Chapter 18 | How Do Dyes Impart Color to Different Components of the Tissues?

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The purpose of imparting color (to "stain") certain components of the tissues is to assist making diagnostic judgements. The diversity of these components, the targets of staining, is indicated in Table 1.

You may be surprised that small-molecule reagents such as dyes can impart color differentially to such a variety of targets, so this chapter provides a broad-brush picture of the physical principles underlying this achievement.

Table 1. Some diverse targets of staining.

How Targets are Defined by the Pathologist	Examples of Such Targets	Examples of Dyes Used to Stain Such Targets
By their chemical character		
	Calcium	Alizarin red S
	Carbohydrates	Periodic acid-Schiff stain
	DNA and RNA	Feulgen nucleal stain, methyl green-pyronin
	Glycosaminoglycans (GAGs)	Alcian blue, colloidal iron
	Hemosiderin	Perls' stain
	Lipids	Oil red O, Sudan black
By their biological character		
Tissue components	Smooth muscle and collagenous or elastic fibers	Gomori's and Masson's trichromes, Verhoeff's stain
	Cartilage matrix	Alcian blue
Whole cells	Mast cells	Toluidine blue metachromasia
	Mucus goblet cells	Alcian blue
Intracellular entities	Cytoplasms	Eosin, light green
	Myelin sheaths	Luxol fast blue
	Nuclei	Al hematoxylin, nuclear fast red Al complex
	Nissl substance (RER)	Cresyl violet
By their pathological character		
	Amyloid	Congo red
	Microorganisms	Carbol fuchsine, Giemsa and Gram stains
	Viral inclusion bodies	Feulgen nucleal stain

Background Concepts

To answer the question of the chapter title, two more specific questions must be addressed. Firstly, how do any components become colored? Secondly, how do certain components (targets) become colored whilst the background remains unstained? Physicochemically this is equivalent to the questions: what are the sources of affinity and selectivity? Given the variety of detectable targets, the variety of sources of affinity and selectivity needed to explain the actions of most special stains is surprisingly small. It is in fact the multiplicity of possible combinations of chemical and physical features of dyes, dye solutions and tissue components that provide the bases of selective staining. A third term which can be clarified here is sensitivity: a highly sensitive stain is one which demonstrates small amounts of a tissue component.

A few complications will be mentioned before proceeding. Firstly, a classical thermodynamic property such as entropy applies rigorously only to systems at equilibrium. Fortunately we are not using such properties quantitatively, so this is not a problem. Secondly, when considering intermolecular effects, dye uptake is often thought of as involving some type of “bonding”, of strong and directed attractions. This is sometimes so, as with covalent bonds. However other intermolecular attractions, such as van der Waals forces, are neither strong nor directed. Finally, note that case examples are presented in summary form. Readers seeking more complete accounts should look elsewhere, eg Horobin (1982), Lyon (1991), Horobin & Bancroft (1998) and Prentø (2009).

How Do Any Components Become Colored?

In physicochemical terms, staining occurs because a dye has an affinity for the tissue components. Affinity is itself dependent both on increasing entropy, with the overall system becoming more disordered, and on decreasing enthalpy, involving such phenomena as dye-tissue attractive forces. Dyes move from their solutions (“dyebaths”) into the unstained tissues and cells because they are moving from regions of high dye concentration to regions containing less dye. Thermodynamically, entry of dye into tissue sections or cell smears is always driven in part by an increase in system entropy, disorder increasing as dye distributes between dyebath and tissue components.

This partial explanation does not explain why dyes can reach higher concentrations in the tissues than in the dyebaths. Such dye accumulation requires additional sources of affinity. Some of these factors are sketched below, starting with those which involve useful decreases in enthalpy.

The most general are the non-directed, short-range intermolecular attractions deriving from weak electronic coupling, e.g., dipole-dipole and dispersion forces. These weak forces are generically termed **van der Waals attractions**. Although occurring with all dyes, they are most significant with dyes having large conjugated systems, such as Congo red shown in Figure 1. Van der Waals attractions can contribute to affinity increases in an intuitively obvious way, by providing dye–tissue attractions. Less intuitively obvious are increases in affinity due to induction of dye–dye binding at certain sites, e.g., metachromatic staining of mast cell granules occurring with basic dyes such as toluidine blue, which dye is shown in Figure 2 .

