

Light colour and different dyestuffs

Dyes and bonding

The components involved in histological staining are dyes and proteins.

The fundamental process involved is the chemical bonding between the carboxyl groups of one and the amino groups of the other.

The commonest bonds involved are ionic bonds, although there are exceptions

What dyes are

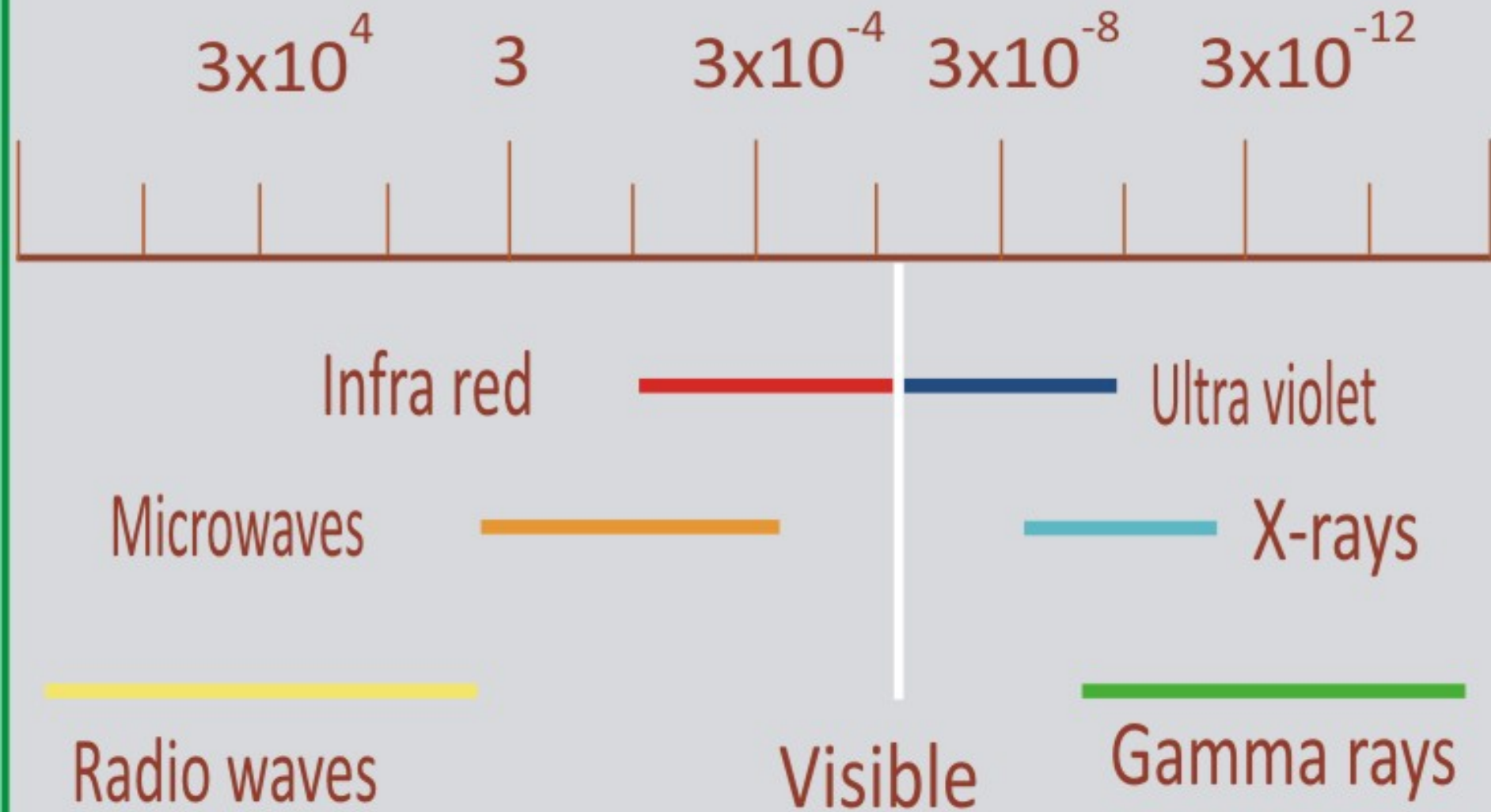
Dyes are coloured, ionising, aromatic organic compounds.

