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DIFFERENCES BETWEEN LIGNIN-LIKE POLYMERS FORMED BY PEROXIDATION OF EUGENOL AND FERULIC ACID IN LEAF SECTIONS OF PHLEUM [1]

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Coniferyl alcohol, eugenol, and ferulic acid can be converted to lignin-like polymers in the presence of H_2O_2 via a peroxidase-catalyzed oxidation. This reaction can be brought about by tissue sections or cell-free extracts of woody and herbaceous plants (6, 10, 14). Furthermore, Neish and his coworkers (5) have shown that ferulic acid-βC^{14} is an efficient precursor of the guaiacyl moiety of lignin of wheat, presumably by conversion to coniferyl alcohol.

Since the position of eugenol in the scheme of lignin biosynthesis has been questioned by Higuchi (10), the lignin-like polymers produced by peroxidation in the presence of eugenol and ferulic acid have been restudied by means of different analytical techniques. The quantitative method for lignin determinations used in the previous studies (10, 14) is based on the weight of the residue insoluble in 72 % H_2SO_4, a technique originally devised for woody tissues. In order to obtain more reliable data for young, herbaceous tissues, determinations used in the present investigation are based on extraction of the lignin in 2 % NaOH (4), followed by an estimation of the free phenolic groups (7), and determination of the ultraviolet difference spectra of the lignin extracts (1, 2, 8). Timothy grass, *Phleum pratense*, was chosen as the test material because it is easy to grow and to study anatomically and biochemically, and mature shoots have been reported to contain as much as 30 % of their dry weight as lignin (13). Results were obtained which show that there are both qualitative and quantitative differences in products from eugenol and ferulic acid.

MATERIALS AND METHODS

Tissue samples were dried first in an oven at 70° C and then in a vacuum desiccator over $CaCl_2$ and H_2SO_4. Dry weight values were either determined directly or were calculated from comparable samples. Lignin was determined as follows: after moistening with distilled water, the samples, containing 30 to 40 mg dry weight, were ground in a mortar with ether until all the chlorophyll was removed, and then were thoroughly extracted with distilled water. The residue was extracted for about 16 hours in 2 to 3 ml of 0.5 N NaOH at about 70° C. This technique is based on the method of Bondi and Meyer (4), who consider the extraction to be quantitative for young annuals. The supernatant and washes of the centrifuged residue were combined, neutralized to about pH 8.5 to 9, and were analyzed within 3 hours for their phenolic and ultraviolet absorbing contents. Some of these components were unstable after about 24 hours under these alkaline conditions. Re-extraction of the residue yielded no significant amount of ultraviolet absorbing compounds. The residue was dried, weighed and tested for materials reacting with phloroglucinol and with Cl_2-Na_2SO_3 (9, 10, 14).

Ultraviolet absorption spectra were determined on aliquots, one diluted with 0.05 N NaOH and the other with 0.05 M phosphate buffer at pH 7, the difference spectrum being obtained by subtraction (1, 2). Optical density readings were made in a Beckman spectrophotometer at intervals of 5 to 10 mμ from 230 to 450 mμ.

Phenol analyses were made by a modification of the method of Gierer for native lignin preparations (7). Suitable aliquots (containing 1 to 3 μg phenol) of the extract in 0.55 ml of distilled water were added to 0.4 ml of 0.5 M tris (hydroxymethyl) aminomethane buffer at pH 9.0 and 0.05 ml of a freshly prepared

