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Eugenol Lignin: its Chemical Properties and Significance

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Observations by several investigators have shown that many different substances may appear to act as precursors of lignin in growing plants or in artificial systems. Stone (1953) has pointed out that deposition of a radioactive lignin from a labelled precursor is not in itself proof that the supposed precursor has been incorporated into the lignin. The radioactivity may be due merely to co-polymerization.

Schubert, Acerbo & Nord (1957) demonstrated the incorporation of labelled *p*-hydroxyphenyl-pyruvic acid into the lignin of sugar cane. Acerbo, Schubert & Nord (1958) showed that the carboxyl-labelled acid gave an active lignin but that the vanillin produced from it was inactive, thus suggesting that the aromatic ring of the acid was incorporated as such into the lignin. Kratzl & Billek (1959) found that the [3-¹⁴C]-labelled acid, when infused into lignifying spruce tissue, gave an active lignin but that the vanillin produced from it was of very low activity. This appears to be a case of co-polymerization. However, Brown, Wright & Neish (1959) found that the wheat plant could synthesize guaiacyl and syringyl radicals from this precursor.

Artificial lignification of *Elodea* sections in solutions containing peroxide and eugenol has been demonstrated by Siegel (1954). In a system with added peroxidase a lignin was deposited on a filter-paper matrix (Siegel, 1956). The lignin formed was similar to natural lignin in ultimate composition (Siegel, 1957). Higuchi (1957), experimenting with

bamboo-shoot and horseradish enzyme preparations, observed the formation of a lignin-like substance from old, but not from freshly distilled, eugenol. He concluded that the lignin-like substance was formed from coniferaldehyde in the eugenol. On the other hand, Stafford (1960) found that a lignin-like substance was deposited in *Phleum* leaf sections even by freshly distilled eugenol. This 'lignin', although giving a positive phloroglucinol test, differed from the natural lignin or that formed from ferulic acid in not being extractable with 2% sodium hydroxide.

Artificial lignification of oat (*Avena*) coleoptiles, *Elodea* internodes and other tissues in the same medium as used by Siegel in his experiments was studied by Wardrop & Davies (1959) mainly from the morphological point of view, and they concluded from electron micrographs and staining reactions of the lignifying tissue that the artificial lignin was deposited between the cellulose microfibrils in the wall in the same way as natural lignin.

These morphological studies leave unanswered the question of whether the lignin deposited is identical with natural lignin. It has been pointed out by Freudenberg (1958) that no eugenol exists in cambial sap and that formation of artificial lignin from this substance has nothing to do with natural lignification. On the other hand, just as an organic substance may be synthesized by different routes, it is conceivable that a living plant could utilize a number of unnatural precursors to lay down a lignin indistinguishable from its natural lignin. For

eugenol this is not such a surprising proposition in view of the possibility of the first step being transformation into coniferyl alcohol or coniferaldehyde, or both, as suggested by Adler (1957).

The present work was undertaken primarily to determine if eugenol lignin could be distinguished chemically from natural lignin, and if so in what way it differed.

EXPERIMENTAL

Analysis and purification of eugenol. Wardrop & Davies (1959) used the phloroglucinol test for lignin in their experiments with coleoptiles. In the present work the eugenol (W. J. Bush and Co., Mentone, Victoria, Australia) was tested for phloroglucinol reaction before and after purification by washing with 20% (w/v) NaHSO_3 solution, followed by water-washing and distillation. Quantitative analyses of coniferaldehyde and coniferyl alcohol present in the original and purified eugenol were made by u.v.-absorption measurements as follows. The absorption curves of both the stock and purified eugenol in the range 240–400 μm were determined by using a 0.005% solution in methanol and in methanolic KOH (0.1N), and the $\Delta\epsilon$ curves derived. $\Delta\epsilon$ was close to zero in the range 320–400 μm . More concentrated solutions (approx. 0.1%) were then taken and $\Delta\epsilon$ was measured at 320 and 400 μm . The percentages of coniferaldehyde and coniferyl alcohol were then calculated with $\Delta\epsilon_{400\mu\text{m}}$ 32 000 for coniferaldehyde and $\Delta\epsilon_{320\mu\text{m}}$ 10 000 for coniferyl alcohol, these values being taken from the curves of Aulin-Erdtman (1953, 1957). Purified eugenol was used throughout the experiments described below.

