22. MILLER, J. S. AND S. A. GORDON. 1962. Auxin relations in illuminated shoots. Plant Physiol. 37: xvi.
23. REINERT, J. 1959. Phototropism and phototaxis. Ann. Rev. Plant Physiol. 10: 441–58.
24. REISENER, H. J. 1958. Untersuchungen über den Phototropismus der Hafer-Koleoptile. Z. Botan. 46: 474–505.
25. STEWART, W. S. AND F. W. WENT. 1940. Light stability of auxin in Avena coleoptiles. Botan. Gaz. 101: 706–14.
26. THIMANN, K. V. AND G. M. CURRY. 1960. Phototropism and Phototaxis. In: Comparative Biochemistry, vol. 1. Florkin and Mason, ed. Academic Press, N.Y.
27. WENT, F. W. 1928. Wuchstoff und Wachstum. Rec. Trav. Botan. Néerl. 25: 1–116.
28. WENT, F. W. AND K. V. THIMANN. 1937. Phytohormones. Macmillan Co., New York.
29. WILDEN, M. 1939. Zur Analyse der positiven und negativen phototropischen Krümmungen. Planta 30: 286–89.
30. ZIMMERMAN, B. K. AND W. R. BRIGGS. 1963. A kinetic model for phototropic responses of oat coleoptiles. Plant Physiol. 38: 253–61.

Comparison of Lignin-Like Products Found Naturally or Induced in Tissues of Phleum, Elodea, and Coleus, and in a Paper Peroxidase System [1, 2]

Helen A. Stafford

Biology Department, Reed College, Portland, Oregon

While the biosynthetic pathway in a grass, wheat, appears to be similiar to that observed in woody materials (3, 12), certain peculiarities of grass lignins or lignin-like products need to be elucidated. One peculiarity is the relative insolubility in alkali of the lignin-like product of peroxidation of an exogenous source of coniferyl alcohol in contrast to that formed by ferulic acid or found naturally within a grass (19, 20). The other is the presence of ferulic and *p*-hydroxycinnamic acids, but not sinapic acid, after alkaline extraction and hydrolysis of cell wall residues containing lignin (21).

In order to explore these problems, comparisons were made of natural and induced lignin-like products in a system containing filter paper infiltrated with peroxidase (17) and in tissue sections of selected herbaceous plants. Tissues studied in detail were Phleum and Coleus, a highly lignified monocot and dicot respectively, and Elodea, an aquatic monocot without detectable lignin.

Materials and Methods

Plants used were generally greenhouse grown from seed or plants obtained commercially. Most of the work here and in preceding papers (19, 20, 21) was done with the hexaploid *Phleum pratense* L. var. climax. In addition, seeds of a tetraploid line of *Phleum pratense* were obtained from E. L. Nielsen. Other plants used were: *Triticum aestivum* L. (wheat), *Phyllostachys aurea* Riv. (bamboo), *Zea mays* L. (corn), *Avena sativa* L. (oats), *Saccharum officinarum* L. (bagasse or sugarcane), *Coleus blumei* Benth., *Pelargonium cultivar* (geranium), *Nicotiana tabacum* L. (tobacco), *Impatiens holstei* Engler and Warb. (balsam), *Helianthus annuus* L. (sunflower), *Tradescantia virginiana* L., *Elodea densa* Planch., *Lemna minor* L., *Apium graveolens* L. (celery). The native lignin preparation from bagasse was the same as used previously (19), but the preparation was hydrolyzed in 0.5 N NaOH as in the regular treatment.

Phenolic compounds were purchased commercially. Ferulic and *p*-hydroxycinnamic acids were recrystallized from ethanol by the addition of water. The eugenol was an aged preparation with a strong phloroglucinol test.

Histochemical tests on sections and biochemical analyses on water and ether extracted tissue (called cell wall residue) were described previously (19, 20, 21). One change was that the fluorometric analyses were done in 0.05 M tris buffer or in 0.05 M NaOH. The change in OD at 350 mμ is a measure of the peak in the ionization difference spectrum in the region near 350 mμ. For this determination, the OD at wave lengths between 340 and 360 mμ of a solution

[1] Received July 31, 1963.
[2] This research has been supported by the National Science Foundation G-15856.

in 0.05 M phosphate buffer at pH 7 was subtracted from that of a solution in 0.05 M NaOH (19). Each determination was made in a 1 ml volume in a cuvette with a light path of 1 cm and results are expressed as per milligram dry weight of the original tissue. Phenol refers to phenolic groups per milligram of original tissue reacting with the quinoneimine reagent to produce a blue color, calculated with guaiacol as a standard. Contrary to the previous report (21), ferulic acid does give a blue color after an initial red-purple one at intermediate concentrations of about 5 to 50 μg per milliliter of test solution.

Solvents used in chromatography were generally the benzene-acetic acid-water mixture followed by butanol-ammonium hydroxide-water as described previously (21), but a sodium formate-formic acid-water mixture (10 g: 1 ml: 200 ml) was substituted for the solvent in the second direction when it was necessary to separate ferulic and sinapic acids.

Incubation of excised tissues with phenolic substrates and H_2O_2 for 24 hours was done as before (20, 21), except that occasionally the incubating mixtures were shaken in a water bath at 25°. First internodes of Phleum were cut from seedlings grown on moist filter paper for 7 days in the dark at 25°. Incubation of excised first internodes with sucrose was for a 7 to 14 day period under sterile conditions. Seeds were initially sterilized with saturated solutions of calcium hypochlorite and Orthocide (California Spray-Chemical Corporation).

The filter paper-peroxidase system was a modification of that reported by Siegel (17). An aqueous solution containing 160 μg of a horse radish peroxidase preparation (Nutritional Biochemicals Corporation) was applied to a 100 mg disc of Whatman No. 1 filter paper and was dried in a stream of cool air. The disc was then cut into 0.3 to 0.5 cm square sections which were subsequently handled as regular sections of tissue, except that incubation was always done in a water bath with shaking at 25° and the alcohol treatment before extraction was omitted. It was impossible to separate some of the precipitate in the medium after incubation from the filter paper shreds. Most of this precipitate, however, was soluble in the ether treatment preceding the alkaline extraction of the lignin.

Products soluble in $NaHCO_3$ were isolated by stirring a cell wall residue in a 5 % solution of $NaHCO_3$ for 10 minutes at room temperature. After centrifugation, the supernatant fluid was decanted and the residue was washed twice each with 0.5 to 2.0 ml of 5 % $NaHCO_3$ and distilled water. All washings were combined with the original supernatant material, and analyses were made on this fraction before and after hydrolysis in 0.5 N NaOH. The residues containing the $NaHCO_3$ insoluble moiety were extracted as usual in 0.5 N NaOH and were analyzed with an untreated sample for comparison.

Acid precipitates were made by acidification of the alkaline extracts to pH 2 to 3 with H_2SO_4. After standing at 4° for 15 minutes, the mixture was centrifuged and the precipitate was washed with small amounts of cold water and then was redissolved in 0.025 N NaOH for the usual analyses. A lignin preparation, partially purified of associated hemicellulose, was prepared according to Bondi and Meyer (2) from mature internodes of Phleum.

