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LIGNIN–CARBOHYDRATE COMPLEX FORMED IN ISOLATED CELL WALLS OF CALLUS*

FRANK W. WHITMORE
Department of Forestry, Ohio Agricultural Research and Development Center, Wooster, OH 44691, U.S.A.

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Key Word Index—*Pinus eliottii*; Pinaceae; slash pine; biosynthesis; lignin.

Abstract—Cell walls of *Pinus elliottii* tissue cultures were isolated and incubated with coniferyl alcohol and H_2O_2. Lignin having physical and chemical properties similar to that prepared from wood was formed by the peroxidase attached to the walls. Fractions of the callus lignin isolated enzymatically or chemically contained bound carbohydrate. The lignin was also strongly bound to a protein containing hydroxyproline, probably extensin. This system may be analogous to the earliest stage of normal lignin formation in which monomers are transported from the protoplast into the primary wall and middle lamella, where peroxidase polymerizes monomers and catalyzes bonds to carbohydrate and protein.

INTRODUCTION

A form of peroxidase (EC 1.11.1.7) is attached to plant cell walls through ionic and possibly covalent bonds [1]. One function of wall-associated peroxidase is the dehydrogenative polymerization of phenolic monomers in the synthesis of lignin. Another may be the establishment of covalent bonds between lignin and carbohydrate in the cell walls. Peroxidase can catalyze covalent bond formation between sucrose and the dehydrogenative polymerization product from coniferyl alcohol [2]. A ferulic acid polymer likewise becomes linked to cell walls or to pure cellulose in a reaction catalyzed by peroxidase [3].

Since peroxidase is attached to the cell wall, it is of interest to know if fragments of walls, isolated from protoplasts, can incorporate and bind lignin from a coniferyl alcohol substrate, and if the lignin formed is similar to that formed in the intact cell wall surrounding active protoplasts. Such an investigation is reported here.

Tissue cultures of *Pinus elliottii* (slash pine) were used in the experiments because their walls are rich in peroxidase but normally contain very little lignin. Phenolic monomers may be incubated with callus cells or isolated walls and their products examined without interference from large amounts of pre-existing lignin.

RESULTS

When intact callus tissue and washed cell wall fragments were incubated separately with [^{14}C]-labelled coniferyl alcohol, lignin was incorporated into the walls in each case. A complex of carbohydrate and lignin was isolated by treating the tissue with a mixture of polysaccharidases. A similar complex was obtained from cambial scrapings of seedling stems previously incubated with cinnamic acid-[2-^{14}C]. This latter experiment represents the system most similar to natural lignin formation, for cinnamic acid must be transported into cells, hydroxylated, methoxylated, reduced and transported out into the wall as a *p*-coumaryl alcohol. Profiles of all 3 complexes obtained by separation on a gel column were similar, having a high MW association of phenolic substances and carbohydrates in the void volume (Fig. 1). Peaks of phenolics and carbohydrates of lower MW were also closely coincident. Pronase treatment of the high MW fractions did not alter the association between carbohydrate and lignin as determined by running the digestion mixture through the gel column.

Lignin thioglycolic acid (LTGA) from isolated callus cell walls incubated with coniferyl alcohol was compared with LTGA from wood of seedling stems by IR and UV spectrophotometry. In each case, spectra were similar, the wall preparations giving the characteristic lignin absorption bands (Fig. 2).

After establishing that lignin can be formed in washed cell wall fragments when treated with coniferyl alcohol and H_2O_2, the association formed between lignin and the pre-existing wall was investigated. Callus cultures were grown in [^{14}C]-labeled glucose or proline in order to label the walls, then washed wall fragments were incubated with or without unlabeled coniferyl alcohol and the resulting lignin preparations counted. LTGA preparations were used because they are considered to be the least contaminated with cocondensed protein [4]. Most lignin preparations are contaminated with carbohydrate and it was expected that the small amount of carbohydrate which might be contained in the LTGA would most likely be covalently bound.

