THE LIGNIN OF POPULUS NIGRA L. CV. 'ITALICA'

A COMPARATIVE STUDY OF THE LIGNIFIED STRUCTURES IN TISSUE CULTURES AND THE TISSUES OF THE TREE

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SUMMARY

1. Anatomical investigation showed that callus cultures of *Populus nigra* L. cv. '*Italica*' contain lignified parenchyma cells and that large amounts of lignin are localized in intercellular spaces. A tissue-culture strain on a medium containing IAA also showed differentiation of tracheids with lignified walls.
2. In the plant, lignin was found in the primary xylem, secondary xylem, phloem, and periderm. The lignin from primary xylem gave a negative Mäule reaction.
3. Lignins from callus cultures and from the plant were compared and quantitatively determined.
4. A method was developed to calculate the lignin content of tissues from the lignothioglycolic acid yield and the absorption at 250 nm in the so-called difference spectrum.
5. Oxidation with nitrobenzene yielded syringaldehyde (S) and vanillin (V) from tissue cultures, young and old secondary xylem, phloem, and periderm; primary xylem gave vanillin but no syringaldehyde.
6. For tissue cultures, young secondary xylem, phloem, and periderm, the S/V ratio is lower than for differentiated wood. For 2,4-D cultures the S/V ratio was lower than for a strain cultured on an IAA medium.
7. The aldehyde yield, calculated as a percentage of the lignin, is appreciably higher for secondary xylem (42%) than for phloem, periderm, and tissue cultures (12–18%).
8. Xylem lignin contains 9–10% esterified *p*-hydroxybenzoic acid. Less of this acid is found in the lignin of young secondary xylem (5.2%) and the tissue cultures (2.8–6.7%). Phloem lignin has a still lower *p*-hydroxybenzoic acid content (0.6%).
9. Lignin rich in *p*-hydroxybenzoic acid is characterized by a very high absorption in the difference spectrum at 296 nm, strong absorption in the infrared spectrum at 1265 cm^{-1}, and an extra absorption band at 766 cm^{-1}. After hydrolyzation, this lignin lacks the 766 band in the IR spectrum and shows much less absorption at 1265 cm^{-1}.
10. Alkali lignin was prepared by a 16-hour extraction with 0.5 N NaOH at 70 °C. Complete extraction of the lignin from the tissue samples was rarely obtained.
11. Lignin preparations deriving from the various tissue regions of the plant and from the

callus cultures show mutual differences. The lignin of the callus cultures shows the greatest resemblance to the lignin of young secondary xylem but is much more resistant to oxidation. In this respect it shows a closer relationship to the lignin from the phloem and primary xylem of the tree.

12. The terms "angiosperm lignin" and "hardwood lignin" applied to a lignin with a high syringaldehyde content are very misleading, because not all the lignified tissues of dicotyledonous trees contain this type of lignin.

1. INTRODUCTION

Lignin studies have mainly concerned woody plants. Extensive research has been done on lignin in the secondary xylem of trees, but there are few data on the lignin found in other tissues of the tree or in herbaceous plants.

During an investigation of xylem differentiation in plant callus cultures, we experienced major difficulties in the determination of lignin, which made further investigation necessary. We wished to know, in the first place, to what extent the lignin formed in callus cultures has properties in common with the lignin formed in the xylem and other tissues of the intact plant. In the second place, we wanted to evaluate several of the routine methods for lignin determination, since we required a quantitative method suitable for a comparative study of the induction in tissue cultures of lignified elements in dependence on various milieu factors.

The study reported in this thesis may be considered as an orientational investigation of lignin in tissue cultures, mainly of material deriving from the Lombardy poplar *(Populus nigra* L. cv. *'Italica')*. In addition to the quantitative determinations, qualitative analyses were performed to permit comparison of the lignin from the cultures with that obtained from various parts of the tree.

2. DISCUSSION OF THE LITERATURE

2.1. The use of *in vitro* cultures in differentiation studies

The morphogenesis of xylem and phloem elements is receiving a great deal of attention at present. On the one hand, attempts are made to influence the differentiation of secondary tissues in various ways, as a result of which normal or abnormal differentiation products arise from the cambium (WAREING *c.s.* 1964; DIGBY & WAREING 1966). On the other hand, various methods are used to induce the differentiation of vascular elements in parenchymatous or meristematic tissue (JACOBS *c.s.* 1952, 1957, 1961). In this connection, use is frequently made of explants or callus cultures (CAMUS 1949; KARSTENS 1965; WETMORE & RIER 1963; JEFFS & NORTHCOTE 1966).

The use of *in vitro* cultures offers certain advantages. A tissue sample or an organ can be removed from the normal coordinated relation to the surrounding tissues and an attempt can be made to replace the influence of the latter by milieu factors. Phytohormones and nutrients can be administered diffusely via the culture medium or by local application in agar cubes or micropipettes.

For this type of study, many investigators prefer explants to callus cultures.

Each of these objects offers certain possibilities and difficulties. The tissues of explants are comparable to those of the intact plant, but the injury associated with their isolation can have a disturbing effect. Callus cultures avoid this difficulty for the most part, but the reproducibility of the experiments is poor and comparison with the tissues of the intact plant presents problems.

The effect of a procedure applied to cambium can often be expressed in the number of (xylem) elements deriving from that cambium, but the effect of the application of a given treatment to a callus culture in order to induce the development of vascular elements, is much more difficult to evaluate. The differentiation is often expressed in the formation of spherical groups of tracheid-like elements, sometimes accompanied by phloem, or in the occurrence of separate small groups of xylem elements. The counting of cells and cell groups is often possible, but an irregular distribution of differentiation products over the tissue as well as variability of the results frequently make it very difficult to sample the material for anatomical study.

During the formation of secondary xylem elements such as tracheids, vessels, and libriform fibres, lignification of the middle lamella and the primary wall occurs in a rather early stage of the differentiation, and at a later period lignin is also deposited in the secondary wall (WARDROP 1957). Only a few cases are known in which the lignification accompanying the differentiation of vessels, tracheids, or libriform fibres is greatly reduced, the best known examples being the incomplete lignification of the secondary wall of fibres in the reaction wood of angiosperms, the "rubbery wood" of apples (SONDHEIMER & SIMPSON 1962), and "*Gummihanf*" in *Cannabis sativa* (TOBLER 1940). But in all these cases some lignin is deposited. Lignification is therefore one of the essential processes belonging to the morphogenesis of the undifferentiated cell into a xylem element. Since differentiated vessel elements and tracheids always contain lignin, the presence of lignin can be used as an indication of the presence of these xylem elements. In the same sense the lignin content of callus cultures can be used for the investigation of the influence of certain factors on xylem differentiation, at least on condition that an anatomical examination is performed, since lignin may be present in other structures than vessel elements or tracheids.

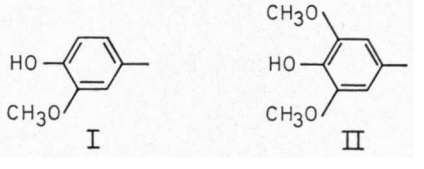
2.2. Lignin

In the study of plant anatomy, lignin is almost a morphological concept. It occurs in the cell walls of plants as an incrusting substance filling the spaces left in the framework formed by polysaccharides, thus strengthening the cell wall. It is therefore a functional component of wood.

From the chemical point of view, the term lignin does not designate a single homogeneous substance but is rather the collective name of a series of compounds having a high degree of structural similarity. In this respect lignin is somewhat comparable to other natural polymers such as cellulose, starch, and perhaps the proteins (SCHUBERT 1965). Lignin is composed of components with a high molecular weight and is to a great extent built up of phenylpropane units. A number of these phenylpropane building units are known. They can

be present variously bound in the lignin molecule. Lignin has a high carbon content and always contains methoxyl groups. It also shows the following properties (BRAUNS 1952a; BRAUNS & BRAUNS 1960): it is not hydrolyzed by acids, it is soluble in hot alkali and bisulfite, it condenses easily with phenols and thio compounds, it is easy to oxidize, *e.g.* with nitrobenzene, and at ethanolysis gives "Hibbert's monomers", a group of phenolic compounds, mainly ketones.

The isolation of lignin is usually accompanied by a change in properties as compared to natural lignin, also called protolignin. During the degradation of lignin, secondary conversions take place due to the great reactivity of some of the released compounds. This led Freudenberg and his collaborators to attempt to determine the structure of lignin via a synthetic approach. They obtained a number of oligomers and dehydrogenation polymers from coniferyl alcohol by the use of enzyme preparations from fungi. FREUDENBERG (1964) also developed a schematic model for the lignin of *Picea*, a species in which much lignin research has been done. This model therefore concerns the so-called gymnosperm lignin, which is characterized by the presence of many units of the guaiacyl type (I) and very few of those of the syringyl type (II). The lignin of deciduous wood shows a different ratio: the syringyl units predominate, as shown, for example, by the higher methoxyl content of the total lignin.



In recent years much research has been done on the biosynthesis of lignin in the plant. Results obtained by BROWN *c.s.* (1959) and HIGUCHI & BROWN (1963) have shown that the synthesis of lignin monomers – as of other phenolic compounds in the plant – occurs via the shikimic acid pathway. The diagram in *fig. 1* presents a scheme of the pathways along which the enzymatic synthesis of the various building units of lignin can occur. The cinnamic acid derivatives probably occur in a free state in only small quantities; in all likelihood they are present mainly as glucosides or esters. Of interest in this connection are the so-called insoluble esters, compounds with a rather high molecular weight, which cannot be extracted with acetone or 80% alcohol and are thought to be important intermediate products of lignin synthesis (EL-BASYOUNI *c.s.* 1964, 1966).

According to FREUDENBERG (1965), in the polymerization of the building units a role is played on the one hand by dehydrogenizing enzymes, and, on the other hand, some of the reactions occur along non-enzymatic pathways (*fig. 2*). A central position is thought to be occupied by the quinone methide, a highly reactive compound (XVI) which may offer possibilities for a linkage with polysaccharides. Although the occurrence of bonds between lignin and cell-wall

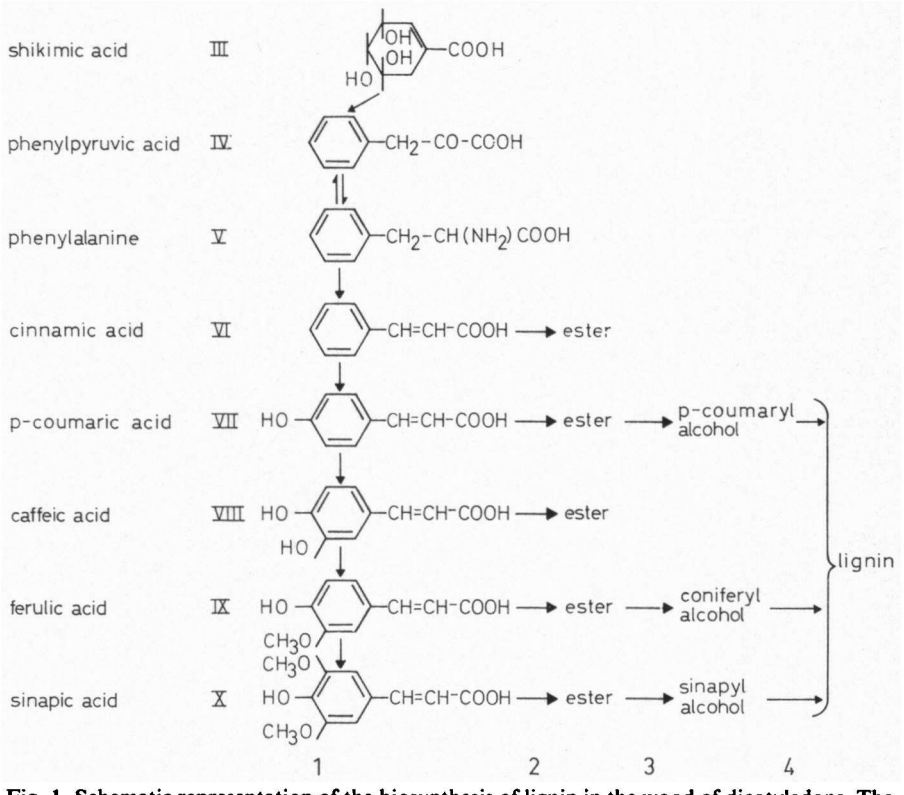


Fig. 1. Schematic representation of the biosynthesis of lignin in the wood of dicotyledons. The formation of cinnamic acid is followed successively by ring hydroxylation and O-methylation (1), formation of esters (2), reduction to alcohols (3), and (co)polymerization, resulting in the formation of oligomeric intermediates and lignin (4). After BROWN 1966, and EL-BASYOUNI *c.s.* 1966.

polysaccharides is not generally accepted, there are strong indications that there is a close association between lignin and these polysaccharides. Synthesis experiments done by SIEGEL (1959) have indicated the importance of the presence of a suitable matrix for the deposition of lignin.

2.3. Discussion of some methods currently used for the investigation of lignin

2.3.1. Sampling

For lignin determinations in wood, the complete secondary xylem is usually employed; separate isolations are made only in exceptional cases, *e.g.* of reaction wood and so-called opposite wood (BLAND 1958a). For work in herbaceous plants, the entire plant is often used. This practice leads to mixing of the lignin from the xylem and that of all the other lignin-containing elements such as bast fibres, sclereids, and epidermal and cork cells. Prior separation of the various

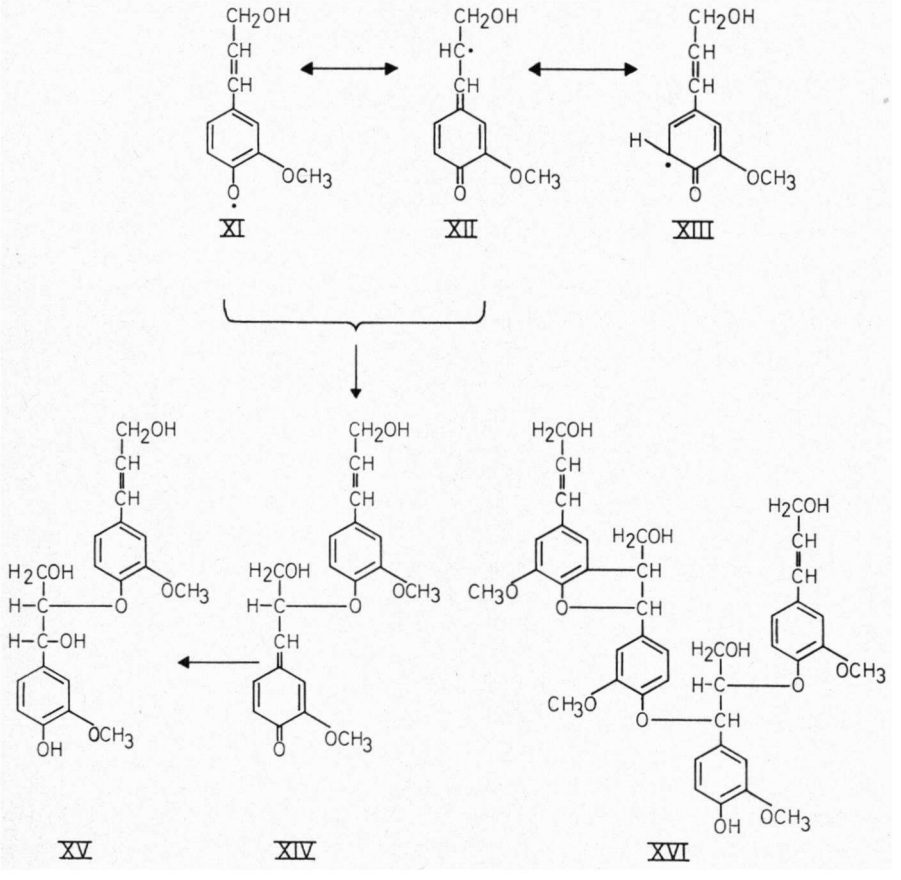


Fig. 2. Polymerization of coniferyl alcohol *in vitro*. The mesomeric free radicals XI, XII, and XIII are formed enzymatically from coniferyl alcohol. Spontaneous condensation leads to the formation of dimers, e.g. the quinone methide (XIV), which combines with water to yield guaiacylglycerol-β-coniferyl ether (XV) or with coniferyl alcohol or a dimer to yield a trilignol or tetralignol, respectively. These are only a few of the ways in which oligomeric intermediates can be formed. After FREUDENBERG 1965.

lignified tissues is often difficult to perform. STAFFORD (1962) attempted to study the lignin composition of the various tissues of the grass *Phleum pratense* by comparison of the lignin from various parts of the plant. Under these conditions the predominant type of tissue more or less determines the lignin composition.

HIGUCHI (1957b) compared the lignin composition of young and older parts of bamboo stalks. The former showed lignin with few syringyl groups and a low methoxyl content, the latter lignin with a high syringyl and methoxyl content. In the absence of anatomical data, however, it is not clear whether the lignin derived from the same type of cells in both cases. It remains possible

that the "young" lignin originated predominantly from an entirely different type of cell than the lignin of the mature stalk. Anatomical investigation is therefore always necessary.

2.3.2. Stabilization and pre-extraction

Wood samples are usually dried and then ground. FREUDENBERG (1955), however, recommends that material from herbaceous plants be boiled first to avoid obtaining insoluble products, the so-called dehydrogenation polymerizates resulting from enzymatic oxidation of phenols and tannins. Stabilization of the fresh material is often obtained by boiling in 80% ethanol. Removal of the extractible material is usually done by extraction with ethanol-benzene, water, and dilute acid. The pre-extraction removes lignin precursors such as free phenolic monomers which might hamper the determination of lignin, but the oligolignols and a highly soluble fraction of the lignin, comparable to "Brauns' native lignin", are also dissolved, and in most cases this soluble lignin fraction amounts to several per cent of the total amount of lignin present. Roadhouse and MACDOUGALL (1956) demonstrated that extractions with ethanol-benzene and water have little harmful effect on lignin, but pre-treatment with acid (1% HCl) must definitely be avoided because it results in radical changes in the lignin. In some cases a short ether extraction is applied to remove chlorophyl that might disturb certain analyses.

2.3.3. Determination and characterization of lignin

2.3.3.1. Quantitative determination

In many quantitative methods the lignin is isolated and then determined gravimetrically. Klason and alkali lignin are determined in this way. With the thioglycolic acid method of Holmberg, the lignothioglycolic acid complex is determined; the lignin content can be calculated from the observed values by applying a correction for thioglycolic acid. However, such methods based on the isolation of lignin have the disadvantage that the resulting preparation may be impure and therefore show too high a lignin content. It is known that the lignin obtained by these methods often retains impurities, the amount depending strongly on the nature of the tissue from which the lignin was isolated. Consequently, certain methods can be suitable for the determination of the lignin content of secondary xylem from trees but completely unsuitable for young tissues with a low lignin and high protein content.

Klason lignin

Isolation methods employing a strong acid, such as Klason's method in which sulfuric acid is used, have proved to be very useful for the determination of lignin in wood, but they carry the danger that the lignin will condense with carbohydrates. In the isolation of lignin from other lignified tissues, the lignin preparation may become contaminated not only with carbohydrates but also with a large amount of protein. Therefore, the Klason method is generally considered unsuitable for the determination of lignin in these tissues.

Alkali lignin

BONDI & MEYER (1948) worked out a method for the isolation of lignin from fodder plants, and called the product alkali lignin, the lignin being dissolved under the action of hot alkali. After being dried the plants are subjected to four successive extractions with hot alkali, and the resulting lignin is freed of hemicelluloses by repeated precipitation. The alkali lignin preparations always contain nitrogen which, according to Bondi and Meyer, is not present in the form of proteins but is an integral part of the lignin of herbaceous plants. For the determination of lignin in such plants as the grass *Phleum pratense*, STAFFORD (1962) used a micromethod in which the samples are heated for 16 hours with alkali.

Both these methods have the disadvantage that the alkali hydrolyzes any ester bonds present in the lignin. Especially in grass lignin, the action of alkali releases large amounts of ferulic acid and *p*-coumaric acid, according to SMITH (1955a) and HIGUCHI *c.s.* (1967) due to the hydrolysis of ester bonds and according to STAFFORD (1964) mainly from a so-called "acid lignin".

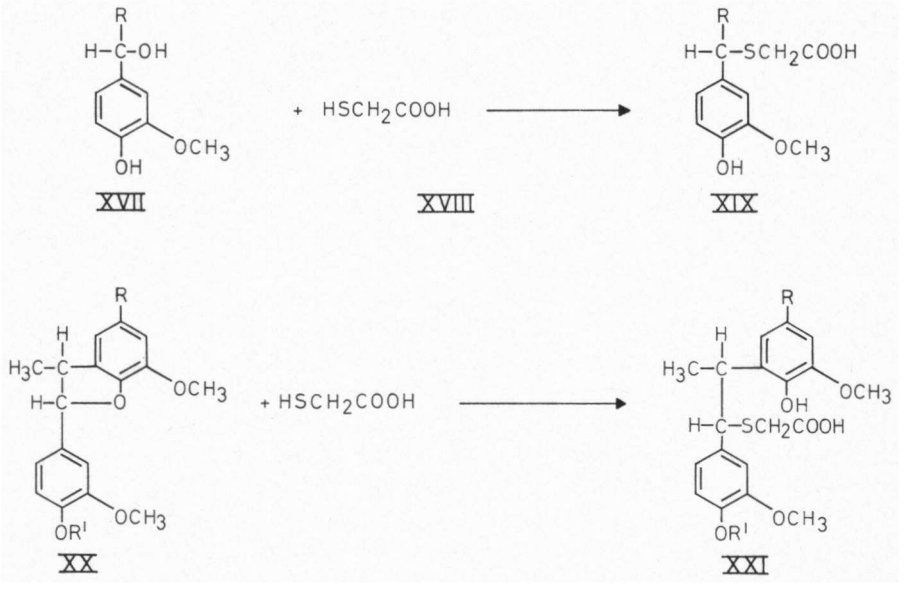
The alkali method has applications, but mainly for the types of lignin containing few esterified phenolic acids. It must be questioned, however, whether the lignin of all herbaceous plants is completely extracted from the tissue with hot alkali. It is possible that in certain cases only a more soluble fraction of the lignin is isolated. This holds particularly for Stafford's method, since it calls for only one alkaline extraction.

In this micromethod, which JEFFS & NORTHCOTE (1966) have also applied to callus cultures, the alkali lignin is determined not gravimetrically but spectrophotometrically, making use of the occurrence of certain groups in the lignin. Phenolic hydroxyl groups are determined from the difference in absorption in alkaline and neutral solution (see p. 252); *p*-hydroxybenzyl alcohol groups are determined from their reactivity with respect to quinoneimine (p. 253).

Thioglycolic acid lignin

The thioglycolic acid method of HOLMBERG (1930, 1934) was recommended by FREUDENBERG (1955) for the determination of lignin in herbaceous plants. By treatment with thioglycolic acid in 2N hydrochloric acid, a solid lignothioglycolic acid is formed. This complex can be isolated by alkaline hydrolysis of the links to other cell-wall components. A soluble lignothioglycolic acid is thus obtained.

The most important binding sites for thioglycolic acid are apparently the benzylhydroxyl groups (cf. XVII and XIX), but the phenylcoumaran structures (XX) also offer suitable sites. In the reaction with the latter structures the heterocyclic ring is opened (XXI). Lignothioglycolic acid preparations contain about 10 per cent sulphur. From the sulphur content, the thioglycolic acid and the true lignin content can be calculated. For this calculation an estimation must be made of the amount of water split off during the reaction with thioglycolic acid. HOLMBERG (1947) assumed the loss of one molecule of water



for each molecule of bound thioglycolic acid; FREUDENBERG *c.s.* (1959) used the assumption that only half this amount of water is lost.