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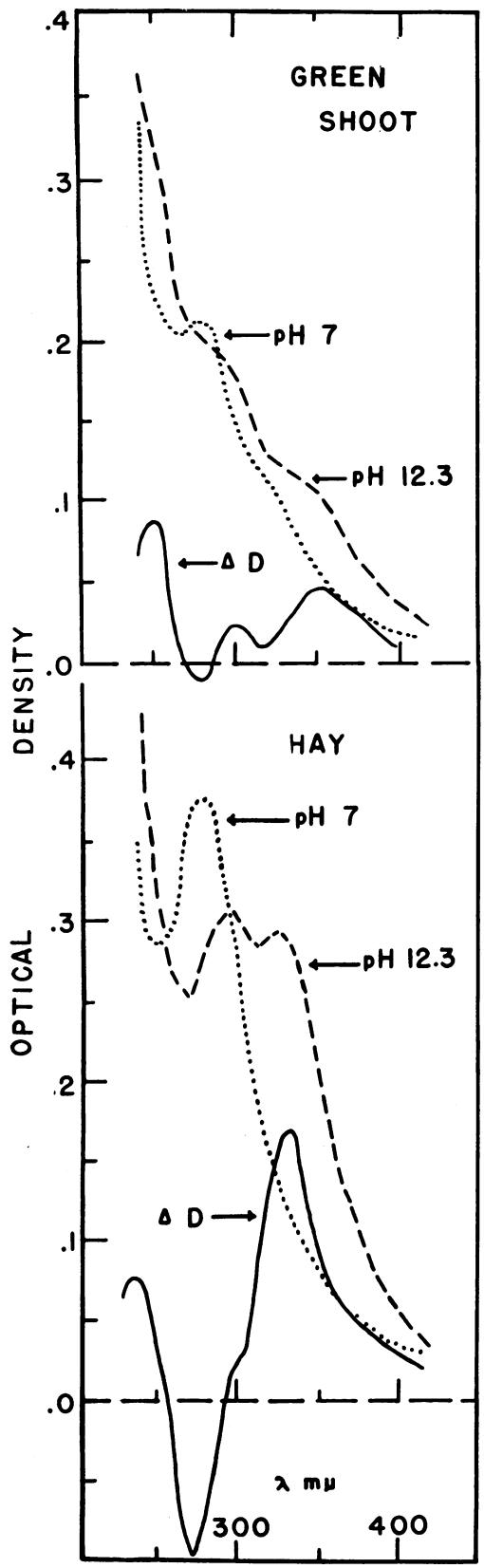


alcoholic solution containing 25 μg of 2,6-dichloroquinonechlorimide (Matheson, Coleman and Bell). After 1 hour at room temperature, optical density readings were made at 610 mμ with a Beckman spectrophotometer, using guaiacol as a standard. Samples of native lignin preparations of birch and bagasse were kindly supplied by Dr. F. F. Nord of Fordham University, and were used as standards for converting the guaiacol values to mg of lignin. A conversion factor of 32 (32 × mg phenol = mg lignin) was calculated from data on the bagasse sample; a similar factor was obtained from birch lignin when it was corrected for impurities (64 % purity according to Nord). Chemical data and ultraviolet absorption spectra for these preparations may be found in papers from Nord's laboratory (16). Klason lignin analyses on hay preparations based on the residue insoluble in 72 % H_2SO_4 were made according to Hägglund (9, p. 326), followed by hot water washes of the final lignin residue.

For the feeding experiments, 3 to 4 laminae (15–25 cm long) from 1 to 3-month-old shoots (20-30 cm high) weighing 300 to 400 mg fresh weight were cut into 5 mm sections. These were floated in dishes on the surface of 10 ml of medium containing 0.05 M KH_2PO_4 at pH 4.5 with 10^{-2} M H_2O_2 and 10^{-2} M eugenol or ferulic acid. Controls are described below. The ferulic acid was dissolved first in 1 ml of 95 % ethyl alcohol, but some recrystallization generally occurred after addition of the buffer to a final volume of 10 ml. Only laminae which were unfolded and reflexed at the point of the ligule attachment were used. After an incubation period of 24 hours at room temperature with occasional stirring, the sections were washed with distilled water and with 95 % alcohol, dried in the oven at 70° C and then in a vacuum desiccator before being extracted as described above. Histochemical tests were made on fresh and lactic acid cleared sections, using the phloroglucinol-HCl and Cl_2-Na_2SO_3 tests (9, 10, 14).

The seeds of *Phleum pratense* variety climax were kindly supplied by M. A. Hein of the U.S.D.A., Beltsville, Maryland. The timothy hay was from an unknown field-grown source. The green plants were grown in the greenhouse with supplementary light to

FIG. 1 (*upper*). Absorption spectra of a crude lignin preparation from young green shoots of *Phleum pratense* under neutral and alkaline conditions and the difference spectrum (Δ D). Each ml of solution in the cuvette contained an aliquot of a lignin extract equivalent to 190 μg dry weight of green shoots, diluted either with 0.05 M phosphate buffer at pH 7 or with 0.05 M NaOH at pH 12.3. Blank cuvettes contained the buffer only.

