# Chapter 17 | "Special Stains" – Influence of Dye Chemistry on Staining

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## What These Words Mean

Special stains are "non-routine" colorants of biological samples applied prior to microscopic examination (i.e., not hematoxylin & eosin staining). This usage of special versus routine works for histology, but is not so clear-cut when routine stains of other diagnostic specialties (e.g., the cytological Papanicolaou stain, or the hematological Wright-Giemsa stain, or the microbiological Gram stain) are used on histological preparations. Nevertheless, since stains such as Sudan black, the Periodic acid-Schiff procedure, or a Ziehl-Neelsen acid-fast stain are so widely termed "special stains" I retain this convention. Occasionally people refer to "usual special stains" to distinguish such methods from immunostaining or in situ hybridization. Here, the phrase "dye chemistry" has the restricted meaning of those physicochemical properties of dyes key to staining. Reactivity, of various types, is briefly considered in a later section on "complications".

"Staining mechanisms" imply accounts of molecular processes involved in selective uptake of dyes into biological specimens during biological staining. Here we restrict discussion to explicating the role in some mechanisms of key physicochemical dye properties. Only simple mechanistic accounts are provided; and all examples illustrate more common special stains. For more substantive accounts of these, often complex, processes, see Dr. John A. Kiernan's paper elsewhere in this Guide, which focuses on carbohydrate histochemistry; or recent reviews by Dapson (2005) and Prentø (2009); or, for thumbnail mechanistic sketches of most types of special stains, Horobin & Bancroft (1998).

## Key Dye Chemistry Factors – Definitions, Examples, Parameterization

Physicochemical factors influencing selective cell and tissue uptake of the special stains are electric charge; size, both overall and that of the conjugated/aromatic system; and the hydro- or lipophilicity. Traditionally some idea of these factors is gained from structural formulae. For instance Figures 1 and 2 provide formulae of dyes present in well known special stains. The colored species illustrated are, respectively, negatively charged (anionic or "acid" dyes) and positively charged (cationic or "basic" dyes). Figure 3 illustrates a non-ionic dye and a metal complex ("mordant") dye.

A casual glance at such formulae does indicate dye size, and more careful inspection reveals their electric charge. However, assessment of the conjugated system size from an inspection of the structural diagrams is not obvious for non-chemists; and even if color-coding is used (see Fig. 1) "overall" hydrophilic or lipophilic character is hard to assess by anyone merely by eyeballing structures. Since structural formulae are limited in what they show us directly, how can we gain such information?

One approach is to use numerical structure parameters. Electric charge (abbreviated as Z) can be directly defined numerically. Other properties may be modeled – overall size by the relative molecular mass (or "molecular weight", or MW), size of the aromatic/conjugated system by the conjugated bond number (CBN), and hydro- or lipophilicity by using the logarithm of the octanol-water partition coefficient (log P). Table 1 gives structure parameters for the dyes shown in Figures 1-3. For more information on these parameters, and their derivation, see Horobin (2004); various alternative structure parameters are discussed by Dapson (2005).

How are these numbers useful to us? First, they let us readily compare dyes, in terms of size or lipophilicity and so on, and indeed compare dyes on the basis of multiple features. We see from Table 1 that azure B is little more than half the size of the orcein component, which in turn is less than half the size of alcian blue: significant differences. But we also see that whilst alcian blue is extremely hydrophilic, the orcein component is lipophilic. And these are not merely curiosities, they impact on mechanisms and practical usage, as seen in Table 1.