Saponification of cell wall residues to detect ester linkages was done according to Brown and Higuchi (11). A sample containing 10 mg of dry cell wall residue from mature internodes of Phleum was refluxed in air for 1 hour in 2 ml of a KOH-ethanol solution (1 g of KOH dissolved in 60 ml of absolute ethanol). The residue was washed several times with

Table I

Natural Lignins of Herbaceous Monocots and Dicots

The values are the average of 2 or more samples from highly lignified areas of stems according to histochemical tests.

Stem tissue	△ OD/mg at 350 mμ*	Peak	Phenol**	Ferulic acid	*p*-hydroxycinnamic acid
			μg/mg dry wt of tissue		
Phleum (hexaploid)	1.4	345	8.1	4	4
Phleum (tetra)	0.8	340–345	4.2	3	3
Oats	1.9	340	7.0	5	8
Corn	1.5	340–345	3.6	1	4
Bagasse N.L. (19)	....	335–340	....	1	100
Wheat	1.2	340–345	5.2	3	6
Bamboo	2.0	340	5.1	1	11
Tradescantia	0.44	340	1.0	0.4	2
Tobacco	0.15	355	1.3	0***	0
Sunflower	0.11	355	1.0	?	0
Geranium	0.17	350	1.1	0	0.2
Coleus	0.15	355	0.84	0	0.4
Balsam	0.09	340	0.91	0	0.1

* ΔOD at the peak of the ionization difference spectrum in the region around 350 mμ per milligram dry weight of original tissue.

** Phenolic groups reacting in the quinoneimine test, calculated as guaiacol.

*** 0 = less than 0.05 μg per milligram.

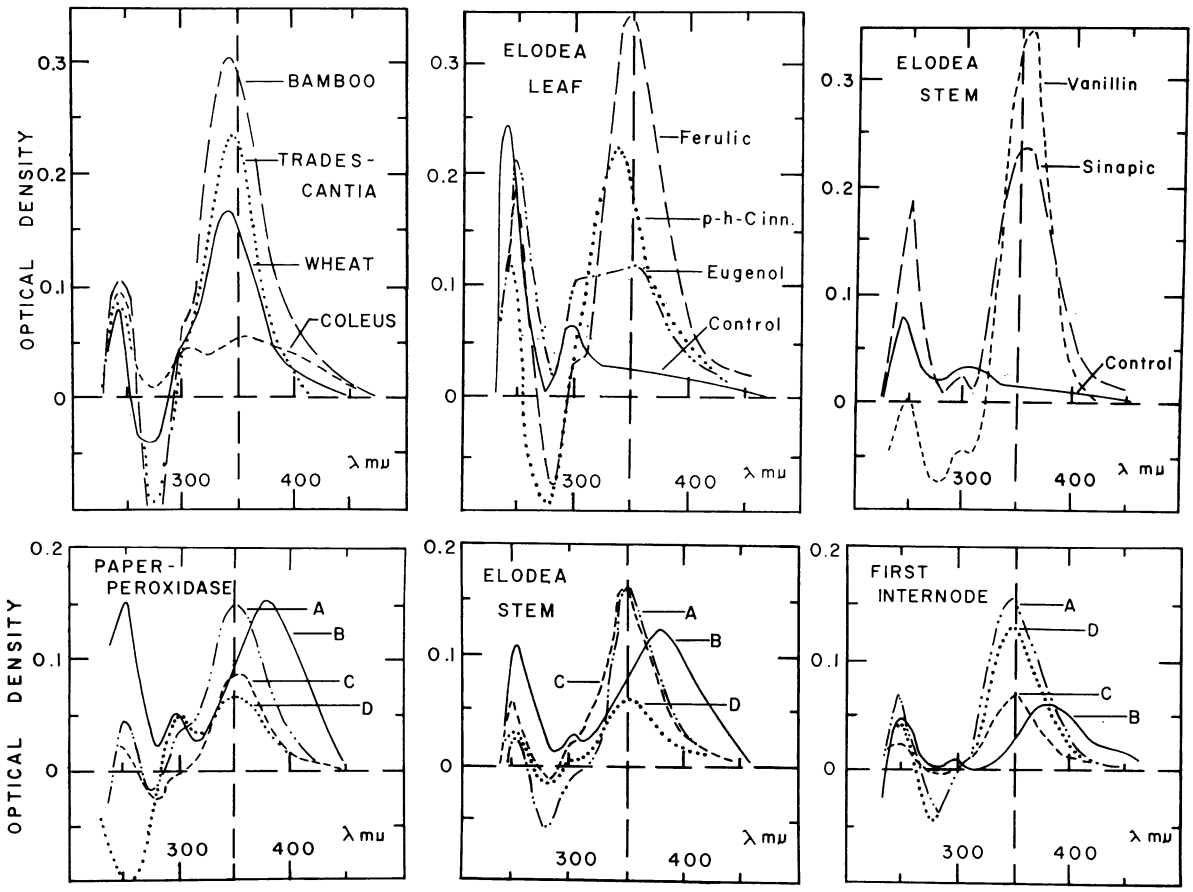


FIG. 1 (*upper left*). Difference spectra of lignin extracts from stems of monocots and dicots. Each milliliter of solution in the cuvette contained an aliquot of a lignin extract equivalent to 140 μg of tissue from bamboo (long dashes), 650 μg from Tradescantia (dotted line), 100 μg from wheat (solid line), and 470 μg from Coleus (short dashes). See table I for other data.

FIG. 2 (*upper middle*). Difference spectra of extracts of leaf sections of Elodea incubated in media described in table II. Each milliliter of solution in the cuvettes contained an aliquot of a lignin extract equivalent to the following dry weight of tissue before incubation: 125 μg when incubated with ferulic acid (long dashes), 143 μg with *p*-hydroxycinnamic acid (dotted line), 125 μg with eugenol (dash-dots), and 580 μg of a control with no added substrate.

FIG. 3 (*upper right*). Difference spectra of extracts of stem sections of Elodea incubated in media described in table II. Each milliliter of solution in the cuvettes contained an aliquot of a lignin extract equivalent to the following dry weights of tissue before incubation: 25 μg when incubated with vanillin (short dashes), 125 μg with sinapic acid (long dashes), and 280 μg from the control without added substrate.

FIG. 4, 5, 6. Difference spectra of extracts from systems incubated with ferulic acid as described in table II. Curve A (dash-dots) represents the total product soluble in NaOH; B (solid line) the $NaHCO_3$ soluble fraction before alkaline hydrolysis; C(short dashes) the same fraction after alkaline hydrolysis; D(dotted line) the $NaHCO_3$ insoluble but NaOH soluble fraction.

FIG. 4 (*lower left*). Paper-peroxidase system. Each milliliter of solution in the cuvettes contained an aliquot of a lignin extract equivalent to 750, 1700, 620, and 6000 μg of paper for A, B, C, and D, respectively. Phenol per milligram equals 1.16, 1.13, and 0.1 μg for A, C, and D, respectively.