FIG. 2 (*lower*). Absorption spectra of a crude lignin preparation from hay of *Phleum pratense* under neutral and alkaline conditions and the difference spectrum. Each ml of solution in the cuvette contained an aliquot of a lignin extract equivalent to 111 μg of dried hay diluted in buffer as in fig 1.

give a total day-length of about 16 hours. None of the green plants used had flowered.

The eugenol (Eastman Kodak Co.) was used directly (i.e., aged) or was distilled under vacuum and the almost colorless fraction boiling at 120° C at 10 mm Hg pressure was used immediately. The ferulic acid (Aldrich Chemical Co.) had a m.p. of 170 to 171° C, and was, therefore, presumably the trans form.

Results

Comparison of Lignin Extracted from Young Green Shoots and from Mature Shoots (Hay): The ultraviolet absorption spectra at pH 7 (phosphate) and pH 12.3 (NaOH), and the difference spectrum are shown in figures 1 and 2 for young green shoots (about 30 cm high and consisting of six leaves) and for mature grass (hay). If only the two neutral curves are compared, little difference would be detected between the extracts except in the sharper and larger maximum at 280 mμ in the case of the hay extract. While the alkaline absorption curves show further variations, the difference spectra, in which the non-ionizable chromophores are eliminated, are much more descriptive. Both curves show peaks or shoulders at 250, 300 and about 350 mμ, but the absolute and relative heights vary greatly. Hay extracts are characterized especially by the large minimum in the region of 270 to 280 mμ which is negative in value, and a large maximum in the region of 350 mμ. Non-conjugated phenols will give the 250 and 300 peaks, while phenols with large conjugated side chains such as the hydroxycinnamic acid derivatives will account for peaks at wave lengths greater than 300 mμ (1, 2, 8). On the basis of these difference spectra, lignin extracts from hay differ from those of green shoots not only in having a greater amount of simple phenolic groups, but by the amount of conjugated side chains as indicated by the major increase in the 350 peak.

Difference spectra made under comparable conditions are shown in figure 3 for the native lignin preparation (see also 16) to illustrate variations in the absorption in the 350 mμ region.

Data comparing the free phenolic groups of these preparations are shown in table I along with values for the difference spectrum (Δ D) in the general region of 340 to 360 mμ ("350" peak). The ratio between the ultraviolet absorption and the phenol content is an arbitrary but useful clue to the presence of contaminating or interfering substances in the preparations analyzed. This ratio is merely the total optical density at 290 mμ at alkaline pH divided by the total μg of phenol calculated as guaiacol. Ratios for extracts of Phleum generally vary from 0.4 to 0.8. Lignin values expressed as the % dry weight for hay from an average of three Klason analyses (72 % H_2SO_4) are shown for comparison along with values obtained from the literature.

Feeding of Eugenol and Ferulic Acid to Green Laminae: Experiments were devised to compare the lignin-like polymers produced by eugenol and by ferulic acid under conditions comparable to those used by Siegel (14) on pea, bean, and Elodea internodes. Laminae were used because of their ease in handling; the first internodes gave similar results. Difference spectra obtained from an ultraviolet analysis of freshly distilled eugenol and ferulic acid are shown in figure 4. Similar spectra of extracts of tissue sections incubated for 24 hours with these substrates in the presence of H_2O_2 are shown in figures 5 and 6. Phenol and lignin values from these three experiments, expressed in terms of the dry weight of the tissue before incubation, are listed in table II.

The eugenol reaction is a very complicated one. Higuchi (10) reported that he did not obtain any lignin-like product with carefully distilled eugenol, but only with aged preparations giving a strong phloroglucinol test. The aged eugenol used here was bright yellow in color and gave a very dark red-purple color with phloroglucinol-HCl. The redistilled fraction was essentially colorless and gave only a very faint pink with phloroglucinol-HCl after standing about 5 minutes with the reagent. The difference spectra, however, were similar except for a small peak in the region of 400 mμ (about 3-4 % of the height of the 300 mμ peak) in the aged preparation. The undistilled residue is dark brown and gives a strong phloroglucinol test. After approximately one week at room temperature, the distilled fraction had already become definitely yellow. Higuchi detected the presence of coniferyl aldehyde in aged eugenol preparations, but presented no quantitative data. The small absorption peak at 400 mμ in the aged preparations would be expected if coniferyl aldehyde were present (2), but it represents only a small percent of the aged eugenol preparation. If the major product of aging

