Factors Controlling the Synthesis of Natural and Induced Lignins in Phleum and Elodea[1,2]

Helen A. Stafford

Biology Department, Reed College, Portland, Oregon

Lignin is peculiar to the cell walls of vascular plants, and is produced only in specific cells at certain developmental periods. It is not detectable in aquatic vascular plants such as *Elodea*, but tissues of these plants can be induced to form lignin-like products if suitable precursors are added (25). Since the products induced in *Elodea* were similar to those found naturally in other monocots such as *Phleum*, a comparison of the metabolism of phenolic compounds in these 2 organisms might aid in an understanding of the factors controlling lignification in plants.

It is generally agreed that the phenolic monomers which are polymerized into lignin products arise from carbohydrates via the shikimic acid pathway (fig 1) (3). Some of the enzymes involved have been demonstrated in cell free extracts of higher plants (5). Mechanisms controlling lignification can be effective at any point of this synthetic pathway. In addition, since lignin is probably attached to specific sites in the wall, the number and type of these sites could be controlling factors.

Possible regulatory agents of the terminal step of lignification have been studied in celery and peas by Siegel et al. (17, 18) and in bamboo by Higuchi (10). The agents, indoleacetic acid (IAA), ascorbic acid and glutathione (GSH), were postulated to act as antioxidants. Possible regulatory mechanisms operating at an earlier level as well as at the peroxidation stage were investigated in this study of the metabolism of phenolic compounds associated with lignin biosynthesis in *Elodea* and *Phleum*. In order to eliminate effects of treatments on growth by elongation of the cell wall, nonelongating but still incompletely lignified tissues from these plants were chosen.

Materials and Methods

Substrates used were similar to those described before (23–25) or were purchased commercially. Neomycin sulfate, gibberellin (75 %), and kinetin were purchased from Nutritional Biochemicals Corporation, and IAA from California Biochemicals Corporation and Distillation Products Incorporated.

Aseptic techniques were used, but since surface sterilization of tissues and seeds was difficult to attain, 25 μg neomycin were added per ml of final solution. Seeds of *Phleum pratense* var. climax were surface sterilized with a saturated solution of calcium hypochlorite followed by a saturated solution of orthocide (California Spray Company), rinsed with sterile water and were placed on sterilized moist filter paper in covered glass trays. After 7 days of growth in the dark at 25°, the first internodes (mesocotyl) were excised. Fifty internodes, equivalent to about 4 mg dry weight, were placed in each 125 ml flask containing 25 ml of nutrient solution. Four to 6 cm portions from the base of lamina from young leaves (just reflexed and with ligules) were cut into 0.3 to 0.5 cm sections. These were surface sterilized with a solution of orthocide (2.5 mg per ml) containing a drop of Aerosol. After rinsing with sterile water, sections equivalent to 150 to 200 mg fresh weight were placed in 25 ml of sterile nutrient solution in 125 ml flasks. *Elodea* stems were treated similarly. Each 25 ml of solution contained 1 or more of the following constituents as designated in tables I and II: 500 mg sucrose, 0.004 to 0.01 M solutions of neutralized amino acid or phenolic precursor, 10 to 50 μmoles of H_2O_2. All solutions were autoclaved for 20 minutes at 20 lbs pressure. Incubation was at 25° for 7 days in the dark or under 40 w cool white fluorescent lamps.

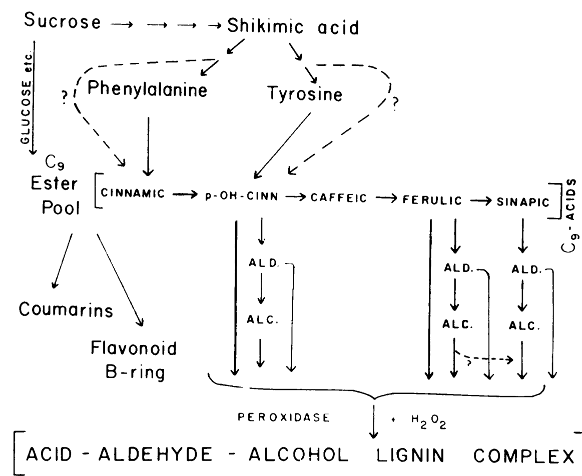

FIG. 1. Summary of proposed pathway of lignin biosynthesis in monocots (3).