FIG. 5 (*lower middle*). Sections of Elodea stem. Each milliliter of solution in the cuvettes contained an aliquot of a lignin extract equivalent to 63, 112, 109, and 125 μg of original tissue for A, B, C, and D, respectively. Phenol per milligram equals 5.0, 1.6, 1.2, and 3.9 μg for A, B, C, and D, respectively.

FIG. 6 (*lower right*). Sections of first internode of Phleum. Each milliliter of solution in the cuvettes contained an aliquot of a lignin extract equivalent to 134, 222, 200, and 134 μg for A, B, C, and D of original tissue respectively. Phenol per milligram equals 12.2, 6.4, and 2.7 μg for A, C, and D, respectively.

0.5 ml aliquots of the KOH-ethanol solution and the washings were added to the original supernatant. This mixture was chromatographed directly, or was evaporated, acidified, and extracted into ether first. The residue was further extracted with 0.5 N NaOH and analyzed by the regular technique.

Enzymic treatments of cell wall residues (10–15 mg dry weight of original tissues) were made with commercial preparations of enzymes. Incubation was for 24 to 48 hours at 30° in 2 ml of 0.05 M

KH_2PO_4 at pH 4.5. After incubation, the mixture was centrifuged and the supernatant fluid was decanted. The residue was reground in water and ether to remove any products made soluble by the enzymes. These water and ether soluble components were added to the original supernatant fluid and the mixture was acidified, extracted into ether, and chromatographed. The residue was extracted as usual in 0.5 N NaOH and analyzed with an untreated sample for comparison. Enzyme preparations used were 10 mg of β-glucosidase (Worthington Biochemical Corporation), 40 mg of hemicellulase or pectinase (Nutritional Biochemical Corporation), 10 mg of anthocyanase D (kindly supplied by Rohm and Haas Co.,). Pectinase and anthocyanase treatments were also made in 0.05 M phosphate buffer at pH 7. The pectinase contained trace amounts (about 1–2 μg 40 mg sample used) of a blue fluorescing compound similar to ferulic acid which were not removed by dialysis against 0.001 M phosphate buffer at pH 7 (14).

Results and Discussion

Natural Lignins. The natural lignin of Phleum was described previously (21). Additional monocot tissues, mainly grasses, have now been examined. All show strikingly similar ionization difference spectra with a high peak in the region of 350 mμ (table I, fig 1). While there were quantitative differences in the amounts of material producing this peak at 350 mμ and in the phenolic groups reacting with the quinoneimine reagent, the ratio of these 2 components was generally similar. Both free ferulic and *p*-hydroxycinnamic acids were found in the ether soluble fraction of all the grasses examined, but the relative amounts varied considerably. The absence of sinapic acid in the ferulic acid area of the chromatograms has been determined only in the case of tissues of Phleum.

All but the aquatic monocot Elodea contained materials reacting in the histochemical tests for lignin (phloroglucinol or Cl_2-Na_2SO_3 tests). Alkaline extracts of stem and leaf tissues of Elodea produced trace amounts of a blue color in the phenol test and no detectable *p*-hydroxycinnamic or ferulic acid in the acidified ether extracts. The difference spectrum showed no definite peak at wave lengths greater than 310 mμ (fig 2, 3). The peaks at 250 and 300 mμ are difficult to interpret since they could be due to the presence of nonlignin moieties in the cell wall residue such as tyrosine from the wall protein. Similar results were obtained with Lemna, another aquatic monocot.

The lignins of herbaceous dicots are also soluble in dilute NaOH (2). A few examples are shown in table I. Coleus exhibits a strong phloroglucinol reaction in its vascular tissue. The difference spectrum differed from that of grasses in having a relatively high peak at 250 mμ and lower ones at 300 and around 350 mμ (fig 1). The phenol values were also lower than that found in grasses. The phenol test with tissues of dicots was more satisfactory if the dye product was extracted into butanol before colorimetric determination to remove interfering dark reaction products. Traces of *p*-hydroxycinnamic acid were found in some of the ether extracts of these dicots. Since tissues such as the stems of geranium gave a very strong phloroglucinol reaction in the vascular bundles and in cylinders of sclerenchyma, the low values for 350 absorption and for phenolic content can not be interpreted as an indication of less lignin, but only of a type with functional groups not detected by these analytical tests.

Induced Lignins. Siegel has shown that lignin-like products can be formed in the presence of high concentrations of phenolic precursors and H_2O_2 in nonlignin containing systems such as tissues of Elodea stems or in a model system made up of filter paper impregnated with peroxidase (16, 17). These systems have been reinvestigated with different analytical methods along with tissues of Coleus and Phleum, and comparisons made of the lignin-like products of peroxidation of various cinnamic acid derivatives (table II, fig 2, 3).

Except for differences in solubility of the product in $NaHCO_3$ and in the production of phloroglucinol reacting groups, these lignins induced in several different systems are strikingly similar when analyzed by the techniques devised for grasses. As has been shown by others (9, 16), peroxidase is able to oxidize a variety of compounds, the products of which are known to polymerize. It is of particular interest that the 3 acids related to the alcohols postulated as the substrates normally polymerized in woody tissues (3, 6) produce products characteristic of the acid itself rather than of its aldehyde or alcohol. This is seen in the position of the long wave length peak in the difference spectrum, the relative increase in the phenol reaction, and the presence of the free acid and its C_6-C_1 degradation products in the acidified ether extract. In ferulic acid (and *p*-hydroxycinnamic acid in part), the product is very similar to that found naturally in Phleum and other grasses.

Although peroxidase was able to oxidize a variety of phenolic compounds, the products of which subsequently polymerize, not all are natural ones or produce products similar to those found in grasses. Eugenol is probably not a natural compound in grasses, and its product, as well as that of coniferyl alcohol, was not entirely similar to that found in a grass like Phleum (19, 20). In tissues of Elodea, both sinapic acid and vanillin were good substrates (table II, fig 3), but the products were partly atypical of lignins found in Phleum. In the case where sinapic acid was used as subtrate the most striking result was the presence of sinapic acid in the ether extracts, identified chromatographically in a benzene-acetic acid-water and sodium formate-formic acid-water system. The products resulting from the use of vanillin as a substrate showed atypically high ratios of the change in OD at 350 mμ to phenolic content. Also, a variety of different products were found in the ether extract. Vanillin may not be a natural product, but

Table II

Lignin-like Products Induced in Paper and Tissue Systems

The incubation mixtures contained 4 × 10^{-2} M H_2O_2, 10^{-2} M phenolic substrate, 0.05 M KH_2PO_4 at pH 4.5, 5 to 20 milligram dry weight of tissue or 100 mg paper in a total volume of 10 ml.

