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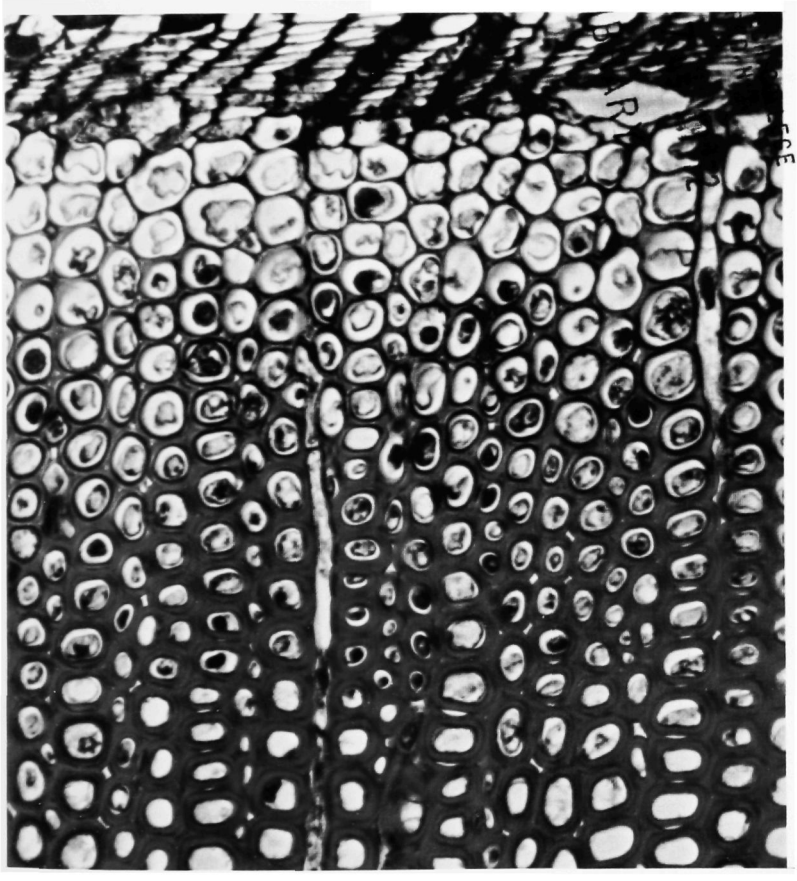
The suitability of certain stains for studying lignification in balsam fir, *Abies balsamea* (L.) Mill

Kutscha and Gray



Technical bulletin 53 March 1972
Life Sciences and Agriculture Experiment Station

Cover photo: Safranin and aniline blue, showing blue unlignified cambium (top of photo) and contrasting red lignified tissue (lower part of photo). Immature, secondary walls appear blue-blue green and can be seen approximately five to eight cells down from the cambial region. Section of FAA-killed and celloidin embedded compression wood sample collected July 6, 1966; X 320.

ABSTRACT

An investigation was conducted to examine the suitability of ten staining reactions for studying lignification in balsam fir, *Abies balsamea* (L.) Mill. Two experiments were carried out on material collected on two different dates. In each experiment slides of fresh, FAA-killed and FAA-killed celloidin-embedded material of normal and compression wood were stained and evaluated.

No significant difference in staining reactions was found between material collected on different dates. In each experiment, the embedded material showed somewhat superior results compared with the fresh and FAA-killed material with at least half of the stains. No marked difference was observed between normal and compression wood.

This study emphasized the need for considering each of the ten staining reactions on an individual basis, since each has particular advantages and disadvantages as emphasized throughout the study. Staining schedules were prepared and tables compiled to determine the suitability and characteristics of the various stains and their color reactions.

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THE SUITABILITY OF CERTAIN STAINS FOR STUDYING LIGNIFICATION IN BALSAM FIR, *ABIES BALSAMEA* (L.) MILL.[1]

Norman P. Kutscha[2] and James R. Gray[3]

INTRODUCTION

Lignin ranks second only to cellulose as the most abundant natural product on earth. As such, it constitutes one of the largest natural resources available to man. It can be found in various forms, depending on where it is obtained and how it is isolated. While its chemical structure is complex, it is now fairly well understood, as are its basic chemical properties.

Wood can be reduced to its individual cellular components by various processes which remove the lignin. On an industrial scale, a large percentage of the lignin removed is discarded and these processes can result in undesirable stream and air pollution. While numerous uses have been developed for lignin, many additional uses are still possible and desirable.

The biochemical formation of lignin and the anatomical aspects of the lignification process within the cell are still not completely known. Neither the distribution of lignin in the cell wall nor its effect on cell wall properties has been completely established. While various microscopical techniques are currently available for distinguishing the lignification process, refinement of these techniques is necessary. Genetic and environmental factors affecting the lignification process are also not well understood at present.

In order to be able to control the production of lignin in nature, one must thoroughly understand the lignification process, including the manner in which lignin is metabolized, how it is layed down in the cell wall, and where it is located in the wall. In order to isolate lignin most easily its chemical structure and its relationship to the other wall components must be resolved. To be able to use lignin more effectively, one must examine its properties in both the natural and isolated state. The most effective use of lignin-containing materials depends on understanding the extent to which the properties of these materials are affected by the properties of lignin.

Perhaps one of the oldest and simplest methods of studying the lignification process is the use of stains in connection with the light microscope. This study was conducted to determine the suitability of certain

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new as well as well-known stains for studying the lignification process of tracheids in fresh, FAA-killed and FAA-killed and embedded tissue of normal and compression wood in balsam fir, *Abies balsamea* (L.) Mill. This work should facilitate further studies on lignification in balsam fir. Balsam fir was the species chosen since this work is a continuation of earlier research initiated on this species (Kutscha, 1968). Also, balsam fir is a commercially important species within the state of Maine and was readily available for sampling purposes. The major staining reactions and some new reactions that have been reported in the literature to be lignin-specific were examined and, where necessary, usable staining schedules for each stain devised. Ten stains were chosen and evaluated, according to predetermined criteria, using the three types of tissue preparation mentioned above. Tables were then compiled indicating the suitability of each stain for the particular types of material tested and the characteristics of the various stains and their color reactions.

It is hoped that the results of this study will be of interest not only to botanists and wood anatomists who deal with lignified plant tissues, but also to organic chemists and biochemists who are concerned with the chemical structure and biosynthesis of lignin.

In this study, the term "stains" will be used to collectively denote stains, dyes, reagents, chemicals and any other similar materials used to detect and delineate lignin.

LITERATURE REVIEW

Staining Reactions of Wood

Almost all the components of wood will produce colored products upon application of selected reagents (Browning, 1967). In the past many workers have felt that these reagents were unreliable as specific indicators for such components as cellulose and lignin, particularly in developing tissue. Currently, however, certain reagents are widely used for location and identification of chemical constituents including polysaccharides, lignin and extractives (Browning, 1967). Care must be taken when applying these reagents to whole wood which contains these constituents in combination. Sometimes it is necessary to remove one or more of these constituents in order to accurately detect the remaining constituents or to use multiple tests. One must also keep in mind that the chemical nature of a particular constituent within the cell wall may be quite different than its nature after removal from the wall.

Problems in Detecting Lignin

Numerous problems arise when trying to use color reactions to specifically detect the [illegible] woody tissue

One problem is that of trying to perform a test for a compound whose chemical structure is not completely known, particularly as it exists in the cell wall in the form of native lignin or protolignin. This is especially true when using a test which reacts to the presence of one particular chemical group.

It is well known that lignin is a highly branched three dimensional polymer of high molecular weight made up of phenol propane units. However, complexity of the structure is such that its presence is difficult to ascertain. Browning (1967) lists seven "lignin criteria" as established by Kratzl (1953, 1956) and Kratzl and Billek (1957) which are required to define a substance as lignin. It has further been suggested that lignin demonstrates certain spectral absorption bands and that its composition lies within the following formula (Freudenberg, Harkin, 1964):

$$C_9H_{8-OCH_3}O_2[H_2O]_{<1}[OCH_3]_{<1.5}$$

Another problem is that of being able to perform the tests on fresh untreated tissue in order to avoid as much change or removal of the lignin as possible (Srivastava, 1966). This is not always possible and often the tissue must be fixed or treated in some other way in order to preserve it until tests and observations can be performed. One technique which should allow few changes is that of using a freezing microtome (Wardrop, 1969).

Still another problem is that of extractives. Since for many color reactions the nature of the reaction products is unknown, it is possible that some of the reactions may be due to the presence of extractives. Brauns (1952b) recommends removal by a benzene-alcohol mixture followed by water.

Problems can arise due to differences in the composition of the lignin present. If the lignin is removed from the tissue or if the tissue is treated in some way, the original composition of the lignin may have been altered. Differences in gymnosperm lignin, dicotyledonous angiosperm lignin and monocotyledonous angiosperm lignin have been established. Work by Northcote (1958) and more recently by Sarkanen and coworkers (1967) has indicated that lignins may vary from one wood species to another and also within the same species. Other work has suggested that there is a difference between the lignin obtained from juvenile and mature wood (Choulet *et al.*, 1965), from sapwood and heartwood, from earlywood and latewood (Goring, 1964) and from inner bark and outer bark (Swan, 1966). With regard to reaction wood, lignin in compression wood, but not in tension wood, differs chemically from normal lignin to [illegible] 1961). Similarly,

Srivastava (1966) concluded that the composition of lignin may vary from plant to plant and among different cells of the same plant, these variations possibly being related to different proportions of aldehyde moieties in the lignin molecule. Many of these differences are significant in the fact that while they are revealed histochemically, they may readily be undetected in chemical analyses of whole bark and wood samples. These differences are also important in any work concerned with the location and biosynthesis of lignin.

As pointed out by Balatinecz and Kennedy (1967), many researchers have cautioned against the use of histological staining techniques to determine the chemical composition of the cell wall, including lignin. The bases for most of the lignin color reactions are not completely understood, and a negative reaction does not necessarily mean that lignin is absent (Jensen, 1962). Discretion must be used with these reactions to obtain meaningful results and it is advisable to use several methods on the same material in order to be reasonably sure of detecting lignin (Srivastava, 1966; Balatinecz, Kennedy, 1967).

Staining Reactions of Lignin

Lignified plant tissues and certain isolated lignin preparations give characteristic color reactions with numerous organic and inorganic reagents. In the early nineteenth century, Runge (1834) treated spruce wood with phenol and hydrochloric acid, which gave a greenish-blue color and with aniline sulfate, which yielded a yellow color. Since then, a large number of color reactions, which are characteristic of lignified material, have been determined. While many of these color reactions have been listed by other authors (Phillips, 1944; Hägglund, 1951; Brauns, 1952a, 1952b; Brauns and Brauns, 1960), examination of the current literature reveals numerous other reagents or variations on previously used reagents. Lignin color reactions have recently been of interest mainly for their assistance in studying lignification, lignin distribution in the cell wall and in elucidation of the chemical nature of lignin.

There are various ways in which lignin color reactions may be classified. Pfoser (1959) indicates that according to Kratzl we can distinguish between wood coloring materials and materials which react with the wood, such as the uncolored agents which chemically change the lignin. According to Brauns (1952b) the coloration produced by dyestuffs is caused, not by the chemical reaction of a specific active group in lignin, but by its amphoteric character and its colloidal properties. Since the assimilation of dyestuffs by organic substrates such as cotton is often caused by a sorbtion reaction, Wedekind and Garre (1928) studied the behavior of lignin toward acid and basic dyestuffs. Their results indicated that lignin poss[illegible] charge, is acidic in nature and

therefore reacts with basic dyes which cannot be washed out with hot water. The acidity is apparently due to its phenolic hydroxyl groups (Roelofsen, 1959). In this regard lignin differs widely from cellulose which does not permanently fix acidic or basic dyes (Brauns, 1952b). Brauns (1952b) and Brauns and Brauns (1960) have classified the various lignin color reactions as being due to organic compounds (aliphatic, phenolic, aromatic amines, heterocyclic), inorganic reagents and dyestuffs. Browning (1967) has classified the reactions as being accounted for by mineral acids, methanol-hydrochloric acid, phenolic compounds, amines, heterocyclic compounds, carbonyl compounds and the Mäule test.

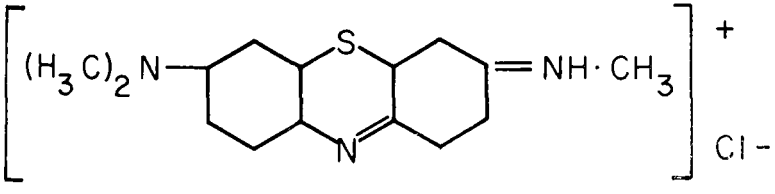
Some Common Lignin Stains

Certain stains have been used more often than others for detecting lignin. Some of the more common ones, or ones that have been used more recently, include:

Azure B
Benzidine
Iodine-Malachite Green
Potassium Permanganate
Mäule Reaction
Phloroglucinol
Safranin-Aniline Blue
Safranin-Celestine Blue B
2-Thiobarbituric Acid
Toluidine Blue O

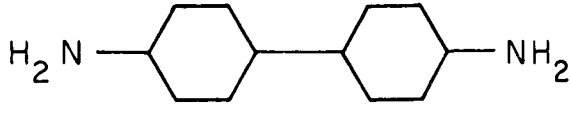
A large number of other stains and reagents used to detect lignin are available. Over 30 are cited in recent literature.

