7. Staining Paraffin Sections

This chapter is not intended to be a comprehensive treatise on the theory and practice of staining. Historical reviews of the evolution of biological staining and critical discussions of the chemistry of dyes and of staining will be found in Conn's *Biological Stains* (1936). For our purposes it is a safe practical assumption that the staining of cellular structures is based on specific affinity between certain dyes and particular cell structures. This specificity is aided in some processes by a mordant, usually a salt, which enters in some manner into a three-way relationship bewteen the mordant, the dye, and some part of the cell.

This chapter presents a graded series of practical exercises in staining, using designated subjects and a limited number of time-tested stains and combinations. Staining procedures are presented in the form of charts. It is easier to follow a series of operations on a chart than in a written account. Staining processes fall into fundamental types, based on the character of the stains used. Each chart should be regarded as a type chart rather than as a rigid set of specific directions. The sequence of operations should be followed closely, but the time element in some operations should be understood to vary widely. If the *function of each operation* is thoroughly understood, variations of the time element are easily made in accordance with the reactions of the material being stained.

Equipment

Paraffin sections affixed to slides are stained and processed by immersion in reagents in staining jars. Note the various types of jars illustrated in catalogues. The most satisfactory type is the Coplin jar, a vertical jar with grooves that hold the slides in a vertical position. Unlike the horizontal type, Coplin jars occupy little table space, the small opening and the ground-glass lid minimize evapora-

tion, and the slides can be handled more easily in the vertical position. A well-built Coplin jar will hold 9 slides, staggered as shown in Fig. 7.1 *A*. Some workers place slides into the grooves in pairs, back to back, but this method does not give the reagents free access to both surfaces of a slide. For quantity production of slides, screw-topped jars can be used in conjunction with various types of racks, holding from 5 to 15 slides (Fig. 7.1 *B*). Staining jars should be cleaned occasionally in chrome-sulphuric cleaning fluid, washed

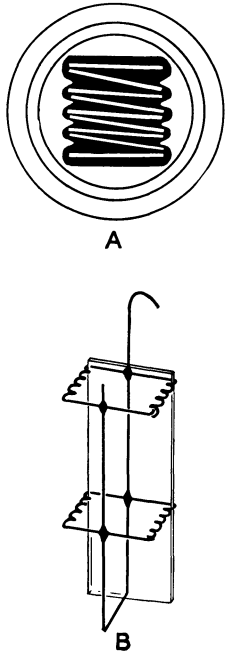

FIG. 7.1—*A*, Top view of Coplin staining jar showing staggered arrangement of nine slides; *B*, wire holder for slides.

thoroughly, and rinsed in distilled water. Jars that are to contain anhydrous reagents must be dried. Assemble a set of at least 15 jars on a shallow wooden tray, on which they can be carried about or put out of the way easily. Label each jar in accordance with the reagents used in Staining Chart I. Do not label water jars, because the same jar is used for several changes of distilled water and tap water by pouring out and refilling. If lids are not readily interchangeable, label each lid to correspond with the matching jar. Do not number your jars. Learn to reason out each step in the staining process rather than to memorize a numerical sequence of operations. Each jar should contain enough reagent to cover the slides completely.

Stain Formulas

The existence of several exhaustive formularies makes unnecessary the compilation of an extensive list of stain formulas in this manual. In actual practice only a few of the large number of known stains are used; therefore, the type formulas and specific formulas of only the most frequently used stains are given here. Detailed methods of using these stains are described in the next section.

THE HEMATOXYLIN STAINS

The hematoxylin formulas rank among the most useful biological stains. Hematoxylin is a natural dye, extracted from logwood, *Hematoxylin campechianum* L. The product is purchased in the form of a pale yellow or brownish powder. The certified dye should be specified. Hematoxylin is not a dye in itself, but in the presence of certain alums, which serve as mordants, hematoxylin stains specific cell structures. Numerous formulas and procedures appear in the literature. There are two principal types of formula: (1) the self-mordanting type, in which the hematoxylin dye, the alum mordant, the oxidizing agent, and a preservative are in the same solution; (2) the separate-mordant type, in which the mordant is first applied to the tissues, followed by the application of the dye.

Three of the most useful self-mordanting formulas are given below:

Mayer's Hemalum (Modified)

Dissolve 20 g. potassium alum in 1 l. boiling water.
Add 1 g. hematoxylin crystals to the above. Remove from the heater when dissolved.
Add 0.2 g. sodium iodate ($NaIO_3$).
The stain is ready to use at once. Filter whenever a metallic scum is visible on the surface of the stain in the staining jar. The stain gradually disintegrates and should be made up fresh every 2 to 3 months.

This stain can be made up more conveniently by using stock solutions, thereby requiring only the relatively rough weighing of the alum. Keep on hand a 5% solution of hematoxylin in 95% ethyl alcohol. The dye dissolves slowly at 35°C. and requires about 2 weeks to attain a deep mahogany-red color. Store this solution in a refrigerator, where it seems to keep its properties indefinitely. Make up the stain as follows:

Dissolve 20 g. potassium alum in 1 l. boiling water.
Remove from the heater and add 20 cc. of the above alcoholic hematoxylin, drop by drop.
Add 10 cc. of 2% $NaIO_3$

Hematein-Alum (Kornhauser)

Hematein, an oxidation product of hematoxylin, is used in the following formula, which is preferred by some workers to the Mayer formula and its modifications.