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Two types of lignin determinations were made on cell wall preparations after ether and water extraction (23). One was based on the ionization difference spectrum in 0.05 N NaOH and in 0.05 M phosphate buffer at pH 7.0, and was expressed as the △ OD at the peak around 350 mμ per ml of solution containing an aliquot of extract equivalent to 1 mg dry weight of the original tissue. The other was based on the phenolic groups reacting with the quinoneimine reagent, and was expressed as μg phenol (with guaiacol as standard) in an aliquot of extract equivalent to 1 mg dry weight of original tissue.

Extraction and Alkaline Hydrolysis of Esters of Hydroxycinnamic Acids. About 0.5 to 1.0 g dry weight of tissues dried at 50° were ground in 70 % ethyl alcohol in an Omnimixer and centrifuged. The supernatant fluid and washings of the residue were evaporated to dryness at 50° under reduced pressure. The residue from green tissues was extracted with petroleum ether (60-100°) to remove interfering pigments. Two ml of 2 N NaOH was added to the residue and the solution was kept for 5 to 6 hours in an evacuated flask to prevent destruction of the caffeic acid. The solution was acidified at pH 2 to 3 with HCl and extracted with about 10 ml of ethyl in 2 ml aliquots. After evaporation with a stream of air, the residue was dissolved in 0.5 ml ethyl alcohol and suitable aliquots were chromatographed with ascending chromatography. The first solvent used was benzene-acetic acid-water (40:10:1, v/v). Each paper was cut into 2 pieces and the upper section containing ferulic and *p*-hydroxycinnamic acids was chromatographed in the second direction in butanol-ammonium hydroxide-water (40:5:5), and the lower part containing caffeic acid was chromatographed in 10 % acetic acid. The isomer of caffeic acid with the lowest R_F was used for analysis. Fluormetric analyses and calculations based on standards after similar chromatography were described previously (24).

Crude homogenates or ammonium sulfate preparations were used for enzyme analyses. Tissues were ground at low speed on an Omnimixer in 0.05 M phosphate buffer at pH 6.0 or 6.8. After low speed centrifugation, the supernatant material was used directly or was treated with 400 g ammonium sulfate per liter of enzyme solution, and the precipitate was resuspended in 0.05 M phosphate buffer. Enzymes bound to cell wall fragments were eluted with saturated ammonium sulfate (27).

Peroxidase was assayed spectrophotometrically at 430 mμ in a 1 ml volume containing 0.05 M phosphate buffer at pH 6.0, 0.005 M pyrrogallol, 0.01 % H_2O_2 and enzyme. Polyphenol oxidase activity in the extracts was negligible at the dilutions used in this assay. Polyphenol oxidase and ascorbic acid oxidase were measured manometrically by following O_2 uptake. For the former, vessels contained 0.05 M phosphate buffer at pH 6.8, 50 μmoles of substrate, and enzyme in a final volume of 3 ml. For the latter, vessels contained 0.05 M phosphate buffer at pH 6.0 and 50 μmoles of ascorbic acid neutralized with NaOH. Suitable blanks were subtracted if necessary.

Results and Discussion

Effects of Substrates in the Formation of Lignin-like Products in Excised Tissues of Elodea and Phleum. Excised tissues of both of these plants produced lignin-like products within 24 hours after incubation with phenolic monomers such as ferulic acid and added H_2O_2 (25). If these same tissues were incubated for a longer period of 4 to 7 days in the presence of sucrose, only those from *Phleum* were capable of the formation of lignin. No substrate has been found that could form detectable quantities of lignin in *Elodea* in the absence of H_2O_2, and the addition of H_2O_2 along with sucrose was also ineffective in producing lignin products.

Excised tissues from both the first internode and the base of young laminae produced more lignin products in the presence of sucrose plus ferulic acid than with sucrose alone (table I). A t-test of the significance of the difference between the means indicated generally a significant difference at the 1 % level or less (19). In the few cases where the level of probability dropped to the 5 or even 10 % level for a value determined by 1 of the 2 lignin analyses, the values obtained with the second type of analysis were always at the 1 % level. In experiments not shown in the tables, the addition of H_2O_2 plus sucrose did not increase lignin production over that with sucrose alone, and concentrations of 100 μmoles of H_2O_2 per flask were inhibitory. Sections incubated with H_2O alone gave values similar to the zero time controls.

The effects of a variety of other substrates are summarized in table II. In all cases the type of lignin products formed were similar to those found in the intact plant. Tissues from the first internode and laminae differed in some of their responses to these precursors.

