HAEMATEIN



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Easily oxidized to haematein by air, sodium iodate etc.

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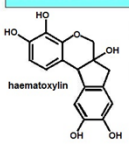

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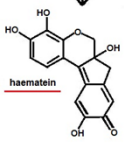

Dirty-yellowish anionic dye

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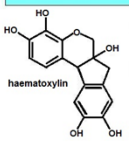

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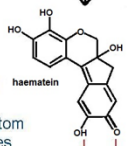

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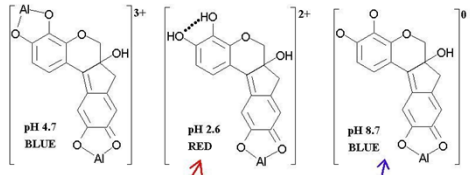
Haematein forms strongly coloured complexes with many metals, notably aluminium and iron(III)

Metal atom displaces this hydrogen and connects to this oxygen, making a stable chelate ring of 5 atoms

HAEMATOXYLIN AND HAEMATEIN

Aluminium chelates of haematein



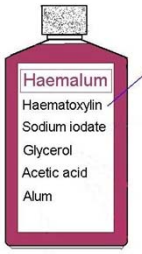
This 1:1 cation is present in haemalum staining solutions.

This uncharged 1:1 complex forms when stained sections are blued in alkalinized water.

[Bettinger C & Zimmermann HW (1991) *Histochemistry* 96: 215-228]

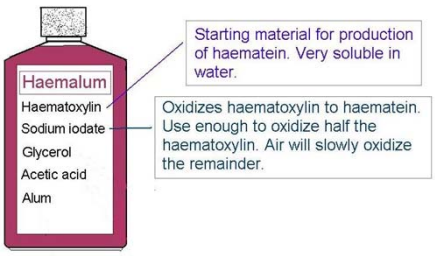
HAEMALUM. What and why?

The H in H & E



Starting material for production of haematein. Very soluble in water.

HAEMALUM. What and why?



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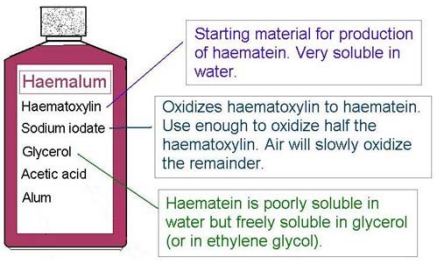

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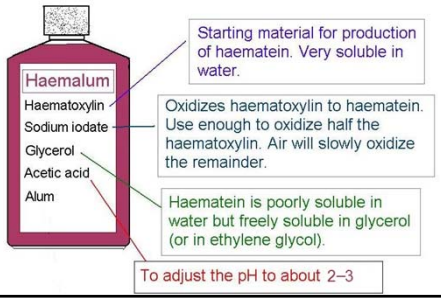
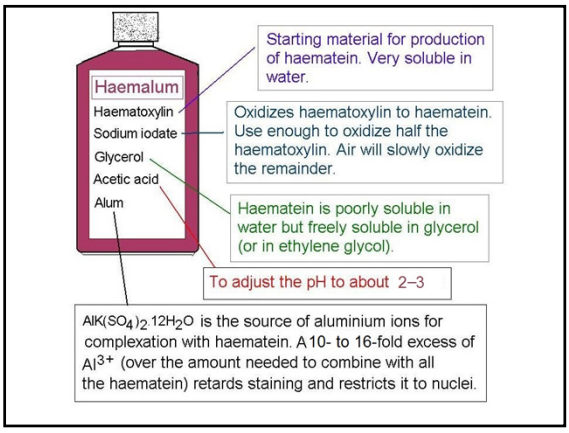
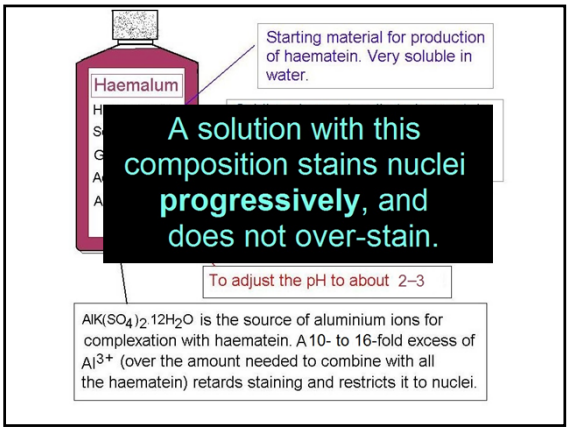
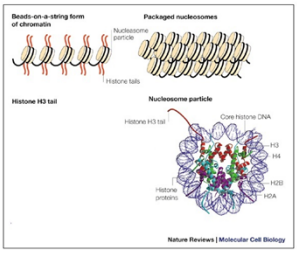






POSSIBLE MECHANISMS FOR PROGRESSIVE NUCLEAR STAINING BY HAEMALUM

Chromatin = DNA + nucleoproteins (mostly histones).