The preparations sometimes contain nitrogen; they are probably contaminated with protein. HOLMBERG (1930, 1934) found good agreement between the elementary composition of the lignin of various species of gymnosperm wood. Angiosperm lignin has roughly the same elementary composition but a higher methoxyl content. HOLMBERG encountered difficulties only in the determination of the lignin content of the stems of certain herbaceous plants. His lignothioglycolic acid preparations from *Lathraea squamaria*, *Solanum tuberosum*, and *Monotropa hypopitys* contained only 4.0, 6.1, and 7.1 per cent sulphur, respectively. The nitrogen content was not determined. Holmberg put forward the hypothesis that in such preparations the lignothioglycolic acid is accompanied by non-lignin-like substances. The lignothioglycolic acid prepared from several other herbaceous plants showed the expected sulphur content of 9 to 10 per cent. HIGUCHI & BARNOUD (1965) used the thioglycolic acid method for the determination of lignification in plant tissue cultures.

2.3.3.2. Qualitative investigations

Infrared spectra

The infrared spectra of various lignin preparations consistently show a strong mutual resemblance. Differences are found mainly in the relative intensity of the absorption bands. Pure lignin preparations from a single species always show a strong mutual agreement, independent of the method by which the isolation is performed.

KAWAMURA & HIGUCHI (1965) compared the IR spectra of milled-wood lignin deriving from a large number of species of woody plants. They found distinct differences between the spectra of angiosperm and gymnosperm species (*table 27*, p. 304). The relative intensity of the absorption bands at 1505 and 1460 cm^{-1} varies. For deciduous wood the absorption is strongest at 1460 cm^{-1}; for gymnosperms the absorption at 1505 is stronger than that at 1460 cm^{-1}. The band at 1275 cm^{-1} is relatively weaker for deciduous wood (1275 $\leqslant$ 1220) than for gymnosperms, in which this band is very strong (1275 $>$ 1220; SOFUE & FUKUHARA 1958). These differences are thought to be related to differences in the number of ether bands and hydroxyl groups. According to Kawamura & Higuchi, it is probably justified to assume on this basis that the degree of polymerization of the lignin from gymnosperms is higher, or at least that relatively more aryl-alkyl ether bands occur. Other differences lie in the relative height of the maxima at 1130 and 1030 cm^{-1} – the former is very high in angiosperm lignin – and the occurrence of a distinct band at 1090 cm^{-1} for gymnosperm lignin. In the 1000 to 800 cm^{-1} region, which mainly represents the substitution on the benzene ring, gymnosperm lignin has bands at 855 and 815 cm^{-1} and angiosperm lignin at 915 and 835 cm^{-1}. But other bands also occur in this region: for example, maple lignin shows distinct absorption at 890 and 870 cm^{-1} (SCHUBERT 1965).

SARKANEN *c.s.* (1967) compared the IR spectra of model substances having guaiacyl groups, condensed guaiacyl groups, and syringyl groups, and found several striking differences between the spectra of lignin models of guaiacyl and syringyl types. For syringyl the absorption at 1500 cm^{-1} is lower than that at 1600 cm^{-1}; for guaiacyl groups the 1500 cm^{-1} band is appreciably stronger. Guaiacyl groups have an absorption band at 1275, condensed guaiacyl groups at 1300, and syringyl groups at 1335 cm^{-1}. Guaiacyl groups show a band at 1160 or 1170 cm^{-1} which is absent for syringyl groups, and they may show an absorption band at 1040 which is not shown by condensed guaiacyl and syringyl groups. According to Sarkanen *c.s.*, the relative proportions of the various components of lignin can be estimated on the basis of such differences.

Ultraviolet spectra

Lignin preparations have a specific absorption maximum in the UV spectrum at about 280 nm. For gymnosperm lignin this maximum lies between 281 and 285 nm, and for angiosperm lignin between 270 and 278 nm. Lignin deriving from grasses shows another maximum at 315 nm. In a very few lignin preparations, such as the native lignin of *Populus tremuloides* (BUCHANAN *c.s.* 1949; PEW 1957) and the methanol lignin of tension wood from *Eucalyptus* species (BLAND 1958b), the maximum at about 280 nm is either not clearly expressed or absent. The extinction coefficient E 1 % 1 cm lies for various lignin preparations at values between 120 and 204 (NORD & DESTEVENS 1958), whereas that of free phenols such as ferulic acid and *p*-coumaric acid is several times higher (825 and 670 respectively) in this region of the spectrum.

Difference spectra

Upon ionization of the phenolic hydroxyl groups of aromatic substances, the absorption maximum in the UV shifts to higher wavelengths and the absorption becomes stronger (LEMON 1947). This property is used for the determination of phenolic hydroxyl groups in lignin preparations by measurement of the absorption in alkaline and neutral milieus and comparison of the difference spectrum with that of known model substances (AULIN-ERDTMAN 1953). In the difference spectrum, maxima are found at 250 and 300 nm for phenolate ions of simple substituted aromatic hydroxyl compounds and at 250 and 350 nm for phenolic compounds in which the hydroxyl group is conjugated through the ring with a carbonyl group of the side chain (GOLDSCHMID 1954). The absorption at 350 nm, or over 300 nm generally, can also be caused by another side-chain chromophore, *i.e.* a conjugated double bond such as is found, for example, in coniferyl alcohol.

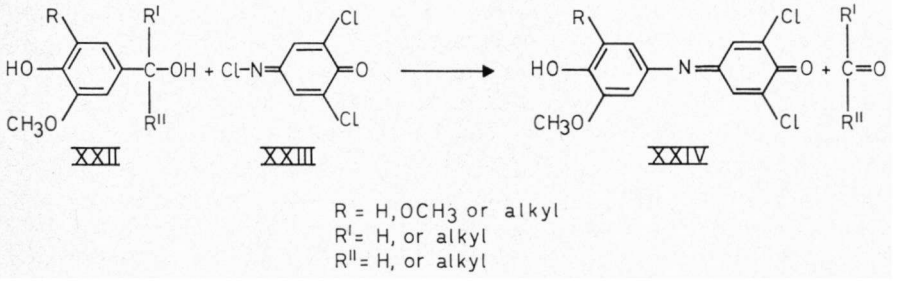
If lignin solutions of comparable composition are investigated, the lignin concentration can be determined on the basis of the absorption in the difference spectrum and the absorption of a pure preparation of known concentration. One great advantage of this method over the use of the absorption at 280 nm in a neutral solution is that non-ionized impurities have no influence on the difference spectrum. What must be determined is whether the various pure lignin preparations really have the same phenolic hydroxyl content and an equally high extinction coefficient E 1 % 1 cm. The phenolic hydroxyl content is perhaps related to the molecular weight of the lignin. After fractionation of lignosulfonates GIERER (1954) found a lower hydroxyl content in the fractions with higher molecular weight, as determined from the absorption at 300 nm in the difference spectrum. AULIN-ERDTMAN (1954) determined difference spectra of lignin preparations from *Picea*, and found maxima in all cases at about 250 and 300 nm, but a maximum at about 350 nm was usually absent for sulphonic acid and thioglycolic acid preparations. Hydrolysis of lignosulfonic acid by boiling in alkali results in a stronger increase of the absorption in this wavelength region, however. The lignin preparations isolated by means of dioxane, methanol, and ethanol always show considerable absorption in the region over 300 nm.

STAFFORD (1964, 1965, 1967) uses the absorption maximum between 340 and 360 nm in the difference spectrum as a measure for the lignin content of alkali lignin preparations. Alkali lignin isolated from *Phleum* and *Elodea* stems after incubation with ferulic acid shows a very high absorption in this region. Stafford ascribes this very high maximum in the difference spectrum of "grass lignin" to a lignin component, the so-called acid lignin, which is assumed to originate from ferulic acid and other phenolic acids, in contrast to the classic lignin, which might be called alcohol-aldehyde lignin. But the maximum at 350 nm does not seem entirely suitable for quantitative measurements, because the lignin absorption in this region is influenced by various factors, including the binding of thioglycolic acid, and, furthermore, the release of ferulic acid and vanillin can also have a disturbing effect here. The maxima at 300 and 250 nm are probably more suitable, but other disturbances may occur in this range, since protein also has two

maxima at corresponding places in the difference spectrum due to the fact that the amino acid tyrosine also has a phenolic hydroxyl group. Protein is often found as an impurity, especially in lignin preparations made from young tissue and tissues of herbaceous plants.

The quinoneimine test

Certain structural elements of the lignin molecule contain a free *para* phenolic hydroxyl group and a free benzyl hydroxyl group (XXII). GIERER (1954, 1956) studied the reaction of quinone monochloroimide with model substances and lignin preparations. The action of quinoneimine in a weakly alkaline solution results in the splitting off of the side chain and the formation of coloured indophenols. Gierer calculated that in soluble native lignin from *Picea*, there is one free *p*-hydroxybenzyl alcohol group for every 7 or 8 lignin building units. This reaction to the *p*-hydroxybenzyl alcohol structure in lignin was applied by STAFFORD (1960 ff.) and JEFFS & NORTHCOTE (1966) as a measure for the lignin content of alkali lignin solutions. Stafford determines the indophenols (XXIV) formed with N,2,6-trichloro-p-benzoquinoneimine (XXIII). The reaction is compared to that of guaiacol and of native lignin from "bagasse" and birch. A weak point of this method lies in the assumption that the alkali lignin of the



material in question contains the same percentage of *p*-hydroxybenzyl alcohol groups as the native lignin of bagasse and birch. As a quantitative method the reaction would only seem to be applicable for the comparative study of lignin preparations with a very constant content of these hydroxyl groups.

Oxidation with nitrobenzene

When preparations containing lignin are subjected to a mild oxidation, about half of the phenylpropane units of the lignin are liberated as phenolic compounds with only one C atom in the side chain. On oxidation with nitrobenzene in alkaline milieu, gymnosperm lignin yields about 27 per cent vanillin (FREUDENBERG *c.s.* 1940). CREIGHTON *c.s.* (1941, 1944) investigated the wood of a large number of gymnosperms and angiosperms, and in the latter found not only vanillin but also large quantities of syringaldehyde after oxidation. The yield of

syringaldehyde is usually about three times greater than that of vanillin. In grasses and herbaceous dicotyledons, not only syringaldehyde and vanillin but also rather large quantities of *p*-hydroxybenzaldehyde are found. Small amounts of other phenolic compounds such as acids are often encountered.

During the oxidation, only a part of the lignin is broken down. LEOPOLD (1952) calculated for gymnosperm lignin that only 45 to 55 per cent of the phenylpropane units are oxidized to vanillin and related compounds. Deciduous wood consistently shows a greater aldehyde yield than does gymnosperm wood; this yield can amount to as much as 56 per cent of the lignin (BLAND *c.s.* 1950). This means that a larger proportion of the building units – up to about 65 per cent – is liberated by oxidation. The nitrobenzene oxidation method is therefore more useful for obtaining information concerning the relative syringyl and guaiacyl content of the lignin than for the quantitative determination of lignin.

In addition to the differences found between taxonomic groups such as gymnosperms and angiosperms (CREIGHTON *c.s.* 1941, 1944) and, for example, between *Lycopodium* and *Selaginella* (WHITE & TOWERS 1967), differences in the syringaldehyde/vanillin (S/V) ratio are also found within one species, for instance in reaction wood as compared to normal wood (BLAND 1958; BLAND & SCURFIELD 1964) and in the lignin of young tissue as compared to that of the mature plant (HIGUCHI 1957a; ROADHOUSE & MACDOUGALL 1956; STONE, BLUNDELL & TANNER 1951).

The aldehyde yield after oxidation with nitrobenzene may be useful for quantitative purposes, *i.e.* for determinations in one particular type of tissue within one species, but only if the S/V ratio is always the same.

Whereas syringaldehyde and vanillin actually originate from lignin or lignin precursors, *p*-hydroxybenzaldehyde can have another source, *i.e.* the amino acid tyrosine. STONE, BLUNDELL & TANNER (1951) obtained 16 per cent *p*-hydroxybenzaldehyde by the oxidation of tyrosine. That a part of the *p*-hydroxybenzaldehyde found on oxidation actually derives from lignin could be demonstrated (DESTEVENS & NORD 1953) for isolated lignin preparations which, although they were nitrogen-free, nevertheless yielded *p*-hydroxybenzaldehyde at oxidation. Another source of extremely high *p*-hydroxybenzaldehyde yield is *p*-coumaric acid, which can be present in esterified state in lignin, particularly that of grasses (HIGUCHI *c.s.* 1967).

Hydrolysis

According to BROWN (1966), a hypothetical lignin model composed of polymerized phenylpropane units, such as Freudenberg's model for *Picea* lignin, is still too simple to be representative for many lignins. In addition, the lignin often contains esterified phenolic acids. In grasses, for instance, ferulic acid and *p*-coumaric acid have often been found (SMITH 1955a; HIGUCHI & BROWN 1963; HIGUCHI *c.s.* 1967a) and small amounts of *p*-coumaric acid were also found in *Asparagus* (HIGUCHI *c.s.* 1967a). SMITH (1955b) subjected a native aspen lignin *(Populus tremula)* to hydrolysis, and obtained more than 10 per cent *p*-hydroxybenzoic acid and limited amounts of vanillic acid, syringic acid, and ferulic acid. He came to the

conclusion that the *p*-hydroxybenzoic acid is esterified to an alifatic hydroxyl group. NAKANO *c.s.* (1961) found in all their samples of wood from *Populus nigra* and *Populus maximowiczii*, 1.3 to 1.6 per cent hydrolizable *p*-hydroxybenzoic acid; "native lignin" of the latter wood gave 7.5 per cent *p*-hydroxybenzoic acid. The results obtained by PEARL *c.s.* (1957) indicate that the occurrence of such *p*-hydroxybenzoate groups is a general characteristic of *Populus* xylem lignin and perhaps of the xylem lignin of all members of the Salicaceae, since wood of the only investigated *Salix* species also yielded *p*-hydroxybenzoic acid on hydrolysis. The wood of all the investigated angiosperms, including the Salicaceae, gave small amounts of vanillin, vanillic acid, syringaldehyde, and syringic acid, and usually also ferulic acid, on hydrolysis. In only a few cases was *p*-coumaric acid found in the hydrolyzate of these wood samples. In the hydrolyzates of phloem of different *Populus* species they found *p*-coumaric acid, *p*-hydroxybenzoic acid, syringic acid, or ferulic acid as the predominating compound (PEARL, BEYER, LASKOWSKI & WHITNEY 1960).

If the hydrolysis is performed on "soluble native lignin" preparations, it will be necessary to determine whether the remaining lignin has the same composition as the soluble fraction. With hydrolysis of tissue samples there can be no certainty that the phenols were actually derived from the lignin. PEARL & BUSCHE (1960) also found rather large quantities of bound *p*-hydroxybenzoic acid in Klason lignin of *Populus tremuloides*.

On the basis of hydrolysis experiments and the *in vitro* synthesis of lignin from ferulic acid, STAFFORD (1962) came to the conclusion that little ferulic acid is esterified in the lignin of *Phleum pratense*. In her opinion, grass lignin is partially composed of an "acid lignin", a polymerization product of phenolic acids.

Lastly, an important problem is formed by the so-called "insoluble esters," which are thought to play a role in the biosynthesis of lignin (EL-BASYOUNI *c.s.* 1964, 1966). These are esters of *p*-hydroxycinnamic acids that cannot be dissolved in 80 per cet methanol or in acetone.

The Wiesner and Mäule reactions

The Wiesner reaction with phloroglucinol and hydrochloric acid is used very widely in anatomical studies to demonstrate lignin. SPURNÝ & SLADKÝ (1955) have correctly pointed out that the intensity of the resulting colour is not necessarily a measure of the degree of lignification, since what is concerned is only a reaction to certain aldehyde groups of the lignin, and these do not always occur in the same numbers. After certain chemical treatments, the aldehyde groups are no longer present and the phloroglucinol reaction is negative even though lignin can still be demonstrated by other methods.

ADLER *c.s.* (1948) found that both coniferyl aldehyde and its methyl ether gave positive results in the phloroglucinol test, whereas the absorption spectrum of the reaction product was similar to that of soluble native lignin. BLACK *c.s.* (1953) showed that not only coniferyl aldehyde gives a reaction with phloroglucinol-hydrochloric acid (red-purple) but also sinapyl aldehyde (blue-purple)

and vanillin and syringaldehyde (peach colour). In deciduous wood, therefore, both coniferyl aldehyde and sinapyl aldehyde groups may react in the phloroglucinol test.

For the Mäule reaction, the material is treated successively with potassium permanganate, hydrochloric acid, and ammonium hydroxide. After this treatment, the secondary xylem elements of angiosperms are stained a purplish red but the wood of conifers shows only a brownish colouring of the lignified walls. CAMPBELL *c.s.* (1938) ascribe the Mäule reaction to a modified pyrogallol nucleus in the lignin. HIGUCHI (1957a) compared the absorption spectra of the coloured reaction products of lignins with those of model substances, and came to the conclusion that the syringyl group (II) is responsible for a positive reaction in the Mäule test.

The Mäule reaction therefore makes it possible to distinguish between so-called angiosperm and gymnosperm lignin, since the latter contains almost no syringyl components (CREIGHTON *c.s.* 1944).

2.3.4. Some other methods for isolating lignin

For many investigations it is necessary to have a lignin whose characteristics agree as much as possible with the lignin in the plant. The lignin isolated by the methods mentioned above (Klason lignin, alkali lignin) has been subject to too many modifications due to the action of strong acid or alkali.

Brauns and collaborators and Björkman have isolated lignin showing much greater agreement with the lignin present in the plant, but neither of these methods gives complete extraction of the lignin in the material under investigation.

Soluble native lignin

With the use of alcohol, 8 to 10 per cent of the lignin of *Picea* can be extracted (BRAUNS 1952b). This "soluble native lignin" or "Brauns' native lignin" is frequently used in research on lignin, because in all probability the isolation method leads to only slight changes in the lignin structure. The solubility in alcohol is ascribed to the low molecular weight of the components concerned.

In this procedure, wood meal is first extracted with cold water and ether and then percolated with ethanol for 8 to 10 days. The dried alcoholic extract is purified by ether extraction and repeated solution in dioxane followed by precipitation. The purified soluble native lignin is a cream-coloured powder.

DESMET (1961) isolated soluble native lignin of *Populus*. This lignin was composed of several components with mutually differing characteristics, the relative quantities of these components varying according to the method used for purification. Therefore, soluble native lignin, too, is not necessarily a homogeneous substance.

Milled wood lignin

BJÖRKMAN (1956) developed a method by which lignin is extracted after prolonged grinding in an oscillating mill. Under favourable circumstances he suc-

ceeded in extracting about half of the lignin of *Picea* with dioxane. The resulting "milled wood lignin" or "Björkman lingin" has probably undergone little change during the isolation procedure. It is relatively pure, but still contains small amounts of carbohydrate.

Milled wood lignin preparations have been used for infrared analysis by such authors as KAWAMURA & HIGUCHI (1965).

3. MATERIAL

3.1. Callus cultures

3.1.1. Isolation and media

The *Populus nigra* L. cv. '*Italica*' strains used in the present study all originated from a single phloem explant torn off along the cambial zone of a thick branch. The isolation was performed in February, 1959. Culturing was done initially on a medium with the following composition: 1 g KNO_3, 0.25 g $MgSO_4$, 0.12 g $CaCl_2$, 0.7 g KH_2PO_4, 0.5 ml Berthelot's solution (GAUTHERET 1942), 10 mg cysteine-HCl, 1 mg thiamine, 10 g agar, 20 glucose per litre. After two months, pieces of callus were inoculated on a medium enriched with 10 mg Ca-pantothenate, 0.01 mg biotine, 100 mg inositol, and 0.1 mg 2.4-D per litre. Culturing was continued on this medium until 1960, when the salts and trace solutions were replaced by the macro- and micro-elements given by HELLER (1953): 0.750 g KCl, 0.075 g $CaCl_2.2H_2O$, 0.125 g $NaH_2PO_4.1H_2O$, 0.600 g $NaNO_3$, 0.250 g $MgSO_4.7H_2O$, 1 mg $FeCl_3.6H_2O$, 1 mg $ZnSO_4.7H_2O$, 1 mg H_3BO_3, 0.1 mg $MnSO_4.4H_2O$, 0.03 mg $CuSO_4.5H_2O$, 0.03 mg $AlCl_3$, 0.03 mg $NiCl_2.6H_2O$, and 0.01 mg KI per litre. Since 1965, agar has been given as 0.8 % Difco agar Noble.

In 1962, tissue from the 16th subculture of the *Populus* strain was transferred to media containing other growth substances in various concentrations. This gave one strain on a medium corresponding to that of the original tissue but with α-naphthalene acetic acid (1.0 mg/litre) instead of 2,4-D and one strain on a medium containing β-indoleacetic acid (10 mg/litre). For the latter, kinetin was added to the medium in some cases (0.1 mg/litre).

The tissues were cultured in tubes with a diameter of 30 (23) mm, containing 25 (16.5) ml medium, at a temperature of 23 °C ± 1 °. The tubes were exposed for 12 hours per day to light provided by Philips TL 55 lamps (40 watt); to make certain that all cultures received the same total amount of light, the tubes were placed vertically on a disc rotating in a plane perpendicular to that of the light tubes. The tissues were transferred to new medium at roughly two-month intervals.

Explants of *Populus* phloem or short pieces of young twigs generally show good callus development in the growing season. The phloem explants show a rich proliferation of green or white callus, several millimetres thick, on the cambial side. The mitotic activity is not limited to the cambial zone but also occurs in the secondary phloem. Transfer of the callus usually gives poor results, however; growth is not resumed, and the tissue dies except in rare cases. Later,

it was found that much better growth can be obtained on media with higher salt concentrations such as those of MURASHIGE & SKOOG (1962) or WOLTER (1968).

The cultures on 2,4-D and NAA media were very similar in appearance and growth rate, and the transfer from 2,4-D to NAA medium gave little difficulty. The culturing of tissue on IAA medium, however, is very troublesome. Of the many attempts with media containing IAA in various concentrations, success was obtained in only one case, that of a culture on a medium with IAA in a concentration of 10 mg per litre. The "substrain" derived from this single culture was maintained on a similar medium with varying succes. No improvement was obtained by the addition of kinetin or pyridoxine, and the same holds for reduction of the IAA concentration.

3.1.2. Brief description of the cultures

The tissue samples grown on media containing 2,4-D and NAA give voluminous, light beige-yellow, rather coherent callus *(fig. 3)*. The moisture content and friability of the cultures can vary per transfer. In some cases the tissue grows in the shape of a cushion with a central cavity on the lower side. For the 29th subculture, *table 1* gives some values for the fresh weight and percentage dry weight of the cultures. The fresh weight reaches more than 5 grams in some cases. The moisture content was often much lower than the values shown in the table. The dry weight can reach 7 per cent of the fresh weight in two months, and may then continue to rise. After two months there is usually little increase in the volume of the cultures.

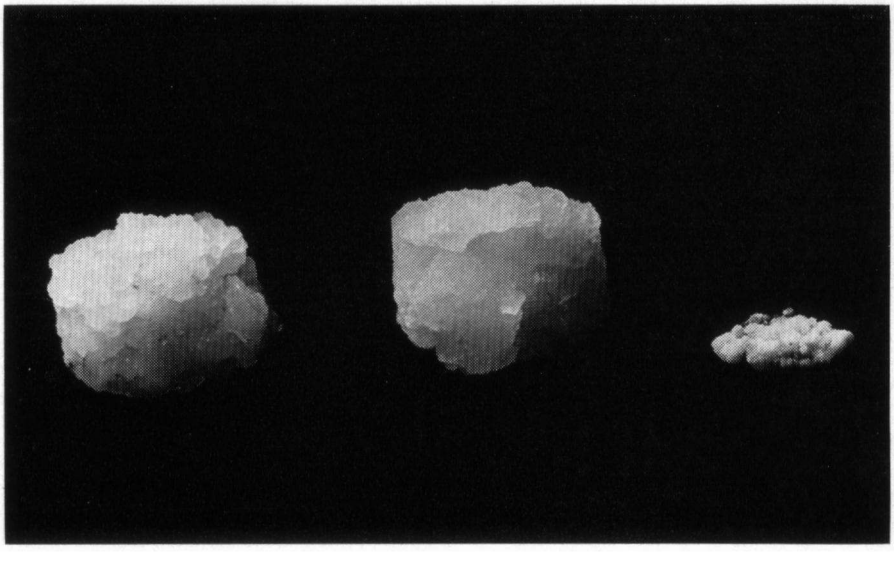


Fig. 3. Tissue cultures (aged two months) from the strains developed on media with IAA (right), NAA (centre), and 2,4-D (left), × 1.1.