Preparation of artificially lignified tissues. Lignified oatcoleptile sections were prepared as described by Wardrop & Davies (1959) with the buffered eugenol solution described by Solberg & Higginbotham (1957), i.e. 4 m-moles of eugenol, 15 ml. of ethanol, 4 m-moles of H_2O_2 and 5 m-moles of KH_2PO_4 in 1 l. of water. In this way it was possible to prepare artificially lignified tissue in small quantities that were sufficient to permit chemical investigation on a limited scale only. The preparation with coleoptiles was extremely tedious and was discarded in favour of potato parenchyma, which could be prepared on a large scale.

Artificially lignified potato parenchyma was prepared as follows. Potatoes (9 kg.) were carefully peeled and all traces of shoots removed. Slices were treated in a Waring Blendor and the resulting pulp was allowed to stand in water (20 l.) until the starch settled to the bottom, when the suspension of pulp was poured off the starch. At this stage addition of eugenol solution to a test portion of the suspension resulted in strong yellowing of the solution presumably caused by the presence of dissolved peroxidase. After four washings, addition of eugenol solution to a test portion caused a barely perceptible yellowing of the solution. The suspended parenchyma was filtered on a Buchner filter and pressed as dry as possible.

The filter cake was broken up and stirred into 20 l. of the eugenol solution described above. After a few minutes the cellular material became a bright yellow but this faded to a dull brown within an hour. After several hours the supernatant liquor was poured off and the treatment repeated, a total of six treatments being given over a week. The preparation was then washed with water (20 l.) four times by decantation and then eight times with warm (50°) ethanol

(5 l.). It was then sucked dry on a Buchner filter, washed with water and spread out to air-dry. This yielded 336 g. of material containing 15% of moisture.

Determination of lignin in artificially lignified tissues. Klason lignin was determined by adapting the procedure of Ritter, Seborg & Mitchell (1932). The tissue was stirred for 2 hr. with 72% (w/w) H_2SO_4 , diluted to 3% (w/v) and boiled for 2 hr. The insoluble residue was collected by filtration on a Norton alundum crucible R.A. 360, washed, dried, weighed and analysed for methoxyl groups and nitrogen. The lignin percentage was corrected on a basis of 16% of methoxyl groups.

An estimate of the lignin present was also made by a u.v.-absorption method. The tissue (about 2 mg.) was placed in a flask containing 10 ml. of 80% (w/w) H_3PO_4 together with ten Teflon balls ($\frac{1}{4}$ in. diam.); the whole was then shaken for 16 hr. at ambient temperature with sufficient vigour to keep the balls in motion but not to cause frothing. Under these conditions the tissue was swollen and dispersed so that unligified tissue gave a low extinction between 240 and 400 μm , but lignified tissue gave a lignin spectrum (Fig. 1). Lignin was determined by measuring extinction at 280 μm , correcting for absorption and scattering of the unligified tissue, and calculating lignin content on the basis of ϵ 3000 for a unit molecular weight of 180.

Isolation of lignin from artificially lignified parenchyma.

(i) An attempt was made to isolate lignin by the Urban (1926) procedure from the eugenol-treated potato parenchyma as follows. Lignified parenchyma (5 g.) was placed in a reagent bottle with 300 ml. of a mixture of conc. HCl (1350 ml.) and 90% (w/w) H_3PO_4 (400 ml.). The bottle was half filled with glass beads and rotated at about 20° for 6 days. The beads were removed by pouring the mixture through a Buchner funnel without a paper, and the lignin was recovered by repeated washing by centrifuging and drying *in vacuo* at 45°.

