intensity of the red colour can be measured by microdensitometry to provide estimates of collagen content in different parts of a tissue (Malkusch et al., 1995; Kratky et al., 1996). When examined through crossed polars the larger collagen fibers are bright yellow or orange, and the thinner ones, including reticular fibers, are green. According to Junqueira et al. (1979) the birefringence is highly specific for collagen. A few materials, including keratohyaline granules and some types of mucus, are stained red but are not birefringent.

It is necessary to rotate the specimen in order to see all the fibres, because in any single orientation the birefringence of some will be extinguished. This minor inconvenience can be circumvented by equipping the microscope for use with circularly rather than plane polarized light (Whittaker et al., 1994; Whittaker, 1995).

Note
The dye benzo blue B (C.I. 22610; Direct blue 6, see Chapter 5) can be substituted for Sirius red F3B, at the same concentration. The birefringence colours are blue–violet for thicker and yellow for thinner collagen fibres (Gitirana and Trindade, 2000).

The name ‘trichrome’ identifies staining techniques in which two or more anionic dyes are used in conjunction with a heteropolyacid: either phosphomolybdic or phosphotungstic acid. These acids are water- and alcohol-soluble crystalline compounds. They may be included in dye solutions or applied to the sections sequentially, between treatments with different dyes. Whatever technique is employed, the result is a selective colouring of collagen by one of the dyes. Cartilage and some mucous secretions acquire the same colour as collagen, but their intensity of staining is usually less. If one other dye is applied, it stains nuclei, cytoplasm and erythrocytes. If two other dyes are used, one imparts its colour to erythrocytes and the other stains the cytoplasm of other types of cell and also cell nuclei. Secretory granules are usually stained; sometimes the same colour as collagen, sometimes the same colour as nuclei or erythrocytes.

The trichrome techniques reveal collagenous and reticular fibres, basement membranes and secretory granules, typically with stronger colour and greater clarity than is possible with van Gieson’s method. With most trichrome techniques the staining is intense enough to obscure structural detail in sections more than 5 μm thick. van Gieson’s and other two-dye methods work well on sections as thick as 15 μm.

8.2.4. The heteropolyacids and their effects on staining
Phosphomolybdic acid (PMA) and phosphotungstic acid (PTA) are known as heteropolyacids. They are formed by coordination of molybdate or tungststate ions with phosphoric acid. They are sold as hydrated crystals, which are freely soluble in water to give strongly acid solutions:

\[
\text{H}_3\text{PO}_4 \cdot 12\text{MoO}_3 \cdot 24\text{H}_2\text{O} \rightarrow 3\text{H}^+ + [\text{PMo}_{12}\text{O}_{40}]^{3-} + 24\text{H}_2\text{O}
\]

phosphomolybdic acid

\[= \text{dodeca-molybdophosphoric acid}\]

phosphomolybdate

\[= \text{anion}\]

\[
\text{H}_3\text{PO}_4 \cdot 12\text{WO}_3 \cdot 24\text{H}_2\text{O} \rightarrow 3\text{H}^+ + [\text{PW}_{12}\text{O}_{40}]^{3-} + 24\text{H}_2\text{O}
\]

phosphotungstic acid

\[= \text{dodeca-tungstophosphoric acid}\]

phosphotungstate

\[= \text{anion}\]
The oxidation number of Mo and W in these compounds is +6. The heteropolyacids are decomposed by alkalis to give molybdate (MoO₄²⁻) or tungstate (WO₄²⁻) and dibasic phosphate ions (see Cotton et al., 1999). In aqueous solutions, PTA forms complex anions, [PO₄(WO₄)₂]⁻ as shown above, but these decompose if the pH rises above 2.0, to give [(PO₄)₃(WO₄)₂]¹⁰⁻ and [PO₆(WO₄)₁]¹²⁻ (Riedl, 1967). The ionic weights of these inorganic anions (2877, 4163 and 2677 respectively) are all greater than those of dyes; the largest of 436 dye ions reviewed by Dapson (2005a) weighed in at 1681. The heteropolyacid ions have approximately spherical structures; different from the large anions of direct cotton dyes, which can assume planar configurations.