Azure B. Azure B is a long established stain which was first recognized by Kehrmann in 1906, but received little recognition until 1940 (Lillie, 1969). It has been little used by botanists but shows great promise as a stain for nucleic acids and for lignin (Jensen, 1962). Azure B, also known as methylene azure, is a basic, violet-blue dye of the thiazine group which is prepared by the oxidation of methylene blue with potassium bichromate. It has a formula of $C_{15}H_{16}N_3SCl$ and the following structure (Gurr, 1960).



Azure B, like toluidine blue O, is a metachromatic dye in that different colors are produced in the same specimen. Metachromatic dyes usually have two absorption peaks, with a third appearing as the concentration of the dye increases (Jensen, 1962). The color of the dye solution is called the orthochromatic shade. The second color, formed in certain parts of the cell, is called the metachromatic shade. With azure B the metachromatic shade is dark blue and the orthochromatic shade is blue-green. For example, RNA will stain blue, DNA blue-green. Cuticle and sieve plates will appear reddish, comprising the third color peak. Cell walls will not stain unless they contain lignin, in which case they appear green. Apparently the metachromatic nature of lignin when using azure B is little known or appreciated (Jensen, 1962). Since lignin is acidic, due to its phenolic hydroxl groups, it reacts with basic dyes such as azure B and safranin (Roelofsen, 1959). Lignin stains a clear blue-green with azure B whereas the other components of the wall do not react (Jensen, 1962).

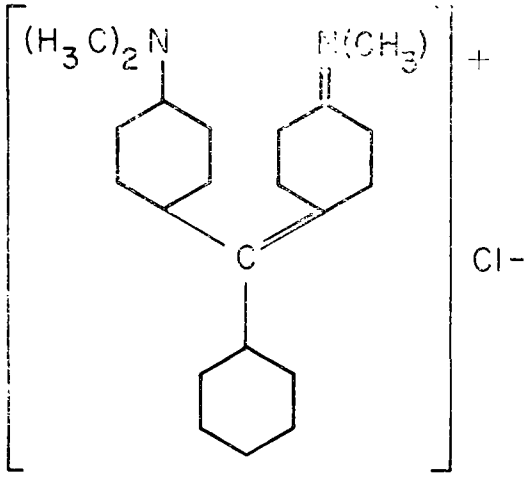
Benzidine. Benzidine was first suggested as a lignin-specific stain by Schneider in 1914 and again by Lominski and Hutchison (1948). Jellum (1960) described a benzidine technique which produced highly satisfactory results in oats and indicated that the technique was also suitable for staining lignin in wheat, barley and other agronomic crops. Benzidine can be classified as an aromatic amine and has the following formula, $(p\text{-}H_2NC_6H_4)_2$ and a structure as follows:



Adler and coworkers (1948, 1948) have felt that in the reaction of amines with lignin, as in the reaction of phloroglucinol with lignin, the coniferyl aldehyde groups are responsible for the color exhibited by wood. Lominski and Hutchison (1948) found that a colorless solution of benzidine gave an orange or yellow color to lignified cell walls and caused no color change in cellulose or in cutinized or suberized walls. Jellum (1960) also found that lignified cell walls stained orange to yellow with the middle lamella staining a dark orange.

Iodine-malachite green. Iodine-malachite green as a staining combination for lignin has been discussed by Bucher (1964, 1968). Apparently malachite green had been known for some time to stain lignin, but Bucher developed a new procedure using iodine together with several other compounds. Malachite green, also known as emerald green, light green E or N, Victoria green WB or China green, is a bluish-green basic

dyestuff of the triphenyl-methane series with a formula $C_{23}H_{25}N_2Cl$ and the following structure (Johansen, 1940; Gurr, 1960):



Lignin binds with basic triphenyl methane dyes such as malachite green. Such stains dye lignified fibers effectively and the color is water proof, whereas nonlignified fibers will not be so colored and the stain can be completely removed by cold water. The procedure worked out by Bucher (1968) is based on the formation of iodine-malachite green as follows: stannic chloride forms a complex compound with that portion of malachite green which is bound to lignin; on addition of iodine-zinc chloride, stannic chloride changes to stannic iodide which in turn transforms the malachite green to the dark brown, opaque, almost insoluble iodine-malachite green. Lignified parts turn brown; cellulosic parts, blue.

Potassium permanganate. Potassium permanganate was probably first used to indicate the presence of lignin in the work of Crocker (1921). More recently, it is commonly used as a fixative for electron microscopy (Mollenhauer, 1959; Bland *et al.*, 1969) although it has also been used in connection with light microscopy (Scurfield, 1967). More recently, Hepler *et al.* (1970) have reported that, when used as a fixative, potassium permanganate reacts with the lignin component of the cell wall and thus can be used as a highly sensitive electron stain to follow the course of lignification during secondary wall deposition. It is well known that the inorganic reagent, potassium permanganate ($KMnO_4$) is a strong oxidizing agent. It is capable of cleaving -C-C- linkages between adjacent CHOH-groups in polysaccharides to produce CHO-groups, and of oxidizing these groups further to COOH groups, thus suggesting that the

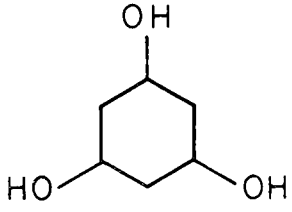
failure of $KMnO_4$ to fix cytoplasmic organelles may be the difficulty with which it penetrates cell walls with which it reacts (Scurfield, 1967). Potassium permanganate is also capable of oxidizing the lignin in which case the potassium permanganate is reduced to manganese dioxide (MnO_2) (Crocker, 1921). In fact, experiments with model compounds showed that phenols, especially di- and trihydroxy phenols, rapidly reduced permanganate to manganese oxides (MnO_2, Mn_2O_3) (Bland *et al.*, 1969). This work also suggested that the most numerous reactive groups in lignin would be the free phenolic syringyl and guaiacyl groups and that fixation of wood sections by potassium permanganate is dependent on the presence of phenolic hydroxyl groups. It is assumed then that the actual electron opacity (for electron microscopy) or color reaction (for light microscopy) is due to the MnO_2 which complexes with the lignin (Hepler *et al.*, 1970). Potassium permanganate fixation of wood shows a high concentration of lignin in the compound middle lamella region as well as a distribution of discrete particles throughout the secondary wall (Bland and Menshun, 1967). Staining of the compound middle lamella even takes place after prior treatment with organic aldehydes (Wardrop, 1965; Cronshaw, 1965). Bland and coworkers (1969) have shown that potassium permanganate does react with compounds such as formaldehyde. Hepler and Newcomb (1963) have shown that the electron density becomes progressively deeper during maturation of the wall in primary xylem. In studying differentiating wound-vessel members, Hepler and coworkers (1970) found that potassium permanganate stained only the secondary wall. Its failure to stain the primary wall, except where the secondary wall was attached, suggests that the permanganate does not stain cellulose, hemicellulose, pectin or protein. When using potassium permanganate in connection with light microscopy of developing tracheids, Scurfield (1967) found that the primary wall and S_1 layer stained a deep brown prior to the development of an S_2 layer, and brown following the presence of an S_2 layer. The S_2 layer stained brown when the protoplast was present and varied between pale brown and brown when the protoplast was absent.

Mäule reaction. The Mäule reaction is commonly used to differentiate the woods of gymnosperms and angiosperms because of a difference in the chemical makeup of their lignins. The basis for the reaction was observed by Mäule in 1900 who used potassium permanganate, hydrochloric acid and ammonia to obtain a deep rose-red color with woods of several species although he seemed not to have realized that the results could be used to distinguish between angiospermous and gymnospermous woods, this being demonstrated by later researchers. The Mäule reaction has found wide practical use and has been studied exten-

sively. It has been used, for example, on large numbers of species (Gibbs, 1957) and to distinguish between hardwood and softwood fibers in mechanical pulp (Spearin, Dressler, 1954). It has also been compared with other lignin reactions (Ullrich, 1955; Srivastava, 1969). Apparently it has been used extensively to study the lignification process.

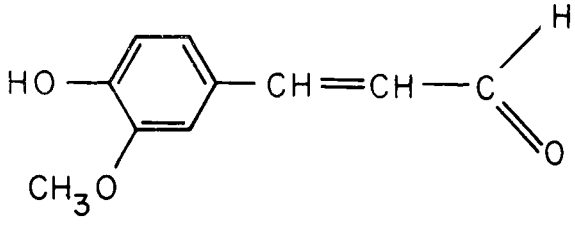
Considerable work has been done on the chemistry of the Mäule reaction, but since several reactions are involved it still has not been fully elucidated (Browning, 1967). A positive test (red coloration) is produced only for those woods which contain syringyl units (Gibbs, 1957). Since all angiosperms contain syringyl units and most gymnosperms do not, it is possible to distinguish between woods from the two different groups. Angiosperm woods produce an intense purple-red color while gymnosperm woods yield an indefinite brownish shade (Brauns, 1952b). No reaction was obtained with cambial cells in aspen (Sultze, 1957) or pine (Srivastava, 1969), but work has shown that the Mäule reaction is good for slightly lignified cell walls, until they become more lignified and reach a stage where the phloroglucinol reaction works better (Pfoser, 1959).

Phloroglucinol. Phloroglucinol has frequently been used by botanists to detect lignin in plant tissue. A solution of phloroglucinol in strong hydrochloric acid is known as Wiesner reagent (Wiesner, 1878). In recent years this reaction has been used in numerous studies on lignification by such workers as Barskaya (1962), Bland (1966), Srivastava (1966), Balatinecz and Kennedy (1967), Scurfield (1967), Wardrop (1969) and Srivastava (1969). Phloroglucinol, also known in the past as phloroglucin, is a phenolic compound with a formula of 3, 5-$(HO)_2C_6H_3OH$ and the following structure:



It is a white or yellowish crystalline powder which becomes darker on exposure to light, as does its solution, although this does not impair its efficiency as a reagent or stain (Gurr, 1960). The phloroglucinol reaction has been ascribed to the formation of a condensation product between ph[illegible] which contain the

coniferyl aldehyde grouping (Pew, 1951; Brauns, Brauns, 1960; Roelofsen, 1959):

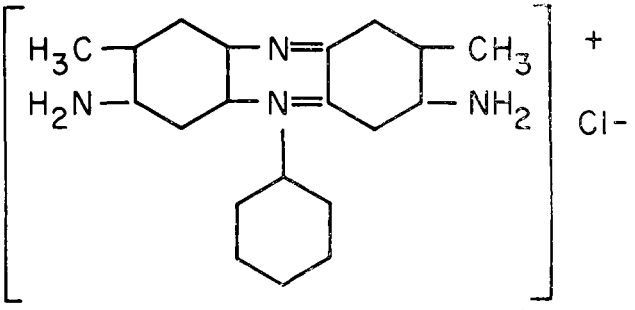


Others have felt that the reaction is an indicator of the side chain -CH = CH-CHO attached to an aromatic nucleus such as occurs in the presumptive lignin precursors coniferaldehyde and *p*-coumaraldehyde (Adler *et al.*, 1948; Bland, 1966). The latter agrees with the fact that a positive reaction is no longer obtained after specimens are treated with aldehyde-blocking agents such as hydroxylamine hydrochloride and phenylhydrazine or oxidizing agents such as osmium tetroxide, potassium permanganate, periodic acid and chlorine water (Scurfield, 1967). While extractives removed from the specimen may yield a positive phloroglucinol reaction, suggesting that extraction may be advisable (Roelofsen, 1959; Brauns, 1952b), in two studies the extraction failed to remove the substances responsible for the phloroglucinol-positive reaction in the specimen (Rassow, Gabriel, 1931; Balatinecz, Kennedy, 1967).