Table I

Comparison of Lignin Values in Extracts of Green Shoots and Hay of Phleum, and Birch and Bagasse Native Lignins

	Lignin		UV/phenol	ΔD/mg @ 350 mμ*
	μg phenol per mg	% dry wt		
Bagasse	31	...	0.58	12.5
Birch	36	...	0.63	3.0
Green shoot	1.8	6**	0.58	0.24
Hay	5.2	17**	0.56	1.9
		16***		

Literature citations: Lignin — % dry wt

Green shoots	8–11***	(13)
Green shoots	3–6***	(12)
Green shoots	6****	(11)
Hay–cut green and dried	30***	(13)
Hay–dried intact	8***	(12)
Hay–dried intact	9****	(11)

* Peak between 340–360 mμ
** Based on phenol
*** Based on 72 % H_2SO_4
**** Based on fuming HCl

is a polymer, however, the spectral characteristics due to the conjugated side chain could be blocked.

Both eugenol preparations gave qualitatively similar but quantitatively different results. Both showed a complicated reaction with the product appearing in three general areas; 1), in the external medium which became milky within 3 to 5 minutes after addition of the green tissue sections; 2), in the water and ether insoluble portion of the tissue which is soluble in 2 % NaOH; and 3), in the water and ether insoluble portion of the tissue which is insoluble in 2 % NaOH.

The difference spectrum of the product precipitated in the external medium is shown in figure 4; no attempt was made to quantitate this product. It could be interpreted as a mixture of products similar to those of a Dehydrierungspolymerizat (DHP) of Freudenberg as analyzed by Aulin-Erdtman (2). Both Higuchi and Siegel (10, 14) indicate the production of a similar cloudiness.

There was a small alkali-soluble product in both the aged and freshly distilled preparations, the difference spectra of the former being about two times that of the latter in the 250 and 300 mμ regions. This alkali-soluble product, however, shows a greater increase in the 300 mμ region than at 350 mμ; in fact, if the difference spectrum of the control is subtracted from that of the experimental one with eugenol and H_2O_2, this derived Δ–Δ spectrum is somewhat similar to the spectrum of the product that appears in the surrounding medium. More experiments would be necessary to determine the possible significance of the slightly higher phenol content of the distilled eugenol extract in comparison to that of the aged eugenol extract. The tissues incubated with eugenol alone did not show any significant increases over that of the controls.

A more dramatic difference appeared in the final residues, which contain mainly cellulose and hemicellulose. These same residues from either green laminae or hay showed no residual phloroglucinol or Cl_2-Na_2SO_3 tests. The residues from incubation mixtures with both the aged and distilled eugenol gave very dark positive phloroglucinol tests which would be difficult to distinguish quantitatively to any degree of accuracy, but the weight difference of the final residues was quite different, with a significant weight increase in the presence of the aged eugenol plus H_2O_2. The slight weight increase in the presence of eugenol alone is probably not significantly greater than that of the controls.

