Schiff's Rainbow

Richard W. Dapson, PhD

In appreciation

- Ralph Lillie (1896 - 1979)
  - BSC Member, Trustee & President
- James Longley (1920 - 2014)
  - BSC Member, Trustee, Editor & President
- Frederick Kasten (1927 - 2014)
  - BSC Member, Trustee & President
- Alton Floyd (1941 - 2015)
  - BSC Member, Trustee & President

All four made contributions to understanding Schiff reagents

In appreciation

- G. Stephen Nettleton
  - BSC Member, Trustee, Editor
- Richard Horobin
  - BSC Member, Trustee, Associate Editor
- John Kiernan
  - BSC Member, Trustee, Secretary)

All made further contributions to understanding Schiff reagents

The story begins...

Once upon a time...

(1861 to be exact)

...in a land far away...

(France)

Charles Lauth (1861)

- Discovered that amines and aldehydes formed a complex
    - Reaction was VERY slow

Later, Charles Lauth returned

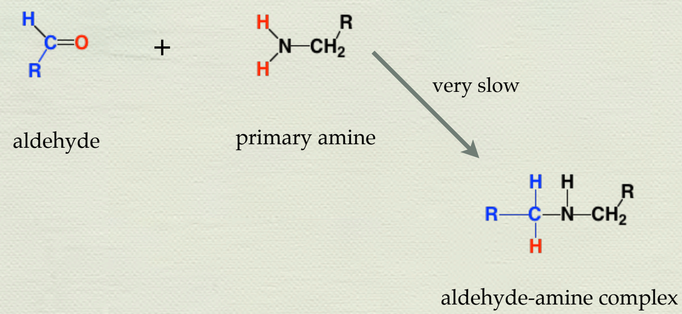
- 1876
    - Invented the dye *Violet de Paris*

      (On the new aniline dye, Violet de Paris. *Laboratory* 1:138-139)

        - became known as Lauth's violet, later called thionine

but that's another story for a later time today...

Lauth’s reaction



Enter Hugo Schiff

Schiff's 1st contribution (1865)

- Hydrochloric acid greatly speeded up the reaction between aldehydes and amines
- Used years later by Gomori to make aldehyde fuchsin, a stain for elastin and pancreatic *beta* cells
- Also used by Horobin & Kevill-Davies to make a sulfite-free reagent for aldyhydes

Schiff's 2nd contribution (1866)

- Sulfurous acid makes the reaction between aldehydes and amines go even faster
  - sulfurous acid is generated in the lab in a 2-step process

Making sulfurous acid - Step 1

- Sulfur dioxide is given off when any of the following SO_2 generators dissolves in water:
  - sodium or potassium bisulfite
  - sodium or potassium metabisulfite
  - thionyl chloride

Making sulfurous acid - Step 2

- SO_2 reacts with water

$$H_2O + SO_2 \longrightarrow HSO_3^- + H^+$$

water, sulfur dioxide, sulfurous acid, hydrogen ion

Making sulfurous acid - Step 2

- Sulfurous acid solution gives off SO_2 to the atmosphere

Phew!!

$$H_2O + SO_2 \longleftarrow HSO_3^- + H^+$$

water, sulfur dioxide, sulfurous acid, hydrogen ion

Schiff's 3rd contribution

- Schiff's original reagent
  - Dissolve pararosaniline in water (red)
  - Add HCl
  - Add sodium metabisulfite
    - The dye is decolorized (Mystery #1)

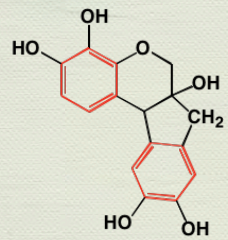
But why is a dye colored?

- Conjugated atoms
  - connected by alternating single & double bonds
  - outer electrons are free to wander all around the conjugated system
  - if the conjugated system is sufficiently large, color is visible
    - *e.g.*, hematoxylin & hematein

Hematoxylin & hematein

Hematoxylin

6 + 6 atoms conjugated

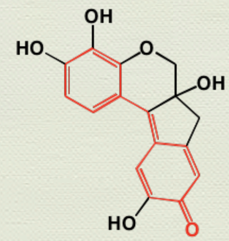


too few atoms in each conjugated system:

essentially colorless

Hematein

14 atoms conjugated



one large conjugated system:

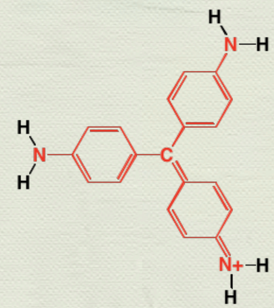
violet

Chromophores

- The conjugated system is called a chromophore if colored
- Shade of the color may be altered by groups attached to, but outside, the conjugated system
  - these alter the distribution of electrons
  - called auxochromes

Schiff's original reagent

Pararosaniline (CI 42500)



What does CI 42500 mean?