System	Substrate	△ OD/mg at 350/mμ*	Phenol**	F, pHC, S***	
				μg/mg dry wt of tissue	
Paper-peroxidase	Ferulic + H_2O_2	0.24	1.2	+	F
	" "	0.19	1.2	+	F
	" "	0.09	0.4	0.3	F
Elodea stem	None	0.06	0.3	0	
	Ferulic + "	5.0	19.8	17	F
	" "	4.7	19.4	18	F
	" "	3.8	9.0	+	F
	p-H-cinn. + "	5.3	3.3	9	C
	" "	3.6	2.8	+	C
	Vanillin + "	13.7	13.5	0	
	Sinapic + "	1.9	13.0	+	S
	" "	1.2	4.7	+	S
	Eugenol + "	0.9	5.2	?	F
Coleus stem	None	0.1	1.0	0	
	Ferulic + "	0.5	2.7	+	F
	" "	1.1	4.4	3	F
	p-H-cinn. + "	0.3	1.1	+	C
	" "	0.5	1.6	2	C
Phleum first internode	None	0.2	1.3	0.4	F
	Ferulic + "	1.2	4.2	+	F
	" "	1.1	5.0	4	F
	Sucrose† + "	1.3	5.1	3	F
	" "	0.9	3.4	2	F

* ΔOD at the peak of the ionization difference spectrum in the region around 350 mμ per milligram dry weight of original tissue.

** Phenolic groups reacting in the quinoneimine test, calculated as guaiacol.

*** Ferulic acid (F) *p*-hydroxycinnamic acid (C), sinapic acid (S), + indicates detected chromatographically, 0 indicates not detected.

† Incubated in 25 ml of solution containing 6 × 10^{-2} M sucrose.

sinapic acid should be if it is the precursor of the syringyl nucleus giving rise to the Cl_2-Na_2SO_3 test and to the syringaldehyde found in the ether extracts. Caffeic acid was not an effective substrate to any of the systems studied, perhaps because it inhibited peroxidase.

Tissues of Elodea were the best site for these polymerizations of all the systems studied, although there was variation among experiments. In contrast to data reported by Siegel (16), leaves of Elodea contained considerable peroxidase (unpublished data and 22) and produced lignin-like materials, although not as effectively as stems on a dry weight basis. Peroxidase, therefore, is not a limiting factor in lignification in this plant. Furthermore, water soluble alkali-sensitive esters of phenolic acids are found in Elodea in amounts comparable to that found in tissues of Phleum (unpublished data). The reason for the lack of typical lignin products in Elodea is still to be explained. Fronds of Lemna gave similar results. Coleus produced definite amounts of lignin-like products similar to those obtained with Elodea or with Phleum, but not as effectively. Celery petioles were even a less satisfactory tissue. It is significant, however, that these dicots produced a product of peroxidation that was typical of a grass lignin and not of their own.

A variety of precursors incubated for 7 to 14 days without added H_2O_2 have been studied with sections of Phleum (unpublished data). Sucrose was one of the best substrates. Data for dark grown first internodes are shown in table II. The lignin formed was greatly in excess of that found in the intact plants and was similar to that found naturally or induced in the presence of ferulic acid. Tissues of Elodea were unable to form lignin products from sucrose under comparable conditions.

The products of peroxidation in the paper-peroxidase system were studied mainly with ferulic acid as the substrate. Except for a greater solubility in $NaHCO_3$, this product was qualitatively similar to that obtained with tissues of Elodea or Phleum. The total amount of product per μmole of ferulic acid fed was as great as with some of these tissues, but

when expressed on a per milligram of paper present, the value was less. More lignin-like materials were produced at pH 4.5 than at 6. Manometric measurements indicated no significant evolution of CO_2 during the first 6 hours of incubation. This would be in contrast to the results of Freudenberg and Richtzenhain, who reported that mushroom oxidase decarboxylated ferulic acid at pH 8 in a soluble system (5, 15). An initial red color was observed on the filter paper just as with tissues of Phleum, possibly indicating an intermediate comparable to the yellow one postulated for coniferyl alcohol (6). This color faded quickly to a yellow and the medium became quite cloudy. About 25% of the total product was still produced after drying the paper disc saturated with peroxidase at 110° for about 12 hours before incubation. No detectable products were obtained when any one of the components was removed from the incubating medium. KCN (10^{-3} M) almost completely inhibited the reaction for about 30 minutes, but ultimately the final product was even greater than that without added cyanide.

Upon incubation with coniferyl alcohol or eugenol, an initial yellow color was observed, followed by the production of a white precipitate. As with tissues of Phleum, some of this product was soluble in NaOH, but the NaOH insoluble residue after incubation with eugenol still gave a phloroglucinol test. Neither ferulic acid nor coniferyl alcohol produced any detectable phloroglucinol positive material even with the total unhydrolyzed product.

Comparison of Natural and Induced Lignins. Two major differences have been detected between these natural and induced lignins when ferulic acid was the substrate. One was the absence of significant amounts of red or red-purple phloroglucinol positive products. There may be a slight increase in the case of ferulic acid fed to tissues of Phleum, but no detectable red color was obtained with Elodea or with the paper-peroxidase system and ferulic acid in contrast to the bright red-purple product in the case of eugenol. This indicates that even in tissues where enzymes other than peroxidase are present, no major conversion to the aldehyde occurred during the incubation period, or if formed, the aldehyde group was no longer free. The other major difference was in the solubility of the products in 5% $NaHCO_3$.

Table III

Products Soluble in $NaHCO_3$ in Lignin-like Preparations

System	Number samples	$NaHCO_3$ soluble*	
		△ OD at 350 mμ %	Phenol %
Paper-peroxidase, ferulic, H_2O_2	2	94	94
Elodea, ferulic, H_2O_2	2	70	68
Phleum-1st internode ferulic, H_2O_2	2	24	28
Sucrose	2	5	12
Phleum-natural			
1st internode	1	< 2	< 1
Upper internode	1	< 2	< 1
Blade	1	< 2	< 1
Sheath	1	< 2	< 1

* Based on values after hydrolysis in 0.5 N NaOH. Percent of total recovered in $NaHCO_3$ soluble plus $NaHCO_3$ insoluble = 100%. Actual recovery values of total NaOH soluble values of comparable samples were about 80 to 100%.

All preparations contained at least small amounts of a lignin-like material soluble in 5% $NaHCO_3$ (table III). While natural lignins in Phleum contained less than 1 to 2% of this product soluble in $NaHCO_3$, the difference spectrum of this material was identical to that obtained in the paper-peroxidase system where approximately 95% of the total product was soluble in $NaHCO_3$. Ferulic acid induced lignin in tissue sections showed varying degrees of solubility, but again the difference spectrum of the soluble portion was similar to the above 2 systems.