Non-directed, longer-range attractions are the strong **electrical forces** arising between ions. These can contribute to affinity when dye and tissue component have charges of opposite sign. For instance tissue components containing glycosaminoglycans (GAGs) can stain metachromatically with basic (cationic) dyes such as toluidine blue. However affinity of routine acid and basic dyeing — involving ionic tissue components becoming preferentially stained by dyes with the opposite charges — is not primarily driven by the electrical effects. Rather, such processes are entropy driven (see above) with the electrical effects controlling selectivity (see below Figure 3). Electrical forces are procedurally adjusted by altering electrolyte content or pH of dyebaths.

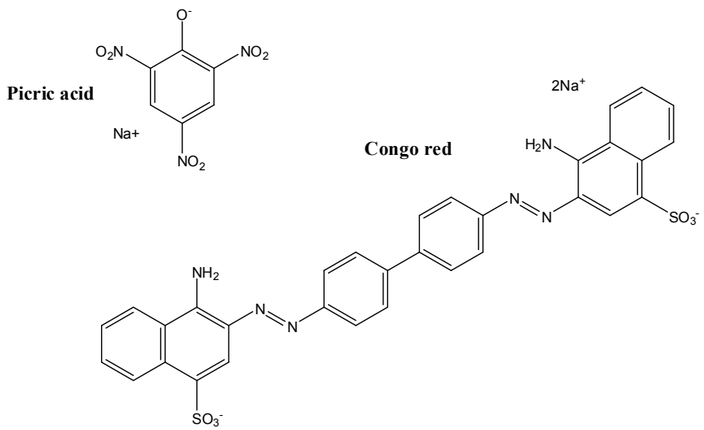

Figure 1. Acid dyes, small and large.

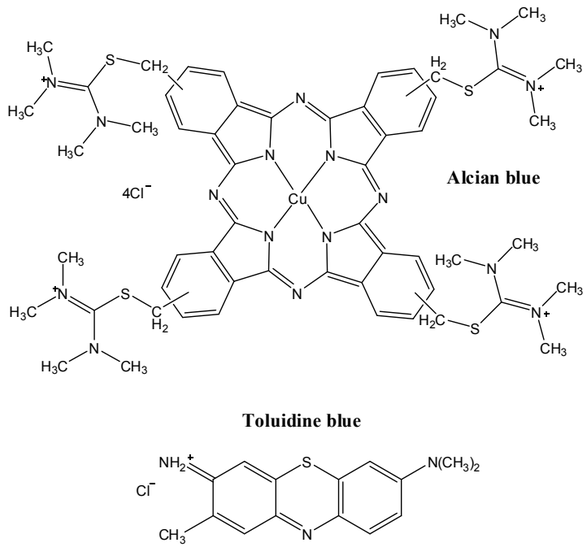

Figure 2. Basic dyes, large and small.

Strong directed attractions arise when dyes interact with tissue components to form **covalent bonds**, e.g., between Schiff reagent and tissue aldehydes; or **polar covalent** bonds, e.g., between alizarin red S and tissue calcium ions. **Hydrogen bonds** are weaker directed bonds, occurring when dye and tissue component carry suitably oriented hydrogen donors and acceptors, as in staining of glycogen with carminic acid.

An additional source of affinity, due to increase in entropy of the aqueous solvent, arises during **hydrophobic bonding**. This occurs when both dyes and tissue biopolymers have substantial hydrophobic domains, and when the solvent is wholly or largely aqueous (Horobin and Bennion 1973).

How Are Targets Colored, Whilst Backgrounds Are Not?

If a tissue component has a high affinity for a dye whilst its surroundings do not, selective staining may occur. An example is staining of elastin — a protein carrying numerous unusual aromatic aminoacid residues — by dyes with large conjugated systems, such as Congo red, which bind by strong van der Waals attractions. Another example is the staining of metal ions such as calcium or copper by reagents which react to form polar covalent bonds with the metal ions, e.g., alizarin red S (discussed below) and p-dimethylaminobenzylinenerhodanine respectively.