Colour Index Number assigned uniquely to each dye by the Society of Dyers & Colourists and the American Association of Textile Chemists and Colorists

22 conjugated atoms

A note from our sponsor

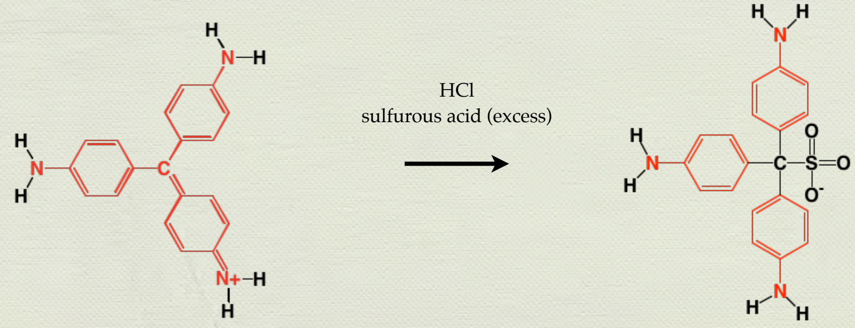
- Pure pararosaniline, certified by the BSC, can be purchased
- Basic fuchsin certified by the BSC contains sufficient pararosaniline to make a good Schiff reagent
- Less pure basic fuchsin contains enough contaminants (similar dyes) to render the mixture unsatisfactory; these are not certified as suitable for Schiff reagent

Back to Schiff's reagent

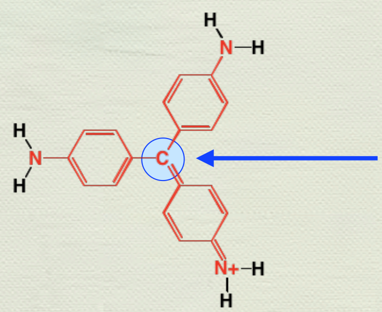
Decolorizing pararosaniline

pararosaniline
22 conjugated atoms
red

leuco-fuchsin
7+7+7 conjugated atoms
colorless

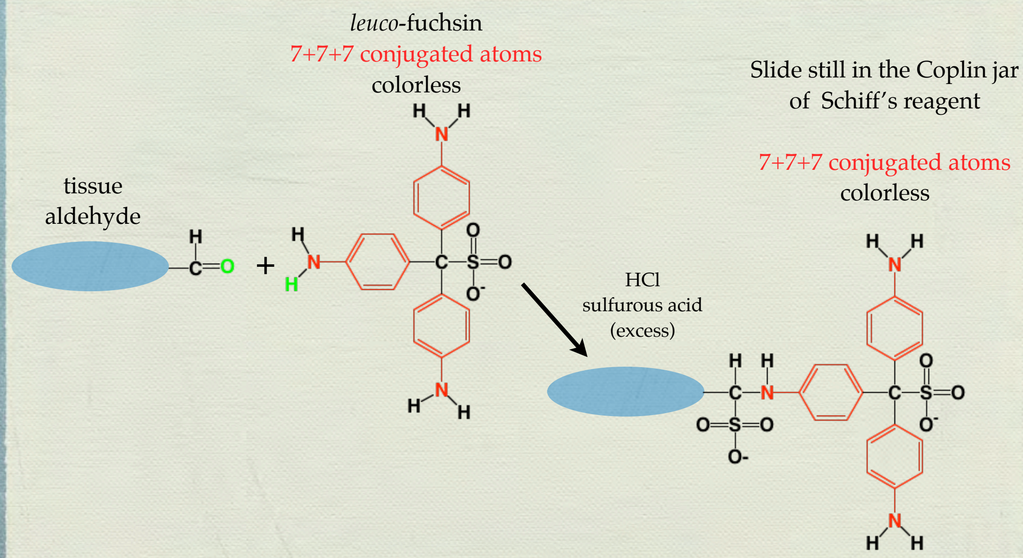


Why does sulfurous acid attach to the central carbon atom?



This carbon atom (C7) is the only available carbon with a partial positive charge (+0.29)

Reaction with aldehyde



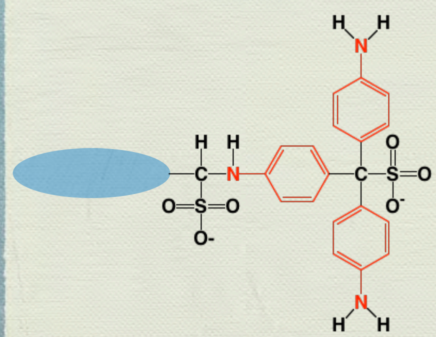
Mystery #2

Color is restored!

Restoring the color

Slide still in the Coplin jar
of Schiff's reagent

7+7+7 conjugated atoms
colorless

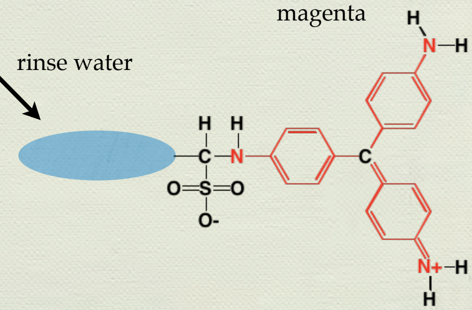


Slide is transferred to rinse water:

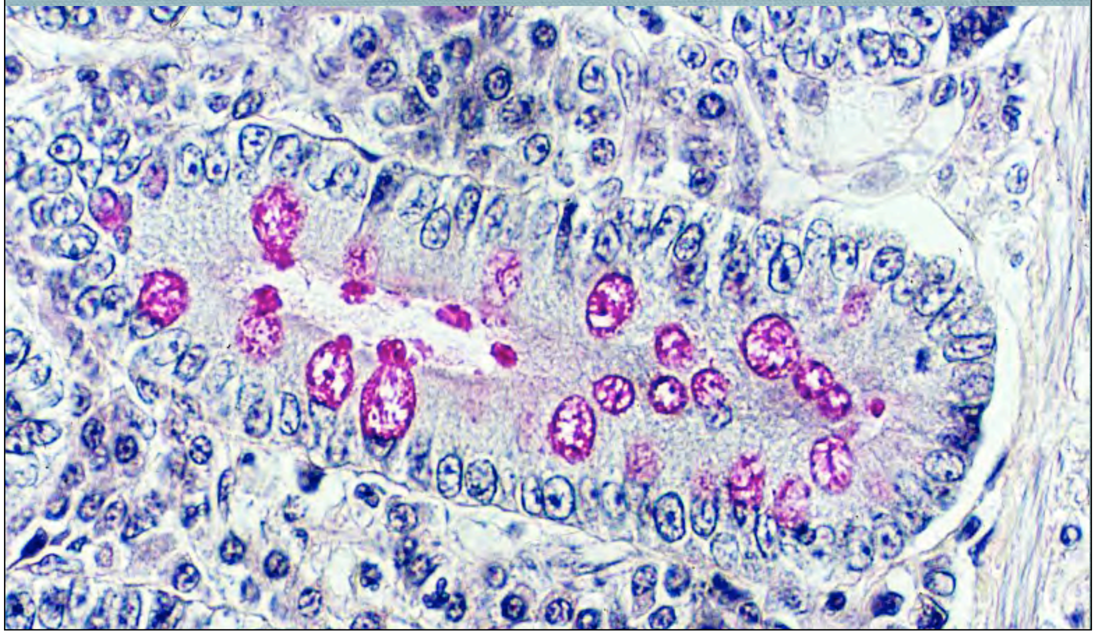
a) sulfurous acid is removed from solution
b) loosely held sulfonic group responds to changed equilibrium and is lost
c) chromophore is restored

rinse water

22 conjugated atoms
magenta



PAS + hematoxylin (duodenum)

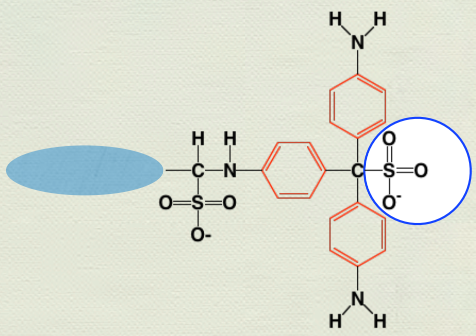


News flash!! (6 pm edition)

- Putting a sulfonic acid onto pararosaniline to decolorize it has nothing to do with the compound reacting with aldehydes. *This is critically important!*
    - The amine is responsible for the reaction with aldehydes *(remember Lauth!)*
    - This sulfonic acid simply decolorizes Schiff's reagent

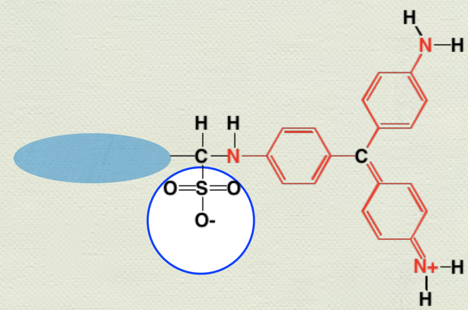
News flash!! (10 pm edition)

- Losing the original sulfonic acid on the Schiff-tissue complex restores the color



News flash!! (10 pm edition)

- The new sulfonic acid at the tissue aldehyde site converts the complex to an acid (anionic) dye

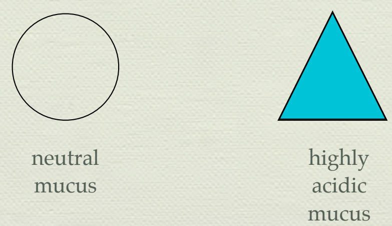


PAS + hematoxylin

- Why not H + PAS
  - Schiff's reagent is highly acidic
  - would destain hematoxylin

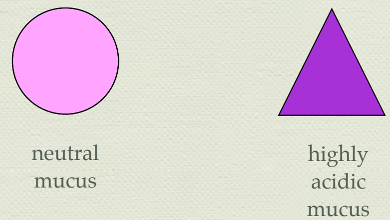
An enigma resolved...

- AB + PAS (done the correct way) differentially stains two types of mucus
    - 1st step: AB first stains highly acidic (sulfonated) mucus blue



An enigma resolved...

