COMPARISON OF LIGNIN-LIKE POLYMERS PRODUCED PEROXIDATIVELY BY CINNAMIC ACID DERIVATIVES IN LEAF SECTIONS OF PHLEUM [1, 2]

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Using the quantitative techniques for lignin determination described previously for herbaceous grasses (14), the study of the production of lignin-like polymers by peroxidation of phenolic compounds such as eugenol and ferulic acid has been extended to include *p*-hydroxycinnamic and sinapic acids and coniferyl alcohol. All of these substrates give slightly different products, some of them more similar than others to the lignin isolated from mature timothy grass, *Phleum pratense* L. This lignin has been characterized by its solubility in 0.5 *N* NaOH, by an ionization difference spectrum with relatively high peaks in the 350 mμ region and smaller ones at 250 mμ and 300 mμ, a negative minimum in the 280 mμ region, and by the presence of hydroxybenzylalcohol groups (14).

These results support the general scheme (fig 5) of immediate precursors of the *p*-hydroxyl phenyl, guaiacyl, and sinapyl moieties of lignins in plants as elucidated by several groups of workers (3, 6, 10), but illustrate possible basic differences between the formation of the more highly polymerized lignin of woody plants and the smaller more soluble polymers typical of herbaceous plants.

MATERIALS AND METHODS

Substrates and material not used in the preceding paper (14) are described below. The *p*-hydroxycinnamic acid (K and K Labs.) had an m.p. of 212 to 214° C, and presumably this and all the other cinnamic acid derivatives are in the trans form. The sinapic acid was from two sources; one was kindly supplied by Dr. S. Masri of the Western Regional Research Labs. at Albany, Cal. (m.p. 186–192° C); the other was purchased from the K and K Labs. (m.p. of 186–189° C). Coniferin, the source of coniferyl alcohol, was either a sample kindly supplied by Dr. O. Goldschmid of Rayonier, Inc. (m.p. 185–188° C) or a sample from A. H. Kahlbaum, Berlin (m.p. 186–188° C). The enzyme β-glucosidase was purchased from Worthington Co., cinnamic acid (m.p 132–133° C, from Paragon Labs. and L-tyrosine from Nutritional Biochemicals Corp.

The ionization difference spectra (1) of these phenolic compounds are shown in figure 1. None of these substrates gave the typical blue color similar to that of the standard guaiacol in the 2,6-dichloroquinonechlorimide test; coniferin, coniferyl alcohol, and *p*-hydroxycinnamic acid gave negative reactions; ferulic acid produced an unstable red color, and sinapic acid formed a red color which turned to purple. Gierer has reported that the C_3 chain para to the free phenolic hydroxyl group must be split off to produce the blue azo dye, and that *p*-hydroxybenzylalcohol groups are responsible for the blue color produced by lignin preparations (7).

Coniferyl alcohol was prepared from coniferin by β-glucosidase activity and was used either directly with the enzyme still present or after ether extraction. About 36 mg of coniferin and 36 mg of β-glucosidase were incubated in 10 ml of 0.05 M KH_2PO_4 solution at pH 4.5 for 24 hours at room temperature under anaerobic conditions. This mixture was extracted with 60 ml of ether in 10 ml portions. The ether was evaporated; the residue, taken up in ethyl alcohol just before use, was analyzed quantitatively, using the data of Aulin-Erdtman (1). About 80 to 90 % of the expected coniferyl alcohol was recovered.

Green laminae (300–400 mg fresh wt) of *Phleum pratense* var. climax were cut into 0.3 to 0.5 cm long sections and floated for 24 hours on the surface of a solution containing 0.05 M KH_2PO_4 at pH 4.5 with 0.01 M phenolic substrate and 0.01 or 0.05 M H_2O_2 in a total volume of 10 ml. No detectable difference was observed between experiments using the different concentrations of H_2O_2. Controls were made with added substrate and buffer, and with buffer only. A few experiments were performed at pH 7 with phosphate buffer.