- It must be appreciated that they are individual chemicals, and like all chemicals, they are similar in their reactions to some other chemicals, and distinctly different from others.
- It may seem that this is a statement of the obvious, but we sometimes appear to view dyes as something other than ordinary chemicals, and I want to stress that the same rules that apply to sodium chloride, acetic acid, benzidine and a host of others also apply to dyes.

What is a colour

- Dyes are aromatic organic compounds, and as such are based fundamentally on the structure of benzene. To us, benzene appears to be a colourless fluid. In fact it absorbs electromagnetic radiation just as dyes do, but it does so at about 200 nm so that we do not see it.
- The perception of colour is an ability of some animals, including humans, to detect some wavelengths of electromagnetic radiation (light) differently from other wavelengths. Normal daylight, or white light, is a mixture of all the wavelengths to which we can respond and some to which we cannot, in particular the infra-red and ultra-violet rays. We respond to wavelengths between about 400-700 nm. When an object absorbs some of the radiation from within that range we see the waves that are left over, and the object appears coloured. In reality this range we see makes up only a very small fraction of the electromagnetic spectrum.

Electromagnetic Radiation



How is color described?

- In scientific terms there is nothing special about the wavelengths in the visible range, other than being the major components of sunlight which are not removed by the earth's atmosphere. Their special importance is based exclusively on the ability of human retinas to respond to them, and to discriminate between them to a significant degree. These discriminations are what we call colour.
- Wavelengths just outside the visible range are considered colourless, even though there is no substantive difference between them and the limiting wavelengths inside the range. Some animals (bees, for example) can see these other wavelengths but, because humans do not, we consider them colourless. The point is that colour is a subjective phenomenon, and thinking of colour as something objective is misleading. For that reason, we should refer to the wavelengths involved rather than describe the human response to them.

Why dyes are coloured

- Colour in dyes is invariably explained as a consequence of the presence of a **Chromophore**. Since, by definition, dyes are aromatic compounds their structure includes aryl rings which have delocalised electron systems.
- These are responsible for the absorption of electromagnetic radiation of varying wavelengths, depending on the energy of the electron clouds.
- For this reason, chromophores do not make dyes coloured in the sense that they confer on them the ability to absorb radiation. Rather, chromophores function by altering the energy in the delocalised electron cloud of the dye, and this alteration results in the compound absorbing radiation from within the visible range instead of outside it.
- Our eyes detect that absorption, and respond to the lack of a complete range of wavelengths by seeing colour.