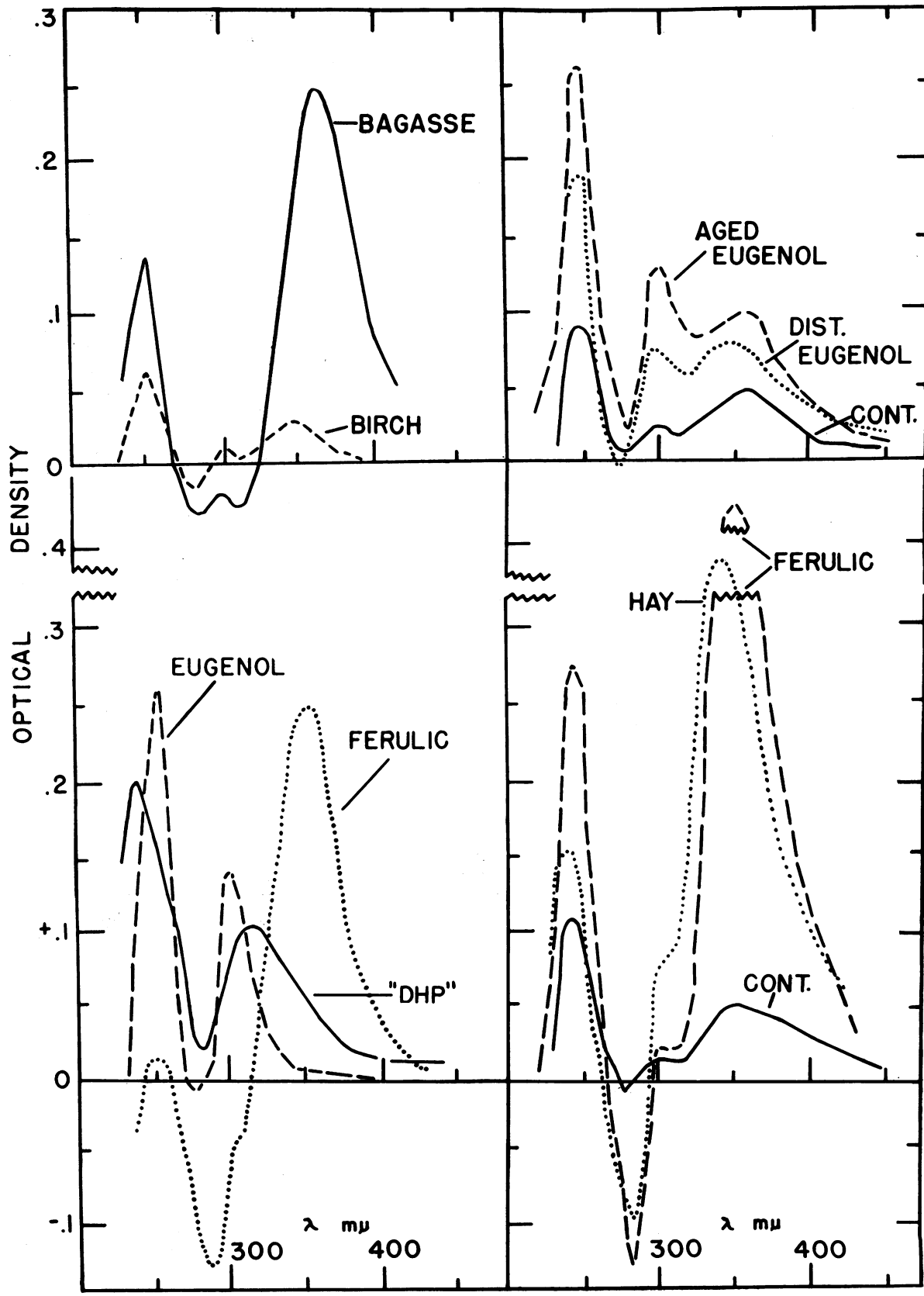
This reaction with eugenol and H_2O_2 was evident to the eye during the incubation period when the tissue sections were examined, with an initial bright yellow color in parts of the sections turning to brown as reported by Siegel (14). Histochemical examination of fresh sections showed a considerable increase in the phloroglucinol test, but unlike the findings of Siegel (14), not in the red color in the Cl_2-Na_2SO_3 test which is considered evidence of the syringyl nucleus (10). Furthermore, the increase in the phloroglucinol reaction was not confined to the vascular tissue, but was observed in the cell walls between veins, especially in the case of the aged eugenol. If the sections are first cleared with lactic acid, which removes some of the lignin, the greater increase in the vascular tissue over that of the non-vascular tissue could then be seen clearly.

The product of an incubation mixture containing ferulic acid and H_2O_2, on the other hand, was confined to the alkali-soluble fraction, and the difference spectrum of this extract mimics that found in untreated extracts of timothy hay (dotted curve of figure 6), with a large negative minimum in the 270 to

TABLE II

EFFECT OF PEROXIDASE ACTIVITY ON LIGNIN SYNTHESIS IN PRESENCE OF EUGENOL AND FERULIC ACID AND H_2O_2 PLUS SECTIONS OF LAMINAE OF PHLEUM PRATENSE

