Characterization of Lignin Deposition in *Pinus taeda* L. Cell Suspension Cultures

by

Thomas Leonard Eberhardt

Dissertation submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Wood Science and Forest Products

APPROVED:

N. G. Lewis

G. Ifju

C. L. Cramer

W. G. Glasser

J. L. Hess

R. H. White

March, 1992

Blacksburg, Virginia

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Committee Chairman and Co-Chairman: G. Ifju and N. G. Lewis

(ABSTRACT)

Pinus taeda L. suspension culture cells were used to develop a model system to study the process of lignification occurring during the early stages of cell wall formation and maturation. Chemical, biochemical and histochemical analyses of the *P. taeda* suspension cultures grown with 2,4-dichlorophenoxyacetic acid (2,4-D) as the growth regulator did not provide conclusive evidence for lignin deposition. On the other hand, cultures in which 2,4-D was substituted with α-naphthaleneacetic acid (NAA) were shown to lignify. During this induction of lignification, limited cell wall thickening occurred since transmission electron microscopy of the 2,4-D grown cells showed only primary walls while the average cell wall thickness of the NAA-grown cells was consistent with secondary (S_1) layer formation. Despite the possibility of only limited lignin deposition in the 2,4-D grown cells, secondary metabolism had occurred as evidenced by reversed-phase and chiral chromatographic separations which revealed the ability of these cells to produce enantiomerically pure (-)-matairesinol. Administrations of [1-^{13}C], [2-^{13}C] and [3-^{13}C] specifically labeled phenylalanines to the *P. taeda* suspension cultures in medium containing NAA allowed the determination of lignin bonding patterns *in situ* by solid-state ^{13}C NMR spectroscopy of the resulting ^{13}C enriched cells. Aqueous and organic solvent extractions and protease treatment yielded ^{13}C enriched cell walls for solid-state ^{13}C NMR spectroscopic analyses of the cell wall bound

lignin component. Subsequently, an isolated lignin derivative from these cell walls was analyzed by solution-state ^{13}C NMR spectroscopy and verified the assignments made in the solid-state. Accordingly, the above experiments represent the first demonstration of lignin bonding patterns *in situ* in a *Pinus* species as well as a suspension culture. This culture system possesses great potential as a model to thoroughly study the early stages of lignification.

ACKNOWLEDGEMENTS

I wish to express my gratitude to Dr. Norman G. Lewis whose efforts made this work possible. To the members of my Committee, Drs. Carole L. Cramer, Wolfgang G. Glasser, John L. Hess, Geza Ifju, and Robert H. White, I express my most sincere appreciation for their assistance and cooperation. The expertise of Dr. J. B. Wooten and the use of the NMR spectroscopy facilities at the Philip Morris USA Research Center are most gratefully acknowledged. I also wish to thank the following: Dr. Lanfang He for providing the electron micrographs; Dr. Wai-Lam Chu for the acquiring of solution-state NMR spectra; Drs. Laurence Davin and Mark Bernards for suggestions and editing; and others, past and present, in the research group who provided assistance. Furthermore I would like to acknowledge the late Herbert F. Eberhardt who had the practical skills and techniques which provided a model and an inspiration to develop my laboratory skills. It deeply saddens me that despite his determination he was not able to see this work to completion. I also acknowledge Eunice B. Eberhardt, my mother, who along with the rest of my family provided support and understanding. The encouragement of Dr. Dana R. Spencer and Marilyn S. Spencer is appreciated. Foremost, I wish to acknowledge Dawn S. Eberhardt, my wife, who gave me the love and understanding I needed to finish this degree.

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1. INTRODUCTION

The formation of plant cell walls is a highly orchestrated process for which our understanding is not yet complete. Although progress has been made concerning certain aspects of cell wall development, in many instances our knowledge is based more on speculation than fact. In this section, our current understanding of the processes involved in cell wall deposition and expansion will be discussed. Lignification of the cell wall is emphasized by a description of its biosynthesis and structural characteristics. Moreover, it will be revealed that previous studies suggesting lignification in plant suspension cultures have not been conclusive thereby necessitating further investigation prior to the use of these plant systems as a model to study lignin deposition.

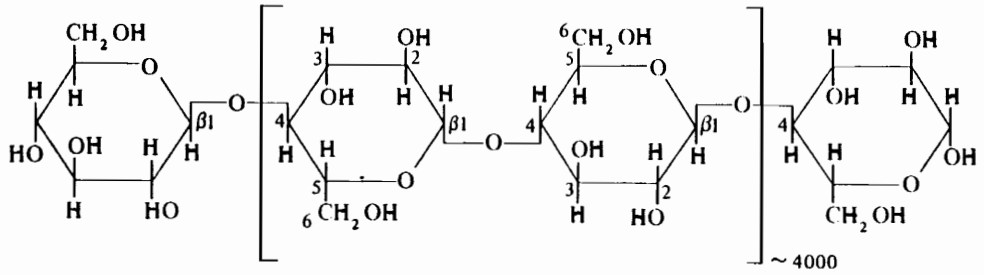
1.1. Plant Cell Wall Maturation Processes

Tissues in roots, stems and leaves of vascular plants are generally comprised of two cell types; one type performs the metabolic functions essential for life while the other is metabolically inactive and provides mechanical support and in some cases, nutrient conduction. A typical mature, metabolically active plant cell (*e.g.* parenchyma, mesophyll or epidermal) possesses a functional protoplast surrounded by a primary cell wall sometimes with secondary thickening. On the other hand, for a mature, metabolically inactive cell (e.g. tracheid, fiber or vessel), the protoplast has ruptured to leave a hollow cell comprised of primary and secondary cell walls. When connected end to end and grouped together such hollow cells form a vascular bundle through which fluids are translocated throughout the plant. Vascular tissues, in which fluid conduction has ceased, continue to provide mechanical support.

Plant growth results from both cell division and expansion. In the meristematic regions of the plant, a single cell can divide to form two daughter cells each having a volume approximately equal to half that of the mother cell (Albersheim, 1975). These daughter cells then expand and increase their "lengths" as much as 100-fold (Albersheim, 1975). During the process of cell expansion the cell walls formed are typically described as being primary in nature. Once cell expansion has ceased, the overall thickness of the cell walls can increase through the deposition of secondary cell wall layers to the interior of the existing primary cell walls. Such secondary thickening, which occurs during plant cell differentiation, provides particular cell types with characteristics optimized for specific functions. For example, tracheids possess thick secondary cell walls which allow for the conduction of fluids and to provide mechanical support.

1.2. Primary Cell Wall Formation.

In primary cell walls, the major organic constituents are the polysaccharides including cellulose and other polyoses (hemicelluloses and pectins). Cellulose is a linear polymer comprised of cellobiose repeating units linked by β-(1$\rightarrow$4) glycosidic linkages (see Figure 1). Since the various methods used during cellulose isolation from woody (angiosperm and gymnosperm) tissues may result in chain cleavage, the average degree of polymerization (D.P.) of 4000 cellobiose residues commonly reported may be a minimum estimate (Delmer, 1983). In plant cell walls, approximately 60-70 of these cellulose chains aggregate by hydrogen bonding to form microfibrils having a cross-sectional diameter estimated to be 4.5 x 8.5 nm (Dey and Brinson, 1984). Highly organized areas of the microfibril are crystalline while other areas are amorphous.



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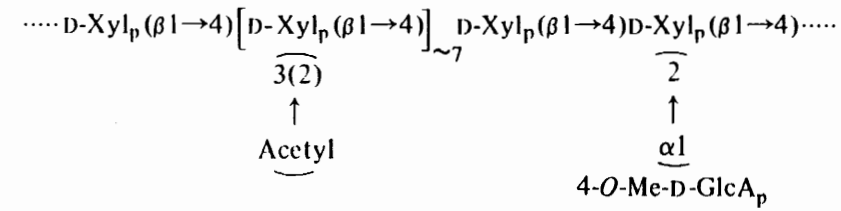

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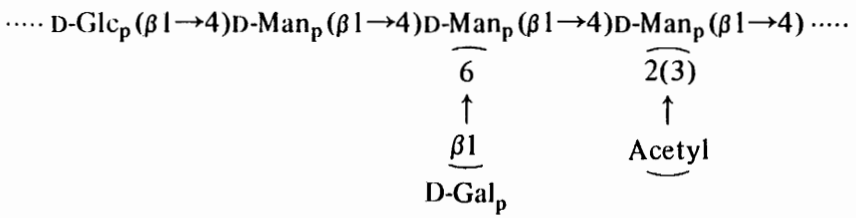

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Figure 1. Cell Wall Polysaccharides: **a**) Cellulose, **b**) Hemicellulose (xylan) **c**) Hemicellulose (mannan) (D-Xyl_p = D-xylopyranose; 4-*O*-Me-D-$GlcA_p$ = 4-*O*-methyl-D-glucuronic acid; D-Glc_p = D-glucopyranose; D-Man_p = D-mannopyranose; D-Gal_p = D-galactopyranose) [Reproduced from T.W. Goodwin and E.I. Mercer, 1988].

Hemicelluloses, on the other hand, are branched heteropolymers containing two or more different sugar moieties (see Figure 1). Two hemicelluloses, the glucomannans and galactoglucomannans, are typically found in gymnosperms and have a β-(1→4)-D-glucan backbone of glucose and mannose units, and in the latter case, single-unit side chains of galactose (Aspinall, 1981; Hori and Elbein, 1985). In angiosperms, xylans are typically found and possess a β-(1→4)-D-xylose backbone with side chains of 4-*O*-methyl glucuronic acid and/or L-arabinofuranose residues (Aspinall, 1981; Hori and Elbein, 1985).

Finally, pectins are also branched heteropolymers but are primarily comprised of glucuronic acid units (see Figure 2) as shown for tobacco (*Nicotiana*) (Sun *et al.* 1987), sugar-beet (*Beta saccharifera*) (Keenan *et al.*, 1985) and sycamore (*Acer pseudoplatanus*) (Goodwin and Mercer, 1983). Other significant sugar constituents in *Beta saccharifera* pectin preparations include arabinose, galactose and rhamnose (Keenan *et al.*, 1985).

The formation of the primary cell wall begins during cell division when vesicles from the Golgi coalesce at an equatorial plane to form a cell plate (Lopez-Saez *et al.*, 1982). The fusion of these vesicles results in new plasma membrane formation on both sides of the cell plate, encapsulating the newly forming daughter cells (Lopez-Saez *et al.*, 1982). As division proceeds, each daughter cell produces a primary wall; the region between these primary cell walls is the middle lamella. It has generally been presumed that the cell plate becomes the middle lamella between two cells and that its composition is primarily pectic (Goodwin and Mercer, 1983).

Recent immunolocalization studies directed towards defining the process of cell plate, middle lamella and primary cell wall formation have confirmed the pectic nature of the middle lamella in red clover (*Trifolium pratense*) (Moore and

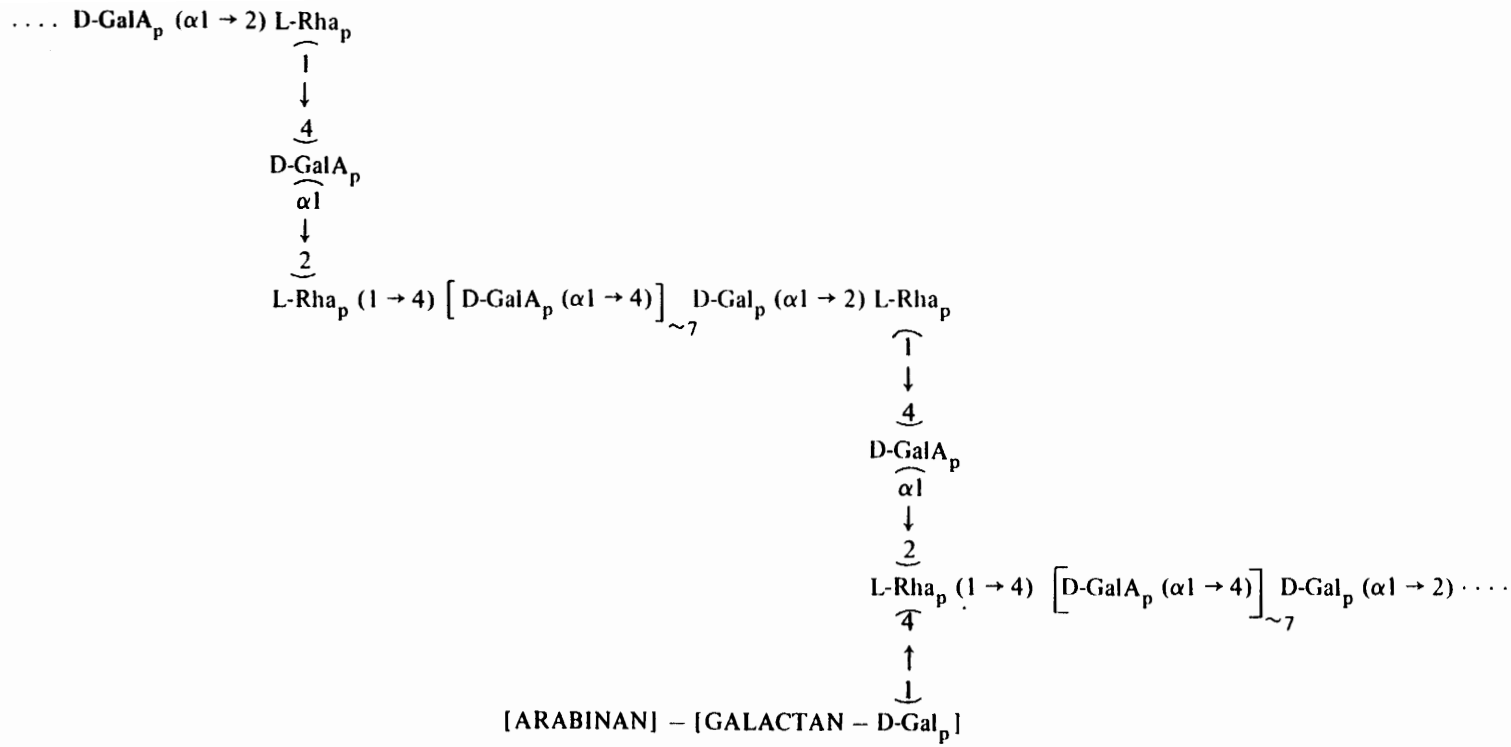

Figure 2. Pectin (rhamnogalacturonan) from Sycamore (D-GalA$_p$ = D-galacturonic acid; L-Rha$_p$ = L-rhamnopyranose; D-Gal$_p$ = D-galactopyranose) [Reproduced from T.W. Goodwin and E.I. Mercer, 1988].

Staehelin, 1988). On the other hand, these studies did not agree with the presumed pectic nature of the cell plate since it was shown that it was comprised mainly of xyloglucan, a hemicellulose; virtually no pectin (rhamnogalacturonan) was detected (Moore and Staehelin, 1988). Moreover, in *Phaseolus vulgaris* and *Zinnia elegans*, only a hemicellulose (arabinogalactan) and callose (β-(1→3)-glucan) were localized in the cell plate (Northcote *et al.*, 1989). Therefore, it appears that the presumed pectic nature of the cell plate may be quite erroneous.