Visible range

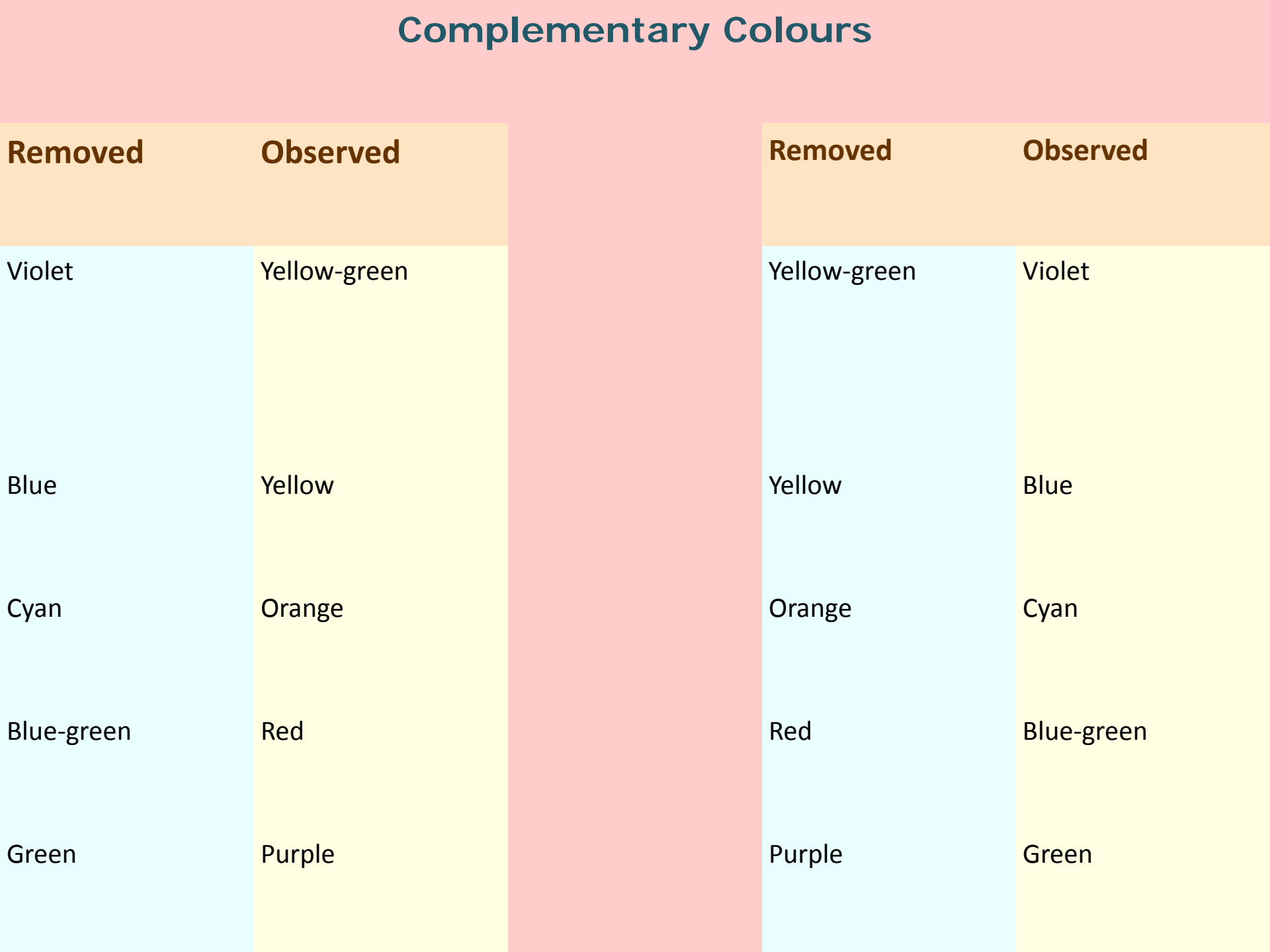
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- When some of the wavelengths found in white light are absorbed, then we see what is left over as coloured light. The colour that we see is referred to as the *complementary colour* of the colour that was removed. For instance, if the red rays are removed from white light, the colour we detect is blue-green. Blue-green is complementary to red, and red is complementary to blue-green.

Chromophore

Chromophores are atomic configurations which can alter the energy in delocalised systems. They are composed of atoms joined in a sequence composed of alternating single and double bonds. Double bonds in organic compounds can be of two types. If the atoms with double bonds are not adjacent, they are termed ***isolated*** double bonds, and exist independently of other double bonds in the same molecule. If adjacent atoms have double bonds they are termed ***conjugated*** double bonds and the bonds interact with each other. Chromophore configurations often exist as multiple units, having conjugated double bonds, and are more effective when they do so. This is due to the interaction between the double bonds, which causes partial delocalisation of the electrons involved in the bonds. In this case, although specific atoms are involved in the bonds, the electrons are distributed over a larger area than the specific atoms and also involve adjacent atoms that have double bonds.