Table 1. Fresh weight and dry weight of some tissue cultures.

	number of cultures	age	mean fr wt/cult (g)	dry wt % of fr wt	dry wt after extr* % of fr wt
29th 2,4-D	106	63 days	2.84	4.0	2.1
29th NAA	110	64 days	3.07	4.7	2.3
23rd IAA	14	165 days	1.20		9.1

* Extraction with 80% alcohol, alcohol-benzene, and water, successively. The percentages are too low due to a loss of dry material during extraction.

The cultures on a medium containing IAA show an entirely different structure. They are very compact, usually flat, and have a beige or brown central area and a greenish-white or beige border. Growth takes place mainly on the margins, but sometimes also on the underside, often in the shape of spherical proliferations. The occurrence of chlorophyl is striking. The IAA strain originated from tissue cultured on a medium containing 2,4-D, which is thought to inhibit the development of chloroplasts (BERGMANN 1967), but in our material the ability to form chloroplasts was maintained throughout 16 transfers on a 2,4-D medium.

In a few of the cultures on IAA medium, root formation was observed.

A detailed anatomical description will be found in section 4.3.

3.2. Tissues from the tree

In the preparation of tissue samples for chemical investigation, an attempt was made to have only one type of lignified tissue in each sample *(table 2)*, but this result was not always achieved. For the isolation of secondary xylem, the pith and probably also the primary xylem were removed. The isolation of the secondary phloem, which contains groups of sclerified elements, also gave no difficulties. But the preparations of the periderm, for which the material was obtained by scraping the surface of branches, were always contaminated by sclerenchyma from the phloem.

For the isolation of primary xylem, a very large number of freshly-collected stem tips were carefully freed of leaves and fixed in alcohol. An attempt was made to collect only pieces containing primary xylem, without lignified fibres or secondary xylem elements. In preparations I, III, and IV this attempt was successful; the lignified tissue consisted mainly of protoxylem. In preparation II, however, some of the twigs had already developed a small amount of lignified secondary xylem and some sclerenchyma.

The sample of young secondary xylem was isolated from 7 to 15 year old branches. After the bark had been stripped away, a very thin layer of the cambial zone and the adjacent xylem was scraped off. The remaining xylem was used for the preparation "old secondary xylem".

The samples of xylem, phloem, and periderm were isolated from the same branch, which had a diameter of 4.5 cm. The periderm was obtained by scraping

Table 2. Tissue samples; origin and isolation

sample	date of isolation	diameter or age of branch	kind of tissue	fixation
xylem	15-6-62	4.5 cm	secondary xylem	drying
xylem twigs	7-7-64	0.5–1.5 cm	secondary xylem	ethanol
young sec. xylem	13-5-65	7–15 yr	cambial zone + young sec. xylem; scraped	ethanol
old sec. xylem	13-5-65	7–15 yr	rest sec. xylem after scraping	ethanol
primary xylem		youngest 1–2 internodes	entire stem tip	
primary xylem I	24-6-63		only primary xylem lignified	drying
primary xylem II	16-6-65		also some lignified sec. xylem elements and fibres	ethanol
primary xylem III	18-6-65		only primary xylem lignified	ethanol
primary xylem IV	5-5-66		only primary xylem lignified	ethanol
phloem	15-6-62	4.5 cm	strands of sclerenchyma	drying
"periderm"	15-6-62	4.5 cm	lignified suberized phellem; sclerenchyma of phellem, cortex, pericycle and phloem	drying
"periderm" twigs	22-1-63	1–2(4) yr	as above but including epidermis	ethanol
1–19th subculture			callus	drying
20th–35th subcult.			callus	ethanol

the surface of the branch. A thin layer of the exposed surface was then scraped off and discarded. The remaining part of the bark was torn off along the cambial zone; this supplied the preparation called "phloem". The wood provided the xylem preparation after removal of the pith. For these last three preparations, the material was cut into small pieces, dried at 80 °C, and then extracted.

The stabilization and pre-extraction procedures are described under 5.2.

4. ANATOMICAL STUDY

4.1. Fixation and staining

The tissue to be sectioned was fixed in a CRAF fixative containing per litre 5 g chromic acid, 35 ml glacial acetic acid, and 150 ml 40% formalin. Before fixation, the cultures were cleared of any adhering agar. After fixation, the tissue was embedded in diglycolstearate (Pegosperse 100S, Glyco Chemicals, New York) and cut into 15μ-thick sections. Some of the sections were stained according to a modified Maácz-Vágás method calling for astra blue (FM, Chroma: 0.5% in 2% tartaric acid), a saturated aqueous solution of auramine (Merck), and a 1% aqueous solution of safranine (Merck). After staining with astra blue and with auramine, the sections were rinsed in distilled water; after staining with safranine, however, acetone was used. The preparations were then treat-

ed with phenol-benzene (1:3) and xylene, and mounted with S.Q.D. balsam (Gurr). In some cases staining for callose was included: after the phenol-benzene bath the sections were placed successively in acetone, distilled water, and a 0.025% solution of aniline blue (Merck) in 0.066 M K_2HPO_4 (1–10 min). After a xylene treatment, these sections were mounted with Fluormount (Gurr).

Another group of sections was stained by other methods, *e.g.* phloroglucinol-hydrochloric acid, Mäule, Sudan III, and Lugol's iodine solution. The Mäule reaction was performed as follows: 1% potassium permanganate for 5 min, rinse with water, 18% hydrochloric acid for 2–10 min, rinse with water, ammonia fumes. The positive result is a red or purplish-red colour; a brownish colour is considered negative. Tannins are stained brown to black. For staining with Sudan III, use was made of a solution in alcohol and glycerine; the preparations were heated together with the reagent to the boiling point of the alcohol.

The Lindt reaction with vanillin and hydrochloric acid, as a test for tannins, could only be used for fresh tissue.

4.2. Types of lignified elements in the tissues of the tree

4.2.1. The young twig

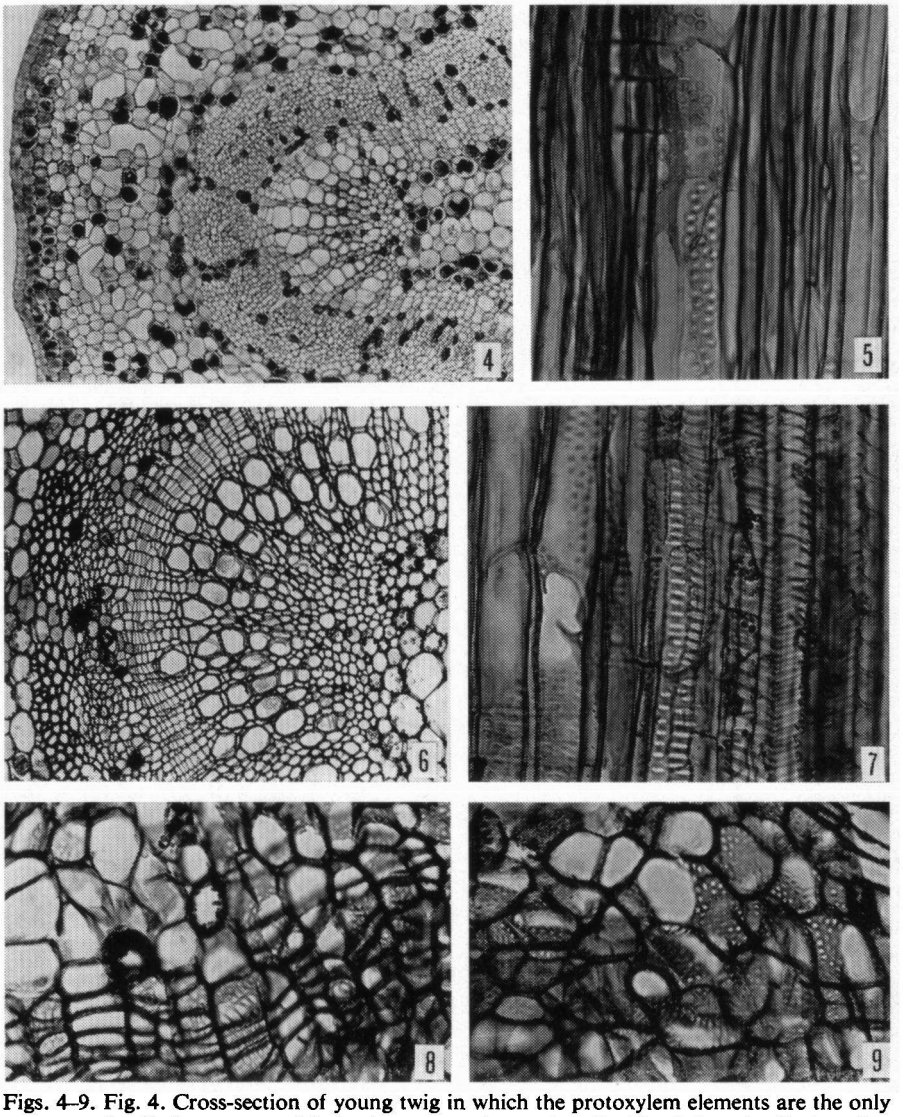
Primary xylem: Very young twigs, like those used for the samples of primary xylem I, III, and IV, were investigated by several histochemical reactions. Some of the samples already showed the onset of cambial activity, but there was as yet no differentiation of secondary elements *(fig. 4)*. The only elements in the twig giving a positive lignin reaction are those of the protoxylem and metaxylem. The compound middle lamella of the vessels is unstained, but the ring and spiral structures show a distinctly positive phloroglucinol reaction. The Mäule test gives only negative results, however.

The pericyclic fibres only differentiate at a later stage *(fig. 6)*. In the primary xylem II sample, which included tissue that already showed a little too much differentiation, there were a few pericyclic fibres with a positive Wiesner and Mäule reaction. In this material some of the most recently formed scalariform pitted vessels showed a weakly positive Mäule reaction. In the ring and spiral elements the Mäule reaction was negative.

4.2.2. The older branch

The situation in older branches is quite different. Lignified cells are present not only in the primary xylem but also in the secondary xylem, secondary phloem, pericycle, cortex, and periderm *(figs. 5–7)*.

Xylem: Within the scope of this paper, only the reaction of the cell walls to staining will be discussed; a detailed description of all the elements of the secondary xylem will not be given. The most striking phenomenon is the lignin reaction of the pitted vessels. Although the walls of the vessels, including the pit borders, give a distinctly positive phloroglucinol reaction, the Mäule reaction of these walls is very weakly positive and is very often restricted to the areas be-



Figs. 4–9. Fig. 4. Cross-section of young twig in which the protoxylem elements are the only lignified structures. All the tissues contain tannin cells. Fig. 5. Longitudinal section of secondary xylem. Pitted vessel with bordered pits, and large pits to the ray parenchyma. Fig. 6. Cross-section of young secondary twig. At this stage, not only primary but also secondary xylem elements with lignified walls are present. The cell walls of the pericyclic fibres are lignified, but still rather thin. Fig. 7. Radial section through the primary and secondary xylem. Between the primary elements with spiral thickenings and the pitted vessels of the secondary xylem there are some metaxylem elements with a scalariform wall pattern. Figs. 8 and 9. Tracheids formed in the IAA culture (35th subculture). They have bordered or simple pits; a more reticulate-scalariform pattern occurs occasionally. The pits are somewhat smaller than those of the pitted vessels from the tree and their borders are narrower. Between the tracheids there are tannin cells. Maácz-Vágás staining. Figs. 4 and 6, × 120; Figs. 5, 7, 8, and 9, × 240.

tween the pits. The rest of the secondary xylem gives positive results for both tests.

The composition of the tissue sample "old secondary xylem" is roughly the same as that of the xylem preparation. The volume of the scraped layer of young secondary xylem is of course insignificant as compared to the entire wood cylinder.

The young secondary xylem tissue sample contains a relatively large amount of wide pitted vessels (sampled in May). The walls of many of the elements show little or no staining with either of the lignin reagents. With the Mäule method, appreciably fewer elements are stained than with the phloroglucinol reaction, and with the former the vessels are consistently less intensely stained than the adjacent cells of other types.

Phloem: In the secondary phloem, new strands of fibres develop anually. These strands are covered with chambered crystal fibres whose walls usually stain darker in the phloroglucinol reaction than those of the ordinary fibres. In the Mäule test, these intensities are often reversed. With the phloroglucinol reaction the compound middle lamella of the fibres stains darker than the thick secondary wall. Here again, in the Mäule reaction the staining of the compound middle lamella is weak and that of the secondary wall rather strong. Sclereids also occur between the fibre bundles, usually in the bast ray tissue. The thick lamellated wall of these sclereids is usually stained completely with phloroglucinol, but the Mäule reaction is often rather weak; some areas in the wall are left unstained, whereas other parts, *e.g.* near pit canals, are well stained.

The local differences in the intensity of staining of walls with the Wiesner and Mäule reactions may indicate the presence of different types of lignin in different parts of the cell wall, but differences in density or swelling capacity of the cell wall could also lead to unequal staining.

Periderm: A branch aged several years is covered with a superficial phellem several cell-layers thick. Usually, the middle lamella of the entirely suberized walls is stained by phloroglucinol. The Mäule reaction is negative. Either diffusely distributed or in tangential bands there are sclereids showing lignified but not suberized walls. In places at which the tissue has been ruptured there are more sclereids and the peridermal cells generally show lignification. The "periderm" sample contained lignified, suberized phellem, but most of the lignin seemed to be present in the walls of sclereids and fibres. The latter are so-called pericyclic fibres or derive from the secondary phloem. The sample must therefore be considered to be a mixture of peridermal cells and sclerenchyma of the cortex, pericycle, and bast; here, however, it will be indicated as "periderm".

4.3. Structure of the tissue cultures

The three tissue-culture strains showed mutual differences in structure and morphogenetic capacity. The original culture on 2,4-D remained capable of

forming tracheids during the first nine transfers. In its third year, however, groups of tracheids were seen only sporadically. After the 13th subculture, no tracheids were found in the 2,4-D strain.

Tissue from the 16th subculture was thereafter cultured on media containing other auxins. The cultures on a medium containing NAA and those on a medium with IAA both showed tracheids again, although the former only during the first few subsequent transfers. The IAA strain, to the contrary, has maintained this characteristic up to the present, and the 4th and 10th subcultures moreover showed development of roots.

4.3.1. The IAA strain

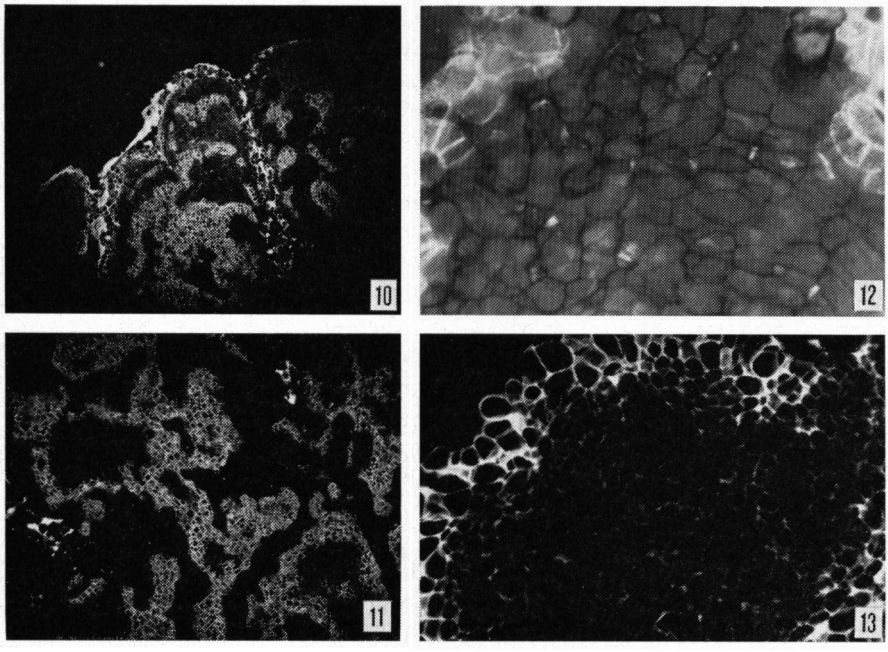
The tissue grown on an IAA medium has consistently shown many tracheids *(figs. 8–12)*. The occurrence of groups of tacheids is apparently not related to the presence of kinetin in the medium, since cultures on a medium in which it is absent can show an abundance of these elements.

The tissues are very firm and compact. Growth occurs mainly on the sides, but new tissue is also formed at the top and bottom. The central old tissue often shows necrosis. At the periphery of the cultures and mainly on the lower surface, there are small, nearly spherical growth regions evidently originating by the mitotic activity of a small group of cells. In some cases the growth is localized in hemispherical lobes with a rather deeply situated meristem, leading to an anatomical structure that presents a fan-shaped pattern in cross-section *(fig. 22)*. There is usually a distinct borderline between adjacent growth zones, because the tissues have a superficial covering of a few or many layers of rather large, somewhat rounded cells. The cell walls and intercellular spaces in this region are often lignified and usually also slightly suberized.

The tissue contains groups of highly lignified tracheids with a central localization or a somewhat diffuse distribution. Beside or between these groups of tracheids, phloem is present *(fig. 12)*. The sieve elements are sometimes found in an arrangement of connected curving chains with sieve plates on the common walls, but they usually occur in more spherical groups. The sieve plates are often covered on both sides by a layer of callose. In the zones with phloem, chloroplasts also occur. Some cultures are unusually rich in starch. Tannin cells occur singly or in groups. The structure and stainability of the content of the tannin cells are highly variable. A tannin-like material is also sometimes found in tracheids.

The following types of lignified structures are present in the tissue cultures:

Tracheids: These structures usually have the same dimensions as the surrounding parenchyma cells, and are isodiametrical to very elongated. The outer surface of cultures sometimes shows rounded tracheids attached only by a small base. The tracheids have round to oval bordered pits or a reticulate-scalariform wall pattern *(figs. 8* and *9)*. Some cells show large simple pits. The thin walls are easily stained with phloroglucinol-hydrochloric acid or safranine. The Mäule reaction is weakly positive or negative. The pit membrane is often stained blue



Figs. 10–13. Primary fluorescence of lignin and fluorescence of callose stained with aniline blue. Fig. 10. Section from upper side of IAA culture. Primary fluorescence of extracellular and intercellular lignin in the surface tissue; groups of tracheids also show fluorescence. × 17. Fig. 11. IAA culture; groups of tracheids and, locally, intercellular lignin. × 33. Fig. 12. IAA culture; chain of sieve elements with callose on the common walls. Fluorescence of tracheids on the left and right. Aniline blue. × 200. Fig. 13. NAA culture; spherical tissue zone showing peripheral parenchyma cells with lignified walls and intercellulars. × 100. The sections in Figs. 10, 11, and 13 were not stained.

by the Maácz-Vágás method, *i.e.* it does not take safranine. In the phloroglucinol test no staining of the pit membrane was observed.

Lignified parenchyma cells: The lignified parenchyma cells occur in groups. The walls are entirely or partially stained by phloroglucinol-hydrochloric acid, by safranine, or by the Mäule reaction. Often a positive lignin reaction is also found in the intercellular spaces of the lignified parenchyma cells.

Intercellular spaces: The lignification of intercellular spaces is a rather frequent phenomenon in tissue cultures, and has been described by BERGMANN (1964) for *Nicotiana* cultures. The middle lamella may be heavily lignified, especially at places where more than two cells are contiguous. "Extracellularly", there may be an entire or partial covering of the cell wall with a stainable substance, as exemplified by the rather loosely connected tissue on the periphery of poplar cultures. The stainable substance often lies as a cap over the wall of projecting cells *(fig. 15)*. Spaces enclosed between the growing lobes of the tissue as well

as wide intercellular canals may also be filled with a stainable substance. This substance either has a homogeneous appearance *(figs. 18, 20)* or is somewhat granular *(fig. 14)*. Even after extensive extraction with alcohol, positive staining reactions are still obtained, and it may therefore be assumed that the substance concerned is not a simple precursor of lignin. In the Mäule reaction a considerable amount of the inter- and extracellular material is also stained red, but in some cases the colour is more orange than red. In general, fewer elements are stained with the Mäule reaction than with the phloroglucinol reaction. In ultraviolet light, the stainable material in the intercellular spaces shows the same greyish-white primary fluorescence as the other lignified structures *(fig. 10)*.

Warts: Warts generally occur on the walls of the cells located on the periphery of the culture *(figs. 16, 17, 19, 21)* in the form of small, often hemispherical structures sometimes showing a positive lignin reaction that may be absent in the rest of the cell wall. Staining is rather variable; there is usually a distinct coloration with Sudan III as well as with ruthenium red. Warts are found on both parenchyma cells and tracheids.

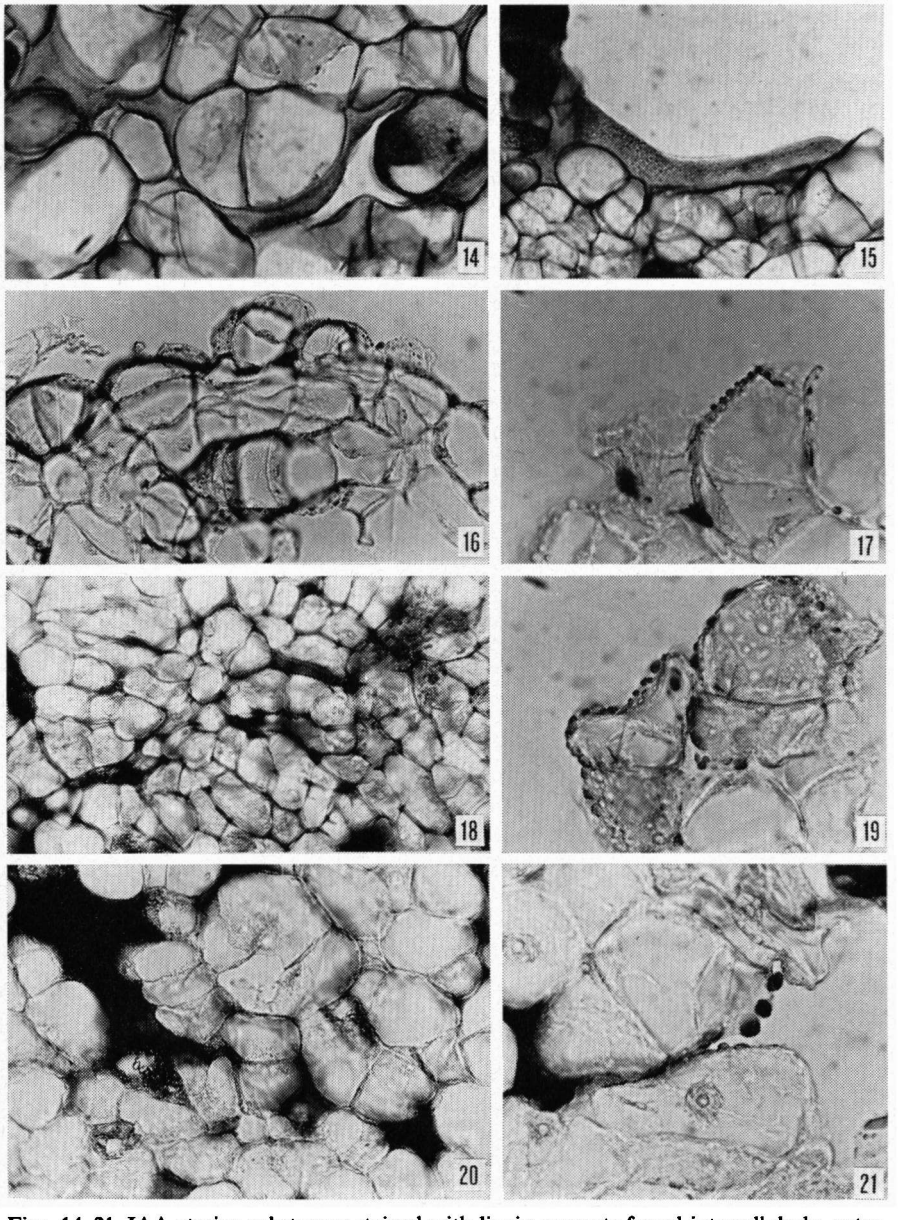
The shape of the warts resembles that of the structures described by BEHR (1959), under the term "*Zapfen*" for the cells of mesocarpal origin found in the core of some apples. Warts were also found in explants of *Sambucus* by CLAUSING & KARSTENS (1955). WHITE (1967) described phloroglucinol-positive droplets on the surface of cells in *Picea* cultures.