In excised tissues of the first internode, the amount of lignin produced after incubation with sucrose plus ferulic acid approached the values obtained with added ferulic acid plus H_2O_2 although longer periods of incubation were necessary. Tissues of the first internode were more sensitive to free hydroxycinnamic acids such as *p*-hydroxycinnamic and caffeic acids in the absence of sucrose, either when added directly or indirectly through phenylalanine or tyrosine. This inhibition was largely prevented by the presence of sucrose in some cases, but not with cinnamic and caffeic acids. Presumably, the addition of sucrose permitted glucosidation or esterification to a less toxic form in the case of *p*-hydroxycinnamic and ferulic acids. Initially, these experiments were done at pH 4.5, but the inhibition was complete with all hydroxycinnamic acids even in the presence of sucrose. At the lower pH values, the free acids

may have penetrated the tissues too rapidly for the esterification step. Lignin production with p-hydroxycinnamic acid plus sucrose was less than that with ferulic acid plus sucrose. This could be due to a residual toxicity at the higher pH of p-hydroxycinnamic acid directly or after its conversion to caffeic acid, or to a rate limiting step between caffeic and ferulic acid. Cinnamic acid may be inhibitory because of the conversion of its ester to that of caffeic acid, bypassing free p-hydroxycinnamic acid (8).

Table I. *Effect of Added Substrates on the Production of Lignin-like Products in Excised Tissues of* Phleum *and a Comparison with the Amounts Found in Mature Tissues*

Incubation was at pH 7.0 for 7 days in all experiments except with ferulic + H_2O_2 which was at pH 4.5 for 1 day. Lignin analyses as in Materials and Methods. All values are based on calculated dry weights before incubation except in intact laminae where values are based on final dry weights. Statistical symbols are defined in ref. (19). N = number of separate experiments; each type of lignin analysis was done in duplicate.

Lignin analyses	△ OD per mg dry wt tissue			Phenol µg per mg dry wt tissue		
Statistical symbols	N	$\bar{X} \pm S_{\bar{x}}$	t	N	$\bar{X} \pm S_{\bar{x}}$	t
First internode						
Excised						
O' Control	7	0.23±.01	7.25* (vs. + Sucrose)	7	1.12±.03	5.1* (vs. + Sucrose)
+ Sucrose	7	0.59±.05	1.86*** (vs. + Sucrose + ferulic)	7	2.86±.30	3.9* (vs. + Sucrose + ferulic)
+ Sucrose + ferulic	3	0.83±.16		3	4.83±.31	
+ Ferulic	3	0.54±.06		3	2.84±.29	
+ Ferulic + H_2O_2	2	1.1		2	4.5	
Intact–mature	2	0.3		2	1.1	
Lamina						
Excised						
O' Control	13	0.37±.03	3.68* (vs. + Sucrose)	13	2.6±.18	4.9* (vs. + Sucrose)
+ Sucrose	8	0.62±.05	3.54* (vs. + Sucrose + ferulic)	8	4.61±.43	2.6** (vs. + Sucrose + ferulic)
+ Sucrose + ferulic	5	0.89±.04	4.35* (vs. + Ferulic)	5	6.34±.48	1.94*** (vs. + Ferulic)
+ Ferulic	3	0.83±.05		3	6.13±.57	
+ Ferulic + H_2O_2	2	2.1		2	13.0	
Intact–mature	2	0.7		2	5.7	

* Significance of t test at level of 1 % or less.
** Significance of t test at level of 2 to 5 %.
*** Significance of t test at level of 5 to 10 %.

Table II. *Summary of Percent Increase in Production of Lignin-like Products in Excised Tissues of* Phleum *after Incubation for 7 Days with Various Substrates*

Lignin analyses as in Materials and Methods. Average of 2 to 13 experiments, each type of lignin analysis being done in duplicate. O = 10 % increase or less over zero time controls.

Substrates added	Lignin analyses: First internode △ OD	First internode Phenol	Lamina △ OD	Lamina Phenol
Sucrose	160	160	70	80
Phenylalanine	0	0	60	40
Tyrosine	0	0	40	60
p-OH-Cinnamic acid	0	0	40	30
Caffeic acid	0	0	0	30
Ferulic acid	160	160	130	130
Sucrose + shikimic acid	150	160	60	50
Sucrose + cinnamic acid	50	40	...	...
Sucrose + p-OH-cinnamic acid	190	130	...	...
Sucrose + caffeic acid	0	0	40	70
Sucrose + ferulic acid	260	330	140	140

Tissues from laminae were not as sensitive to free hydroxycinnamic acids, and produced as much product with ferulic acid alone as with ferulic acid plus sucrose. But caffeic acid still showed some inhibition in the presence of sucrose, and none of the precursors preceding ferulic acid were as effective as ferulic acid in the production of lignin. Older laminae lost the ability to form lignin products from sucrose alone, although they were still capable of lignin formation after incubation with ferulic acid plus H_2O_2. Unlike the first internode, the production of lignin in the presence of ferulic acid plus sucrose did not approach that obtained with added ferulic acid plus H_2O_2.