Both xyloglucan and rhamnogalacturonan biosynthesis in *Trifolium pratense* occurs in association with the Golgi apparatus as demonstrated by immunogold localization of these polysaccharides on this organelle (Moore and Staehelin, 1988). Similarly, localization of xylan in Golgi vesicles in *Phaseolus vulgaris* suggests that the mechanism of hemicellulose transport is vesicular (Northcote, 1989). Therefore, the non-cellulosic polysaccharides translocated within vesicles derived from the Golgi apparatus presumably form an amorphous matrix in the newly forming cell wall (see Figure 3).

The exact temporal stage at which cellulose deposition occurs during primary cell wall formation is not yet well understood. In immunolocalization experiments in *Phaseolus vulgaris* and *Zinnia elegans* mentioned previously, efforts to localize cellulose deposition failed (Northcote *et al.*, 1989). Antigens to cellobiose and a β-(1→4) glucan (D.P., 6-8) did not recognize cellulose within the cell wall where it is known to occur. It was concluded by the authors that the cellulose chains are highly organized and tightly bound by intermolecular hydrogen bonds thereby preventing antigenic recognition. Unfortunately, an appropriate control experiment was not described such as the determination of antigen binding to a cellulose

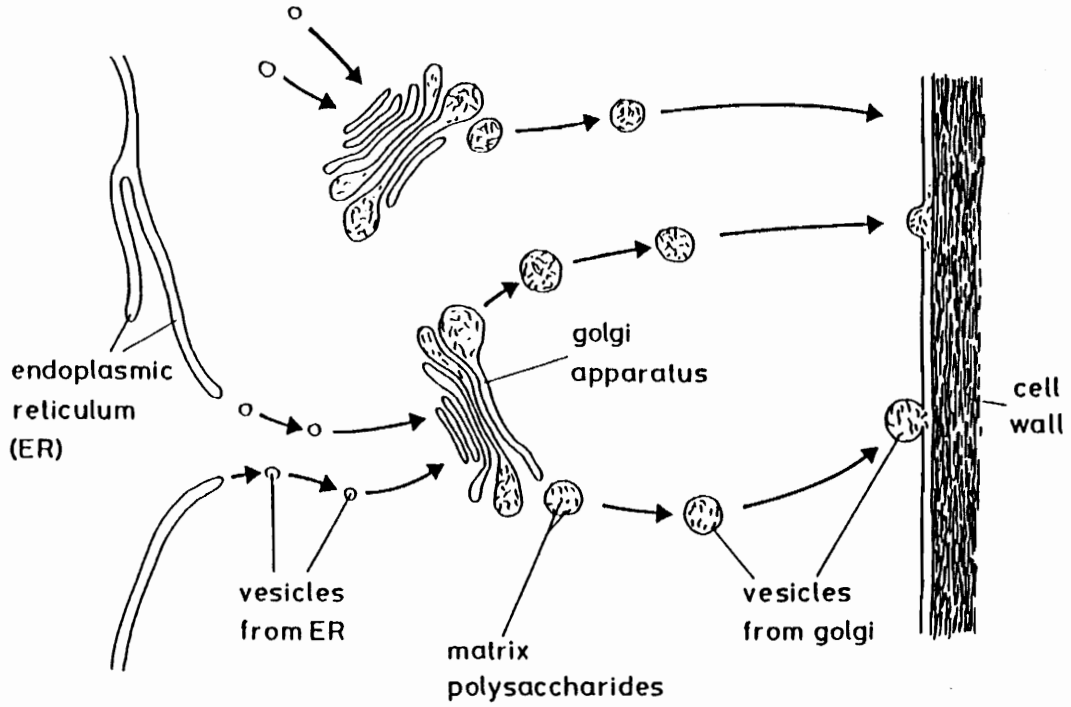

Figure 3. Model of Cell Wall Matrix Polysaccharide Transport from Golgi Apparatus to Cell Wall Via Golgi-derived Vesicles [Reproduced from C. Brett and K. Waldron, 1990].

preparation. Therefore, the lack of antibody binding to cellulose in the cell wall cannot yet be attributed to the properties of cellulose alone.

Cellulose biosynthesis has been widely studied in the bacterium *Acetobacter xylinum* where it is synthesized as an extracellular secretion (D.P. 2000-6000) (Delmer, 1987). Synthesis of a β-(1→4) glucan from UDP-glucose can be accomplished *in vitro* using membranes isolated from *A. xylinum* (Delmer, 1987). Although this β-(1→4) glucan may represent cellulose, this has yet to be established, hence the identification of the product as a glucan rather than cellulose. Regulation apparently occurs through the coupling of two molecules of guanosine triphosphate (GTP) to form one molecule of cyclic guanylic acid which has been shown to be an activator of the presumed cellulose synthase (Ross *et al.*, 1987). This activator has been suggested to be degraded by another membrane bound enzyme which has its activity inhibited by Ca^{2+} (Ross *et al.*, 1987).

A digitonin-solubilized "cellulose" synthase reported for *A. xylinum* has been shown to synthesize a β-(1→4) glucan which was considered to resemble cellulose since it was able to bind a gold-labelled cellobiose hydrolase and had electron diffraction pattern typical for cellulose (Lin *et al.*, 1985). During these experiments, the product was shown to be formed *de novo* as evidenced by incorporation of [^{3}H]-UDP-glucose into the polymer. However, it was not typical of native *A. xylinum* cellulose but rather resembled that produced by this organism when grown in the presence of chemical reagents which interfere with crystallization. One such reagent, Calcofluor White ST, when used to treat *A. xylinum* cells during microfibril formation, resulted in a 4-fold increase in glucose polymerization (Benziman *et al.*, 1980). After the removal of the Calcofluor, formation of typical cellulose microfibrils resumed. It therefore

appears that formation of crystalline regions in cellulose microfibrils retards the rate of polymer synthesis.

In higher plants, attempts to synthesize cellulose *in vitro* with a membrane preparation and radioactive UDP-glucose as substrate have resulted in the synthesis of callose (β-(1→3)-D-glucan) and not cellulose (Delmer, 1987). The inability to isolate cellulose synthase in higher plants may be related to the instability of cellulose synthase or to the loss of a specific activator or regulatory protein. Although cellulose abounds in the plant cell wall, the enzyme(s) responsible for its synthesis awaits detection and characterization.

Despite this lack of knowledge, cellulose biosynthesis has been presumed to occur at the plasma membrane. This presumption is primarily based on the observation of globular structures (terminal complexes) in association with striations on the plasma membrane comparable in diameter to microfibrils (see Figure 4) (Delmer, 1987). Visualization of these intra-membranous terminal complexes by scanning electron microscopy can be made after specimen preparation by the freeze-fracture technique whereby the membrane lipid bilayer is separated. In various species of vascular plants and green algae, the terminal complexes appear as rosettes having six particles arranged hexagonally on the protoplasmic face with a complementary globule on the exoplasmic face of the separated membrane (Hotchkiss, 1989).

The above-mentioned terminal complexes have been observed to show variable degrees of consolidation on the plasma membrane. In this respect, terminal complexes that are solitary or occur in rows have been suggested to be associated with microfibril assembly during primary cell wall formation (Hotchkiss, 1989). On the other hand, microfibril formation during secondary wall formation may be

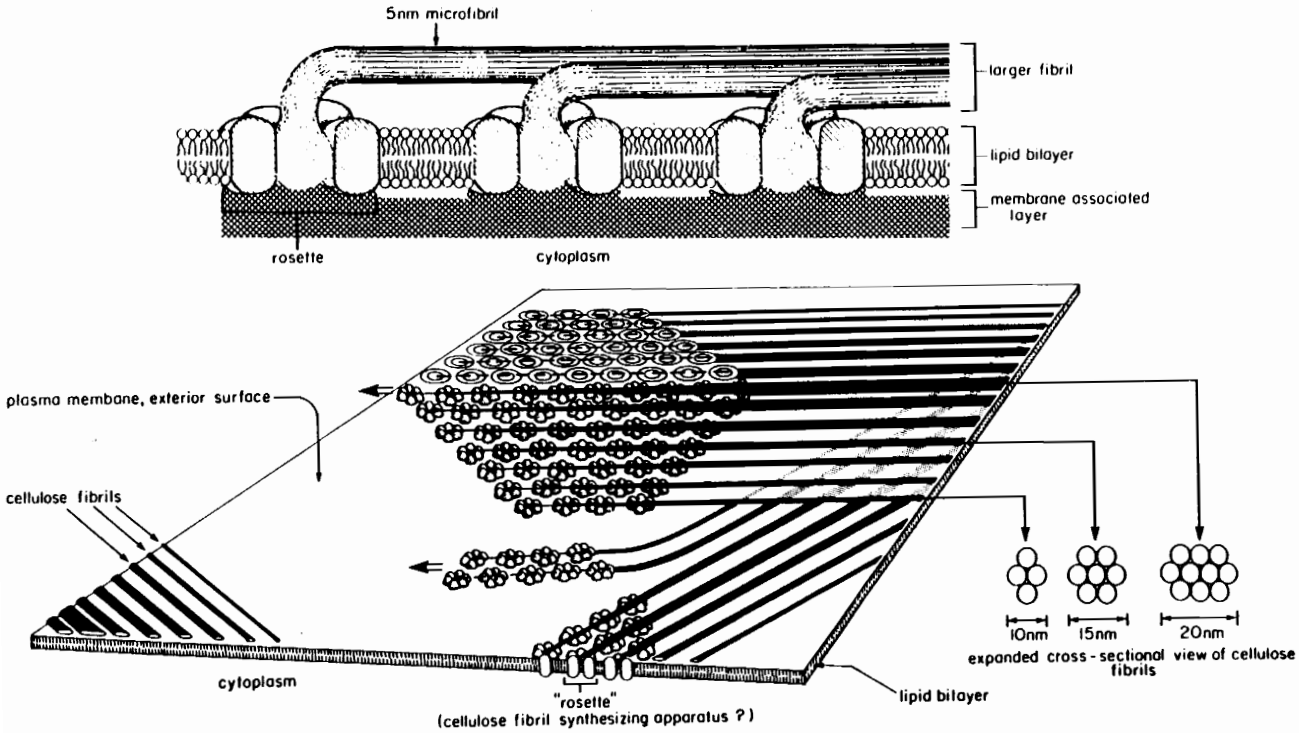

Figure 4. Model of Cellulase Synthase on Plasma Membrane [Reproduced from C. Brett and K. Waldron, 1990].

from hexagonal arrays of terminal complexes (Hotchkiss, 1989). Accordingly, the degree of terminal complex consolidation has been suggested to be related to the width of the microfibril produced.

Direct evidence for the involvement of the terminal complexes in cellulose biosynthesis is not yet available. Problems with defining the exact function of these terminal complexes may be related to their apparent instability. In one study, terminal complexes were observed on the plasma membrane of *Glycine max* suspension cultures by freeze-fracture and electron microscopy with the samples directly transferred to the cell holder (Herth and Weber, 1984). Interestingly, cells collected by centrifugation prior to placement on the cell holder did not show any terminal complexes (Herth and Weber, 1984). Therefore, if the observed terminal complexes catalyze the biosynthesis of cellulose in plants, such experiments demonstrate that the inaccessibility of the cellulose biosynthetic system is likely related to its apparent instability.

As the cell wall expands, polysaccharide deposition continues since during this expansion process the thickness and composition of the primary cell wall does not appear to change (Albersheim, 1975). The rate of cell expansion, presumably driven by osmosis, is limited by the rate at which the cell wall can stretch (Albersheim, 1975). Efforts to explain the phenomenon of cell expansion have resulted in a variety of hypotheses regarding the structure of the primary cell wall. Such hypotheses have been used to formulate diagrammatic models of the primary cell wall. Models put forward by the research groups of Albersheim, Lamport and Fry are discussed below.

1.3. Models of the Primary Cell Wall.

Albersheim's Model: The model proposed by Albersheim and coworkers (see Figure 5) was based on data obtained with suspension cultures of sycamore (*Acer pseudoplatanus*), which were chosen because unlike intact plants, it is possible to obtain a relatively homogeneous population of cells with only primary cell walls (Albersheim, 1975). In this model, each cellulose microfibril is coated with a xyloglucan monolayer linked via hydrogen bonding. The xyloglucan is purportedly covalently linked to another hemicellulose, arabinogalactan, which in turn is thought to be covalently bonded to pectin. Finally, cell wall structural proteins such as extensins (hydroxyproline rich glycoproteins) are covalently bonded to the cell wall pectins. [When this model was first suggested, the involvement of proteins as a structural component of the primary cell wall had not been identified. Accordingly, they were absent from initial representations of the primary cell wall model.]

Evidence for Albersheim's model was derived from various extractions and enzymatic degradations of cell walls from sycamore suspension cultures. Treatment of sycamore cell walls with 8 M urea resulted in the release of "small amounts of a 4-linked glucan" as characterized by methylation analyses (Bauer *et al.*, 1973). Since the glucan showed only negligible degradation by treatments with amylase it was assumed to be β-(1→4) linked and suggested to be cellulosic. Other cell wall polysaccharides (e.g. hemicelluloses, pectins) were not removed by this urea treatment. Since urea is widely used to disrupt hydrogen bonds, the above results were interpreted as meaning that the non-cellulosic polysaccharides were covalently linked to each other and associated with the isolated β-(1→4) glucan (cellulose) via hydrogen bonding. Next, treatment of endopolygalacturonase-pretreated cell walls with endoglucanase or 0.5 M NaOH resulted in the release of

