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Investigations on Lignins and Lignification
Pt. XXXI. Characterization of Metasequoia "Milled-Wood" Lignin
by Michael J. Reale, Donald D. Clarke, Walter J. Schubert and F. F. Nord
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Introduction
In 1941 the Japanese botanist, Shigeru Miki, conducted an investigation of certain native fossil materials of the Pliocene age. Although these specimens had earlier been attributed to the genus Sequoia, Miki noticed some discrepancies. Most striking was the finding that some of the samples were of deciduous habit, whereas the Sequoia are evergreen. Consequently, he established the genus Metasequoia (meta, "beyond"), implying a possible relationship of the plant species to the genus Sequoia (1).

In the same year, a forest survey was conducted in the mountainous interior of China. The existence of a large, unknown coniferous tree in eastern Szechuan Province was reported. However, no samples of the tree were removed until 1944. Examination of this material then revealed that it was a distinct species, not previously discovered in China. Further work identified it as a member of the recently proposed fossil genus, Metasequoia. In 1948 the plant was described and named Metasequoia glyptostroboides because of a similarity to the genus Glyptostrobus (2).

Thus the newly discovered plant species is considered to be a "missing link" in the phylogeny of the modern Sequoia, the famed redwoods of California and Oregon. Perhaps more important is the fact that this is an extant, not extinct, plant, or a "living fossil" (3). A fossil usually exhibits only fragmentary features, while a living plant can be studied in all its aspects, biological as well as chemical.

From the time of its discovery, Metasequoia has been studied quite extensively in its various botanical aspects (4) and has been taxonomically classified as a gymnosperm. However, botanical taxonomy depends on a comparison and evaluation of morphological characteristics — the forms and structures of plants, and the organization and coordination of their parts and organs, particularly those which are concerned with sexual reproduction. This basis of classification may be regarded as somewhat artificial.

Over 60 years ago, Mäule discovered that the woods of angiosperms produce a red color when treated successively with aqueous potassium permanganate, hydrochloric acid and ammonium hydroxide, whereas the woods of conifers exhibit a brown or orange color (5). This represented the first observations in chemical taxonomy of woody plants.

Later, an attempt was made chemically to classify plants on the basis of their metabolic products (6), such as alkaloids and volatile oils. In this connection, Erdt-
been investigated (14). Hence, it was thought of interest chemically to study this plant species from other viewpoints. Thus, we first sought to provide a chemical basis for the, taxonomic classification of Metasequoia glyptostroboides by relating its response in the Mättle reaction to the products of its alkaline nitrobenzene oxidation, and of its catalytic hydrogenation-hydrogenolysis.

Secondly, previous investigations in this laboratory had revealed that isolated lignins may differ not only from one species to another, but also within the same species (15). To further study this phenomenon, we isolated "milled-wood" lignin from Metasequoia sapwood. This lignin was characterized in the following ways (16): elementary and methoxyl group analyses, acetate and phenylhydrazone derivatives, solubility, color reactions, infrared and ultraviolet absorption spectroscopy, catalytic hydrogenation-hydrogenolysis and alkaline nitrobenzene oxidation.

Experimental

A young Metasequoia tree was debarked; the smaller branches were removed and the residue was cut into small pieces. The heartwood was separated wherever possible. Each of these separate parts was then individually ground to 80 mesh and air-dried.

The moisture contents of these samples were determined by drying at 115°C; their lignin contents were determined by the TAPPi Standard T 13 m method (17).

Milled-wood lignin was isolated from the Metasequoia sapwood according to the procedure of Björkman (18); its solubility in various solvents and its color reactions were studied. An acetylated derivative and a phenylhydrazone of this lignin were prepared according to well known procedures (19).

The Metasequoia Björkman lignin and its derivatives were characterized spectroscopically. The ultraviolet absorption spectra were determined in dioxane solutions with a Cary model 15 double-beam recording spectrophotometer. The infrared absorption spectra were measured as KBr pellets with a Perkin Elmer model 21 double-beam recording spectrophotometer.

Alkaline nitrobenzene oxidation (19b) was performed on the lignin on a micro scale (20). The oxidation mixture was subjected to preliminary washings with methylene chloride before and after acidification, and then separated by a recently developed procedure involving thin-layer chromatography on silica gel (21). Multiple unidimensional development (22) was employed, with methylene chloride-acetone (99:1 by volume) as solvent. After visualization under ultraviolet light (3660 Å), aldehyde-containing zones were scraped from the plate, and the resulting powders extracted with ethanol. For quantitative estimation, the ethanol extracts were compared with standard curves relating optical density and concentration.