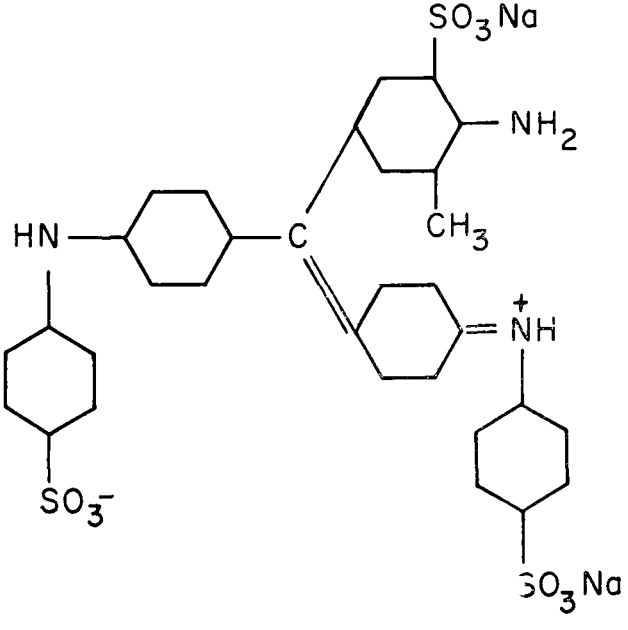
Numerous workers have reported on the color produced by the phloroglucinol reaction. Generally, the color is a red-violet (Johansen, 1940). Black and co-workers (1953) have shown that the reaction produces a red-purple with coniferyl aldehyde, a blue-purple, with sinapyl aldehyde and a peach color with syringaldehyde. In studying developing tissues, cambial cells produce no color (Srivastava, 1969). The chemical changes associated with lignin deposition do not spread throughout the S_2 layer by the time the cell protoplast disappears, though substances which give a red color with the phloroglucinol reaction have done so (Scurfield, 1967). In developing xylem, the color reaction of lignified xylem elements begins with bright red and ends with a particularly low intensity cherry red (Wimmer, 1948). In studying collenchyma, Wardrop (1969) found that the secondary wall gave a strong positive reaction for lignin by the Coppick and Fowler method and an intense red with the chlorine water-sodium sulphite method but only a pale pink with the phloroglucinol method.

Safranin-aniline blue. Safranin-aniline blue utilizes safranin which is perhaps the most important stain known to botanists (Johansen, 1940). Ehrlich and co-workers [illegible] to

microtechnique by Hermann (1893). It has been used to stain lignified tissue for many years (Jensen, 1962) and can be employed in combination with a variety of contrast stains (Lillie, 1969). It is often used in combination with fast green (Balatinecz, Kennedy, 1967) but may also be effectively used with aniline blue, particularly for gymnosperm tissue (Johansen, 1940; Jensen, 1962). Hyland (1970) has used it extensively with aniline blue. Safranin, also known as safranin O or carthamine, is a red, basic, cationic dye of the azine group with a formula of $C_{20}H_{19}N_4Cl$ and the following structure (Gurr, 1960):

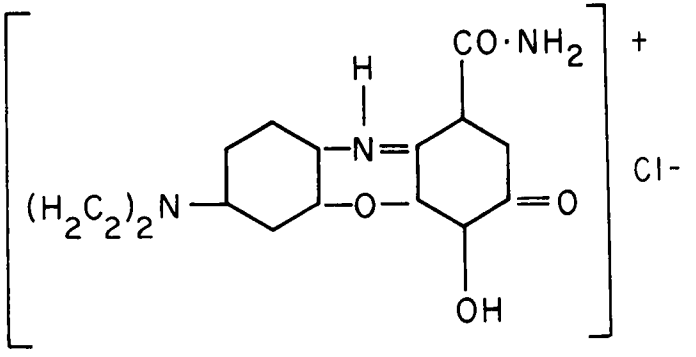


Aniline blue (aqueous) is an acid anionic dye of the triphenyl-methane series with a formula of $C_{32}H_{25}N_3O_9S_3Na_2$ and a structure of (Gurr, 1960):



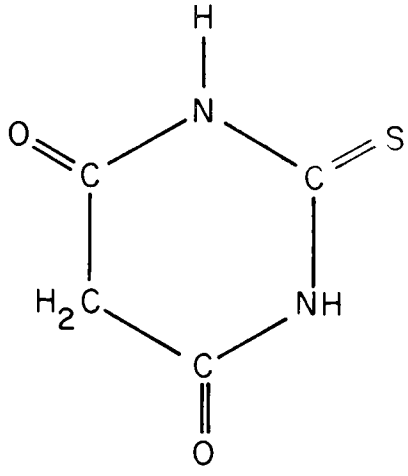
Since lignin is acidic, due to its phenolic hydroxyl groups and safranin is basic, one would expect lignified tissue to stain with safranin (Roelofsen, 1959). However, since acid alcohol will effectively remove all the safranin if applied long enough (Jensen, 1962), one would expect that there would be no chemical reaction between the two resulting in a permanently colored product. Furthermore, safranin is certainly not specific for only lignin since it will also stain nucleoli and chromosomes as well as cutinized, suberized and chitinized structures (Johansen, 1940). Nevertheless, there does seem to be a close correlation between tests with safranin and other more specific methods (Wilcox, 1964); also, Dadswell (1940) found a high correlation between the amount of lignin in the secondary wall and its affinity for safranin. Certainly, while not being used as a specific test, it should be of value in routine examination in connection with lignification studies (Wardrop, 1969). When using safranin with aniline blue, lignified and cutinized cell walls, nucleoli and chromosomes stain bright red while cellulose cell walls and cytoplasm stain blue. At certain developmental stages, some cell walls are stained sharply with the safranin while other portions will appear a faint blue. Scurfield (1967) noted that the extent to which the S_2 layer stained increased with increasing distance of the cells from the cambium.

Safranin-celestine blue B. Safranin-celestine blue B is a unique combination in that it employs a mordant for safranin, which apparently has not been commonly recommended and it utilizes one of the oxazine dyes, none of which appears to have been used for plant histology until the work of Gray and Pickle (1956). Celestine blue B, also known as celestine blue R, is a basic dye of the oxazine group with a formula of $C_{17}H_{18}N_3O_4Cl$ and the following structure (Gurr, 1960:



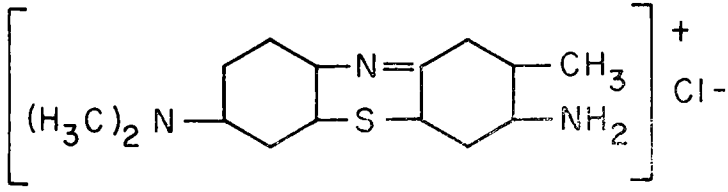
Lignified cell walls stain scarlet while cellulose cell walls are blue-black (Gray, Pickle, 1956).

2-thiobarbituric acid. T-thiobarbituric acid has been used to detect ignified tissue by Bernheim and coworkers (1951) and more recently ›y Drozdz (1964). This acid is heterocyclic compounl with a formula ›f $C_4H_4O_2N_2S$ and the following structure:



Bernheim and coworkers (1951) feel that the reaction is due to the ›resence of aldehyde groups in the lignin. They obtained an orange, ›range-red or red color with a large number of lignified plant tissues. Drozdz (1964) ind.cated that lignified walls turned orange to orange-red.

Tolu.dine blue O. Toluidine blue O, like azure B, is a metachroma-.ic stain which has the ability to impart d.fferent colors to different cell ›tructures (Johansen, 1940; Jensen, 1962). It is commonly used in ›nimal histology but to a lesser extent with plants (Johansen, 1940; Jensen, 1962; O'Brien *et al.,* 1964). The abil.ty of toluidine blue O to ›olychromatically stain plant cell walls has been known for many years (Czaja, 1934). It has most recently been used by O'Brien and co-workers (1964) and Srivastava (1969). Toluidine blue O, also known as methy-ene blue T50 or methylene blue T extra, is a bluish-violet basic stain of he thiazine series with a formula of $C_{15}H_{16}N_3SCl$ and a structure of (Gurr, 1960; Conn, 1961):



O'Brien and co-workers (1964) found generally that lignified walls of tracheary elements stained green or bluish green while unlignified compound middle lamellae stained reddish purple or red. Srivastava (1969) found that cambial cells stained dark red.

MATERIALS AND METHODS

Collection, Processing and Sectioning of Samples

Embedded material. The celloidin-embedded samples used in this study were originally collected in conjunction with an earlier investigation carried out in Syracuse, New York (Kutscha, 1968). These samples were collected approximately each week throughout an entire growing season between March 28 and September 30 from trees producing both normal and compression wood. The samples used in this study were those collected on May 30 and June 21, 1966, in the Heiberg Forest, Tully, New York. The normal wood samples were taken from the same tree in a spiral fashion, starting at a height of approximately two meters and moving downward. This technique was used to disturb the cambium as little as possible in succeeding samples. By sampling the same tree, the possible factor of tree-to-tree variation was eliminated. The compression wood samples, however, were taken from different trees since there is only a small area of compression wood on each tree from which samples could be cut. If succeeding samples were cut on the same tree, they would have to be cut above and below the first sample and thus include tissue from a markedly disturbed cambium. The samples were cut from the tree by first taping a 3 x 5 inch card, in which a 20 x 40 mm. hole had been cut, on the tree. This card served as a template for razor blade cuts made through the bark, cambium and developing xylem. The sample was then excised by removing the surrounding area with a sharpened screwdriver and splitting out the sample with a chisel.

The samples were subdivided and either fixed in the field or placed in an insulated container, to prevent any radical change in moisture content and temperature, and fixed in the laboratory. The samples were killed and fixed in FAA (formalin, acetic acid and ethanol), and embedded in celloidin. This method of preparing bark-cambium-wood specimens has been a preferred technique for many years (Johansen, 1940). The particular celloidin embedding schedule used has previously been outlined (Kutscha, 1968). After celloidin embedding, all the samples were stored in capped shell vials in a thick celloidin solution to allow further impregnation of the samples. The celloidin solution eventually solidified with loss of solvent.

Before sectioning, the samples had to be affixed to wooden pegs by celloidin in order to facilitate sectioning with the sliding microtome. One-

half inch wooden dowelling was sawed into approximately one inch pegs. These pegs were progressively embedded in celloidin by transferring them in celloidin solutions of 2, 4, 8, 12, 16 and 20% with a 24-hour impregnation time in each solution. It was only necessary to impregnate the pegs a few millimeters on the end. Once the pegs were impregnated, the samples to be sectioned were taken out of the shell vials and the excess of hardened celloidin carefully cut away. A drop of 20% celloidin solution was placed on both the peg and the sample, and the two were worked together to form the best bond possible. In all cases, the sample was considerably smaller than the peg it was being fastened to, which enabled one to pour a jacket of heavy celloidin over the sample to help hold it firm during sectioning. To accomplish this, a piece of paper, approximately 2 x 4 inches, was wrapped around the peg so that it was higher than the sample and was held by a rubber band (Hyland, 1970). It was then an easy matter to pour the celloidin in around the sample until it was completely covered. The fresh celloidin was allowed to harden in the air for 15 to 30 minutes to shrink the celloidin. The samples were then placed in chloroform for 24 hours before being stored in a glycerin-100% alcohol (1:1) mixture.

Sections were cut at 15 microns on a sliding microtome and stored in glycerin on glass slides in dust-free boxes.

Fresh material. Fresh samples of normal and compression wood were cut from three healthy trees from the University of Maine Forest on Marsh Island, Old Town, Maine, on two separate dates, June 4 and July 1, 1970. The normal wood tree, which was used for both collection dates, was selected by considering such factors as diameter, site, straightness and percent of live crown. It was desirable to have a tree large enough that a sample could be cut from it without having too much curvature in the sample and so it would be possible to get a sample large enough to cut several specimens from it without causing severe injury. Site and live crown ratio gave an indication of the growth rate without actually cutting into it. A relatively fast growing tree was desired in order to be able to study the suitability of selected stains in regard to actively lignifying tissue. A slow growing tree could have a cambium producing so few cells that the lignifying tissue might be reduced to a region of one or two cells, thus making it impossible to evaluate a stain's potential for differentiating lignin in developing tracheids. Straightness of the bole would suggest that normal wood was being produced. The same techniques were used to locate compression wood trees, one for each collection date. Considerable time was spent to select trees that were leaning, or that had bent over for several years, thus having a curved portion near their [illegible]od.

Once a tree had been selected, the samples were taken by making two parallel cuts perpendicular to the grain approximately 1½ inches apart and 1½ inches deep. This was done with a freshly sharpened bow saw, being very careful to cut rather than rip, the bark and gelatinous cambium. The sample was then extracted with a sharp wood chisel and immediately put in distilled water for return to the laboratory. These samples were kept in distilled water in a refrigerator at 9°C until staining was conducted. Four or five smaller blocks, approximately ¼ x ¼ x ½ inches, were cut from each of the larger samples, using a small laboratory band saw, and then sectioned.

Sections of fresh wood were cut on a sliding microtome at 15 microns. Thus, the sections were thin enough to allow good resolution at high magnification and yet strong enough to remain intact during cutting. Throughout the remainder of this discussion, the angle between the edge of the knife and the side of the block will be referred to as the knife angle. The angle formed between the top of the block to be cut and the cutting facet of the blade will be referred to as tilt.

All samples were cut with a tilt of one and one-half units as indicated by the scale on the carriage of the microtome, and a knife angle adjusted so that cutting proceeded throughout as much of the blade's travel as possible. Furthermore, the block was positioned in the chuck so that the bark, cambium and wood were cut simultaneously, with the bark before the wood so that the knife motion held the bark against the gelatinous cambium. In this position, the bark, which was the hardest to cut because of the abundance of sclereids, had a lesser tendency to separate from the cambium because it was being pushed toward it instead of being pulled away from it. Even with the proper blade position and sample orientation, however, it was difficult to cut an intact sample with bark, cambium and developing xylem from the fresh unembedded material because of the soft gelatinous cambial region. For this study, however, it was not necessary to have the bark or the complete cambium as long as enough new developing xylem cells were present to enable a valid evaluation of a stain's usefulness in indentifying lignification.