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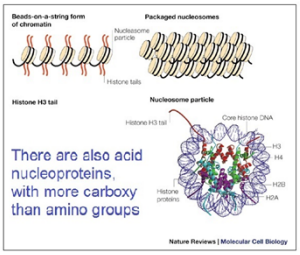

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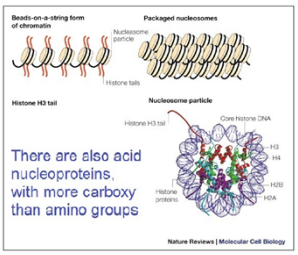

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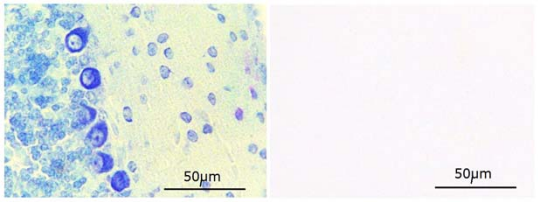
Evidence: After extraction of nucleic acids from sections by strong acids, or enzymatic digestion (DNase, RNase), chromatin cannot be stained by simple cationic dyes.



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More evidence: An inorganic salt (NaCl or $MgCl_2$) added to the staining solution inhibits coloration because Na^+ or Mg^{2+} competes with Dye^+.

When a small, planar dye cation has been attracted to $(phosphate)^-$ of DNA, weaker forces maintain adhesion between ring systems of dye and the cyclic bases of DNA.

Goldstein DJ (1962) *Quart. J. Microsc. Sci.* **103**: 477-492.

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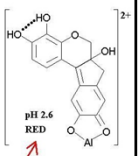

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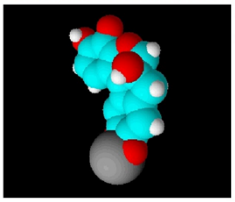
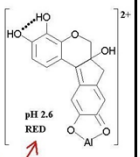


THIS SHAPE, AND THE LOW pH, PREDICT WEAK BINDING OF HAEMALUM TO THE PHOSPHATE POLYANIONS OF DNA



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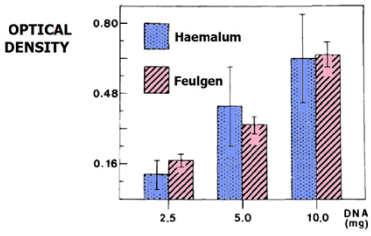
DOES HAEMALUM STAIN NUCLEIC ACIDS?

Histochemical testing can be carried out on films containing known substances.



DNA SPOTS IN POLYACRYLAMIDE GEL FILM

EKW Schulte & DK Fink 1995. *Anal. Cell. Pathol.* **9**: 257-268.

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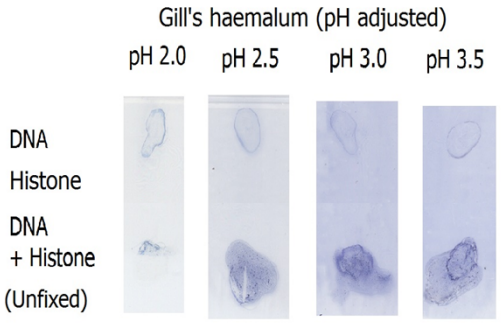

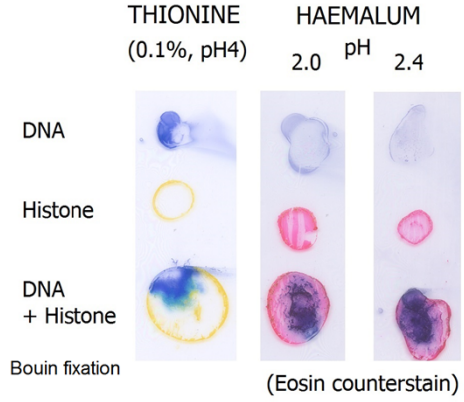

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RESULTS VARY!