Data obtained with excised tissues of the upper internodes were highly variable, probably due to the difficulty in selecting internodes at a comparable state of lignification. The data, however, showed a trend similar to that obtained with laminae.

Alkali-Sensitive Esters of Hydroxycinnamic Acids and Flavonoids. Since the lack of lignin in *Elodea* could be due to a block preventing the formation of C_9 phenolic acid, tissues of *Elodea* and *Phleum* were compared as to the level of hydroxycinnamic acids and flavonoids. While the level of phenolic compounds at the site of lignification in the cell wall is not known, *Elodea* accumulates alkali-sensitive esters of *p*-hydroxycinnamic, caffeic, and ferulic acids in amounts comparable to those found in *Phleum* (table III). But the total amounts are small, and while they do indicate the presence of the shikimic pathway, the production of these phenolic precursors could still be a major limiting factor in lignification.

Table III. *Presence of Alkali-Sensitive Esters in Tissues of* Phleum *and* Elodea

Average of 2 to 3 extractions. Values are minimal since they are not corrected for loss prior to chromatography.

Tissues analyzed	μg per g dry wt		
	p-OH-Cinnamic	Caffeic	Ferulic
Elodea			
Leaf	320	1600	120
Stem	210	300	100
Phleum			
First internode	720	2300	140
Young green shoots 1–2 leaves	300	450	300
Mature lamina	630	1750	830
Mature upper internodes	130	140	460

Major flavonoids in *Elodea* are an anthocyanin, a cyanidin glycoside, and an unidentified flavone according to Reznik (15). Preliminary work associated with the present studies indicated that the spectrum of the major flavone in *Elodea* is similar to that of luteolin. Traces of 2 flavones and a cyanidin derivative could be detected in *Phleum*. Chromatography of methanol-HCl extracts indicated that there was a greater complexity and abundance of flavonoids and other phenolic compounds in *Elodea* than in *Phleum*.

Extraction of Key Enzymes Involved in Lignification. Peroxidase. The fact that tissues can be induced to form lignin-like products in the presence of phenolic precursors and H_2O_2 can be considered as indirect evidence of the presence of peroxidase in *Elodea* (25). Direct evidence of peroxidase activity is shown in table IV. While leaves of *Elodea* do have less activity than the stems on a dry or fresh weight basis, the amount is comparable to that found in tissues of *Phleum*. In both cases, about 10 to 20 % of the total activity was associated with a well-washed fraction, and could be eluted from the cell wall by treatment with saturated ammonium sulfate (27).

Table IV. *Peroxidase Activity in* Phleum, Elodea *and* Lemna

Tissues analyzed	OD × min^{-1} × g dry wt^{-1} of original tissue
Elodea	
Stem	0.550*
Leaf	0.210
Phleum	
First internode	0.230
Lamina-young	0.430**
Lemna	
Fronds	0.181

* 1.3 × min^{-1} × mg $protein^{-1}$ in a crude ammonium sulfate fraction.

** 0.79 × min^{-1} × mg $protein^{-1}$ in a crude ammonium sulfate fraction.

Since caffeic acid appeared to be an inhibitor in lignification, its effect on peroxidase in the spectrophotometric assay method was determined with a crystalline preparation of peroxidase (Nutritional Biochemicals Corporation). Preincubation of the enzyme for 5 minutes with either caffeic or chlorogenic acid at 2 times the concentration of the pyrrogallol inhibited the subsequent rate of oxidation of pyrrogallol about 50 %.

Hydroxylating Enzymes. The presence of detectable amounts of alkali sensitive esters of hydroxycinnamic acids (table III) in tissues of *Elodea* and *Phleum* is indirect evidence of the existence of hydroxylating enzymes capable of converting cinnamic to caffeic via *p*-hydroxycinnamic acid.

The natural hydroxylating agents are not definitely known (5). One possible contender for this role is the peroxidase-dihydroxyfumarate system, capable of converting cinnamic to both *o*- and *p*-hydroxycinnamic acids (4). No substitute for the requirement of dihydroxyfumarate is known, and the presence of this acid in plants has never been proven although there are enzymes capable of metabolizing it and its products (22).