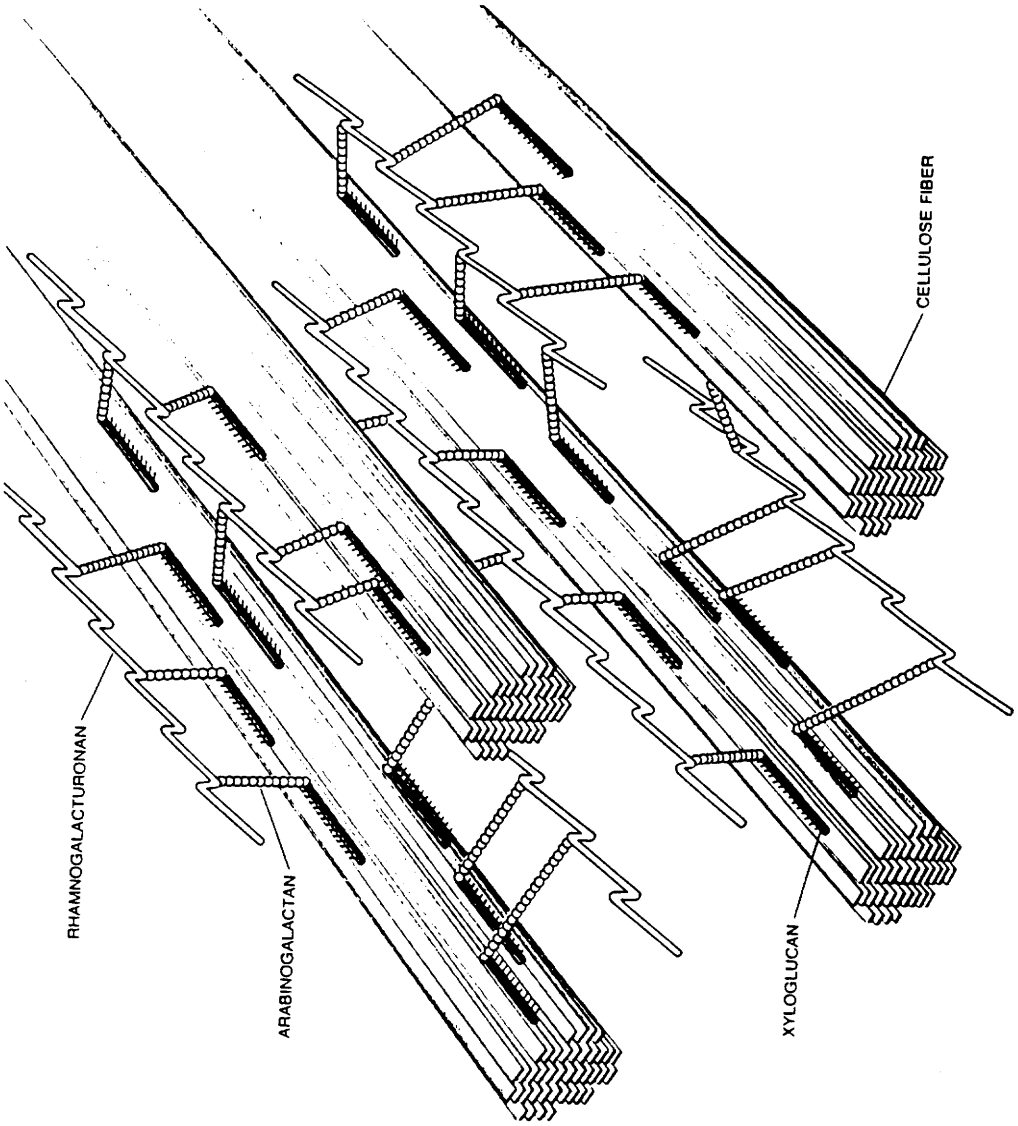

Figure 5. Model of Primary Cell Wall as Proposed by Albersheim and Coworkers [Reproduced from Albersheim, 1975].

xyloglucan (or xyloglucan fragments). In relation to this result, it was proposed that a xyloglucan was hydrogen bonded to the cellulose microfibrils. Covalent linkages between the cell wall matrix polysaccharides such as xyloglucan and pectin were suggested by the isolation of oligosaccharides possessing sugar moieties reflecting both constituents. Finally, treatment of endopolygalacturonase-endoglucanase-pretreated cell walls with pronase released oligosaccharide fragments thereby suggesting covalent linkages between cell wall proteins and matrix polysaccharides (Keegstra *et al.*, 1973).

To allow for cell wall expansion in the Abersheim model, bond cleavage between or within existing polysaccharide chains and the synthesis of new polysaccharides would likely be required. In relation to the latter point, autoradiographic studies involving the incorporation of radiolabeled (^{3}H) sucrose showed uniform labeling of cell walls in expanding cells (Albersheim, 1975). Accordingly, *de novo* polysaccharide synthesis appeared to occur uniformly throughout the expanding cell wall matrix. Moreover, since it had already been noted that cell wall thickness did not change during expansion, this implied that polysaccharide cleavage also occurs uniformly during cell wall expansion. This cleavage of covalent polysaccharide bonds was presumed to be enzyme mediated although at that time the identity of the enzyme involved or the specific polysaccharides cleaved was not yet known.

Subsequent studies on lupin (*Lupinus*) and mung bean (*Phaseolus aureus*) hypocotyls yielded results which were suggested to be inconsistent with Albersheim's model (Monro *et al.*, 1976). In these experiments the treatment of the hypocotyl cell walls with 10% KOH (20-24°C) removed hemicelluloses but neither pectins or proteins. If pectins were glycosidically linked to the hemicelluloses as depicted in the model it could be assumed that pectin would

accompany the removal of these hemicelluloses. The contradictory result above suggested that in lupin and mung bean an alternative linkage which is alkali-labile may be responsible for the attachment of the hemicelluloses to pectins. One possible linkage suggested was an ester bond of hemicelluloses with pectin uronic acid moieties. Accordingly, it has been indicated that the model developed with sycamore suspension culture cells may not be completely applicable to other systems such as mung bean and lupin hypocotyls.

Lamport's Model : This model proposes that the primary cell wall is organized in a fashion similar to that of a woven fabric where the cellulose microfibrils represent the "warp" and the cell wall protein the "weft" (see Figure 6) (Lamport, 1986). In this case, cell wall proteins which can represent up to 10% of the primary cell wall (Kieliszewski and Lamport, 1988) are essential to maintain the structural integrity of the primary cell wall. These structural proteins, called extensins, are highly basic hydroxyproline-rich glycoproteins that contain approximately 33 to 50% protein (Kieliszewski and Lamport, 1988); the carbohydrate fraction is comprised of arabinoside oligosaccharides *O*-linked to the hydroxyproline moieties (Kieliszewski and Lamport, 1988).

In Lamport's model, cellulose microfibrils are interwoven into a highly organized matrix of extensin (Lamport, 1986). Through coupling of the extensin chains by isodityrosine linkages (see Figure 6), pores are formed through which the cellulose microfibrils pass. Interaction between the cellulose microfibrils and extensin is a mechanical restraint rather than a chemical linkage or association. Thus, during cell wall expansion the cellulose microfibrils are envisaged to be free to slide through the pores of the extensin network. Expansion of the cell wall in a

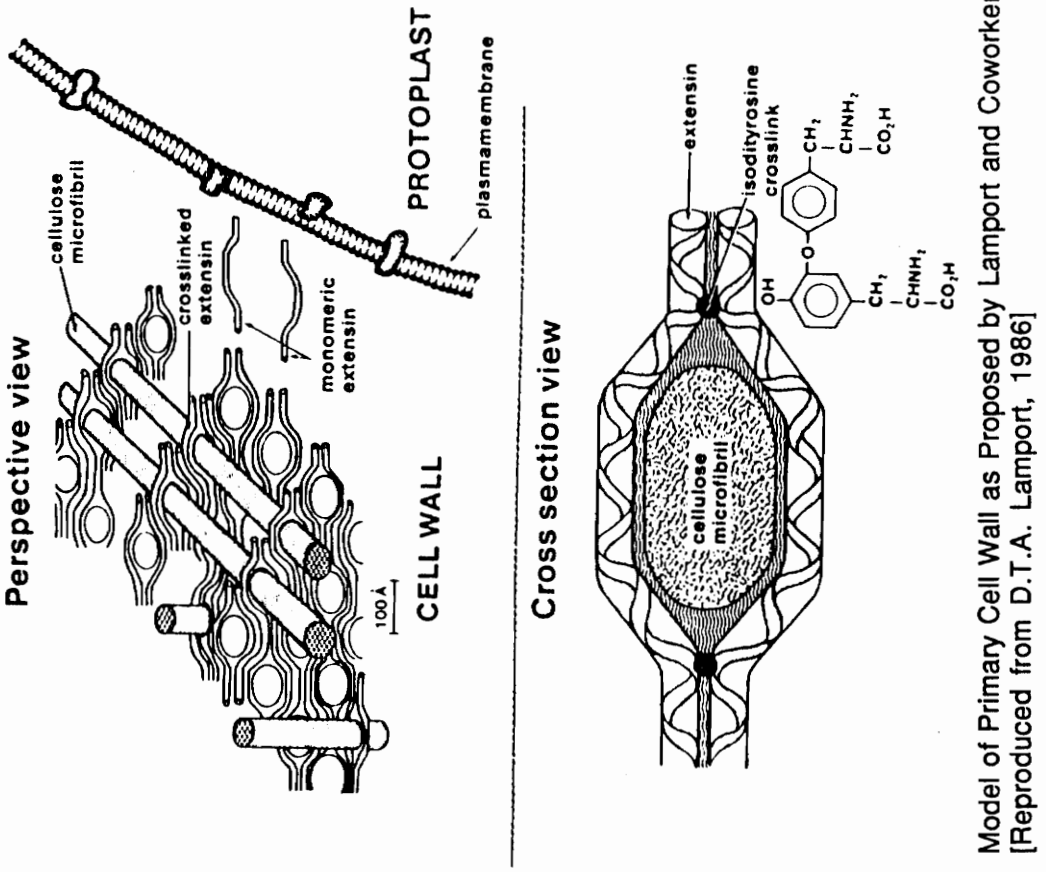

Figure 6. Model of Primary Cell Wall as Proposed by Lamport and Coworkers [Reproduced from D.T.A. Lamport, 1986]

direction transverse to the cellulose microfibrils is presumed to occur through cleavage of the existing extensin network and deposition of new extensin chains.

This model is purely speculative since no direct evidence is available to support that extensin functions as such in the primary cell wall. Instead, all that has been demonstrated is the nature of one of the likely crosslinks between the extensin chains which may account for extensin insolubility during certain acid hydrolyses of cell walls. Treatment of glycoproteins with anhydrous hydrofluoric acid under certain conditions (1h, 0°C) has been shown to cleave glycosidic but not peptide linkages (Mort and Lamport, 1977). Under said conditions, treatment of tomato (*Lycopersicon*) suspension culture cells with anhydrous hydrofluoric acid showed that the cell wall proteins remained insoluble thereby suggesting that glycosidic linkages were not involved in the binding of extensin to the cell wall matrix (Mort and Lamport, 1977).

Subsequently, extensin insolubility in the cell wall was suggested to be due to the isodityrosine phenolic ether linkage between extensin chains; this was proposed following the isolation and characterization of isodityrosine from potato (*Solanum tuberosum*) acid hydrolysates from cell walls treated with 6 M HCl (Fry, 1982). Cleavage of the isodityrosine linkage has been demonstrated with acid sodium chlorite which has long been known to cleave phenolic ether linkages (Biggs and Fry, 1990). Under conditions where this treatment does not cleave peptide bonds, extensin was released from the cell walls of *Capsicum frutescens* (Biggs and Fry, 1990). The release of extensin from *Phaseolus coccineus* cell walls has also been reported using a comparable acid sodium chlorite treatment (O'Neill and Selvendran, 1980). Therefore, it has been suggested that the isodityrosine linkage is responsible for the observed insolubility of extensin in the cell wall.

Presumably, these isodityrosine linkages are formed within the cell wall matrix since amino acid analyses of tomato (*Lycopersicon esculentum*) suspension culture cell walls showed the release of extensin chains with little or no isodityrosine moieties (Smith *et al.*, 1984). This result, along with those described above, suggest that the observed extensin insolubility in the cell wall matrix is the result of the coupling of extensin chains in the cell wall by the isodityrosine linkage. There is no evidence, however, that the isodityrosine linkages between extensin chains mechanically restrain the cellulose microfibrils as depicted by Lamport's model of the primary cell wall.

Fry's Model : The isodityrosine linkages between extensin chains is also an important component of the primary cell wall model for Dicotyledenous plants put forward by Fry and coworkers (see Figure 7)(Fry and Miller, 1989). In this model, xyloglucan chains are randomly associated across a cellulose framework and association occurs through hydrogen bonding. The xyloglucan chains restrict lateral expansion since they straddle more than one cellulose microfibril. Loops of extensin and/or pectin with associated phenolic moieties, formed via phenolic coupling, tether a particular cellulose microfibril and its associated xyloglucan. In the case of extensin, the phenolic coupling is presumed to be through the isodityrosine linkage.

Only indirect evidence is available to support this model and it is solely based upon specificity of the mode of phenolic coupling. *In vitro* coupling of tyrosine moieties in protein gives isodityrosine (as opposed to dityrosine) using plant cell walls at pH 6 (Fry and Miller, 1989). In contrast, animal structural proteins contain dityrosine (rather than isodityrosine). Likewise dityrosine is produced from extensin treated *in vitro* with peroxidase and H_2O_2 under more alkaline

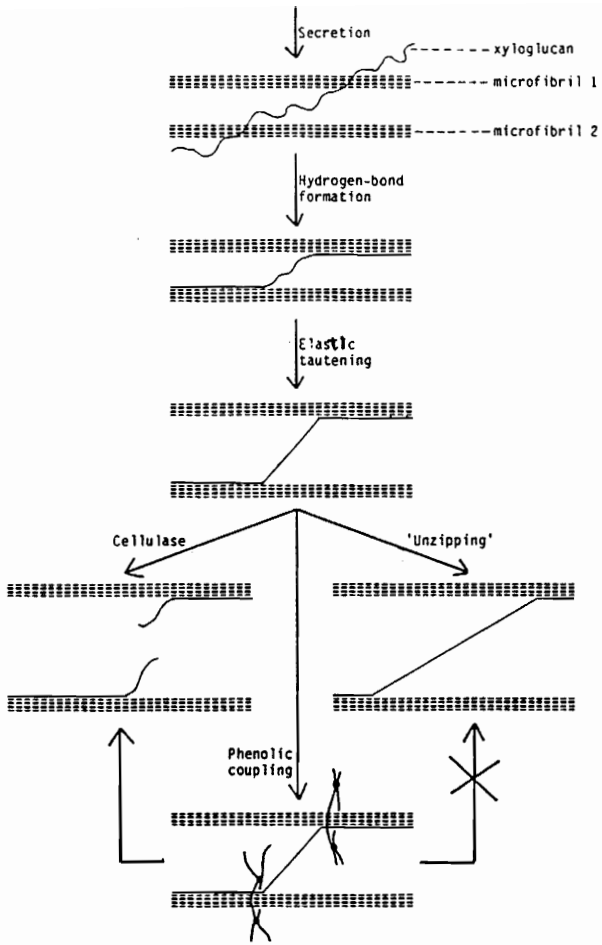

Figure 7. Model of Primary Cell Wall as Proposed by Fry and Coworkers [Reproduced from S.C. Fry and J. G. Miller, 1989]

conditions (pH 9). Unfortunately, no data is available to explain how formation of the isodityrosine linkage occurs in plant cell walls.

Despite its scant foundation, the model has been used in an attempt to explain the process of cell wall expansion (Figure 7). During expansion, a presumed "tautening" of the xyloglucan chains between two cellulose microfibrils occurs. Further expansion of the cell wall would then result in a breakage of hydrogen bonds between the xyloglucan and cellulose microfibrils, or the cleavage of the xyloglucan chain itself. Extensin loops surrounding the microfibril and xyloglucan provide mechanical support and prevent hydrogen bond cleavage resulting from the pulling of the xyloglucan chain away from the microfibrils. In this situation, further expansion would occur through the cleavage of the restrained xyloglucan chains.