Conjugated systems

- The point of this is that conjugated systems have partially delocalised electrons, and the energy in these delocalised electrons can impact on the energy of the delocalised electrons of the parent aromatic compound by extending the number of electrons involved in the system and the energy needed to keep the whole system in place.
- Conjugated double bonds
- Another common chromophore is the nitro group. This chromophore is a nitrogen with two oxygen atoms attached. One oxygen is shown attached with a single bond, the other with a double bond. In fact, like the carbon atoms in benzene, these two oxygen atoms are attached to the nitrogen with bonds of equal strength. The extra electrons are delocalised between the three atoms.



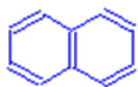
Complementary Colours

Removed	Observed	Removed	Observed
Violet	Yellow-green	Yellow-green	Violet
Blue	Yellow	Yellow	Blue
Cyan	Orange	Orange	Cyan
Blue-green	Red	Red	Blue-green
Green	Purple	Purple	Green

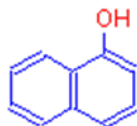
Auxochromes

- Auxochromes are groups which attach to non ionising compounds yet retain their ability to ionise. While this definition is largely correct, it is also inadequate. This is because it restricts the definition of the auxochrome to ionisation, and does not comment on the effect of auxochromes on the absorbance of the resulting compound.
- The word **auxochrome** is derived from two roots. The prefix **auxo** is from **auxein**, and means **increased**. The second part, **chrome** means **colour**, so the basic meaning of the word auxochrome is **colour increaser**. This word was coined because it was noted originally that the addition of ionising groups resulted in a deepening and intensifying of the colour of compounds.

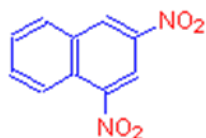
Colour enhancing by an auxochrome



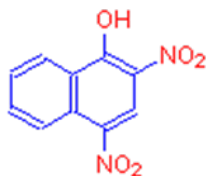
To the left is **naphthalene**, a colourless compound.



The addition of a single hydroxyl group to naphthalene produces **1-naphthol** which is also a colourless compound, but one which can ionise.



If instead of a hydroxyl group we add the nitro group, which is a chromophore, we get the compound **2,4-dinitronaphthalene**. The addition of this chromophore has caused it to become pale yellow.



If instead of a hydroxyl **or** nitro groups, **both** a hydroxy**and** nitro groups are added, we get the deep yellow dye, **martius yellow**.

The addition of **both** an auxochrome **and** a chromophore results in a much stronger alteration of the absorption maximum of the compound. The hydroxyl group must have deepened the colour, showing that auxochromes are also chromophores.

Trinitrobenzene

Trinitrobenzene, although coloured, is still not a dye, as it will not bind to tissues. Treating the section with trinitrobenzene will temporarily colour it yellow in the same way that a plastic sponge appears coloured when it is soaked in a coloured liquid but the colour will wash out as soon as the tissue is rinsed in a solvent.

To turn a coloured compound into a dye requires the addition of an ionizable group that will allow binding to the tissues. Such binding groups are called auxochromes.

The addition of an ionizable OH group turns trinitrobenzene into the dye trinitrophenol, which is more commonly called picric acid in histology. Picric acid is an acid dye (the OH group is phenolic and ionizes by losing a hydrogen ion) and is very useful

Colour

- The colour of the dye is caused by the absorbance of electromagnetic radiation. We have constantly referred to the **wavelength that is absorbed** in the singular, but a simple scan of a dye solution with a spectrophotometer shows that dyes do not remove a single wavelength. Rather they absorb radiation on either side of the wavelength most completely removed (the absorption maximum). Plotting the wavelength absorbed against the degree of absorbance usually results in a display resembling a bell curve. If any part of this curve is in the visible range, the dye will appear coloured.
- White light is a mixture of wavelengths. Some of these have a relationship to the energy in the delocalised electron cloud of the dye molecule. By the process of resonance, previously described, the electron cloud will respond to the energy contained in that radiation by absorbing it, and removing it from the spectrum. As a consequence the white light will cease to be white and will display the colours of the wavelengths left over. The transmitted light will have the complementary colour to the wavelengths removed.