Baker 1962. Staining greatly reduced (TCA)

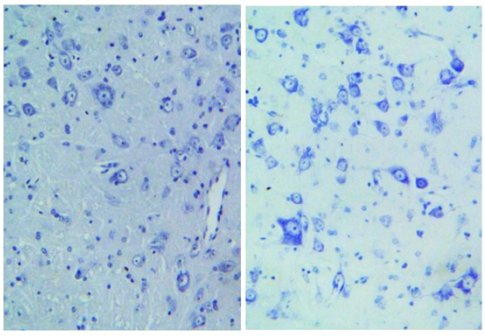
Marshall & Horobin 1973. Staining only slightly reduced (TCA)

Lillie, Pizzolato & Donaldson 1976a. Staining only slightly reduced (HCl)

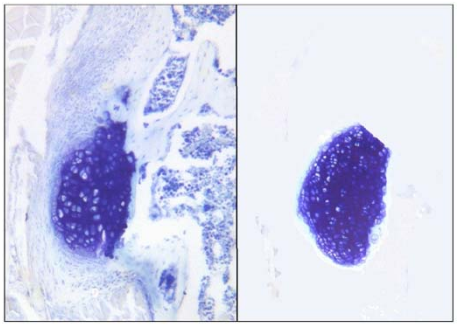
Lillie, Pizzolato & Donaldson 1976b. Staining weakened, not prevented (HNO_3; also DNase)

Bettinger & Zimmermann 1991. Staining prevented (DNase and RNase)

Controls:



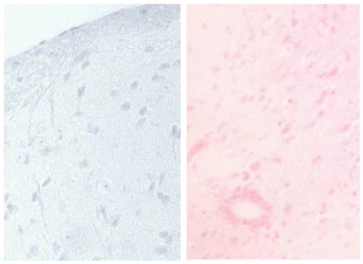
Haemalum pH2.4 5min. 0.1% thionine pH4 5min.



Control section After DNase + RNase

Thionine (0.1%, pH 4.0, 5 minutes)

Perchloric acid (5%, 60°C, 30m)



Gill's haemalum (pH 2.4)

Alkaline eosin Y (0.2%, pH 9.5)

My experiments with DNA extraction from sections agree with some earlier studies (Baker 1962, Bettinger & Zimmermann 1991) **but not with others** (Marshall & Horobin 1973; Lillie *et al.* 1976; Puchtler *et al.* 1986).

Haemalum staining of spots on slides (DNA and the DNA+histone precipitate), but no staining of spots containing only histone, supports the notion of DNA as the major substrate of progressive haemalum staining of chromatin.

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These simple tests show that haemalum does not bind to the major protein of chromatin, even when the histone has been made insoluble by coagulation and cross-linking (Bouin fixation).

But chromatin also contains non-histone proteins, some with long sequences of glutamic and aspartic acid residues (MacGillivray et al. 1972; Kuehl et al. 1986).
These need to be investigated as potential binding sites for haemalum.

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Chromatin = DNA + nucleoproteins (mostly histones).

Holde Puchtler (1920-2006) was a pathologist familiar with the chemistry of textile dyeing. She cited evidence for **hydrogen bonding** of adjacent –OH groups of unoxidized haematoxylin (or of haematein) to oxygen atoms of proteins (–COOH as in silk) and carbohydrates (–OH as in cellulose). Metal ions compete for tissue oxygens and also combine with bound haematein.

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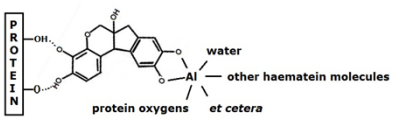
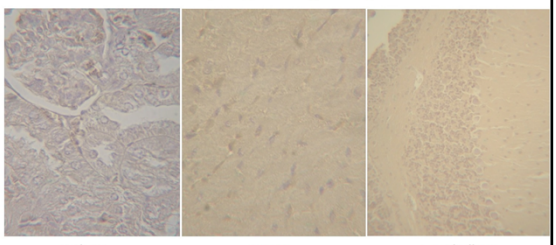


TESTING PUCHTLER'S HYPOTHESIS

Apply haematein to sections and detect bound dye by subsequent application of an Al^{3+} solution, and blueing.

Haematein, 0.0017M = Gill's 1974 ("#1") without the aluminium sulphate; pH=2.8), 5 min. Rinse in water, then aluminium sulphate, 0.026M (pH=3.4), 5 min.