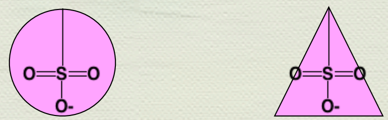
- 2nd step: PAS then stains most mucus (neutral & acidic) magenta



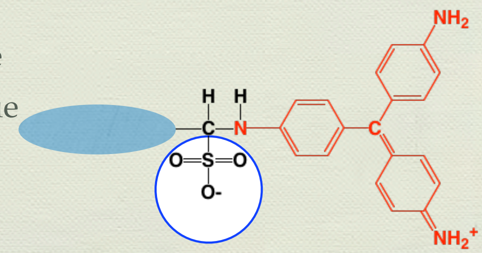
- Acidic mucus stained with both AB & PAS appears purple

Staining backwards...

- PAS + AB stains all mucus purple
    - Schiff's reagent attaches to most mucus

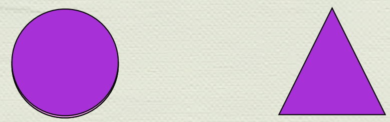


- Sulfonic acids on Schiff-tissue complexes act as anionic tissue sites



Staining backwards...

- AB then stains natural sulfonic acids in tissue as well as those on Schiff-tissue complexes



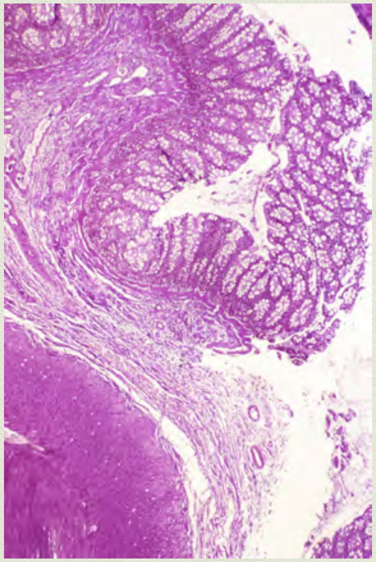
neutral mucus

highly acidic mucus

Ooops!

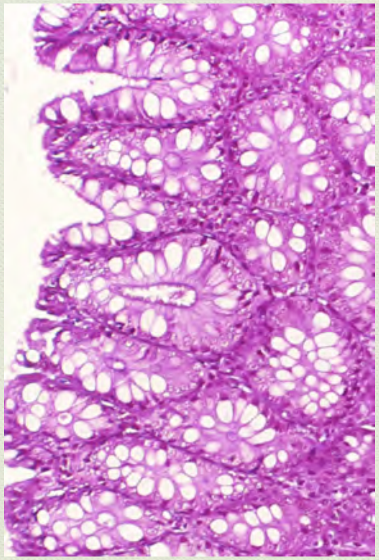
A fun experiment

- Add Schiff's reagent to formalin (formALDEHYDE solution)
  - Solution turns magenta
- Use this as a stain on tissue containing muscle & collagen
  - Tissue stains dark magenta as if it had been stained with a red acid dye *(actually, it has!)*



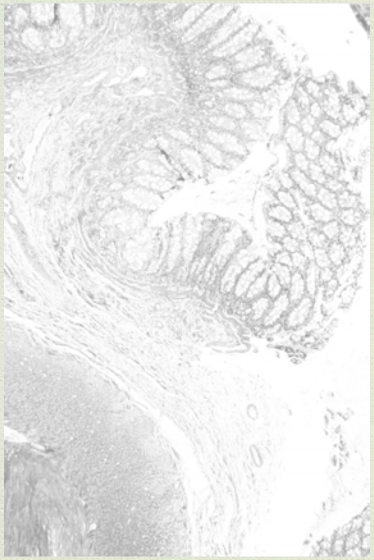
A fun experiment

- Note: goblet cells are unstained



A fun experiment (cont.)

- Destain with an alkaline solution (*e.g.,* bluing agent)
  - Tissue becomes colorless

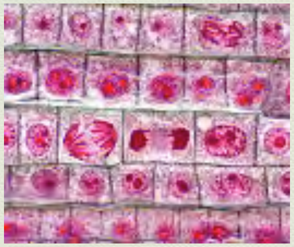


Another Schiff reaction

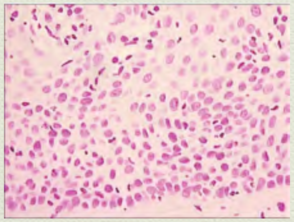
- The Feulgen reaction (Robert Feulgen, 1924)
  - No periodic acid needed
  - Hydrolyze DNA with HCl
    - Removes purines (adenine & guanine), exposing aldehydes on the DNA backbone
  - Stain with Schiff's reagent

Fuelgen reaction for DNA

- With conventional Schiff's, chromosomes stain magenta
  - obvious on onion root tips

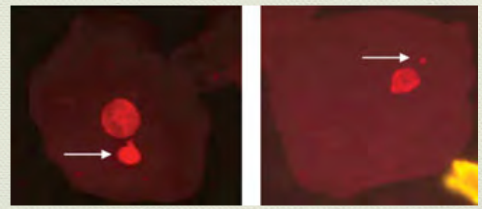


  - rather weak on oral mucosa



Fluorescent Feulgen

- Use acridine yellow, acriflavine, auramine O or many other dyes
- Treat with HCl and SO_2 just like with pararosaniline
- Chromosomes stain faintly with visible light, but fluoresce intensely under UV light