Washed cells incubated with coniferyl alcohol yielded *ca* twice as much radioactivity in LTGA preparations as the controls with no coniferyl alcohol (Table 1). The radioactivity could only have come from pre-existing wall constituents. Some, of course, may have arisen from lignin synthesized from labeled glucose, to which the unlabeled coniferyl alcohol was attached during

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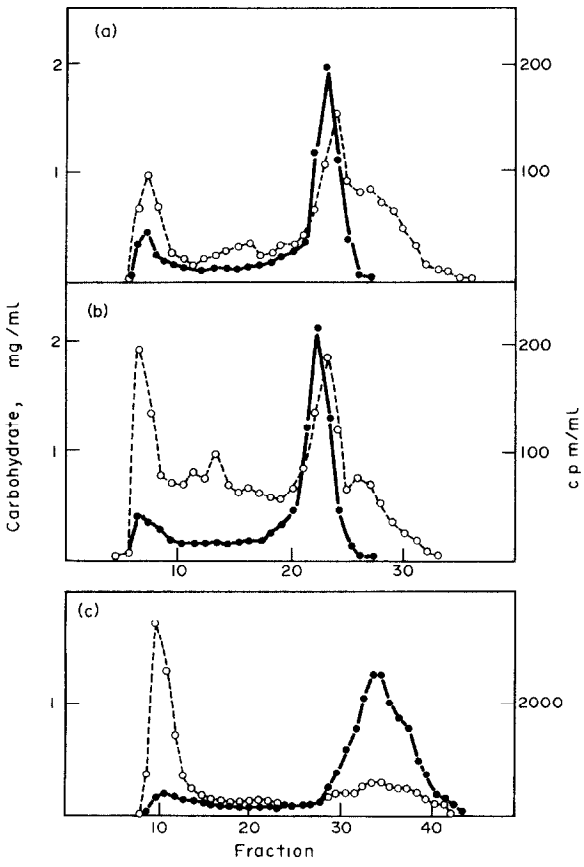

Fig. 1. Association of carbohydrate and phenol in polysaccharidase digestion of cell walls (a) Washed callus cell walls incubated with coniferyl alcohol-[^{14}C] and H_2O_2. (b) Whole callus cells incubated with coniferyl alcohol-[^{14}C] and H_2O_2. (c) Seedling stem sections incubated with cinnamic acid-[^{14}C]. Digestions were separated on Bio-Gel P-100. ●—carbohydrate; ○—phenol.

Table 1. Complexing of lignin with previously labeled, washed cell walls

Labeled substrate	Treatment	LTGA (μg/g)	Radioactivity of lignin complex (cpm/g)	Ara (μg/g)	Xyl (μg/g)
Glucose-[^{14}C]	Control	670	840	1	4
	Coniferyl alcohol	3754	1610	16	8
Proline-[^{14}C]	Control	1780	24480	nd	nd
	Coniferyl alcohol	9078	52170	nd	nd

Callus was first grown on media containing the labeled substrate. Washed cell walls from the cultures were then incubated with or without coniferyl alcohol and H_2O_2, and lignin thioglycolic acid prepared from the walls. Values are based on fr. wt of callus. nd not determined.

incubation. In the glucose experiment, however, considerably more arabinose and xylose were found in the LTGA from tissue with added coniferyl alcohol.

Labeling of walls with proline-[^{14}C] was included because of the finding of arabinose in LTGA from glucose-labelled tissue, suggesting the possibility that the cell wall glycoprotein, extensin, was involved in the lignin–carbohydrate complex [5]. Proline could be catabolized into compounds giving rise to cell wall carbohydrates, but it is likely that it would contribute more heavily to cell wall protein. Thus, the significant increase in radioactivity of LTGA from proline-labeled walls indicated the presence of protein in the lignin–carbohydrate complex.

LTGA preparations from tissue incubated with and without coniferyl alcohol were analyzed for protein N and hydroxyproline, the presence of which would indicate extension in the lignin–carbohydrate complex. Three times more hydroxyproline was found in LTGA