A dispersion of Metasequoia sapwood sawdust in 50% aqueous dioxane was subjected to hydrogenation-hydrogenolysis over Raney nickel at an initial hydrogen pressure of 500 p. s. i. g. and a temperature of 202°C for five hours. The chloroform-soluble products were analyzed with a standard Aerograph model 110-C gas chromatograph. A 130-disk integrator, mounted on a one mv. one second pen speed Brown recorder, was used for quantitative measurements.

Optimum separation of the mixture was achieved by employing a 1/8" x 5' column packed with 5% 6-ring poly-α-phenyl ether on Chromosorb-W, precoated with 5% silver nitrate, with an oven temperature of 200°C and a helium flow rate of 30 ml/min.

Identification of the components of the chloroform-soluble fraction was established by comparing their retention times with those of authentic samples. The effluents of the major components were collected as they emerged from the chromatograph. Further proof of their identities was obtained by comparing their infrared absorption spectra with those of authentic samples determined in the same manner, viz., collection from the gas chromatograph. (This was necessary because the stationary phase was somewhat volatile, and hence, the infrared spectra could contain peaks attributable to it.)

Results and Discussion

The moisture contents of the various parts of the Metasequoia tree are recorded in Table 1. In Table 2 are listed the lignin contents of the same samples; the data obtained by Koshyma et al. (14) are presented for comparison. Although the values are higher than those usually observed for certain other species, they do fall within the range of values found for soft-woods (23).

Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial Weight of Sample (gms)</th>
<th>Loss of Weight (gms)</th>
<th>Moisture Content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twigs</td>
<td>1.0315</td>
<td>0.0930</td>
<td>9.02</td>
</tr>
<tr>
<td></td>
<td>0.9917</td>
<td>0.0886</td>
<td>8.94</td>
</tr>
<tr>
<td>Sapwood</td>
<td>0.9994</td>
<td>0.0827</td>
<td>8.27</td>
</tr>
<tr>
<td></td>
<td>1.0016</td>
<td>0.0820</td>
<td>8.19</td>
</tr>
<tr>
<td>Heartwood</td>
<td>0.9981</td>
<td>0.0837</td>
<td>8.39</td>
</tr>
<tr>
<td></td>
<td>1.0044</td>
<td>0.0836</td>
<td>8.32</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight of Wood Sample (gms)</th>
<th>Weight of Lignin (gms)</th>
<th>Lignin Content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twigs</td>
<td>0.9103</td>
<td>0.3078</td>
<td>33.81</td>
</tr>
<tr>
<td></td>
<td>0.9108</td>
<td>0.3071</td>
<td>33.72</td>
</tr>
<tr>
<td>Sapwood</td>
<td>0.9223</td>
<td>0.2924</td>
<td>31.70</td>
</tr>
<tr>
<td></td>
<td>0.9098</td>
<td>0.2850</td>
<td>31.31</td>
</tr>
<tr>
<td>Heartwood</td>
<td>0.9186</td>
<td>0.2954</td>
<td>32.15</td>
</tr>
<tr>
<td></td>
<td>0.9196</td>
<td>0.2976</td>
<td>32.37</td>
</tr>
<tr>
<td>Wood</td>
<td>—</td>
<td>—</td>
<td>29.58*</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>—</td>
<td>32.30*</td>
</tr>
</tbody>
</table>

*a) Value for a 40 year-old tree (14)

*b) Value for a five year-old tree (ibid.)

When pre-extracted Metasequoia sapwood powder was subjected to the Mättle reaction, only an indefinite brown color was obtained, with no trace of red. That this is indicative of a coniferous wood is well established (5, 8, 10, 24, 25).
Metasequoia Björkman lignin was isolated from the sapwood in 30% yield, based on the Klason lignin content. It was found to be soluble in 2 N sodium hydroxide, aqueous dioxane, aqueous acetic acid and pyridine; it was insoluble in 6 N hydrochloric acid, water, benzene, petroleum ether and ethanol.

The color reactions of Metasequoia Björkman lignin are indicated in Table 3. It is generally accepted that these colors are produced by the reaction of phenols or amines with aldehyde groups present in the lignin (17a). Adler proposed that coniferyl aldehyde moieties are part of the lignin molecule, and the colors (26). However, Brauns (27) suggested that, although the color reactions may be due to this group, coniferyl aldehyde itself, need not form part of the lignin molecule. An \(\alpha\)-hydroxydihydroconiferyl aldehyde structure could become dehydrated under the influence of the mineral acid used in the reactions, thereby producing coniferyl aldehyde (28). We also have some evidence for this possibility in conjunction with the formation of a phenylhydrazone derivative of the Metasequoia Björkman lignin (see below). This lignin gave a positive Fehling's test.