0.5 g. hematein.
Grind in a glass mortar with 10 cc. 95% alcohol.
Add to 500 cc. potassium aluminum sulphate, saturated aqueous solution.
The stain is ready to use at once and has good keeping qualities.

Harris' Hematoxylin

1 liter 50% alcohol; 1 g. aluminum chloride; 2 g. hematoxylin crystals.
Heat on a water bath until dissolved.
Add 6 g. mercuric oxide; filter.
Add 1 cc. HCl.

Delafield's Hematoxylin (Slow-Ripening Formula)

100 cc. saturated solution of ammonia alum; add drop by drop 6 cc. absolute alcohol containing 1 g. hematoxylin.
Expose to light in open bottle for 1 week.
Filter, and add 2.5 cc. glycerin and 2.5 cc. methyl alcohol.
Allow to ripen at least 2 months. Filter as needed.

Delafield's Hematoxylin (Rapidly-Ripened Formula, Kohl and James)

Prepare the complete formula as above. Pour into a shallow open dish and expose to a quartz mercury-vapor lamp for 2 hr. Another method of ripening (Neild) consists of exposing the liquid to a Cooper-Hewitt light, for 1 hr., 15 cm. from the light, at 140 volts, 3.3 amp.

The most widely used separate-mordant staining procedure uses the following reagents:

Iron-Hematoxylin (Heidenhain's), (Iron-Alum Hematoxylin)

The mordant consists of a freshly made 4% solution of iron alum (ferric ammonium sulphate). Select clean, transparent, violet-colored crystals, especially avoiding crystals with a rusty coating. Discard the solution when a yellow precipitate develops in the bottle.

The following mordant will keep for months (Lang):

4% iron alum	500 cc.
Acetic acid (glacial)	5 cc.
10% H_2SO_4	6 cc.

The stock solution of stain is a 0.5% aqueous solution of hematoxylin. Measure the required volume of distilled water, add a pinch of sodium bicarbonate, about as large as a match head, to a liter of water. Bring the water to the boiling point, remove from the heater and add the dye. Do not boil the solution! Cool promptly and store in a refrigerator. Dilute the stock solution with twice its volume of water for the 4-hr. schedule and with 4 parts of water for a 12-hr. stain. Although the new stain will give satisfactory results, it improves after 2 or 3 days. The stain begins to deteriorate in a few months.

Another type of stock solution consists of a 5 or 10% solution of hematoxylin in absolute ethyl alcohol or 95% alcohol. Dilute to 0.5% in water as needed.

Some satisfactory destaining agents are:

1. Mordant diluted with an equal volume of water.
2. Saturated aqueous solution of picric acid.
3. Equal volumes of mordant and the above picric acid.

Mordanting is sometimes necessary with the synthetic dyes described in the following section. The complex problem of mordanting is well summarized by Popham (1949), and specific suggestions are made throughout the present chapter.

THE COAL-TAR DYES

The coal-tar dyes comprise a large and highly diverse class of synthetic dyes. Their derivation, chemical composition, and properties are discussed in great detail by Conn (1936). Specify dyes that are certified by the Commission on Biological Stains (Conn, 1936). Only the members of this group of stains that are in common use for botanical work will be presented here. Coal-tar dyes are used in a variety of solvents, and the general formulas for the most common stock solutions are as follows:

(1) O.5 to 1% solution in water, with 5% methyl alcohol optional, as a preservative.

(2) 0.5 to 1% solution in ethyl alcohol, with alcohol concentrations of 50, 70, and 95% and absolute alcohol preferred by various workers.

(3) Saturated solution in clove oil, or
in equal volumes of clove oil and anhydrous ethyl alcohol, or
in methyl Cellosolve, or
equal volumes of clove oil, anhydrous alcohol and methyl Cellosolve.

The following table shows the usual solvents (x) in which the best known coal-tar dyes are used.

Dye	Water	Alcohol, %	Clove oil or Cellosolve
Acid fuchsin (acid)	x	70	
Aniline blue (acid) (= cotton blue)	x	50	
Bismarck brown Y (basic)	.....	70	
Crystal violet (basic)	x	.....	x
Eosin Y (acid)	.....	95	
Erythrosin (acid)	.....	95	x
Fast green *FCF* (acid)	.....	95	x
Orange G or gold orange (acid)	.....	100	x
Safranin O (basic)	x	50 to 95	x

The principal botanical uses for the common stains are indicated in the following tabulation:

Cellulose cell walls.
- Hematoxylin (self-mordanting type).
- Fast green *FCF*.
- Aniline blue.
- Bismarck brown Y.
- Acid fuchsin.

Lignified cell walls.
- Safranin.
- Crystal violet.

Cutinized cell walls.
- Safranin.
- Crystal violet.
- Erythrosin.

Middle lamella.
- Iron hematoxylin.
- Ruthenium red (material cut fresh).

Chromosomes.
- Iron hematoxylin.
- Safranin.
- Crystal violet.
- Carmine (for acetocarmine smears).
- Orcein

Mitochondria.
- Iron hematoxylin.