Details of the extraction techniques and the quantitative analyses are described in the preceding paper (14). After exhaustive extraction with ether and then with water, the residues were extracted overnight with 0.5 *N* NaOH at 70° C to solubilize the lignin (2). Ionization difference spectra of the alkaline extracts (1, 8) were made by subtracting the ultraviolet absorption at neutral pH (0.05 M phosphate buffer) from that at an alkaline pH (0.05 *N* NaOH); the peaks at 250 and 300 mμ indicate the presence of simple phenolic compounds, while peaks at longer wave lengths are dependent upon the presence of double bonds of the 3-carbon sidechain in conjugation with the benzene nucleus. Estimates of the free phenolic content (*p*-hydroxybenzylalcohol groups) (7) were made by means of the colorimetric 2,6-dichlorquinonechlorimide (N, 2,6-trichloro-*p*-benzequineimine) test, and the phenol values were converted to lignin values by multiplying the mg of phenol by the factor 32, derived from data on native lignin

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preparations of bagasse (14). Analyses were always made within 1 to 3 hours after the extraction was completed since some of the products were unstable after about 24 hours under alkaline conditions.

Experimental Results

Incubation With *p*-Hydroxycinnamic Acid: When *p*-hydroxycinnamic acid and H_2O_2 were added to sections of laminae of Phleum at pH 4.5, there was very little macroscopic evidence of any reaction, except for a pale brown color along the edges of the sections in the mesophyll and vascular tissue, and a slight cloudiness in the medium. When the fresh sections were viewed microscopically, brown globules could be seen in the mesophyll cells. There was only a questionable increase in the phloroglucinol histochemical test in the vascular tissue and in a pale orange-brown color in the mesophyll in the Cl_2-Na_2SO_3 test. A striking characteristic of the polymer produced from this substrate was the absence of any increase in free phenolic groups reacting in the quinonechlorimide test, a lack which was reflected in the great change in the uv/phenol ratio (table I). The difference spectrum was characterized by a negative minimum in the 280 mμ region and a high maximum at 345 mμ (fig 2), in addition to the 250 and 300 mμ peaks.

Incubation With Ferulic Acid: This reaction has been described previously (14); further data are supplied here to indicate the variability (table I and fig 2). Besides the appearance of a red color in the veins during the incubation period, pink and brown globules could be seen microscopically in many of the cells. These globules did not stain in the phloroglucinol test, and there was sometimes a slight increase in the intensity of the red color of the Cl_2-Na_2SO_3 test in the walls of the vascular tissue. Since the red color in this test is unstable and turns brown, it is

Table I

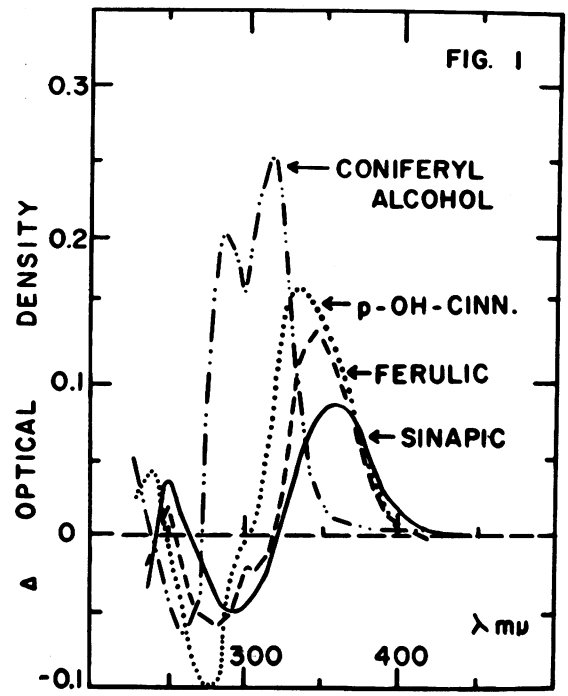
Effect of Phenolic Substrates on Alkali-Soluble Lignin Content of Phleum