The model by Fry, as well as those by Lamport and Albersheim, provides descriptions of the primary cell wall and attempts to explain cell wall expansion. Unfortunately, as the true complexity of the architecture of the primary cell wall is hardly understood, these models have little sound experimental foundation. Although not incorporated into formal models, several hypotheses for the mechanism(s) of cell wall expansion have been proposed. These hypotheses are described below, but as with the models above, there is little definitive experimental evidence at present.

1.4. Cell Wall Expansion.

Wall extensibility has been correlated with changes in cell wall pH which can occur during the treatment of a variety of plant stems with auxin (Jarvis, 1984, Cleland, 1981). Two mechanisms have been proposed to explain this phenomenon of cell extensibility and reduction in pH: in the first, the reduction in cell wall pH provides conditions which are more suitable for enzyme catalyzed hydrolysis of cell

wall polysaccharides, whereas in the second, it results in the release of cell wall bound calcium which is considered to form ionic crosslinks between the organic acid moieties of pectins.

In elongating *Pisum sativum* internode sections in acidic buffers, rates of hemicellulose (xyloglucan) and pectin degradation were reduced by low temperature (0 °C) thereby indicating enzymatically mediated hydrolysis (Terry *et al.*, 1981). Cleavage of the xyloglucan chains is presumably catalyzed by cellulases within the cell wall. Cellulases are a group of enzymes that cleave -D-glucopyranosyl-β-(1→4)-D-glucopyranosyl- glycosidic linkages within polysaccharide chains (Fry, 1989). [Other enzymes often described incorrectly as cellulases include exo-cellobiohydrolase and β-glucosidase which cleave cellobiose and glucose moieties from the non-reducing end of the polysaccharide, respectively; note that the Nomenclature Committee of the International Union of Biochemistry disapproves of describing these as cellulases.] For the true cellulases, one might assume that they only effectively catalyze cellulose degradation. To the contrary, β-(1→4) glucans such as xyloglucan are also hydrolyzed by cellulases (Fry, 1989). Indeed, the relatively amorphous nature of xyloglucan as compared to cellulose may facilitate its degradation. Such was demonstrated during the treatment of etiolated pea (*Pisum sativum*) stems with cellulase where a facile degradation of xyloglucan was accompanied by negligible cellulose degradation (Hayashi and Maclachlan, 1984). Accordingly, it has been suggested that the crystalline nature of cellulose may exclude the enzyme thereby limiting degradation (Hayashi and Maclachlan, 1984).

Through partial degradation of the cell wall xyloglucan, cell wall expansion may therefore be facilitated. Etiolated *Pisum sativum* stem segments treated with auxin (2,4-D) underwent elongation (McDougall and Fry, 1990), which may have

resulted from cell wall loosening due to synthesis of new cellulases or to the activation of those already present in the cell wall. Interestingly, treatment of the *Pisum sativum* stem sections with xyloglucan oligosaccharide fragments resulted in about 33% of the elongation observed during auxin treatments. It was suggested by the authors that the oligosaccharides activated endogenous cellulases as evidenced by increased xyloglucan hydrolysis with an *in vitro* visometric assay for cellulase activity. Using another assay, ^{3}H labeled xyloglucan degradation to ethanol soluble products was not promoted by the same oligosaccharides. Such results suggested that for the stem sections, in the presence of the oligosaccharides, a greater proportion of mid-chain hydrolyses occurred resulting in reduced viscosity as opposed to hydrolysis of the ends of the polysaccharide chains. Through dispersed hydrolyses of the cell wall polysaccharides it is presumed that cell wall loosening occurs thereby facilitating cell wall expansion.

Another possible mechanism of cell wall expansion in relation to decreased cell wall pH may involve pectins which possess acidic moieties that form ionic bridges via calcium ions (Baydoun and Brett, 1984). Incubation of etiolated *Pisum sativum* seedling cell walls in medium in which the pH was lowered showed decreased binding of Ca^{2+}. Preincubation of the cell walls with a polygalacturonase resulted in a 60% removal of calcium binding capacity; only small decreases in calcium binding capacity were observed for cell walls preincubated with protease, nucleases, phospholipase and hemicellulase. Accordingly, more than half of the calcium binding capacity was attributed to pectins. Presumably, calcium forms ionic bridges thereby reducing cell wall extensibility. Replacement of the bound calcium with protons as the pH of the cell wall decreases has been suggested to facilitate extension.

During an immunolocalization study with *Daucus carota*, an antibody to an un-esterified pectin epitope labelled the inner surface of the primary cell wall and the middle lamella (Knox *et al.*, 1990). On the other hand, an antibody to a methyl-esterified pectin showed labelling uniformly across the cell wall. To what extent these variations in methyl esterification of the pectins influences cell wall extensibility and architecture is not known. Presumably, the degree of pectin esterification would determine the number of ionic bridges with calcium that could be formed. This could suggest that the mechanism by which cell wall extensibility is limited by bound calcium is enzymatically mediated by pectin methyl esterase activity. Despite the uncertainties, the spatial distribution of these two pectin forms further demonstrates the complexity of primary cell wall formation and expansion.

1.5. Secondary Cell Wall Formation.

Up to this point, the extent of our understanding of primary cell wall formation has been outlined. Unfortunately, the process of secondary cell wall deposition is also poorly understood. After cell wall expansion has ceased, certain cells continue to produce cell wall constituents (e.g. cellulose, hemicelluloses) as they differentiate into various cell types. In such cases, the protoplast shrinks as secondary cell wall deposition proceeds. With cells involved in fluid conduction (e.g. vessels, tracheids), the latter stages of secondary wall formation are followed by the death of the protoplast leaving behind a hollow cell.

The xylem (wood) of angiosperms is comprised of a variety of cells with thick secondary walls including ray parenchyma, vessels, fibers and tracheids. Gymnosperms do not possess vessels and therefore utilize tracheids for fluid

conduction. Through analyses of angiosperm and gymnosperm xylem by electron microscopy, distinguishable layers have been observed in the secondary walls. A model by Cote (1977) representing the typically observed layers of the entire cell wall (primary and secondary) is shown in Figure 8. In the order of their deposition, the layers of the secondary cell wall are identified as S_1, S_2 and S_3. The ability to distinguish between these distinct secondary cell wall layers is related to differences in their chemical composition and arrangements of structural elements (Fengel and Wegener, 1983). For example, transmission electron microscopy of ultra-thin sections of *Picea abies* revealed different orientation of the cellulose microfibrils in the individual wall layers (Fengel and Wegener, 1983); the helicoidal arrangement of the cellulose microfibrils in a typical woody cell is approximated by the striations shown in the model.

Efforts to determine the chemical composition of the individual secondary cell wall layers have been limited by the inability to obtain reasonable amounts of each for detailed analysis. As described earlier, studies related to the chemistry of the primary cell wall were facilitated by the use of those tissues rich in cells with only primary cell walls. Since plant cells cannot produce specific secondary cell wall layers without first producing those layers preceding, it is almost impossible to obtain significant quantities of cell wall material from specific secondary cell wall layers.

Much of our understanding of the polysaccharide composition of secondary cell wall layers has resulted from a technique developed by Meier and coworkers (Cote, 1977). Using a microscope and a micromanipulator, cells at different degrees of cell wall development were separated and their carbohydrate composition determined. By this technique it was assumed that each cell wall layer was deposited

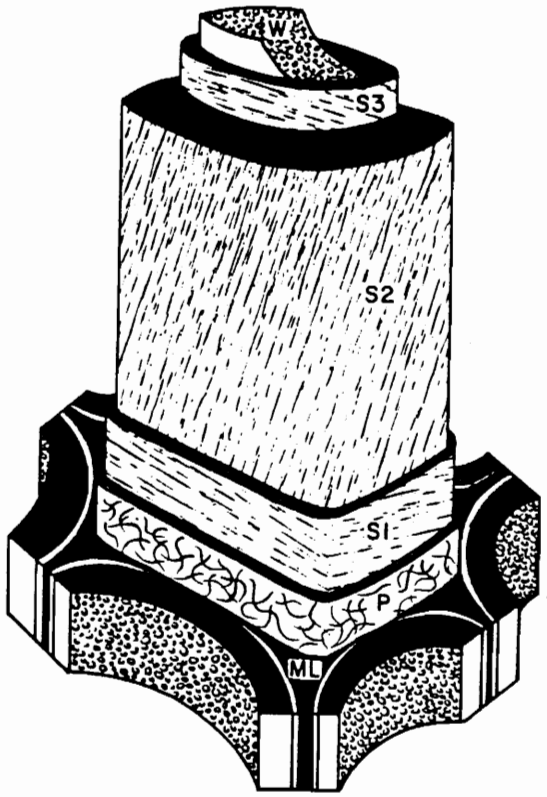

Figure 8. Model of Wood Cell as Proposed by Cote [Reproduced from Cote, 1977].

sequentially and that once a layer had been formed its composition would not change. Thus by determining the changes in carbohydrate composition with increases in cell wall thickness, the composition of individual layers were estimated.

Recently, an adaptation of the above method was used to determine the carbohydrate composition of the secondary cell wall layers of tracheids from a gymnosperm (*Cryptomeria japonica*)(Takabe *et al.*, 1989). In this experiment, the monosaccharide content of each of 12 layers of cells at different degrees of maturation was determined. The results obtained suggested that during secondary cell wall formation, cellulose deposition occurred most actively during the deposition of the S_2 layer. On the other hand, hemicelluloses (xylans, mannans) are deposited between the end of S_1 and beginning of S_2 layer formation as well as towards the end of S_2 and S_3 layer formation. Unfortunately, these values represent only estimates based on the assumptions mentioned above. Even if these assumptions are eventually proven to be correct, the accuracy of the results for the S_3 layer may be questionable because of the thinness of this layer relative to the others attached. For this reason, an earlier determination of the carbohydrate composition of the S_3 layer in *Abies balsamea* by the same general method was considered to be "uncertain" (Cote *et al.*, 1968). Accordingly, questions relating to the temporal and spatial deposition of the cell wall polysaccharides are far from resolved.

Despite the limitation in the analyses of the carbohydrate composition in specific cell wall layers, it is quite apparent that the relative proportions of the cell wall polysaccharides can vary from layer to layer. Also variable from layer to layer is cellulose microfibril orientation to the longitudinal axis of the cell. Observations by electron microscopy have shown microfibrils to run parallel to microtubules in

plant cells (Hori and Elbein, 1985). Based on such results it has been concluded that the orientation of microfibrils within the cell wall are mediated by microtubules within the protoplast. The actual mechanism by which this occurs is not known. Nevertheless, it has been suggested that the microtubules associate with the plasma membrane and form grooves through which the terminal complexes of cellulose synthase move during cellulose biosynthesis (Delmer, 1987).

During recent studies, antibodies to yeast α-tubulin were used against cells in *Pisum sativum* stems to visualize the entire cortical tubulin network by immunofluorescence (Roberts *et al.*, 1985). Observation of the tubulin network were also made after treatments with ethylene which has long been known to terminate cell elongation. Upon the addition of ethylene, the orientation of the microtubules showed a definite shift which was subsequently mimicked by the cellulose microfibrils. Therefore it has been suggested that the microtubule network in the plant cell is dynamic and through alterations to its helical pitch it mediates the pitch of the cellulose helices within the cell wall.

In contrast, through other studies it has been suggested that the helical orientation of the cellulose microfibrils occurs through a spontaneous self-assembly process similar to that observed for cholesteric liquid crystal systems (Vian *et al.*, 1986). For such a self-assembly to occur it is usually necessary that the polymer structure be rigid with short and flexible side-chains. Cellulose coated with xyloglucan has been suggested to satisfy these criteria. Recently, the helicoidal orientation of the microfibrils was found to occur in regions of the plant cell where xyloglucan was also present thereby suggesting this mechanism of assembly.

During development of the secondary cell wall, another important cell wall constituent, lignin, is deposited. Lignin, a phenylpropanoid polymer, encrusts

vascular tissues at the cell corners and proceeds into the intercellular spaces during the deposition of the polysaccharide matrix of the S_1 cell wall layer (Higuchi, 1985). Lagging behind cell wall thickening resulting from the deposition of the cell wall polysaccharide matrix, lignin progressively encrusts the secondary cell wall after the S_1 layer is formed (Takabe *et al.*, 1989). Primarily, lignin deposition occurs after the formation of the S_3 layer (Takabe *et al.*, 1989).

It is generally accepted that lignification is essential for vascular plants to develop into large upright forms (Freudenberg and Neish, 1968). This is because the lignified cell wall is not only rigid but also has decreased permeability thereby facilitating fluid conduction in vascular plants (Higuchi, 1985). Lignified tissues also show resistance to microorganisms by slowing the penetration of pathogens and their enzymes (Sarkanen and Ludwig, 1971). This suggests that lignin is involved in plant defense mechanisms.

A typical softwood tracheid contains approximately 28% lignin with the remainder being cellulose (approx. 40%), hemicelluloses (approx. 30%) and extractives (Sjöström, 1981). However, the actual lignin content at specific morphological regions within the cell (*e.g.* middle lamella) can be quite different than the above-mentioned value. For example, analyses of black spruce (*Picea mariana*) and Douglas fir (*Pseudotsuga menziesii*) tracheids by UV microscopy demonstrated that the lignin concentration (g/g) in the secondary walls was less than half that in the compound middle lamella (Fergus *et al.*, 1969; Wood and Goring, 1971). Taking into consideration that the secondary wall occupies approximately 90% of the cell volume leads to the conclusion that the majority of lignin in wood is in the secondary walls (Fergus *et al.*, 1969). Other analyses involving bromination (Saka and Thomas, 1982; Saka and Goring, 1988) or

mercurization (Westermark *et al.*, 1988; Eriksson *et al.*, 1988) followed by scanning electron microscopy (SEM) coupled with energy dispersive X-ray analysis (EDXA) have led to similar results although in the case of bromination the exact values may differ with the method used (Westermark, 1985).

Lignin (Latin: lignum, meaning wood) is a complex phenylpropanoid polymer which typically comprises 15-36% of stem tissue of woody plants (Sarkanen and Ludwig, 1971). During the last century when wood was considered to be a uniform substance, De Candolle coined the term "la lignine" in 1819 to describe that part of wood remaining after solvent and mild acid treatments (Monties, 1989). The composite nature of wood was not realized until 1838 when Payen established that there were two distinct components, "la cellulose" and "l'incrustation ligneuse" (Monties, 1989, Alder, 1977). Lignin not only occurs in wood (xylem tissue), but is also found in the cell walls of pith, roots, fruits, buds and bark (Pearl, 1967, Goodwin and Mercer, 1983).