Another postulated hydroxylating enzyme is the phenolase complex which converts monophenols to diphenols. A potato extract rich in polyphenolase activity has been reported that converts an ester of *p*-hydroxycinnamic acid to one of caffeic acid (8). Both *Phleum* and *Elodea* contained active polyphenolases which differed in their relative specificity for phenolic substrates (table V). This difference was

Table V. *Polyphenol Oxidase Activity of Extracts from Young, Green Shoots of* Phleum *and* Elodea

Substrate	Relative activity	
	Phleum	*Elodea*
Pyrrogallol	++++	+
Catechol	++++	+
Chlorogenic acid	++++*	++++**
Caffeic acid	++	+++
Ferulic acid	0	0
p-OH-Cinnamic acid	0	0
Guaiacol	0	+
Tyrosine	0	+***
p-Cresol	0	++

* 7.0 μl O_2 $\times$ hr^{-1} $\times$ mg^{-1} protein in a crude homogenate.
** 2.4 μl O_2 $\times$ hr^{-1} $\times$ mg^{-1} protein in a crude homogenate.
*** After 30 minutes lag.

first detected when tyrosine was fed to both tissues. No visible pigments accumulated in tissues of *Phleum*, but traces of a brown-black pigment accumulated in tissues of *Elodea*. Catechol was a more potent inhibitor of phenolase activity in *Elodea* than in *Phleum*, and completely inhibited the O_2 uptake in the presence of added tyrosine. Both extracts showed strong activities with chlorogenic acid, and the activity with diphenols in *Elodea* was greater than that with monophenols such as *p*-cresol.

Ascorbic Acid Oxidase. The enzyme has not yet been implicated in the process of lignification, but since its substrate, ascorbic acid, serves as an inhibitor of phenol oxidations (10), it could control the ratio of oxidized to reduced ascorbic acid. Both *Phleum* and *Elodea* have detectable ascorbic acid oxidase activity which is completely inhibited by 10^{-3} M diethyldithiocarbamate. In the case of *Phleum*, most of the activity was associated with the cell wall fraction, and treatment with saturated ammonium sulfate was necessary to obtain highly active preparations (27). In the case of *Elodea*, the enzyme was more soluble.

Site of Synthesis of Induced Lignins. Siegel had shown that lignin-like products induced by eugenol and H_2O_2 were partly associated with cell wall fractions (17). This was also true in tissues of *Phleum* incubated with sucrose. Leaf tissues from *Phleum* were ground in an omnimixer in 0.05 M phosphate buffer at pH 7.0 in an ice bath. The cell wall mat obtained was washed several times with buffer, and

Table VI. *Effect of 7 Days of Light or Darkness on Lignin and Anthocyanin Biosynthesis in 7-Day Old Dark Grown First Internodes of* Phleum

Lignin analyses as in Materials and Methods. Average of 2 separate experiments with each lignin analysis in duplicate. Based on calculated dry weight at 0′. Anthocyanin values based on visual observations.

Experimental conditions	Lignin* Δ OD	Phenol	Anthocyanin**
Control 0′	0.23	1.3	0
Excised			
Darkness* + sucrose	0.53	2.2	0
Light** + sucrose	0.56	2.5	++++
Intact			
Darkness* + sucrose	0.22	1.8	0
Light** + sucrose	0.36	1.9	++++
Light** + no sucrose	0.30	1.1	+

* About 1 hour white light to stop internode growth.
** About 200 ft-c from cool, white fluorescent lamp.

still contained most of the activity capable of forming lignin products from sucrose. This would indicate that enzymes of the shikimic acid pathway and subsequent enzymes leading to the lignin polymer are a part of the cell wall or are associated with cytoplasm tightly bound to the cell wall.

Effect of Light on the Synthesis of Lignin and Flavonoids in the First Internode of Phleum. Light (about 200 ft-c from cool white fluorescent lamps) had no detectable effect on lignin synthesis in excised tissues of the first internode incubated with sucrose, but the same light intensity increased the synthesis of a red anthocyanin (table VI). If intact seedlings were placed in a sucrose medium, there was little or no increase in lignin products in either the dark or the light, but there was considerable production of the red anthocyanin in light. If the seedlings were placed instead on moist filter paper without added sucrose, both lignin and anthocyanin synthesis were limited.