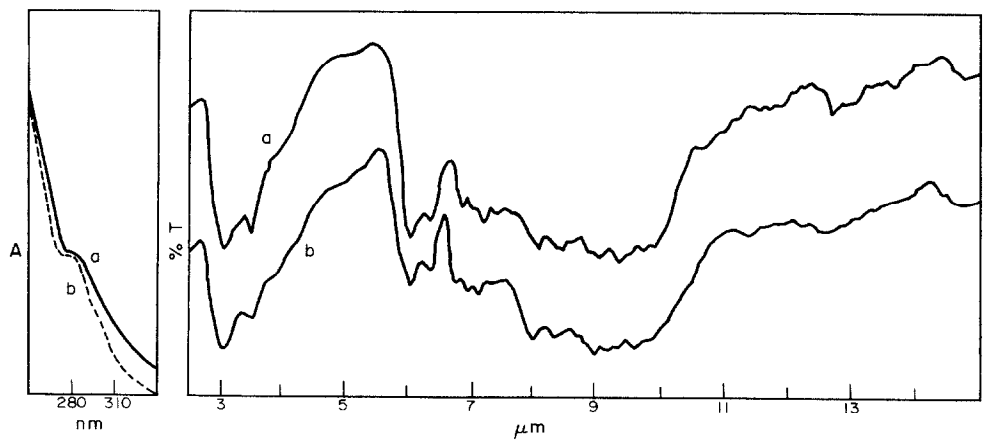

Fig. 2. UV and IR spectra of lignin thioglycolic acid prepared from (a) washed callus cell walls of slash pine incubated with coniferyl alcohol and H_2O_2, and (b) wood of slash pine seedling stems. UV spectrum from lignin dissolved in 0.5 N NaOH; IR spectrum from KBr disks.

from callus treated with coniferyl alcohol than from untreated tissue (Table 2), approximately the same ratio as protein content.

It is apparent that gravimetric determinations of LTGA from this tissue would lead to gross overestimation of lignin. Lignin can more accurately be determined in tissue cultures by a photometric analysis of LTGA preparations. LTGA is soluble in 0.5 N NaOH, in which its A at 280 nm is 12 times that of bovine serum albumin. In the case mentioned above, where LTGA contained 36% protein, the contribution to absorption by protein would be only about 3%, and could probably be ignored.

In a more detailed analysis of the lignin–carbohydrate complex formed by washed cell walls incubated in coniferyl alcohol, tissue so treated was ball milled for 15 days, then extracted with water. The extract was separated on a Bio-Glas column with an exclusion of MW 500000 for globular protein. Fractions of the extract were analyzed for protein N as well as carbohydrate and lignin. As Fig. 3a shows, the radioactivity peak coincided closely but not exactly with carbohydrate. Protein distribution was similar to carbohydrate. Only 7.9% of the bound radioactivity was removed by water extraction.

The tissue was next extracted with pronase, which removed 4.7% of the radioactivity. Separation on the Bio-Glas column gave a similar distribution of lignin and carbohydrate (Fig. 3b). Digestion of the tissue in a mixture of macerase and cellulysin for 4 days released only 2% of the radioactivity, and the mixture was not separated on the column.

Following polysaccharidase treatment, the residue was extracted with a mixture of dioxane and water for 10 days. This treatment removed 13% of the radioactivity. The dioxane extract was separated on a LH-20 column, yielding a coincident peak of lignin and a small amount of carbohydrate in the void volume.

The remaining cell wall material was used to prepare LTGA, which was dissolved in 0.5 M NH_4OH and separated on the same Bio-Glas column used before (Fig. 3c). A lignin–carbohydrate association was evident. No protein was detected in any of the LTGA fractions.

To test the possibility that the peroxidase system was not involved in catalysis of bonds between lignin and cell wall components, treatments known to inhibit peroxidase activity were applied to the incubation system, namely KCN and the absence of peroxide. Inclusion of 1 mM KCN or no H_2O_2 resulted in 61 and 58% inhibition, respectively (Table 3). Incomplete inhibition of binding in the absence of H_2O_2 suggests either the presence of phenolase in the wall or production of H_2O_2 by wall tissue [6]. LTGA made from the tissue showed similar inhibition; however, the counts were considerably lower in all treatments. The drop in radioactivity of LTGA from the total bound during incubation was found in all experiments and is interpreted as the loss of incompletely polymerized coniferyl alcohol in the extraction process.

Table 2. Protein and hydroxyproline in lignin thioglycolic acid

Treatment	LTGA (μg)	Protein (μg)	Protein/ LTGA (percent)	Hydroxyproline (μg)
Control	754	280	37	14
Coniferyl alcohol	5270	658	12	10

Washed cell walls from 10 g callus were divided into 5 g portions. One part was incubated with 25 mg coniferyl alcohol and H_2O_2; control part with H_2O_2 only. LTGA was prepared from both samples.