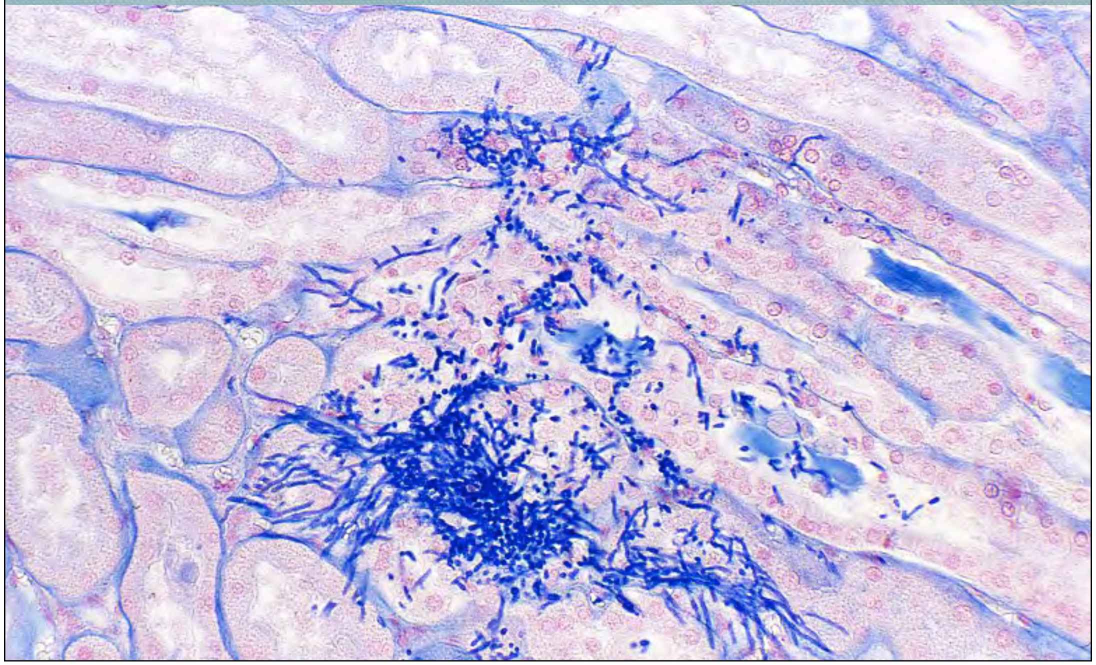


Fluorescent Fuelgen on buccal smear, showing micronucleus

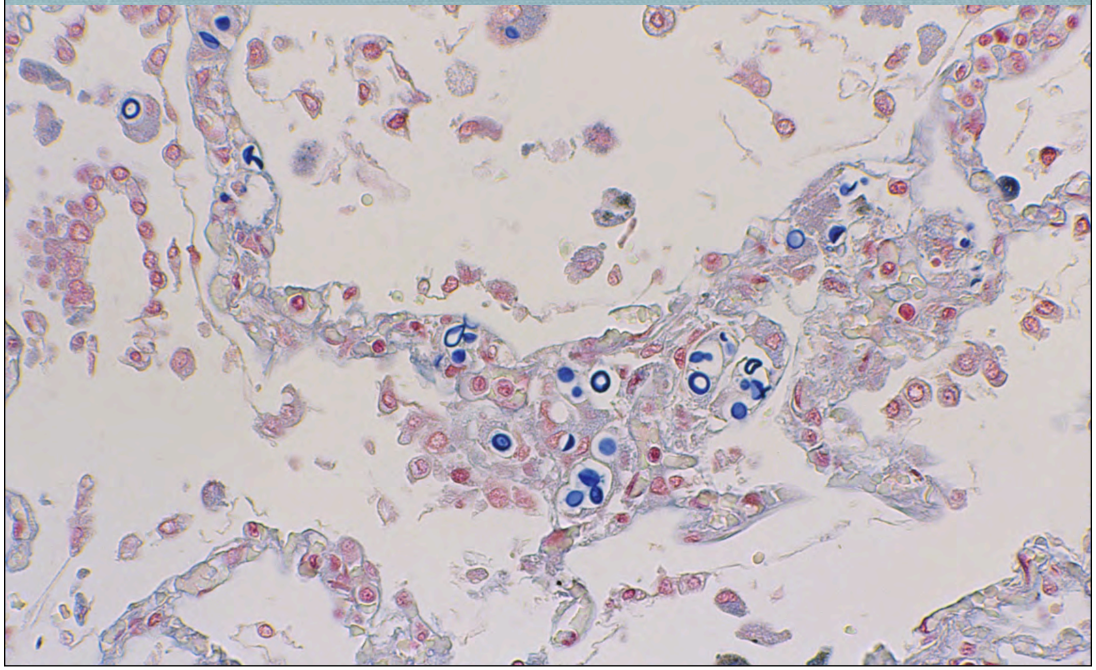
Let's change the color (again)

- A different Schiff reagent: thionine-Schiff
  - Greater intensity of staining in visible light (the eye is especially sensitive to blue)
  - Ideal for very small objects