Sections of fresh and embedded material were cut in 30% alcohol which appeared to serve as a better lubricant than water, since the alcohol would flow on the blade surface with less tendency to bead, due to the lower surface tension of the alcohol.

Section curling was controlled by allowing the sections to lay flat on the knife for a few minutes after cutting. They were then transferred directly to the slide, with five sections per slide. Under extreme curling conditions, some sections would roll up very tightly when transferred to the first solutions, even [illegible] on the knife for as long as

15 minutes. To prevent the sections from curling under these conditions, a small piece of stainless steel screening was placed directly over the sections and tied onto the slide with a piece of waxed thread (Hyland, 1970). Although some sections were destroyed, with practice and a moderate amount of caution, this technique was found very beneficial. The entire staining schedule could be completed with the screen in place, unless some step in the particular schedule was very acidic or in some way reactant with the screen material. This technique allowed more rapid solution transfer and less chance of washing a section off of a slide.

Staining Procedure

Choice of stains. After a literature search (see Literature Review) and preliminary laboratory experiments with a number of staining techniques, it was decided to examine in detail ten particular stains. Some of the stains chosen have been well established in the literature for many years and were included as a control for the more recently reported stains.

Phloroglucinol, as mentioned earlier, is probably the most widely used of the lignin-reactive stains. It is reported to be reasonably specific but not too sensitive to lignin (Jensen, 1962). The second well-established stain is the Mäule reaction, commonly used to distinguish between softwood and hardwood lignin (Gibbs, 1957). Two other less well established stains were also compared: potassium permanganate and azure B (Jensen, 1962). The more recently reported color reactions that were examined were benzidine (Jellum, 1960), iodine-malachite green (Bucher, 1968), safranin-aniline blue (Hyland, 1970), safranin-celestine blue B (Gray, Pickle, 1956), 2-thiobarbituric acid (Drozdz, 1964) and toluidine blue O (O'Brien *et al.*, 1964).

General staining sequence. As mentioned earlier, each stain was evaluated using fresh, FAA-killed and FAA-killed celloidin-embedded material on both normal and compression wood; thus a total of six different types of material was used with each stain evaluated (see Appendix C).

When the fresh material was sectioned, half of the samples were put in water for immediate staining. The other half was immersed in FAA-killing solution for 24 hours prior to staining (Sass, 1958).

After soaking the sections cut from embedded material 12 hours in absolute ethanol and ether (1:1) to remove the celloidin, they were stained exactly as the fresh and FAA-killed material. This procedure was followed throughout the study in order to keep the number of variables at a minimum (see Appendix A).

Two complete experiments were conducted using fresh material collected c nd experiment),

1970, and selected embedded material collected on May 30 (first experiment) and June 2 (second experiment), 1966. Rather than section embedded material that was collected on the same date as the fresh samples, the embedded samples had been chosen according to the number of new xylem cells laid off. In this way, all samples within one experiment were in equivalent stages of development (see Appendix C).

Preparation and Application of Stains

Azure B. (see Appendix A) The azure B solution was prepared according to Jensen (1962) by dissolving 0.25 mg./ml. azure B in citrate buffer, pH 4. The citrate buffer solution was prepared by mixing 65 ml. of solution A, which consisted of 4.2 gm. citric acid in 100 ml. of water, with 35 ml. of solution B, which was a solution of 5.9 gm. sodium citrate in 100 ml. of water.

Following hydration, several drops of the stain were applied to each slide, which was then placed in a warm oven at 50°C for two hours. The excess stain was subsequently washed off with tap water before being run through three 30 minute changes of 100% butanol. If the sections appeared too deeply stained, they were left in the butanol for a period up to 24 hours in order to destain them. When the staining action was judged complete, the sections were passed through three changes of xylene and mounted in balsam.

Benzidine. (see Appendix A) The preparation of the stain and the technique used was carried out according to Jellum (1960).

Benzidine stain was prepared by dissolving one gm. of benzidine in 10 ml. of glacial acetic acid. Although it was reported that the concentration was unimportant because overstaining is impossible, it was nevertheless kept constant throughout this study.

The sections were dehydrated to 95% ethanol, and four to five drops of benzidine solution then placed on the slide and immediately diluted with a full eye dropper of absolute ethanol. This diluted staining solution was drained off and sections were stained for 15 to 30 minutes. Following dehydration, they were run through three changes of xylene; balsam was then added and a cover slip applied.

Iodine-malachite green. (see Appendix A) A number of solutions are needed for the procedure (Bucher, 1968). The malachite green solution was prepared by mixing one gm. in 100 ml. of water plus 9 to 10 drops of concentrated acetic acid. A stannic chloride solution, 48% aqueous (65 gm. stannic chloride, $SnCl_4 \cdot 5H_2O$ in 100 ml. distilled water) is also necessary and must be stabilized against hydrolysis by acidifying it with one ml. hydrochloric acid followed by slow filtration through a fritted glass filter. A white precipitate forms within 24 hours, and the clear usable lic[illegible] zinc iodine

(Herzberg's stain) is prepared by mixing two solutions together. Solution A consists of 20.0 gm. dry zinc chloride plus 10 ml. of water. Solution B consists of 2.1 gm. potassium iodide, 0.1 gm. iodine plus 5 ml. of water. When added together, these also formed a precipitate after a few hours, and the clear brown liquid was decanted off the top and stored in a brown glass bottle to prevent rapid deterioration. This stain can be further stabilized by adding a few flakes of iodine to the mixture. The last solution required in the procedure is the same as solution B above but made up with 25 ml., instead of 5 ml., of water.

For best results, the malachite green should be heated. Bucher suggested heating over a Bunsen flame one minute or in a water bath for one hour. Using the Bunsen flame created many control problems, which will be discussed later, and a water bath was preferred. The slide with sections was placed in a petri dish half full of stain, and the petri dish was then floated on the boiling water in the water bath. Using this method, the stain required two hours to develop the correct intensity. After two hours, the excess stain was rinsed off with tap water, and the slide was blotted dry with filter paper. Next, several drops of potassium iodide plus iodine were placed on the section for two minutes to intensify the reaction. This was again rinsed with water and blotted dry with filter paper. If a screen was used to flatten the sections, it was removed at this point to avoid reaction with the highly acidic stannic chloride which was applied next. The stannic chloride solution was left on the slide for 15 minutes, then one or two drops of the Herzberg stain was added and the cover slip applied. The lignin turned brown and the remaining material green if the procedure was successful. However, some difficulty was experienced in coordinating all the stages of the staining process. If the lignin also turned green, it was probably because there was too much water present, and more care was needed to dry the sections before adding the stannic chloride.

Potassium permanganate. (see Appendix A) The test for lignin using potassium permanganate is relatively simple. The material was hydrated to distilled water, and immersed in the potassium permanganate solution for 10 to 20 minutes. The solution used was 1% potassium permanganate in distilled water. The compression wood samples seemed to pick up the stain faster than the normal wood, perhaps simply because of the higher concentration of lignin. The staining time, therefore, was adjusted depending on the type of sample. After staining, the samples were washed in distilled water and dehydrated to absolute ethanol. Three changes of xylene were followed by balsam and a cover slip.

Mäule reaction. (see Appendix A) Following hydration, the tissue was stained [illegible] permanganate for about

10 to 12 minutes (Browning, 1967). After staining, it was washed with water, treated with 12% hydrochloric acid, again washed with water, and then moistened with ammonium hydroxide for 30 seconds to one minute. This was then followed by dehydration, three changes of xylene, balsam and the cover slip.

Phloroglucinol. (see Appendix A) The phloroglucinol procedure consists of simply placing a few drops of a saturated aqueous solution of phloroglucinol, followed by 20% hydrochloric acid, over the sections and applying a cover glass (Jensen, 1962). Some difficulty was experienced in dissolving enough phloroglucinol to get a completely saturated solution, even after long periods of constant stirring. This was solved by heating the solution to about 40°C while constantly stirring. By doing this, it was possible to create a super-saturated solution. When this mixture was cooled, the excess phloroglucinol precipitated out, leaving the desired saturated solution of phloroglucinol at room temperature. The color imparted to the specimen normally lasted from 15 to 30 minutes, but it could usually be intensified by simply adding a drop or two of the 20% hydrochloric acid at the edge of the cover slip.

Safranin-aniline blue. (see Appendix A) After hydrating the sections to water, they were stained in a 2% aqueous safranin solution for 24 hours, then washed in distilled water and dehydrated to 90% ethanol (Hyland, 1970). During dehydration, the safranin was washed out rapidly in 30% and 50% ethanol so that time spent in these solutions was as brief as possible. Several drops of the aniline blue at a concentration of 1% in 90% ethanol were then applied. The aniline blue was not left on the sections over two minutes, and often less. This stain is removed by the higher alcohols, so again the time in the 95% and absolute ethanol during dehydration was brief before immersing in xylene and mounting in balsam.

Safranin-celestine blue B. (see Appendix A) All three types of solution preparations used by Gray and Pickle (1956) were prepared and tried on the material in this study, but the actual experiments used the second preparation where both the safranin and celestine blue B were mordanted and applied individually.

In order to prepare the safranin, 100 ml. of distilled water were heated to about 50°C. Fourteen ml. of glycerol and 5 gm. of crystal ferric alum were added to this. The mixture was stirred on a magnetic stirrer to form a solution before 2 gm. of safranin O were added. The celestine blue B was prepared in essentially the same way. One hundred ml. of distilled water were heated to about 50°C. Fourteen ml. of glycerol were added and then 5 gm. crystal ferric alum. Next, one gm. celestine blue B was p[illegible] accumulated in as small

an area as possible, and 0.5 ml. concentrated sulfuric acid was poured directly on the dye. This mixture was stirred to a paste and left until the evolution of gas ceased. After the reaction stopped, the friable mass was broken up before the hot iron alum solution was added. The resultant mixture was cooled to room temperature, and the pH adjusted to 1.0 with concentrated sulfuric acid. Gray and Pickle pointed out that this solution is probably a colloidal dispersion, and that the method of preparation should be followed exactly. Either solution may be used first.

In this study, the sections were hydrated to tap water and rinsed at least five minutes before being placed in the safranin solution. The length of time required for this stain varied between normal and compression wood. Normal wood samples required one to two hours, whereas compression wood usually stained dark enough within 20 minutes. Following safranin staining, the samples were again rinsed in tap water for about 30 seconds, or just long enough to remove excess stain, and quickly dipped in 70% ethanol before the mordanted celestine blue B was applied for one to two minutes. Again the samples were rinsed quickly in 70% ethanol and transferred immediately to absolute ethanol for two changes. Neither color changed in xylene, and the slide was examined here but was watched closely because of the rapid xylene evaporation. If either color was weak, it could be taken back through the sequence and intensified. However, the subsequent solutions usually washed out the satisfactory stain. Therefore, if restaining was necessary, it had to be taken back to safranin and the entire procedure repeated. The sections were mounted in balsam.

2-thiobarbituric acid. (see Appendix A) The staining solution was prepared by mixing 0.3 gm. of 2-thiobarbituric acid, at room temperature, with 40 ml. glacial acetic acid plus 10 ml. lactic acid (Drozdz, 1964). The stability of the color reaction can be increased up to six months if 20% lactic acid is added to the solution of 2-thiobarbituric acid in glacial acetic acid immediately before staining.

The schedule starts by hydrating the samples to water. The sections were dried and exposed to chloral hydrate for two to three minutes. Then two to three drops of the 2-thiobarbituric acid solution were added and the slide was heated over a Bunsen burner for 10 to 20 seconds. The solution evaporated very rapidly during heating and had to be watched closely. Furthermore, the solution was flammable if allowed to come in direct contact with the flame. The lignified walls take on an orange-red color; this color will eventually appear without heating but not for 15 to 30 minutes.

Toluidine blue O. (see Appendix A) The staining solution contained 0.05% [illegible] in 0.1 M phosphate buffer at pH 6.8

(O'Brien *et al.*, 1964). The 0.1 M phosphate buffer was prepared by mixing two solutions (Glick, 1961). The primary solution contained 13.800 gm. sodium orthophosphate, di-H (NaH_2PO_4 H_2O) per liter and mixed with the secondary solution which contained 26.825 gm. sodium orthophosphate, mono-H (Na_2HPO_4 $7H_2O$) per liter in a proportion of 51 ml. of primary to 49 ml. of secondary solution. The toluidine blue O dissolved rather slowly in the phosphate buffer and had to be mixed for about 15 minutes.

The staining schedule used was relatively simple. After hydrating to water, the section was stained in the solution for about one minute, then rinsed in tap water for two to three minutes. It did require about 30 minutes after staining before the full colors developed in the sections.

Stain Evaluation Procedure

As each slide was completed, it was individually judged according to predetermined criteria (see example, Appendix B; for complete data see Gray, 1971). Four criteria were used. The first criterion was the ability of the stain to differentiate between lignin and the cellulose in young developing tracheids, *i.e.*, the progression of developing lignin distinguishable in the newly formed tracheids. To receive a high rating in this category, the slide would have to show the lignin first developing in the cell corners, a few tracheids away from the cambium, followed by middle lamella and primary wall incrustation, with the developing secondary wall still unlignified. The second criterion was the distinctness of color shades in the mature wall layers. After the secondary wall is completely formed, lignin is present in the composite layers in varying concentration; if the stain showed this variation by different color intensities in the separate wall layers, it would receive a high rating. Third consideration was the uniform reproducibility of results for a particular dye and material on different sections. Fourth was the resolution on high power.