However there are additional and very different sources of selectivity. Widely discussed are various **rate of staining effects**. For instance, even in the absence of affinity differences, if a tissue component takes up dye more rapidly than does its surroundings, and if staining is terminated before equilibrium is reached, then the component will become selectively colored. This is **progressive staining**, and an example is the selective coloration of goblet cell mucin by a large dye such as alcian blue. Conversely, both a tissue component target and its surroundings may be stained. Then exposure to a dye solvent (differentiator) results in selective staining if the target loses dye more slowly than its surroundings, and if destaining is terminated before too much dye has been lost. This is **regressive staining**, and an example is the staining of mycobacteria with carbol fuchsine.

Such effects depend on differing rates of dye diffusion through different tissue components. This is predictable, since tissue components can be ranked in terms of their permeabilities, see Table 2. Rates of staining may be accelerated by various procedural manoeuvres such as increasing dye concentration or temperature, adding swelling agents such as phenols, or using extremes of pH in staining solutions. For more detail see Horobin (1982, pages 49 ff and 97 ff).

Table 2. Permeabilities of some common tissue components.

Type of Component	
Acidophilic components	
Collagen fibers	Most permeable
Smooth muscle and other cytoplasms	↕
Red blood cells	Least permeable
Basophilic components	
Goblet cell mucin	Most permeable
Chromatin	↕
Nissl substance	
Elastic fibres	Least permeable

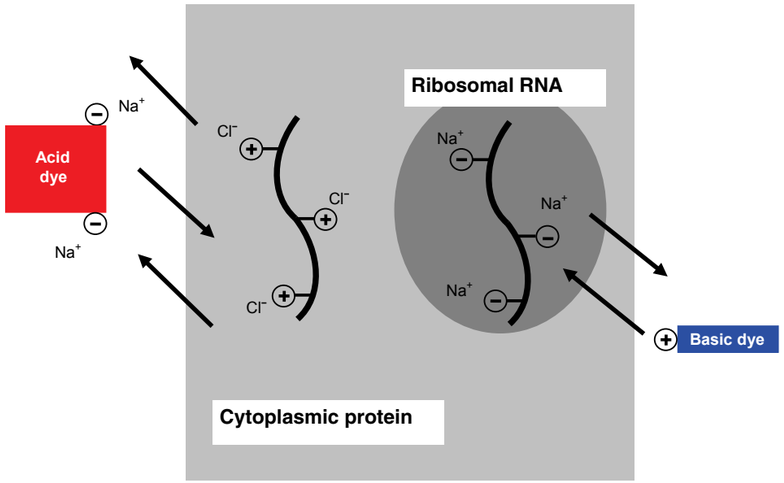

Figure 3. The ion-exchange selectivity mechanism of acid and basic dyeing. The phosphate groups of the RNA and the protonated amines of the protein are represented by their charge signs. The mobile ions are shown as nominal chloride and sodium ions, although the actual ionic species present will depend on the composition of the staining solution.

Case Examples Illustrating the Bases of Affinity and Selectivity

As entropy effects contribute to affinity in all staining procedures, they are not always mentioned. Another common effect which does call for comment is that selective staining often depends both on factors favoring staining of the target **and** on factors favoring the non-staining of the background.

Acid Dyes Giving General Oversight Staining of Cells and Tissues

Such dyes are anionic, vary from small (picric acid, see Figure 1 for structure) to moderate (e.g, light green) in size; and also vary from very hydrophilic (acid fuchsine) to weakly lipophilic (eosin Y). The generalised background staining of tissue resulting from binding of such dyes to proteins can be conceptualized as follows.

Sources of affinity — Dye–protein van der Waals attractions contribute significantly, and in aqueous solutions hydrophobic bonding also occurs.

Sources of selectivity — Such dyes bind to most proteins, and the near-universal distribution of these biopolymers results in non-selective background coloration. Staining is typically carried out from acidic dyebaths, in which proteins are protonated and carry overall positive charges. Acid dye anions exchange with mobile tissue anions associated with the protein polycations, a process termed “acid dyeing”. This ion-exchange is entropy driven, as it increases the randomness of the system, and is illustrated in Figure 3.