	LIGNIN		UV/ PHENOL	ΔD/MG @ 350 mμ	RESIDUE	
	μG PHENOL PER MG	% DRY WT			% DRY WT	PHLORO-GLUCINOL
Buffer only	1.9	6	0.5	0.29	13	...
Aged eugenol	2.0	6	0.5	0.23	22	?
Aged eugenol + 10^{-2} M H_2O_2	2.8	9	0.67	0.52	30	++++
Buffer only	1.7	6	0.75	0.25	17	...
Distilled eugenol	1.8	6	0.65	0.22	21	?
Distilled eugenol + 10^{-2} M H_2O_2	3.8	12	0.54	0.40	18	+++
Buffer only	2.5	8	0.43	0.28	20	...
Ferulic	3.0	10	0.44	0.35	25	...
Ferulic + 1×10^{-4} M H_2O_2	3.3	11	0.46	0.37	24	...
+ 1×10^{-3} M H_2O_2	3.1	10	0.56	0.55	29	...
+ 5×10^{-3} M H_2O_2	5.0	16	0.49	0.95	24	...
+ 1×10^{-2} M H_2O_2	13.5	43	0.40	2.1	25	...


.3
.2
.1
0
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DENSITY
BAGASSE
BIRCH
AGED
EUGENOL
DIST.
EUGENOL
CONT.
FERULIC
HAY
OPTICAL
.3
.2
+.1
0
-.1
EUGENOL
FERULIC
"DHP"
CONT.
λ mμ
300
400
λ mμ
300
400

280 mμ region and an increase in the maximum at 350 mμ. When alcohol was not added to the incubation mixture to solubilize the ferulic acid, a similar but slightly smaller product was obtained. Again, as with eugenol, the tissues incubated with ferulic acid alone produced little extra lignin. Until more is known about the composition of the broad peak in the 350 mμ region, the slight shift in the absolute position of this peak when compared with that of the hay extracts should not be unduly emphasized.

This increase in the absorption at 350 mμ is compared with the data obtained by the phenol test in table II, and the dependency of the reaction on high concentrations of H_2O_2 is shown by the series of extracts incubated with increasing amounts of H_2O_2. The increase in free phenolic groups at 10^{-2} M H_2O_2 was considerable, with little change in the uv/phenol ratio. If a correction is made for the increased weight of the tissues due to the new lignin, a value of about 31 % of the original dry weight of the tissue is obtained. Approximately 60 % of the original ferulic acid added was converted into this new alkali-soluble lignin. This compares with a figure of not more than 10 % for the eugenol.

The reaction with ferulic acid and H_2O_2 could be followed with time by the observation of a red color which appeared in the veins first along the cut edge of the sections and subsequently throughout the veins or in isolated patches along them. A brown discoloration could be detected in the tissue in between the veins. The red color of the veins turned to brown during the drying process, but when the alkali was added to the water and ether extracted residue, a transitory reddish-orange color appeared which turned to a yellow-brown. Hay extracts. on the other hand, showed an initial deep yellow color which likewise turned to a yellow-brown. When the veins were tested for lignin histochemically, a slight increase was observed of the red-purple color of the phloroglucinol test, and a more definite increase in the brown color in the Cl_2-Na_2SO_3 test typical of the guaiacyl nucleus.

Discussion

Studying the physiology of lignin formation has been hindered by lack of a suitable quantitative method and incomplete knowledge concerning the actual structure of lignin. The methods used in this study are more sensitive and supply more information than the Klason technique used by previous workers studying the peroxidase-catalyzed polymerization of phenolic compounds. Like all other methods, however, interference with contaminants is a definite problem. The major contaminants in the alkali extracts which contain mainly lignin and hemicelluloses are protein and flavanoids not removed in the water and ether extracts. Since probably they are present only in small amounts in these lignin extracts, it is felt that they do not account for any major fraction of the ultraviolet absorption measured. Tyrosine from protein would react in the phenol test if present in high concentrations, but it forms a red color rather than a blue one. Since ferulic acid in an unknown ester form is common in grass leaves (3), contamination with this in a crude lignin preparation is also a problem.

Aside from the peaks at 250 and 300 mμ in the difference spectra, which can be attributed to ionizable groups in simple phenols, the maxima in the general region of 350 mμ are probably composite curves representing a complex series of peaks due to different conjugated phenols. No attempt has been made to analyze these components (cf. 1, 2). Furthermore, quantitation of the 300 mμ peak is often difficult because of interference by the minimum at 280 mμ.

The purity of eugenol is a major problem, and the apparent instability of a freshly distilled sample makes it a difficult compound to use with long incubation periods and heterogeneous enzyme systems. The lignin-like products are apparently complex and the major reaction product is atypical for Phleum in the sense that it is insoluble in the alkali which usually removes all phloroglucinol reacting components. The polymerization product of coniferyl alcohol will be studied with these analytical techniques in order to assess the validity of the scheme proposed by Higuchi; i.e., that eugenol must first be converted to a product similar to coniferyl alcohol before being polymerized by peroxidase.