Agar: In sections, the agar often found adhering to the lower part of the cultures sometimes stains red in the phloroglucinol reaction. The cell-free surface layer of the agar substrate on which a culture has grown for some time is also usually phloroglucinol-positive in the area on which the culture rested. An investigation to determine whether this reaction indicates the presence of a true lignin is in progress.

The tissue of the 7th subculture on an IAA medium was used for the lignin determinations. The pre-extracted powder contains all of the above-described lignified cellular structures, although relatively few tracheids. The lignin of intercellular spaces and parenchyma cells predominates. The material from the 7th subculture on an IAA medium (representing the 23rd subculture in total) had distincly fewer tracheids than material from other subcultures. The photographs in *figs. 8–12* show callus of the 35th subculture, which had an abundance of tracheids.

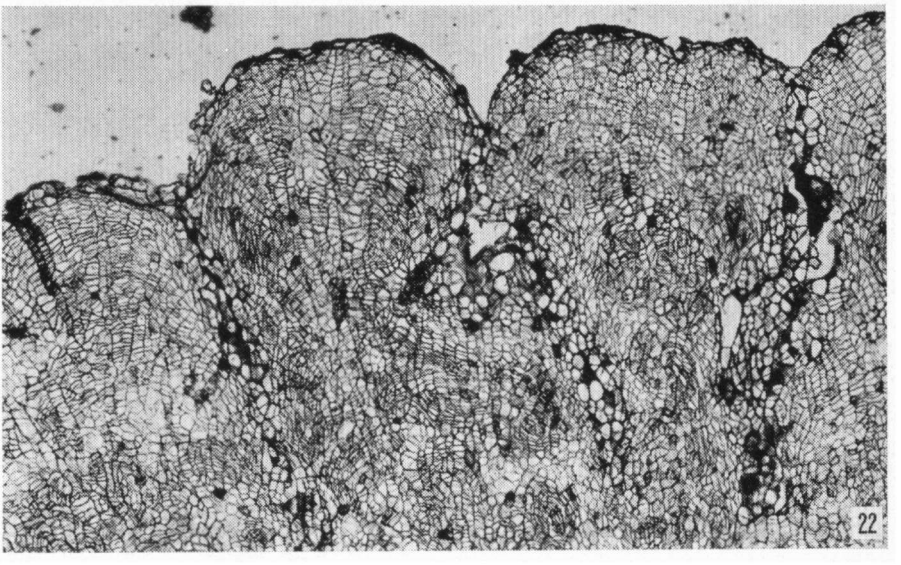
4.3.2. NAA and 2,4-D cultures

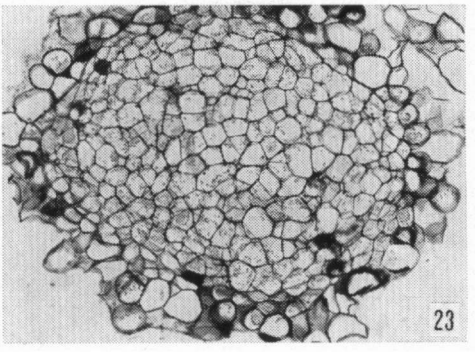
The later subcultures show no essential anatomical differences between the tissues grown on NAA and 2,4-D media. The cultures of both strains have a structure differing completely from that of the IAA strain. Mitotic activity often occurs in superficially located foci, which develop into spherical structures, but only the outermost layers of these spherical structures form a coherent tissue;



Figs. 14–21. IAA strain; substances stained with lignin reagents found intercellularly, extracellularly, or in warts. Fig. 14. Large intercellular spaces filled with a fine granular substance stained with auramine. Fig. 15. Granular layer stained with auramine and safranine covering some peripheral cells. Figs. 16, 17, 19, and 21. Warts on walls of peripheral cells. The warts are stained with phloroglucinol-HCl; the cell walls under the warts are sometimes stained. Figs. 18 and 20. Large and small intercellulars stained with phloroglucinol-HCl. Figs. 14 and 15, Maácz-Vágás staining. Figs. 14, 15, 17, 19, and 21, × 330; Figs. 16, 18, and 20, × 210.

the central area shows marked elongation of the cells. The middle lamellae probably dissolve to a large extent. Thin-walled, detached cellular filaments resembling hyphae are present. The cells vary widely in size *(fig. 24)*. Tannins and starch are sometimes found locally in the tissue, but chloroplasts are absent. There are no tracheids. A positive lignin reaction is usually found in the walls and intercellular spaces of the tissue covering the spherical structures *(fig. 13)*, but zones of lignified cells also occur at other places. Fewer elements are stained





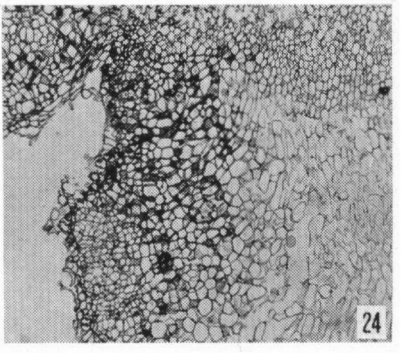


Fig. 22. IAA culture; lobed structure at the top of the callus. Safranine staining of material in spaces between the tissue lobes and at the surface (cf. Fig. 10). To the left, a row of tannin cells. Maácz-Vágás staining. × 50.

Fig. 23. 2, 4–D culture; section through a spherical group of isodiametric cells. Walls and intercellulars of the outer cell layers are lignified (cf. Fig. 13). Maácz-Vágás staining. × 110.

Fig. 24. Part of a 2, 4–D culture with a surface layer of isodiametric cells and a central zone of large cells in filaments. Maácz-Vágás staining. × 35.

in the Mäule than in the phloroglucinol reaction, and the intensity of the staining is often weak. As in the IAA cultures, extracellular material is sometimes also stained by the lignin reagents.

Cells with warts are seldom seen. On the periphery of the cultures there are sometimes small groups of cells with a thicker wall. Cells with a suberized wall are rare.

In the extracted powder from the cultures on NAA and 2,4-D media used for the chemical investigations, cells and intercellular material again show positive lignin reactions.

5. CHEMICAL INVESTIGATION

5.1. Sampling

The sampling of tissues from the tree has been described on page 259. The tissue cultures were normally used two months after the last transfer, but in a few cases older cultures were used. The tissue was freed of agar remnants, weighed, and extracted. Details of the tissue samples are shown in *table 2.*

5.2. Stabilization and pre-extraction

Initially, the tissues were cut into pieces and then dried at 80 °C. This slow fixation was applied to the samples of xylem, phloem, "periderm", primary xylem I, and all tissue cultures through the 19th subculture. The dried samples were ground before extraction with ethanol-benzene and water. To avoid undesirable reactions, this procedure was later replaced by a more rapid method in which the tissues were cut into pieces, fixed for 10 minutes in boiling 80 per cent ethanol, ground in a Serval Omnimixer, given two 10-minute extractions with 80 per cent ethanol, and then extracted for 8 to 10 hours in a Soxhlet apparatus with alcohol-benzene (1:2). In most of the cases the air-dried material was then extracted for 5 to 6 hours with water ($\pm$ 100 °C). The extracted material was filtered and rinsed successively in hot water, 96% alcohol, acetone, and ether. Drying was done in a desiccator over P_2O_5. All determinations were calculated on the basis of the dry weight (at 100 °C) of the material extracted in this way. For the determination of the moisture content, separate samples were used in all cases.

5.3. Methods used for the determination and characterization of lignin

Alkali lignin

About 50 mg air-dry, extracted material was heated with 2 ml 0.5N NaOH for 16 hours at 70 °C in a stoppered tube. The hydrolyzate was then cooled and filtered. The filter was rinsed with NaOH (0.5 ml; twice)and a small amount of water. The filtrate was neutralized with 2N HCl to partial decoloration of the yellow or brown solution, and then made up to 5 ml to form the total alkali-lignin preparation. In most cases, however, only alkali lignin A is obtained; *i.e.* the lignin fraction that can be precipitated by acidification of the filtrate. Several hours after the acidification, the precipitate was separated by filtration

and redissolved in NaOH 1/12 N to a volume of 5 ml. The resulting alkali lignin or alkali lignin A solution was used in the quinoneimine test. In addition, the absorption spectra in alkaline and neutral solution and the difference spectrum were determined for subsequent calculation of the lignin content (see page 271).

Soluble native lignin

From the wood of *Populus nigra* cv. *'Italica'*, a soluble native lignin was isolated. A sample (100 g) of the same material from which the "young secondary xylem" and "old secondary xylem" had been prepared was cut up and then ground until a particle size passing a sieve with a pore diameter of 0.5 mm was obtained. Extraction was done with 96% ethanol at room temperature for a total of 30 days. Fresh alcohol was substituted several times. The alcoholic extracts were filtered, combined, and concentrated in the presence of a small amount of calcium carbonate under reduced pressure at a bath temperature not above 40 °C. Distillation was continued until the sample was almost dry, after which a few millilitres of water were added and distillation was continued to remove the remainder of the alcohol. The residue was transferred to a G4 glass-filter, filtered, and then rinsed with 10 ml ether. The remaining mass was dried over P_2O_5 and treated on the filter for several days with dried ether. The residue (970 mg) was brought into a tenfold volume of dioxane (p.a.) to which a few drops of water had been added. This gave a brownish-yellow solution, but a rather large portion remained undissolved. This portion was removed by centrifugation. Under stirring, the dioxane solution was added dropwise to a fifteenfold volume of water, after which a cream-white precipitate appeared. After filtration and drying, 365 mg of a light-coloured powder was obtained; this powder showed strongly positive phloroglucinol and Mäule reactions.

Thioglycolic acid lignin

A 500 mg air-dry, extracted sample of tissue was heated with 15 ml thioglycolic acid-HCl (10 g thioglycolic acid to 100 ml 2N HCl) in a water bath at 100 °C. When less material was available, smaller samples were used (*e.g.* 200 mg). After heating for 1 hour, the mass was stirred and the walls of the vessel were rinsed off with an additional 7 ml of the thioglycolic acid-HCl mixture. After a total of 4 hours of heating, the material was cooled and filtered. The residue was thoroughly rinsed with about 40 ml water and then dried for 1 hour in an air current. The lignothioglycolic acid fraction I was obtained by percolation with ethanol for 24 hours and drying the alcoholic extract. The residue on the filter was allowed to dry for some time to remove the alcohol, and then brought into 10 ml 0.5 N NaOH, into which it was mixed by stirring. After 24 hours the now dissolved lignothioglycolic acid was filtered and precipitated with HCl 5 N. On the following day, the precipitate was collected on a previously weighed filter and then dried for 4 hours at 100 °C (lignothioglycolic acid fraction II). The remaining cell-wall material was checked with the Wiesner and Mäule reactions for the presence of stainable substances, and in some cases treated again with

thioglycolic acid. After filtration, treatment with alkali, filtration, and precipitation, the lignothioglycolic acid fraction III was obtained.

For the determination of the absorption spectra, the lignothioglycolic acid preparations were dissolved in 1/12 N NaOH and diluted with NaOH or phosphate buffer (pH 7.0).

The elementary analyses were done by the Department of Microanalysis of the Organic Chemistry Laboratory of the University of Amsterdam.

Ultraviolet and difference spectra

The alkali-soluble lignin preparations (lignothioglycolic acid, native lignin, and alkali lignin) were dissolved in 1/12 N NaOH and diluted with NaOH (pH 12) or phosphate buffer (pH 7.0) to a buffer concentration of 0.05 M; when necessary, neutralization was done with 2 N HCl. The concentration of the lignin preparation in the solutions to be measured amounted to 0.04 mg/ml.

The absorption spectra at pH 7 and pH 12 were measured separately, and an ionization difference spectrum was obtained by automatic registration of the difference in absorption between the two solutions. When the difference spectra were used for quantitative calculations, a correction was made for the absorption of the buffer and NaOH solutions.

All the measurements were made in a Beckman DB spectrophotometer provided with a recorder.

Infrared spectra

The infrared spectra were determined with a Beckman IR 10 apparatus of the Organic Chemistry Laboratory in Leiden[1]. A 3 mg sample or less of the dried lignin preparation was powdered and mixed with about 300 mg dried KBr and stamped to form a pellet (diameter 16 mm). The absorption was measured in the range of 4000 to 300 cm^{-1} (2.5 to 40 μ).

Quinoneimine test

The method given by STAFFORD (1960) was modified such that the spectrophotometric determination could be done in a reaction mixture of 2.4 ml. To 1 ml of the lignin sample in a neutral or weakly alkaline solution, 0.4 ml of a 0.5 M tris (hydroxymethyl)-aminomethane buffer and 1 ml of a freshly prepared alcoholic solution of N,2,6-trichloro-p-benzoquinoneimine (Fluka; 50 μg/ml) were added. These solutions were shaken briefly in a stoppered flask. After one hour, the absorption was measured against a blank composed of distilled water, buffer, and the reagent. Guaiacol solutions (1 to 10 μg/ml) were used as standard. For the alkali lignin of xylem, about 2 mg of the original tissue sample was required per determination.

The method cannot be used in this form for the analysis of the dark solutions of the lignin from periderm and the tissue cultures, because the solutions themselves show absorption in the range of the measurements. The samples were

[1] I wish to take this opportunity to express my sincere appreciation for all the facilities put at my disposal during this study.

therefore shaken with 2.4 ml n-butanol to extract the indophenols (STAFFORD 1964). In this case the 2,6-dichloroquinone-4-chloroimide was diluted not with alcohol but with water, to permit extraction with butanol. The measurements were performed at the absorption maximum (± 636 nm). This method has the disadvantage, however, that the absorption maximum in butanol of the 2,6-dimethoxyphenolindophenol lies at a slightly lower wavelength (± 615 nm) than that of the 2-methoxyphenolindophenol (XXIV).

Oxidation with nitrobenzene

Air-dry tissue samples weighing between 10 and 180 mg were shaken with 2 ml NaOH 2N and 0.2 ml nitrobenzene in a stainless steel bomb for 3 hours at 170 °C in an incubator requiring about 50 minutes to raise the temperature from 100 to 170 °C. In a few cases other times and temperatures were used. The reaction mixture was cooled and brought with distilled water into a separating funnel. The nitrobenzene was removed by shaking with 3 to 4 portions of 100 ml ether. After acidification, the aldehydes were extracted with 4 portions of 50 ml ether. The ether was then evaporated over a waterbath and the residue taken up in alcohol to a total volume of 5 ml. With a micrometer syringe, 0.500 ml of this solution was streaked onto the 15 cm wide middle zone of the chromatograms. In addition, a mixture containing the pure aldehydes was spotted alongside the streak. The Whatman 1 chromatography paper had been given a thorough prior washing in alcohol to remove any vanillin present in the paper. The chromatograms were developed with ligroin (100–120 °C) – n-butylether (6:1; water saturated; descending). To insure good water saturation, a damp filter paper was hung in the tank.

After development, the lateral test strips of the chromatogram were removed and sprayed with a saturated solution of 2,4-dinitrophenylhydrazine in 1% hydrochloric acid to localize the aldehydes. For the quantitative determination of vanillin, syringaldehyde, and *p*-hydroxybenzaldehyde, bands of a given width containing the desired materials were cut out of the middle zone and eluated in small Soxhlet extractors with 18 ml 96% alcohol. After the addition of 2 ml 0.2% KOH, the solutions were made up to 25 ml. The absorption spectra were determined in a Beckman DB spectrophotometer with recorder; the aldehyde content was determined by measurement at 353, 368, and 335 nm, respectively. In a few cases a strong contamination of the aldehyde zones of the chromatogram necessitated the use of difference spectra (LEMON 1947). A more accurate result can be obtained with this method, because the contamination usually shows no absorption in the difference spectrum. The absorption values were corrected on the basis of measurements performed with blanks subjected to the complete procedure, including heating, extraction, and chromatography.

When the aldehydes themselves are subjected to oxidation with nitrobenzene in an alkaline medium, the recovery is always below 100 per cent; for syringaldehyde this value is usually not more than 50 per cent and for vanillin and *p*-hydroxybenzaldehyde it is higher. Only a small proportion of the observed loss is to be ascribed to the chromatographic procedure, since extraction and

chromatography of freshly prepared aldehyde solutions give a loss of only a few per cent (*table 14*, page 288). Heating with alkali and nitrobenzene results in a very high aldehyde loss, however. For heating at 170 °C for 3 hours, this loss is 4/3 greater than that given by heating to 160 °C for 2 hours. A large proportion of the missing vanillin and *p*-hydroxybenzaldehyde can be recovered as vanillic acid and *p*-hydroxybenzoic acid. During the oxidation of samples containing lignin, the aldehydes are gradually formed after a period of heating. The loss of aldehydes during the oxidation period is strongly dependent on the characteristics of the sample, and it is therefore impossible to calculate a correction for this loss.

In samples with a high tyrosine (protein) content, a high yield of *p*-hydroxybenzaldehyde is to be expected. At oxidation, 3.6 per cent *p*-hydroxybenzaldehyde was obtained from 1.0 mg tyrosine, which means that about 5 per cent of the tyrosine was converted into *p*-hydroxybenzaldehyde. Oxidation of 100 mg egg albumin gave a yield of 0.24 mg *p*-hydroxybenzaldehyde. Egg albumin contains about 3.7 per cent tyrosine. This means that about 9 per cent of the tyrosine was oxidized into *p*-hydroxybenzaldehyde. The tyrosine content of proteins is variable; for example, globulin from peas contains 3.8 per cent tyrosine and zein from maize 5.2 per cent. It may be concluded that tyrosine can only have a disturbing influence in samples with a low lignin and a high protein content.

Hydrolysis

For the hydrolysis of lignin preparations and tissue samples, 10 mg lignothioglycolic acid (or a corresponding amount of tissue) was brought into 7 ml 1/12 N NaOH and hydrolyzed for 18 hours at 80 °C with 7 ml 5 N KOH. For the later determinations a smaller volume of liquid was used, but hydrolysis was always performed in about 2.5 N alkali. After cooling, the solution was filtered if necessary and acidified with 5 N HCl. After about 3 hours, the precipitate was collected by filtration (fraction A). The filtrate was shaken with 4 portions of 50 ml ether. Fraction B is found in the aqueous phase; fraction C, containing the phenolic acids and the aldehydes, is found in the ether.

The acids and aldehydes were demonstrated by paper chromatography in which the spots were compared with spots representing known quantities. Certain acids, *i.e. p*-hydroxybenzoic acid, vanillic acid, syringic acid, and ferulic acid, were also determined quantitatively. For this purpose, chromatogram zones were eluted with alcohol and the absorption in the alcoholic difference spectrum was compared with that of solutions of known concentration. The aldehydes were determined quantitatively according to the method already described (page 272). For the separation of the phenolic acids, the following solvents were used: benzene saturated with formic acid (descending); benzene-acetic acid-water (40:10:2, upper layer; descending); *n*-butanol-ammonium hydroxide-water (8:1:1). The last of these solvents was combined with one of the first two for two-dimensional chromatography. The chromatograms were examined in the light of a Chromatolite UV lamp with and without am-

monia fumes and sprayed with a saturated solution of the diazonium salt of *p*-nitroanilin (Light & Co.) in water, and again, after drying, with a saturated solution of sodium carbonate (PEARL & MCCOY 1960). The fluorescence, staining, and R_f values of the phenolic compounds are shown in *table 3*. Certain acids (*e.g.* ferulic acid and protocatechuic acid) cannot be chromatographed with the butanol – ammonium hydroxide mixture because of instability.

5.4. Results

5.4.1. Thioglycolic acid lignin

5.4.1.1. Quantitative determination of lignothioglycolic acid

Lignothioglycolic acid was isolated according to Holmberg, a method giving quantitative data with fairly good reproducibility. Two fractions were usually isolated, an alcohol-soluble lignothioglycolic acid fraction (Lth I) and a fraction soluble in alkali but not in alcohol (Lth II). Both fractions contain complexes of thioglycolic acid and lignin, and both are soluble in dilute alkali;

Table 3. Chromatographic properties of phenolic compounds.

	UV	UV + NH_4OH	diazo[1]	diazo + Na_2CO_3	phlor + HCl	$R_f \times 100$[2] 1	2	3	4
gallic acid	v		br-y	br	–	0	0	0	0
coniferyl alcohol	(v)		be	v	(v)	0–4	0	±4	76
caffeic acid	bl		v-br	gr-br	–	0	0	12	±15
protocatechuic acid	d-v		be	br-v	–	0	1	12	
protocatechualdehyde	d-v			ysh	s	1	2	20	
p-hydroxybenzoic acid (Pa)	d-v		ysh	r	–	0	11	39	6
p-coumaric acid	d-v		oc	bl	–	1	18	42	14
p-hydroxybenzaldehyde (P)	d-v		ysh	r	y!	17	24	47	43
sinapic acid	l-bl	l-gr!	r	(v-bl)	gy-gr	0	31	58	6
syringic acid (Sa)	v		s	bl	(br)	0	37	62	4
sinapyl aldehyde[3]	gr	gr-y		y	bl-v	1	47		
vanillic acid (Va)	v		y	v	–	1	48	63	4
ferulic acid (F)	v-bl	l-bl	p	gy-bl	–	1	49	65	11
syringaldehyde (S)	(d-v)		be	l-y	p	9	59	69	40
coniferyl aldehyde[3]	gsh	ysh		y	r-v	16	63		
vanillin (V)	d-v		(be)	(v-r)	s	29	71	72	46
salicylic acid	v		–	br	–	12	74	75	74
cinnamic acid	d-v		–	–	–	24–40	88	86	59

Abbreviations: be = beige, bl = blue, br = brown, gr = green, gsh = greenish, gy = grey, oc = ochre, p = pink, r = red, s = salmon, v = violet, y = yellow, ysh = yellowish; l = light, d = dark; ! = intense colour, ( ) = weak colour.

[1] diazonium salt of *p*-nitroanilin

[2] solvents: 1 ligroin (100–120 °C): *n*-butylether = 6:1, water saturated; descending
2 benzene saturated with formic acid; descending
3 benzene: acetic acid: water = 40:10: (2); descending
4 n-butanol: ammonia: water = 8:1:1; ascending

[3] Obtained by alcoholic extraction from xylem

Table 4. Thioglycolic acid lignin yield of tissues from the tree.