A Small Basic Dye Giving Metachromatic Staining of Mast Cell Granules — Toluidine Blue (TB)

TB is a small, weakly hydrophilic cationic dye. Attached to DNA or RNA, in chromatin or Nissl substance, this dye has a blue color. Attached to GAGs, in mast cell granules or cartilage matrix, TB displays a purple "metachromatic" color. The dye's structure is shown in Figure 2.

Sources of affinity — Van der Waals attractions between TB and polyanions contribute to affinity when binding to DNA and RNA, as does hydrophobic bonding. However flexible, high charge density GAGs permit charge neutralization of dye aggregates or "stacks" formed due to dye–dye van der Waals attractions and hydrophobic bonding.

Sources of selectivity for mast cell granules —TB is typically applied from weakly acidic aqueous solutions. DNA, RNA and GAGs are then polyanionic, whilst most proteins are protonated and so polycationic. Thus basic dye cations exchange with mobile tissue cations associated with the various polyanions, a process termed "basic dyeing". This ion-exchange is entropy driven as it increases the randomness of the system. Since polycationic proteins are not associated with mobile cations, ion exchange cannot occur, minimising background staining. For a cartoon illustrating basic dyeing see Figure 3.

TB does however distinguish GAGs from other polyanions. TB stacks, only occurring with GAGs, are of a different "metachromatic" color to the monomeric dye present in nuclei and Nissl substance.

A Large Acid Dye which Stains Amyloid Selectively — Congo Red (CR)

CR is a large, hydrophilic anionic dye with a linear molecule, see Figure 1a.

Source of affinity — The large conjugated system of CR results in substantial dye-tissue van der Waals attractions.

Sources of selectivity for amyloid —The staining solution (and/or pre-staining wash and post-staining differentiator, dependent on variant) is typically of high pH. Consequently most tissue proteins are anionic, and acid dyeing of background proteins does not occur since proteins contain no mobile exchangeable anions. In some variants, acid dyeing is also inhibited by the presence of large amounts of sodium chloride, and hydrophobic bonding inhibited by use of a non-aqueous solvent. Proteins stain under these conditions only if: they are cationic at high pH (the basic proteins of eosinophil granules); they contain unusual aromatic aminoacid residues (elastin); or have linear binding sites matching the linear structure of CR so facilitating van der Waals attractions, as do the β-pleated sheet proteins of amyloids.

A Large Basic Dye Giving Selective Staining of GAGs — Alcian Blue (AB)

AB is a large, hydrophilic cationic dye; see Figure 2 for the structural formula.

Source of affinity — The large conjugated system of AB results in a significant contribution from dye–tissue van der Waals attractions.

Source of selectivity for GAGs — AB is applied from acidic aqueous solutions. Various tissue polyanions are present — DNA, RNA and GAGs — whose associated mobile cations could exchange with AB in a basic dyeing process. However AB stains GAGs very much faster than other polyanions, as tissue components containing GAGs are more permeable than chromatin or Nissl substance, see Table 2. Background staining of proteins is minimal as these biopolymers are protonated, and hence have no mobile exchangeable cations.

A Reactive Dye Selective for Calcium Deposits — Alizarin Red S (ARS)

ARS is a small, hydrophilic anionic dye which can chelate a variety of metal ions; see Figure 4 for the structural formula.

Source of affinity — This is usually regarded as the formation of dye–metal ion polar covalent bonds.

Sources of selectivity for calcium deposits — Background acid dyeing of proteins is reduced by the small conjugated system size and hydrophilic character of ARS minimising van der Waals attractions and hydrophobic bonding. In the pH 6.5 variant, acid dyeing by anionic ARS is further reduced, since tissue proteins then have reduced cationic character and so contain few mobile exchangeable anions.

Sensitivity for calcium deposits — Increasing pHs and staining times increases staining intensity, since these maximise the number of dye phenolate groups and accommodate the slow staining reaction rate respectively.