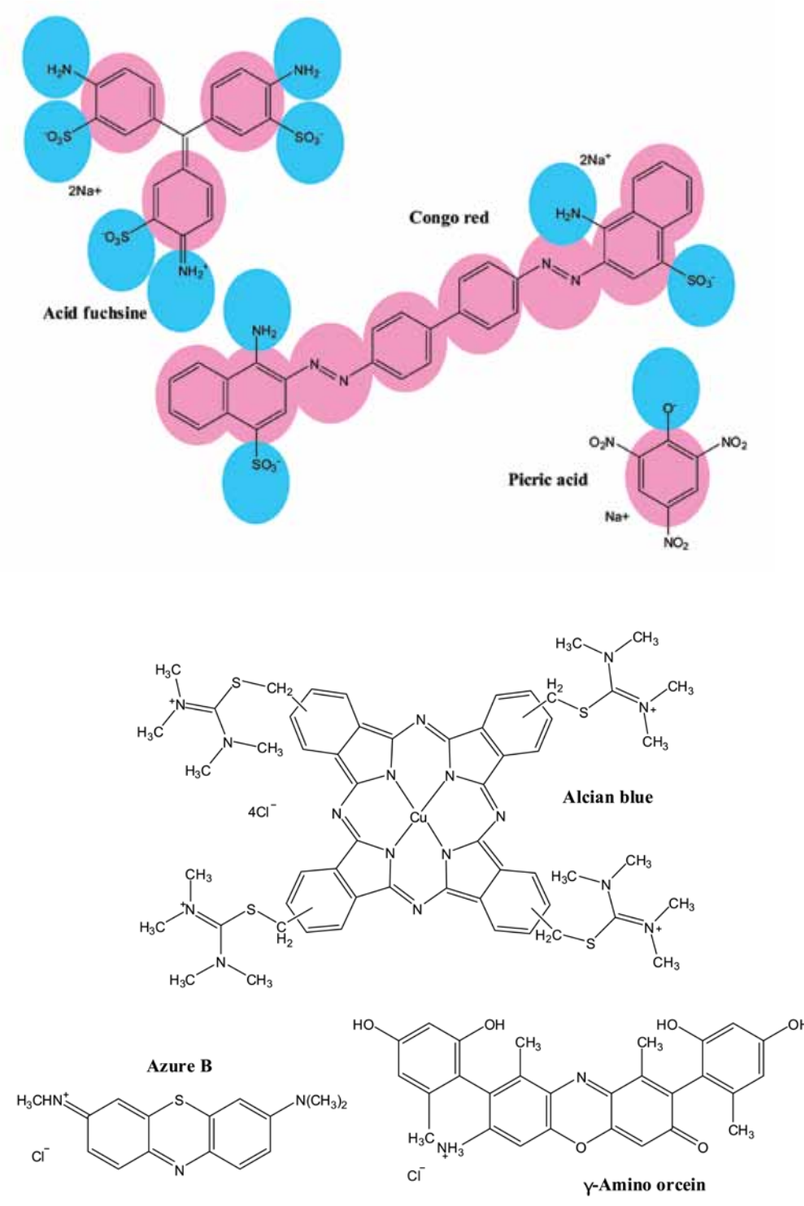


**Figure 1.** Structures of exemplar anionic (“acid”) dyes used in special stains. Blue blobs indicate the more hydrophilic structural fragments, pink blobs the more lipophilic. Counterions are shown as nominal sodium ions.

**Figure 2.** Structures of exemplar cationic (“basic”) dyes used in special stains. Counterions are shown as nominal chloride ions.





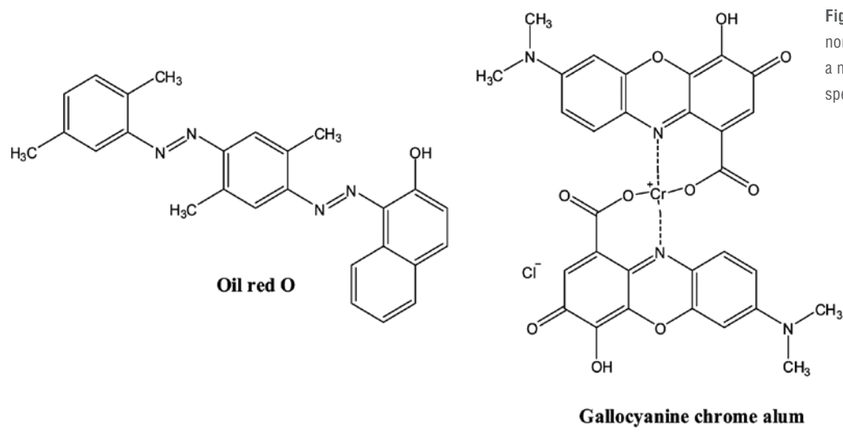


**Figure 3.** Structures of Oil Red O, a non-ionic, and gallocyanine chrome alum, a metal-complex (“mordant”) dye, used in special stains.