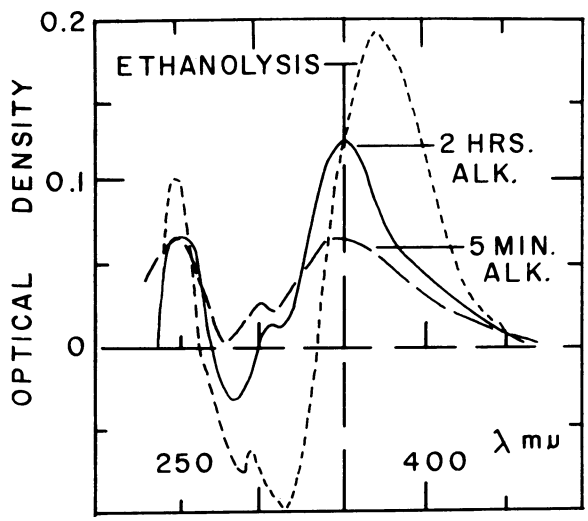
This fraction soluble in $NaHCO_3$ was studied mainly in the paper-peroxidase system because this contained the greatest percentage of such material. The major peak in the difference spectrum was at about 370 to 380 mμ. The alkaline solution was unstable, with a slow increase at 350 mμ occurring during the measurements. After alkaline hydrolysis under conditions comparable to the usual NaOH extraction, this 370 to 380 peak shifted to 350 mμ (fig 4, 5, 6). The ether extract contained ferulic acid, vanillin, and vanillic acid along with unidentified compounds. Chromatography of the unhydrolyzed solution indicated a product(s) close to the origin detected by a green-white fluorescence and a purple color with Fast Red and Na_2CO_3. Materials in this area were insoluble in 95% ethanol or ether, but were soluble in dilute alkali. After alkaline hydrolysis and acidification, ferulic acid was detected chromatographically along with a variety of other products including vanillin and possibly vanillic acid in ether extracts.

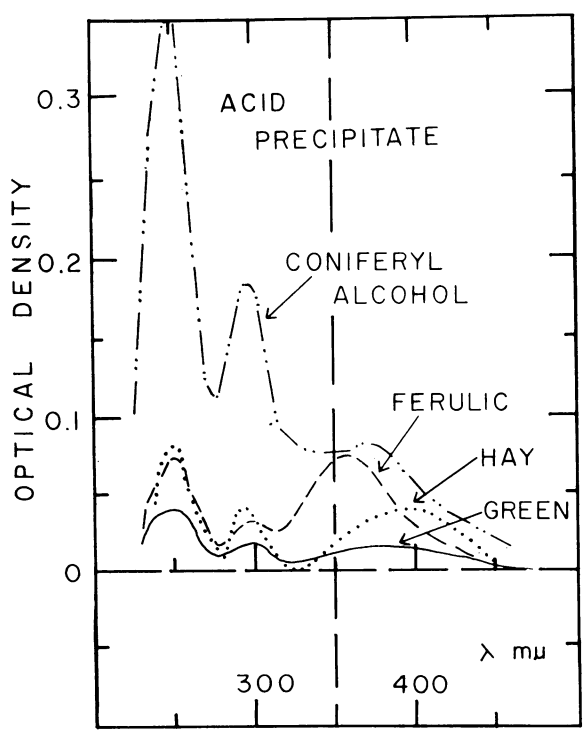
Even the paper-peroxidase system had products insoluble in $NaHCO_3$ but soluble in NaOH. The paper residue turned orange with the addition of $NaHCO_3$ and a deep orange yellow when the 0.5 N NaOH was added. The difference spectrum (fig 4) showed a typical peak at 350 mμ, a slightly smaller one at 300 mμ and a highly modified spectrum in the 250 mμ region. This latter modification was not evident in the insoluble product in the other system (fig 5, 6).

Effect of Various Treatments on Natural and Induced Lignins. The complexity of linkages in both natural and induced lignins is indicated by the difference spectra obtained after various treatments of cell wall residues. Some of these are shown in figures 7 and 8.

A study of the effect of time on the solubilization by NaOH of lignins from mature internodes of

Phleum showed that about 6% of the total product absorbing at 350 mμ was removed in the first 5 minutes of hydrolysis in cold 0.5 N NaOH. The difference spectrum lacked the high peak relative to that at 250 mμ which is characteristic of the product of a longer extraction with NaOH (fig 7). Little change occurred in this first soluble portion upon standing in alkali. If the residue from this first treatment was allowed to remain in alkali for a further 2 hours, about 40% of the total products absorbing at 350 mμ was removed, and the spectrum now was similar to that obtained with a longer treatment. Considerable time is required, therefore, to break the linkages





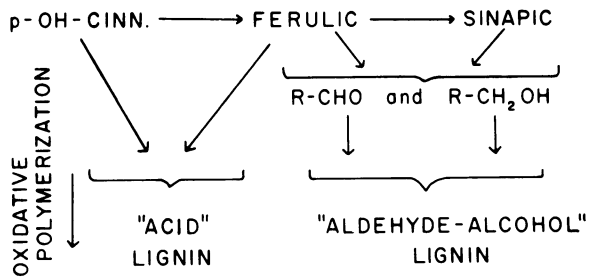


FIG. 9. Postulated scheme of biosynthesis of acid and classical or aldehyde-alcohol lignin.

either to carbohydrate in the wall or to other parts of the lignin complex or both.

Ethanolysis of cell wall residues of Phleum internodes (refluxed in ethanol containing 3% w/w HCl for 0.5 hour) produced a solution exhibiting a peak in the difference spectrum around 375 mμ (fig 7). After alkaline hydrolysis the peak shifted to about 350 mμ, and this represented only about 20% of the total product soluble in NaOH. Before alkaline hydrolysis, the solution exhibited a strong red color with phloroglucinol, and about 60% of the total products reacting in the phenol test were recovered. The residue was still phloroglucinol positive and contained approximately 40% of materials producing both the peak at 350 mμ and reacting in the phenol test. Products made soluble by ethanolysis of bamboo tissues were varied (10). These products in Phleum were not examined chromatographically, but the peak at 375 mμ could be due to the presence of diketones, syringaldehyde or to ester linkages between free acid groups and the ethanol in the medium (8, 13).

Acid precipitation of lignin products in alkaline extracts of induced and natural lignins likewise produced an array of different spectra (fig 8). The

FIG. 7 (*top*). Difference spectra of extracts of cell wall residues of mature internodes of Phleum after ethanolysis (short dashes), after 5 minutes in NaOH at room temperature (long dashes), after another 2 hours in NaOH at room temperature (solid line). Each milliliter of solution in the cuvettes contained an aliquot of a lignin extract equivalent to 225, 1620, and 260 μg of original tissue respectively.

FIG. 8 (*bottom*). Difference spectra of acid precipitates of alkaline hydrolyzates of cell wall residues in leaf sections of Phleum incubated with coniferyl alcohol (dash-dots), ferulic acid (dashes), and no substrate (green laminae) (solid line). Each milliliter of solution in the cuvette contained an aliquot of a lignin extract equivalent to 190, 240, and 300 μg of original tissue for the above spectra respectively. A spectrum similar to that obtained with young green laminae was produced by an acid precipitate from mature internodes of Phleum with an aliquot of 148 μg of tissue per milliliter. The spectrum for hay (dots) is based on 12.5 μg per milliliter of lignin isolated according to Bondi and Meyer (2) from mature internodes of Phleum.

acid precipitate for leaf tissue of Phleum incubated with coniferyl alcohol represented about 40% of the total free phenolic groups recovered, the others about 25%. These spectra are somewhat similar to that obtained from the product soluble in NaOH from tissues of herbaceous dicots such as Coleus. This acid precipitate may represent a part of that studied by Bondi and Meyer (2), although their precipitation step was done after considerable concentration of their extracts. The spectrum for such a preparation from mature internodes of Phleum is shown. There is a definite shift to the longer wave lengths in the difference spectrum.