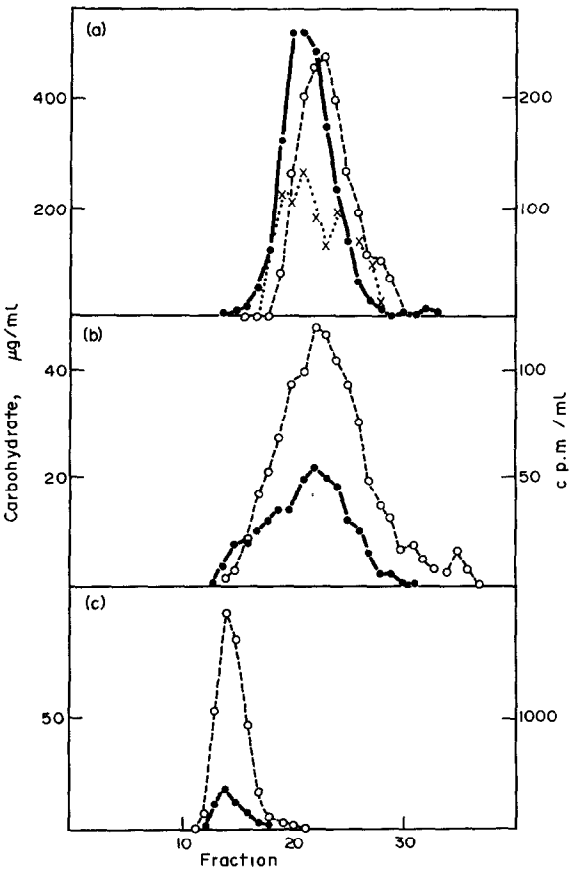

Fig. 3. Separation of fractions of callus cell walls incubated with coniferyl alcohol-[^{14}C] and H_2O_2, on Bio-Glas 1000. After incubation, wall tissue was ball-milled 15 days. (a) Water extract. (b) pronase extract. (c) Lignin thioglycolic acid. ●—carbohydrate; ○—phenol; ×—protein (same scale as carbohydrate).

DISCUSSION

The various methods used in these experiments to extract parts of the callus wall yield evidence of a strong bond, probably covalent, between lignin and carbohydrate. Indeed, the great difficulty, if not impossibility, of isolating lignin free of carbohydrate has led to general acceptance of the existence of covalent lignin–carbohydrate bonds in wood. Evidence indicates that the bonds are ether linkages [7] and the present results indicate that peroxidase in cell walls can catalyze the formation of these bonds.

The strongest evidence for bond formation comes from the experiment in which addition of coniferyl alcohol causes an increase in pre-existing wall material extracted along with LTGA. The LTGA complex also

Table 3. Inhibition of peroxidase-catalyzed lignin formation and binding to callus cell walls

Treatment	Radioactivity in walls (cpm)	Inhibition (percent)	Radioactivity in LTGA (cpm)	Inhibition (percent)
Control	14700		1890	
1×10^{-3} M KCN	5650	61	1010	47
$-H_2O_2$	6200	58	1310	31

Washed callus cell walls were incubated with coniferyl alcohol-[^{14}C] under given conditions. Control and KCN treatments contained H_2O_2. Radioactivity bound to walls determined after washing with H_2O and EtOH

contains protein and hydroxyproline, suggesting a complexing of extensin with the polymerization of coniferyl alcohol. That the hydroxyproline does not arise from the peroxidase bound to the walls is shown from the experiments of Liu and Lamport [8]. A lignin–carbohydrate–protein complex containing hydroxyproline was recently isolated from poplar callus tissue [9].

The only other tenable hypothesis which could explain the inseparability of lignin from carbohydrate without requiring covalent bonds is the 'snake cage' effect discussed by Goring [10]. In this concept, pieces of hemicellulose are trapped by physical entanglement in a lignin matrix. Such a possibility cannot be ruled out by the present experiments, for the peroxidase-catalyzed polymerization of coniferyl alcohol could take place around the hemicellulose and glycoprotein structures during the incubation.

The lignin produced in these experiments may be representative of the first steps in lignification of living cells which produce secondary, lignified walls (the tissue cultures used do not produce secondary walls and do not form much lignin, normally less than 1% of the total wall). When the first lignin monomers, such as coniferyl alcohol, are transported out of the protoplast, they encounter the thin primary wall and middle lamella, rich in peroxidase and extensin. Presumably some monomer units become covalently bound to either carbohydrate or protein, as well as to other phenolic units as lignin is formed. As the formation of secondary walls progresses in the cell, lignification would continue but lignin monomers would become bonded to earlier formed lignin or to carbohydrates, since the process would have moved out of the region containing glycoprotein.