**Table 1.** Structure parameters specifying or modeling key dye chemistry properties. See text for abbreviations.

| Dye Name | Z | CBN | MW | log P |
|---|---|---|---|---|
| Acid fuchsine | 2- | 24 | 540 | -11.5 |
| Alcian blue 8G | 4+ | 48 | 1157 | -9.7 |
| Azure B | 1+ | 18 | 269 | -0.7 |
| Congo red | 2- | 43 | 651 | -1.5 |
| Gallocyanin chrome alum | 1+ | 44 | 618 | -3.9 |
| Oil Red O | 0 | 30 | 409 | 9.4 |
| γ-Amino orcein | 1+ | 36 | 485 | 1.8 |
| Picric acid | 1- | 16 | 229 | -1.9 |

## How Dye Chemistry Impacts on Staining Mechanism – Case Examples

**Example 1 – Acid and basic dyeing.** Most special stains use anionic ("acid") or cationic ("basic") dyes, one at a time or in multicolored combination. This enables selective staining of electrically charged cell and tissue components. Thus anionic biopolymers such as DNA, RNA and glycosaminoglycans are selectively stained by cationic dyes, such as azure B and alcian blue; and cationic polymers such as proteins in acidic dyebaths by anionic dyes, such as acid fuchsine and picric acid. Note that some metal complex ("mordant") dyes, such as gallocyanine chrome alum, are also cationic and also selectively stain anionic biopolymers. However their staining mechanisms are more complex and are not always dominated by electrical effects.

**Example 2 – Staining rates, dye size, and mucin staining.** Large dyes diffuse through the tissue sections or into cell smears much slower than small dyes. This influences selectivity of several special stains using staining protocols in which large dyes only have time to reach the faster staining tissue sites. An example, discussed further by Kiernan elsewhere in this Guide, is the selective staining of glycosaminoglycans by alcian blue. As indicated by Table 1 this dye is much larger than azure B; which latter also stains mucins but in addition stains polyanionic nucleic acids present in the less permeable nuclear chromatin and ribosomes. Other mucin stains, such as alcian yellow and mucicarmine, are also very large cationic species.

**Example 3 – Conjugated system size, the basis of amyloid and elastic stain selectivity.** Most acid (anionic) dyes used in special stains, including acid fuchsine and picric acid, stain proteins most strongly from acid dyebaths, when the targeted biopolymers are cationic. Analogously, basic (cationic) dyes stain proteins from alkaline (i.e. high pH) but not acid (low pH) dyebaths. However some dyes stain certain proteins strongly even from dyebaths of the "wrong" pH; indeed even when the solvent used is largely non-aqueous, which also usually inhibits acid and basic dyeing of proteins. Such unusual coloration patterns include the selective staining of amyloid by dyes such as Congo red, and the selective staining of elastin by dyes such as orcein. As seen in Table 1, these dyes have unusually large conjugated systems, and hence large conjugated bond numbers (CBNs). The atypical dye-protein binding is due to various non-polar attractive forces, which are stronger with the dyes possessing large aromatic (conjugated) systems.

This effect is illustrated in Figure 4, which compares "normal" acid dyes with acid dyes giving selective staining of amyloid and elastin. Analogous, unillustrated, effects arise with basic dyes. Of course amyloid and elastin are themselves unusual proteins. Amyloid forms β–pleated sheets, facilitating close approach by linear high CBN dyes, which give the best staining. Elastin is unusually hydrophobic, with numerous aromatic residues.

**Example 4 – Lipophilicity and staining of lipids.** Since staining of fat and lipid droplets by non-ionic dyes from aqueous-alcoholic solutions is mechanistically understood as partitioning of hydrophobic dyes between "wet" and "dry" environments, the log P parameter should predict which dyes are effective – and, from Figure 5, this is apparent. For currently recommended lipid stains of this type log P > 7; whilst quinoline blue, a dye so used in the nineteenth century, has a log P value of only 2.2; and note that for all fat stains log P > 0.