The heteropolyacids are able to bind to tissues from aqueous or alcoholic solutions. Baker (1958) called them 'colourless anionic dyes'. Sites of attachment of PMA are easily demonstrated by subsequent treatment of the sections with either ultraviolet radiation or a chemical reducing agent such as stannous chloride. A blue mixture of insoluble oxides of Mo(V) and Mo(VI), with compositions such as MoO₂(OH) and MoO₂.₅(OH)₀.₅, is formed. It is known as molybdenum blue. A corresponding but less intensely coloured tungsten blue, formulated as WO₂.₇, can also be produced. Sites of binding of PTA to tissues have been studied under the electron microscope, with which the electron-dense tungsten-containing deposits can be accurately localized.

The various studies of the binding of heteropolyacids to tissues (Baker, 1958; Puchter and Isler, 1958; Bulmer, 1962; Puchter and Sweat, 1964b; Everett and Miller, 1974; Hayat, 1975, 1993; Allison and Tanswell, 1993; Reid et al., 1993) are not all in agreement, but the following facts appear to be undisputed:

(1) Chemical studies indicate that PTA binds to proteins and amino acids but not to carbohydrates. Both the heteropolyacids are used as precipitants for proteins, amino acids, and alkaloids. The heteropolyacid anions are held by ionic attraction to protonated amino and guanidino groups of proteins.

(2) Applied at pH <1.5, PTA imparts electron density to carbohydrate-containing structures, but at pH > 1.5 it binds to proteins. PTA can oxidize carbohydrate hydroxyl groups to aldehydes, so the electron-dense deposits resulting from staining at pH <1.5 may be insoluble compounds in which the oxidation state of the tungsten is less than +6.

(3) Collagen fibres bind large amounts of PMA. Cytoplasm binds smaller amounts. Nuclei of cells have very little affinity for PMA. PTA behaves similarly.

(4) Affinity for PMA and PTA is depressed or abolished if amino groups in the tissue are first removed by treatment with nitrous acid or esterified by reaction with acetic anhydride or benzoyl chloride. In light microscopy there is no evidence for binding of PMA or PTA to carbohydrates or to hydroxyl groups of amino acids.

(5) Methylation of sections results in increased attachment of PMA to all parts of the tissue, including erythrocytes. Methylation agents add methyl groups to amine nitrogen atoms (increasing their basicity) and to hydroxyl oxygen atoms (forming ethers or glycosides).

(6) Structures that have bound PMA or PTA become stainable by cationic dyes. [This change is exploited in Monroe and Frommer's (1967) variant of Twort's stain (See Chapter 6, Section 6.4.2).]

(7) Treatment with PMA or PTA affects stainability by anionic dyes. The effects are variable:

(a) There is considerable suppression of the staining of all parts of the tissue by some anionic dyes, including ones with small molecules, such as
picric acid, Martius yellow, eosin, orange G, and biebrich scarlet. The amount of suppression is greater in collagen than in cytoplasm.

(b) There is similar suppression of cytoplasmic staining by dyes that have large molecules, including aniline blue, light green SF, fast green FCF, and acid fuchsin, but collagen is stained with only slightly reduced intensity by these dyes after treatment of the sections with a heteropolyacid.

(c) Treatment of sections with PMA or PTA either before or at the same time as staining with aniline blue, light green SF, or fast green FCF has the effect of preventing the attachment of these dyes to materials other than collagen, cartilage matrix, and certain carbohydrate-containing secretory products.

(d) If sections are treated with PTA, stained with aniline blue, and then exposed to 6 M urea, a reagent which disrupts hydrogen bonds, the dye is removed, but the PTA remains attached to the collagen in the tissue. The treatment with urea may not, however, be a specific test for hydrogen bonding.

(e) Freezing and thawing before fixation of a tissue changes the colours imparted by trichrome staining methods, such that the cytoplasm of some cells are atypically stained by the dye with the larger molecules.

8.2.4.2. How do trichrome methods work?
Three hypotheses have been advanced to account for the differential colouring of tissues by anionic dyes used in association with heteropolyacids.