	% lignothioglycolic acid				E 1% 1 cm			% lignin			
	I	II	III	I+II +III	I	II	III	I	II	III	I+II +III
xylem	3.84	18.4		22.2	53.7	56.2		2.5	12.3		14.8
	4.12	16.7		20.8	57.0	54.5		2.8	10.8		13.6
xylem twigs	3.71	17.7		21.4	64.5	59.0		2.8	12.4		15.2
	2.91	16.2		19.1	58.5	55.0		2.0	10.6		12.6
old sec. xylem	3.44	15.0		18.4	60.0	62.5		2.5	11.2		13.7
	3.48	15.6		19.1	61.7	66.0		2.6	12.3		14.9
	3.66	15.2		18.9	53.0	62.0		2.3	11.2		13.5
	4.26	16.1		20.4	53.5	59.7		2.4	11.4		13.8
	4.26	15.3		19.6	53.5	63.0		2.4	11.5		13.9
young sec. xylem	0.46	8.8		9.3	27.0	42.5		0.1	4.5		4.6
	0.67	8.27		8.9	21.0	41.7		0.2	4.1		4.3
	0.41	5.93		6.3	18.0	54.5		0.1	3.8		3.9
	0.22	7.43		7.6	24.5	51.7		0.1	4.6		4.7
	0.22	5.63		5.8	30.7	62.5		0.1	4.2		4.3
prim. xylem I	+	6.9		6.9	−	14.2			1.2		1.2
III	+	4.6		4.6	−	32.7			1.8		1.8
IV	+	3.0		3.0	+	34.7			1.1		1.1
phloem	1.55	12.0		13.5	47.0	49.5		0.9	7.1		8.0
	1.55	12.5		14.0	61.2	43.7		1.1	6.5		7.6
	1.3	12.5		13.8	69.2	56.0		1.1	8.3		9.4
	1.5	13.8		15.3	85.0	53.5		1.5	8.8		10.3
periderm	9.3	15.9	3.5	28.7	9.0	39.0	12.2	1.0	7.4	0.5	8.9

Lth II can be precipitated by acidification, but this is rarely the case for Lth I. The alcohol-soluble Lth I probably represents a lignin with a low molecular weight. Lth I always forms only a small proportion of the total amount of lignothioglycolic acid. In cases in which the residue was not entirely free of lignin (colour reactions), a second treatment with thioglycolic acid yielded a third fraction. Only in a few cases was an appreciable amount of this Lth III obtained. The total lignothioglycolic acid content was calculated by addition of the fractions Lth I, Lth II, and Lth III. *Table 4* gives the results for tissues from the tree, *table 5* for the tissue cultures. The percentages of lignothioglycolic acid are shown in the first four columns.

To check the purity of the preparations, the sulphur and nitrogen content was determined in several cases, and a complete elementary analysis was performed for one lignothioglycolic acid preparation of xylem and one of tissue-culture material. Both preparations were redissolved in diluted alkali and precipitated by acidification. The results are shown in *table 6*, in which the values obtained by Holmberg for *Populus tremula* L. are given for purposes of comparison. For *Populus nigra* cv. '*Italica*', the ash content of the preparations was not determined. The values obtained for the xylem preparation show good agreement with Holmberg's results, although the carbon content is lower. The tissue-culture preparation, however, shows a highly divergent composition. The very low

Table 5. Thioglycolic acid lignin yield of tissue cultures.

	% lignothioglycolic acid				E 1% 1 cm			% lignin			
	I	II	III	I+II +III	I	II	III	I	II	III	I+II +III
2,4-D 21st	0.64	4.86	0.7	6.2	+	24.5	10	+	1.4	0.1	1.5
	0.60	6.98	0.7	8.3	+	9.0	10	+	0.7	0.1	0.8
	0.54	4.12	—	4.7	13.5	32.7		0.1	1.6	—	1.7
2,4-D 23rd	0.76	4.90	0.89	6.6	—	24.0	16.5	—	1.4	0.2	1.6
2,4-D 29th	0.30	3.92	0.37	4.6	—	20.5	41.5	—	1.0	0.2	1.2
	0.39	6.83	0.37	7.6	—	13.5	41.5	—	1.1	0.2	1.3
	0.20	4.38	0.14	4.7	—	19.5	76*	—	1.0	0.1	1.1
	0.20	5.34	0.14	5.6	—	17.5	76*	—	1.1	0.1	1.2
	0.15	4.94	0.18	5.3	30*	21.7	+	—	1.3	+	1.3
	0.16	2.37	0.34	2.9	34*	42.0	+	0.1	1.2	+	1.3
	0.11	3.66	—	3.8	—	39.7		—	1.7	—	1.7
NAA 21st	0.24	11.5	1.0	12.7	9.2	32.0	53.0	—	4.4	0.6	5.0
NAA 23rd	0.92	12.0	2.5	15.4	27	33.5	58.7	0.3	4.8	1.7	6.8
NAA 29th	0.37	7.55	0.95	8.9	9.0	21.2	53.0	—	1.9	0.6	2.5
	0.45	8.36	0.72	9.5	13.2	20.5	41.7	0.1	2.0	0.4	2.5
	0.24	7.61	0.91	8.8	7.5	23.5	43.7	—	2.1	0.5	2.6
	0.20	6.55	0.91	7.7	+	26.7	43.7	—	2.1	0.5	2.6
	0.28	6.91	0.39	7.6	28.5	42.2	+	0.1	3.5	+	3.6
	0.31	9.39	0.24	9.9	20.5	22.5	+	0.1	2.5	+	2.6
	0.27	6.14	—	6.4	16.5	46.0		0.1	3.4	—	3.5
	0.10	7.85	+	7.9	39.0	24.5	+	—	2.3	+	2.3
	0.20	5.78	—	6.0	+	51.0		—	3.5	—	3.5
IAA 23rd	2.33	17.8	2.9	23.0	32.5	41.2	58.2	0.9	8.7	2.0	11.6
	1.76	18.3	1.61	21.7	23.2	47.5	63.7	0.5	10.3	1.2	12.0

* The shape of the spectrum implies that impurities contributed to the measured extinction.

Table 6. Elementary analysis of some lignothioglycolic acids.

		C %	H %	O %	S %	N %	OCH_3 %	
old sec. xylem	Lth II*	52.88	5.66	30.32	9.62	0	15.5	
xylem	Lth II	53.35	5.57	31.02	8.83	0		
NAA 29th	Lth II	46.94	5.44	40.31	3.47	2.89	4.3	
xylem	Lth II	55.32	5.61	30.12	8.95		16.9	Holmberg 1934
xylem	Lth II	54.52	5.56		10.32		15.5	Holmberg 1947

* purified by dissolving in dioxane and precipitation in water

carbon content and the very high oxygen content indicate a marked degree of contamination of the lignin. The sulphur content is extremely low, and the presence of a large quantity of nitrogen also indicates severe contamination, probably in the form of protein. The nitrogen, sulphur, and methoxyl content of several other preparations is shown in *table 7*. The lignothioglycolic acid preparations of secondary xylem show a high sulphur content. Nitrogen is absent or occurs only in a negligible quantity.

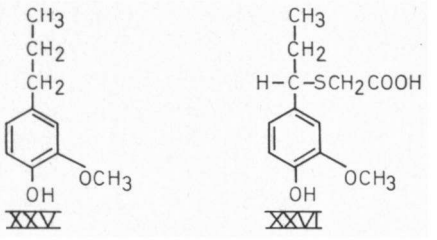
The preparations of young secondary xylem have a slightly lower sulphur content. The lignothioglycolic acid derived from the cultures shows a low (IAA culture) or very low sulphur content in all cases.

Lignothioglycolic acid from the primary xylem of the tree also has a low sulphur content. These data do not indicate a different ratio between lignin and thioglycolic acid for the lignothioglycolic acid derived from the tissue cultures. The data on the nitrogen content suggest rather that the low sulphur percentage of the lignothioglycolic acid preparations may be ascribed to contamination, *e.g.* by proteins. Holmberg also found abnormally low sulphur contents for the lignothioglycolic acid of several herbaceous plants, although others showed the normal value of about 9 per cent.

It is exactly for the tissues from which little lignothioglycolic acid is obtained, such as the primary xylem and the cultures on media containing NAA and 2,4-D, that the sulphur content of the lignothioglycolic acid is low and the nitrogen content high. These cases therefore concern a relatively strong contamination of the lignothioglycolic acid. A 9 per cent nitrogen contamination could mean that the preparation contained about 54 per cent protein. It is clear that the observed lignothioglycolic acid percentages require correction. It did not seem permissible to base this correction on the sulphur content, since there is no certainty that the lignin from cells still in the differentiation phase really bind as much thioglycolic

Table 7. Sulphur, nitrogen, and methoxyl content of some lignothioglycolic acids.

	Lth I or II	Lth % of sample	S %	N %	OCH_3 %
old sec. xylem	I	4	10.57	0	18 3
			10.30		15.9
old sec. xylem	II	15	9.10		18.5
			9.78	0.59	16.2
xylem	II	17	8.83	0	
					16.0
young sec. xylem	II	7	5.20	4.62	
			7.77	0.68	
			7.09	0.93	
primary xylem IV	II	3	2.39	9.40	1.82
IAA 23rd	II	18	5.73		
NAA 29th	II	7	3.47	2.84	
			4.73	3.23	6.17
2,4-D 29th	II	4	2.42	2.53	2.77
			4.25	3.68	



acid as lignin from mature secondary xylem. It therefore seemed preferable to develop a method that would make it possible to investigate the true lignin content of the lignothioglycolic acid preparations on the basis of the ultraviolet absorption of lignin. Furthermore, the influence of non-phenolic impurities can be avoided by the use of ionization or difference spectra according to AULIN-ERDTMAN (1953).

5.4.1.2. Absorption spectra of lignothioglycolic acid preparations

The influence exerted by thioglycolic acid on the lignin spectrum is probably limited. AULIN-ERDTMAN (1953, 1954) compared dihydroeugenol (XXV) and compound XXVI, which has a thioglycolic acid group in the side chain, and found a slightly higher specific extinction for the thioglycolic acid derivative. The ratio of the absorption at the two maxima in the difference spectrum was the same (0.45) for both compounds, but the higher maximum for dihydroeugenol (in alcohol) lay at 247 nm and for the thioglycolic acid compound (in water) at 255 nm.

FREUDENBERG, SEIB & DALL (1959) determined difference spectra of several other model substances containing thioglycolic acid, and found an important influence of the carboxyl group of the thioglycolic acid component on the absorption in the difference spectrum.

For comparison of the absorption spectra of lignin with and without thioglycolic acid, a soluble native lignin extracted with alcohol from the secondary xylem of *Populus* was used (page 270). This only slightly purified preparation still contained 1.31 per cent nitrogen. Treated with thioglycolic acid, this lignin yielded 107 per cent Lth I, and thereafter no Lth II. When the treatment with alcohol (Lth I) was omitted, a large proportion of the lignin was lost, and only 43 per cent Lth II was obtained. This considerable loss must be explained on the basis of the characteristics of native lignin, which represents an easily soluble fraction probably comprising components with a low molecular weight. This lignin cannot be precipitated easily in a weakly acid medium. But the preparations made by the first method also showed a loss, since the expected yield was 135 per cent. This value was calculated for a lignothioglycolic acid containing 10 per cent sulphur and having lost during its formation one molecule of water in binding two molecules of thioglycolic acid.

The absorption spectra of native lignin and those of lignothioglycolic acid from native lignin in neutral and alkaline solution were compared next. Both

materials were dissolved in 1/12 N NaOH. To obtain a neutral solution, dilution was done with phosphate buffer with a pH of 7; for an alkaline solution 1/12 N NaOH was used. In addition, the difference spectrum was determined. The difference spectrum of Lth I is in general agreement with that of native lignin *(fig. 25; table 8)*. The maximum at 297 nm, however, is relatively lower and that in the low-wavelength region shows a shift from 247 to 260 nm. The lignothioglycolic acid, moreover, has a much stronger absorption at the "250 nm" peak. The absorption of the Lth I is consistently higher per weight unit than that of the native lignin itself. Since a 7 per cent weight increase resulted from the formation of Lth I, we can calculate that the Lth I derived from a quantity of native lignin that would give a 1 per cent solution, will finally give an extinction of $107/100 \times 70.5 = 80$. Because there is probably some loss during the production of Lth I, the extinction of a lignothioglycolic acid solution containing 1 per cent pure lignin probably lies still higher. Sulphur was not determined. For a sulphur content of 10 per cent, the true lignin content of the lignothioglycolic acid would be 74.1 per cent (see page 283). From this we can calculate for a lignothioglycolic acid solution containing 1 per cent pure lignin, an ex-

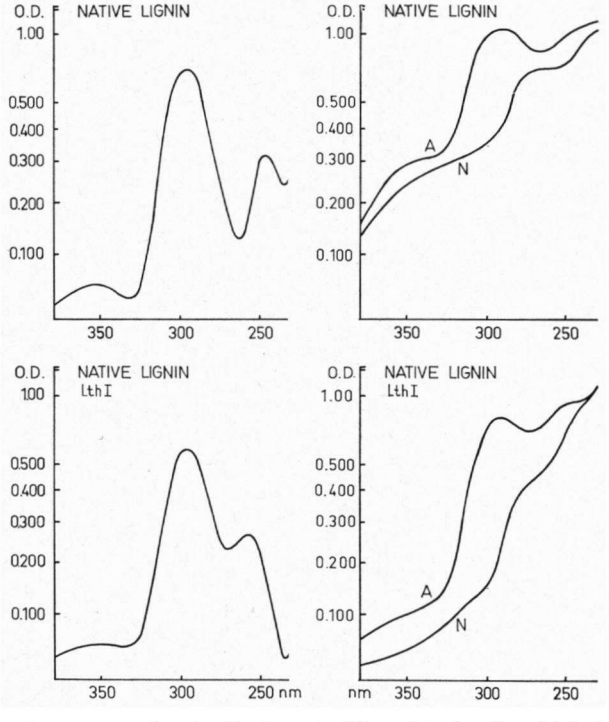


Fig. 25. Absorption spectra of native lignin and of lignothioglycolic acid derived from native lignin. On the right, the spectra in alkaline (A) and neutral (N) solutions; on the left, the difference spectra.

Table 8. Difference spectra. Comparison of the properties of native lignin with those of lignothioglycolic acid derived from native lignin.

	maxima (nm)		E 1% 1 cm Lth "250 nm"	E 1% 1 cm lignin "250 nm"	absorption ratio 350	300	250 nm
native lignin	295	247		64	0.2:	2.7:	1.0
Lth I native lignin	297	260	70.5	80*	0.2:	2.1:	1.0
Lth II native lignin	297	256	79.2		0.2:	1.3:	1.0

* The calculation is based on the supposition that all of the native lignin is transformed into lignothioglycolic acid.

tinction of $\frac{100}{74.1} \times 70.5 = 95$. Thus, although the shape of the absorption curve shows little change due to the binding of thioglycolic acid, there has been an appreciable increase in absorption. Since a large part of the native lignin is lost during the isolation of Lth II, comparison of the specific extinction of Lth II and native lignin is meaningless.

Thioglycolic acid itself has a rather strong absorption in the region below

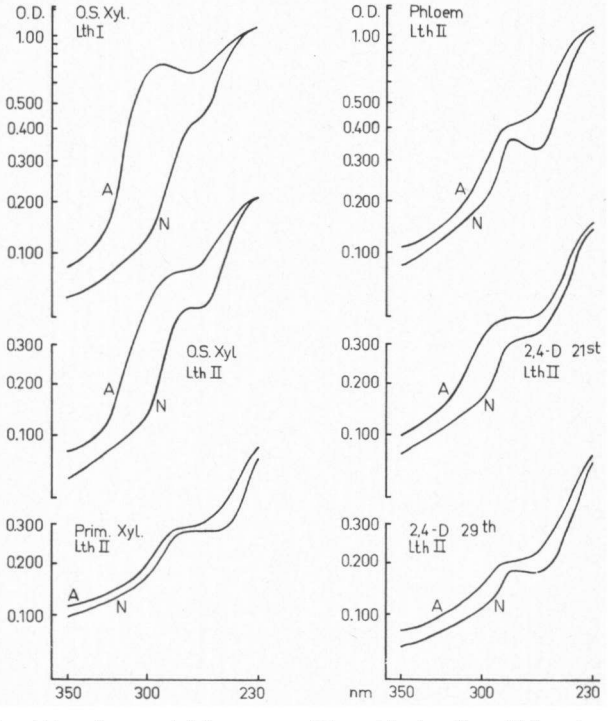


Fig. 26. Alkaline (A) and neutral (N) spectra of lignothioglycolic acid fractions I and II.

280 nm, and can therefore disturb the measurements when it is present in a free form.

The absorption spectra of a large number of lignothioglycolic acid preparations, including tissue culture material, were measured in a concentration of 0.04 mg lignothioglycolic acid per millilitre in neutral and alkaline solution *(fig. 26)*. The absorption spectra of these solutions have characteristics typical for lignin, *i.e.* a peak at about 275 nm that shifts to higher wavelengths in alkaline solution. The difference spectra consistently show a double-peaked curve, one peak lying in the region of 294 to 300 nm and the other between 247 and 262 nm. The relative height of the first maximum and the location of the second always show differences according to the type of tissue sampled for the preparations *(table 9; figs. 27* and *28)*. A third maximum at about 350 nm is usually not found for the lignothioglycolic acid preparations until after hydrolysis has been performed. *Table 9* also shows the absorption ratio of the first two of these maxima in the difference spectrum.

The often unusually high maximum around 300 nm is typical of poplar lignin, especially that originating from xylem. This strong absorption around 300 nm is shown not only by lignothioglycolic acid preparations but also by native lignin *(fig. 25)*. A discussion of the correlation between the strong absorption in this region in the difference spectrum and the presence of *p*-hydroxybenzoate

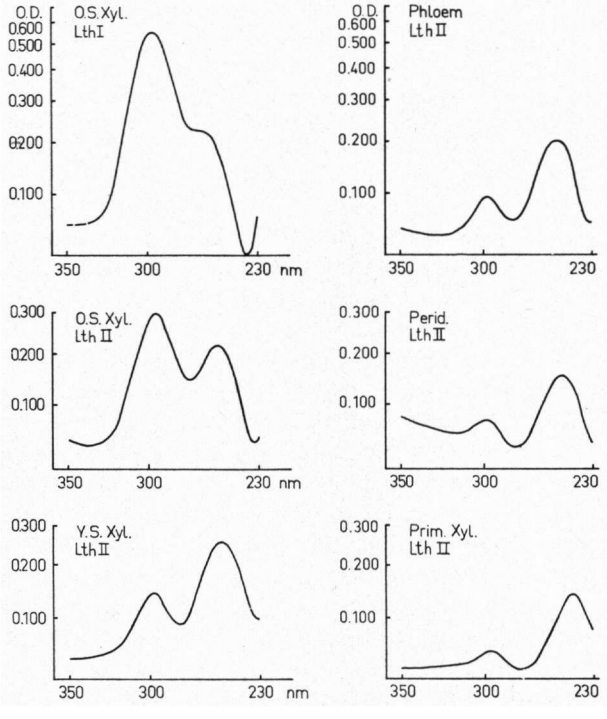


Fig. 27. Difference spectra of lignothioglycolic acid I and II from tissues from the tree.

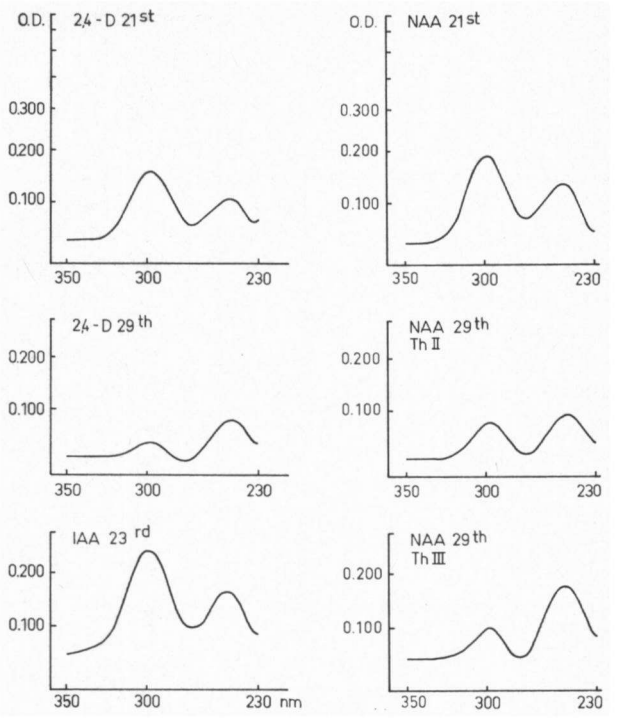


Fig. 28. Difference spectra of lignothioglycolic acid II from tissue cultures, and (lower right) one difference spectrum of a Lth III fraction showing a relatively lower 296 nm maximum than fraction II of the same sample.

groups in the lignin is given on page 308. The height of the 300 nm peak is strongly dependent on the method by which the solutions are prepared. As a result of the alkali treatment preceding the extraction of Lth II, the absorption in this range is greatly modified. The absorption ratio at the maxima of about 250 and 300 nm in the difference spectrum is appreciably lower for Lth II preparations than for Lth I of the same tissues *(table 9)*. The region around 300 nm is therefore unsuitable for quantitative lignin determinations in *Populus*.

The most favourable region for measurements seems to be the maximum around 250 nm. In this region both the phenols with a double bond in the side chain and those without this feature show an absorption maximum in the difference spectrum. Since the wavelength at which the maximum occurs differs to some extent from one preparation to another, the absorption was measured at the maximum rather than at a particular wavelength. *Figures 27* and *28* show the difference spectra of several samples, all with a concentration of 0.04 mg lignothioglycolic acid per millilitre. If the absorption at the 250 nm peak is a good measure for the true lignin content of these preparations, a contaminated lignothioglycolic acid should show a lower absorption than a pure preparation. The low maxima

Table 9. Some characteristics of ionization difference spectra of lignothioglycolic acid and other lignin preparations.

	preparation	wavelength 2nd max (nm)	absorption ratio 1st and 2nd max
old sec. xylem	Lth I	262	2.4
old sec. xylem	Lth II	256	1.3
young sec. xylem	Lth II	252	0.7
phloem	Lth II	254	0.4
periderm	Lth II	251	0.5
primary xylem	Lth II	243	0.3
IAA 23rd	Lth I	258	3.0
IAA 23rd	Lth II	250	1.5
NAA 21st	Lth II	251	1.4
NAA 23rd	Lth I	254	1.3
NAA 23rd	Lth II	249	0.9
NAA 23rd	Lth III	250	0.7
NAA 29th	Lth II	249	0.9
NAA 29th	Lth III	250	0.6
2,4-D 21st	Lth II	248	1.5
2.4-D 29th	Lth II	247	0.5
xylem	N.L.	247	2.1
phloem	A.L.	244	0.6

N.L. = native lignin
A.L. = alkali lignin

for primary xylem and the tissue culture preparations agree fairly well with the contamination assumed on the basis of the nitrogen and sulphur contents. *Tables 4* and *5* show the extinction in the difference spectrum of 1 per cent solutions through a liquid layer of 1 cm for all the lignothioglycolic acid preparations. If this extinction coefficient E 1 % 1 cm is known for a pure lignothioglycolic acid, the true lignin content of the samples can be calculated from the known percentages of lignothioglycolic acid.

5.4.1.3. Quantitative lignin determination on the basis of the absorption of lignothioglycolic acid

The preparations of old secondary xylem were chosen as standard for a pure lignothioglycolic acid, since they give the highest extinction at the 250 nm maximum in the difference spectrum. The thioglycolic acid content was calculated from the sulphur content according to FREUDENBERG *c.s.* (1959), with the assumption that in binding two molecules of thioglycolic acid one molecule of water is lost. For each molecule of bound thioglycolic acid, therefore, $C_2H_4O_2S_1 - {}^1/_2H_2O = C_2H_3O_{1.5}S_1$ was subtracted *(table 10)*.

The first determination shown in this table was done with re-dissolved (dioxane) and precipitated lignothioglycolic acid. But this treatment, which was intended as purification, did not provide lignothioglycolic acid with a higher ex-

Table 10. Extinction coefficient and lignin content of lignothioglycolic acid.