Achromatic figure.
- Crystal violet.
- Fast green *FCF*.

Filamentous fungi in host tissues.
- Iron hematoxylin.
- Safranin O.
- Fast green *FCF*.

Cytoplasm.
- Eosin Y.
- Erythrosin B.
- Fast green *FCF*.
- Orange G or gold orange.

The above tabulations indicate some relationship between the acid or basic character of a stain and its specificity. A basic stain is one in which the *color bearer* is a basic radical; in an acid stain the color bearer is an acid radical. As a rule basic stains are selective for nuclear structures and, in some processes, for lignified cell wall. Acid stains usually are selective for components of the cytoplasm and for unlignified cell wall.

The common clearing oils (clove oil, cedar oil, bergamot oil, and wintergreen oil) usually are used in concentrated form as purchased

or thinned slightly with xylene. An inexpensive and highly satisfactory clearing agent, known as *carbol-xylene,* consists of 1 volume of melted c.p. phenol (carbolic acid) and 3 to 4 volumes of xylene.

Staining Processes

To meet the needs of teachers and beginners, staining processes are arranged in a graded sequence, beginning with the simplest processes, in which the variables and possibilities for errors are reduced to a minimum. The simplest type of stain is a *progressive* stain, in which the intensity of the color imparted to the tissues is proportional to the length of immersion in the stain. Some of the most useful stains of this type have hematoxylin as the active ingredient. In this category of self-mordanting stains, the most important are Delafield's hematoxylin, Harris' hematoxylin, and Mayer's hemalum. Many modifications may be found in the literature. The term "hemalum" is used in this manual to refer to any of the self-mordanting alum hematoxylins. The choice among these stains is a matter of personal preference.

HEMALUM (PROGRESSIVE)

The modification of Mayer's hemalum, on which staining Chart I is based, is selective for cellulose, pectin, fungus mycelium in many cases, weakly selective for chloroplasts, strongly selective for metabolic (resting) nuclei, and moderately selective for chromosomes in some subjects. Hemalum may be used without any other stain for meristematic organs, for anther and ovary slides in which a critical chromosome stain is not necessary, and for subjects having but little strongly lignified or differentiated tissues. This stain develops a "metallic" scum on standing. The particles of this scum adhere to the adhesive and to the sections on the slide, therefore the stain should be filtered before using.

The preliminary processing of slides, prior to immersion in stain, is essentially the same regardless of the stain used. This prestaining process will now be outlined and the procedure is understood to apply when an aqueous stain is used. After the affixed sections have been dried in the 53°C. oven, the sections and adjacent parts of the slide are found to be coated with melted paraffin from the ribbon. Obviously, the first operation is to dissolve this paraffin by immersing the slide in a jar of xylene. If slides are taken directly from the oven,

the paraffin dissolves in 1 or 2 min. With cold slides it is better to allow 5 min. The slide is now in a very dilute solution of paraffin in xylene, which is removed by immersing the slide in anhydrous alcohol. As outlined in Staining Chart I, progressive transfer to water is then made through the indicated grades of ethyl alcohol. Isopropyl alcohol or acetone also may be used in most of the staining charts in this chapter. The slide has been *run down* to water, and is now ready to be stained in an aqueous dye. Transfers should be made quickly so that the slides do not become dry. The intervals can be shortened to 30 sec. by moving the slides up and down in the solution with forceps.

The series of reagents in which slides are deparaffined should be replaced when the 30% alcohol becomes cloudy or when the fluid drains from the slides as if the glass were oily, indicating that paraffin and xylene have been carried down the series until the 70% and 30% cannot hold the xylene-paraffin contaminant in solution. The addition of 10% *n*-butyl alcohol to the anhydrous and 95% grades prolongs the useful life of the series.

The correct staining interval for a given subject must be determined by trial. An experienced worker can make a good guess for a trial slide and make corrections for subsequent slides. One collection of lily ovary killed in Bouin's solution required only 10 min. for a brilliant stain, whereas another collection, fixed in Craf required 1 hr. A collection of lily anther in the microspore stage yielded excellent slides with a 30-min. stain. To determine the correct interval, stain three slides of a subject for three intervals, *i.e.,* 10, 20, and 40 min., respectively. Mark the slides before staining. The sample slides may be held in distilled water and put into the stain at intervals, or they may be put into the stain simultaneously and removed after the desired intervals. After staining, rinse the slides in two or more changes of distilled water, then rinse in three changes of tap water, or in running tap water for 2–5 min. Note that the color in the tissues changes from purple to blue after the transfer into tap water. Hematoxylin gives a reddish-purple color when acid and a blue color when alkaline. The latter color is preferred for the subjects recommended for this first exercise. If the city water in your community does not produce the bluish tinge in tissues that have been stained in hemalum, use 0.1% sodium carbonate for the last rinse. This process may be called *alkalizing.*

At this stage, examine the three test slides that were stained as

suggested above. Use a smear microscope, preferably one that has only 10x (16 mm.) and 20x (8mm.) objectives and no condenser. The magnification is adequate and the objectives have such long working distance that they are not likely to be dipped into reagents on the slide. The tissues must not become dry during this quick examination. Nuclei should be blue-black. Cellulose cell walls should be black, whereas lignified cell walls should be nearly colorless. Plastids may be pale blue to blue-black, and cytoplasm blue-gray. If the foregoing structures do not have a deep enough color, transfer the slide from the water to hemalum and give it another interval in the stain, usually as long as the first immersion. Rinse and wash in tap water, examine again and if satisfactory, proceed with dehydration as in Chart I.