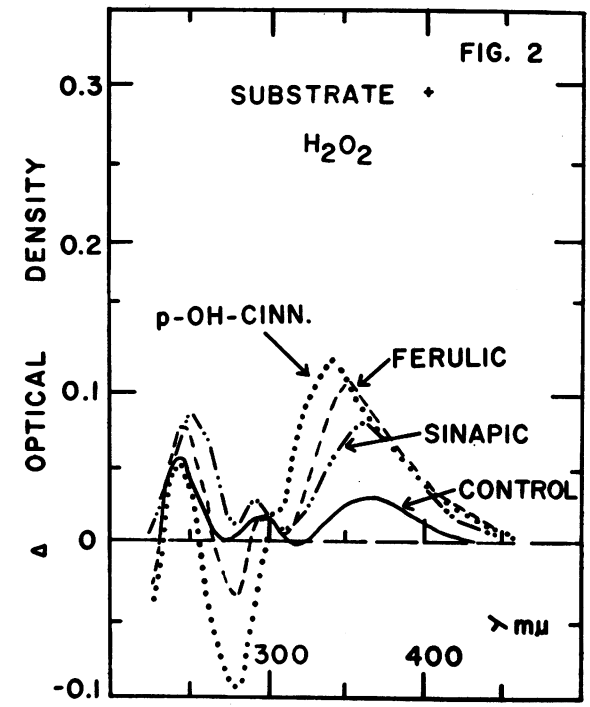
Substrate	Lignin % original dry wt	uv*/phenol	ΔD/mg @ 300 mμ	ΔD/mg @ 350 mμ	Residue % original dry wt
Buffer controls					
Avg 10	11	0.43	0.21	0.38	17
Range	(8–13)	(0.3–0.5)	(0.18–0.23)	(0.27–0.45)	(12–19)
p-OH-cinnamic	11	0.39	...**	0.22	26
"	10	0.43	...	0.29	20
" + H_2O_2	10	1.4	...	1.2	31
" "	12	1.2	...	1.5	26
" "	8	1.8	...	1.2	22
Ferulic + H_2O_2	43	0.40	...**	2.1	24
" "	30	0.41	...	1.4	22
" "	29	0.43	...	1.4	22
" "	14	0.68	...	1.1	23
" "	18	0.61	...	1.8	18
Sinapic	11	0.38	0.29	0.35	19
" + H_2O_2	11	0.48	0.20	0.41	11
" "	17	0.41	0.21	0.81	19
" "	15	0.43	0.22	0.78	19
Coniferyl alcohol	14	0.38	0.31	0.43	13
" "	20	0.38	0.36	0.78	20
" " + H_2O_2	17	0.37	0.47	0.41	...
" " "	46	0.41	1.4	1.1	30
" " "	21	0.30	0.39	0.56	23
" " "	21	0.38	0.50	0.62	19
Coniferin + glucosidase + H_2O_2 ***	13	0.55	0.45	0.45	16
	12	0.49	0.40	0.44	...