	Lth I or II	S %	$C_2H_3O_{1.5}S_1$ %	lignin %	Lth E 1% 1 cm
old sec. xylem	II*	9.62	25.0	75.0	59.2
old sec. xylem	II	9.10	23.6	76.4	62.5
old sec. xylem	II	9.78	25.4	74.6	63.0
old sec. xylem	I	10.57	27.4	72.6	60.0

* purified by dissolving in dioxane and precipitation in water

tinction coefficient. The values of the next two determinations shown in *table* 10 were averaged. The lignothioglycolic acid contains about 75 per cent lignin and has an average extinction coefficient of 63. The extinction coefficient of the lignin in the lignothioglycolic acid is therefore $^4/_3 \times 63 = 84$. The percentage of pure lignin (L) can now be calculated by multiplying the percentage of lignothioglycolic acid (x) by the extinction coefficient of that lignothioglycolic acid (y) and dividing the result by 84:

$$L = xy/84.$$

It is assumed here that the sulphur content and the extinction coefficient of pure lignothioglycolic acid from the other tissues are the same as those of the xylem. All the percentages of lignothioglycolic acid shown in *tables 4* and *5* were converted in this way to percentages of pure lignin. The preparations with the lowest extinction coefficient, such as the primary xylem, periderm, and the 2,4-D cultures, of course show the strongest decrease in the percentage on the basis of the assumed contamination.

The lignin content of old secondary xylem calculated by this method averages 14.0 per cent. This value is rather low as compared to the data in the literature. HOLMBERG (1947) found 16.0 per cent lignin for *Populus tremula* and 14.2 per cent for the "Gigas" variety. In calculating the lignin content, however, Holmberg assumed that one molecule of water is lost at the binding of one molecule of thioglycolic acid. According to him, with the thioglycolic acid method part of the lignin is lost due to the formation of soluble compounds. Furthermore, for poplar lignin some of the bound *p*-hydroxybenzoic acid is split off during the isolation (see 5.4.3.). BRAUNS (1952b) mentions percentages of 20.3 to 22.1 for *Populus nigra* obtained by methods employing mineral acids. PEARL & BUSCHE (1960) isolated 18.1 per cent Klason lignin from *Populus tremuloides*, but calculated a total lignin content of about 21 per cent on the basis of the loss of an acid-soluble fraction.

The preparation "young secondary xylem" – cambial zone with the youngest lignified elements – contains only 4.4 per cent lignin on the average; the primary xylem preparations even give values below 2 per cent. The secondary phloem, because of its abundance of fibres, shows a rather high lignin content averaging 8.8 per cent. The periderm preparations, which contain not only suberized and lignified phellem cells but also a rather large amount of pericyclic

and even bast fibres, also show a rather high lignin content. Of the tissue cultures, only the one on a medium containing IAA, which had many tracheids and a large amount of lignified parenchyma, showed a high lignin content. The lignin content of the tissue cultures on a medium containing NAA is much lower. Generally, the early subcultures show a somewhat higher lignin content than the 29th subculture. However, differences in the age of the subcultures themselves complicate comparison. All the cultures on 2,4-D medium show a very low lignin content. A pepsin or trypsin pretreatment had no distinct influence on the lignin yield; these treatments were performed to remove proteins and thus obtain a purer lignothioglycolic acid, but the results were not distinctly better than those obtained without a pretreatment.

Whereas for the xylem samples the residue after the first thioglycolic acid and alkali treatment was entirely free of lignin, the residue of the callus cultures almost always contained lignin, as demonstrated by the Mäule and phloroglucinol-hydrochloric acid reactions. Even the residue after isolation of the Lth III fraction sometimes still showed a weakly positive lignin reaction. This limited solubility of a portion of the lignin or lignothioglycolic acid of the tissue cultures might be due to a different type of chemical linkage between the lignin and other cell wall components. This might be the case for intercellular lignin.

5.4.2. Oxidation with nitrobenzene

5.4.2.1. Quantitative determinations

Initially, all the oxidations were performed at 170 °C for 3 hours. This time and temperature were chosen to approach as closely as possible the optimal conditions found by KAVANAGH & PEPPER (1955) for *Populus tremuloides* xylem. These authors obtained a maximum yield of vanillin and syringaldehyde by heating for $2^1/_2$ hours at 170 °C, with a rather long interval for reaching this temperature. A distinct optimum in the temperature curve was found only for vanillin; the yield of syringaldehyde showed little variation between the results of heating at 150°, 160°, 170°, and 180 °C for $2^1/_2$ hours.

In our study great difficulties were encountered with the determinations in phloem from *Populus nigra* cv. *'Italica'*. The aldehyde yields were not reproducible, and the percentages seemed to be dependent on the quantity of oxidized material. Because of these difficulties, a series of oxidations was done at various times and temperatures in samples of xylem, phloem, and a tissue culture on a NAA medium. The results are shown in *table 11*. Xylem gave the highest yield of aldehyde for $1^1/_2$ hours oxidation at 170 °C, but the yield was only slightly lower for 2 hours at 160 °C. Phloem shows much sharper optima, especially in the temperature curves, the best results being obtained with a period of 2 hours at 160 °C. At both 150° and 170° the values were much lower.

It is clear that the time of 3 hours and temperature of 170 °C used throughout most of the present study give suboptimal aldhyde yields for phloem and xylem. Although 84 per cent of the optimal yield was obtained for the xylem sample, for phloem this value was only 50%. For the only tissue culture investigated, 3 hours of heating at 170 °C gave appreciably higher and more reproducible re-

Table 11. Nitrobenzene oxidation at various oxidation times and temperatures.

	sample (mg)	temp (°C)	time (hr)	number of determ	S:V:P	S %	V %	P %	S+V+P %	%of max
xylem	52	170	3	2	2.1:1:0.03	5.02	2.39	0.08	7.5	86
		170	2	1	2.4:1:0.03	5.82	2.40	0.07	8.3	95
		170	1.5	2	2.5:1:0.02	6.20	2.47	0.06	8.7	100
		170	1	1	2.6:1:0.02	5.91	2.29	0.04	8.2	94
		160	3	1	1.8:1:0.02	4.03	2.25	0.05	6.3	72
		160	2	1	2.5:1:0.02	5.99	2.37	0.04	8.4	97
		160	1.5	1	2.4:1:0.01	5.46	2.31	0.03	7.8	90
		150	2	1	2.4:1:0.01	5.56	2.30	0.03	7.9	91
phloem	93	170	3	3	0.9:1:0.07	0.30	0.34	0.02	0.66	50
		170	2	1	0.8:1:0.07	0.44	0.55	0.04	1.03	77
		160	2.5	2	1.0:1:0.05	0.59	0.58	0.03	1.20	90
		160	2	4	0.9:1:0.04	0.62	0.68	0.03	1.33	100
		160	1.5	2	1.1:1:0.04	0.62	0.56	0.02	1.20	90
		160	1	1	1.1:1:0.04	0.56	0.53	0.02	1.11	83
		150	2	1	0.8:1:0.04	0.44	0.54	0.02	1.00	75
NAA29th	150	170	3	3	0.6:1:0.5	0.15	0.24	0.12	0.51	
					0.7:1:0.4	0.17	0.26	0.11	0.54	100
					0.7:1:0.4	0.18	0.27	0.12	0.57	
		160	2	5	0.7:1:0.2	0.09	0.13	0.03	0.25	
					0.7:1:0.3	0.12	0.17	0.05	0.34	
					0.8:1:0.3	0.14	0.18	0.06	0.38	69
					0.8:1:0.3	0.15	0.19	0.05	0.39	
					0.7:1:0.3	0.17	0.24	0.07	0.48	

sults than 2 hours at 160 °C. For phloem, the values obtained by oxidation for 2 hours at 160 °C were used; for all the other samples, the aldehyde yield after 3 hours of oxidation at 170 °C are shown (*tables 12* and *13*). The low yields obtained with high temperatures and long oxidation times must be ascribed to breakdown of the free aldehydes, probably under the influence of certain other substances present in the sample. As a matter of fact, Roadhouse and MacDougall found a greater loss at the oxidation of added pure aldehydes in the presence of young tissues than in the presence of older plant material.

To obtain an idea of possible breakdown of aldehydes during the oxidation period, the pure aldehydes were subjected to a complete oxidation in the presence of nitrobenzene and alkali. The results, which are shown in *table 14*, have already been discussed (section 5.3). The total loss per aldehyde by oxidation for 3 hours at 170 °C can amount to 44 to 56 per cent; with 2 hours at 160 °C the loss was much lower (23 to 42 per cent). It is impossible, however, to apply corrections for aldehyde loss during oxidation on the basis of these values, since the rate at which the aldehydes are formed apparently varies from sample to sample. Consequently, the maximal yields are probably found for the oxidation time at which the breakdown of aldehydes tends to surpass their formation

Table 12. Nitrobenzene oxidation of tissues from the tree.

	sample (mg)	ratio aldehydes S:V:P	% aldehydes			
			S	V	P	S+V+P
xylem	52	2.1:1:0.03	5.14	2.45	0.07	7.7
	50	2.1:1:0.04	4.90	2.35	0.10	7.3
xylem twigs	51	1.9:1:0.03	4.78	2.48	0.06	7.3
o.s.xylem	51	2.1:1:0.03	4.37	2.11	0.07	6.5
	52	1.9:1:0.03	3.51	1.83	0.06	5.4
	49	2.0:1:0.04	3.87	1.97	0.07	5.9
	50	1.9:1:0.03	3.91	2.01	0.06	6.0
y.s. xylem	49	1.1:1:0.3	0.54	0.50	0.14	1.18
	86	1.2:1:0.3	0.55	0.48	0.13	1.16
prim. xylem I	180	– 1:1.6	–	0.062	0.097	0.16
	180	– 1:1.4	–	0.072	0.101	0.17
	179	– 1:1.4	–	0.055	0.077	0.13
prim. xylem III	46	– 1:2.5	–	0.06	0.15	0.21
prim. xylem IV	179	– 1:3.3	–	0.054	0.178	0.23
phloem*	93	1.0:1:0.05	0.60	0.63	0.03	1.26
	93	0.8:1:0.03	0.61	0.72	0.02	1.35
	93	1.0:1:0.03	0.63	0.65	0.03	1.31
periderm	61	0.16:1:0.14	0.14	0.86	0.12	1.12
	80	0.24:1:0.14	0.21	0.87	0.12	1.20
periderm twigs	89	0.1:1:0.2	0.08	0.54	0.11	0.73
	98	0.2:1:0.2	0.09	0.45	0.09	0.63
	107	0.2:1:0.2	0.08	0.45	0.10	0.63
N.L. sec. xylem	10	1.3:1:0.06	12.5	9.4	0.5	22.4

N.L. = native lignin
* oxidation: 2 hr at 160 °C

Table 13. Nitrobenzene oxidation of tissue cultures.

	age*	sample (mg)	ratio aldehydes S:V:P	% aldehydes			
				S	V	P	S+V+P
2,4-D 21st	7	90	0.8:1:0.4				0.5
		96	0.9:1:0.4	0.17	0.19	0.07	0.43
2,4-D 23rd	2	121	0.4:1:0.8	0.06	0.15	0.12	0.33
		105	0.4:1:0.8	0.05	0.13	0.10	0.28
2,4-D 29th	2	155	0.6:1:1.2	0.05	0.08	0.10	0.23
		156	0.6:1:1.0	0.05	0.09	0.09	0.23
NAA 23rd	2	132	0.5:1:0.3	0.36	0.66	0.17	1.19
		102	0.5:1:0.3	0.32	0.66	0.18	1.16
NAA 29th	2	156	0.6:1:0.5	0.15	0.24	0.12	0.51
		156	0.5:1:0.4	0.11	0.21	0.09	0.41
IAA 23rd	5	72	0.8:1:0.3	0.76	0.90	0.24	1.90
		82	0.7:1:0.3	0.60	0.83	0.23	1.66

* age of cultures given in months since last subculture

Table 14. Recovery of samples of 0.8 mg syringaldehyde (S), vanillin (V), and *p*-hydroxybenzaldehyde (P). Influence of extraction, chromatography, and oxidation.

	S %	V %	P %
ether extraction, chromatography	95	84	94
oxidation 2 hr at 160°, extr., chrom.	58	65	77
oxidation 3 hr at 170°, extr., chrom.	44	48	56

from lignin. An indication for the degree of aldhyde breakdown may lie in the amount of vanillic acid formed, since in the experiments with pure aldehydes, part of the vanillin and *p*-hydroxybenzaldehyde was oxidized to vanillic acid and *p*-hydroxybenzoic acid.

Oxidation of protein-containing samples can give rise to the formation of *p*-hydroxybenzaldehyde via tyrosine (page 273). In general, however, tyrosine will only have a disturbing effect in lignin-poor preparations with a high protein content.

Table 12 shows the results obtained for the tissues from the tree; *table 13* gives those for the tissue cultures. The percentages of aldehyde were calculated for the material extracted with alcohol-benzene and water; no corrections were applied for the loss of aldehydes during oxidation and chromatography. The indicated ratio between the amounts of syringaldehyde (S), vanillin (V), and *p*-hydroxybenzaldehyde (P) represents not the molecular but the weight ratio.

The mature secondary xylem shows an S/V ratio of about 2.0 This is entirely in accordance with expectation for "hardwood" lignin. The young secondary xylem shows a relatively much lower S yield. The native lignin prepared from secondary xylem also shows a lower S/V ratio after oxidation than the xylem. Very little or no syringaldehyde was found for the primary xylem, which is in good agreement with the negative Mäule reaction of the protoxylem. It is highly probable that this concerns not an early phase of the tissue but a permanent situation, because the protoxylem in old branches also showed a negative Mäule reaction.

The young secondary xylem isolated in the month of May, to the contrary, is to be considered as representing an early phase in the differentiation of the secondary xylem. This may mean that the lignin showing an S/V ratio of 1.1 in this analysis belongs to the early stages of lignification and that the lignin deposited in the later stages of lignification, *i.e.* further away from the cambium, has a higher syringyl content. Differences between early and late wood cannot be entirely excluded here, however. The differences in the relative *p*-hydroxybenzaldehyde content between young and old secondary xylem may also be related to the lignin. This means we may have here a "young lignin" with a lower syringyl and a higher *p*-hydroxyphenyl content, *i.e.* with a lower methoxyl content. This is in agreement with the results of investigations in other species and the conclusions drawn from them (HIGUCHI 1957b).

The *Populus* tissue cultures gave measurable quantities of vanillin, syringaldehyde, and *p*-hydroxybenzaldehyde after nitrobenzene oxidation in all cases *(table 13)*. The percentages vary somewhat for all the subcultures. The callus grown on a medium containing naphthalene acetic acid shows about the same S/V ratio as the 2,4-D cultures. For the callus on an IAA medium the ratio is higher and the total aldehyde value is also larger. The high S/V ratio found for the IAA cultures may be related to the age of the cultures.

For the callus cultures in this study the S/V ratio is consistently lower than that for the mature secondary xylem, and even lower than that for the young secondary xylem. Most of the lignin in the callus cultures is located in and on the walls of parenchyma cells and in intercellular spaces, often mainly on the periphery of the culture. From the anatomical point of view, it would seem logical to compare the tissue of such regions in the cultures with the periderm from the tree. The "periderm" samples of old and also of young branches from the tree, however, show an entirely different S/V ratio. The best agreement is found between the aldehyde ratio of callus cultures and phloem.

The *p*-hydroxybenzaldehyde of primary xylem and of the 29th subculture on a 2,4-D medium is probably not derived exclusively from lignin. If nitrobenzene oxidation is applied to isolated lignothioglycolic acid, the results show the same S/V ratio but a much lower P/V ratio *(table 15)*. Two other samples (*i.e.* periderm and an NAA culture), both with a rather low P yield on oxidation, do show a high degree of agreement between the P/V ratio of the isolated lignin and that of the total preparation, but the aldehyde yield from lignothioglycolic acid is very low in all cases. These results offer some support for the assumption that the high P yields from primary xylem and the 2,4-D culture are mainly due to the oxidation of protein. For a sample containing 40 per cent protein, we may expect a yield of about 0.1 per cent P with respect to the dry weight of the sample if this protein has the same composition as egg albumin (page 273). It is therefore indeed possible that the high values are to be attributed to the oxidation of protein.

Table 15. Comparison of the relative p-hydroxybenzaldehyde yield from oxidation of tissue and of isolated lignothioglycolic acid.

type of tissue sampled	oxidized material	S:V:P	S %	V %	P %	S+V+P %
primary xylem II	tissue	0.3:1:1.1	0.033	0.127	0.146	0.3
	Lth II	0.3:1:0.2	0.015	0.056	0.014	0.08
2,4-D 29th	tissue	0.6:1:1.2	0.049	0.084	0.097	0.2
	Lth II	±0.4:1:0.3	±0.022	0.060	0.020	0.1
periderm	tissue	0.2:1:0.1	0.17	0.86	0.12	1.2
	Lth II	0.2:1:0.1	0.056	0.30	0.034	0.4
NAA 23rd	tissue	0.5:1:0.3	0.34	0.66	0.17	1.2
	Lth II	0.6:1:0.2	0.12	0.21	0.045	0 4

5.4.2.2. Comparison of the aldehyde yield and the lignin content

It is obvious to compare the amount of aldehydes present after oxidation and the percentages of lignothioglycolic acid. Starting from the assumption that the lignin deriving from the tissues of the tree and that from the callus cultures have the same general composition, we would expect a constant ratio between the aldehyde yield and the thioglycolic acid lignin content. *Table 16* shows these data for several preparations; all the figures represent average values calculated from the data in *tables 4*, *5*, *12* and *13*, except those for primary xylem, which concern only a single thioglycolic acid determination. Column B shows the uncorrected lignothioglycolic acid percentages, column C the percentages of pure lignin calculated from them (5.4.1.3.) There are very striking differences between the amount of the aldehydes as percentage of the lignothioglycolic acid (A/B × 100) for old secondary xylem, young secondary xylem, and the other preparations. It is evident from the last column (A/C ×100) that the large difference between the lignin of secondary xylem and that of the other preparations is not solely dependent on impurities in the lignothioglycolic acid. The data fall into three groups, the first being made up of old secondary xylem (42%); the second of young secondary xylem (27%) and native lignin from secondary xylem (22% of the native lignin, 28% of the thioglycolic acid lignin); and the third of the callus cultures and the primary xylem, phloem, and periderm from the tree (12–18%).

Under conditions of optimal oxidation, the value found for the mature secondary xylem would be about 51 per cent, in complete agreement with expecta-

Table 16. Comparison between the yield of aldehydes after oxidation and the percentage of thioglycolic acid lignin

	A	B	A/B × 100	C	A/C × 100
	aldehydes %[1]	Lth %	aldehydes % of Lth	lignin %[4]	aldehydes % of lignin
old sec. xylem	5.9	19.3	31	14.0	42
young sec. xylem	1.17	7.6	15	4.4	27
primary xylem I	0.15	6.9	2	1.2	12
phloem	1.30[2]	14.1	9	8.8	15[3]
periderm	1.16	28.7	4	8.9	13
2,4-D 29 th	0.23	4.9	5	1.3	18
NAA 29 th	0.46	8.1	6	2.9	16
IAA 23 th	1.78	22.3	8	11.8	15
N.L. sec. xylem	22.4	107	21	80	28

[1] oxidation 3 hr at 170 °C
[2] oxidation 2 hr at 160 °C
[3] % is lower after oxidation for 3 hr at 170 °C
[4] calculated from the lignothioglycolic acid percentage

N.L. = native lignin

tion. PEPPER *c.s.* (1959) found for the wood of *Populus tremuloides* an aldehyde percentage (V+S) of 50.5 in relation to the lignin content determined according to Klason. But the percentages for all our other preparations are much lower and, with the exception of young secondary xylem and native lignin the values lie very close together. The question arises of whether this concerns an experimental error or fundamental differences in the lignin. It is possible that the corrected lignin values (column C) are still somewhat too high, due to the presence of protein contaminations in the lignin preparations. But it is of course also conceivable that the specific extinction is not a proper measure of the lignin content, for example because the lignin of the preparations of the second and third groups might have a much higher phenolic hydroxyl content than the lignin of secondary xylem. Several possible errors could lead to an excessively low aldehyde value, one being a higher aldehyde loss in the tissues of these two groups during oxidation. It is also possible that a higher percentage of the lignin is converted into other, undetermined compounds during the oxidation process, for instance syringic acid and vanillic acid or acetoguaiacon and protocatechualdehyde. In fact, all these possible sources of error indicate a more or less deviational structure of the lignin of the second and third groups. The size of the differences between the values for the first and third groups also point to essential differences in the lignin.

In the discussion of the literature, reference has already been made to the relationship between the syringyl content of a lignin and the aldehyde yield. Whereas the syringyl components of lignin are readily oxidized to syringaldehyde, some of the guaiacyl components – those of the condensed type – are difficult to oxidize because of the C-C bond (LEOPOLD 1952). A high syringyl content is generally associated with a high aldehyde percentage. *Table 17* shows a few relevant data, most of them concerning secondary xylem. In a few cases divergent types of wood, such as reaction wood and rubbery wood, are compared with normal wood. Usually, there is a distinct correlation between the S/V ratio and the aldehyde percentage within a given taxonomic group. In isolated cases the correlation is sometimes absent, for instance between Eucalyptus species. The dicotyledons with an S/V ratio of 2 to 4 have an aldehyde content of 31–60%. A few species with a very low S/V ratio also have a rather low aldehyde content (30–33%). Among the monocotyledons the species with a high S/V ratio also show a high aldehyde percentage. On the average, the aldehyde yield for the gymnosperms is much lower than that for the angiosperms. The genus *Podocarpus* is of special interest because it contains species with and without syringyl groups in the lignin, which results in differences in the aldehyde yield. The differences found between opposite wood and normal wood in angiosperms with reaction wood and those found in apple trees between rubbery wood and normal wood, result in aldehyde yields that are lower by 10 and 20 per cent, respectively.

The only group with an aldehyde content as low as that of the callus cultures and the phloem of *Populus* is the Gramineae family, and among them particularly the herbaceous grasses. Herbaceous dicotyledons, to the contrary, apparently

Table 17. Nitrobenzene oxidation of the xylem of different species. Comparison of S/V ratio with the yield of aldehydes.

species or type of wood	S/V	aldehydes (% of Klason lignin)	author
Gymnosperms			
Picea	–	28	PEPPER *c.s.* 1959
Picea, Pinus, etc.	–	20–25	CREIGHTON *c.s.* 1944
Podocarpus, 2 species	–	17–20	CREIGHTON *c.s.* 1944
Podocarpus, 3 species	0.8–1.0	29–32	CREIGHTON *c.s.* 1944
Monocotyledons			
Gramineae	0.4–1.7	16–30	CREIGHTON *c.s.* 1944
Phyllostachys	1.2	26.5	HIGUCHI 1958
Triticum	1.2	51	ROADHOUSE & M. 1956
Zea stalks	0.5	8.5	CREIGHTON & H. 1944
Dracaena, Aloe	3.3–3.8	35–37	CREIGHTON *c.s.* 1944
Dicotyledons			
Belliolum, Zygogynum	1.1–1.2	30–33	CREIGHTON *c.s.* 1944
Drimys	2.7	35	CREIGHTON *c.s.* 1944
several trees	2.5–3.5	39–49	CREIGHTON *c.s.* 1944
Populus tremuloides	3.7	43.3	CREIGHTON *c.s.* 1944
Populus tremuloides	2	50.5	PEPPER *c.s.* 1959
several trees	±3	40–47	LEOPOLD & M. 1952
Eucalyptus, 2 species	1.6–1.8	21–23	BLAND *c.s.* 1950
Eucalyptus, other species	2.8–6.2	31–56	BLAND *c.s.* 1950
Medicago sativa	3.7	29	ROADHOUSE & M. 1956
opposite w. *Euc. regnans*	3.2	36.9	BLAND 1958a
side wood *Euc. regnans*	4.1	40.8	BLAND 1958a
opposite w. *Euc. goniocalyx*	4.0	55.0	BLAND 1958a
side wood *Euc. goniocalyx*	4.9	59.3	BLAND 1958a
rubbery wood *Malus*	2.8	45.5	SONDHEIMER & S. 1962
normal wood *Malus*	4.0	55.5	SONDHEIMER & S. 1962

give a rather high aldehyde value: Roadhouse and MacDougall found, for mature plants of *Medicago sativa*, 29 per cent aldehydes in relation to the Klason lignin, and for young plants the values are still higher.