The infrared absorption spectrum of the lignin is depicted in Fig. 2 (32, 33). It shows the following characteristic peaks (34): the absorption at 2.90 \(\mu\) is due to hydroxyl groups, which may be classified as aliphatic primary and secondary hydroxyl and phenolic hydroxyl groups, because of the C–O stretching band at 8.19 \(\mu\) (phenolic), the C–O deformations at 9.23 \(\mu\) (secondary hydroxyl), and at 9.69 \(\mu\) (primary hydroxyl). The carbonyl absorption at 5.79 \(\mu\), together with the shoulder in the C–H stretching region at 3.48 \(\mu\) suggest a non-conjugated aldehyde. The C–H stretching absorption at 3.28 \(\mu\) and the C–C skeletal vibrations at 6.24 and 6.66 \(\mu\) show the presence of aromatic rings. That aromatic ether groups are present is indicated by the C–O stretching band at 7.88 \(\mu\). This spectrum is similar to those of pine and hemlock "milled wood" lignins (34).

The results of elemental and group analyses of Metasequoia Björkman lignin, its acetate and phenylhydrazone derivatives are recorded in Table 4. As may be seen in the table, the empirical formula calculated for the lignin itself is \(C_{102}H_{110}O_{39}\), indicating a minimum molecular weight of 1920 for the "building unit". Although this is more than double the values reported for different lignins (19, 19a), this might be anticipated, since "native lignin" is the fraction most easily extracted from any wood sample, and hence would be expected to have the lowest molecular weight. Björkman determined the molecular weight of "ordinary" milled-wood lignin from spruce by means of the ultracentrifuge, and obtained a value of 11,000 (35, 36); therefore it would appear that the Metasequoia lignin contains six building units per "molecule" based upon Björkman's molecular weight value. The value found for the methoxyl content of the Metasequoia lignin

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**Table 3**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Color Produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolglucinol-HCl</td>
<td>Violet</td>
</tr>
<tr>
<td>Phenol</td>
<td>Green</td>
</tr>
<tr>
<td>Phenol reagent</td>
<td>Blue</td>
</tr>
<tr>
<td>Resorcinol</td>
<td>Grey-green</td>
</tr>
<tr>
<td>2,6-Diamino pyridine</td>
<td>Yellow-orange</td>
</tr>
<tr>
<td>2,4-Diamino pyridine</td>
<td>Red-brown</td>
</tr>
</tbody>
</table>

**Table 4**

<table>
<thead>
<tr>
<th>Lignin</th>
<th>Acetate of Lignin</th>
<th>Phenylhydrazone of Lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculated</td>
<td>Found</td>
<td>Calculated</td>
</tr>
<tr>
<td>C</td>
<td>62.50  62.74</td>
<td>61.32  61.61</td>
</tr>
<tr>
<td>H</td>
<td>5.67   5.65</td>
<td>5.47   5.68</td>
</tr>
<tr>
<td>O(\alpha)</td>
<td>31.84  31.61</td>
<td>33.19  31.71</td>
</tr>
<tr>
<td>N</td>
<td>22.82  22.82</td>
<td>12.37  12.06</td>
</tr>
<tr>
<td>OCH(_3)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>COCH(_3)</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* by difference
is in accord with those values usually obtained for gymnosperm lignins (17a), and corresponds to ten such groups per building unit, or 60 per “molecule”.

The data obtained for the acetylated derivative indicate that there are 13 acetylable hydroxyl groups for each methoxyl group. The infrared absorption spectrum of this product is shown in Fig. 3, and reveals bands at 5.75 and 8.15 μ, which are characteristic of acetates (29a). The large hydroxyl absorption at 2.85 μ indicates that unacetylated hydroxyl groups remained in this material.

The analytical data for the phenylhydrazone derivative of *Metasequoia* Björkman lignin, also reported in Table 4, indicate that there are two carbonyl groups per ten methoxyls. To correlate the analytical data with the calculated values, it is necessary to assume the loss of two molecules of water, in assigning an empirical formula to the phenylhydrazone. These water molecules might originate from dehydration of alcoholic hydroxyl groups beta to each of the two carbonyl groups, under the influence of the pyridine used as solvent. Such a dehydration was mentioned above in relation to the colors produced when lignin is treated with amines and phenols in the presence of dehydrating acids. The infrared spectrum of this derivative, shown in Fig. 4, reveals the absence of carbonyl absorption.

The average yield of vanillin formed on alkaline nitrobenzene oxidation of *Metasequoia* Björkman lignin was 22.0 ± 1.2%. Although our procedure would have detected as little as 1% of either p-hydroxybenzaldehyde or syringaldehyde in samples of the size we employed, neither compound was observed, although some shadowing appeared in the region of p-hydroxybenzaldehyde. When the chromatogram was sprayed with a solution of 2,4-dinitrophenyldihydrazine, a light yellow color appeared in the zone assigned to it. In a further effort to determine whether p-hydroxybenzaldehyde was indeed present, another chromatogram was developed; the aldehyde-containing zones were scraped from the plate, and the ultraviolet absorption spectra measured from 300 to 400 mμ in the presence of added base. The spectrum of the p-hydroxybenzaldehyde zone gave no absorption in addition to that of the blank.