(ii) Isolation of the lignin by fine grinding in toluene as in the preparation of milled wood lignin according to Björkman (1956) was also attempted. Owing to the small yield obtained, purification was not carried further than the initial precipitation into water from acetic acid solution. The i.r.-absorption spectrum of the eugenol lignin isolated was determined in an NaCl disk by the method of Higgins (1957).

(iii) Methanol-soluble lignin was obtained by heating 50 g. of the lignified parenchyma in 400 ml. of methanol at 150° for 16 hr. The filtered methanol extract was concentrated to 20 ml. and added dropwise to cold (–5°) ether. The precipitated lignin was collected by filtration and dried *in vacuo*.

(iv) A Klason lignin was prepared as in the determination described above from the lignified parenchyma after methanol extraction.

(v) An alkali-soluble lignin was recovered by boiling a specimen of the lignified parenchyma in 0.5N-NaOH for 1 hr. and acidifying the filtrate. This gave a gelatinous precipitate, which was hydrolysed with 3% (w/v) H_2SO_4 to leave a lignin residue.

(vi) An ethanol lignin was recovered by refluxing 5 g. of the parenchyma in 50 ml. of 3.0% (w/v) HCl in ethanol for 48 hr. After precipitation of the lignin in water, the ether-soluble oils were recovered from the filtrate and examined by the paper-chromatographic method of Kratzl & Schweers (1954).

Nitrobenzene oxidation. Specimens of the lignified parenchyma (0.3 g.) were oxidized with nitrobenzene (0.2 ml.) in 2N-NaOH (7 ml.) for 3 hr. at 160° in stainless-steel bombs. Parallel experiments were done with eugenol, dehydrodieugenol and *Pinus radiata* wood. The vanillin produced was determined by separation on borate-impregnated paper by the method of Bland & Stamp (1955). The insoluble residue left after acidification of the oxidation mixture was extracted with ether, and the ether-soluble matter separated by two-dimensional chromatography on borate-impregnated paper.

Table 1. *Impurities in eugenol before and after purification*

	Coniferaldehyde (%)	Coniferyl alcohol + isoeugenol (%)
Eugenol (stock)	0.18	1.0
Purified eugenol	0.004	0.7

Fig. 1. Ultraviolet-absorption curves of oat-coleoptile tissue in phosphoric acid. 1, Eugenol lignified; 2, eugenol lignified, ethanol-extracted; 3, untreated, ethanol-extracted.

Estimation of p-hydroxybenzyl alcohol groups. These were tested for by the *p*-quinonemonochloroimide method of Gierer (1954).

RESULTS

The phloroglucinol-hydrochloric acid colour test was positive for the stock eugenol but negative for the purified material. The results of the analyses given in Table 1 confirm that it contained a significant quantity of coniferaldehyde but that the purification process reduced this to a negligible amount. The artificially lignified tissues stained strongly with phloroglucinol-hydrochloric acid reagent. The u.v.-absorption spectrum of a dispersion of lignified oat-coleoptile tissue in phosphoric acid is shown in Fig. 1.

Determination of lignin by the Klason method on both the oat coleoptiles and the potato parenchyma gave lignin percentages of over 12% in both cases, but Table 2 shows that the so-called lignins contained nitrogen and that the methoxyl-group percentage was low in each case. The corrected Klason lignin value is based on a methoxyl-group content of 16.0% for uncontaminated lignin. The lignin values arrived at by u.v. absorption agree better with the corrected lignin figures than with the original yields of Klason lignin for both tissues.

From the corrected Klason lignin value it may be calculated that the potato parenchyma contained artificial lignin corresponding to 39% of the eugenol added. This shows that the lignin was formed from the eugenol and not only from the coniferaldehyde impurity; however, coniferaldehyde could be an intermediate.

The yield and composition of the isolated lignins are shown in Table 3. They all contained nitrogen.