Effect of Indoleacetic Acid, Gibberellin, and Kinetin. Experiments with excised tissues of the first internode of *Phleum* incubated with sucrose indicated that IAA completely inhibited lignification at 10^{-4} M, but no acceleration was detected at lower concentrations. Addition of kinetin (0.1, 1 or 10 mg/liter) or gibberellin (1, 10 or 100 mg/liter) with or without the addition of IAA (0.1 mg/liter) showed no detectable effect, except for a slight inhibition with high concentrations of gibberellin.

Discussion and Conclusions

There is no adequate evidence that any of the enzymes involved in lignification which have been demonstrated in cell free extracts (5) are induced enzymes. Peroxidase associated with cell walls has been considered to be induced (11), but this may just be an activation or indirect effect (14), and evidence of de novo synthesis of protein has not been

demonstrated. Peroxidase activity was increased in tips of growing roots of *Vicia,* indicating that this enzyme or sites in the wall for attachment may be a limiting factor in lignification in growing tissues. But peroxidase does not appear to be a major limiting factor in any older tissue studied, although it has been reported to be absent in some sources of *Elodea* (16).

Tissues of *Elodea* used in this study contained sufficient peroxidase to produce considerable lignin-like products when incubated with H_2O_2 and hydroxycinnamic acids such as ferulic acid or its alcohol. Since the concentration of the esters of hydroxycinnamic acids in *Elodea* were comparable to those in *Phleum* and relatively large amounts of flavonoids were present, the block to lignin synthesis does not lie in the absence of the enzymes of the shikimic acid pathway. Unless some sort of compartmentalization prevents these C_9 esters from reaching the cell wall sites (7), a more likely point for a complete block is in the production or the availability of the necessary H_2O_2. But attempts to demonstrate this requirement directly by the addition of H_2O_2 along with sucrose have been unsuccessful. However, either an inhibition by H_2O_2 or excess catalase activity could produce such negative results. The extent of competition for C_9 precursors from flavonoid pathways is unknown. The significance of the differing specificities of the polyphenolase activities of *Elodea* and *Phleum,* and the recently reported absence of phenol glucosylation reactions in *Elodea* are not clear (13).

A lack of lignin appears to be common in aquatic plants. Since a supporting function is not necessary for such plants, some step in the biosynthetic pathway to lignin such as one producing H_2O_2 at the cell wall site could have been eliminated without harm to the species. Some factor characteristic of the aquatic environment might also be limiting. But oxygen is probably not a major limiting factor, because *Elodea* is a photosynthetic organism with considerable air spaces, and tissues of *Phleum* produce ample lignin when submersed in stationary fluid.

In contrast to *Elodea,* there is no absolute block to lignification in either intact or excised tissues of *Phleum.* Control mechanisms limiting lignification must be in operation in the intact plant, since most of the tissues do not attain the maximum lignification that can be induced in excised tissues after incubation with excess phenolic substrates and H_2O_2. In addition, histochemical tests frequently show that lignification is only partial in the sclerenchyma cells of the larger bundle caps. Intact tissues of the mature laminae ultimately attained levels of lignification close to those produced by excised tissue when incubated with sucrose plus ferulic acid. This was not the case with intact first internodes which formed little lignin even when the entire seedling was incubated with sucrose. Control mechanisms in the seedling may divert the carbohydrate supply to the upper growing parts (1).

Excision probably removes the tissues from some of the control mechanisms present in the intact plant. Lignin was not formed in significant amounts without the addition of exogenous substrates. Sucrose was an effective exogenous substrate presumably by being metabolized via the shikimic acid pathway, but it also serves as a glucosyl donor for esterification of the C_9-acids, and possibly as a precursor to substrates giving rise to H_2O_2 needed in the peroxidation step (fig 1). In the presence of sucrose, some step between sucrose and the hydroxycinnamic acids may become limiting, since the addition of ferulic acid plus sucrose gave an increase over that of sucrose alone. In first internodes, the formation of anthocyanins with added sucrose competes for C_9 precursors, but the amount of anthocyanin formed would account for an insignificant amount of total lignin. After incubation with sucrose plus ferulic acid, these tissues produced lignin in amounts comparable to that formed with excess ferulic acid and H_2O_2, possibly indicating a saturation of sites for attachment of the lignin products to the cell walls. Such a saturation had not occurred in tissues from the laminae. H_2O_2 was probably limiting in both tissues only in the presence of sucrose plus ferulic acid.