Earlier, the alkaline nitrobenzene oxidation products of, among others, five species of *Taxodiaceae*, the family of which *Metasequoia* is a member, was investigated. It was observed that each of the species produced p-hydroxybenzaldehyde to the extent of 2.0–3.5% (37). Since this assay method consisted of a visual comparison of the intensities of color produced when the chromatogram was sprayed with 2,4-dinitrophenyldihydrazine with the intensities produced by a series of standard solutions of known concentration, *Leopold* may possibly have been observing the same material referred to above.

Gas chromatographic analysis of the chloroform-soluble catalytic hydrogenation products of the *Metasequoia* sapwood showed that guaiacol, 4-methylguaiacol, 4-ethylguaiacol, 4-n-propylguaiacol and dihydroconiferyl alcohol were the only detectable products, as may be seen from the data in Table 5. Since hydrogenolysis of alpha and beta C—O bonds present on a phenylpropane side-chain is a facile reaction, the formation of dihydroconiferyl alcohol is readily interpretable (13), if lignin contains linkages of the guaiacyl-glycerol-β-aryl ether type, as has already been proposed (38, 39). The other degradation products may arise by a cleavage of the side chain (40, 41). The isolation of guaiacyl compounds is anticipated in the case of a coniferous wood (12). However, dicotyledons yield the five guaiacyl products mentioned above, in addition to the corresponding syringyl analogs (13, 40); there is evidence that monocotyledons yield all of the above-mentioned products and the corresponding p-hydroxyphenyl analogs as well (42).

**Conclusion**

Since the time of its discovery, *Metasequoia* has been classified as a monotypic genus of the family *Taxodiaceae*, belonging to the class Gymnospermae. The
lignins of gymnosperms are believed to be derived almost entirely of guaiacylpropane units, and they are chemically characterized by the following properties. They give no response to the Mäule test (since this reaction depends on the presence of syringyl units in the lignin), and in general, they yield only vanillin upon alkaline nitrobenzene oxidation. Catalytic hydrogenation-hydrogenolysis of these lignins produces only guaiacol, 4-methylguaiacol, 4-ethylguaiacol, 4-n-propylguaiacol and dihydroconiferyl alcohol. The lignins from conifers have methoxyl contents in the range 15—16%, whereas those of dicotyledons are in the range 20.5—21.5%. The ultraviolet absorption spectra of the sawdust lignins show a maximum absorption in the region 281—285 mμ; hardwood lignins absorb in the region 274—276 mμ.

When Metasequoia sapwood was subjected to the Mäule reaction, an indefinite brown color was observed. Vanillin was the only aldehyde detected after its alkaline nitrobenzene oxidation. Hydrogenation-hydrogenolysis of the pulverized sawdust of this tree yielded the above-mentioned guaiacyl compounds; the corresponding p-hydroxyphenyl or syringyl analogs were not detectable. The "milled-wood" lignin from Metasequoia has a methoxyl content of 15.82% and its ultraviolet spectrum revealed an absorption maximum at 281 mμ. Thus all the chemical evidence supports the previous biological classification of Metasequoia glyptostroboideae as a member of the class Gymnospermae.

Acknowledgement

The Metasequoia tree was obtained through the courtesy of Mr. L. Politi of the New York Botanical Garden. This investigation was supported in part by a grant of the National Science Foundation to Dr. F. F. Nord.

Summary

A young Metasequoia tree was debarked; its branches and heartwood were removed. The branches, sapwood and heartwood were separately ground to 80 mesh and air-dried. The moisture and lignin contents of these were determined.

"Milled-wood" lignin was isolated from the sawdust, and was characterized by means of its ultraviolet and infrared absorption spectra. As a means of determining the empirical formula and the number of certain functional groups present in this lignin, an acetylated derivative and a phenylhydrazone were prepared. These were also studied spectrophotometrically. Elemental, methoxyl and acetoxyl analyses were performed on the original lignin and on its derivatives. The solubility of Metasequoia Björkman lignin in various solvents and its color reactions were also determined.

Alkaline nitrobenzene oxidation was applied to the lignin, and the average yield of vanillin present in the oxidation mixture was 22.0 ± 1.2%o. Neither p-hydroxybenzaldehyde nor syringaldehyde was detected.

Hydrogenation-hydrogenolysis of Metasequoia sawdust was also conducted. The products were separated by gas-liquid chromatography, and the following compounds were identified by their retention times and/or infrared absorption spectra: guaiacol, 4-methylguaiacol, 4-ethylguaiacol, 4-n-propylguaiacol and dihydroconiferyl alcohol. Thus all the chemical evidence supports the previous biological classification of Metasequoia glyptostroboideae as a member of the class Gymnospermae.

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