Blue PAS (fungus, kidney)



Blue PAS (*Cryptococcus*, lung)



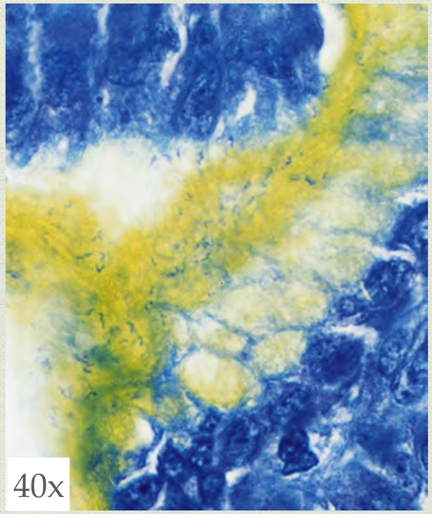
Another color, another practical application

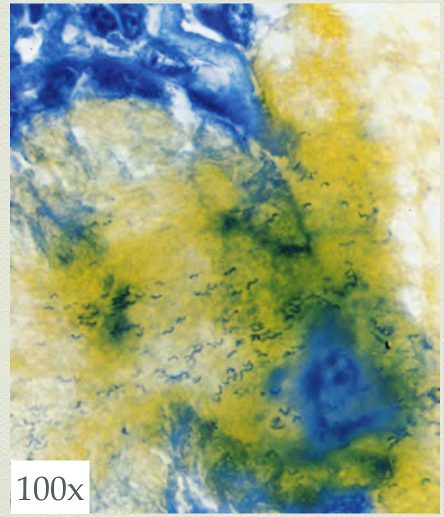
Finding *Helicobacter* (original)

- *Helicobacter* lies embedded in mucus in the stomach lining
- Formerly, the best way to demonstrate it was Alcian yellow + toluidine blue (AY+TB)
  - offered the best contrast (dark blue against bright yellow)
  - Alcian yellow not available after 2000

Finding *Helicobacter* (today)

Periodic acid + Yellow Schiff + blue basic dye





Yellow Schiff

- A mixture of acridine derivatives
- Cannot be decolorized
- Otherwise it combines with tissue aldehydes

What exactly is a Schiff reagent?

Let's ask Hugo Schiff...

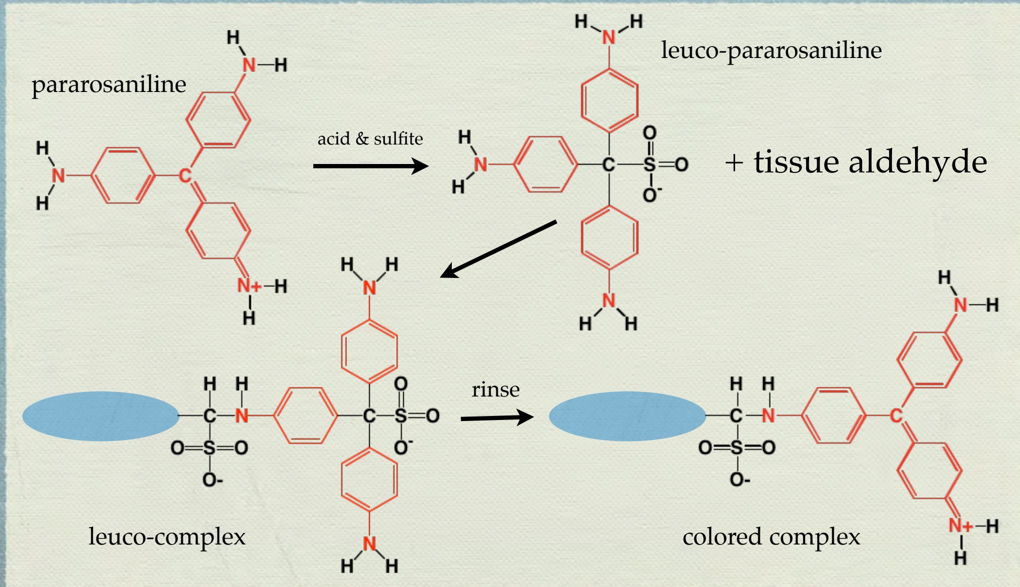
- His original reagent was an amine in a highly acidic solution that formed a complex with aldehydes (1865)
- He improved on this by adding sulfurous acid to an amine dye that was decolorized before reacting with an aldehyde (1866)
  - This is considered the true Schiff reagent today

But there are
lots of variants

Type I. Triphenylmethanes (true Schiff reagents)

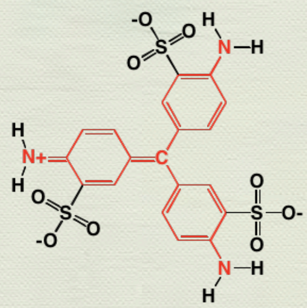
- An amine bearing dye
  - decolorized by the addition of sulfurous acid to a positively charged carbon atom, then
  - reacts with an aldehyde to form a complex bearing a second sulfurous acid; and finally
  - becomes colored again upon washing because the first sulfurous acid is lost
- Examples: pararosaniline (CI 42500), acid fuchsin (CI 42685), methyl violet (CI 42535)