In the first theory, championed by Baker (1958), and upheld by Horobin (1982, 1988) as an example of 'rate controlled' staining, it is held that the anions of the dyes and of the heteropolyacids compete with one another for cationic binding sites and that the textures of the various structural components of a tissue determine their penetration by molecules of different size. PMA and PTA anions are assumed to be intermediate in size between those of the dyes generally used as cytoplasmic stains and those which stain collagen. Large dye ions exist in solution as aggregates, so this is a reasonable supposition despite the ionic weights of PMA and PTA being greater than those of large dye ions. The smallest dye anions would enter and bind to the supposedly dense network of haemoglobin and other protein molecules forming the stroma of the erythrocyte, which would not be penetrated by the heteropolyacid. The collagen fibre is considered to be more porous, and able to accommodate the ions of PMA or PTA and those of a dye with large molecules such as aniline blue or light green SF. Small dye ions could also penetrate the collagen fibres but, because they diffuse rapidly, they would enter and leave freely. The larger, more slowly diffusing particles of the heteropolyacids and of such dyes as aniline blue would remain in the collagen and become attached to cationic sites there. Dyes with molecules of intermediate size (e.g. acid fuchsin) would compete with PMA or PTA for binding sites in the cytoplasm of muscle fibres and other cells. These dye molecules would be too big to enter erythrocytes in the presence of dyes with small molecules, but they would be small enough to escape from collagen fibres more quickly than the largest dye molecules. Bulmer (1962) demonstrated that heteropolyacids were bound to tissues only when the latter contained protonated amino groups, but agreed with Baker (1958) in attributing the trichrome staining effects to differential permeability of cytoplasm and collagen to large and small molecules.

A similar postulated mechanism was discussed earlier in relation to the differential staining of cytoplasm and collagen in van Gieson's and related methods. The objections raised there also apply to the application of this hypothesis to the trichrome techniques. Furthermore, it is difficult, in terms of this theory, to account for the fact
that treatment with heteropolyacids induces basophilia, as well as selective though depressed affinity for certain anionic dyes, in collagen. Control of staining by different rates of diffusion receives some support from the atypical staining of cells by dyes of high molecular weight in previously frozen and thawed specimens (Allison and Tanswell, 1993). Tiny holes made by ice crystals can be expected to cause increased porosity in cytoplasm.

The second hypothesis accounts adequately for the basophilia produced by treatment of collagen with PMA or PTA. Puchtler and Isler (1958) proposed that cationic dyes were attracted by the free negatively charged groups of the collagen-bound ions of heteropolyacid. For example:

\[
\text{COLLAGEN} + \text{NH}_2^+ + \text{H}^+ + [\text{PMA}]^{3-} \rightarrow \text{COLLAGEN} - \text{NH}_2^+ - [\text{PMA}]^{2-}
\]

\[
\text{COLLAGEN} - \text{NH}_2^+ - [\text{PMA}]^{2-} + 2 \text{DYE}^+ \rightarrow \text{COLLAGEN} - \text{NH}_2^+ - [\text{PMA}]^{2-} + \text{DYE} + \text{DYE}^+
\]

Thus, a function similar to that of a classical mordant (Chapter 5) is attributed to the heteropolyacid. Puchtler and Isler noted that the dyes used to stain collagen in trichrome techniques were all amphoteric. They suggested that these dyes were bound by ionic forces to the PMA or PTA, which was itself attached electrovalently to protonated amino and guanidino groups of collagen. The cytoplasmic stains used in trichrome techniques are wholly anionic dyes, so they would not attach to the free negatively charged sites of the bound heteropolyacid molecules.

Several objections can be made to this hypothesis. The staining of collagen is attributed to its content of amino acids with basic side-chains: lysine, arginine, and histidine. Haemoglobin, the principal protein of erythrocytes, has a higher proportion of these basic amino acids than collagen but erythrocytes are not similarly stained in the trichrome procedures. Classical mordanting by PMA or PTA would be expected to result in intensification of staining by amphoteric dyes: two molecules of dye would attach to each of the bound (trivalent) anions of the heteropolyacid. All investigators agree, however, that pre-treatment with PMA or PTA reduces the intensity of staining by aniline blue and similar dyes, even in collagen (Baker, 1958). The matrix of cartilage and the granules of mast cells, although they are composed of strongly acid proteoglycans (Chapter 11), are not ordinarily stained strongly by amphoteric dyes such as acid fuchsin and aniline blue, so these dyes do not behave as if they were cationic. Finally, the effect of 6 M urea on trichrome-stained sections indicates that the dye is bound to collagen by non-ionic forces whereas the heteropolyacid is held in place by a different and stronger force, probably ionic attraction as proposed by Puchtler and Isler (1958).