Each slide was judged and assigned a value from one to five for each criterion above. Further, it was felt that the criteria were not all equally important, and each was assigned a weight factor which varied from one through ten (specifically 10, 6, 5 and 3). The value assigned in each case was multiplied by the predetermined weight factor, thus giving a weighted total for each criterion which, when added together, gave a total for each stain on each material. These totals were further compiled to give a quality rating from A through E, A indicating the highest or best rating for a stain used on a particular type of tissue (Appendix C).

The first two charts in Appendix C represent the data obtained in the first and second [illegible] chart is an

average of the first and second experiments, thus giving a more accurate representation of the stain's usefulness on the material tested.

In addition to the above evaluation of the stain's suitability in studying the lignification process, each stain was given a second rating of its individual characteristics using values zero through five (Appendix D). The characteristics judged in this section were those that are inherent in the process itself and, therefore, would probably not be changed by the material it is applied to. The first characteristic of the evaluation was the amount of color contrast produced, with a high value indicating high contrast. Second was ease of applications; *i.e.*, how intricate and involved is the staining procedure, where a high value indicates a relatively simple technique. Third is the length of time required to complete the standard procedure, with a high value indicating a short time requirement. Finally, fourth is the permanence of the finished slides, with a high value indicating a permanent finished slide. The values (one through five) applied to each of the four criteria were added together to get a total value for each stain. The final values ranged from 10 to 18; however, this is a very relative figure and an examination of the individual ratings is probably more beneficial.

Photomicrography

Photomicrographs were taken with a Zeiss Photomicroscope equipped with a built-in 35 mm. camera and photo-electric exposure meter.

Bright-field microscopy was chosen to minimize distortion of the actual color of the cells. The light source was a built-in incandescent illuminator maintained at a brightness level sufficient to give all the wave lengths of light needed for accurate color photography.

The three objectives used were: Neofluar 16X, N.A. 0.40; Neofluar 40X, N.A. 0.75; and Neofluar 100X, N.A. 1.30, oil immersion.

The film used was Kodachrome II for daylight color slides, ASA 25. The three light-balancing filters included a Kodak 80B, a Kodak 82B and a Tiffen CC-10-Y.

Exposure times were between 3 and 60 seconds.

OBSERVATIONS AND DISCUSSION

Superior Stains for Lignin

On the basis of this study, four staining techniques were judged superior to the others: safranin-aniline blue, safranin-celestine blue B, 2-thiobarbituric acid and toluidine blue O. All of these stains received an "A" rating for at least some of the material tested (Appendix C). However [illegible] for a particular

type of material or feature to be studied. Therefore, they are not described in any particular order of priority.

2-thiobarbituric acid. One of the best stains for general use seems to be 2-thiobarbituric acid, which was rated high for each material. 2-thiobarbituric acid is a quick and effective stain than can be used on freehand, as well as microtome, sections and seems to be effective on fresh, FAA-killed or embedded sections of both normal and compression wood (Appendix C). Neither the fresh nor the FAA-killed normal wood samples developed clear color shades in the mature cell wall as distinct as the normal embedded or any of the compression wood samples. Generally, the contrast between cellulose and lignin was quite good even though there were no contrasting colors, but merely dark and light shades of orange. As with some of the other single stains, the lignin was hard to detect in early stages of tracheid development because the very light orange in the cell corners did not contrast well with the clear cellulose walls. Later stages of development were much better in this respect. This stain differentiated the individual layers of the mature cell walls of the compression wood embedded material very well (Fig. 1, 2). Another highlight is its ease of application and a very high percentage of reproducibility.

Besides its temporary nature, perhaps the major disadvantage of 2-thiobarbituric acid is the lack of distinctly contrasting colors between lignin and cellulose which the other three stains in this category exhibit. Drozdz also noted that the orange to orange-red color he obtained was slightly inferior to phloroglucinol from the viewpoint of color contrast (Drozdz, 1964). Perhaps because of this poor color contrast, in this study it seemed to indicate initiation of lignification in normal wood slightly later than the phloroglucinol reaction. On further comparison with the phloroglucinol reaction, Drozdz indicates that 2-thiobarbituric acid does provide a more stable coloration, does not affect calcium oxalate as does phloroglucinol and is not as potentially harmful to the microscope since it does not involve the use of hydrochloric acid.

Safranin with aniline blue and celestine blue B. Two slightly more involved but equally useful techniques utilize safranin in combination with aniline blue or celestine blue B. These produce permanent slides with excellent contrast between cellulose and lignin even during the development of the secondary wall (Fig. 3, 4). Apparently, they can both be used on most types of material, but again, results indicate some improvement in the embedded material over the fresh and FAA-killed material (Appendix C). Other workers have found the combination of safranin with fast green to give a rapid qualitative indication of lignification (Balatinecz, Kennedy, 1967), although Johansen (1940)

recommends that aniline blue may be a little more precise than fast green for some materials.

Many workers have felt that safranin is a valid indicator of lignified tissue since the results obtained correlate well with other methods. Wilcox (1964) felt results obtained correlated well with the expected chemical composition of decayed wood while other workers found a good correlation using ultraviolet absorption (Scurfield, Wardrop, 1963). Results of this study also indicated a good correlation with results from other stains such as phloroglucinol.

Some additional problems, when using safranin as a lignin indicator, not encountered in this study have been noted by other workers. In studying hardwoods, in connection with fast green, Harlow (1941) indicated that cell walls may stain entirely red, or entirely green, and some may show both colors in the same wall. Also, in using fast green, Balatinecz and Kennedy (1967) found that the intensity of the red color varied with both section thickness and length of time in the fast green. If nicks were present in the microtome knife, minute scratches developed in the section which subsequently stained almost exclusively green in the localized area of the defect. In using aniline and safranin it has been noted that the composition of both these dyes may be somewhat variable from batch to batch, consequently producing variable results (Johansen, 1940; Conn, 1953).

In this study, which used unextracted material, both the aniline blue and the celestine blue B received almost the same ratings (Appendix C) and showed some improvement when used with embedded material as compared to unembedded material. Although safranin-aniline blue had a somewhat more uniform reproducibility, safranin-celestine blue B produced a slightly more distinct color outline and showed lignin distribution better in the developing secondary wall layers (Fig. 4). This might have been due to the fact that aniline blue color is sometimes less permanent if the balsam is acidic (Johansen, 1940). The lack of uniformity of results of the celestine blue B is due, perhaps, to the fact that safranin and celestine stains are both basic and iron mordanted and can mask each other if either is allowed to overstain. The observed masking could just be the mutual affinity of the dyes to the ferric alum used as the mordant although the mechanism of mordanting is still little understood (Gurr, 1965). Mordanting is not common for safranin, but in this case, it allows more rapid staining and also leaves the stain deposited in a form which was not as easily removed by prolonged soaking in ethanol, a point also observed by Gray and Pickle (1956). While using mordanted safranin, Gray and Pickle reported, "Five-minute staining, even of chrome fixed material, gave bright red, sharply differentiated

xylem. The color was brighter and far more stable in alcohol than sections from the same block stained for six days in conventional safranin of Johansen (1940)."

Both safranin stains gave slightly better results with compression wood than with normal wood for all types of material. This is in agreement with Scurfield (1967) who concluded that basic dyes, without exception, stained the walls of reaction wood cells much more intensely than those of normal cells. One might expect this considering the higher lignin content of compression wood as compared to normal wood.

Although safranin seems to indicate the presence of lignin, it also stains other structures or materials (nucleoli, chromosomes, cutin, suberin, chitin) and therefore one must be careful in saying just with what the safranin is reacting. If one is looking at the cell wall, which of course lacks nucleoli and chromosomes, and from other tests one determines that cutin, suberin, chitin or other extractives are absent, then one might assume that the safranin is preferentially staining the lignin and not the cellulose.

Toluidine blue O. In agreement with O'Brien and co-workers (1964), this stain produced a simple, quick, lignin-cellulose differentiation in fresh, FAA-killed and embedded material capable of resolution on high power.

O'Brien *et al.* (1964) reported the use of toluidine blue O in staining cell walls of various plant species. They found that not only could tissue sections be resolved into their component cell types, but that in some instances the color resolution extended to different layers of the wall of one cell. They indicated that in the tracheary elements of pea epicotyl the lignified secondary wall stained bluish-green while the unlignified compound middle lamella, separating two such adjacent elements, stained a brilliant reddish purple. In fibers of the same tissue they reported that toluidine blue O showed the lignin first developing in the primary wall by coloring it a clear blue-green, while the unlignified middle lamella and young secondary wall stained an intense reddish purple. Then, as lignification proceeded, the blue-green staining progressed into the middle lamella and finally the secondary wall.

The results obtained in this study of balsam fir correspond very well with O'Brien's results on pea epicotyl. The cambium, young unlignified tracheids and the phloem cells stained a light reddish purple. The blue first showed in the cell corners of the primary wall, then progressed to the middle lamella and secondary wall. The individual cell layers were fairly distinct, especially in the embedded material. At an early stage of development, a green layer could be seen, perhaps part of the S_2 layer, while a thin pink layer could be seen just inside it, perhaps the S_3 layer

or maybe just remnants of the cell cytoplasm. Later, the secondary wall still appeared greenish while the area that had been pink was now stained a darker blue. This could indicate the presence of a heavier concentration of lignin in the inner S_2 or S_3 layers.

The use of toluidine blue O has certain disadvantages. One is the temporary nature of some of the colors produced. For example, the pink color mentioned above faded in less than 15 minutes, though not completely for almost an hour. O'Brien and coworkers do describe a procedure for preparing permanent slides although it was not tried for this study. Another problem is the use of certain fixatives which can cause a pronounced shift in color and fading. The biggest disadvantage, as with certain other lignin stains is that, while results compare favorably with the phloroglucinol reaction, until the chemistry of both reactions is better understood it is impossible to decide with certainty how reliable toluidine blue O is for the identification of lignin (O'Brien *et al.*, 1964).

Satisfactory Stains for Lignin

The results of this study indicated that four stains yielded good to fair results. These stains were phloroglucinol, the Mäule reaction, potassium permanganate and azure B. The choice of one of these stains should be made according to what phase of the lignification process the microscopist wants to look at, as each has distinct advantages over the others depending on the phase studied.

Phloroglucinol. Phloroglucinol is a quick, useful, relatively accurate lignin stain which has probably been satisfactorily used with more combinations of fixatives and materials than any other lignin stain (Fig. 5, 6). It gave very good results with all types of material used in this study and reportedly may also be used with freeze-substituted and freeze-dried material (Jensen, 1962). This study indicated phloroglucinol to have distinct advantages with regard to ease of application, the short time required for staining and the extreme uniformity of results. It has been found to be superior to the Mäule reaction for later stages of lignification (Ullrich, 1955).

When using phloroglucinol, a number of problems may be encountered such as fading and variable results. Still other questions arise when using phloroglucinol for developmental studies and quantitative work.

The color produced in the sections fades in such a way that it is not possible to make permanent slides. In this study the color lasted from 15 to 30 minutes. Balatinecz and Kennedy (1967) found that observations were possible up to two hours. Once the color has faded, it may be revitalized by addition of hydrochloric acid. The hydrochloric

acid will also disrupt any calcium oxalate crystals present (Drozdz, 1964).

Another problem with phloroglucinol is the considerable variability in the reaction within a species. These variations are particularly common among angiosperms and extend to different elements and to different wall layers of the same cell (Harlow, 1933; Srivastava, 1966). Some of this variability, particularly in hardwoods, may be due to the presence of extractives or wound gum, both of which are known to react with phloroglucinol (Brauns, Brauns, 1960; Esau, 1965). This variability within a plant supposedly does not allow clear correlation between a positive reaction and age af cells, age of tissues and their mode of origin (Srivastava, 1966).

Various problems arise when attempting to study developing tissue using phloroglucinol. When considering the first criterion in this study (the ability to differentiate between lignin and cellulose in developing tracheids), phloroglucinol rated less than the highest value. In the newly developing cells, the low sensitivity of the phloroglucinol permitted only slight reaction to the initial lignin development. Thus, it was difficult, though not impossible, to detect the first signs of lignin fixation in the cell corners. These results compare well with those of Wimmer (1948), Ullrich (1955) and Pfoser (1959). Wimmer found that the phloroglucinol reaction is only visible at a later stage of development than can be shown using fluorescence. Ullrich found that at the beginning of membrane thickening one gets a reaction with the Mäule reaction but the phloroglucinol reaction is only evident later. That is, initial lignification may be taking place even though one gets no reaction with phloroglucinol. Pfoser concluded that phloroglucinol reacted poorly with slightly lignified material and that the groups in the lignin molecule which react in the phloroglucinol test only become evident when the cell wall begins to thicken. This study showed phloroglucinol was slightly inferior to some of the other stains in its ability to show lignin distribution in the thickened wall layers. Although shading definitely developed in the secondary wall layers, it was not as pronounced as in some of the other stains.