Lignins are comprised of up to three monomer units, those being *p*-coumaryl **1**, coniferyl **2** and sinapyl **3** alcohols (see Figure 9). Accordingly, these polymers consist of phenolic moieties containing *p*-hyroxyphenyl **4**, guaiacyl **5** and sinapyl **6** units, respectively. The variability of the monomeric composition of lignin has also been studied by the above-mentioned microscopy methods to determine lignin heterogeneity within morphological regions of the cell wall as well as between cell types. For example, in hardwoods (angiosperms), studies with UV microscopy and SEM-EDXA have suggested lignin monomer compositions having greater syringyl **6**:guaiacyl **5** ratios in the secondary cell wall as opposed to the middle lamella (Saka and Goring, 1988; Musha and Goring, 1975; Fergus and Goring, 1970). Similar results were obtained by autoradiography of tissues previously administered ^{3}H

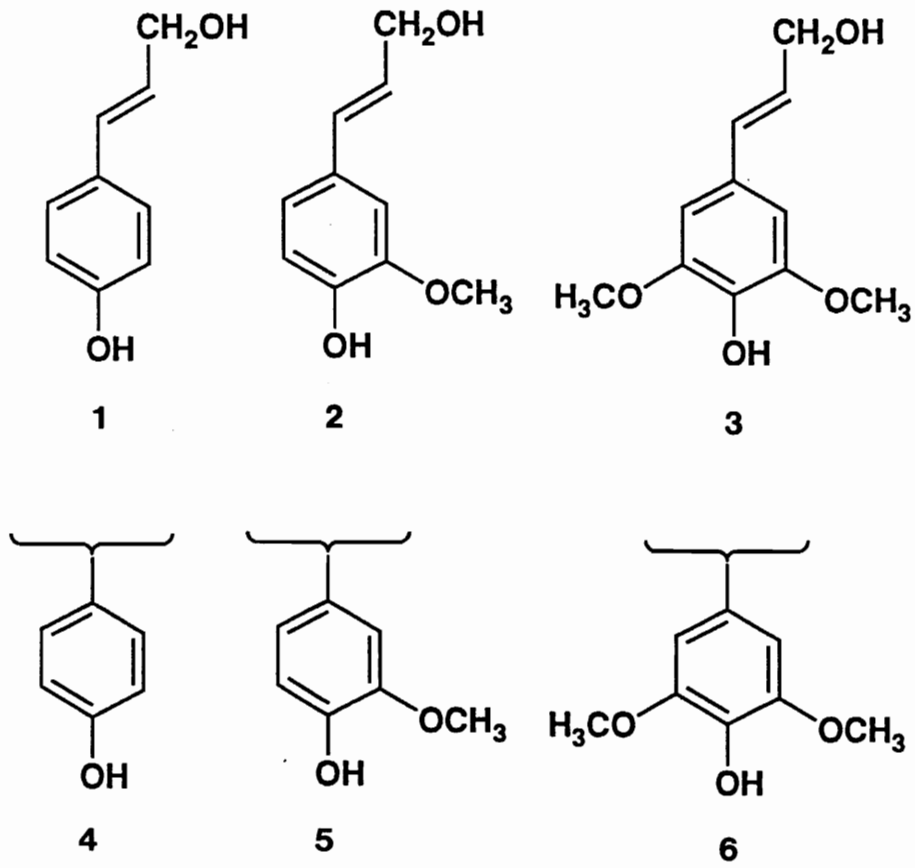

Figure 9. Monolignol and their Respective Phenolic Moieties.

labelled guiaicyl **5** and syringyl **6** model compounds (Fukushima *et al.*, 1990; Terashima *et al.*, 1986, 1988). Through tissue fractionation followed by functional group analyses, greater methoxyl contents in the secondary wall as compared to the middle lamella was shown in a softwood (*Picea mariana*) (Whiting and Goring, 1982). Accordingly softwood lignin apparently possesses a greater *p*-hydroxyphenyl **4**:guaiacyl **5** ratio in the middle lamella than that in the secondary wall. The underlying reasons for such differences have never satisfactorily been explained, but adds to the difficulty in rationally explaining cell wall maturation.

1.6. Overview of Lignin Biosynthesis.

Lignins are biosynthesized from combinations of two or three of the E-monolignols, *p*-coumaryl **1**, coniferyl **2** or sinapyl **3** alcohols. The biochemical pathway to the E-monolignols was determined following ^{14}C tracer experiments and enzymatic studies. It has been established that monolignols are produced from the shikimate-chorismate pathway and a main branch of the phenylpropanoid pathway. During the process of lignification, the monomers are polymerized in the cell walls and middle lamellae through an enzyme-catalyzed reaction.

1.6.1. Shikimic/Chorismic Acid Pathway: The shikimic acid pathway derives its name from shikimic acid **7** which was first isolated in 1885 by Eykmann from the oriental plant *Ilicium religiosum* (Ganem, 1978). At that time, the structure of this substance as well as its metabolic importance were unknown. About 20 years after the exact structure of shikimic acid **7** was determined in the 1930's (Ganem, 1978), Davis and coworkers showed that it was an intermediate in the biosynthesis of the aromatic amino acids tyrosine **8**, phenylalanine **9** and

tryptophan (Davis, 1955). Since then, studies have shown that shikimic acid **7** plays a critical role as an intermediate in the biosynthesis of a variety of natural products such as the lignins as well as some alkaloids and vitamins; it also contributes in part to the molecular structure of flavonoids, coumarins and indole derivatives such as growth regulator indole acetic acid (Gilchrist and Kosuge, 1980). Experimentation with a mutant strain of *Aerobacter aerogenes* which was unable to synthesize tyrosine **8**, phenylalanine **9** and tryptophan showed an accumulation of a new intermediate, chorismic acid **10**, when grown with an exogenous supply of these amino acids (Edwards and Jackman, 1965; Gibson, 1964; Gibson and Gibson, 1964). Since then, it has been established that chorismic (Greek derivation, meaning "separating") acid **10** is the main branch point intermediate of this pathway from which five main biosynthetic routes diverge (Ganem, 1978). The pivotal role of chorismic acid **10** leads one to refer to this pathway as the shikimic/chorismic acid pathway.

The first committed step in the pathway leading to shikimic **7** and chorismic **10** acids is the coupling of erythrose-4-phosphate **11** and phosphoenol pyruvate **12** which are derived from the pentose phosphate and glycolytic pathways, respectively (see Figure 10)(Gilchrist and Kosuge, 1980). This reaction which results in the formation of 3-deoxy-D-arabino heptulosonic acid phosphate (DAHP) **13** is mediated by the enzyme DAHP synthase. Regulation of the shikimic/chorismic acid pathway results, in part, from the regulation of this enzyme. For example, in *Norcordia* sp 239, DAHP synthase was shown to be regulated through feedback inhibition by phenylalanine **9**, tyrosine **8** and tryptophan (de Boer *et al.*, 1989). Accordingly, feedback inhibition of DAHP synthase in *Nisseria gonorrhoeae* by

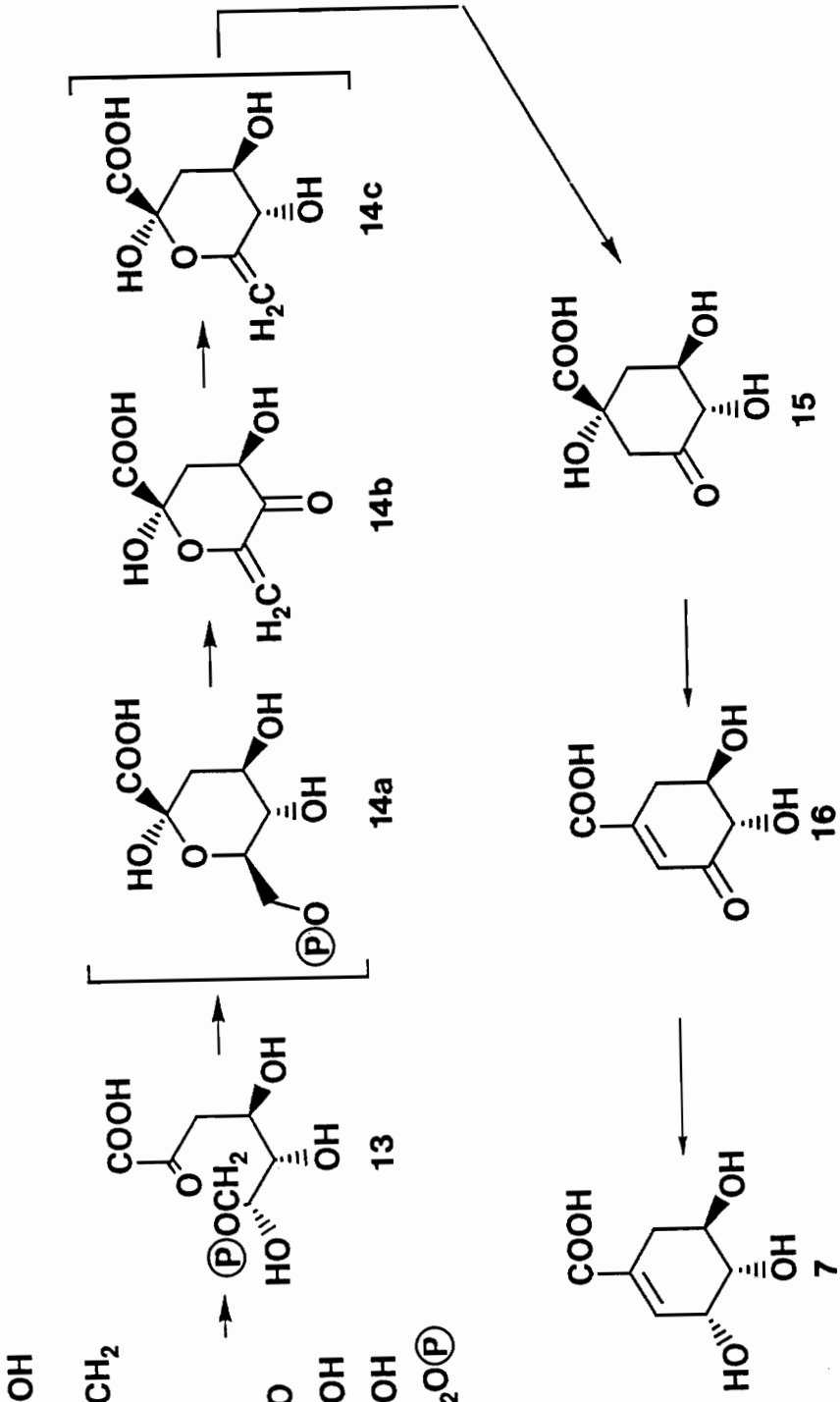

Figure 10. Biosynthesis of Shikimic Acid.

phenylalanine **9** resulted in the inhibition of growth as well as tyrosine **8** biosynthesis (Bhatnagar *et al.*, 1989).

In plant systems such as parsley (*Petroselinum crispum*) suspension cultures, DAHP synthase activity has been found to be affected by light (McCue and Conn, 1990) as well as fungal elicitors (McCue and Conn, 1989). After exposure to either light or an elicitor, activity for a plastidic isoform (DS-Mn) of DAHP synthase was induced while on the other hand, activity for a cytosolic isoform (DS-Co) remained unchanged. It is generally accepted that an intact metabolic pathway leading to the aromatic amino acids occurs in the plastids (Jensen *et al.*, 1989). In the cytosol, a less regulated pathway has also been suggested to occur (Jensen *et al.*, 1989). Although a complete cytosolic pathway has yet to be demonstrated, it has been implicated as the source of aromatic amino acids needed for phenylpropanoid metabolism presumed to occur on enzyme complexes on the cytosolic face of the endoplasmic reticulum (Hrazdina and Wagner, 1985). Since phenylpropanoid metabolism is induced by fungal elicitation it might be expected that the cytosolic form of DAHP would also be induced. The induction of the plastidic isoform (DS-Mn) as opposed to the cytosolic isoform (DS-Co) therefore warrants careful consideration and further investigation. Nevertheless, based on the results mentioned above it has been suggested that the plastidic isoform (DS-Mn) is involved in some manner in the production of precursors for secondary metabolism (McCue and Conn, 1990).

In the next step of the pathway, DAHP **13** is converted to 3-dehydroquinate **15** by an apparently complex mechanism of ring closure which is only beginning to be understood. The enzyme catalyzing the formation of this first cyclic precursor of the shikimic acid pathway is 3-dehydroquinate synthase. The first report of

activity for this enzyme in higher plants was demonstrated with cell-free extracts of *Phaseolus mungo* seedlings where a requirement for NAD^+ and Co^{+2} for enzyme activity was observed (Yamamoto and Minamikawa, 1976). Since then, studies have focussed in greater detail on the mechanism of the reaction.

Studies have suggested that this reaction mechanism begins with the oxidation of the α-pyranose form **14a** of DAHP by NAD^+ at C_5 (Bender *et al.*, 1989). This was demonstrated in experiments using various potential substrates of DAHP synthase with molecular stuctures similar to that of DAHP. Depending on the functional group modification for the substrate analog used, the rate of reaction catalyzed by the enzyme was altered or the reaction was prohibited altogether. Analogs to DAHP in its α-pyranose form **14a** as opposed to an analog in the open form **13** were bound by 3-dehydroquinate synthase thereby suggesting that DAHP in its α-pyranose form **14a** is utilized by this enzyme (Widlanski *et al.*, 1989); other analogs of DAHP which favor oxidation at C_5 facilitated the binding of the resulting ketone intermediate and NADH (Bender *et al.*, 1989). For the ketone intermediate, enolization occurs at C_6 as suggested by the lack of proton exchange at C_6 in substrate analogs which are blocked from the elimination step (Bender *et al.*, 1989). A subsequent *syn*-elimination of phosphate could afford an intermediate **14b** which would be reduced by NADH to give the enol-pyranose intermediate **14c** (Widlanski et al., 1989). Enzymatic conversion of an analog of DAHP with C_7 specifically labelled with deuterium demonstrated that the β-elimination of phosphate occurs with *syn*-stereochemistry (Widlanski *et al.*, 1989). The enol-pyranose intermediate **14c**, after being released from the enzyme, presumably undergoes a spontaneous reorganization to give 3-dehydroquinate **15**. Accordingly, this mechanism suggests that DAHP synthase does not catalyze a complex ring

closure but instead performs a more simple function as an oxidoreductase (Widlanski *et al.*, 1989).

The 3-dehydroquinate **15** so formed is next converted to 3-dehydroshikimate **16** through an unusual *syn*-elimination of water catalyzed by 3-dehydroquinate hydrolyase as shown with *Escherichia coli* (Smith *et al.*, 1970; Turner *et al.*, 1975). Subsequently, 3-dehydroshikimate **16** is reduced to afford shikimic acid **7** in a reaction catalyzed by shikimate oxidoreductase. Through experimentation with spinach (*Spinacia oleracea*) chloroplasts, it has been shown that these enzyme activities purify together on the same polypeptide (Fiedler and Schultz, 1985). Additionally, non-photosynthetic tissue such as roots from *Spinacia oleracea* and *Brassica rapa* possess dehydroquinate hydroxylase-shikimate oxidoreductase activities which may be plastidic (Schmidt *et al.*, 1991).

The biosynthesis of chorismic acid **10** from shikimic acid **7** begins with the phosphorylation of shikimic acid **7** to form the intermediate shikimate 3-phosphate **17** (see Figure 11) through a reaction catalyzed by shikimate kinase as demonstrated in cell-free extracts of *Sorghum bicolor* (Bowen and Kosuge, 1977) and *Phaseolus mungo* (Koshiba, 1978). The detection of shikimate kinase in spinach (*Spinacia oleracea*) chloroplasts further demonstrates the generally accepted view of the presence of the shikimic/chorismic acid pathway in plastids (Schmidt *et al.*, 1990).