Most workers assume that the hydroxycinnamic acids associated with lignin residues are linked as esters to already formed lignin (11, 18). Natural and induced lignins were saponified or hydrolyzed by esterases to detect such ester linkages. Both methods discussed below indicated that most of the ferulic and *p*-hydroxycinnamic acids are not attached as simple terminal esters in these materials.

Cell wall residues of mature internodes were saponified in a KOH-absolute ethanol mixture. The difference spectrum of the resulting solution had a major peak at 355 mμ and represented about 8% of the total material absorbing around 350 mμ in the total residue. Only possible traces of ferulic and *p*-hydroxycinnamic acid (about 2 μg per 13 mg original tissue) were detected chromatographically in ether extracts of this acidified fraction. Furthermore, analyses of the residues from the saponification indicated no significant loss of ferulic and *p*-hydroxycinnamic acid in the subsequent ether extract when compared with comparable nonsaponified residues. Therefore, the lignin products analyzed by the methods described in this and previous papers (19, 20, 21) are included in a fraction comparable to that studied by Higuchi and Brown (11), which contained 97% of the total C^{14} incorporated into a cell wall fraction in wheat after administration of ferulic acid-$2C^{14}$.

The effect of crude enzyme preparations containing esterases on the lignin induced in the paper-peroxidase and in Elodea tissue systems and on the natural lignins of Phleum was examined. Both the anthocyanase D and pectinase preparations were capable of completely hydrolyzing 800 μg of chlorogenic acid. No *p*-hydroxycinnamic and only possible traces of ferulic acid were detected chromatographically in the ether extract of products released by the enzyme treatment. In the case of the natural lignins of Phleum or the ferulic acid induced lignin in Elodea, there was also no detectable loss in the ferulic and *p*-hydroxycinnamic acid recovered in the cell wall residues after the enzyme treatment. This was not true with the paper-peroxidase system where in some experiments only 50% of the total ferulic acid of a nonenzyme treated sample was recovered in the residue after enzyme treatment. The fate of the rest of the ferulic acid was not determined. Higuchi and Brown reported the liberation of small amounts of ferulic acid after pectinase treatment of wall residues of wheat (11). The possible trace amounts of ferulic acid released from a cell wall residue of internodes of Phleum represented no more than 1 to 3% of the total amount found in the residue after alkaline hydrolysis.

Although the crude enzyme preparation of anthocyanase D did not release a significant proportion of the hydroxycinnamic acid derivatives associated with lignins in grasses, it did affect in other ways the cell wall residues in some of the systems studied. In the case of mature tissues of Phleum, there was little significant change in any of the analytical tests before and after enzyme treatment, and practically no phenolic compounds were detected in the products of enzymic activity. When tissues of Elodea incubated with ferulic acid were examined, there was a slight loss of about 15% of the materials producing the 350 mμ peak and reacting in the phenol test, and detectable amounts of unidentified phenolic compounds were removed by the enzyme treatment. The most drastic alterations occurred in the case of the product of the paper-peroxidase system. Approximately 50% of the materials producing the 350 mμ peak and the blue color in the phenol test were removed from the lignin-like products by treatment with anthocyanase D. As with Elodea, the ether soluble material released by enzyme treatment showed a number of unidentified phenolic compounds upon chromatography. In addition, the difference spectrum of extracts of the residue was modified by the appearance of a very high peak at 300 mμ.

Preparations containing hemicellulase and β-glucosidase had no effect on subsequent lignin analyses of cell wall residues of mature internodes of Phleum.

Conclusion

Any concept of the structure of grass or possibly monocot lignins must account for the following characteristics: all 3 basic phenolic nuclei; solubility in NaOH; peak around 350 mμ in the ionization difference spectrum after alkaline hydrolysis; peaks at longer wave lengths after ethanolysis, acid precipitation, or in a $NaHCO_3$ soluble fraction studied in Phleum; free *p*-hydroxycinnamic and ferulic acids and their C_6-C_1 aldehydes and acids in ether extracts after acidification of an alkaline hydrolyzate; the absence of sinapic acid in a comparable fraction, but the presence of the C_6-C_1 aldehyde, syringaldehyde as in tissues of Phleum; free aldehyde groups in the propane side chain to account for the phloroglucinol test; free phenolic groups capable of reacting with the quinoneimine reagent: β-guaiacyl ether units and other degradation products studied by Higuchi in bamboo (10); pattern of incorporation of radioactivity found in degradation products after incubation of wheat with C^{14}-labeled precursors (3, 11, 12).

Peroxidase acts on a wide variety of phenolic compounds in the presence of a suitable site for polymerization and a source of H_2O_2. When analyzed by the methods used here, the induced products exhibit char-

acteristics typical of the phenolic monomer itself. Upon comparison with the products of natural lignin, one can eliminate certain compounds such as vanillin and possibly sinapic acid as major natural substrates for peroxidation.

With any one phenolic substrate, similar lignin-like products are produced after alkaline hydrolysis regardless of the source of peroxidase, the site for polymerization, or the type of natural product: i.e., in the model paper-peroxidase system, in excised tissues of nonlignin-containing plants like Elodea and Lemna, or in lignified plants such as the herbaceous dicot Coleus and the monocot, Phleum.

While no significant differences were detected between lignins of Phleum and the lignin products induced after incubation with sucrose alone as substrate, 2 major differences between the ferulic acid induced and natural lignins were observed before alkaline hydrolysis of the cell wall residues. One was the weak or nondetectable increase in phloroglucinol reacting groups in a ferulic acid induced lignin. This would be expected if peroxidase is the only enzyme functioning and the polymer is one of ferulic acid and its oxidation products.

The other major difference was in the increased solubility of the products in $NaHCO_3$, studied with ferulic acid induced lignin products. While almost all of the products were soluble in $NaHCO_3$ in the paper-peroxidase system, only 70 and 25% were soluble in the induced systems in Elodea and Phleum, respectively. This was in contrast to the less than 2% in mature tissues of Phleum. The significance of this solubility and these graded differences is not clear since the final products after alkaline hydrolysis were similar. This solubility could be due to the presence of free acid groups in the propane side chain, but the difference spectrum was not characteristic of free ferulic acid nor was any detected chromatographically before alkaline hydrolysis. A possible additional factor is that the linkage to the carbohydrate site may be different in the case of the moiety soluble in $NaHCO_3$. NaOH may be necessary to break the linkage to the more natural site on the hemicellulose.

In spite of the inability of saponification or of esterase preparations to release significant amounts of ferulic acid from any of these lignin products, it is still possible to attribute the peak in the difference spectrum of the products soluble in $NaHCO_3$ to ester linkages (11, 18). For instance, the C_6-C_3 acids may still be attached to other units in nonester but alkali sensitive linkages. The peak of the $NaHCO_3$ soluble product was similar in all cases regardless of the percent of the total product present as such. Esters of common phenolic compounds produce peaks in the region of 370 to 380 mμ and they typically give greater bathochromic shifts than their free acids (8). Other compounds characterized by peaks in this general area are 7-hydroxycoumarin, syringaldehyde, and several phenylpropane derivatives (13). Alkaline sensitive ether and cyclic ether linkages might also be considered (4, 7).