Lack of lignification in callus tissue may result from the lack of enzyme systems to produce the phenolic monomers. I have found, for instance, very low phenylalanine-ammonia lyase activity in slash pine callus relative to slash pine stem cambium. The other phenol hydroxylating, methoxylating and reducing enzymes [11] may also be lacking in callus. The evidence from these experiments shows that the cell wall by itself, containing bound peroxidase and possibly phenolase, is capable of forming lignin if provided with coniferyl alcohol and H_2O_2. Peroxide may not be absolutely necessary; but without it the rate of lignin formation is greatly reduced.

Although the LTGA has been recommended for estimating lignin in tissue culture walls [4], caution should be exercised in its use. LTGA prepared from wood is reported to be nearly free of protein but this may not be the case in tissue culture preparations (Table 2). The lignin determinations reported for *Acer pseudoplatanus* cell cultures [12], ranging from 20 to 60% of the dry wt of walls, appear suspiciously high. Those values are for Klason lignin which is extremely susceptible to protein contamination, but the values were backed up by LTGA determinations which gave even higher yields. LTGA preparations from tissues rich in cell wall protein should be corrected for protein N or the UV absorbance used rather than weight.

EXPERIMENTAL

Preparation and incubation of callus cell walls. Callus tissue of slash pine (*Pinus elliottii*), grown on Murashige and Skoog medium [13], was ground first in a mortar with H_2O, followed by homogenization in a Polytron to disrupt cells. The tissue was washed and centrifuged 5× with H_2O. The tissue was incubated with either coniferyl alcohol-[^{14}C] or non-radioactive coniferyl alcohol. Coniferyl alcohol-[^{14}C] was synthesized by the method of ref. [14], using malonic acid-[2-^{14}C]. Non-radioactive coniferyl alcohol was purchased commercially or synthesized by the same method. The incubation medium was 0·05 M Pi buffer, pH 5.4, containing various amounts of [^{14}C]-labeled or unlabeled coniferyl alcohol and 30% H_2O_2, 0.01 ml/ml sol. Incubation was at 25° for 18 hr. Where whole cells were incubated, solns and conditions were the same as for washed cell wall tissue. After incubation, tissue was washed 2× with H_2O and 3× with EtOH. Tissue was resuspended in EtOH and samples were withdrawn for counting in a gas-flow planchet system.

Incubation of seedling stems with cinnamic acid. Bark was removed from 15 cm stem sections of 1-yr-old slash pine seedlings. Three sections were incubated in an aerated test tube containing 45 ml soln of 0.5% sucrose and 5 μCi cinnamic and 5 μCi cinnamic acid-[2-^{14}C] (0·18 mg), for 18 hr. The sections were washed with H_2O and the cambial layers scraped. Scrapings were ground in a mortar with H_2O and sand, then washed with H_2O followed by EtOH, extracted with C_6H_6–EtOH (2·1) for 2 hr, EtOH for 2 hr, and washed with H_2O.

Extraction with polysaccharidases. Cell walls of callus and seedlings were incubated in 3 ml Pi buffer, pH 5.4, 0.05 M containing 50 mg each of Macerase and Cellulysin (Calbiochem) for 18 hr at 25°. The extract was removed by centrifugation, heated at 100° for 10 min, centrifuged, then separated on a Bio-Gel P-100 column with 0.05 M NH_4 formate buffer, pH 7, as solvent. Phenolic material was determined by counting portions of the 4 ml fractions in planchets, and carbohydrates by a phenol–H_2SO_4 method [15].

Labeling of cell walls. Callus explants were grown on the medium described above, but with 2 μCi glucose-[U-^{14}C] per bottle. After 6 weeks, 2.5 g callus tissue was removed for preparation of washed cell walls as described above. The tissue was divided into 4 equal parts each containing 29500 cpm, and placed in flasks containing 25 ml of Pi buffer, pH 6.4, 0.05 M. Coniferyl alcohol, 5 mg in 0.5 ml EtOH was added to 2 flasks.

H_2O_2, 0.05 ml, was added to all flasks, which were incubated 18 hr at 25°. Tissue was washed twice with H_2O, 3× with EtOH, and lignin thioglycolic acid (LTGA) was prepared For proline-labeled walls, 2 g callus tissue was incubated for 5 days in 25 ml of the culture medium minus agar, to which was added 25 μCi proline-[U-^{14}C]. Washing of cell walls and incubation with coniferyl alcohol was done as described above for the glucose experiment.