Ferulic acid, on the other hand, produced a much more abundant lignin-like polymer which was soluble in alkali and was similar in spectral qualities to the extract from untreated timothy hay. Under the present conditions, nearly four times as much alkali-

Fig. 3 (*upper left*). Difference spectra of native lignin preparations of birch and bagasse obtained under conditions comparable to those described for the Phleum preparations. The cuvette contained 10 μg per ml of the bagasse lignin and 8 μg per ml of the birch (the latter corrected for impurity).

Fig. 4 (*lower left*). Difference spectra of ferulic acid (3 μg/ml) (*dotted line*), freshly distilled eugenol (4.3 μg/ml) (*dashed line*), and the precipitate in the external medium in the presence of Phleum tissue, distilled eugenol, and H_2O_2 (DHP) (*solid line*).

Fig. 5 (*upper right*). Difference spectra of the controls with buffer only and with eugenol only (*solid line*), the experimental mixture with freshly distilled eugenol and H_2O_2 (*dotted line*) and the mixture with aged eugenol plus H_2O_2 (*dashed line*). Each ml of solution in the cuvette contained an aliquot of a lignin extract equivalent to 200 μg dry weight of the original tissue.

Fig. 6 (*lower right*). Difference spectra of tissues of Phleum incubated in buffer only (*solid line*), and with ferulic acid plus H_2O_2 (*dashed line*), and of the extract of hay (*dotted line*). Each ml of solution in the cuvette contained an aliquot of a lignin extract equivalent to 200 μg dry weight of the original tissue.

soluble lignin was produced from ferulic acid as from eugenol. The product from ferulic acid is not highly reactive with phloroglucinol, indicating a low proportion of free aldehyde groups (9). Unpublished results indicate that the rate of reaction with ferulic acid is dependent on time up to 16 to 20 hours of incubation, and does not occur in the presence of boiled tissues. The dependency of the reaction on H_2O_2 indicates that peroxidase is presumably the enzyme catalyzing these oxidations as indicated by previous investigators (6, 10, 14).

Although ferulic acid forms a product strikingly similar to that found in hay, the polymers formed in these experiments and in those of Siegel (14) and Higuchi (10) are subject to at least two interpretations, especially concerning the dependency upon such high concentrations of H_2O_2. Although it is difficult to determine the rate of peroxidase genesis with accuracy, data on peas obtained by Siegel and Galston (15) indicate that only about 10^{-4} M H_2O_2 would be produced over a 24 hour period by an amount of tissue comparable to those used in the present experiments. This is well below the concentration used in these phenol feeding experiments for a maximum response. One interpretation would be that the tissues have been subjected to non-physiological conditions both in regard to the rate of lignification and competition for the H_2O_2 by other enzyme systems. Another interpretation, however, is that the peroxidase of the tissue will form by means of a non-specific peroxidative action on a variety of phenolic compounds, a series of lignin-like polymers some of which are natural and some unnatural to the plants. Further work from a physiological point of view is necessary to determine which of these interpretations is the correct one. Until then, it is perhaps better to speak of the peroxidative production of "lignin" or of lignin-like polymers.

Summary

The lignin-like polymers produced by peroxidase activity of tissue sections in the presence of eugenol and ferulic acid have been restudied by means of improved analytical methods. These methods show qualitative and quantitative differences between the products of this oxidative polymerization. Lignin analyses were based on extracting the lignin in 2% NaOH followed by an estimate of the free phenolic groups by the quinonemonochlorimide method, and a study of the ultraviolet difference spectra of these crude lignin extracts. While freshly distilled eugenol still produced a lignin-like polymer in the presence of leaf sections of *Phleum pratense*, the amounts were small and a major product giving a positive phloroglucinol test was insoluble in alkali; in general, aged eugenol gave a larger reaction, especially in the alkali-insoluble form. Ferulic acid, on the other hand, produced a very large amount of a lignin-like polymer soluble in alkali similar to that found in mature timothy grass or hay. It is concluded that ferulic acid is much nearer to being a natural precursor of true lignin than is eugenol. The possible interpretations of these peroxidase catalyzed polymerizations are discussed.

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