If a slide is left in hemalum longer than the optimum period, the contents of the cell may become black, and the details of the wall and protoplast may be obscured. The slide can be destained by brief immersion in dilute acid. The preferred destaining agents are 1 to 5% acetic acid, 0.5% hydrochloric acid, or a saturated aqueous solution of picric acid. Try one minute in acid, wash, alkalize and re-examine with a microscope. When the stain is satisfactory, proceed with dehydration according to Chart I.

Staining Chart I now calls for progressive dehydration of the tissues and the surface of the slide, followed by "clearing." Consult the reference manuals for the various clearing agents in common use. An inexpensive agent is carbol-xylene, the formula of which is given on page 61. Both ingredients must be of high purity. Phenol has a great affinity for water and removes the last traces of water from the preparation. Xylene has nearly the same index of refraction as glass, thus rendering the tissues transparent. High-grade phenol and xylene should not affect the stain even after several days of immersion. Equal volumes of xylene and cedar oil may replace the carbol-xylene.

The final operation consists of cementing a cover glass on the preparation. Have ready a supply of newly cleaned and dried cover glasses. Use a cover glass of generous, but not wasteful, size, with shape and dimensions in keeping with the material to be covered (Fig. 6.6). Discoloration of resin and fading of stain with age proceed from the edges of the cover inward. Have a margin of at least 5 mm. between the sections and the edge of the cover glass. For mounting one section on a slide, or a few sections in a single row, use a ½-, ¾-,

STAINING CHART I *

Progressive Hemalum

Xylene
2–5 min.
(de-waxing)
↓
absolute
(anhydrous)
alcohol
2–5 min.
↓
95%
alcohol
2–5 min.
↓
70%
alcohol
2–5 min.
↓
50%
alcohol
2–5 min.
↓
30%
alcohol
2–5 min.
↓
distilled
water
1–2 min.
↓
Hemalum
5–30 min.
↓
distilled
water
1 min.
↓
Tap water
(see page 62) ——→

resin and
cover glass
↑
xylene III
5 min.
↑
xylene II
5 min.
↑
xylene I
5 min.
↑
carbol-
xylene
5–10 min.
↑
absolute
alcohol II
5–10 min.
↑
absolute
alcohol I
5–10 min.
↑
95%
alcohol
5–10 min.
↑
70%
alcohol
5–10 min.
↑
50%
alcohol
5–10 min.
↑
30%
alcohol
2–5 min.

* The beginner is advised to copy each staining chart on a large card. By means of colored arrows, indicate the sequence of operations used to correct overstaining or understaining.

or 7/8-in. cover glass. For large longitudinal sections of rectangular outline, or for covering several rows of sections on a slide, use a square or long cover glass of such size that there is a margin of at least 5 mm. Caliper all cover glasses, using only those that fall within 0.15 to 0.20 mm. in thickness.

Canada balsam has been the most widely used mounting medium

for many years. Stained sections mounted in balsam may remain in perfect condition for 25 years. However, it is much more likely that the stain will fade, the balsam will become dark yellow, and may even become cracked and opaque like dried varnish. In recent years, numerous synthetic resins have been tried as mounting media. (Lillie, Winkle and Zirkle, 1950). Further experimentation can be expected in the future and the many possible polymers will be tested. The reader is advised to consult the catalogues of biological supply dealers for the currently recommended resins.

The affixing of cover glasses should be accomplished quickly and neatly. Remove a slide from the last xylene, and place with tissue upward on a sheet of dry blotting paper. Working rapidly to avoid drying of the tissues, wipe excess xylene from around the sections, put a drop of resin on the tissues and lower a cover glass obliquely onto the resin. A black background aids in seeing and expelling bubbles. If the size of the drop of resin is correctly gauged, there should be no excess resin squeezed out around the edges or over the cover glass. Newly covered preparations must be used with care because the cover glass is easy to dislodge and the tissues may be damaged. Drying new slides in the 53°C. oven for one or more days hardens the resin somewhat and permits safer handling of the slides.

This is a convenient point at which to discuss the repair of damaged slides. It is possible to salvage a slide that has some sound sections as well as some sections that have been damaged by misuse. Place the slide upside down under a low-power objective and locate the damaged sections. Place a mark over each broken section with India ink. Allow the ink to dry thoroughly, and drop the slide into a jar of xylene. After the cover glass has slid off, rub off the damaged section with a matchstick, rinse in xylene, and mount a new cover glass.

Destaining and Restaining

Slides may be examined for color at several stages in the staining process, in fact from any reagent that is not so highly volatile that the preparation becomes dry during a brief examination. See page 63 for the procedures used to increase or decrease the intensity of the color imparted by hemalum. If the slide is examined out of xylene or carbol-xylene and the stain intensity needs to be increased or decreased, transfer the slide backwards through the dehydrating series to water, and proceed with corrective measures.