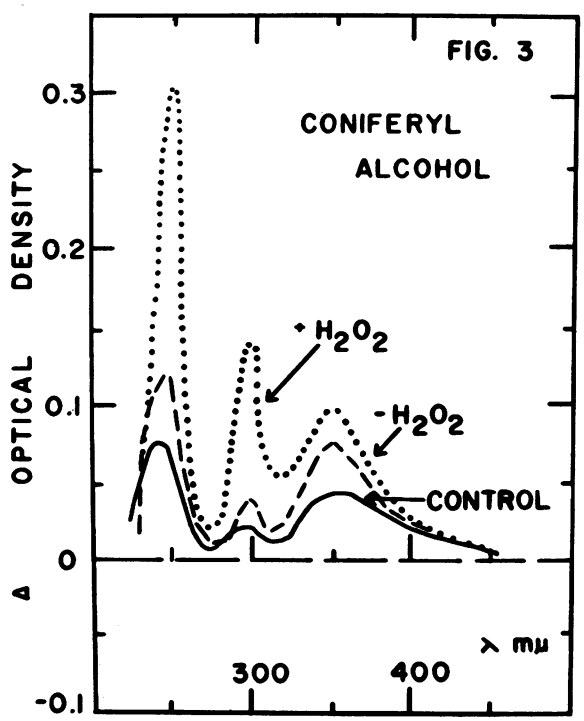
* Total O.D. @ 290 mμ in 0.05 *N* NaOH/total μg of phenol calculated as guaiacol.

** Not estimated for these cinnamic acid derivatives because of the interference of the negative minimum at 280 mμ.

*** Added after preincubation with glucosidase.







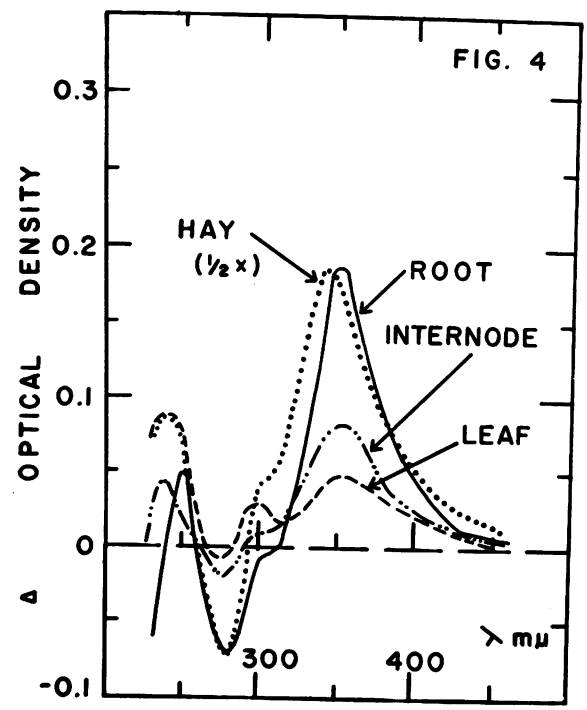


FIG. 1. Difference spectra of phenolic substrates. The Beckman cuvette contained 3.7 μg/ml of coniferyl alcohol (dashed-dotted line) or 1.5 μg/ml of *p*-hydroxycinnamic (dotted line), ferulic (dashed line), or sinapic acid (solid line).

FIG. 2. Difference spectra of extracts of leaf tissues incubated with 0.01 M *p*-hydroxycinnamic, ferulic, or sinapic acid in the presence of 0.01 M H_2O_2 at pH 4.5. Each ml of the solution in the cuvette contained an aliquot of a lignin extract equivalent to 100 μg dry weight of the original tissue.

very difficult to distinguish between the red of the syringyl-positive test and the orange-brown of the guaiacyl-positive test which likewise fades to a brown color. The major peak in the difference spectrum is generally at about 350 to 355 mμ, in between those of tissues incubated with *p*-hydroxycinnamic and sinapic acids (fig 2).

While the incubating medium with added ferulic acid at pH 4.5 remained clear and colorless during the 24 hour incubation period, the same medium at pH 7 slowly turned to a bright yellow-brown color. The difference spectrum of the two media showed no striking variations, both being similar to that of the initial substrate alone except for a slight increase in the region between 380 to 450 mμ. In contrast to the coniferyl alcohol experiment at the higher pH value, the yield of alkali-soluble product was significantly less (about half) at the higher pH.