Two interrelated mechanisms may control lignin formation in intact tissues of *Phleum.* One is the level of caffeic acid esters, the other is the level of IAA. Caffeic acid and its esters inhibited peroxidase activity and sucrose-induced lignification. Caffeic acid was also the 1 phenolic intermediate incapable of inducing lignin-like products in the presence of H_2O_2 (23), and C^{14}-labelled caffeic acid was a less effective precursor of aldehyde lignin than *p*-hydroxycinnamic or ferulic acids (7). If peroxidase is involved in the hydroxylation of cinnamic acid, this step might also be inhibited. Caffeic acid may not only be a key intermediate in lignin biosynthesis, therefore, but may play a role as inhibitor of an enzyme in the pathway preceding its formation and in the final peroxidation step.

Derivatives of caffeic acid were the major components in the pool of esters of hydroxycinnamic acids in most of the tissues studied. The significance of these levels is not evident since the intracellular localization of these compounds is not known. The concentration of hydroxycinnamic acids is either not related to lignin formation (7), or there is an inverse relationship (table III). The sum of the soluble ester content and that of the alkaline sensitive acids associated with the lignin in different tissues of *Phleum* (24) was remarkably constant (about 6 mg per g dry weight).

Caffeic and chlorogenic acids were both excellent substrates of the classical diphenol activity of polyphenol oxidase, but monophenol activity could not be demonstrated in crude extracts of *Phleum.* Since

this monophenol activity has been postulated as the source of the hydroxylating step in the conversion of *p*-hydroxycinnamic to caffeic acid, further work with this enzyme complex is necessary.

Peroxidase is responsible for the enzymatic activity known as IAA oxidase, and hydroxycinnamic acids and their esters may play a role in the regulation of this activity (28). Although the significance of the activity of IAA oxidase in vivo is questioned (20), this enzyme would alter the level of IAA. The inhibition by 10^{-5} M IAA could be a direct or an indirect effect through its control of the reducing agents ascorbic acid and GSH (10, 12, 18). The lack of acceleration of the sucrose-induced lignification at concentrations of IAA less than 10^{-5} M does not corroborate the work of Jensen (11), but peroxidase may be a limiting factor only in elongating tissues.

The use of nonelongating tissues in the present study may also explain the lack of an effect of added kinetin or gibberellin (2, 21, 26). Similarly, the effects of boron on peroxidase activity and lignification reported by others might be dependent on indirect effects detectable only in growing tissues (6).

It is significant that the concentration of IAA (10^{-5} M) that accelerates growth and possibly deposition of hemicelluloses and cellulose in tissues such as the first internode is that which inhibits lignification (9, 18). As growth of the wall ceases and the level of IAA within a cell or in a localized area of the cell wall decreases, possibly through the conversion of the ester of caffeic acid to that of ferulic acid, the process of lignification can compete for phenolic precursors and the supply of H_2O_2. Although some lignification occurs relatively early in the history of a cell (protoxylem), the majority of it is formed during a later phase of maturity and even during senescence. Since protein would be broken down under these conditions, the phenyalanine and tyrosine released would be a source of precursors that could account for the heavy lignification that occurs in grasses just before flowering.

Summary

Factors controlling lignin biosynthesis have been studied in nonelongating tissues of *Elodea* and *Phleum pratense*. Since *Elodea* can accumulate significant amounts of flavonoids and alkali-sensitive esters of hydroxycinnamic acids, and excised tissues can produce lignin-like compounds only in the presence of added hydroxycinnamic acids and H_2O_2, the block to lignification may lie in the pathway of the production or utilization of H_2O_2. Incubation of tissues with H_2O_2 and sucrose, however, was ineffective in producing lignin.

There was no complete block to lignification in tissues of *Phleum*, but none of the intact tissues attained the maximum lignification that could be induced in the presence of excess hydroxycinnamic acids and H_2O_2. In excised tissues incubated with sucrose, the major limiting factor appeared to be some step between the level of sucrose and ferulic acid, and possibly one just preceding the formation of the latter compound. Light had no detectable effect on the production of lignin in tissues incubated with sucrose.

Activities of peroxidase, polyphenoloxidase and ascorbic acid oxidase in *Elodea* were similar to those found in *Phleum*. The only major difference was in details of the substrate specificity of the polyphenolase activity.

While IAA at a concentration of 10^{-4} M inhibited the production of lignin in excised tissues of the first internode of *Phleum* when incubated with sucrose, there was no detectable effect of kinetin or gibberellin singly or in combination with indoleacetic acid, except for a slight inhibition with high concentrations of gibberellin.

Two possible interrelated mechanisms controlling lignification in intact tissues of *Phleum* are discussed; one is the level of caffeic acid esters, the other is the level of IAA.