The question of quantitative work with phloroglucinol has been considered by other workers. Harlow (1933) felt that since only a small fraction of the lignin is responsible for the phloroglucinol reaction that this color test is not dependable as a quantitative measure of lignin present. While DeBaun and Nord (1951) developed a quantitative technique using a photo-electric colorimeter, Spurny and Sladky (1955) have shown that the intensity of the resulting color is not necessarily a measure of the degree of lignification, since what is concerned is only a reaction to certain alde [illegible] these aldehyde groups

On the following pages are photomicrographs illustrating the application of certain stains as referred to in the text. A description of the results obtainable with the stain, method and time of sample preparation and magnification of photomicrograph are given. Bulletin must be rotated 90 degrees to obtain proper viewing position.

Figure 1. 2-thiobarbituric acid showing unstained phloem (top of photo), cambial region, and initiation of the lignification process (middle of photo). Immature unlignified (unstained) secondary walls are discernible approximately five cells down from the cambial region and practically fully lignified mature xylem cells are evident at the bottom of the photo. Section of FAA-killed and celloidin embedded compression wood sample collected July 6, 1966; X 320.

Figure 2. 2-thiobarbituric acid showing progressive lignification of the maturing secondary wall. Cells at the top of the photo are approximately five to seven cells from the cambial region, while cells near the bottom can be considered practically mature. Section of FAA-killed and celloidin embedded compression wood sample collected July 6, 1966; X 320.

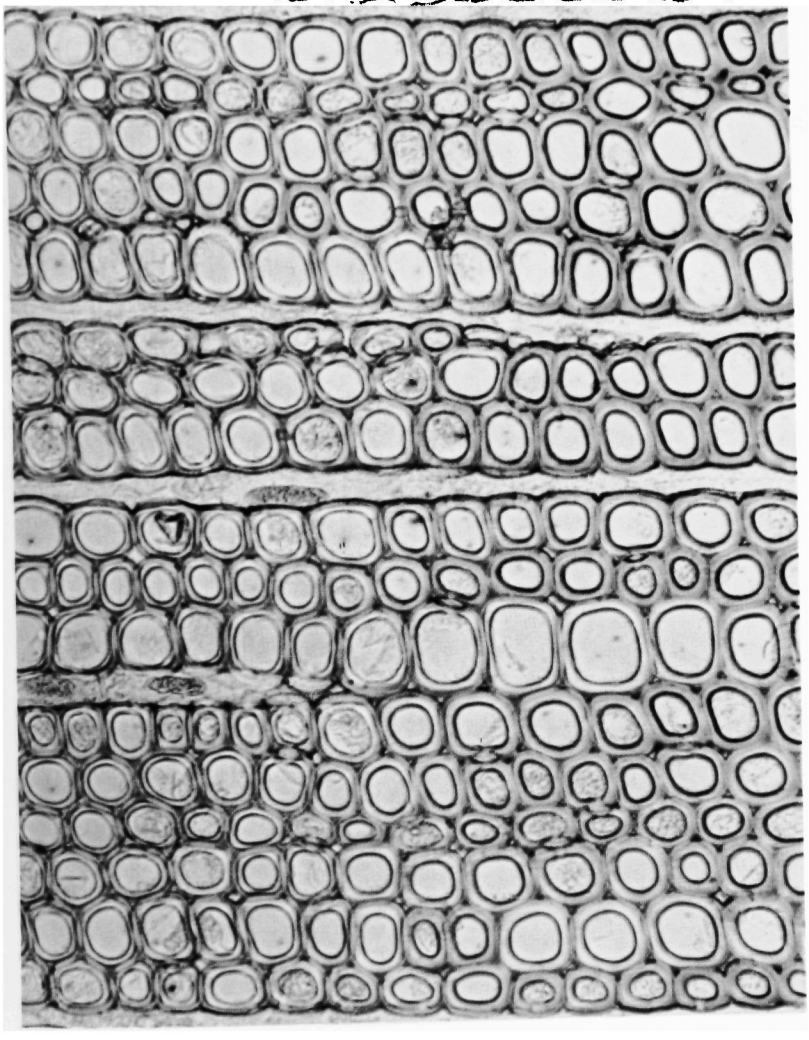


Figure 3. Safranin and aniline blue, showing blue unlignified cambium (top of photo) and contrasting red lignified tissue (lower part of photo). Immature, secondary walls appear blue-blue green and can be seen approximately five to eight cells down from the cambial region. Section of FAA-killed and celloidin embedded compression wood sample collected July 6, 1966; X 320.

Figure 4. Safranin and celestine blue B, showing blue unlignified phloem and cambium (top of photo) and contrasting red of lignified tissue (lower part of photo). Immature, incompletely lignified, secondary walls appear blue (center of photo), and almost fully lignified xylem cells are red. Section of FAA-killed and celloidin embedded compression wood sample collected July 6, 1966; X 320.

Figure 5. Phloroglucinol showing unlignified phloem and cambial region (top of photo). Progressive lignification of the immature secondary wall can be seen starting about three cells from the cambium where it is almost completely unlignified to where it is practically fully lignified (lower part of photo). Section of FAA-killed and celloidin embedded compression wood sample collected July 6, 1966; X 320.

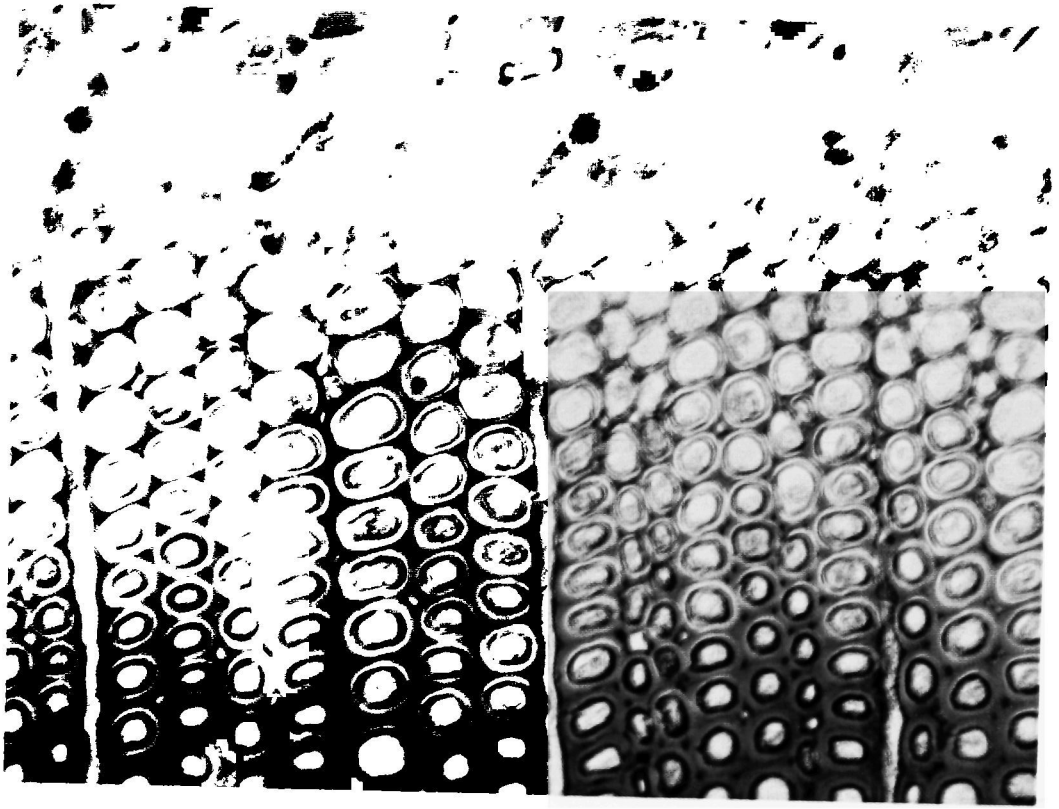


Figure 6. Phloroglucinol showing unlignified cambial region (top of photo). Lignification of the primary wall and immature secondary wall seems to be evident across a larger number of cells from the cambium in this normal wood sample as compared to compression wood (see Fig. 5). Section of fresh, normal wood sample collected July 1, 1970; X 320.

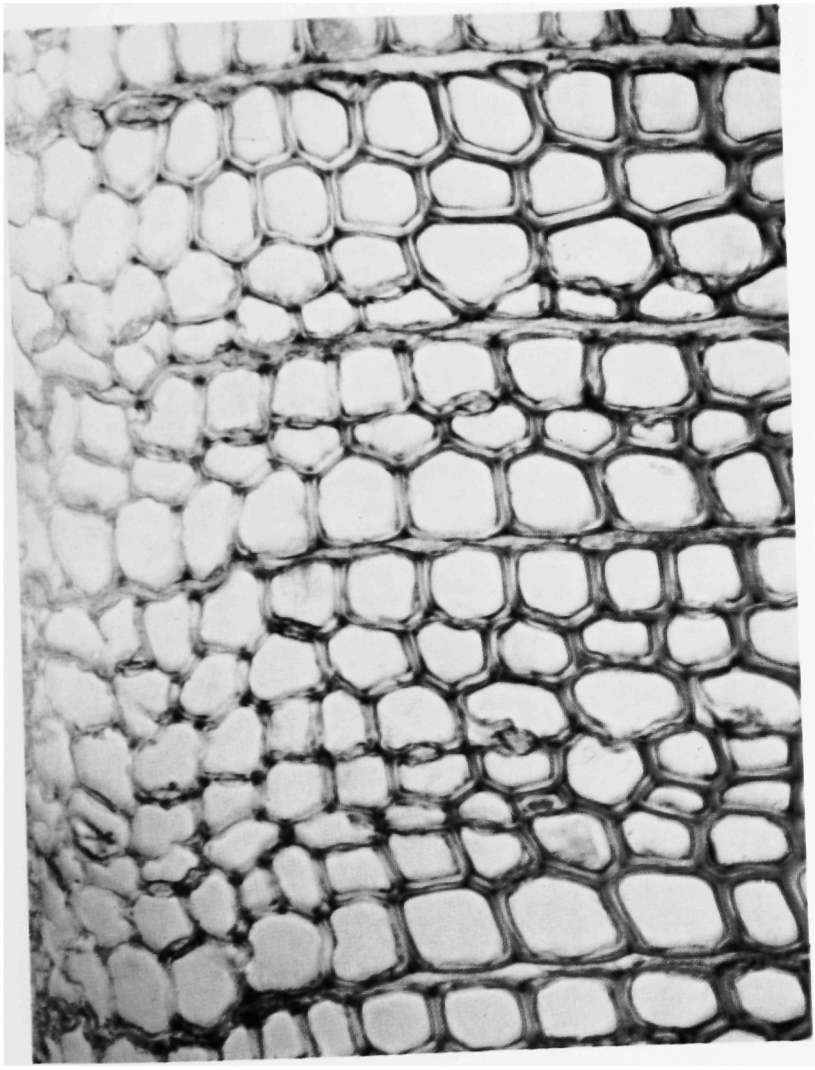


Figure 7. Mäule reaction, showing young xylem cells with lignin concentrated in cells corners (orange), compound middle lamella almost completely lignified, and lignification of secondary walls still incomplete. Section of fresh normal wood sample collected July 1, 1970; X 800.

Figure 8. Potassium permanganate showing lignin distribution in wall layers of nearly mature tracheids. Note darker staining portion of tracheid walls inside lighter-staining portion (center of photo). Section of FAA-killed and celloidin embedded compression wood sample collected July 6, 1966; X 800

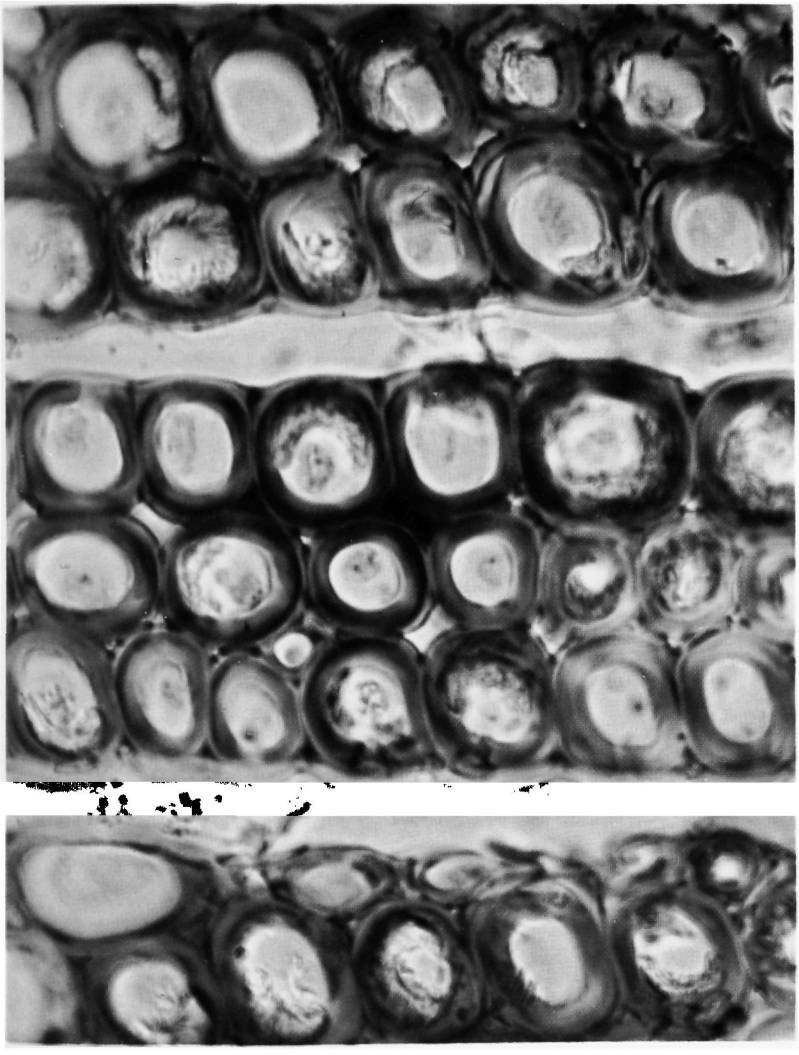


Figure 9. Azure B, showing distinct color shading across cell wall layers in mature tissue. Section of FAA-killed and celloidin embedded compression wood sample collected June 21, 1966; X 320.