Even in the paper-peroxidase system, the linkages are probably complex if Freudenberg's theory of oxidative polymerization is valid for the phenolic acid as well as for its alcohol (5). Evidence of the complexity of linkages in natural lignins of Phleum are shown in the different spectra obtained after ethanolysis, alkaline hydrolysis, and acid precipitation. Higuchi also found a varied pattern of linkages in lignins of bamboo (10).

Theories of the structure of lignins of woody materials do not adequately explain some of the peculiarities of lignins of grasses. The data presented here and in earlier papers can be accounted for by the following hypothesis of the structure of grass lignin and the final stages in its biosynthesis. Peroxidase oxidizes with subsequent polymerization of the products not only the C_6-C_3 aldehydes or alcohols as postulated by Freudenberg (5, 6), but also the acids. This gives rise to 2 major portions of the lignin complex. Basically one is a polymer of ferulic acid and its oxidation products, associated with varying amounts of a comparable polymer of *p*-hydroxycinnamic acid. This acid lignin may be peculiar to grasses or monocots. The other portion of the lignin complex is the more classical type made up of a polymer of coniferaldehyde or its alcohol and their oxidation products, associated with varying amounts of a comparable polymer of syringyl units. Linkages might be similar in both types and they are probably complex. At least some of the ester or ether linkages are alkali sensitive. Both types of lignins are soluble in NaOH.

This acid polymer may be formed by peroxidase only when the hydroxycinnamic acids are present in relatively high concentrations at the cell wall site. Preliminary evidence with the paper-peroxidase system showed that coniferyl alcohol and eugenol were more effectively converted to a polymer than the corresponding acids at low concentrations of peroxidase. This requirement for a high concentration of substrate could explain the lack of any detectable sinapic acid polymer and the lag in incorporation of the *p*-hydroxycinnamic acid moiety (fig 9). Any sinapic acid produced may always be too rapidly converted to its aldehyde and alcohol for sufficient accumulation to occur. Initially *p*-hydroxycinnamic acid may be rapidly methoxylated to ferulic acid. The latter accumulates because it is only slowly converted to coniferaldehyde or to sinapic acid. Later in development or in sclerenchyma tissue, the level of *p*-hydroxycinnamic acid may increase sufficiently for direct peroxidation. Upon alkaline hydrolysis, free ferulic and *p*-hydroxycinnamic acids are released from ester and possibly ether linkages, along with the formation of vanillin, vanillic acid, and a complex of unidentified fragments that are soluble in ether after acidification of the alkaline extract. This acid polymer is presumably the only type produced in the paper-peroxidase system, and forms a significant part in the tissue systems. Although it is mainly soluble in $NaHCO_3$ in the paper-peroxidase model, at least

a part must be insoluble in $NaHCO_3$ in tissues. The terminal acid group need not be free in tissues, but may become highly methylated as in the galacturonic acid polymer of pectins. This acid lignin accounts for the high peak at 350 mμ relative to that at 250 or 300 mμ in the difference spectrum. It may be responsible for the majority of the more acid soluble portion of the alkaline hydrolyzate.

The more classical aldehyde and alcohol lignin moiety would account for the positive reactions in the phloroglucinol (free aldehyde group) and the Cl_2-Na_2SO_3 (syringyl nucleus) histochemical tests. While coniferyl alcohol incubated with the paper-peroxidase or a tissue system produced a polymer much more insoluble in NaOH than that formed with ferulic acid, there was a detectable alkaline soluble portion. This soluble portion and the type found in Coleus may be good examples of this classical form of lignin found in grasses. The reason for the complete solubility in NaOH in herbaceous plants is not clear, but concentration levels of precursors and the nature of the linkage to carbohydrate may be involved. Compared with the acid lignin moiety, this classical type has a lower peak in the 350 mμ range relative to that at 250 and 300 mμ in the difference spectrum. This type may be responsible for most of the acid insoluble portion of the alkaline hydrolyzate. The ether soluble portion gives rise to vanillin (1, 21), vanillic acid, syringaldehyde, possibly syringic acid and a number of unknown compounds as degradation products.

At present, it is difficult to assess the relative proportions of these 2 types of lignin since both are detected in alkaline extracts by the difference spectrum technique and the quinoneimine phenol test, and give some of the same ether soluble products. The acid precipitable fraction vs. the acid soluble one may be a possible method to explore further, but the effect of the acid on linkages or subsequent polymerization is not known.

Summary

While lignins of grasses are similar to those of herbaceous dicots in their solubility in NaOH, they differ in the type of ionization difference spectrum, in having a greater amount of quinoneimine reacting groups, and in the presence of relatively large quantities of *p*-hydroxycinnamic and ferulic acids associated with the lignin complex.

After hydrolysis in 0.5 N NaOH, the lignin-like products of peroxidation of a variety of phenolic compounds were strikingly similar in the following systems: a paper-peroxidase model, tissue sections of lignified and nonlignified monocots such as *Phleum pratense* L. and *Elodea densa* Planch., and herbaceous dicots like *Coleus blumei* Banth. with a definite but a different type of lignin. The lignin-like products always showed characteristics typical of the phenolic monomer itself.

Lignins induced after incubation with ferulic acid and H_2O_2 in the above systems differed from natural lignins in 2 major characteristics detectable before alkaline hydrolysis. One was the absence of or low amount of phloroglucinol reacting groups. The other was the solubility of the product in $NaHCO_3$. The product in the paper peroxidase system was about 95% soluble, about 70% in Elodea tissues, 25% in Phleum tissues, compared with 1% in natural lignins of Phleum. This product soluble in $NaHCO_3$ was similar in all systems studied and was characterized by a peak around 370 to 380 mμ in the difference spectrum. After alkaline hydrolysis, this fraction was indistinguishable from that of the total NaOH soluble product.

Saponification in absolute ethanol containing KOH and hydrolysis carried out with pectinase and anthocyanase did not release significant amounts of the ferulic and *p*-hydroxycinnamic acids associated with lignin of Phleum or in the product of ferulic acid peroxidation in tissues of Elodea or in the paper-peroxidase system.

The following hypothesis concerning lignins of grasses is presented. Peroxidase oxidizes not only the C_6-C_3 aldehydes and alcohols, but also the acids such as ferulic acid, depending on the relative concentration of these precursors at the cell wall site. This gives rise in grasses to 2 major portions of the lignin complex. One, an acid lignin, is a polymer of ferulic acid and its oxidation products, associated with varying amounts of a comparable *p*-hydroxycinnamic lignin. The other, a classical lignin, is a polymer of coniferyl aldehyde or its alcohol and their oxidation products, associated with varying amounts of syringyl units.

Acknowledgments

The author is grateful for the assistance of Miss Karen Hamburger, Mrs. Maryanna Warmoth, and Mr. Tom Emmel during these studies. The initial work with Elodea was performed by Miss Irene Heller as a part of her Reed College Senior Thesis program.