INCUBATION WITH SINAPIC ACID: The macroscopically visible red color noted in the veins of the ferulic acid-fed-leaves was even more apparent in the case of the sinapic acid-fed-leaves. This color was also evident during the later stages of incubation with sinapic acid in the absence of added H_2O_2, but only in the internal part of the sections and never along the edges. Pink and brown globules could be observed microscopically. The incubation medium became slightly yellow and the sections showed considerable chlorophyll demage in contrast to the control sections. There was no significant increase in the phloroglucinol test, but the Cl_2-Na_2SO_3 test yielded a strong red color in the veins and an orange color in the mesophyll cells. The first wash with water removed a large proportion of the brown color of the dried sections, the orange-brown solution turning brown upon standing at room temperature. The spectrum of this aqueous solution is discussed below. The water and ether washed residue was a rich chocolate brown; this color was removed by alkaline treatment. The difference spectrum of this alkaline extract was characterized by peaks at about 250, 300, and 355 mμ and a complete absence of any negative minimum in the region of 280 mμ (fig 2).

INCUBATION WITH CONIFERYL ALCOHOL: When coniferyl alcohol and H_2O_2 were incubated with sections of laminae, there was little visual evidence of a reaction, but there was a large increase in materials reacting with phloroglucinol both in the vascular tissue as well as in the mesophyll. In the early part of the reaction, globules which stained a bright red with phloroglucinol were observed in the mesophyll cells (5). During the incubation a definite cloudiness occurred in the medium, although this was probably less than in the case of the eugenol-treated sections (14).

The feeding of coniferin for 24 hours followed by another 24 hour incubation period with added H_2O_2 produced only a small increase in the phloroglucinol-positive materials, while the addition of an external source of glucosidase greatly enhanced this reaction. These results would imply that the tissue was weak in glucosidase activity, or that the coniferin does not penetrate the cells.

In table I, data are shown for extracts of tissues pre-incubated with coniferin and β-glucosidase and then with H_2O_2, and for extracts from experiments in which coniferyl alcohol, derived from the coniferin, was added directly to the tissues in the presence of H_2O_2. The relatively high values even in the absence of added substrate and H_2O_2 should be noted; the highest set of values should be compared with the highest ones obtained in the presence of added substrate and H_2O_2 since the tissues used for incubation were selected from the same plants. As indicated by the percentage of lignin values, the product showed an increase in the free phenolic groups comparable to that obtained with ferulic acid. This alkali-soluble product from coniferyl alcohol and H_2O_2, however, differed from that obtained with ferulic acid in that there was a greater increase in the 250 and 300 mμ region of the difference spectrum than at 350 mμ (fig 3). Furthermore, a significant amount of phloroglucinol-positive polymer was insoluble in the alkaline extraction technique, a result similar to that found with eugenol (14).

When the incubation was carried out at pH 7 instead of pH 4.5 there was no significant qualitative or quantitative difference in the analysis of the alkali-soluble product. There were fundamental differences, however, in the incubation medium, with the production of more precipitate and an intense yellow color at the higher pH. The difference spectrum of this precipitate was characterized by large peaks at 250 and 300 mμ and a definite shoulder at about 320 mμ. The spectrum of the supernatant showed a major peak at 300 mμ, secondary ones at 250 and 320 mμ, and a low but definite peak at about 400 mμ. This indicates a definite change from the original spectrum of coniferyl alcohol with peaks at about 230, 290, and 320 mμ (fig 1). The medium at pH 4.5 showed a difference spectrum approximately intermediate between that of coniferyl alcohol and the pH 7 medium. Siegel (11)

FIG. 3. Difference spectra of extracts of leaf tissue incubated with 0.01 M coniferyl alcohol with (dotted line) and without (dashed line) H_2O_2 and the control with buffer alone (solid line). Each ml of solution in the cuvette contained an aliquot of lignin extract equivalent to 100 μg dry weight of the original tissue.