Next, another molecule of phosphoenol pyruvate **12** is integrated into the pathway through a reversible condensation with shikimate 3-phosphate **17** (Dewick, 1985; Levin and Sprinson, 1964). The resulting product, 5-enolpyruvyl shikimate 3-phosphate **18** is then dephosphorylated to give chorismic acid **10** by a stereo-specific 1,4 anti-elimination of phosphoric acid (Floss *et al.*,

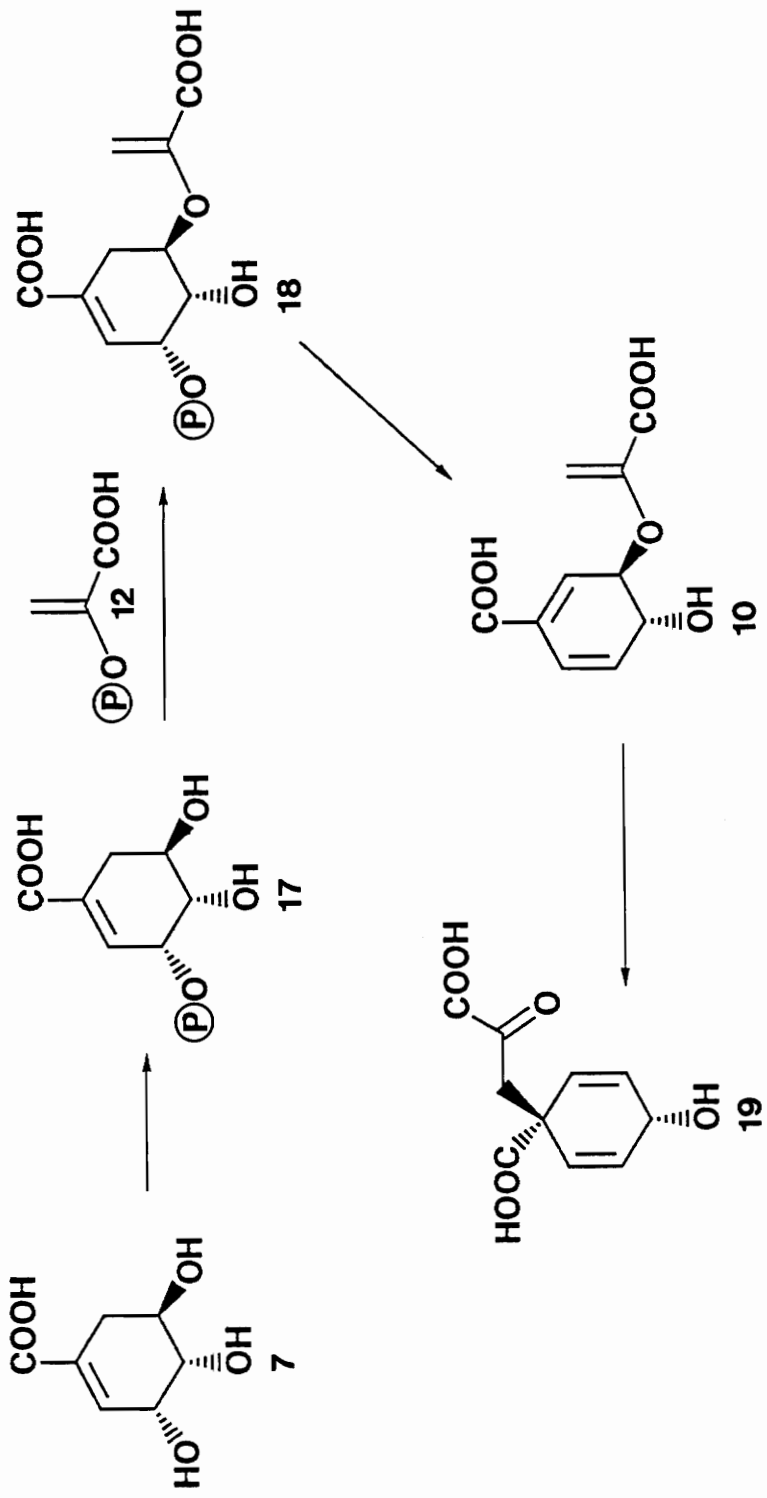

Figure 11. Biosynthesis of Prephenic Acid from Shikimic Acid.

1972; Hill and Newkome, 1969; Onderka and Floss, 1969) which may be concerted (Balasubramanian, 1990). The enzyme which catalyzes this reaction, chorismate synthase, has been detected in *Pisum sativum* (Mousdale, 1986) and purified from *Corydalis sempervirens* (Schaller *et al.*, 1991).

The shikimic/chorismic acid pathway is undoubtedly a critical metabolic pathway in higher plants when one considers the massive quantity of metabolites ultimately produced. One of the major avenues of chorismate metabolism is its conversion to prephenate **18** leading to among many products, the lignins. In plants, chorismate mutase was first demonstrated in *Pisum sativum* (Cotton and Gibson, 1968). Since then, it has been detected in a wide variety of plants and in most cases 2 isozymes were observed (Poulson and Verpoorte, 1991). One of the isozymes is located in the plastids (CM-1) and is regulated by feedback inhibition by phenylalanine **9** and tyrosine **8**. On the other hand, the second isozyme is located in the cytosol (CM-2) and appears to be unregulated by aromatic amino acids and secondary metabolites (*e.g. p*-coumarate, ferulate). The conversion of prephenate to chorismate represents one of two examples of a [3,3]-sigmatropic Claisen rearrangement in primary metabolism (Ganem, 1978), and has been suggested to pass through a chair-like transition state intermediate (Andrews *et al.*, 1977).

The subsequent conversion of prephenate **19** to phenylalanine **9** or tyrosine **8** in bacteria was initially viewed to occur via the intermediates phenylpyruvate **20** and 4-hydroxyphenyl pyruvate **21**, respectively (see Figure 12) (Ganem, 1978). The formation of these intermediates are catalyzed either by prephenate dehydratase or prephenate dehydrogenase. The aminotransferases which convert phenylpyruvate **20** to phenylalanine **9** and 4-hydroxyphenylpyruvate **21** to tyrosine **8** are considered to be non-specific (Wightman and Forest, 1978).

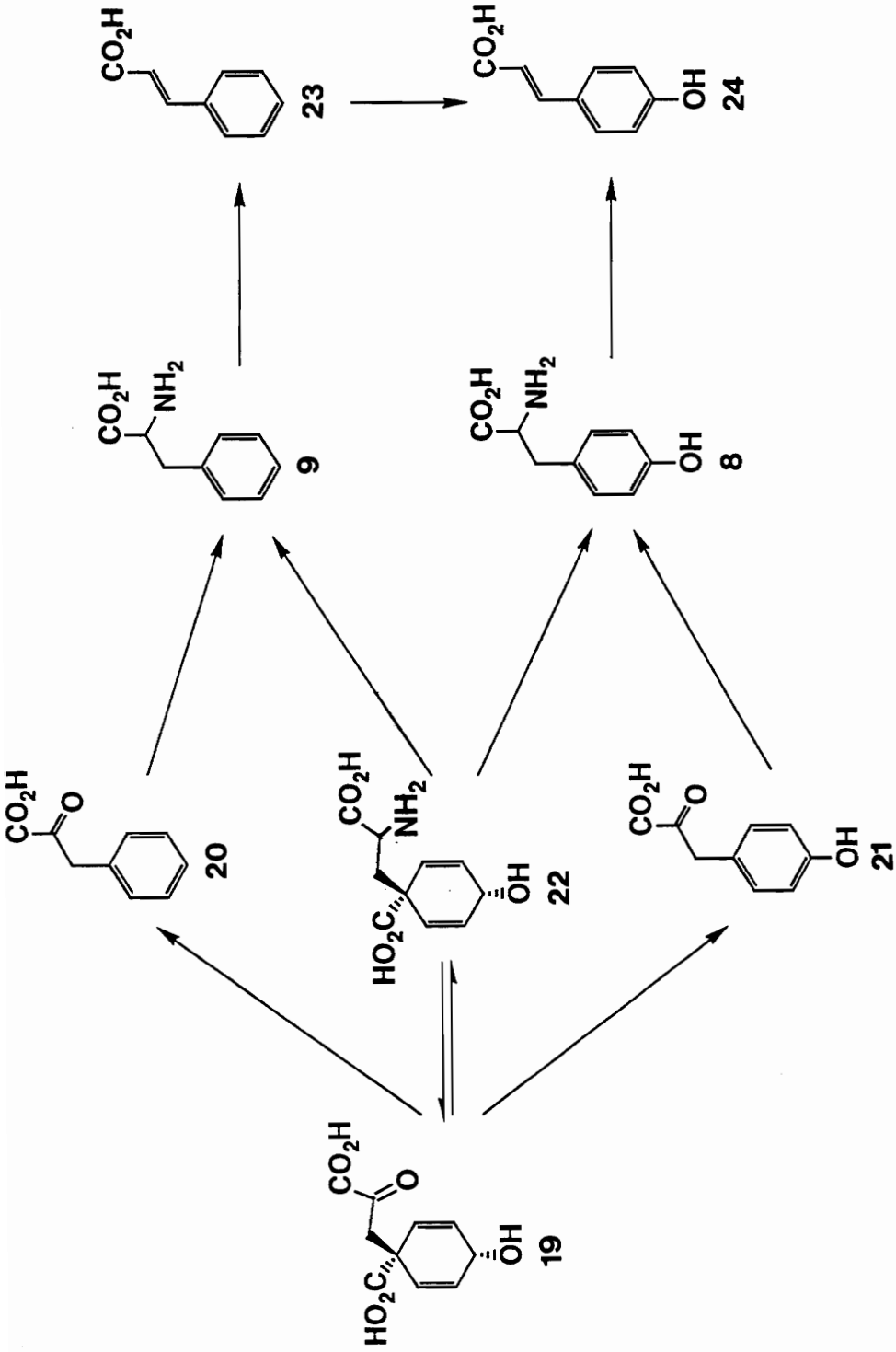

Figure 12. Biosynthesis of *p*-Coumaric Acid from Prephenic Acid.

Interestingly, in blue-green bacteria (*Agmenellum quadruplicatum*) it was discovered that the biosynthesis of tyrosine **8** did not occur through the intermediate 4-hydroxyphenylpyruvate **21** but was instead formed from arogenic acid **22** (Stenmark *et al.*, 1974). The evidence for this new pathway was the lack of detectable prephenate dehydrogenase activity and the ability to detect activities for prephenate aminotransferase and arogenate dehydrogenase (Stenmark *et al.*, 1974). The exact structure of this new intermediate, arogenic acid **22**, was later proven by UV, ^{1}H NMR, ^{13}C NMR and mass spectrometry (Zamir *et al.*, 1980). Since the discovery in blue-green bacteria, mung bean (*Vigna radiata* [L.] Wilczek) seedlings were shown to have the potential to synthesize tyrosine **8** through the intermediates 4-hydroxyphenylpyruvate **21** or arogenic acid **22** (Rubin and Jensen, 1979). The significance of the arogenic acid pathway in higher plants has been demonstrated through the detection of arogenate dehydrogenase and not prephenate dehydrogenase in *Nicotiana silvestris* (Gaines *et al.*, 1982), *Sorghum bicolor* (Connelly and Conn, 1986) and *Zea mays* (Byng *et al.*, 1981). Subsequently, phenylalanine **9** biosynthesis in higher plants by the arogenic acid pathway was further demonstrated in *Nicotiana silvestris* (Jung *et al.*, 1986) and *Sorghum bicolor* (Siehl and Conn, 1988) by activity for arogenate dehydratase and not prephenate dehydratase. Thus, the biosynthesis of L-tyrosine **8** and L-phenylalanine **9** in higher plants apparently occurs through the same intermediate, arogenic acid **22**.

1.6.2. Phenylpropanoid Pathway: The first committed step for phenylpropanoid metabolism is the *anti*-elimination of the elements of ammonia from L-phenylalanine **9** to give E-(trans) cinnamic acid **23** (Hanson and Havir, 1981; Jones, 1984). This reaction which does not require any cofactors is

catalyzed by phenylalanine ammonia-lyase (PAL) (Gross, 1985). Through appropriate labelling experiments it was shown that the pro-3S hydrogen is removed during the deamination of phenylalanine **9** (Hanson *et al.*, 1971; Ife and Haslam, 1971). In a similar manner, tyrosine **8** is enzymatically deaminated to give *p*-coumaric acid **24**. The enyzme which catalyzes this reaction, tyrosine ammonia-lyase has been mainly found in grasses (Neish, 1961) and shows specificity for the Pro-3S hydrogen during deamination (Ellis *et al.*, 1973). Since tyrosine ammonia-lyase activity is always accompanied by significant levels of phenylalanine ammonia-lyase activity, it has been suggested that tyrosine ammonia-lyase activity is the result of a less specific phenylalanine ammonia-lyase species (Gross, 1985).

The cinnamic acid **23** produced by phenylalanine **9** deamination is subsequently hydroxylated to form *p*-coumaric acid **24**. This reaction, catalyzed by cinnamate 4-hydroxylase, a cytochrome P-450 dependent monooxygenase, is stereospecific for E-cinnamic acid **23** and not its corresponding Z-analog. Activity for this enzyme was first shown using a microsomal fraction from parsley cell suspension cultures (*Petroselinum*) (Pfändler *et al.*, 1977) and more recently from Jerusalem artichoke (*Helianthus tuberosus*) (Gabriac *et al.*, 1991).