It may be necessary to modify the stain intensity of a finished slide

that has had a cover glass affixed with balsam or synthetic resin. The cover can be loosened and allowed to slide off by immersing in a jar of xylene as long as necessary. After the cover has slid off, transfer the slide to the absolute alcohol after the de-waxing xylene, run down to water and proceed with the restaining or destaining process.

HEMALUM (REGRESSIVE)

The destaining action of acids on hematoxylin is selective,—cytoplasm is destained more rapidly than cell walls, plastids, and nuclear structures. This fact makes it possible to use a self-mordanting hematoxylin as a stain that is adequately differential for many subjects. The slides are purposely overstained in Delafield's, Harris', or Mayer's hemalum, then destained in acid until the proper contrast is obtained.

The foregoing single stain, using a self-mordanting hematoxylin formula, either as a progressive or regressive stain, deserves more extensive use for routine diagnostic examination of research material. An enormous amount of time and energy can be spent in applying elaborate multiple stains to large numbers of slides, many of which are discarded after a moment's examination. In such a series of slides, stained with a single stain, the few slides having the desired stage can be easily restained if a more diagnostic differential stain is needed.

HEMALUM WITH A "GENERAL" COUNTERSTAIN

The foregoing hemalum stain can be supplemented by a *counterstain,* a stain that has little specific selectivity, but furnishes optical contrast for the principal stain. A counterstain is introduced into the staining series at a place having approximately the same water concentration as the solvent of the counterstain. One of the most useful counterstains is erythrosin. The stock solution contains ½% stain dissolved in 95% alcohol. Referring to Staining Chart II, note that the slide, previously stained to the correct intensity in hemalum, rinsed and alkalized, is put into erythrosin after 95% alcohol. The interval in erythrosin must be determined by trial and may range from a few seconds to 1 hr. This counterstain is removed from different types of material in variable degree by the subsequent dehydration. The final intensity of the pink counterstain depends on the tenacity with which the tissues retain the stain. If the pink color is too dark, it will obscure some of the details stained blue by the hematoxylin. Excess counterstain can be removed by running the slide back to 50% alcohol. More pink can be added as shown on

Staining Chart II. The same slide can be repeatedly destained or restained in the counterstain until exactly the desired effect is obtained. The hematoxylin is not affected during this manipulation.

STAINING CHART II

Hemalum With "General" Counterstain
Pre-Staining Operations and Intervals as in Chart I

Hemalum		resin and
to correct		cover glass
intensity		↑
↓		xylene III
30%		↑
alcohol		xylene II
↓		↑
50%		xylene I
alcohol		↑
↓		carbol-
70%		xylene
alcohol		↑
↓		absolute
95%		alcohol II
alcohol		↑
↓		absolute
erythrosin		alcohol I
(see text)	——→	

Other common counterstains used with the above hematoxylins are orange G, gold orange, eosin, fast green, and light green. The underlying principle for applying other counterstains is the same as for erythrosin. Counterstains may also be dissolved in clove oil and applied after the last dehydrating step, omitting carbol-xylene because clove oil is an excellent clearing agent. Counterstains may also be dissolved in water, 50% to absolute alcohol, or Cellosolve and introduced into the series at the corresponding point of dehydration.

HEMALUM AND SAFRANIN

After acceptable results have been obtained with the foregoing single stain and the double stain, undertake the mastery of a double stain having two selective components. One component of the next double stain to be discussed is a self-mordanting hematoxylin; the second component is safranin, which is highly selective for chromosomes, lignin, cutin, and in some cases for hemicellulose. An important feature of this combination is that the hemalum is applied to the desired intensity and remains fixed throughout subsequent processing, whereas the safranin is applied until the material is strongly overstained and then differentially destained.

Staining Chart III begins with a slide that has been stained in hemalum as shown in Chart I; the slide is then immersed in safranin. The interval in safranin ranges from a few minutes to 12 hr. Some collections of young corn stem require at least 1 hr. in safranin. Wood sections cut in celloidin may take up enough safranin in 5 min. to make destaining difficult. Untested material should be tried at intervals of 10, 30, and 60 min. and 8 to 12 hr. After removal from safranin and rinsing in water, all cells of the section are found to be stained deep red, the blue color of the hemalum being masked. Dehydration and differential destaining are accomplished simultaneously by passage through the alcohol series. Safranin is removed from