FIG. 4. Difference spectra of extracts of different organs of Phleum. Each ml of solution in the cuvette contained an aliquot of lignin extract equivalent to 100 μg of the original dry weight of the hay, or 200 μg of the original dry weight of young roots, first internodes, and leaves. The lignin content, expressed in percent of the original dry weight and based on the quinonechlorimide test, was 16 % for hay, 14 % for the roots, 4 % for the first internode, and 6 % for the green leaves.

reported a smaller yield and an intensification of a yellow coloration in the medium with eugenol at pH values higher than 4.5. Freudenberg (4) indicated that there are major differences in the type of dimeric precursor and the subsequent polymer produced at pH 7 and pH 5.5; he generally prepared his synthetic lignin or "DHP" at the higher pH value.

INACTIVE SUBSTRATES: When L-tyrosine and cinnamic acid were used as the substrates in these incubation experiments, no detectable lignin products were obtained. Since the radioactive tracer work of Brown and Neish (3) demonstrated that these are precursors of lignins in grasses, these negative results may indicate that the conversion of these compounds to *p*-hydroxycinnamic acid is not rapid enough, that the enzymatic reactions involved are inhibited by the high concentration of H_2O_2, or that the substrates are being metabolized to a non-lignin product.

WATER SOLUBLE PRODUCTS: When sinapic acid was incubated with laminae, a large proportion of the product was soluble in water; this result led to further examination of these extracts (table II). The difference spectrum showed a large peak in the 260 mμ region with a slightly smaller one at 380 mμ. Extracts from control experiments showed small, irregular peaks at 250 mμ and 300 mμ with a slightly higher peak in the 380 mμ region. When extracts of ferulic acid-fed-leaves were examined, some of these showed a definite increase in the 380 mμ region; this increase in a water-soluble component was generally associated with lower values for the 350 mμ peak in the alkaline extracts. Extracts of coniferyl alcohol-fed-leaves showed slight increases in the 250 and 300 mμ peaks, but no significant change in the 380 mμ region. The ether extracted materials have not been analyzed.

THE 350 MΜ PEAK: Since the high peak at approximately 350 mμ in the difference spectrum is characteristic of the lignin of mature timothy, the difference spectra of extracts from various organs of the growing grass plant were examined.

While young and old laminae had a less pronounced peak at 350 mμ, the internodes and especially the roots of young Phleum seedlings (2–3 weeks old) exhibited the pronounced absorption at 350 mμ found in mature shoots or hay (fig 4). In all cases, the high peak at 350 mμ was associated with a pronounced minimum at 280 mμ, an increase in the phenol-reacting groups in the quinonechlorimide test, and tissue having the strongest phloroglucinol and Cl_2-Na_2SO_3 tests.

A definite but variable amount of the product producing the absorption in the difference spectra at regions greater than 320 mμ could be precipitated with acid as described in the method of Bondi and Meyer (2). The difference spectrum of the precipitate had peaks which were broader and were shifted toward the longer wave lengths up to 400 mμ, and the phenolic content was lower and the uv/phenol ratio was higher than that of the original extract. The product of the reaction with coniferyl alcohol and H_2O_2 was the most easily precipitated with acid.

When a hay sample was exhaustively extracted with ethyl alcohol prior to the alkaline extraction, there was no significant difference in the subsequent analyses of this alcohol-extracted residue from the non-alcohol extracted material. The alcohol extracts, on the other hand, showed no indication of any lignin since the major peak of the difference spectrum was at 410 mμ with a smaller one at 270 mμ. The spectrum remained unchanged even if this alcohol extract was heated at 70° C under alkaline conditions.

DISCUSSION

The characteristics of the lignin-like polymers produced by peroxidation of five closely related phenolic compounds (fig 5) are summarized in table III, and are compared with those of the lignin isolated from timothy hay. On the basis of these characteristics, ferulic acid produces the most natural lignin, but the product reacts weakly in the histochemical phloroglucinol test. Coniferyl alcohol, on the other hand, is very effective in increasing the histochemical phloroglucinol reaction in both mesophyll and vascular tissue, but a major portion of this product is not soluble in 0.5 *N* NaOH, and therefore, may be considered an unnatural form of lignin in timothy. Similar results were obtained previously with eugenol (14).