Structure and colour depend

- The explanation of the relationship between structure and colour depends on the basic atomic structure of the aryl ring, and the shared or delocalised electrons that this atomic arrangement has. The ability to absorb radiation is inherent in this structure. The effect of other atomic configurations is to modify the energy contained in the delocalised electron cloud so that the compound absorbs electromagnetic radiation at a wavelength in the visible range. Some also ionise, enabling the compound to chemically react with ionising tissue groups.
- Colour, fading, fluorescence and phosphorescence are all seen to be different effects of the same fundamental process.

Effect of light

- When light illuminates a dye, some of it is absorbed as energy. Since energy is not destroyed, something must then happen. We could use an analogy of heating water - the water's temperature rises, molecular vibration increases. However, we do not see anything else, as the water just sits there being water.
- The same can happen with dyes, we may not observe anything particular as the effect may be at the atomic level. There are several possibilities, however.
- The energy level in the electrons in an unaffected dye is called the ground state. When electromagnetic radiation, as light energy, is absorbed the electrons become more energised.
- With most dyes, there is then a gradual decay and the electrons return to the ground state. We do not see anything. Nevertheless, something may happen that we do not see. Possibly there is an increase in temperature, or some chemical changes occur which disrupt the dye's structure and cause it to lose colour - *fading*.

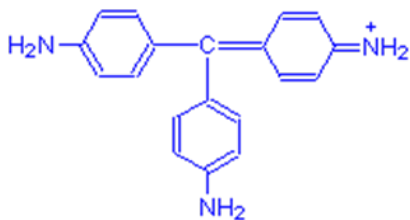
Effect of light

- Another possibility is that the return to the ground state is not gradual, but sudden. If this is accompanied by emission of any residual energy in the form of light, we observe the dye glowing - ***fluorescence***. Since the emitted light must always contain less energy than the absorbed light, as some was used to energise the electrons, the emitted radiation is always at longer wavelengths than the absorbed radiation. By manipulating the light available, we can cause ultra-violet light to be absorbed and visible light to be emitted.
- A third possibility is that the electrons stabilise in their newly energised state. After a passage of time they then return to the ground state. If this happens gradually, we may observe nothing, with the same possibilities regarding fading and temperature increase as before.
- If return to the ground state happens suddenly, and the residual energy is emitted as light, we once again see the dye glowing - ***phosphorescence***. As with fluorescence, the light emitted is always a longer wavelength than the light absorbed, but the disparity is greater with phosphorescence due to the greater energy consumed in keeping the electrons in the excited state.
- Note that the difference between fluorescence and phosphorescence is in whether the electrons stabilise in the excited state before returning to the ground state. With any stability, no matter how long (or short), it is considered phosphorescence.

Color Modifiers

Colour modifiers such as methyl or ethyl groups alter the colour of dyes by altering the energy in the delocalised electrons. By themselves they cannot do this enough to cause absorption in the visible range, but they can affect the shade significantly when absorption is already in that range. Adding more of a particular modifier results in a progressive alteration of colour. Compounds that differ from each other in this kind of regular fashion are called ***homologues***. A very good example is seen with the Methyl violet series.

Alteration of colour by modifiers



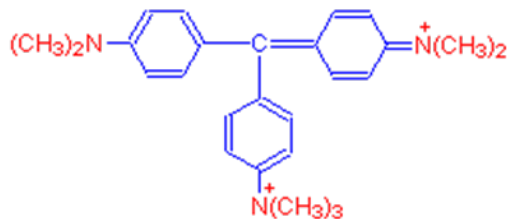
Without any methyl groups the parent dye is called **pararosanilin** and is red.



When four methyl groups are added we get the reddish purple dye **methyl violet**.



As more methyl groups are added we get the purple blue dye **crystal violet** which has six such groups.