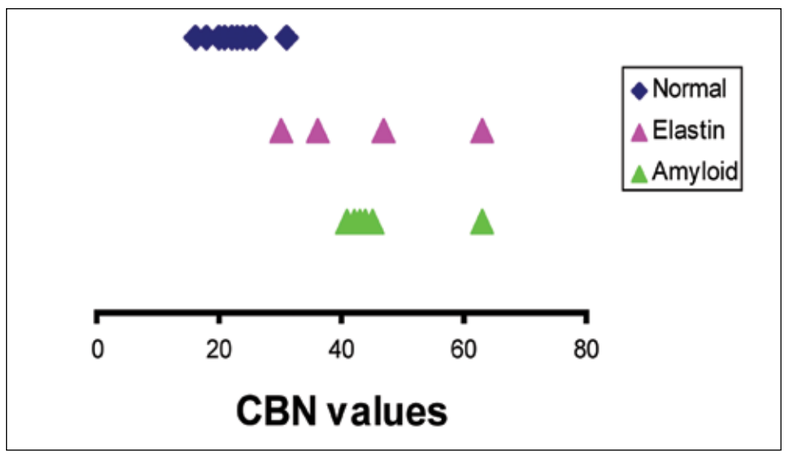


**Figure 4.** Influence of the size of the aromatic/conjugated system (modeled by CBN) of dyes on staining of proteins, in particular elastin and amyloid. Unbiased samples of "normal", elastin and amyloid staining "acid" (anionic) dyes were obtained from Lillie & Fullmer (1976: being the first 10 dyes listed on page 138, and relevant dyes from page 666 and page 707 respectively).

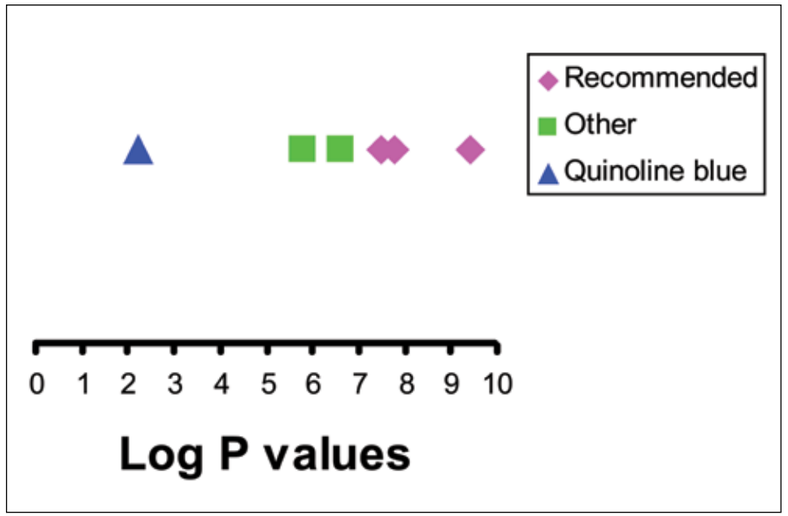


**Figure 5.** Influence of the lipophilicity (modeled by log P) of dyes on staining of fat and lipid droplets. Unbiased sample of fat staining dyes taken from Lillie & Fullmer (1976, p. 565).

## Is That It? Complications and Puzzles

Yes, it is more complicated! One complication is "reactive staining", involving making or breaking of covalent bonds, or significant oxidation-reduction changes, or formation of insoluble salts or complexes. This is not usual for the uptake of dyes – except for "metachromatic" dyeing, and the "Gram" and "Romanowsky" stains. However some special stains do involve reactivity, of various types. Procedures such as the "periodic acid-Schiff" or "Feulgen nucleal" stains are organic chemistry on a slide, with making and breaking of covalencies. Biochemical processes involved in "enzyme histochemistry" also involve changes in covalencies. Formation of Prussian blue in the "Perls'" stain for iron involves polar covalencies. "Metal impregnation" or "silver stains" involve substantial redox changes, and precipitation of metal microcrystals or insoluble metal sulfides. Mechanistic details of phenomena within quotes in this paragraph can be found via the indices of various monographs, e.g., Horobin & Bancroft (1998), Kiernan (2008) and Lyon (1991).

But don't let this appeal to documentation deceive you, because there are still puzzles concerning mechanisms of special stains. Consider the trichromes. In sequence stains such as Masson's, it is typically the larger acid dyes which stain collagen fibers. The experimentally-grounded interpretation, that this is because access of slow diffusing dyes is limited to the most readily penetrated tissue substrate, dates back to Mann (1902). Application of this mechanism to one-bath trichromes, such as that of Gomori, has been argued elegantly by Baker (1958) and, using the structure-parameter approach, by Horobin & Flemming (1988). Nevertheless this is not a universal mechanism, as clearly demonstrated by Prentø (1993) for the widely used picro-Sirius red variant of van Gieson's stain.

## Conclusion

The general principles of the staining mechanisms of most special stains are now understood. Staining selectivity is surprisingly often dominated by a limited number of dye chemical factors – such as electric charge (Z) for acid and basic dyeing; overall molecular size (MW) and charge for mucin stains; size of the aromatic/conjugated system (CBN) for amyloid and elastin stains; and lipophilicity (log P) for fat stains. Nevertheless some complications exist, and some puzzles remain, even for some widely used methods.

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