Literature Cited

1. Adler, E. and S. Hagorth. 1949. Über die Bildung von Vanillin und Acetaldehyde aus Lignin und Lignosulfonsäure mittels Alkali. Acta Chem. Scand. 3: 86–88.
2. Bondi, A. and H. Meyer. 1948. Lignins in young plants. Biochem. J. 43: 248–56.
3. Brown, S. A. and A. C. Neish. 1959. Studies of lignin biosynthesis using isotopic carbon. VIII. Isolation of radioactive hydrogenolysis products of lignin. J. Am. Chem. Soc. 81: 2419–24.
4. De Stevens, G. and F. F. Nord. 1953. Investigations on lignin and lignification. XI. Structural studies on Bagasse native lignin. J. Am. Chem. Soc. 75: 305–09.
5. Freudenberg, K. and H. Richtzenhain. 1943. Enzymatische Versuche zur Entstehung des Lignins. Chem. Ber. 76: 997–1006.
6. Freudenberg, K., G. Grion, and J. Harkin. 1958. Nachweis von Chinonmethiden beider enzymatische Bildung des Lignins. Angew. Chem. 70: 743–4.
7. Gierer, J. and I. Norén. 1962. Über die Reak-

tionen des lignins bei der Sulfatkochung. II. Modellversuche zur Spaltung von Arylalkyllatherbindingungen durch Alkali. Acta Chem. Scand. 16: 1713–29.
8. HANSON, K. R. AND M. ZUCKER. 1963. The biosynthesis of chlorogenic acid and related conjugates of the hydroxycinnamic acids. J. Biol. Chem. 238: 1105–15.
9. HIGUCHI, T. 1957. Biochemical studies of lignin formation. II. Physiol. Plantarum 10: 621–32.
10. HIGUCHI, T. 1958. Studies on the chemical properties of bamboo stalk. J. Biochem. (Tokyo) 45: 675–85.
11. HIGUCHI, T. AND S. A. BROWN. 1963. Studies on lignin biosynthesis using isotopic carbon. XI. Reactions relating to lignification in young wheat plants. Can. J. Biochem. Physiol. 41: 65–76.
12. HIGUCHI, T. AND S. A. BROWN. 1963. Studies of lignin biosynthesis using isotopic carbon. XIII. The phenylpropanoid system in lignification. Can. J. Biochem. Physiol. 41: 621–8.
13. LEMON, H. W. 1947. The effect of alkali on the ultraviolet absorption spectra of hydroxyaldehydes, hydroxyketones, and other phenolic compounds. J. Am. Chem. Soc. 69: 2998–3000.
14. LEVY, C. C. AND M. ZUCKER. 1960. Cinnamic and *p*-coumaryl esters as intermediates in the biosynthesis of chlorogenic acid. J. Biol. Chem. 235: 2418–25.
15. RICHTZENHAIN, H. 1949. Enzymatische Versuche zur Entstehung des Lignins. IV. Mittel Dehydrierung in der Guajacolreihe. Chem. Ber. 82: 447–53.
16. SIEGEL, S. M. 1954. Studies on the biosynthesis of lignins. Physiol. Plantarum 7: 41–50.
17. SIEGEL, S. M. 1957. Non-enzymic macromolecules as matrices in biological synthesis: The role of polysaccharides in peroxidase catalyzed lignin polymer formation from eugenol. J. Am. Chem. Soc. 79: 1628–32.
18. SMITH, D. D. C. 1955. Ester groups in lignin. Nature 176: 267–68.
19. STAFFORD, H. A. 1960. Differences between lignin-like polymers formed by peroxidation of eugenol and ferulic acid in leaf sections of Phleum. Plant Physiol. 35: 108–14.
20. STAFFORD, H. A. 1960. Comparison of lignin-like polymers produced peroxidatively by cinnamic acid derivatives in leaf sections of Phleum. Plant Physiol. 35: 612–18.
21. STAFFORD, H. A. 1962. Histochemical and biochemical differences between lignin-like materials in *Phleum pratense* L. Plant Physiol. 37: 643–49.
22. STAFFORD, H. A. AND I. HELLER. 1963. Lignin production in tissues of Elodea and Phleum. Plant Physiol. 37: lvi.

Enhancement by Auxin of Ribonucleic Acid Synthesis in Excised Soybean Hypocotyl Tissue [1, 2, 3]

Joe L. Key and Jack C. Shannon

Department of Botany and Plant Pathology, Purdue University, Lafayette, Indiana

Many changes in RNA metabolism occur during normal (3, 4, 9, 16, 27, 28) and auxin-modified growth (1, 2, 7, 8, 10, 11, 12, 13, 14, 25) of plant cells. Skoog (18) suggested in 1954 that the action of auxin in regulating growth is concerned with nucleic acid metabolism. Yet, there is no evidence that the influence of auxin on RNA metabolism is more primary than other metabolic responses of auxin.

The experiments reported in this and the following paper (11) were done to gain additional information on the regulation by auxin of growth and development of plant cells as related to RNA metabolism. In general, growth promotive concentrations of auxin stimulated C^{14}-nucleotide incorporation into RNA of elongating cells whereas inhibitory concentrations decreased incorporation. In fully elongated cells, auxin induced a net synthesis of RNA, the increase occurring primarily in the ribosomal fraction. The data reported provide evidence for the DNA dependence of ribosomal RNA synthesis.

Materials and Methods

Soybean seeds (*Glycine max*, var. Hawkeye) were planted between layers of Krum moistened with distilled water in 22 × 33 cm Pyrex baking dishes covered with Saran wrap (perforated for aeration). After 48 hours at 27 to 29° in the dark, the Saran cover was removed, and 150 ml of solution containing 1×10^{-3} M $CaCl_2$, 3×10^{-4} M $MgCl_2$, and 3×10^{-3} M KCl were added to each tray. Experimental tissue was harvested after an additional 24 hours of growth.

Tissue sections were incubated in Erlenmeyer flasks in a 30° water-bath shaker. After incubation, the sections were blotted to remove excess moisture, weighed, and homogenized in ice-cold deionized water containing one drop of antifoam, unless otherwise stated. The homogenates were filtered through glass wool, and aliquots removed for RNA determinations. In experiments where C^{14}-nucleotide was added to the incubation medium, aliquots of the homogenates were removed, dried, and counted for total nucleotide uptake. RNA analyses were made by a modified Smillie and Krotkov procedure (19). The aliquots were made to 0.2 N with respect to $HClO_4$, thoroughly mixed, and centrifuged at 1100 × *g* for 10 minutes. The pellets were then suspended and twice washed in 0.2 N $HClO_4$ and once in methanol containing 0.02 M formic acid. All steps including centrifugation were carried out at 2 to 4°. The washed pellets were twice extracted at 37° for 30 minutes in a 2: 2: 1 mixture of ethanol-ether-chloroform to remove lipids. RNA was hydrolyzed in 0.3 N KOH for 18 hours at 37°.

[1] Aug. 5, 1963.
[2] This work was supported by Public Health Service Research Grant GM 10157–03 from the National Institutes of Health.
[3] Purdue Agriculture Experiment Station Journal Paper 2186.