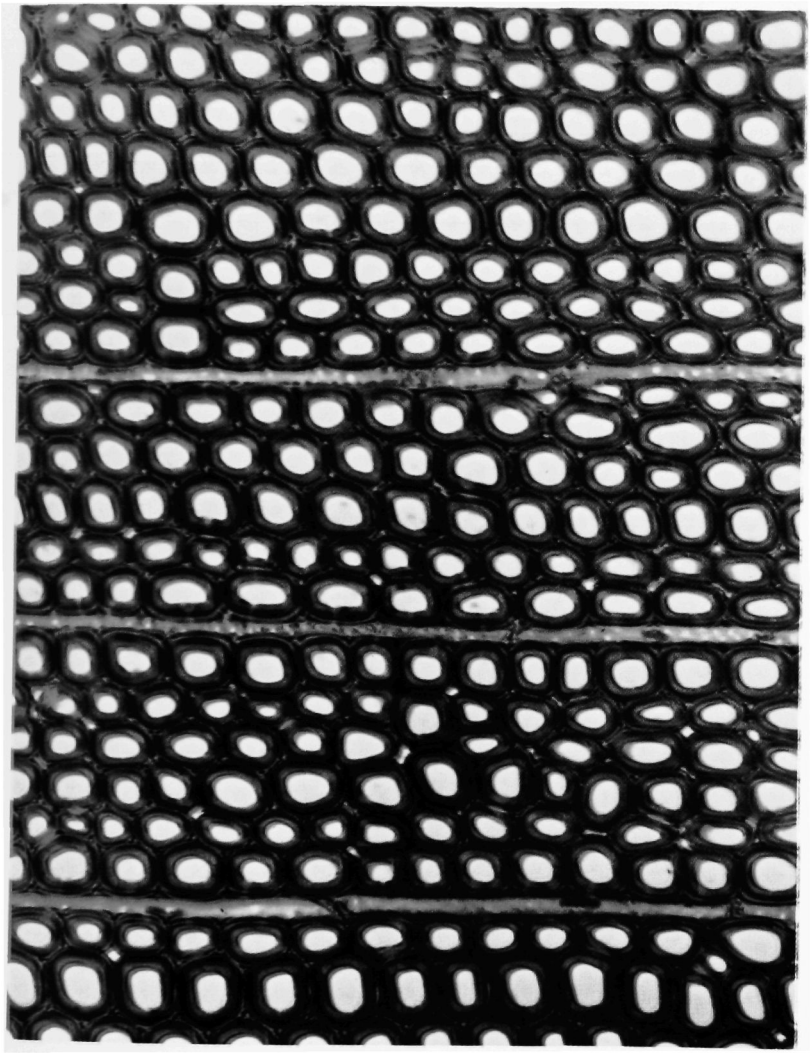


Figure 10. Azure B, showing possible lignin distribution across the mature secondary wall. Higher lignin content, dark greenish-blue; lower lignin content, light green. Section of FAA-killed and celloidin embedded compression wood sample collected June 21, 1966; X 2000.

do not always occur in the same numbers. If the aldehyde groups are chemically blocked, there is no reaction to phloroglucinol even though lignin can still be verified by other methods.

From the above, one can see that while the phloroglucinol test is a commonly used lignin detection technique, the interpretation does involve some difficulties. Though Gurr (1960) indicates it is extremely sensitive, it is probably more accurate to say that the test is reasonably specific (Jensen, 1962) but not too sensitive to early stages of lignification. The chlorine-sulfite test (Siegel, 1953) which is reported to be more sensitive than phloroglucinol did not perform well during initial screening of tests and was therefore not investigated in detail. Jensen (1962) indicates that a negative phloroglucinol reaction does not necessarily mean that lignin is absent. Undoubtedly since the reaction involves only a small part of the lignin molecule, one cannot conclusively prove the existence or absence of lignin with this reaction alone.

Mäule Reaction. The Mäule reaction gave results very similar to phloroglucinol except that it was much better for early stages of development. This is in agreement with the work of Ullrich (1955) and Pfoser (1959) who indicated that the Mäule reaction is good for slightly lignified cell walls which, as they become more lignified, reach a stage where the phloroglucinol reaction works better.

A number of problems arise in using the Mäule reaction as a lignin indicator. As one might expect, from a gymnosperm wood such as balsam fir, relatively poor color contrast was obtained between lignin and cellulose, the lignified regions being indicated by orange or light brown (Fig. 7). Gibbs (1957) has indicated that when a red color is obtained, such as with angiospermous woods, it frequently diffuses out of the tissue. Results of the present study indicate that the Mäule reaction is not particularly useful to study older, heavily lignified material, since the entire section develops a brown color with lignin distribution visible only by shade differences. The reaction may even give negative results in very heavily lignified material such as sclereids and completely mature secondary walls. This is in agreement with Ullrich (1955) who thought this lack of reaction might be due to compaction of the lignified cell wall or incrustation with gum-like substances. As with the phloroglucinol reaction, the Mäule reaction exhibits considerable variation within a species and down to different wall layers of the same cell resulting in age-of-cell correlation problems (Srivastava, 1966).

The extra steps necessary to use this stain and lack of contrast do not seem justified unless it is desirable to distinguish between gymnosperm lignin, which turns brown, and angiosperm lignin, which turns red, or unless one is strictly interested in only the newly formed, slightly lignified

Potassium permanganate. A similar procedure to the Mäule reaction is the potassium permanganate schedule. Potassium permanganate staining requires very little time and satisfactory results were obtained on all the material tested.

Results with young differentiating cells were not very good. In the early stages of development next to the cambium, the cells took on a light brown appearance throughout and initiation of lignification was somewhat indistinct. Although there was some variation, where distinct, the potassium permanganate indicated lignin initiation at about the same distance back from the cambium as phloroglucinol. Somewhat in contrast, Helper and co-workers (1970), when using potassium permanganate as a fixative for electron microscopy, found that it was effective in showing the beginning of lignification in the earliest detectable stages of secondary wall thickening. This might indicate that potassium permanganate is more effective as an electron dense stain for detecting lignin than as a coloring material for light microscopy. Certainly the added resolution of the electron microscope is a distinct advantage.

Potassium permanganate was rated higher for its ability to show lignin distribution by distinct shadings in the wall layers of nearly mature tracheids (Fig. 8). The lignin seems to respond to the potassium permanganate faster than the cellulose; although the entire section stains brown, the darker lignin is rather pronounced. This is in good agreement with the work of Hepler and co-workers (1970). They also found that the potassium permanganate selectively reacts with lignin and negatively stains the cellulose microfibrils, revealing their orientation and dimensions. In addition, they found good correlation between the patterns of staining and fluorescence, indicating that the permanganate provides much greater detail on the location of lignin deposition than the fluorescence technique. Bland *et al.* (1969) also found that, in general, the electron dense regions correspond to those parts of unfixed material which are strongly ultraviolet absorbing. A final advantage of potassium permanganate is its simple progressive staining qualities which allow one to adjust the intensity of the reaction to bring out particular features.

Azure B. As with other stains tested, azure B yielded somewhat better results with embedded material than with fresh or FAA-killed material (Appendix C). However, even in the embedded material the reaction did not work well in the developing cells close to the cambium. The very light blue-green color shades were indistinct in this area; therefore, the differentiation between lignin and cellulose in developing tracheids was poor. Conversely, the mature secondary walls showed distinct color shades across the wall layers, especially in sections of embedded compressio [illegible] eared

dark green to black. Next to the middle lamella was a thin layer which stained a light greenish-blue. The coloration in the thick secondary wall varied from a dark greenish-blue near the primary wall to a very light green next to the lumen (Fig. 10). If one assumes that the basic azure B reacts with the acidic lignin, then the dark greenish-blue color of the secondary wall close to the primary wall may be an indication of higher lignin concentration as compared to a lower lignin concentration in the light green area next to the lumen. This is in agreement with evidence from electron microscopy (Kutscha, 1968).

Unsatisfactory Stains for Lignin

Two of the ten stains examined were found to be unsatisfactory for studying the lignification process. Benzidine proved to be too insensitive and lightly colored to be used with microtome sections, and iodine-malachite green produced variable results, even under close control.

Benzidine. Benzidine was found to be a very fast easy method with excellent reproducibility of results. In addition, others have found that the reaction does not overstain, the color is unaffected by alcohol and xylene, and that the color resists fading for up to three to six months (Lominski, Hutchison, 1948; Jellum, 1960).

A major draw-back was that poor differentiation between lignin and cellulose in developing tracheids was obtained, especially in fresh material. The reaction essentially provided poor contrasting colors between lignin (yellow-orange) and cellulose (unstained). It was also found that, although some color was imparted, microtome sections contained too little material to produce a sufficiently dark coloration. This is in agreement with Jellum's work which indicated that the color shows up distinctly in hand-sectioned material but on very thin tissues, such as microtome sections or macerated cells, the color is not intense and does not show up readily.

Iodine-malachite green. Control difficulties with iodine-malachite green were complicated as well as somewhat time consuming. Results produced were variable.

When satisfactory results were obtained, a pronounced color difference was evident between lignin which turned brown, and cellulose which turned green. Jayme and Harders-Steinhäuser (1954) indicate that malachite green alone bonds with the protolignin of native wood fibers as well as with the lignin-sulfonic acid of fibers from sulfite pulp and also with the sulfur lignin from unbleached sulfate pulp. When used with iodine according to the technique developed by Bucher (1968), very thin preparations may be used under high magnifications.

In this study, the iodine-malachite green appeared to be better for studying [illegible] of lignin in mature

cell walls. The lignin stained distinctly brown in the cell corners in early stages of tracheid growth. At a later stage, the entire primary wall and middle lamella appeared brown with the newly formed secondary wall green (unlignified). As the subsequent secondary wall layers developed, they rapidly took on a relatively uniform greenish tone. The individual secondary wall layers of a mature tracheid were not easily distinguishable. This is somewhat in opposition to the work of Bucher (1968) which showed it was possible to distinguish concentric layers in reaction wood cells as well as lignin distribution across the cell wall.

Evaluation of Stain Characteristics

In choosing a stain that will best suit his needs, one primarily looks at the end results that the stain is capable of producing. However, inherent characteristics of the coloring agent and the steps involved in the procedure should not be ignored. Very little has been said about these particular stain characteristics which are not changed by the material it is applied to.

These data for the stains used are compiled in Appendix D. The total values ranged from 18 for safranin-celestine blue B down to 10 points for iodine-malachite green. The total number of points is of far less importance than where they were gained or lost. For example, although iodine-malachite green received the lowest total score for all the stains tested (10), it rated the highest score possible for distinct color differences. Therefore, a person might want to use this procedure if he needed high contrast for photomicrographs. Conversely, the same individual probably would not be satisfied with potassium permanganate which received a higher overall rating (17) but a much lower rating for contrast.

Examination of Appendix D shows that iodine-malachite green, safranin-aniline blue and safranin-celestine blue B have the most distinct color contrast of all the stains tested. The simplest and least time consuming stains were potassium permanganate, phloroglucinol and toluidine blue O. The most difficult one to use was iodine-malachite green.

The colors generally obtained for the various stains are listed in Appendix E. The stains are listed in the approximate order of their usefulness on the types of material studied with the most useful listed first as determined by Appendix C. It should be emphasized, however, that this priority rating is questionable since each stain has its own particular advantages and disadvantages, as described throughout this study, for particular uses.

CONCLUSIONS

On the basis of this study, the following conclusions may be drawn:

1. No major difference in staining reactions was noted between the two experiments which used material collected on different dates. In each experiment, the embedded material exhibited somewhat superior results compared to the fresh and FAA-killed material with at least half of the stains. No marked difference was observed between normal and compression wood.