Pararosaniline

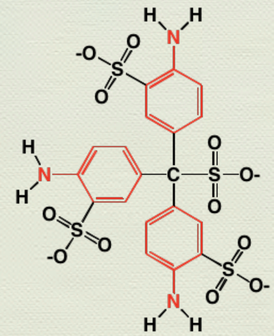


Acid fuchsin

acid fuchsin
(trisulfonated pararosaniline)
CI 42685



leuco acid fuchsin

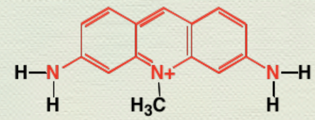


Reactions are the same as for pararosaniline

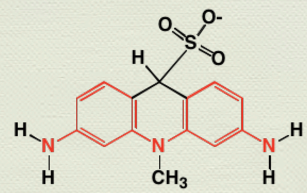
Type II. Acridines

- An amine bearing dye
  - sulfurous acid attaches to a positively charged carbon atom without disrupting the conjugated system
  - dye then reacts with an aldehyde to form a complex bearing a second sulfurous acid (as in Type I)
- Examples: acriflavine (CI 46000), acridine yellow G (CI 46025), proflavine (CI 490), phosphine (CI 46045), chrysoidin Y (CI 11270), chrysoidin R (CI 11320)

Acriflavine



Addition of sulfurous acid eliminates only 1 atom from the conjugated system



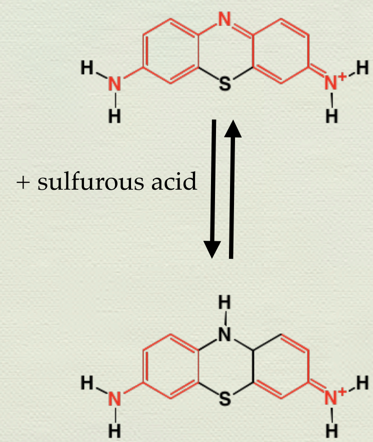
Type III. Thiazines, oxazines, azines, indamines

- An amine bearing dye
  - decolorized by reduction (addition of hydrogen), not by the addition of sulfurous acid
  - reacts with an aldehyde to form a complex bearing a sulfurous acid (as in Type I)
- Examples: thionine (CI 52000), brilliant cresyl blue (CI 51010), neutral red (CI 50040), toluylene blue (CI 49410)

Thionine (a thiazine)

Sulfurous acid reduces the dye without combining with it, in a reversible process

colored

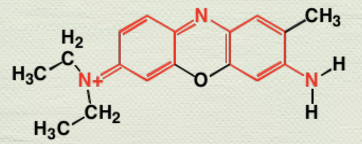


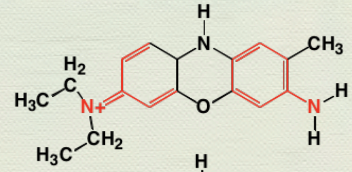
not colored

Other Type III Schiff dyes

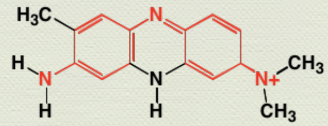
colored form | leuco form

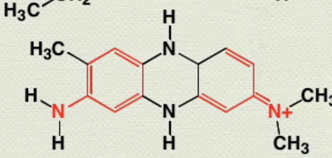
Brilliant cresyl blue
(oxazine)



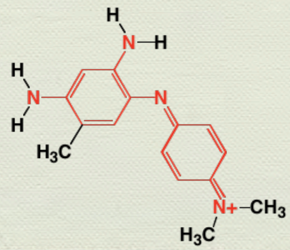


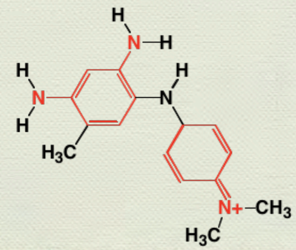
Neutral red
(azine)





Toluylene blue
(indamine)



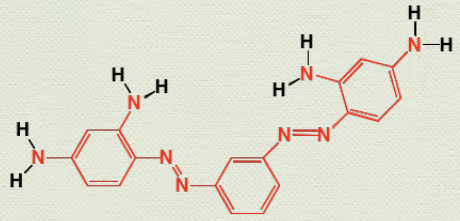


Type IV. Azo

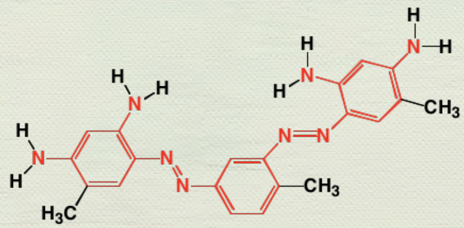
- An amine bearing dye
  - not decolorized by sulfurous acid (it cannot attach or if it does, does not break the conjugated system)
  - neither is it reduced to a *leuco*- form
  - reacts with an aldehyde to form a complex bearing a sulfurous acid (as in Type I)
- Examples: Bismarck brown Y (CI 21000), Bismarck brown R (CI 21010), chrysoidin (CI 11270), chrysoidin R (CI 11320)