STAINING CHART III

Hemalum and "Specific" Counterstain

Pre-Staining Operations as in Chart I

Hemalum to correct intensity
↓
safranin 1–12 hr.
↓
wash
↓
30% alcohol
↓
50% alcohol
↓
70% alcohol
↓
95% alcohol ——→ absolute alcohol
↑
absolute alcohol
↑
carbol-xylene
↑
xylene I
↑
xylene II
↑
xylene III
↑
resin and cover glass

cytoplasm and unlignified tissues by 50 and 70% alcohol and at a slower rate by similar grades of acetone. Higher concentrations of alcohol and anhydrous alcohol also dissolve the safranin, but 90% acetone and anhydrous acetone have slight destaining action. Acetone, therefore, permits easier control of destaining than does alcohol. Lignified tissues, cutin, and plastids retain safranin throughout suitably rapid dehydration. The correct stain has been attained when lignified cell walls are a clear, transparent red and unlignified walls are blue, with little or no reddish tinge. Chloroplasts may be blue, violet, or red. In order to make chloroplasts red enough to show up

clearly, it may be necessary to compromise by leaving too much red in the cellulose walls. If a finished preparation is found to be unsatisfactory, the cover glass can be removed, and the material destained or restained. However, alterations in the intensity of the safranin can be made best after the slide has been examined from carbol-xylene. Carbol-xylene has a very slow destaining action on safranin. Preparations left in carbol-xylene for 4 to 12 hr. show highly critical differentiation of structures having varying degrees of lignification, such as the stratifications in the walls of xylem cells and sclerenchyma.

SAFRANIN-FAST GREEN

The next type of stain combination to be considered has two components, both of which are subject to differential destaining and which react upon each other during dehydration. This staining process is obviously more difficult to control than the preceding processes. As shown in Staining Chart IV, the first stain to be applied is aqueous safranin, in which the preparation is strongly overstained. One hour in safranin is occasionally enough; some woody materials stain well in 5 min. Your previous experience with the hemalum-safranin combination will indicate the safranin-holding capacity of tested materials. The safranin begins to dissolve out during passage through 30, 50, and 95% alcohol. The counterstain, fast green *FCF* in 95% alcohol, is now applied. Both the green stain and its solvent have a differential solvent action on the safranin, and remove it from the unlignified tissues more rapidly than from the lignin, cutin, and chromatin. The interval in green is usually a matter of seconds, rarely as much as 2 min. Correct contrast has been attained when lignin, chromatin, and in some cases cutin are brilliant red, chloroplasts pink to red, and cellulose walls and cytoplasm are green.

The two stains of this combination can be manipulated until the desired contrast and intensities are obtained. If alcohol is used in the dehydrating series, the slide may be placed on a microscope, kept wet with 50% alcohol, and observed until only the lignified elements remain red. The slide is then rapidly carried through the subsequent processes. Acetone is too volatile to permit such examination. With some experience it is possible to judge when the safranin has been destained sufficiently to add the green counterstain. If the stock solution of fast green acts too rapidly for a given subject, the green color will mask or remove the red, and all cells may become stained deep green. In such cases dilute the green stain with 1 to 5 volumes of 95% ethyl alcohol. The slide may be examined best out of carbol-

xylene. If red color is still evident in cellulose walls and cytoplasm, carry the slide backward through the series to fast green, double the previous interval in fast green, run upward again to carbol-xylene and examine. This process can be repeated until the desired color contrast between chromatin, lignified walls, cellulose and cytoplasm is obtained.

If the red color is too pale when the slide is examined out of carbol-xylene, transfer to the de-waxing xylene and proceed as with a new slide. The green will be removed in the down series. If the green is too intense at the carbol-xylene stage, back downward to 70% alcohol, in which the green is removed rapidly. Try 10 seconds and carry up to carbol-xylene again and examine.

Several stains can be substituted for fast green in Chart IV. The most commonly used other green stains are light green and malachite green. Several excellent blue counterstains are cotton blue, methylene blue, gentian violet (crystal violet), and aniline blue. Any of these green or blue counterstains can be used in solution in 95% alcohol, in the sequence shown in the chart, or they may be dissolved in 50% alcohol or in clove oil and introduced at the appropriate place in the series. The above safranin-green or safranin-blue combinations

STAINING CHART IV

Safranin-Fast Green

Pre-Staining Operations and Intervals as in Chart I

Aqueous safranin 1–12 hr.	resin and cover glass
↓	↑
water, change until colorless	xylene III
↓	↑
30% alcohol	xylene II
↓	↑
50% alcohol	xylene I
↓	↑
70% alcohol	carbol-xylene
↓	↑
95% alcohol	absolute alcohol III (optional)
↓	↑
fast green in 95% alcohol 5–30 sec. ⟶	absolute alcohol II
	↑
	absolute alcohol I

serve as excellent cytological stains for many subjects, primarily for the preparation of classroom materials.

THE TRIPLE STAIN (FLEMMING)

The triple stain is of considerable historical interest and is still in high favor in some laboratories. The three components are safranin, crystal (gentian) violet, and orange G (or gold orange). Safranin is intended to stain chromatin, lignin, cutin, and in some cases chloroplasts. Gentian violet should stain spindle fibers, nucleoli during some phases, and cellulose walls. The orange dye acts as a differentiating agent, serves as a general background stain, and stains cytoplasm and in some subjects cellulose walls. All three components are highly soluble in the reagents used in the staining process and are subject to changes of intensity and mutual interaction during most of the process. The correct balance of relative intensities is, therefore, very difficult to control. The process yields spectacularly beautiful slides from the hands of an expert. However, an attractive or gaudy polychrome effect is not adequate justification for the use of an elaborate and time-consuming process. The real test of the desirability of a multiple stain is the specific selectivity of its color components for definite morphological or chemical entities in the cell.