A third explanation for the actions of PMA and PTA in trichrome procedures was offered by Everett and Miller (1974), who provided evidence of two different modes of binding of these acids to tissues. Ionic attraction of the heteropolyacid ions to cytoplasmic proteins was thought to inhibit there the binding of anionic dyes. The binding of the heteropolyacids to collagen was believed to be non-ionic, so that staining by anionic dyes was not prevented. The principal objections to this hypothesis are that it does not account for either the fact that PMA cannot be removed from collagen by 6 M urea or the fact that staining of collagen after treatment with PMA or PTA can be effected by some anionic dyes but not by others.
In summary, there is much evidence to support the idea that the stainability of tissues is determined by rates of diffusion within parts of the tissue that are differently permeable to large and small anions, but this proposed mechanism does not explain how the dyes and the PMA or PTA are bound to the cytoplasm and collagen. The second and third of the postulated mechanisms cannot fully account for the differential staining obtained with the trichrome methods, but they do shed some light on the action of heteropolyacids, which evidently are held to tissue proteins by electrostatic attraction, and on the attachment of larger dyes to collagen, which appears to be by way of non-ionic forces, which may include hydrogen bonds and van der Waals forces.

The techniques of Masson, Mallory, and Heidenhain are done in stages. This allows some control of the intensity of color in cytoplasm and collagen. Two one-step procedures (Cason and Gabe) are also described here. They are technically simpler than the classical trichromes, but do not always work as well. See Luna (1968), Gabe (1976), Clark (1981) and Bancroft and Gamble (2002) for detailed technical instructions for these and other trichrome methods.

The fixative for tissues to be stained by any trichrome method should not be a simple formaldehyde solution with no other active ingredients, and it should not contain glutaraldehyde. Non-aqueous fixatives are not recommended. Mercury-containing mixtures such as SUSA give excellent results, and Bouin is also satisfactory. If you used a mercury-containing fixative, do not forget to treat the sections with iodine and thiosulphate before staining (Chapter 4, Section 4.4.1). According to Churukian et al. (2000), zinc–formalin (Chapter 2) is also a satisfactory fixative for trichrome staining. The staining properties of neutral-formaldehyde-fixed tissue, especially of cytoplasm, can be improved by immersing hydrated paraffin sections overnight in fixative solutions such as Bouin, Zenker or zinc–formalin, either overnight at room temperature or for 10–15 min at 55–60°C. Saturated aqueous picric acid is as effective as Bouin (unpublished observations). Yu and Chapman (2003) pre-treated slides with either an iodine solution (0.33% I₂ in 0.67% aqueous KI) or a citrate buffer at pH 4 prior to staining by Masson’s method. The mechanisms of action of pretreatments that enhance trichrome staining are in need of investigation.

Nuclei are stained by the dye with molecules of intermediate size (red in the methods that follow), but it is often desirable to stain them black instead, with an iron–haematoxylin (Chapter 6). This is always done with Masson’s technique. Prior nuclear staining reduces the brilliance of the colours in cytoplasm and collagen, but it often improves the overall morphological clarity of the stained preparation.

8.2.5.1. Masson’s trichrome
This is the simplest trichrome, because only two anionic dyes are used, after staining the nuclei with iron–haematoxylin. There are many variants of this method, using different dyes or a different heteropolyacid solution (see Note below). The following version is that of Luna (1968).

Solutions required
A. Iodine and sodium thiosulphate
For removing mercury deposits (Chapter 4).