The relationship between the occurrence of oxidizable syringyl components in the lignin and the relative aldehyde yield was also studied for the xylem of a herbaceous dicotyledon (tobacco) and cedar wood, using the thioglycolic acid method instead of the Klason method for lignin isolation *(table 18)*. No corrections for bound thioglycolic acid have been applied. The xylem of tobacco gives a relative aldehyde yield roughly as high as that found for the xylem of dicotyledonous trees. For the cedar, on the contrary, the relative aldehyde yield is very low. With a correction for thioglycolic acid the yield would amount to about 18 per cent, which is low even for gymnosperm wood. Cedar wood therefore shows a relative aldehyde yield as low as that of the tissues of the third group, in which, however, the lignin generally contains syringyl nuclei. From

Table 18. Nitrobenzene oxidation. Comparison of S/V ratio with the yield of aldehydes.

	A aldehydes[1] %	B Lth[2] %	A/B × 100	S/V
Nicotiana tabacum	4.0	11.8	34	1.1
Cedrus libani	5.6	41.5	13	<0.05

[1] oxidation: 2 hr at 160 °C
[2] lignothioglycolic acid as percentage of dry weight

the data in *tables 17* and *18* we may conclude that for xylem lignin there is a certain correlation between the syringyl content and the aldehyde yield. In lignins with a low syringyl content the relative amount of condensed or non-oxidizable guaiacyl units varies strongly.

The low relative aldehyde yield found for the tissues of the third group seems to point to essential differences between the lignin of this group and that of secondary xylem.

5.4.3. Esterified *p*-hydroxybenzoic acid and other phenolic acids and aldehydes in *Populus* lignin

Upon hydrolysis of lignin or lignin-containing tissue samples of *Populus*, *p*-hydroxybenzoic acid is liberated, as well as smaller quantities of some other phenolic compounds. All the investigated tissue samples of *Populus nigra* cv. '*Italica*' yielded *p*-hydroxybenzoic acid (Pa), vanillic acid (Va), and vanillin upon hydrolysis, and in most of the cases syringic acid (Sa), syringaldehyde, and *p*-hydroxybenzaldehyde were also liberated in measurable quantities. The same holds for the isolated lignothioglycolic acid fractions I and II.

When whole samples are hydrolized, the origin of the *p*-hydroxybenzoic acid and the other phenolic compounds cannot be determined. The preceding extractions, *e.g.* with alcohol-benzene, remove the free acids and aldehydes but not the so-called insoluble esters (EL-BASYOUNI *c.s.* 1964, 1966), which may occur apart from the lignin. The use of isolated lignin is therefore considered preferable. The Lth II fraction, however, is not ideal for this purpose due to the isolation method. Since the lignothioglycolic acid is held for 24 hours in 0.5 N NaOH at 20 °C, some hydrolysis has already taken place during the preparation of the Lth II. Lth I is in principle better suited for hydrolysis experiments, but a sufficient amount of this fraction was only available for a few kinds of tissue. *Table 19* shows the composition of the hydrolyzates of lignothioglycolic acid preparations of *Populus nigra* cv. '*Italica*'. The aldehydes were determined quantitatively and the acids semiquantitatively, by chromatographic comparison with spots of known quantity. The percentages were calculated on the basis of the uncorrected lignothioglycolic acid. Any ferulic acid, caffeic acid, *p*-coumaric acid, and sinapic acid present is not taken into consideration. Xylem hydrolyzates are very rich in *p*-hydroxybenzoic acid and syringaldehyde. The

Table 19. Alkaline hydrolysis of lignothioglycolic acids

	Sa	Va	Pa	S	V	P	% of Lth		
							acids	ald	total
Lth II									
xylem	++	+	+++	+++	++	+	1.8	1.8	3.6
old sec. xylem	++	+	+++	++	++	+	2.7	1.3	4.0
young sec. xylem	++	+	++	++	++	+	1.0	1.5	2.5
prim. xylem III	−	+	+	−	(+)	−	0.1	(0.2)	(0.3)
phloem	++	+	++	++	++	+	0.7	0.7	1.4
periderm	+	+	+	++	++	+	0.4	0.9	1.3
2,4-D 29th	+	+	++	+	+	+	0.4	0.2	0.6
NAA 29th	+	+	++	+	+	+	0.9	0.3	1.2
IAA 23rd	++	+	+++	++	++	+	2.8	0.8	3.6
Lth I									
xylem	++	+	+++	+++	++	+	5.5	1.7	7.2
old sec. xylem	++	+	+++	+++	++	+	5.7	2.0	7.7
phloem	++	+	++				1.1		
2,4-D 29th	+	+	++	++					
NAA 29th	+	+	+	+					
IAA 23rd	++	++	+++	++	++	+	5.6	1.5	7.2

\+ = < 0.2%
++ = 0.2–1.0%
+++ = > 1.0%

Sa = syringic acid
Va = vanillic acid
Pa = *p*-hydroxybenzoic acid

high *p*-hydroxybenzoic acid content is particularly striking in the case of Lth I (5%). As a check, this Lth I was also subjected to direct ether extraction. According to the chromatographic results, less than 0.04 per cent Pa was obtained in this extraction, a negligible amount. It may therefore be concluded that hydrolysis of Lth I is necessary to obtain free *p*-hydroxybenzoic acid. The lignin of young secondary xylem yields slightly less Pa upon hydrolysis. In the hydrolyzates from primary xylem lignin only very small quantities of phenolic compounds are found; syringaldehyde and syringic acid were not present. The primary xylem lignin does give a *p*-coumaric acid spot on the chromatogram, but this lignin preparation is very impure. In a few cases ferulic acid was demonstrated, and sinapic acid and caffeic acid are probably present in some of the preparations.

The lignin preparations deriving from callus cultures also yielded phenolic acids and aldehydes on hydrolysis. The yield was generally somewhat lower than that for the lignin preparations from tissues from the tree, even taking into account the appreciable impurity of the preparations. The Pa yield was always high, however, almost equalling the values found for the xylem lignin from the tree. The hydrolyzate of lignothioglycolic acid from the IAA culture closely resembles that of secondary xylem. Among the cultures, a reliable result for Lth I could only be obtained for the tissue grown on an IAA medium, which

Table 20. Yield of *p*-hydroxybenzoic acid upon alkaline hydrolysis[1].

type of tissue	lignin %	*p*-hydroxybenzoic acid			
		hydrolysis of tissue		hydrolysis of Lth I	
		% of dry wt	% of lignin	% of Lth I	% of lignin
xylem	14.2	1.44	10.1	(5)[3]	
		1.42	10.0		
		1.50[2]	10.6		
old sec. xylem	14.0	1.23	8.8	(5)	(8)
		1.28	9.1	4.9	8.8
young sec. xylem	4.4	0.23	5.2		
phloem	8.8	0.053	0.6		
		0.049	0.6		
periderm	8.9	0.16	1.8		
2,4-D 8+9th	7.0	0.41	5.9	1.7	6.3
2,4-D 14th	5.6	0.23	4.1	1.3	4.4
2,4-D 29th	1.3	0.037	2.8	+	
NAA 29th	2.9	0.14	4.8	+	
IAA 23rd	11.8	0.79	6.7	(5)	(15)
native lignin			9.3[4]	6.1	

[1] hydrolysis: 18 hr at 80 °C
[2] hydrolysis: 16 hr at 70 °C
[3] numbers between brackets calculated from the dimensions of the spots on the chromatogram
[4] % of native lignin

showed a large amount of Pa and a strong general resemblance to xylem hydrolyzate.

For the hydrolyzates not of lignin but of tissues, the values are somewhat higher. In this series the acids were also determined quantitatively. *Table 20* shows the quantities of liberated Pa, first as a percentage of the dry weight of the extracted tissue sample and then as a percentage of the pure lignin content of the tissue. It was therefore assumed here that the Pa was entirely derived from the lignin. The last column gives, for the sake of comparison, the percentages of Pa from lignothioglycolic acid I, here expressed as percentages of the pure lignin. The Pa from the lignothioglycolic acid was in most of the cases only determined semiquantitatively. With the exception of the culture on the IAA medium, there is reasonable agreement between the values for the tissue and for Lth I, *i.e.* the lignin fraction isolated as Lth I does not seem to contain a higher percentage of *p*-hydroxybenzoic acid than the total lignin.

Hydrolysis of the native lignin preparation made from secondary xylem gave 9.3 per cent Pa, whereas hydrolysis of an Lth I prepared from this native lignin yielded only 6.1 per cent.

In the hydrolyzates of tissue samples a few other phenolic aldehydes and acids were also determined *(table 21)*; the amounts were generally very small. The

Table 21. Alkaline hydrolysis of total samples.

	lignin %	Sa	Va	Pa	S	V	P	acids %	ald %	total %
		% of lignin			% of lignin					
xylem	14.2	0.1	—	10.1	0.6	0.4	0.03	10.2	1.0	11.2
		+	+	10.0	0.5	0.3	0.02	10.0	0.8	10.8
o.s. xylem	14.0	0.1	—	8.8	0.7	0.4	0.03	8.9	1.1	10.0
y.s. xylem	4.4	—	—	5.2	0.5	0.4	0.2	5.2	1.1	6.3
phloem	8.8	0.1	—	0.6	0.2	0.2	0.05	0.7	0.4	1.1
		+	+	0.6	0.2	0.1	0.02	0.6	0.3	0.9
periderm	8.9		+	1.8	0.4	0.6	0.1	1.8*	1.1	2.9*
2,4-D 8+9th	7.0	0.03		5.9	0.4	0.2	0.1	5.9	0.7	6.6
2,4-D 14th	5.6	0.1	0.2	4.1	0.1	0.1	0.1	4.4	0.3	4.7
2,4-D 29th	1.3	—	—	2.8	0.3	0.5	0.5	2.8	1.3	4.1
NAA 29th	2.9	—	—	4.8	0.6	0.6	0.3	4.8	1.5	6.3
IAA 23rd	11.8	+	+	6.7	0.3	0.3	0.1	6.7	0.7	7.4

* also present 3.8 % ferulic acid; total acids 5.6 % of the lignin

same aldehydes as those found at the oxidation of lignin were demonstrated. For xylem, larger amounts of syringaldehyde than of vanillin are found, and in the other preparations about as much or less S than V. Mature xylem, phloem, and some of the cultures yielded only small amounts of *p*-hydroxybenzaldehyde, but for young xylem and especially for the last subcultures of the callus material the quantities were slightly larger. The very low percentage of phenolic compounds found for the phloem is very striking: not only is the *p*-hydroxybenzoic acid content of the phloem lignin very low but, in addition, the phenolic aldehydes obtained by hydrolysis constituted only a very small proportion of the lignin. The periderm hydrolyzate showed a very large amount of ferulic acid. The latter was not determined exactly, because of unsatisfactory separation on the chromatogram. The origin of the ferulic acid was not investigated further.

After ether extraction of the acidified hydrolyzate, the remaining part of the lignin is present partially as precipitate (A) and partially in the aqueous solution (B). Chromatography of Lth IIB of young and old secondary xylem showed that this fraction contained almost no free acids or aldehydes except for a limited amount of syringaldehyde. The spectra and difference spectra of fractions A and B can be compared with those of the lignothioglycolic acid from which these fractions were derived. In *figs. 29* and *30* the spectra and difference spectra of the lignothioglycolic acid before and after hydrolysis are compared. All these spectra pertain to concentrations of 0.04 mg/ml lignothioglycolic acid or hydrolyzates derived from the same amount Lth. The most radical change is seen in the difference spectra of xylem. The high (Lth II) or very high (Lth I) maximum at 296 nm, which is specific for poplar lignin, has returned to normal proportions after hydrolysis. This decrease of the maximum at 296 nm can also be observed for the combined difference spectra of A and B *(fig. 30)*; the maximum at a lower wavelength, however, keeps its original height. After hy-

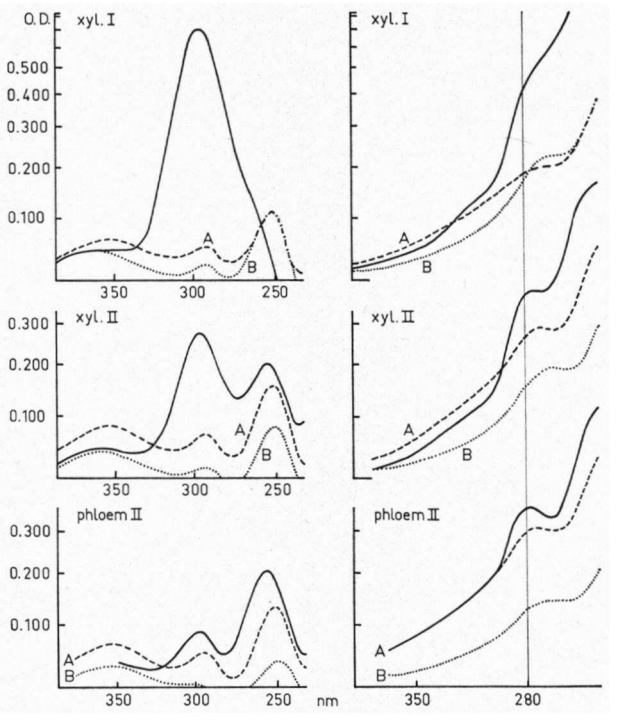


Fig. 29. Comparison of the absorption spectra of lignothioglycolic acid I and II before and after hydrolysis. The lignothioglycolic acid is shown by a solid line (——). After alkaline hydrolysis the lignin is found in fractions A (– – –) and B (. . . . . .), which are respectively not soluble and soluble in diluted acid. On the left, the difference spectra; on the right, the neutral spectra.

drolysis the lignin of the IAA culture also shows a marked decrease of the maximum at 296 *(fig. 30)*. The maximum at about 250 nm has shifted for all the hydrolyzates towards the region of the lower wavelengths *(table 22)*. After hydrolysis there is a third maximum in the difference spectrum at about 350 nm. This maximum is generally absent in the non-hydrolized lignin preparations, but an exception is formed by Lth II prepared from periderm from the tree.

Studies with model substances have shown that the 350 nm maximum in the difference spectrum must be ascribed to a so-called side-chain chromophore: a carbonyl group or double band (AULIN-ERDTMAN 1953). In most lignothioglycolic acid preparations this side-chain chromophore will only appear after hydrolysis, possibly after the splitting off of thioglycolic acid or the breaking of other bonds.

The shift of the 250 nm maximum to a lower wavelength, amounting to 4 to 11 nm, is probably to be ascribed to the splitting off of part of the bound thioglycolic acid. Native lignin of *Populus*, for example, has a maximum in the

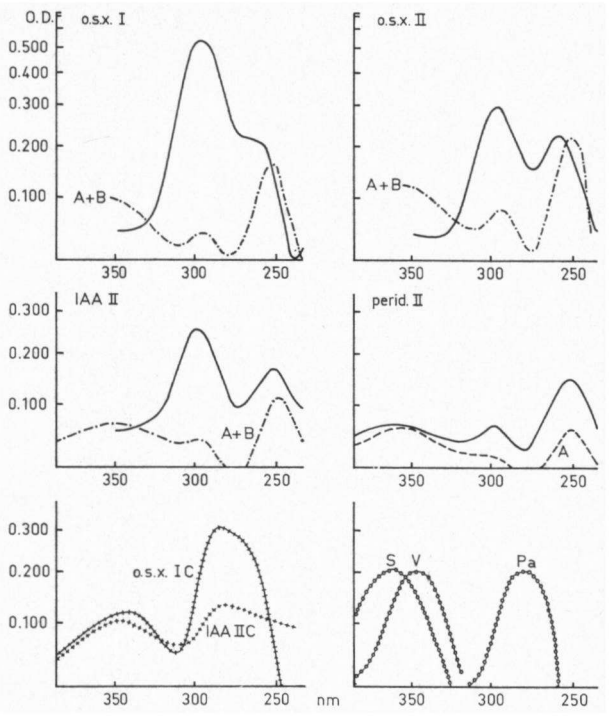


Fig. 30. Comparison of difference spectra before and after hydrolysis of the lignin. The lignothioglycolic acid is shown by a solid line (——). The absorption spectra of the hydrolized lignin are shown for fractions A and B together (– . – . – . – . –), or for fraction A separately (– – – –). The C-fraction of the hydrolyzate consists of ether-soluble phenolic aldehydes and acids. For purposes of comparison, the maxima in the difference spectra of syringaldehyde (S), vanillin (V), and *p*-hydroxybenzoic acid (Pa) are given (lower right).

difference spectrum at 247 nm; the Lth I prepared from native lignin has a maximum at 260 nm.

The *p*-hydroxybenzoic acid and syringaldehyde yielded upon hydrolysis have their maxima in the difference spectrum at 282 + 276 and 363 nm, respectively. Although it is clear that there is a correlation between the decrease in the absorption at about 300 nm and the liberation of *p*-hydroxybenzoic acid, a broader investigation, preferably with model substances, will be required for a better understanding of the role of *p*-hydroxybenzoic acid in the poplar lignin.

The lignothioglycolic acid spectra at pH 7 also show differences before and after hydrolysis. The B-hydrolyzate consistently shows a maximum at a lower wavelength than the Lth sample or the A-hydrolyzate (*table 23*). This holds not only for lignothioglycolic acid preparations but also for lignin isolated by other methods. The difference spectra for A and B hydrolyzates show marked agreement, although the maximum at 300 nm is lower for the B hydrolyzate. Since the B fraction does not precipitate in an acid medium and the A fraction does,

Table 22. Location of the absorption maximum in the low-wavelength region of the difference spectrum of lignothioglycolic acid before and after hydrolysis.

	Lth I or II	before hydrolysis (nm)	after hydrolysis (nm)
old sec. xylem	I	262	251
old sec. xylem	II	256	250
phloem	II	254	250
IAA 23rd	II	250	246–247

Table 23. Location of the absorption maximum in the neutral spectrum of lignin preparations before and after hydrolysis.

	preparation	before hydrolysis (nm)	after hydrolysis	
			A (nm)	B (nm)
xylem	N.L.	272	272	265
xylem	Lth I	272	272	264
xylem	Lth II	274	272	262
phloem	Lth II	280	276	268
IAA 23rd	Lth II	274	±272	±268

N.L. = native lignin

we may assume that the difference lies primarily in the degree of polymerization. The B fraction probably consists of smaller fragments of lignin.

5.4.4. Results obtained with other methods

5.4.4.1. Alkali lignin

An attempt was made to dissolve the lignin of the *Populus* tissue samples in an alkaline medium so that the quinoneimine test could be performed on the resulting alkali lignin. The alkali lignin was extracted according to Stafford (16 hr at 70°C; see 5.3). However, it proved impossible to dissolve the lignin quantitatively. The residue gave a strongly positive reaction in the Mäule test, not only for the secondary xylem but also for other samples, including those from tissue cultures. Confirmation of the results obtained with this histochemical reaction was obtained by a thioglycolic acid determination in the residue. Lignothioglycolic acid was prepared (II or I + II) and the pure lignin content was calculated from the difference spectra. For the samples of xylem, phloem, periderm, and the 14th subculture on 2,4-D medium, half or more of the lignin proved to be still present in the residue *(table 24)*, the only exception being the lignin of the 29th subculture on 2,4-D, all of which was extracted by the alkali treatment.

It is evident from the foregoing that alkali lignin does not form a good basis for quantitative lignin determinations in *Populus* preparations from the tree itself; and the method also seems to be unsuitable for tissue cultures. It is gener-

ally assumed that the lignin of herbaceous plants, in contrast to that of the wood of trees, can be more or less quantitatively extracted at temperatures that are not too extreme (BRAUNS 1952a). In this respect the lignin of the tissue cultures of *Populus* material resembles not that of herbaceous plants but that of secondary xylem.

The alkali lignin itself can be investigated spectrophotometrically. Direct determination of the dissolved alkali lignin proved to be impossible due to the disturbing effect of proteins and *p*-hydroxybenzoic acid in the solution. The lignin was therefore precipitated by acidification and then filtered. The resulting alkali lignin A was again dissolved in $^1/_{12}$ N NaOH, and part of this solution was diluted for measurement of the difference spectrum (see 5.3); another part was used for the quinoneimine test.

Alkali lignin has, like native lignin, a lower extinction coefficient than lignothioglycolic acid. For the alkali lignin A of old secondary xylem the extinction coefficient E 1% 1 cm of a weighed sample was determined. Meaurement of the difference spectrum can be difficult because, with the buffer concentration used, the neutral solution sometimes becomes turbid. The calculated extinction coefficient was 52, a lower value than the average of 64 found for soluble native lignin prepared from secondary xylem.

From the extinction coefficient of xylem alkali lignin A and the measured absorption of the alkali lignin solutions, the alkali lignin content of these solutions can be calculated. The alkali lignin A yield from the tissues and tissue cultures is lower or about equal to the calculated thioglycolic acid lignin percentages. *Table 24* shows these results, which give the impression that a somewhat higher lignin yield is reached by the combination of alkali lignin and thioglycolic acid lignin determinations than for a thioglycolic acid determination alone. The values in *table 24* are based on only one or two determinations, however, and much value therefore cannot be attached to them.

Table 24. Assay of residual lignin after extraction of alkali lignin. Comparison of the yield of this residual lignin with the quantity of alkali lignin A and the thioglycolic acid lignin yield of the total sample.*

	1	2		3	
	thioglycolic acid lignin	thioglycolic acid lignin residue		alkali lignin A	
	%	%	%of1	%	%of1
xylem	14.2	11.6	82	4.9	35
phloem	8.8	6.6	75	1.3	15
periderm	8.9	4.4	49	5.9	66
2,4-D 14th	5.6	3.4	61	0.7	12
2,4-D 29th	1.3	—	—	1.7	±130

* Lignin content calculated from the extinction. Assumed extinction coefficients: alkali lignin E 1% 1 cm = 52; lignin in lignothioglycolic acid E 1% 1 cm = 84.

5.4.4.2. Quinoneimine test

The quinoneimine test was performed on various alkali lignin samples and native lignin. Lignothioglycolic acid preparations show only a very low reactivity in this test, probably because most of the reactive groups have disappeared during the isolation.

This test was chosen not so much as a quantitative lignin determination but rather for comparison of the amount of reactive groups in the alkali lignin of the various preparations. As a parameter for the lignin content, the absorption at about 250 nm in the difference spectrum of the alkali lignin was used. Most of the determinations were done in alkali lignin A fractions and not in the total alkali lignin (see 5.4.4.1.); this has the advantage that the phenolic acids liberated by the treatment with alkali cannot participate in the reaction, *i.e.*, it is really the activity of the lignin that is measured.

The standard chosen for the determinations was guaiacol, which shows a linear relation between concentration and absorption of the reaction product. *Table 25* shows the number of μg lignin giving the same absorption as 1 μg guaiacol. By comparison of the reactivity of lignin with that of guaicol it is possible to calculate how many of the phenylpropane units of the lignin have a reactive group. For these calculations a molecular weight of 210 was assumed for the phenylpropane unit (GIERER 1954). All the values were multiplied by $\frac{124}{210}$ (mol wt guaiacol = 124). The result for xylem lignin is one reactive group to 16 phenylpropane units.

For native lignin of secondary xylem, 10.4 μg gave the same reaction as 1 μg guaiacol. This corresponds to about one reactive group per 6 phenylpropane units. The bound *p*-hydroxybenzoic acid of the native lignin probably plays only a minor role, since *p*-hydroxybenzoic acid itself shows little activity in the

Table 25. Reactivity of lignin samples in the quinoneimine test.