The sphere of usefulness of the triple stain may be judged by a consideration of the stains used in modern cytological research. It is noteworthy that the most critical modern work on chromosome structure and behavior has been done with the iron-hematoxylin stain, with the gentian violet-iodine stain, and with acetocarmine smears. The most reliable work on the spindle-fiber mechanism and spindle-fiber attachment also has been done with the first two stains. As an illustration in the field of anatomy, it will be obvious that in studies of vascular tissues a stain is required primarily to show a xylem-phloem contrast, distinguishing between lignified and unlignified cell walls. This usually is done adequately with a two-stain combination. There is no special virtue in having a delicate orange background for a study of the organization of a vascular bundle or in a section of pine lumber. However, in many cytological problems involving the entire cell rather than merely the actively dividing chromosomes, the triple stain is an indispensable tool. Another legitimate sphere is in pathological studies in which it is desirable to produce polychrome contrasts between a parasite and its host. The object of the above discussion is to emphasize again the view that any elaboration that does not serve a definite, useful function is a waste

of time. The triple stain should be kept in its proper place among the diverse tools of the technician.

The three stains used in the conventional process are the following standard stock solutions:

Safranin O, aqueous, or in 50% alcohol.
Crystal violet, or gentian violet, 0.5 or 1.0% in water.
Orange G, or gold orange, saturated solution in clove oil.

Mordanting is necessary for some subjects. After killing in fluids that contain osmic and chromic acids, mordanting is usually not necessary. For materials that do not retain the stains, mordant for 1 to 12 hr. in 1% aqueous chromic acid or in an aqueous solution containing 2% chromic acid and 0.5% osmic acid.

Staining Chart V is intended primarily to show the sequence of operations in a typical schedule. The variability of the time element

STAINING CHART V

Triple Stain

Pre-Staining Operations and Intervals as in Chart I

Safranin
4–24 hr.
↓
water, 3 changes
↓
crystal violet
10 min. to 1 hr.
↓
water, 3 changes
↓
dip into
50% alcohol
↓
rinse with
95% alcohol
(pipette)
↓
dip into
95% alcohol
↓
2–3 changes anhydrous alcohol → flood sections with orange G → drain off orange G
↑
rinse with used clove oil (pipette)
↑
flood with new clove oil
↑
differentiate under microscope
↑
rinse with clove oil-xylene (pipette)
↑
rinse with xylene (pipette)
↑
xylene
2 changes
(jars)
↑
resin and cover glass

in any of the operations hardly can be overemphasized. The suggested intervals merely furnish a starting point for experimentation to determine the optimum time schedule for any specific subject.

Many modifications of schedule have appeared in the literature.

Variations in the composition and purity of the component stains have necessitated revisions of schedule to suit the currently available stains. Some workers prefer to differentiate the safranin with very dilute HCl or with acidified alcohol before adding the violet. The acid must subsequently be thoroughly washed out with water.

The gentian violet may be almost fully differentiated in clove oil containing no orange G. The orange can then be added progressively and the violet brought to final differentiation in xylene containing 1/10 to ¼ (by volume) clove oil saturated with orange G or gold orange.

Quadruple-stain combinations using four coal-tar dyes are available in some excellent commercial slides. Conant (Triarch) uses a combination of safranin, crystal violet, fast green, and gold orange; Johansen (California Botanical Materials Co.) uses safranin, methyl violet 2B, fast green, and orange G. These complex processes yield striking preparations but are probably unnecessarily elaborate for most tasks. The advanced worker can obtain details of procedure from the excellent service leaflets of the above manufacturers and from the Johansen manual (1940).

Staining processes using coal-tar dyes are entering a most interesting and important phase of development. Many new organic solvents are being produced by synthetic methods. Solvents that have been little more than chemists' curiosities are now being produced in large quantities and are available at reasonable cost. Some illustrations are the higher alcohols, such as the butyl, propyl, and amyl series, ethyl and methyl Cellosolve, trichloroethylene, and many other solvents. The stains themselves are undergoing constant study and improvement. The possibilities of systematic study, or just plain dabbling, should gratify the heart of the most inveterate experimenter.

IRON HEMATOXYLIN

The next stain to be considered is known as Heidenhain's, or iron-alum hematoxylin. The history of this stain and the names of several investigators who have contributed to its development may be found in the literature. This stain is primarily a cytological stain, used especially for chromosome studies, but it is useful for studies on the cell wall, plastids, and in some studies in pathological histology. The formulas of the mordant (iron alum), the stain (hematoxylin), and the destaining agent are given in the stain formulary. The schedule advocated here and outlined in Staining Chart VI is known as the short schedule, or 4–4 schedule; *i.e.*, 4 hr. in mordant, thorough but

quick rinsing in five changes of distilled water at 1-min. intervals, then 4 hr. in stain. The material becomes stained solid black and must be differentially destained. The destaining solution removes the stain rapidly from cytoplasm, less rapidly from plastids, and slowly from chromatin and from the active tips of mycelium. The destaining action should be observed under a microscope and stopped by

STAINING CHART VI

Iron Hematoxylin

Pre-Staining Operations as in Chart I

4% iron alum
4 hr.
↓
dist. water
5 changes
1 min. intervals
↓
hematoxylin
4 hr.
↓
dist. water
3 changes
↓
destaining reagent
until differentiated
↓
dist. water
3 changes
↓
running
tap water
5 min. ——→