2. 2-thiobarbituric acid is a quick lignin stain of good specificity for lignin that is effective for studying lignification with all three types of material studied and is especially suited for studying progressive lignification of the maturing secondary wall.

3. Safranin-aniline blue and safranin-celestine blue B produce similar results with all three types of material. While both have average specificity for indicating the presence of lignin, they provide excellent color contrast in all stages of cell development and are suitable for the preparation of permanent slides.

4. Toluidine blue O, while of unknown specificity, is a quick procedure that provides good contrast and can be used on all three types of material. It is especially good for studying lignification in young developing cells.

5. Phloroglucinol is a quick procedure of good specificity that can be used on all three types of material. It is particularly good for studying later stages of lignification in young developing tracheids.

6. The Mäule reaction has good specificity and can be used on all three types of material. It is best for studying early stages of lignification in young developing tracheids.

7. Potassium permanganate is a quick method of good specificity which can be used on all three types of material. It is suitable for studying lignification in nearly mature tracheid walls.

8. Azure B has average specificity and shows good color shades in mature wall layers.

9. Benzidine is a quick method with good specificity but is unsatisfactory for use with thin microtome sections.

10. Iodine-malachite green, of average specificity, is better for young developing tracheids than for mature cells. It presents control problems.

11. Since the chemistry of most lignin color reactions is poorly understood, and considering the large number of possible variables involved, one must be cautious in utilizing any one of the above stains alone to positively identify lignified material. The use of more than one stain, as well as other chemical or physical methods, is highly recommended.

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APPENDICES

APPENDIX A
STAIN SCHEDULE

AZURE B

STEP	SOLUTION	EXPLANATION
1	1:1 Absolute Ethanol-Ether	12 Hours—To remove celloidin, if embedded
2	80% Ethanol	Most easily miscible with 1:1 alcohol-ether
3	Hydrate to Water (Distilled)	
4	Azure B	2 Hours at 50°C in citrate buffer pH 4
5	Rinse (Tap Water)	
6	Tertiary Butanol (3 Changes)	30 Minutes each
7	Xylene (3 Changes)	
8	Balsam—Cover Slip	

BENZIDINE

1	1:1 Absolute Ethanol-Ether	12 Hours—To remove celloidin, if embedded
2	80% Ethanol	Most easily miscible with 1:1 alcohol-ether
3	95% Ethanol	
4	Benzidine	4-5 drops
5	Absolute Ethanol	Dilute the above 4-5 drops with eye dropper full
6	Benzidine (Pure solution)	15-30 Minutes
7	Absolute Ethanol (2 Changes)	5 Minutes
8	Xylene (3 Changes)	5 Minutes
9	Balsam—Cover Slip	

APPENDIX A, continued
STAIN SCHEDULE

IODINE-MALACHITE GREEN

STEP	SOLUTION	EXPLANATION
1	1:1 Absolute Ethanol-Ether	12 Hours—To remove celloidin, if embedded
2	80% Ethanol	Most easily miscible with 1:1 alcohol-ether
3	Hydrate to Water (Distilled)	
4	Malachite Green	Flood sections with stain and leave for 2 hours. 90-95°C
5	Rinse (Tap Water)	Remove excess stain
6	Dry	Blot dry with filter paper
7	Potassium Iodide & Iodine	2 Minutes
8	Rinse (Tap Water)	
9	Dry	Same as above
10	Stannic Chloride	9-10 Drops on Slide—15 Minutes
11	Herzberg's Stain	1 Drop—Added to the stannic chloride
12	Cover Slip	Draw out excess stain with filter paper

POTASSIUM PERMANGANATE

1	1:1 Absolute Ethanol-Ether	12 Hours—To remove the celloidin, if embedded
2	80% Ethanol	Most easily miscible with 1:1 alcohol-ether
3	Hydrate to Water (Distilled)	
4	1% Potassium Permanganate	15 Minutes
5	Rinse (Distilled Water, 3 Changes)	
6	Dehydrate to Absolute Ethanol	
7	Xylene (3 Changes)	
8	I	

APPENDIX A, continued
STAIN SCHEDULE

MÄULE REACTION

STEP	SOLUTION	EXPLANATION
1	1:1 Absolute Ethanol-Ether	12 Hours—To remove celloidin, if embedded
2	80% Ethanol	Most easily miscible with the 1:1 alcohol-ether
3	Hydrate to Water (Distilled)	
4	1% Potassium Permanganate	10-12 Minutes
5	Rinse (Distilled Water)	
6	12% HCl	30 Seconds-1 Minute
7	Rinse (Distilled Water)	
8	Moisten with Ammonium Hydroxide	30 Seconds-1 Minute
9	Dehydrate to Absolute Ethanol	
10	Xylene (3 Changes)	
11	Balsam—Cover Slip	

PHLOROGLUCINOL

1	1:1 Absolute Ethanol-Ether	12 Hours—To remove celloidin, if embedded
2	80% Ethanol	Most easily miscible with 1:1 alcohol-ether
3	Hydrate to Water (Distilled)	
4	Phloroglucinol	2-3 Minutes
5	20% Hydrochloric Acid	
6	Cover Slip	

APPENDIX A, continued
STAIN SCHEDULE

SAFRANIN-ANILINE BLUE

STEP	SOLUTION	EXPLANATION
1	1:1 Absolute Ethanol-Ether	12 Hours—To remove celloidin, if embedded
2	80% Ethanol	Most miscible with 1:1 alcohol-ether
3	Hydrate to Water (Distilled)	
4	2% Safranin in Water	24 Hours
5	Rinse (Distilled Water, 3 Changes)	
6	Dehydrate to 90% Ethanol	
7	1% Aniline Blue in 90% Ethanol	1-2 Minutes
8	Dehydrate to Absolute Ethanol	
9	Xylene (3 Changes)	
10	Balsam—Cover Slip	

SAFRANIN-CELESTINE BLUE B

1	1:1 Absolute Ethanol Ether	12 Hours—To remove celloidin, if embedded
2	80% Ethanol	Most easily miscible with 1:1 alcohol-ether
3	Hydrate to Water (Tap)	
4	Rinse (Tap Water)	5-10 Minutes
5	Safranin (Mordanted)	20 Minutes-2 Hours
6	Rinse (Tap Water)	Quick
7	70% Ethanol	30 Seconds-1 Minute
8	Celestine Blue B (Mordanted)	1-2 Minutes
9	70% Ethanol	Quick
10	Absolute Ethanol (2 Changes)	
11	Xylene (3 Changes)	15 Minutes each
12	E	

APPENDIX A, continued
STAIN SCHEDULE

2-THIOBARBITURIC ACID

STEP	SOLUTION	EXPLANATION
1	1:1 Absolute Ethanol-Ether	12 Hours—To remove celloidin, if embedded
2	80% Ethanol	Most easily miscible with 1:1 alcohol-ether
3	Hydrate to Water (Distilled)	
4	Dry	
5	Cloral Hydrate	2-3 Minutes
6	2-Thiobarbituric Acid	2 or 3 drops
7	Heat	Over Bunsen burner for 10-20 seconds
8	Cover Slip	

TOLUIDINE BLUE O

1	1:1 Absolute Ethanol-Ether	12 Hours—To remove celloidin, if embedded
2	80% Ethanol	Most easily miscible with 1:1 alcohol-ether
3	Hydrate to Water (Distilled)	
4	Toluidine Blue O	1 Minute
5	Rinse (Tap Water)	2-3 Minutes
6	Cover Slip	

APPENDIX B

EXAMPLE OF CRITERIA FOR EVALUATION

AZURE B, FIRST EXPERIMENT

Features Used in Evaluation	Reference Number
Differentiates between lignin and cellulose in developing tracheids	1
Color shades distinct in mature wall layers, showing mature lignin distribution	2
Uniformity (reproducibility) of results for this dye on this material	3
Resolution on high power	4

NORMAL WOOD

Material	Reference Number	Value 1-5	Weight 1-10	Total
Fresh	1	3	10	30
	2	3	6	18
	3	5	5	25
	4	3	3	9
Total for fresh material				82
FAA-killed	1	3	10	30
	2	3	6	18
	3	5	5	25
	4	3	3	9
Total for FAA-killed material				82
Embedded material	1	3	10	30
	2	3	6	18
	3	5	5	25
	4	3	3	9
Total for er				82

APPENDIX C

STAIN SUITABILITY CHART*

FIRST EXPERIMENT, JUNE 4, OR EQUIVALENT STAGE OF DEVELOPMENT

EXPERIMENTAL STAIN	NORMAL WOOD			COMPRESSION WOOD		
	Fresh	FAA-killed	Embedded	Fresh	FAA-killed	Embedded
Azure B	C+	C+	C+	D+	D+	B−
Benzidine	E−	D	D	E−	D	D
Iodine-Malachite Green	D+	D+	D+	D+	D+	D+
Potassium Permanganate	D	D	C+	D	D	C+
Mäule Reaction	C+	C+	C+	C+	C+	C+
Phloroglucinol	B+	B+	B+	B+	B+	B+
Safranin-Aniline Blue	B−	B−	A−	B−	B−	A+
Safranin-Celestine Blue B	B−	B−	A	B−	B−	A
2-Thiobarbituric Acid	A−	A−	A+	A+	A+	A+
Toluidine Blue O	B−	B−	A	A−	A−	A

*A = 109 - 98 (Most suitable)
B = 97 - 86
C = 85 - 74
D = 73 - 62
E = 61 - 50 (Least suitable)

SECOND EXPERIMENT, JULY 1, OR EQUIVALENT STAGE OF DEVELOPMENT

Azure B	C+	C+	B	C+	C+	B+
Benzidine	E−	D	C−	E−	D	C−
Iodine-Malachite Green	C	C	C	C	C	C
Potassium Permanganate	C	C	B+	C	C	A
Mäule Reaction	A−	A−	A−	A−	A−	A−
Phloroglucinol	B	B	B	B	B	B
Safranin-Aniline Blue	B+	B+	A−	A−	A−	A+
Safranin-Celestine Blue B	B	B	A	A	A	A+
2-Thiobarbituric Acid	A−	A−	A+	A+	A+	A+
Toluidine Blue O	B	B	A	B	B	A

*A = 107 - 94 (Most suitable)
B = 93 - 80
C = 79 - 66
D = 65 - 52
E = 52 - 45 (Least suitat

APPENDIX C, continued

STAIN SUITABILITY CHART*

AVERAGE OF FIRST AND SECOND EXPERIMENT

EXPERIMENTAL STAIN	NORMAL WOOD			COMPRESSION WOOD		
	Fresh	FAA-killed	Embedded	Fresh	FAA-killed	Embedded
Azure B	C+	C+	B−	C	C	B
Benzidine	E−	D	D+	E−	D	D+
Iodine-Malachite Green	C−	C−	C−	C−	C−	C−
Potassium Permanganate	C	C	B	C	C	B+
Mäule Reaction	B	B	B	B	B	B
Phloroglucinol	B	B	B	B	B	B
Safranin-Aniline Blue	B	B	A−	B+	B+	A+
Safranin-Celestine Blue B	B	B	A	B+	B+	A
2-Thiobarbituric Acid	A−	A−	A+	A+	A+	A+
Toluidine Blue O	B	B	A	B+	B+	A

*A = 107 - 95 (Most suitable)
B = 94 - 81
C = 80 - 67
D = 66 - 53
E = 52 - 45 (Least suitable)

APPENDIX D

EVALUATION OF STAIN CHARACTERISTICS (STAIN FACTORS NOT AFFECTED BY THE MATERIAL)

CHARACTERISTICS*	Identification Letter
Distinctness of color differences between lignin and cellulose	A
Ease of application	B
Length of time required to apply standard procedure	C
Permanency	D

NAME OF STAIN	A	B	C	D	TOTAL VALUE
Azure B	2	4	4	4	14
Benzidine	2	4	5	4	15
Iodine-Malachite Green	5	0	3	2	10
Potassium Permanganate	3	5	5	4	17
Mäule Reaction	2	4	5	4	15
Phloroglucinol	3	5	5	2	15
Safranin-Aniline Blue	5	5	3	4	17
Safranin-Celestine Blue B	5	4	5	4	18
2-Thiobarbituric Acid	2	4	5	2	13
Toluidine Blue O	4	5	5	2	16

*See text f

APPENDIX E

COLOR REACTIONS OF STAINS WITH MOST USEFUL STAIN LISTED FIRST

	STAIN	LIGNIFIED TISSUE	UNLIGNIFIED TISSUE
1.	2-Thiobarbituric Acid	orange	clear
2.	Safranin-Celestine Blue	red	blue
3.	Safranin-Aniline Blue	red	blue
4.	Toluidine Blue O	blue-green	reddish purple
5.	Phloroglucinol	red	unstained
6.	Mäule Reaction	brown	unstained
7.	Azure B	blue-green	unstained
8.	Potassium Permanganate	dark brown	light brown
9.	Iodine-Malachite Green	brown	green
10.	Benzidine	yellow-orange	unstained

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