Literature Cited

1. Ball, N. G. and G. B. Newcombe. 1961. The relationship between the growth of the primary leaf and of the coleoptile in seedlings of *Avena* and *Triticum*. J. Exptl. Botany 12: 114–28.
2. Bergmann, L. 1964. Der einfluss von Kinetin auf die ligninbildung und differenzierung in Gewebe kulturen von *Nicotiana tabacum*. Planta 62: 211–54.
3. Brown, S. A. 1964. Lignin and tannin biosynthesis. In: Biochemistry of Phenolic Compounds. J. B. Harborne, ed. Academic Press. p 361–98.
4. Buhler, D. R. and H. S. Mason. 1961. Hydroxylation catalyzed by peroxidase. Arch. Biochem. Biophys. 92: 424–37.
5. Conn, E. E. 1964. Enzymology of phenolic biosynthesis. In: Biochemistry of Phenolic Compounds. J. B. Harborne, ed. Academic Press. p 399–435.
6. Dutta, T. R. and W. J. McIlrath. 1964. Effects of boron on growth and lignification in sunflower tissue and organ cultures. Botan. Gaz. 125: 89–96.
7. El-Basyouni, S., A. C. Neish, and G. H. N. Towers. 1964. The phenolic acids in wheat. II. Insoluble derivatives of phenolic cinnamic acids as natural intermediates in lignin biosynthesis. Phytochem. 3: 627–39.
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. Henderson, J. H. M. and J. P. Nitsch. 1962. Effect of certain phenolic acids on the elongation of *Avena* first internodes in the presence of auxins and tryptophan. Nature 195: 780–82.
10. Higuchi, T. 1957. Biochemical studies of lignin formation II. Physiol. Plantarum 10: 621–32.

11. JENSEN, W. A. 1955. The histochemical localization of peroxidase in roots and its induction by indoleacetic acid. Plant Physiol. 30: 426–32.
12. KEY, J. L. 1962. Changes in ascorbic acid metabolism associated with auxin-induced growth. Plant Physiol. 37: 349–55.
13. PRIDHAM, J. B. 1964. The phenol glucosylation reaction in the plant kingdom. Phytochem. 3: 493–97.
14. RAY, P. M. 1958. Destruction of auxin. Ann. Rev. Plant Physiol. 9: 81–118.
15. REZNIK, H. AND R. NEUHÄUSEL. 1964. Farblose Anthocyanine bei submersen Wasserpflanzen. Z. Botan. 47: 471–89.
16. SIEGEL, S. M. 1954. Studies on the biosynthesis of lignins. Physiol. Plantarum 7: 41–50.
17. SIEGEL, S. M. 1955. The biochemistry of lignin formation. Physiol. Plantarum 8: 20–32.
18. SIEGEL, S., P. FROST, AND F. PORTO. 1960. Effects of indoleacetic acid and other oxidation regulators on in vitro peroxidation and experimental conversion of eugenol to lignin. Plant Physiol. 35: 163–67.
19. SIMPSON, G. C., A. ROE, AND R. C. LEWONTIN. 1960. Quantitative Zoology. Harcourt, Brace and Company, New York.
20. SONDHEIMER, E. 1964. Chlorogenic acid. Botan. Rev. 30: 667–712.
21. SOROKIN, H. P., S. N. MATHUR, AND K. V. THIMANN. 1962. The effects of auxins and kinetins on xylem differentiation in the pea epicotyl. Am. J. Botany 49: 444–54.
22. STAFFORD, H. A. 1957. Tartaric acid dehydrogenase activity in higher plants. Plant Physiol. 32: 338–45.
23. 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.
24. STAFFORD, H. A. 1962. Histochemical and biochemical differences between lignin-like materials in *Phleum pratense* L. Plant Physiol. 37: 643–49.
25. STAFFORD, H. A. 1964. Comparison of lignin-like products found naturally or induced in tissues of *Phleum, Elodea,* and *Coleus,* and in a paper peroxidase system. Plant Physiol. 39: 350–60.
26. WAREING, P. F. 1958. Interaction between indoleacetic acid and gibberellic acid in cambial activity. Nature 181: 1744–45.
27. WAYGOOD, E. R. 1950. Physiological and biochemical studies in plant metabolism II. Respiratory enzymes in wheat. Can. J. Res. C. 28: 7–62.
28. ZENK, M. H. AND G. MULLER. 1963. In vivo destruction of exogenously applied indolyl-3-acetic acid as influenced by naturally occurring phenolic acids. Nature 200: 761–63.