In spinach beet (*Beta vulgaris*) early reports suggested that the formation of caffeic acid **25** from *p*-coumaric acid **24** (Figure 13) was catalyzed by a phenolase (Vaughan *et al.*, 1975). Since then it has been shown that elimination of phenolase activity in mung bean by tentoxin, a chlorosis-inducing fungal toxin, did not result in diminished levels of caffeic acid **25** and flavonoids such as rutin (Duke and Vaughn, 1989). Subsequently, specific *p*-coumaric acid **24** hydroxylation in mung bean seedlings treated with tentoxin suggested that not phenolase but a specific

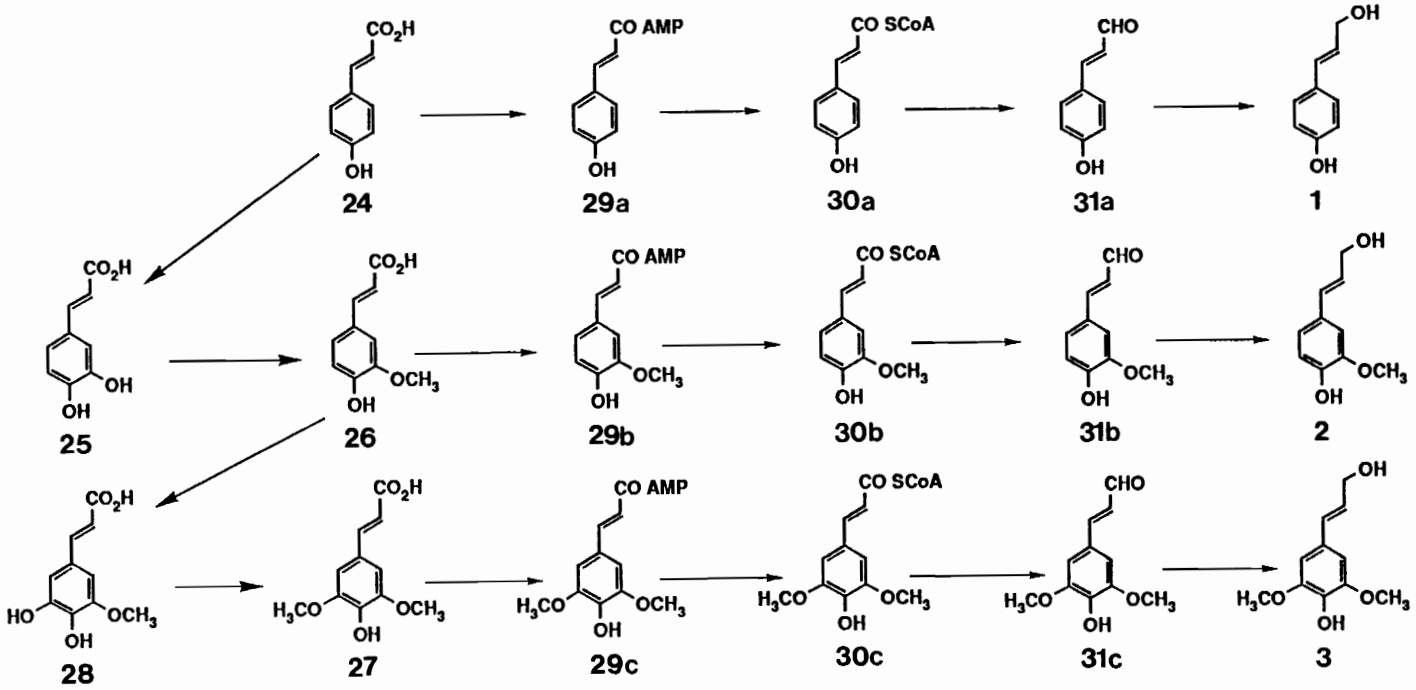

Figure 13. Biosynthesis of Monolignols from *p*-Coumaric Acid.

enyzme, *p*-coumarate-3-hydroxylase, catalyzes the conversion of *p*-coumaric acid **24** to caffeic acid **25** (Kojima and Takeuchi, 1889). Unfortunately, in these experiments it has only been shown that *p*-coumarate-3-hydroxylase catalyzes the production of caffeic acid for flavonoid biosynthesis. Further experimentation with tentoxin is needed to determine if the elimination of phenolase activity affects the biosynthesis of other phenylpropanoids such as lignin. Until these studies are completed the role of phenolase in caffeic acid biosynthesis cannot be disregarded.

The caffeic acid **25** so formed is then methylated by meta-specific *O*-methyltransferases using *S*-adenosyl-L-methionine as the methyl donor to form ferulic acid **26** (Poulton, 1981). In *Brassica oleracea* *O*-methyltransferase has been purified from the soluble fraction (De Carolis and Ibrahim, 1989). Interestingly, although activity of *O*-methyltransferase in Douglas fir saplings is soluble, that in the respective callus culture was found to be associated with membranes and cell walls as evidenced by its release through digitonin treatment of the crude homogenate (Monroe and Johnson, 1984). This is a curious result since in another suspension culture (alfalfa, *Medicago sativa*) the soluble nature of *O*-methyltransferase was verified (Edwards and Dixon, 1991).

The biosynthesis of sinapic acid **27** has been suggested to occur through the hydroxylation of caffeic acid **25** with subsequent methylation of a trihydroxy intermediate or through a hydroxylation of ferulic acid **26** to give 5-hydroxy-ferulic acid **28** which in turn is methylated. Administration of radiolabelled 5-hydroxyferulic acid **28** to wheat (*Triticum aestivum*) showed incorporation of radioactivity into sinapic acid **27** thereby suggesting that sinapic acid **27** biosynthesis occurs through this intermediate (Higuchi and Brown, 1963). Subsequently, a microsomal fraction from *Populus X euroamericana* was shown to

exhibit ferulic acid 5-hydroxylase activity (Grand, 1984). Purified isoforms of *O*-methyltransferase from *Brassica oleracea* have been shown to methylate 5-hydroxyferulic acid **28** to afford sinapic acid **27** (De Carolis and Ibrahim, 1989). The isolation of 5-hydroxyferulic acid **28** from corn (*Zea mays*) (Ohashi *et al.*, 1987) along with the evidence mentioned above defines a pathway leading to sinapic acid **27** which involves the intermediate 5-hydroxyferulic acid **28**. Although there is limited evidence for the pathway involving 3,4,5-trihydroxycinnamic acid, it cannot be stated that the only pathway to sinapic acid **27** is through 5-hydroxyferulic acid **28**.

As stated above, the methylation of 5-hydroxyferulic acid **28** is mediated by *O*-methyltransferases. Interestingly, 5-hydroxyferulic acid **28** was a poor substrate for *O*-methytransferases from Japanese black pine (*Pinus thunbergii*) (Kuroda *et al.*, 1975, Shimada *et al.*, 1972) whereas on the other hand it was a good substrate for *O*-methyltransferase from bamboo (*Bambusa*) (Shimada *et al.*, 1973). This apparent substrate specificity between angiosperm and gymnosperm *O*-methyltransferases has been suggested to be partly responsible for the greater proportion of syringyl **6** units in angiosperm lignin (Grisebach, 1981).

The three hydroxycinnamic acids, *p*-coumaric **24**, ferulic **26** and sinapic **27** acids, are reduced to give the three monolignols, *p*-coumaryl **1**, coniferyl **2** and sinapyl **3** alcohols, respectively. The first step of these parallel conversions is the formation of acyl adenylate intermediates **29a-c** which further react with coenzyme-A to form thioesters **30a-c**. The formation of the thioesters **30a-c**, catalyzed by cinnamate: CoA ligase, occurs through a reaction requiring ATP as a cosubstrate and Mg^{2+} (or other divalent cation) as a cofactor (Gross, 1985). Studies on cinnamate:CoA ligase have shown it to have a substrate preference for

hydroxycinnamic acids (Hahlbrock and Grisebach, 1970) in their trans-configuration (Knobloch and Hahlbroch, 1975). Further characterizations of hydroxycinnamate:CoA ligase have suggested a role for this enzyme in controlling the lignin monomeric composition between species (Kutsuki *et al.*, 1982b; Luderitz *et al.*, 1982; Maule and Ride, 1983) as well as between different tissues of the same species (Grand *et al.*, 1983) by its substrate specificities toward the hydroxycinnamic acids.

The metabolism of the thioesters **30a-c** formed has been studied through tracer experiments where cell-free extracts from soybean (*Glycine max*) have shown incorporation of radioactivity into *p*-coumaryl alcohol **1** and coniferyl alcohol **2** after incubations with radiolabelled *p*-coumaryl-CoA 30a and feruoyl-CoA **30b**, respectively (Ebel and Grisebach, 1973). After that discovery, cell-free extracts from Forsythia suggested that the cinnamoyl-CoA thioesters **30a-b** were converted into the respective aldehydes **31a-c** which accumulated in the absence of cinnamyl alcohol dehydrogenase activity which catalyzes the reduction of the aldehydes **31a-c** to alcohols **1-3** (Gross and Kreiten, 1975; Stöckigt *et al.*, 1973). The enzyme catalyzing the reduction to the aldehydes **31a-c**, cinnamoyl-CoA reductase requires NADPH **32** as a cosubstrate. During this reductive step, the B hydrogen (H_S) of NADPH **32** is transferred to the cinnamyl moiety and therefore this enzyme is classified as a type B oxidoreductase (see Figure 14) (Grisebach, 1981). Although feruoyl-CoA **30b** has been shown to be a substrate for cinnamoyl-CoA reductase from spruce (*Picea abies* L.) and soybean (*Glycine max* L.), sinapyl-CoA **30c** was shown to be a substrate only for the enzyme from soybean (Lüderitz and Grisebach, 1981). For cinnamoyl-CoA reductase from both species, *p*-coumaryl-CoA **30a** was shown to be a poor substrate (Lüderitz *et al.*, 1982). Since these data are

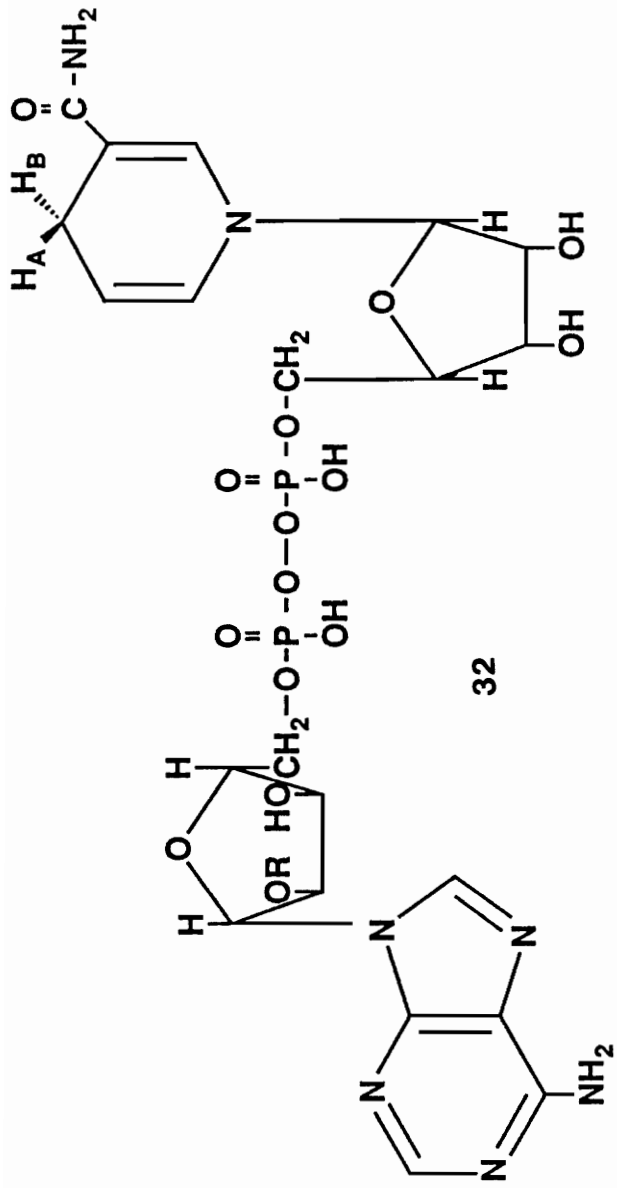

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Figure 14. Structure of NADP(H).

consistent with the monomer composition in the two species, a role for cinnamoyl-CoA reductase in controlling the ratio of lignin monomers may be possible.

Determination of the substrate specificity of the enzyme responsible for reducing the cinnamyl aldehydes **31a-c** to the monolignols **1-3**, cinnamyl alcohol dehydrogenase, has also been conducted. Cinnamyl alcohol dehydrogenase has been studied in a wide variety of species in the Bryophyta, Pterophyta, gymnosperms and angiosperms and shown to be specific for NADP(H) **32** (Mansell *et al.*, 1976) and the cinnamyl moiety (Mansell *et al.*, 1974). This reduction utilizes the A hydrogen (H_R) of NADPH **32** and therefore the enzyme is classified as a type A oxidoreductase (Grisebach, 1981). Studies on cinnamyl alcohol dehydrogenase from spruce (*Abies picea*), soybean (*Glycine max*) and Japanese black pine (*Pinus thunbergii*) have shown that sinapaldehyde **31c**, as compared to *p*-courmaraldehyde **31a** and coniferaldehyde **31b**, was a good substrate only if the source of the enzyme was an angiosperm (Kutsuki *et al.*, 1982a, Lüderitz and Grisebach, 1981). In contrast, cinnamyl alcohol dehydrogenase from xylem and sclerenchyma from poplar (*Populus X euroamericana*) showed the same substrate specificity even though the lignin in the sclerenchyma cells possesses more syringyl units **6** than that in the xylem (Sarni *et al.*, 1984). Such results therefore raise questions as to the involvement of cinnamyl alcohol dehydrogenase in regulating the monomer composition of lignin (Sarni *et al.*, 1984). Interestingly, American beech (*Fagus grandifolia*) has been shown to accumulate the Z-monolignols **33a-b** (and respective glucosides **34a-c**) in bark tissue (see Figure 15) (Lewis *et al.*, 1988a). Radiotracer experiments suggested that the formation of the Z-coniferyl alcohol in the bark arose from the isomerization of E-coniferyl alcohol **2** (Lewis *et al.*, 1987a). In *Menyanthes trifoliata* the enzymatically mediated interconversion

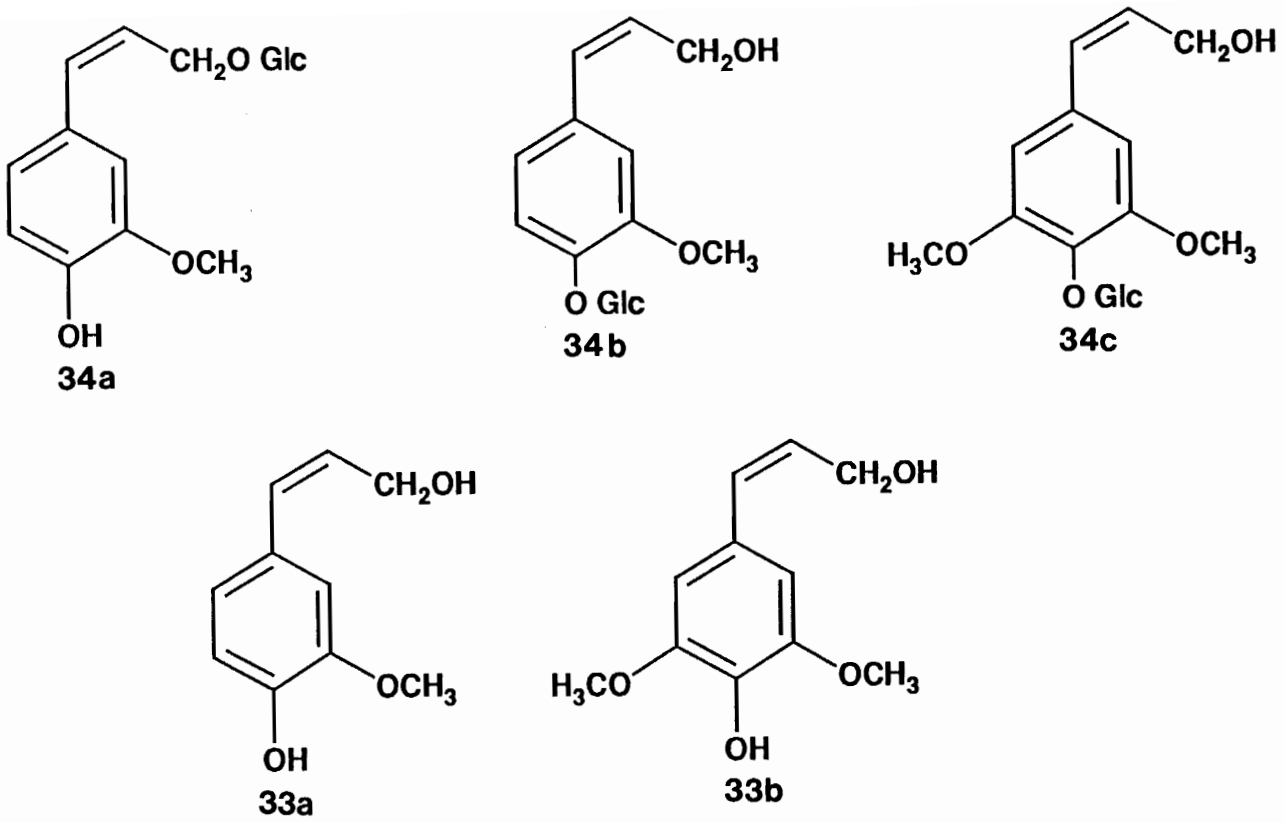


Figure 15. Cis-Monolignols and Glucosides Found in Beech Bark.

of the allylic alcohols, geraniol and nerol, was shown to occur through the cis/trans interconversion of the respective aldehydes (Arigoni, 1975). This was apparently not the case for the isomerization occurring in beech bark where cinnamyl alcohol dehydrogenase activity in cell-free extracts showed a substrate preference for the E-isomer of coniferyl alcohol **2** thereby further suggesting that E-coniferyl alcohol **2** was directly isomerized to Z-coniferyl alcohol **33a** (Lewis *et al.*, 1987a).