Table 2. *'Lignin' content of eugenol-treated tissues*

Material	Klason lignin yield (%)	Composition of Klason lignin		Klason lignin yield (% corr. on a basis of 16.0% of OMe)	Lignin by u.v. absorption (%)
		N (%)	OMe (%)		
Oat coleoptiles I	14.0	3.88	8.36	7.32	8.5
II	12.0	—	—	—	5.5
Potato parenchyma I	12.7	2.92	12.27	9.71	7.7
II	12.6	1.66	13.79	10.88	—

Table 3. *Yield and composition of isolated potato (eugenol) lignins*

Lignin	Yield as % of parenchyma	C (%)	H (%)	O (%)	N (%)	Total CHON (%)	OMe (%)	OMe* (% corr.)
Urban	15.4	53.54	6.33	34.7	2.22	96.79	7.99	9.28
Klason (after methanol)	8.7	62.04	6.09	25.7	3.84	97.67	11.55	15.20
Ball-mill	0.6	61.66	6.65	28.8	1.64	98.75	11.12	12.39
Alkali	1.1	—	—	—	3.54	—	11.78	15.13
Ethanolysis	0.9	65.47	5.94	25.2	0.86	97.47	13.04	13.78

* Corrected for presence of protein, see text.

The low carbon and methoxyl-group content of the Urban lignin suggest that it is heavily contaminated with carbohydrate material. The yield indicates that it does in fact contain all the lignin but the excessive carbohydrate contamination renders it valueless for chemical investigation. The methanol lignin was obtained in low yield and the ash and nitrogen contents were so high that it also was of no value. The result of the Klason determination after the methanol treatment shows that a large part of the lignin was resistant to the methanol treatment and that it had not been dissolved or degraded to ether-soluble oils.

The ball-mill lignin, recovered by grinding in toluene and extracting with dioxan according to Björkman (1956), shows high carbon, relatively high methoxyl-group content and low ash and nitrogen. These results point to its being a fairly

pure lignin but the yield as shown in Table 3 was very low. The i.r.-absorption spectrum of this lignin is shown in Fig. 2 together with those of eugenol and lignin obtained from milled wood of *Pinus radiata*. That of the eugenol lignin clearly resembles that of the *P. radiata* lignin.

Lignin isolated by ethanolysis had the lowest nitrogen content but the yield was also low. Table 3 shows that the total alkoxy-group content of this lignin, corrected on the assumption that the contaminant is protein containing 16% of nitrogen, does not indicate that any considerable ethylation had taken place, although α -ethoxypropionyl-guaiacone was present in the ether-soluble oil.

The results of the nitrobenzene oxidation of the artificially lignified potato parenchyma together with those of parallel experiments on *P. radiata* wood, eugenol (I) and dehydrodieugenol (II) are

Fig. 2. Infrared-absorption spectra of: 1, eugenol; 2, milled potato-parenchyma eugenol lignin; 3, milled *P. radiata* wood lignin.

Table 4. Products obtained from artificially lignified potato parenchyma, *Pinus radiata* wood, eugenol and dehydrodieugenol oxidized with nitrobenzene

	Lignified potato (% of corrected Klason lignin)	<i>P. radiata</i> (% of Klason lignin)	Eugenol	Dehydrodieugenol
Vanillin	5.2	19.9	87	0.0
	5.4	20.2	—	—
Dehydrodivanillin	1.1	0.9	0.0	63
	0.7	0.7	—	—

Fig. 3. (I), Eugenol; (II), dehydrodieugenol; (III), dehydrodivanillin; (IV), vanillin; (V), *p*-hydroxybenzyl alcohol type group of gymnosperm lignin; (VI), arylcarbinol group of gymnosperm lignin. Arrows show approximate conversions obtained on nitrobenzene oxidation in the present experiments.

shown in Table 4 and Fig. 3. The yield of vanillin (IV) based on the corrected percentage of eugenol lignin in the potato was about one-fourth of that obtained from *P. radiata* lignin in the same way in both the present and earlier experiments (Bland, 1958). The high yield from eugenol and the zero yield from dehydrodieugenol are in agreement with the results of Pew (1955). The high yield of dehydrodivanillin (III) from dehydrodieugenol also confirms the result of Pew.