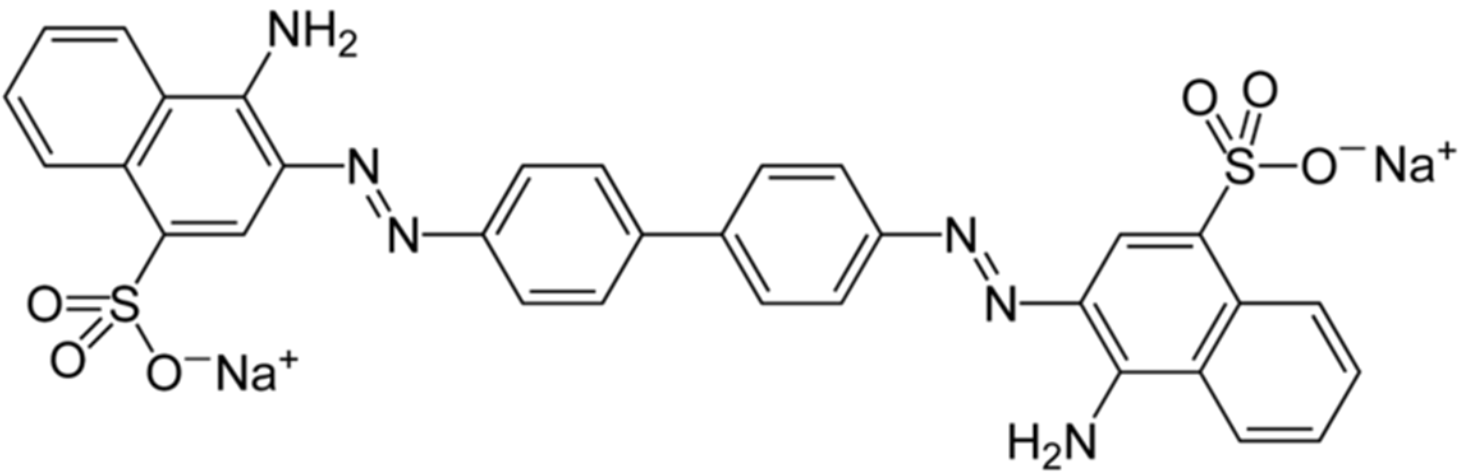


If a seventh methyl group is added, the resulting dye is **methyl green**.

Resonance

- The process by which electrons are stimulated by radiation is ***resonance***. It should be made clear at the outset that resonance is not the same as vibration.
- Resonance is the induction of a response in one energy system from another energy system in close proximity, which is operating at the same energy level (frequency). In aromatic organic compounds, including dyes, the two energy systems are ***electromagnetic radiation***, and the ***delocalised electron cloud***. Do not confuse resonance with the imaginary *resonance hybrids* used in an older explanation for the structure of benzene.

Dyes are produced mainly for industrial uses such as textile dyeing, so a wide variety of different dyes have been synthesized to give a large range of colours. Dye manufacturers usually give the dyes they produce common names such as eosin or Congo red rather than their full chemical name and some of these names are copyrighted. The dye structure shown below illustrates the complexity of many of these dyes. The full chemical name of this structure is 3,3'-((biphenyl)-4,4'-diylbis(azo))-bis(4-amino-1-naphthalenesulphonic acid) disodium salt, whilst its common name is Congo red. The common name is easier to remember and to say, so most histologists stick to using these.



The Colour Index

To overcome all of the confusion there is a standard list of all dyes, their synonyms and their structures. This is called the Colour Index (CI). This is a monumental work of reference produced by The Society of Dyers and Colourists and each dye is given an individual number and listed along with its name(s) and properties. Since each dye on the list has a unique number to identify it, this list is the most reliable way of identifying a dye. When naming a dye in the description of a technique, the CI number should be given to avoid ambiguity, e.g. eosin Y (CI 45380). CI numbers are arranged according to their structure, with the most important feature being their chromophoric group. For example, all nitroso dyes have numbers between 10000 and 10299, nitro dyes have numbers between 10300 and 10999, monoazo dyes have numbers between 11000 and 19999, and so on. There are 31 groups in all, with CI numbers up to 78000. Not all of these groups include important histological dyes; a few of the more important

groups are listed below with examples of histological dyes from the group.