↑
30% alcohol
↑
50% alcohol
↑
70% alcohol
↑
95% alcohol
↑
absolute alcohol I
↑
absolute alcohol II
↑
carbol-xylene
↑
xylene I
↑
xylene II
↑
xylene III
↑
resin and cover glass

washing first in distilled water, then by prolonged washing in running tap water. Dehydration and subsequent processing follow as shown on the chart. In the finished slide, chromosomes, the chromatin of resting nuclei, middle lamella, and active mycelia should be blue-black, whereas the cell walls and cytoplasm should be practically colorless. A finished, covered preparation may be destained by soaking off the cover glass, running the slide back through the series into water, then immersing in the destaining solution. If a preparation is

found to be destained too much, it can be run back into water and mordanted and stained again. As in some other staining combinations, restained preparations are seldom as clear in detail as slides stained correctly the first time.

THE CRYSTAL VIOLET-IODINE STAIN

The next stain to be considered is another cytological stain. It is included here because it yields preparations that are valuable in the teaching of some topics. In teaching mitosis at the elementary college

STAINING CHART VII

Crystal (Gentian) Violet-Iodine
Pre-Staining Operations as in Chart I

IKI
15 min.
↓
rinse in water
↓
crystal violet
1–4 hr.
↓
rinse
↓
IKI
15 min.
↓
rinse
↓
50% alcohol
picric acid
30 sec.
↓
95%
alcohol
10–60 sec. ⟶

resin and
cover glass
↑
xylene III
↑
xylene II
↑
xylene I
↑
examine
↑
⅓ absolute alcohol
⅓ xylene
⅓ cedar oil
30–60 sec.
↑
absolute
alcohol II
30 sec.
↑
absolute
alcohol I
30 sec.

level, most teachers prefer longitudinal and transverse paraffin sections, stained to show cell walls and tissue organization as well as the more prominent features of nuclear division. Crystal violet stains the chromosomes a brilliant blue-black, on a colorless, almost invisible, cellular background. Such preparations are a valuable supplement to the standard classroom slide.

The procedure given in staining Chart VII is but one of the numerous variants of the process. Probably no other staining procedure is as responsive to the individual touch of the technician, and a specific procedure should be regarded as a point of departure for developing a personal routine.

The following version recognizes that it is cheaper to rinse out stains and mordants in water than in alcohol, and also prolongs the usefulness of the dehydrating series. The stain is a ¼ to ½% aqueous solution of crystal (gentian) violet. The mordant is aqueous IKI (Chap. 9). The picric acid solution is a saturated solution in 50% alcohol. De-waxing and running down to water are carried out in Coplin jars. However, the slides must be carried one by one through the dehydrating series, which must not contain other stains as contaminants. Therefore, a special dehydrating set should be maintained for the violet process, preferably in wide-mouth screw-capped jars.

Differentiation is accomplished in the alcohol dehydrating series. The slide should be agitated in the fluids with forceps. Observe closely in the 95% alcohol, and when visible color no longer comes out of the sections, move rapidly through anhydrous alcohol. Differentiation should be complete when the alcohol-xylene-cedar oil clearing solution is reached. The function of the oil is to retard evaporation to permit examination. If the cytoplasm is too blue, back down to 95% alcohol. If the blue has been lost from the chromosomes, carry back to water and IKI and restain.

The Tannic Acid-Ferric Chloride Stain (Foster)

This stain is used for meristematic tissues, in which it stains the thin cell walls. Because of the simplicity of the schedule, no chart is necessary. The reagents that bring about the staining are as follows:

1. Tannic acid, 1% aqueous, with 1% sodium benzoate as a preservative.
2. Ferric chloride, 3% aqueous solution.

The procedure from water is as follows:

1. Tannic acid 10 min.
2. Wash thoroughly in water.
3. Ferric chloride, 2–5 min.
4. Wash in water, and examine with microscope.

Repeat steps 1 to 4 inclusive until the cell walls are sharply outlined. Nuclei may be stained in safranin if desired, using aqueous safranin, or safranin in 50% alcohol.

Variations of the fundamental method are described by Northen (1936). This stain is likely to undergo further modification and will probably become one of our most useful histological stains.

The foregoing outline of the elements of staining processes is likely to be adequate for the average needs of students and teachers

and for many research problems. The beginner is warned not to dabble in a wide variety of processes but to gain a mastery of a few fundamental methods. Ability to analyze and remedy difficulties should be cultivated. The advanced worker who finds that a research problem requires more specialized methods should turn to the literature and search out methods that have been used for similar investigations.

Some of the processes described in this chapter have been used in cytological research for many years. These methods have been replaced by smear and squash techniques, ultra-thin sectioning, and electron microscopy in studies that are concerned primarily with the structure and behavior of chromosomes within a cell. Nevertheless, the well-established "thick" sectioning and staining techniques are still indispensable for cytological studies in which it is imperative to keep intact the organization of tissues and organs. Some examples are the study of meristems, ovules, embryology, as well as tumors and other aberrations induced by chemical agents, irradiation, or invading organisms.