Bismarck brown Y

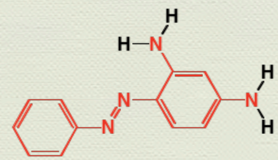
There are no carbon atoms bearing a positive charge



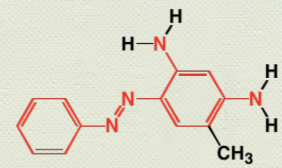
Other Type IV Schiff dyes



Bismarck brown R



Chrysoidin

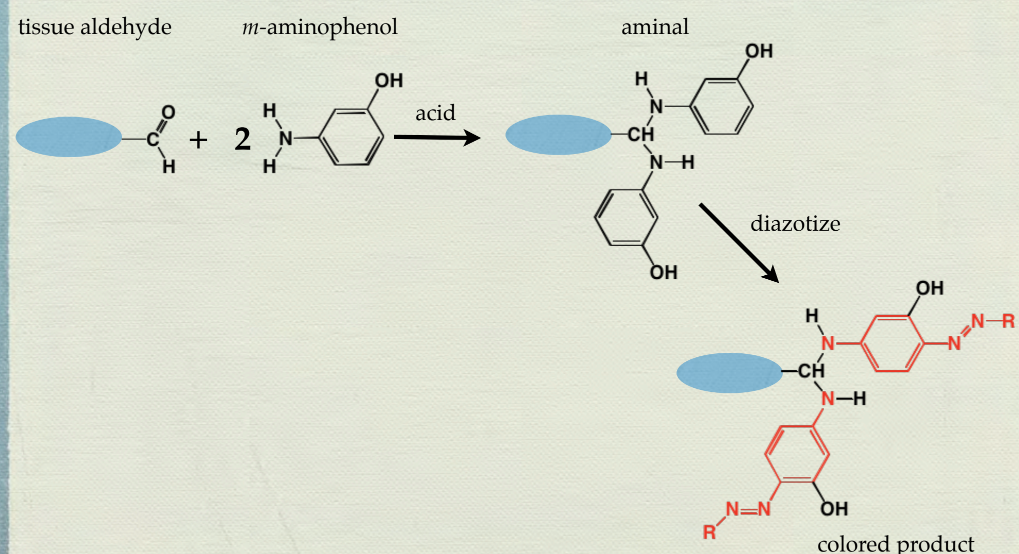


Chrysoidin R

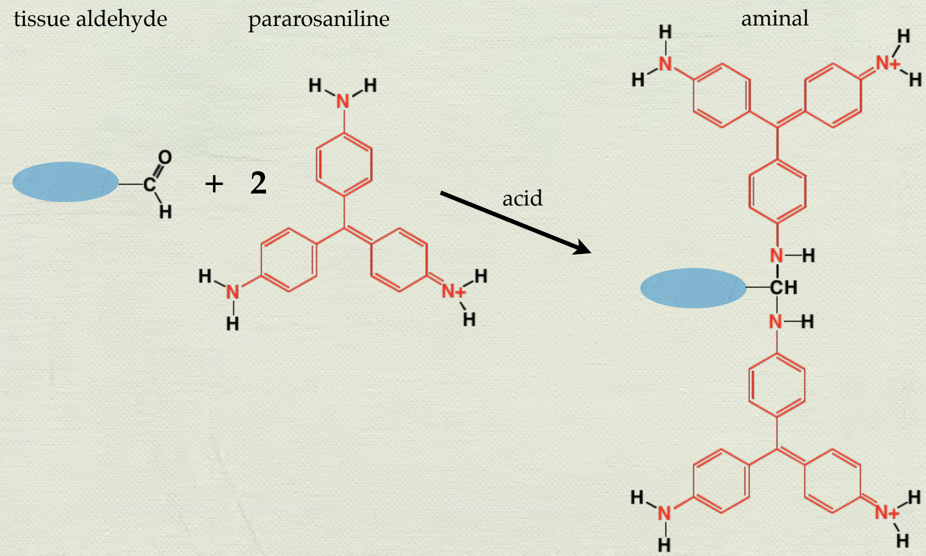
Type V. Aminal reaction

- An amine bearing dye or dye precursor
  - used in highly acidic solution without sulfurous acid
  - reacts with an aldehyde to form a complex (as in Schiff's finding in 1865)
  - Example: Lillie's diphenamine bases, & acidified alcoholic basic fuchsin (Horobin & Kevill-Davies 1971)

Lillie's diphenamine reaction



Acidic alcoholic basic fuchsin



Summary

Five classes of Schiff reagents

Type	Dye classes	Sulfite to decolorize	Sulfite to assist reaction with aldehyde	Acid to assist reaction with aldehyde
I	triphenyl methanes	attaches & decolorizes	yes	yes
II	acridines	attaches but does not decolorize	yes	yes
III	azines, thiazines, oxazines, indamines	decolorizes by reduction	yes	yes
IV	azo	does not attach or decolorize	yes	yes
IV	aminals	no: not present	no: not present	yes

I guess we can say...

...there is no end to Schiff's rainbows