The paper-chromatographic examination of ether-soluble material present in the residue obtained after nitrobenzene oxidation disclosed some material (visible in u.v.) at or near the starting line, when the upper phase of *isopropyl ether*-methanol-water (1:1:1, by vol.) was used as solvent. Irrigation in the second direction with methanol-water (1:1, v/v) showed the presence of two materials, one at R_f about 0.7 and one at R_f 0.8. The former corresponded in u.v.-absorption spectrum and R_f with dehydrodivanillin. A sufficient quantity of the compound with higher R_f was isolated by means of thick paper chromatograms to demonstrate that it contained nitrogen and no methoxyl group. This has been identified as *p*-phenylazobenzoic acid and was not derived from the lignin (Bland & Szilagyi, 1960).

The yields of dehydrodivanillin shown in Table 4 suggest that there are about the same number of

diphenyl groups in the eugenol lignin as in *P. radiata* lignin.

The artificially lignified potato parenchyma gave no colour reaction with the *p*-quinonemonochloroimide. This indicates that *p*-hydroxybenzyl alcohol groups are absent.

DISCUSSION

The colour reaction with phloroglucinol, the insolubility in 72% (w/w) sulphuric acid, the infrared and ultraviolet spectra, as well as the ultimate composition of the 'lignin' formed by potato parenchyma from eugenol in the presence of peroxide, all indicate that the eugenol 'lignin' is similar to gymnosperm lignin. On the other hand, its failure to give any colour reaction with *p*-quinonemonochloroimide shows that it contains none of the *p*-hydroxybenzyl alcohol groups (V), which are characteristic of natural gymnosperm lignin (Gierer, 1954). Similarly, the fact that the total alkoxyl-group content of the ethanol lignin did not indicate appreciable ethylation points to the absence of arylcarbinol groups (VI) and their ethers, which are probably the principal groups of natural lignin that can be alkylated (Adler, 1958). The nitrobenzene oxidation experiments of Wacek & Kratzl (1947) and of Kratzl, Hoyes & Silbernagel (1960) with reference compounds show that the low yield of vanillin is in agreement with the

absence of arylcarbinol groups and may be taken to indicate a large number of C-C bonds between the phenylpropane units of the artificial lignin. In view of the readiness with which eugenol forms dehydrodieugenol (II) it might be suspected that a large number of diphenyl links would occur in eugenol lignin, but the low yield of dehydrodivanillin (III) shows that this is not the case. Despite similarities of the eugenol lignin to gymnosperm lignin it does differ from natural lignin in the very important aspects mentioned above and certainly cannot be accepted as a true lignin. This should emphasize the danger of drawing conclusions about natural lignification from experiments in artificial systems.

SUMMARY

1. Artificial lignin has been produced on oat-coleoptile segments and potato parenchyma from purified eugenol in buffered aqueous solution containing hydrogen peroxide.

2. The colour reaction of the eugenol lignin with phloroglucinol, its behaviour in the 72 % (w/w) sulphuric acid lignin determination and its ultimate composition were all similar to these characteristics of gymnosperm lignin. Its ultraviolet- and infrared-absorption spectra were similar to those of gymnosperm lignin.

3. Unlike natural lignin, the eugenol lignin has no *p*-hydroxybenzyl alcohol or other arylcarbinol groups that can be ethylated.

4. On oxidation with nitrobenzene the eugenol lignin gave only a low yield of vanillin (5 %). The yield of dehydrodivanillin was about the same as from lignin.

5. It is concluded that eugenol lignin is a lignin-like substance, but that it differs from natural lignin in important respects. Conclusions about natural lignin and lignification drawn from experiments with eugenol lignin must therefore be made with the utmost caution.

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Biosynthesis of Griseofulvin: the Methylated Benzophenone Intermediates

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Consideration of structural evidence led Birch & Donovan (1953) to suggest that the skeletons of many natural phenolic compounds were derived, at least in part, by the head-to-tail linkage of acetic acid units. Two types of ring closure were dis-

tinguished: an aldol type, leading notably to orcinol derivatives, and a *C*-acylation type, leading principally to phloroglucinol derivatives. It was suggested that griseofulvin arose from the head-to-tail linkage of seven acetic acid units and sub-