B. Acid–alcohol
May be needed for differentiation of the nuclear stain.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (95% or 100%)</td>
<td>140 ml</td>
</tr>
<tr>
<td>Concentrated hydrochloric acid:</td>
<td>1 ml</td>
</tr>
<tr>
<td>Water:</td>
<td></td>
</tr>
</tbody>
</table>

Mix before using and use only once.
C. Acidified water
This is water with about 5 ml of glacial acetic acid added to each litre.

D. Weigert's or Lillie's iron–hematoxylin
See Chapter 6.

E. Biebrich scarlet-acid fuchsine
   Biebrich scarlet (CI. 26905):
   Acid fuchsine (CI. 42685):
   Water:
   Glacial acetic acid:
   4.5 g
   0.5 g
   495 ml
   5 ml
   Keeps for several months and may be used repeatedly. Filter before using.

F. PMA–PTA solution
   Phosphomolybdic acid (also known as molybdophosphoric acid):
   Phosphotungstic acid (also known as tungstophosphoric acid):
   Water:
   5 g
   5 g
   200 ml
   Keeps for about 5 years, and goes on working despite a change in color from yellow to green.

G. Fast green FCF, 2%
   Fast green FCF (CI. 42053):
   Water:
   Glacial acetic acid:
   4.0 g
   195 ml
   2.0 ml
   Keeps for several years and may be used repeatedly.

Procedure
(1) De-wax sections and bring to 70% alcohol. The next two steps are necessary if the fixative contained mercuric chloride. Otherwise they may be skipped.
(2) Remove mercury deposits by immersion in Gram's iodine (1% I₂ in 2% aqueous KI) for 30 s. (Alternatively, use 0.5% iodine in 70% alcohol, which needs 3 min.)
(3) Remove iodine stain by immersion in 5% sodium thiosulphate solution until sections are no longer yellow or brown (about 15 s). Proceed to Step 5.
(4) If the fixative did not contain picric acid or mercuric chloride, place the slides in either saturated aqueous picric acid or Bouin's fluid or a zinc–formalin fixative solution. Duration of the pretreatment may be overnight at room temperature or 2 h at 55–60°C.
(5) Wash in tap water (3 changes, or running water for 2 min), then in distilled water, 30–60 s. (If the fixative did not contain mercuric chloride, and the iodine-thiosulphate treatment was omitted, simply place in distilled water for 1 min with agitation for the first 30 s.)
(6) Stain nuclei in a working iron–hematoxylin (Solution D) for 3 min. Rinse in tap water and check wet slide under a microscope. Only nuclei should be stained. See Chapter 6 for action to be taken if nuclear staining is unsatisfactory.
(7) Wash in running tap water for 1 min.
(8) Stain for 4 min in biebrich scarlet–acid fuchsine (Solution E).
(9) Rinse in slightly acidified water (Solution C) to remove excess dye.
(10) Immerser in PMA–PTA (Solution F) for 10 min. Rinse in acidified water and check a slide under a microscope to ensure that the red dye has been removed from collagen. Return to the PMA–PTA solution for another 5 min if necessary.
(11) Stain for 4 min in 2% fast green FCF (Solution G).
(12) Immerse in 2 changes of slightly acidified water, each for about 30 s. (A longer time in the second change does no harm.)
(13) Dehydrate in 3 changes of 100% alcohol, clear in xylene and apply cover-slip, using a resinous mounting medium.

Result

Note
Masson (1929) used a more complicated iron–haematoxylin nuclear stain. For red dyes he used a mixture of acid fuchsin with an azo dye that was probably ponceau 2R (C.I. 16150). He used 1% PMA for the heteropolyacid and either aniline blue or light green for the collagen stain. In the present variant, following Lillie (1945), fast green FCF is used instead of light green because the latter fades with time (Chapter 5).

8.2.5.2. Mallory's trichrome
Mallory described at least three trichrome procedures. This one (Mallory, 1905) is the simplest.

Solutions required
A. Iodine and sodium thiosulphate
For removing mercury deposits (Chapter 4).

B. Acidified water
This is water with about 5 ml of glacial acetic acid added to each litre.

C. Acid fuchsin, 0.5%
Acid fuchsin (C.I. 42685):
Water: 1.0 g 200 ml

Keeps indefinitely and may be reused many times.