	μg lignin[1] equivalent to 1 μg guaiacol	reactive phenylpropane units
alkali lignin A		
native lignin xylem	25	1 : 15
xylem	27	1 : 16
young sec. xylem	112	1 : 66
phloem	207	1 : 122
periderm	297	1 : 175
NAA 18th	488	1 : 288
2.4-D cultures	200	+
native lignin		
xylem	10.4[2]	1 : 6

[1] lignin content calculated from the extinction (E 1% 1 cm = 52)
[2] based on the weight of the native lignin

quinoneimine test. During the treatment with alkali, bound *p*-hydroxybenzoic acid is liberated but at the same time the number of groups available for the quinoneimine reaction drops sharply. It is obvious to assume that the *p*-hydroxybenzoic acid was initially esterified to *p*-hydroxybenzylalcohol groups in the lignin, as SMITH (1955b) assumed for native lignin of *Populus tremula*. After hydrolysis of the ester bonds, only some of the *p*-hydroxybenzyl alcohol groups remain intact. In milled wood lignin of *Picea*, BJÖRKMAN (1956) found less reactivity than in the native lignin preparation; the former showed a sharp decrease in reactivity after various simple treatments that are supposed to have little influence on lignin. *Table 26* gives some data from the literature on the reactivity of various lignin preparations in the quinoneimine test.

The alkali lignin deriving from phloem contains far fewer reactive groups than that from xylem, and the periderm alkali lignin even fewer. The tissue-culture alkali lignin also shows only a very weak reactivity in the quinoneimine test.

It is therefore clear that lignin, at least the alkali lignin of these samples, varies widely in behaviour; there is a wide range from lignin with many reactive groups to lignin with very few reactive groups. For *Populus*, in any case, the quinoneimine test certainly cannot be accepted as a suitable method for the quantitative determination of lignin.

Comparison of the amount of *p*-hydroxybenzoic acid in the native lignin of xylem and the reactivity of the native lignin in the quinoneimine test shows that there are roughly the same number of reactive groups as molecules of bound acid. For 1 gram native lignin, a *p*-hydroxybenzoic acid content of 9.3 per cent was determined, or $6.7 \cdot 10^{-4}$ gmol. In view of the loss involved in chromatography, this value is probably too low. The reactivity of 1 gram of native lignin in the quinoneimine test corresponds to that of $1/10.4$ g $= 7.8 \cdot 10^{-4}$ gmol guaiacol. These values show such good agreement that they support the view that the same group is responsible for the reactivity to quinoneimine and the binding of *p*-hydroxybenzoic acid.

Table 26. Quinoneimine test. Frequency of reactive groups in various lignin preparations calculated from data in the literature.

lignin sample		author	reactive phenylpropane units
Picea xylem	N.L.	Gierer 1954	1 : 7–8*
Picea xylem	N.L.	Björkman 1957	1 : 11
Picea xylem	M.W.L.	Björkman 1957	1 : 18
Betula xylem	N.L.	Stafford 1960	1 : 24*
Saccharum (bagasse)	N.L.	Stafford 1960	1 : 18*

N.L. = native lignin
M.W.L. = milled wood lignin

* molecular weight of the phenylpropane unit assumed to be 210

5.4.5. Infrared spectra

The thioglycolic acid lignin preparations give good infrared spectra with the potassium bromide method *(fig. 31)*. The preparations of primary xylem and of some of the tissue cultures, however, do not show distinct bands as a result of excessive contamination. Thioglycolic acid itself has a strong absorption in the region above 2900 cm^{-1}, a band at about 1700, and a weak absorption in the region of 1400 to 1375 and at 1280 and 1150 cm^{-1}.

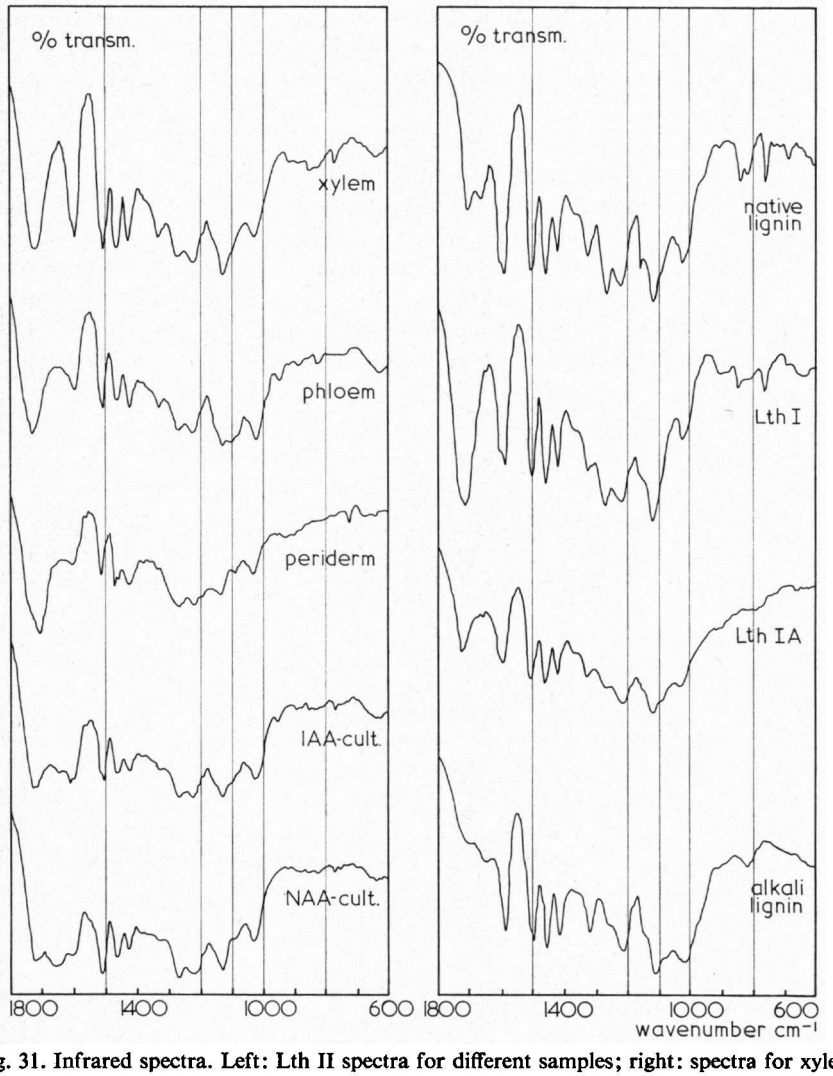


Fig. 31. Infrared spectra. Left: Lth II spectra for different samples; right: spectra for xylem lignin isolated by various methods. The alkali lignin and the Lth II had undergone alkaline hydrolysis during isolation; the native lignin and the Lth I were not exposed to alkali.

For purposes of comparison, spectra were also measured of lignin isolated by other methods, *i.e.* alkali lignin A (precipitated fraction) and soluble native lignin, both prepared from *Populus* xylem.

Table 27 shows the data on the occurrence of a few characteristic bands in the region of 1800–600 cm^{-1} and of the absorption ratios at certain wave numbers. The criteria applied are those of KAWAMURA & HIGUCHI (1965) for milled wood lignin preparations of gymnosperm and angiosperm xylem (see p. 251). According to these criteria, the secondary xylem shows mainly angiosperm-lignin characteristics. The absorption at 1505 cm^{-1} of Lth II is on the high side but it is still much lower than the 1505 cm^{-1} band for gymnosperm lignin.

The 1325 cm^{-1} band, which according to SARKANEN *c.s.* (1967) originates from syringyl groups (1335 cm^{-1}) is rather pronounced, especially for the preparations of secondary xylem, but is also recognizable in other preparations.

The 1265 cm^{-1} band is present in all preparations and is even very strong for the xylem lignin. For the hydrolized lignothioglycolic acid I A and alkali lignin the absorption at 1265 is much lower, however. Sarkanen *c.s.* ascribed this band (1275 cm^{-1}) to non-condensed guaiacyl groups. The stronger absorption of non-hydrolized xylem lignin could possibly be caused by *p*-hydroxybenzoate groups. The absorption at 1265 cm^{-1}, which is exceedingly high for an angiosperm lignin, would then be partially due to the esterified *p*-hydroxybenzoic

Table 27. Some characteristic absorption bands in the infrared spectra of lignothioglycolic acid and other lignin preparations

	preparation	1600 <or> 1505	1505 <or> 1460	1325	1265 <or> 1215	1120 <or> 1025	1090	766 cm^{-1}
xylem	N.L.	>	<	++	>	>	s?	++
xylem	A.L.	<	<	++	<	>	–	–
old sec. xylem	Lth I	<	<	+	≧	>	s?	++
old sec. xylem	Lth IA	<	<	+	<	>	–	–
young sec. xylem	Lth I	<	<	+	>	>	–	+
old sec. xylem	Lth II	<	≧	++	<	>	–	(+)
young sec. xylem	Lth II	<	>	+	≦	>	–	?
phloem	Lth II	<	>	+	<	>	+	–
periderm	Lth II	<	<	(+)	>	>	(+)	–
NAA 29th	Lth II	<	>	+	>	>	+	+.
IAA 23rd	Lth II	>	>	(+)	=	>	–	+
gymnosperms*	M.W.L.		>	weak	>	<	+	
angiosperms (S)	M.W.L.		<	strong	<	>	-or s	
(T)	M.W.L.		>		≦		+	

N.L. = native lignin
A.L. = alkali lignin
M.W.L. = milled wood lignin
s = shoulder
*Data from KAWAMURA and HIGUCHI for milled wood lignin of gymnosperms and angiosperms.
S = standard type; T = tropical species (some *Dipterocarpaceae*)

acid of the poplar lignin and not to a lignin "core" with a high proportion of non-condensed guaiacyl groups as in gymnosperms.

Another striking observation is the absorption at about 766 cm^{-1} found for the xylem preparations and the IAA and NAA cultures. This band was strongest in the spectra of native lignin and lignothioglycolic acid I. It again seems obvious to seek a connection between the presence of this band at 766 cm^{-1} and esterified *p*-hydroxybenzoic acid in the lignin. Free *p*-hydroxybenzoic acid has a band at 767 cm^{-1}, syringic acid one at 768, and vanillic acid at 752 and 760 cm^{-1}. The methylester of *p*-hydroxybenzoic acid too has an absorption band at 770 cm^{-1} (BARBIER *c.s.* 1960). Alkali lignin lacks such a band, and after hydrolysis of Lth I, during which a large amount of *p*-hydroxybenzoic acid is split off, the band at 766 was no longer present. Thus, evidently there is indeed a relationship between the occurrence of this extra band in the *Populus* lignin spectrum and the presence of large quantities of *p*-hydroxybenzoic acid in the lignin.

The periderm preparation showed a distinct band at about 719 cm^{-1} not found for any of the other preparations.

In the spectra of lignothioglycolic acid the absorption of thioglycolic acid predominates in the region of 1700 cm^{-1}, thus blocking the observation of any lignin carbonyl bands that might be present in this region. The alkali lignin has no distinct bands in this zone, but the native lignin shows bands at 1707 and 1665 cm^{-1}. Perhaps these bands must be considered to correspond to the absorption at 1694 and 1658 cm^{-1} found for native lignin of *Populus tremula* dissolved in dioxane by SMITH (1955b), who ascribed the 1694 cm^{-1} band to ester carbonyl groups, probably from the *p*-hydroxybenzoate groups in the aspen lignin.

6. DISCUSSION

In the third year of growth on a medium containing 2,4-D, the *Populus* culture gradually lost the capacity to form tracheids. That this capacity had not entirely disappeared was demonstrated by the subcultures of this material on other media. On a medium containing NAA a few tracheids were formed by the first subcultures, but the strain developed on an IAA medium has continued to form tracheids. Furthermore, in the 10th subculture of the IAA strain, *i.e.* 6 years after the isolation of the *Populus* tissue, the formation of roots was observed. At that time, consequently, the IAA tissue was still capable of producing apical meristems. Meristematic activity is still observable at other levels; besides xylem elements, more or less orientated sieve elements are formed, which may indicate cambial activity. Later attempts to subculture tissue of the 2,4-D and NAA strains on a medium containing IAA and no other auxins were unsuccessful. In all probability, the only successful transplantation to an IAA medium concerned a sample of tissue still possessing the capacity for differentiation. Due to the poor growth of the IAA strain, it only recently became possible to study the growth and differentiation of this tissue on other media.

The cultures show two types of lignified cells: tracheids and lignified paren-

chyma cells. In addition, an extracellular substance stained by lignin reagents was found. To study the influence of certain factors on lignin synthesis, it must be kept in mind that stimulation of this synthesis can be brought about directly as well as indirectly. The indirect pathway involves the induction of cambium or the formation of cells with a predisposition for lignification of their walls. A more direct induction of lignin synthesis could be caused by a shift in the carbohydrate metabolism in favour of the pentose phosphate cycle or later, at the aromatic level, by a shift of the synthesis of phenolic substances in favour of direct lignin precursors.

In the tracheids, the formation of a secondary cell-wall pattern is always accompanied by the deposition of substances stainable with lignin reagents. There is no reason to assume that the phenolic lignin precursors are not formed in the tracheids themselves. In some cases, differentiating tracheids even show a phloroglucinol-positive material in their lumina.

For the parenchyma it is not certain at what stage in the cytogenesis of a given cell the differentiation pattern, including lignification, is determined; it may, as in the tracheids, be a very early one. The peripheral tissue covering the spherical growth zones often shows lignification and suberization. This tissue might be comparable to the periderm, and the differentiation pattern is probably fixed very early here too. Elsewhere in the tissue there are zones with lignified cell walls and intercellular material, but the cells do not otherwise differ distinctly in type or arrangement from those in the adjacent tissue zones. Since lignification is often restricted to intercellular areas, there is some question of whether a 'passive lignification' takes place here, for instance because, in the presence of a suitable matrix and an oxidizing system, a precursor supply from elsewhere initiates the lignification. In this connection the role played by the intercellular spaces and canals requires closer examination. Callus cultures do not have a well-developed transport system; but the intercellular system may serve in this way. Certain cellular excretions undoubtedly reach the intercellular areas (WHITE 1967). LIPETZ (1965) has demonstrated peroxydase excretion by tissue cultures, and it is known that many other enzymes can be recovered from the culture medium. In addition, phenolic compounds can often be demonstrated in the medium under the cultures. If the assumption of mobility of enzymes and precursors is correct, it would mean that the localization of lignin deposition must be mainly dependent on the presence of a matrix and on the accessibility by means of the transport channels. The parts of the cell walls enclosing the intercellular spaces seem to offer a good matrix for the deposition of lignin, especially in the furthest corners, which is roughly where Wardrop observed the first lignification in *Pinus* tracheids. The incorporation of radioactively labelled lignin precursors in secondary xylem is also strongest in corners between cells (SALEH *c.s.* 1967). The larger intercellular canals in the cultures are often filled with a homogeneous or granular substance, and a somewhat fibrillar structure is also sometimes seen. On the basis of the staining in sections and lignin determination in whole cultures, we may assume that a lignin has been deposited in these intercellular masses. Many of these cases concern tissues in which there

are no tracheids, and rather few cell walls are stained in the phloroglucinol test. If a large amount of lignin can be extracted from whole cultures, we may assume that some of this lignin has an intercellular origin. It is not certain whether the intercellular areas contain a substance that can function as a lignin matrix. WHITE (1967) describes an extracellular substance in cultures of *Picea glauca* which he called "matrix" and which "evidently consists of lignin and cellulose precursors in a wide range of degrees of polymerization".

It is not yet known whether the positive phloroglucinol reaction obtained in the agar under the cultures results from the presence of precursors or true lignin. It would be interesting to know whether agar can function as a kind of lignin matrix.

The deposition of lignin in the tissue cultures of *Populus* follows a completely different pattern than the lignification of the xylem of the tree. In all the cultures studied, tracheids were either absent or played a minor role. It seems conceivable that quite different macromolecules serve as matrix in tissue cultures than in tissue from the tree; this could have a radical influence on the composition of the lignin. Different types of linkage between lignin and components of the cell could greatly influence the lignin yield obtained with various isolation methods.

The lignin found in tissue cultures differs in certain respects from that of secondary xylem. In the first place, the methoxyl and syringyl contents are lower than in xylem lignin. At oxidation with nitrobenzene, however, callus lignin yields an appreciable amount of syringylaldehyde; in respect of its syringylaldehyde/vanillin ratio it is comparable to the lignin of young secondary xylem. A still better agreement in the S/V ratio would probably be obtained if isolation was performed on only the youngest stages of the secondary xylem. It is also likely that in the metaxylem there are elements with a persisting low S/V ratio. As we have seen, the lignin of the protoxylem does not contain a measurable amount of syringyl groups, but some reticulate-scalariform elements developing later often show a weakly positive Mäule reaction of the cell walls. In the tissue cultures it seems probable that over the years there was a change in the composition of the lignin resulting in a lowering of the S/V ratio. Comparison of the differences in the ratios is difficult, however, because the culture period of the earlier subcultures was often longer than two months.

The S/V ratios for the *Populus* cultures lie appreciably higher than those for the cultures of *Paulownia tomentosa*, *Syringa vulgaris*, and *Rosa wichuraiana* studied by HIGUCHI & BARNOUD (1965). The indication '*lignine immature*' for the lignin in tissue cultures (BARNOUD 1965) does not seem to be an entirely fortunate choice. Although comparison of the S/V ratio in young and old secondary xylem suggests a later enrichment of the lignin with phenylpropane units having a higher methoxyl content, it is equally likely that various types of lignin with an increasingly high methoxyl content are successively deposited. Furthermore, low S/V ratios cannot justifiably be identified with lignin of immature elements since completely differentiated elements in the tree can also show a lignin with a low syringyl content.

The relative aldehyde yield is consistently low for callus lignin, and amounts to only one- half to one- third of the aldehyde yielded by the oxidation of lignin of young or old secondary xylem. Since only part of the lignin is broken down by nitrobenzene oxidation, it seems obvious to assume that the lignin in the callus cultures is very highly resistant to oxidation. This might be due to differences in localization in the cell wall, in binding to the cell wall, or in the structure of the lignin itself. During the isolations with thioglycolic acid it was observed that after the first treatment cell-wall material of the callus cultures often still contained lignin. Another treatment with thioglycolic acid and alkali was necessary to remove this residual lignin.

A typical property of the poplar xylem lignin is the very high *p*-hydroxybenzoic acid (Pa) content. According to SMITH (1955b) and NAKANO *c.s.* (1961) this *p*-hydroxybenzoic acid is esterified to an alifatic hydroxyl group, probably at the first C-atom of the side chain of the phenylpropane unit. The formation of indophenol with quinoneimine is considered to be a reaction to such groups, *i.e.* the *p*-hydroxybenzyl alcohol groups in the lignin. In native lignin of the Lombardy poplar the number of reactive groups in the quinoneimine test is almost equal to the number of molecules of bound *p*-hydroxybenzoic acid. It therefore seems probable that the same alifatic hydroxyl groups are indeed concerned in both the binding of *p*-hydroxybenzoic acid and the reaction with quinoneimine.

The hydrolysis of lignothioglycolic acid also yields *p*-hydroxybenzoic acid. The callus cultures gave a lignothioglycolic acid with a rather high *p*-hydroxybenzoic acid content. The differences in the UV spectra before and after hydrolysis suggest that the callus lignin, like the xylem lignin, contains a rather large amount of esterified *p*-hydroxybenzoic acid. Calculation showed that the *p*-hydroxybenzoic acid in the callus cultures can amount to more than 6 per cent of the lignin; for xylem and native lignin prepared from xylem, this value is 9 to 10 per cent.

Lignin rich in *p*-hydroxybenzoic acid has a strikingly high maximum in the ionization difference spectrum at about 296 nm. For xylem preparations the absorption at this maximum is more than twice as high as that at the maximum at about 250 nm. There is a distinct correlation between the *p*-hydroxybenzoic acid content of a preparation and the absorption at 296 nm. Lignin freed of *p*-hydroxybenzoic acid by hydrolysis or phloem lignin, which contains almost no *p*-hydroxybenzoic acid, have an absorption ratio of only 0.4 for the maxima at about 300 and 250 nm. The *p*-hydroxybenzoic acid-rich *Populus* lignin is in a sense comparable to "grass lignin", which contains esterified *p*-coumaric acid (HIGUCHI 1967b). In the neutral spectrum of the non-hydrolyzed grass lignin there is an additional maximum at 315 nm, and in the difference spectrum there is high absorption at 355 nm. Both maxima are absent in the hydrolyzed lignin. Since *p*-coumaric acid itself has an absorption maximum in the difference spectrum at about 330 nm, it seems, therefore, that esterification of *p*-coumaric acid causes a shift of this maximum to a higher wavelength. This phenomenon is also seen in *p*-hydroxybenzoic acid; for the free acid the maxi-

mum lies at about 282 nm in the difference spectrum and for the esterified acid at about 296 nm.

In the infrared spectrum xylem lignin has a distinct band at about 766 cm^{-1}. The absorption at this wave number is probably related to the presence of *p*-hydroxybenzoic acid in the lignin, because a distinct band at 766 cm^{-1} is shown only by lignin preparations giving *p*-hydroxybenzoic acid upon hydrolysis. Furthermore, not only the free acid but also its methylester shows absorption in this region. The relatively strong absorption at 1265 cm^{-1} must probably also be ascribed to the *p*-hydroxybenzoate groups in the lignin.

The lignothioglycolic acid preparations and the native lignin of *Populus*, when in neutral solution, consistently show a more or less distinct absorption maximum in the region between 272 and 280 nm. The hydrolyzed fraction (B) of lignothioglycolic acid that is not precipitated by acidification usually has a maximum at a lower wavelength (264 nm).

In the difference spectrum, native lignin has an absorption maximum at 247 nm. Binding of thioglycolic acid gives an upward shift of from 9 to 13 nm. Lignothioglycolic acid preparations of tissue from the tree and from callus cultures have this difference-spectrum maximum between 262 and 243 nm. For the tissue cultures there is always a maximum at a rather low wavelength; for the lignin of primary xylem this maximum in the difference spectrum lies at a still lower wavelength.

The infrared spectrum of lignothioglycolic acid from tissue cultures shows agreement at many points with the infrared spectrum of xylem lignin. The differences are found mainly in the relative height of certain maxima. The spectra of periderm, phloem, and young secondary xylem also show similar divergence with respect to the xylem lignin spectrum. The spectrum of soluble native lignin differs slightly from the spectrum of xylem lignothioglycolic acid showing a relatively higher maximum at 1265 cm^{-1}. This high maximum at 1265 is also found for the lignothioglycolic acid preparations of young secondary xylem, periderm, and the NAA culture. Direct comparison with the results of Kawamura and Higuchi is not possible, because these authors worked with millled wood lignin. Milled wood lignin offers great advantages because of its purity but often represents only part of the lignin and it therefore remains a question whether this sample is representative of the total lignin, in every respect.

The quinoneimine test proved to be entirely unsuitable for the quantitative determination of alkali lignin from tissue cultures because of the extremely low numbers of reactive groups. The lignin from wood showed a strong reaction in the quinoneimine test; the number of reactive groups proved to be influenced by the method used to isolate the lignin, however. With the alkali treatment, for instance, a large proportion of the reactive groups was lost.

The comparative lignin investigation of the tissues of the tree clearly showed that many different types of lignin can be deposited simultaneously in a single organ. We must even conclude that lignins with different compositions are formed within a single type of tissue. The analyses of the lignin from young

secondary xylem and of soluble native lignin from secondary xylem and the staining reactions point in this direction.

The lignin isolated from callus cultures showed agreement in many respects with the lignin from mature secondary xylem or lignin deposited in the walls of secondary xylem elements in the initial phase of lignification. Nevertheless, there are important differences between xylem lignin and tissue culture lignin, *e.g.* in the relative aldehyde yield and syringaldehyde/vanillin ratio at oxidation and in reactivity in the quinoneimine test. It is interesting that the lignin of primary xylem, phloem, and periderm also differs in this repect from the lignin of secondary xylem.

The terms "hardwood lignin" or "angiosperm lignin" found in the literature seem to imply that all lignins from dicotyledonous trees show the same or a very similar composition, but actually this only holds for secondary xylem. Wood has always received the most attention in the study of lignin because of its enormous economic importance. It is now evident, however, that data on solubility, frequency of certain groups, and other characteristics cannot be generalized for lignin in other kinds of tissue and callus cultures. Therefore, more attention should be given to the analysis of lignin from these sources and to appropriate determination methods for each object.

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