Kidney Heart Cerebellum

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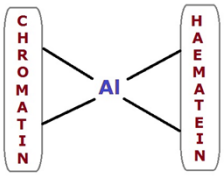
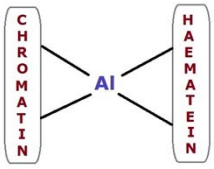
The traditional explanation of haemalum staining has coordinate bonds: tissue-metal and metal-haematein:



The bonds account for strong dye binding (fastness). The substrate in chromatin could be DNA or protein.



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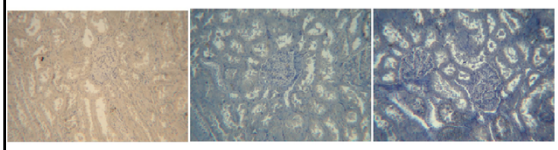
Baker (1962) used a haematein solution to detect Al^{3+} previously applied to sections [at pH~3.5]. Everything stained. Acid was applied after the Al^{3+}, and then only nuclei were stained by haematein. Acid differentiation breaks the tissue-metal bonds, not metal-dye bonds.

TESTING TRADITION. Was Baker (1962) right?

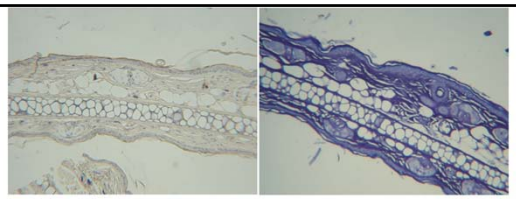
Sequential staining with Al^{3+} (range of pH, 2.0-3.5) followed by water rinse then haematein at pH~3.5, which should detect all bound metal.

TESTING TRADITION. Was Baker (1962) right?

Sequential staining with Al^{3+} (range of pH, 2.0-3.5) followed by water rinse then haematein at pH~3.5, which should detect all bound metal.



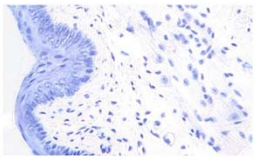
$Al_2(SO_4)_3$ pH2.6 $Al_2(SO_4)_3$ pH3.0 $Al_2(SO_4)_3$ pH3.6



$Al_2(SO_4)_3$ pH 2.0 PINNA OF EAR $Al_2(SO_4)_3$ pH 3.4

BLUE NUCLEI AT pH2, BUT NOT THE SAME!

Gill's haemalum at pH 2.0



PHARYNX

CONCLUSIONS.

Baker (1962) was probably right in considering that DNA was the principal substrate of selective nuclear staining by alum-haematein.

Lillie *et al.* (1976a,b) and Puchtler *et al.* (1986) were probably wrong in identifying nucleoproteins as the substances in chromatin stained by haemalum.

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Bettinger & Zimmermann (1991), using laboratory-purified haematoxylin and haematein (not commercially available) obtained convincing staining of DNA and RNA in methanol-fixed monolayers of HeLa cell cultures. Nucleases prevented all staining. Their haemalum solutions were very dilute, and were used at pH3.2 and pH4.7, for 2 hours — conditions greatly different from regular practice! B&Z did not do tests on sections of tissues, and they did not speculate about the forces binding the aluminium-haematein complex to nucleic acids.

Baker JR (1962) Experiments on the action of mordants. 2. Aluminium-haematein. *Quart. J. Microsc. Sci.* **103**: 493-517.
Bettinger C, Zimmermann HW (1991) New investigations on hematoxylin, hematein, and hematein-aluminium complexes. 2. Hematein-aluminium complexes and hemalum staining. *Histochemistry* **96**: 215-228.
Lillie RD, Donaldson PT, Pizzolato P (1976a) The effect of graded 60C nitric acid extraction and of deoxyribonuclease digestion on nuclear staining by metachrome mordant dye metal salt mixtures. *Histochemistry* **46**: 297-306.
Lillie RD, Pizzolato P, Donaldson PT (1976b) Nuclear stains with soluble metachrome metal mordant lake dyes. The effect of chemical endgroup blocking reactions and the artificial introduction of acid groups into tissues. *Histochemistry* **49**: 23-35.
Puchtler H, Meloan SN, Waldrop FS (1986) Application of current chemical concepts to metal-hematein and -brazilein stains. *Histochemistry* **85**: 353-364.