D. Aniline blue–orange G-PTA solution
Aniline blue (C.I. 42755):
Alternatively use methyl blue (C.I. 42780). These dyes are sometimes labeled soluble blue, water blue or aniline blue, water soluble. Aniline blue, alcohol-soluble (spirit blue; C.I. 42775) cannot be used in this solution.

Orange G (C.I. 16230):
Phosphotungstic acid
(also called tungstophosphoric acid):
Water: 4.0 g 2.0 g 200 ml

Stable for about 2 years, but deteriorates with repeated use.

Procedure
(1) De-wax sections and bring to 70% alcohol. The next two steps are necessary if the fixative contained mercuric chloride. Otherwise they may be skipped.
(2) Remove mercury deposits by immersion in Gram's iodine (1% I₂ in 2% aqueous KI) for 30 s. (Alternatively, use 0.5% iodine in 70% alcohol, which needs 3 min.)
(3) Remove iodine stain by immersion in 5% sodium thiosulphate solution until sections are no longer yellow or brown (about 15 s). Proceed to Step 5.
(4) If the fixative did not contain picric acid or mercuric chloride, place the slides in either saturated aqueous picric acid or Bouin's fluid or a zinc–formalin fixative solution. Duration of the pretreatment may be overnight at room
temperature or 2 h at 55–60°C. (This option was not included in Mallory's original method.)

(5) Wash in tap water (3 changes, or running water for 2 min), then in distilled water, 30–60 s. (If the fixative did not contain mercuric chloride, and the iodine-thiosulphate treatment was omitted, simply place in distilled water for 1 min with agitation for the first 30 s.)

(6) Immerse in 0.5% acid fuchsine for 2 min.

(7) Shake off excess dye solution and transfer the slides directly into the aniline blue–orange G-PTA solution. Leave there for 30 min.

(8) Immerse in acidified water to wash off excess dye, shake the slides and transfer them to a clean, dry staining rack or Coplin jar.

(9) Dehydrate in 3 changes of 100% alcohol, clear in xylene and apply coverslips, using a resinous mounting medium.

**Result**

**Note**
If Step 6 is omitted, blue and orange nuclei will be be seen in sections 6 μm and thicker, with the percentage of yellow nuclei increasing with section thickness. Lison (1955) deduced that aniline blue enters and stains only nuclei that have been cut by the microtome knife whereas orange G stains nuclei that are entirely contained in the section. Baccari et al. (1992a,b) found that treatment with RNase increased the proportion of orange nuclei and postulated that the blue nuclear staining occurred in cells with high rates of RNA synthesis.

### 8.2.5.3. Heidenhain's AZAN

This is the trichrome method that gives the user maximum control of the colours, because there are two destaining (differentiation) steps. AZAN (the acronym refers to *Azokarmin* and *Anilinblau*) is useful for showing cytoplasmic and extracellular structures, and it displays fine collagen and reticular fibers with brilliant clarity, even in sections as thick as 10 μm. The method takes 3 h, and is too troublesome for routine use with large numbers of slides.

For optimal staining of secretory products and other cytoplasmic components the tissue should have been fixed in a mixture containing mercuric chloride and either potassium dichromate or formaldehyde or both these compounds (Chapter 2). Treatment of sections of formaldehyde-fixed tissue with Bouin's fluid or saturated aqueous picric acid (as described for the Masson and Mallory techniques, in place of steps 2 and 3 of the procedure below) improves the colour contrast between cytoplasm and connective tissue but does not provide the sharply defined cytoplasmic detail that can be seen in well fixed cells stained by the AZAN method (my unpublished observations).

The most critical stage of the AZAN procedure is the first differentiation (in aniline–alcohol). The prescribed times for the other stages may be exceeded but cannot be shortened. This is the method as adapted by Gabe (1976).

**Solutions required**

**A. Acidified water**
This is water with about 5 ml of glacial acetic acid added to each litre.

**B. Aniline–alcohol**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% ethanol:</td>
<td>400 ml</td>
</tr>
<tr>
<td>Aniline:</td>
<td>4 ml</td>
</tr>
</tbody>
</table>