LTGA preparation. LTGA was isolated by a modification of the method of ref [16]. EtOH-washed cell wall tissue was heated at 100° in 5 ml 2 N HCl and 0.5 ml thioglycolic acid for 4 hr. After cooling, the tissue was washed twice with H_2O. The tissue was extracted at 25° in 2 ml 0.5 N NaOH for 18 hr. The NaOH soln was collected by centrifugation, the tissue washed with 2 ml H_2O which was added to the NaOH extract, and LTGA ppd by addition of 4 drops of conc HCl. The ppd was washed with 0.1 N HCl, then dissolved in 0.5 N NaOH for spectrophotometric determination at 280 nm. Protein N was determined by Nesslerization and hydroxyproline by the method of ref. [17]. Monosaccharides were determined in some LTGA samples by autoclaving LTGA in 3% H_2SO_4 for 2 hr, neutralizing the soln with $Ba(OH)_2$, and preparing alditol acetates which were separated and identified by GLC [18].

Analysis of ball-milled cell walls. A sample of callus of 4.5 g was ground and washed as above. It was incubated with 10 mg coniferyl alcohol containing 500000 cpm, and H_2O_2 in the system previously described. The tissue was washed in H_2O and EtOH, dried at 75°, then ball-milled for 15 days with glass beads. Cell wall powder was washed from the beads with H_2O, then extracted with the same H_2O for 18 hr. Extract was concd to 2 ml and separated on a Bio-Glas 1000 column with 0.02 M NH_4 formate buffer, pH 7, as solvent. The Bio-Glas was treated with polyethylene glycol 20000 to prevent retardation by active groups on the glass surfaces. Carbohydrates in the 4 ml fractions were estimated by phenol–H_2SO_4 and N by Nesslerization. The tissue residue was extracted with 6 mg pronase in 3 ml H_2O for 18 hr at 38°. The extract was heated to 100° for 5 min, centrifuged, and separated on the Bio-Glas column described above. After pronase treatment, the residue was extracted for 4 days in 3 ml Pi buffer, pH 5.4, 0.05 M containing 50 mg each of Macerase and Cellulysin. A drop of toluene was added to inhibit growth of microorganisms. Because of the small amount of radioactivity removed, the extract was not separated. The tissue was next extracted with 3 ml dioxane–H_2O (9:1) for 10 days at 25°. The extract was separated on a column of Sephadex LH-20 with the same dioxane–H_2O as solvent. The remaining tissue was used to prepare LTGA, which was dissolved in 0.05 M NH_4OH and separated on the Bio-Glas column with the NH_4OH soln as the solvent.

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REFERENCES

1. Ridge, I. and Osborne, D. J. (1971) *Nature* **229**, 205.
2. Freudenberg, K. and Harkin, J. M. (1960) *Chem. Ber.* **93**, 2814.
3. Whitmore, F. W. (1976) *Phytochemistry* **15**, 375.
4. Lai, Y. Z. and Sarkanen, K. V. (1971) *Lignins* (Sarkenen, K. V. and Ludwig, C. H. eds) pp. 165–240. Wiley Interscience, New York.
5. Lamport, D. T. A. (1969) *Biochemistry* **8**, 1155.
6. Gross, G. G. (1977) *Phytochemistry* **16**, 319.
7. Brownell, H. H. (1971) *Tappi* **54**, 66.
8. Liu, E. H. and Lamport, D. T. A. (1974) *Plant Physiol.* **54**, 870.
9. Fukuda, T. and Kanda, T. (1976) *Mokuzai Gakkaishi* **22**, 564.
10. Goring, D. A. I. (1971) *Lignins* (Sarkanen, K. V. and Ludwig, C. H. eds) pp. 695–768. Wiley Interscience, New York.
11. Mansell, R. L., Stockigt, J. and Zenk, M. H. (1972) *Z. Pflanzenphysiol.* **68**, 286.
12. Carceller, M., Davey, M. R., Fowler, M. W. and Street, H. E. (1971) *Protoplasma* **73**, 367.
13. Murashige, T. and Skoog, F. (1962) *Physiol. Plantarum* **15**, 473.
14. Freudenberg, K. and Niedercorn, F. (1956) *Chem. Ber.* **89**, 2168.
15. Hodge, J. E. and Hofreiter, B. T. (1962) *Methods in Carbohydrate Chemistry* (Whistler, R. L. and Wolfrom, M. L. eds) Vol. I, pp. 380–394. Academic Press, New York.
16. Brauns, F. E. (1952) *The Chemistry of Lignin* p. 87. Academic Press, New York.
17. Leach, A. A. (1960) *Biochem. J.* **74**, 70.
18. Borchardt, L. G. and Piper, C. V. (1970) *Tappi* **53**, 257.