The discrepancy between the results obtained with ferulic acid and coniferyl alcohol is of considerable interest, because the former substrate is converted to its alcohol (i.e., coniferyl alcohol) before it is oxi-

TABLE II

WATER SOLUBLE PRODUCTS OF PEROXIDATIC ACTION ON PHENOLIC SUBSTRATES

	H_2O SOLUBLE	ALKALI SOLUBLE
	ΔD/MG @ 380–390 MΜ	ΔD/MG @ 350 MΜ
Buffer	0.23	0.34
p-Hydroxycinnamic acid + H_2O_2	0.19	1.2
Ferulic acid + H_2O_2	0.38	1.1
" " "	0.14	1.8
Sinapic acid + H_2O_2	0.42	0.78
" " "	0.54	0.81

TABLE III

COMPARISON OF MAJOR CHARACTERISTICS OF LIGNIN-LIKE POLYMERS PRODUCED PEROXIDATIVELY AT pH 4.5 BY GREEN LEAVES AND THE LIGNIN OF HAY

	SUBSTRATES FED TO GREEN LEAVES					HAY
	p-OH-CINNAMIC	FERULIC	SINAPIC	CONIFERYL ALCOHOL	EUGENOL	
Color reaction in veins						
Visible red	...	++	++++	...	...	...
Phloroglucinol test	?	+	?	++++	++++	++*
Cl_2-Na_2SO_3 test**	...	?	+++	...	...	++
H_2O Extract						
Δ D @ 380 mμ	...	++	++++	...	...	( )***
Alkaline extract						
Δ D @ 250 mμ	++	++	++	++++	+++	++
Δ D @ 300 mμ	++	++	++	++++	+++	++
Δ D @ 350 mμ	++++	++++	+++	++	+	++++
Δ D @ 270 mμ†	+++	+++	...	...	...	++++
Quinonechlorimide test	...	++++	++	++++	++	++++
Alkali insoluble residue						
Phloroglucinol test	...	...	...	+++?	++++	...

* As compared with gymnosperm wood (++++)
** Red color
*** H_2O extract very dark brown; not analyzed
† Negative minimum

datively polymerized according to the scheme elucidated by Brown and Neish (3) and Freudenberg (6) (fig 5). The major differences in the products of these two substrates are in the amount of the phloroglucinol-reacting substances, the amount of material absorbing in the region above 310 to 320 mμ in the difference spectrum, and the solubility of the polymer produced. These variations may reflect a basic difference between the lignins of gymnosperm wood and the herbaceous grasses, for although coniferyl alcohol yields some measurable amount of alkali-soluble product in Phleum, its difference spectrum is closer to that found in the enzymatically produced dehydrierungspolymerizat (DHP) and to that of lignin extracts of gymnosperm woods (5). Likewise, the solubility characteristics of the phloroglucinol-reacting material are closer to those expected of woody gymnosperm tissues.

Several interpretations of these experimental results can be considered. First, some of the ferulic acid may be converted to sinapic acid before polymerization via sinapyl alcohol, producing a more soluble polymer, such as the one described by Freudenberg (5, 6). Second, ferulic acid may be converted into lignanes (4) or directly into a more soluble ferulic acid lignin in the case of the Phleum tissue. Freudenberg reports that such a direct conversion is not the case at least for some of the lignin produced in gymnosperm tissue (4). Third, the polymer formed may differ with the concentration of free coniferyl alcohol at any one time, the concentration being initially higher in the case of the added coniferyl alcohol than with added ferulic acid. Again, Freudenberg reports differences in the characteristics of DHP depending on whether the substrate is added all at once or drop by drop (4).