- Nitro dyes. These have the nitro group -NO_2 as the chromophore, e.g. picric acid, martius yellow.
- Azo dyes. These have the -N=N- group (azo) as the chromophore, e.g. orange G

Triaryl methane dyes

- Triaryl methane dyes. These include the quinonoid arrangement as the actual chromophore. The quinonoid ring but since all three benzene rings are equivalent there can be rearrangement of the bonds and any of the benzene rings could take up this arrangement.
- There are a large number of dyes used in histology that fall into this category; a few examples are fuchsins, methyl violet, methyl blue and aniline blue

- Anthraquinone. Here the quinonoid ring is seen as the middle of the three fused rings. Examples are alizarin and carmine.
- Xanthene. Here the quinonoid ring is the right hand one of the three fused rings and the ring is tilted compared with the previous example. Examples include eosin and xanthene.
- Thiazine. This is very similar to the previous example in overall structure, but the middle ring now has S and N as constituent atoms. This group contains many important metachromatic dyes, such as toluidine blue, methylene blue and azure A.

Basic Dyes

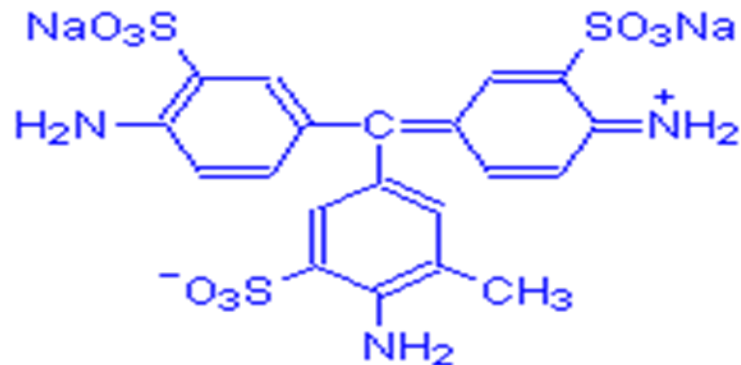
- Basic dyes are cationic and will stain anionic or acidic materials such as carboxylates, sulphates (many complex carbohydrates are sulphated) and phosphates (particularly the phosphates in nucleic acids). Most are used as nuclear stains and staining of cytoplasmic carboxyl groups is deliberately suppressed by using a slightly acid pH. Acidic substances that stain with basic dyes are termed basophilic.

Acidic and Neutral dyes

- Acidic dyes are anionic and will color cationic or basic groups such as amino groups. Most are used to stain proteins in the cytoplasm and connective tissues. Substances that stain with acid dyes are called acidophilic.
- Neutral dyes are simply compounds of basic and acid dyes. In this case, both ions are coloured. Such dye complexes will stain both nucleus and cytoplasm from a single dye bath. Romanowsky stains are neutral dyes made from more complex mixtures. These are the commonest dyes used in haematology. They are less common in histology but still very useful and include Giemsa, Leishman and Wright's stains.

Amphoteric dyes

- Amphoteric dyes also have both anionic and cationic groups, but these are on the same ion.
- Amphoteric dyes have both positively chargeable groups and negatively chargeable groups present on the molecule. Depending on the charge actually present, these dyes may interact as either positively charged ions basic dyes or negatively charged ions acid dyes



Natural dyes

Natural dyes are simply dye substances extracted from natural sources. Although the main source of dyes for early times, they have largely been replaced by synthetic dyes, which are usually more reliable, cheaper and can be supplied more readily. Natural dyes still in use include haematoxylin, carmine, orcein and litmus, although synthetic varieties are also available for some of these.