A Non-ionic Dye Selective for Lipids — Sudan Black B (SBB)

SBB is a non-ionic, hydrophobic dye of moderate size; see Figure 4 for the structural formula.

Source of affinity — Staining is primarily entropy driven. In some variants affinity is enhanced by manoeuvres such as increasing the proportion of water in the aqueous-alcoholic solvent, or using supersaturated SBB solutions.

Source of selectivity for lipid — The non-lipid components of tissues, being largely comprised of polysaccharides, proteins and nucleic acids, are significantly hydrated. Consequently entry of SBB would be entropically unfavorable — it would result in increased water structure — so accumulation does not occur. Unbound background is readily removed by post-staining solvent washes.

Sensitivity for lipids — Triglyceride deposits near their melting point are fluid and permeable, and stain readily; whereas crystalline deposits such as cholesterol remain unstained in routine procedures.

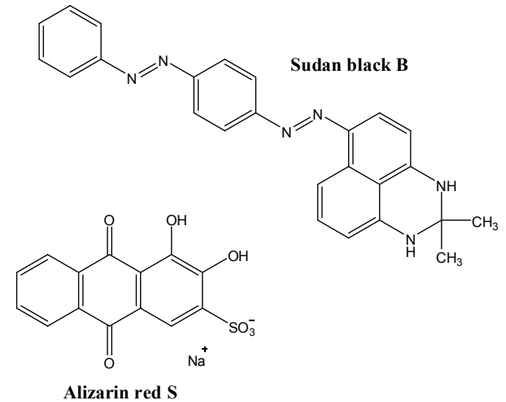

Figure 4. A non-ionic dye (Sudan black B) and a "reactive" dye (alizarin red S).

A Tailpiece: Hematoxylin & Eosin (H & E)

A special case, though not a special stain. H & E is so widely used that no account of staining mechanisms can fail to mention it — the more so as H & E still puzzles us in various ways.

What is H & E? — The easy bit is to say what "eosin" is. Eosin Y is a normal acid (anionic) dye of moderate size and well studied chemistry. "Hematoxylin" however is a misnomer, hematoxylin being a colorless natural product whose oxidation can produce hematein. And "hematoxylin" in H & E is the product of the reaction of hematein with aluminium ions, so is better termed Al-hematein. But to ask "what is this?" is to encounter puzzle number one. The nature of the Al-hematein complex or complexes in staining solutions remains uncertain. Both cationic and anionic complexes could be present; for experimental data and summary of prior work see Bettinger & Zimmermann (1991) and Puchtler et al (1986). Moreover the Al-hematein complex present in tissue sections following "blueing" is probably different again, perhaps a poorly soluble olation polymer (Puchtler et al 1986).

How does H & E work? — Again starting with the easy bit: eosin stains sections by acid dyeing. The Al-hematein staining mechanism however is puzzle number two. Its staining behavior differs from nuclear and Nissl staining basic dyes such as cresyl violet or toluidine blue. Extraction of DNA from cell nuclei inhibits nuclear staining by such basic dyes, but does not eliminate Al-hematein staining. Routine basic dyes readily wash out of sections if over-enthusiastically dehydrated by alcohol, but "hematoxylin" does not. Nuclear staining by hemalum after extraction of DNA may be attributable to histones, the strongly basic proteins of eukaryotic nuclei. It has been argued (but not proved) that hemalum solutions contain anionic dye-metal complexes that would be attracted to the protonated lysine and arginine side-chains of histones. It has also been suggested that Al-hematein is a large hydrophilic basic dye, with resistance to alcohol extraction due to insolubility in that solvent; an alternative speculation notes that olation polymers are typically insoluble.

Conclusions — "H" remains a puzzle in some respects, although "E" is easy. Turning speculations into knowledge will require renewed chemical investigation of the actual Al-hematein complexes present in the dyebaths, and of the actual nature of "blued hematoxylin".

Further Reading

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.

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Lyon H (1991) *Theory and strategy in histochemistry.* Springer-Verlag, Berlin.

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Puchtler H, Meloan SN & Waldrop FS (1986) Application of current chemical concepts to metal-hematein and –brazilein stains. *Histochemistry* 85: 353-364.