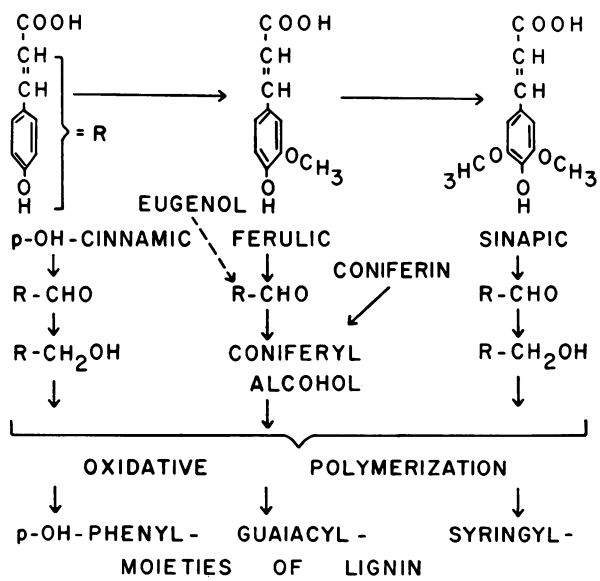


FIG. 5. Summary of the postulated pathway of lignin biosynthesis as elucidated by several groups of workers (3, 6, 10).

Another basic problem is the significance of the difference spectrum in the region of 350 mμ. Such high peaks at wave lengths greater than 310 mμ are not typical of woody plants, although small peaks are sometimes evident (8, 14). Similar peaks, however, have been found in other grasses, i.e., in native lignin preparations from bagasse and wheat (13, 14). Absorption above 310 mμ could be due to the presence of double bonds in the side chains in conjugation with free phenolic nuclei, indicating less highly polymerized molecules or ester linkages (12). Preliminary work on lignins precipitated by acid indicates that the materials absorbing in this portion of the spectrum are complex. If any of this absorption at 350 mμ is due to a contaminating non-cell wall material, this contaminant is insoluble in alcohol, ether, and water. The degree of alteration of the original lignin polymer during the alkaline extraction is not known.

Besides the problem of the non-physiological conditions necessary to produce these lignin-like polymers (14), and the lignification in some cases of non-vascular tissue, further difficulties in the interpretation of these results may be due to confusion concerning the nature of lignin, our knowledge of which is based largely on data from the highly polymerized lignins of woody tissues. Beyond the characterization of lignin as a constituent of cell walls composed of polymers of phenylpropane units with some phloroglucinol-reacting groups, there may be taxonomic differences besides the variation in the amount of the three basic phenylpropane units (9). Furthermore, the various histochemical stains used to identify lignin, such as safranin, phloroglucinol, and Cl_2-Na_2SO_3, do not always agree when tracheids and sclerenchyma, and when tissues produced by primary and by secondary growth, are compared (15). More work is necessary with lignins from sources other than woods to bridge the gap between our morphological and chemical information concerning this aromatic polymer.

Summary

A series of cinnamic acid derivatives and related compounds postulated as lignin precursors were incubated with sections of the lamina of *Phleum pratense* (timothy grass) in the presence of H_2O_2 at pH 4.5. Cinnamic acid and tyrosine were inactive, while *p*-hydroxycinnamic, ferulic, and sinapic acids, and coniferyl alcohol produced lignin-like polymers, presumably by peroxidative polymerization. The actual products, however, differed in their reaction to histochemical tests for lignin, in their solubility in water and in 0.5 *N* NaOH, in the free phenolic groups detected by the quinonechlorimide test, and in their ionization difference spectra. When compared with the lignin extracted from mature timothy shoots or hay, ferulic acid produced the most natural product. Although these results support the general scheme implicating these compounds as immediate precursors of the three different moieties found in grass lignins, the variations in the polymers formed may reflect basic differences in the solubility and polymerization of lignins from woody plants and from herbaceous grasses.

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