Additional characterization of cinnamyl alcohol dehydrogenase have involved efforts to isolate the cDNA clone encoding this enzyme. Treatment of bean (*Phaseolus vulgaris*) suspension cultures with a fungal elicitor resulted in the induction of cinnamyl alcohol dehydrogenase activity as well as cinnamyl alcohol dehydrogenase mRNA presumably through the activation of a stress-induced CAD gene (Walter *et al.*, 1988). Unfortunately the isolated and sequenced cDNA was later shown to have considerable homology with that of malic enzyme from corn (Walter *et al.*, 1990) and poplar (Van Doorsselaere *et al.*, 1991). Since this cDNA clone does not represent the gene for cinnamyl alcohol dehydrogenase, a new effort is needed to isolate and sequence a cDNA clone encoding this enzyme catalyzing the final step in monolignol biosynthesis.

1.6.3. Lignin Deposition: During lignification the monolignols produced in the protoplast are polymerized in the cell wall matrix. The enzymes in the protoplast involved in monolignol biosynthesis have been shown in some cases to exhibit substrate preferences which have been correlated to the monolignol composition of the lignin in a particular plant species or even tissue. Typically, softwood (gymnosperm) lignins are primarily comprised of coniferyl alcohol **2** (~90%) with smaller amounts of *p*-coumaryl alcohol **1** (Gross, 1979). In

contrast, hardwood (angiosperm) lignins are polymers of coniferyl **2** and sinapyl **3** alcohols with lower levels of *p*-coumaryl alcohol **1** such as that observed in softwood lignins (Higuchi, 1980). Grasses (monocots) such as bagasse have been shown to contain considerably more *p*-coumaryl alcohol **5** units (~30%) with the remainder being coniferyl **2** (~30%) and sinapyl **3** (~40%) alcohols (Glasser *et al.*, 1983). In addition to the monolignols, it has been suggested that grass lignins may also contain ether or ester linked hydroxycinnamic acids (Lewis and Yamamoto, 1990). This further exemplifies that the monomer composition of lignin between plant species is not homogeneous. In fact, lignin heterogeneity has been demonstrated within species in relation to geographical location, local climate and soil conditions (Harkin, 1969).

Light also has an effect on the monomer composition since it has been demonstrated that xylem and intact stems from poplar grown in the dark as compared to in the light produce significantly lower levels of radiolabeled guaiacyl **5** units from administrations of radiolabelled phenylalanine (Grand *et al.*, 1982). These alterations of monomer composition have been suggested to be related to the effects of light on membrane permeability (Grand *et al.*, 1982).

The monolignols incorporated into the lignin polymer are assumed to be exclusively in their E-configuration. As noted before, beech (*Fagus grandifolia*) bark accumulates significant amounts of Z-coniferyl **33a** and Z-sinapyl **33b** alcohols. It has been suggested that in this tissue, the Z-monolignols **33a-b** may play a role in lignification (Morelli *et al.*, 1986). Since the degradation of synthetic lignins (DHP) produced from E- **2** or Z- **33a** coniferyl alcohols gave similar products, it is possible that in some cases the Z-monolignols **33a-b** are also involved in the lignification process (Morelli *et al.*, 1986).

Lignifying tissues typically contain the monolignols not in their free form but as their β-D glucosides (Gross, 1985). These glucosides (e.g. coniferin **35**, syringin) have been suggested to play a role in monolignol transport and storage (see Figure 16). In this respect, the glucose is transferred from UDP-glucose to the monolignol through a reaction catalyzed by UDP-glucose:monolignol-glucosyltransferase. The monolignol is retrieved from the glucoside through a reaction catalyzed by β-glucosidases attached to the cell wall matrix by presumably ionic linkages (Gross, 1985). Although the glucosides may be involved in lignin biosynthesis, the turnover of coniferin **35** in pine seedlings was not as high as the rate of lignification thereby suggesting that the transport of the monolignols **1-3** to the lignifying cell wall does not occur exclusively via the glucosides (Marcinowski and Grisebach, 1977).

In the cell wall, the monolignols **1-3** are dehydrogenatively polymerized to form the lignin macromolecule. This process is initiated by the enzymatic dehydrogenation of the monolignols to afford phenoxy radicals, in mesomeric forms (see Figure 17), which couple to form dimeric quinone methides (Higuchi, 1985). Dilignols are formed from these quinone methides through the addition of water or intramolecular nucleophilic attacks on the benzylic carbons by quinone or alcohol groups (Higuchi, 1985). The lignin macromolecule is formed through continuation of the enzymatic dehydrogenations and non-enzymatic coupling/nucleophilic attacks.

The idea of a dehydrogenative polymerization of C_6C_3 units such as coniferyl alcohol **2** was first proposed by Erdtman in 1933 (Erdtman, 1950). Working on the dehydrogenation of isoeugenol he noted that the product, dehydrodi-isoeugenol possessed a phenylcoumarin structure similar to that observed by Karl Freudenberg

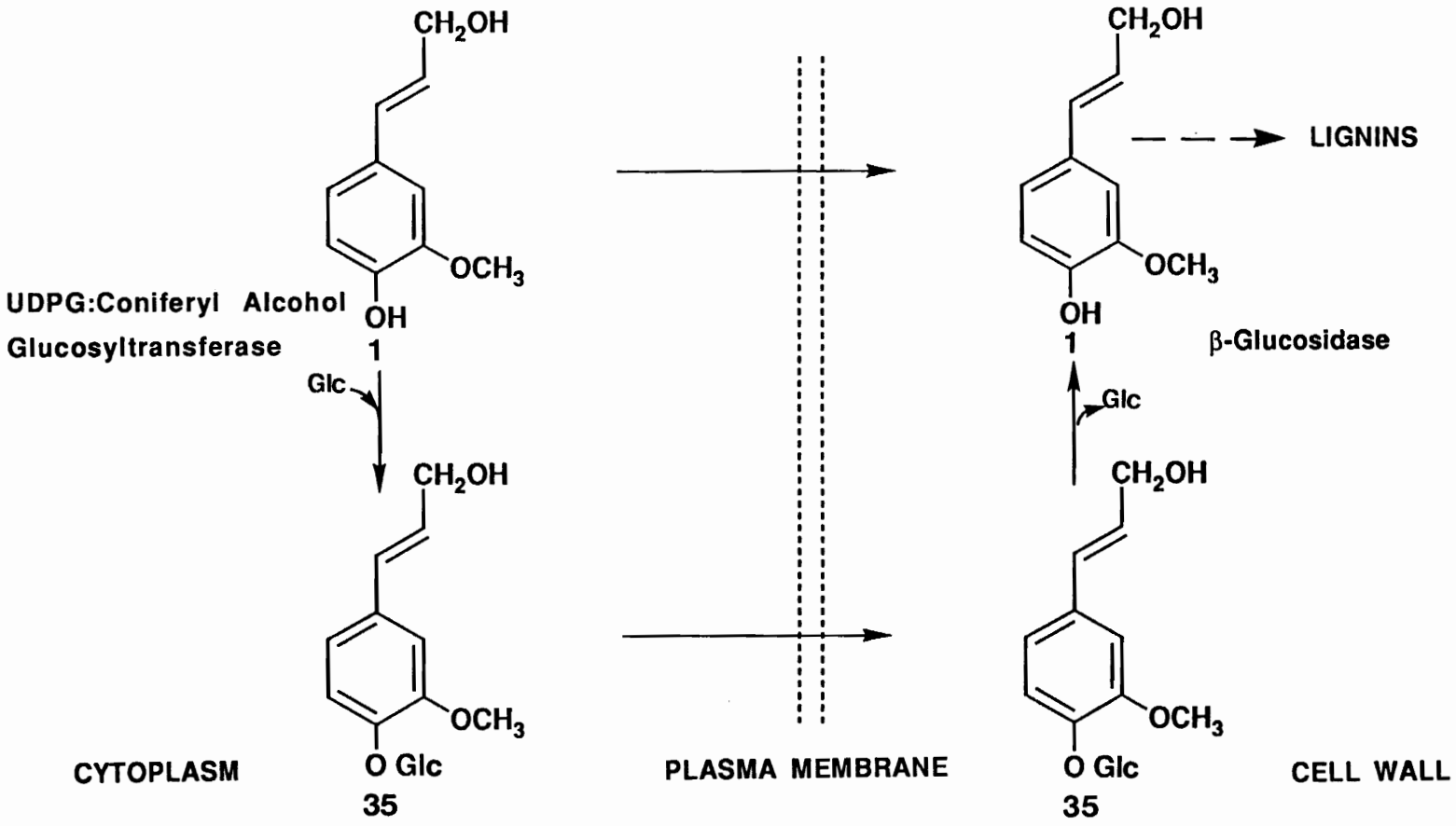


Figure 16. Transport of Coniferyl Alcohol to the Cell Wall.

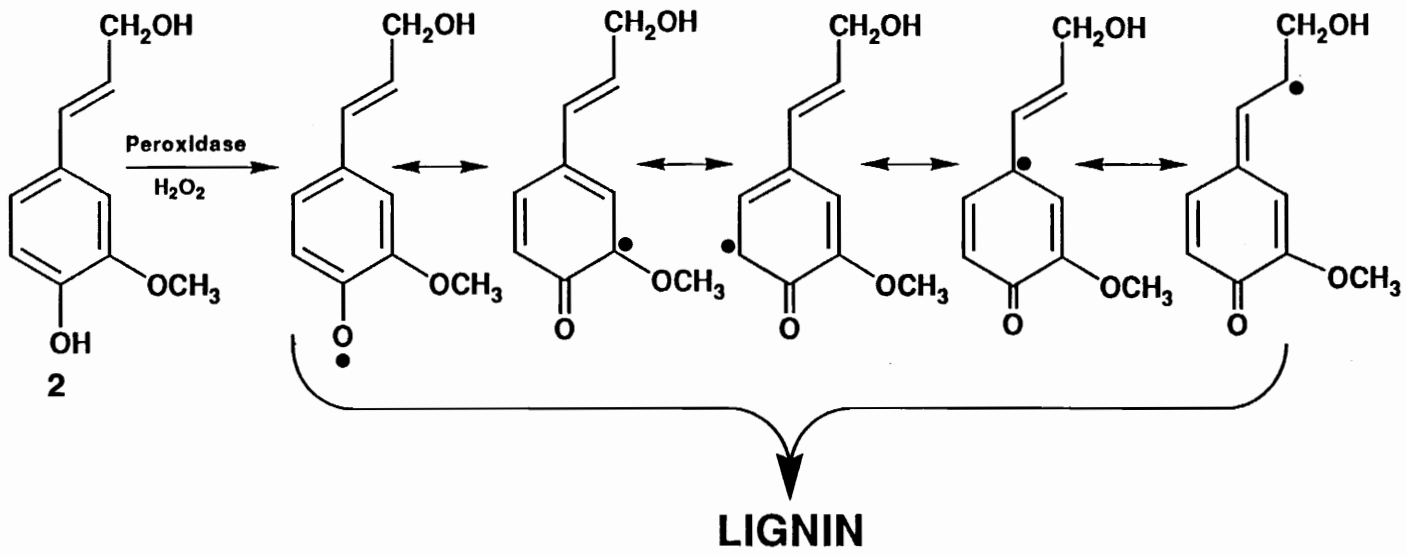


Figure 17. Mesomeric Forms of Coniferyl Alcohol after Dehydrogenation Through Peroxidase and H_2O_2.

to occur in lignin (Erdtman, 1950). Such a mechanism was plausible because it could explain why lignin was observed to be optically inactive (Erdtman, 1949).

The first enzymatic dehydrogenation of coniferyl alcohol **2** was reported by Freudenberg in 1943 (Freudenberg, 1965). Artificial lignins or dehydrogenatively polymerized (DHP) lignins could be produced by reactions of dilute solutions of the monolignols with either a laccase purified from the mushroom *Agaricus campestris* or culture filtrate from *Polyporus versicolor* or with commercial horseradish peroxidase and H_2O_2 (Freudenberg 1965). The lignins so formed were shown to possess similar properties to Bjorkman MWL preparations from spruce by oxidative degradation (permanganate) and IR spectroscopy (Freudenberg, 1965). With the additions of coniferyl alcohol **2** at once to a peroxidase/H_2O_2 solution (Zulaufverfahren) a DHP lignin was obtained with greater amounts of the phenylcoumarin and pinoresinol bonding patterns than the β-O-4 linkage which is the main bonding pattern in lignin based on degradative analyses (Higuchi, 1985; Sjöström, 1981). Dropwise addition of the coniferyl alcohol **2** over long periods of time (Zutropverfahren) resulted in a relative increase in the number of β-O-4 linkages (Higuchi, 1985). The Zulaufverfahren method has been suggested to result in the formation of dilignols which in turn polymerize, whereas the Zutropverfahren method may involve the endwise addition of coniferyl alcohol **2** moieties to a growing lignin polymer (Higuchi, 1985).

Evidence that peroxidase, and not laccase, was the enzyme responsible for the dehydrogenation during lignification was provided through a study by Harkin and Obst (1973). Using histochemical methods they demonstrated that lignifying woody tissues possesses peroxidase but not laccase activity (Harkin and Obst, 1973). Experiments with horseradish (*Armoracia lapathiforil* Gilig.) later showed that the

H_2O_2 needed for the dehydrogenative polymerization of the monolignols involved peroxidases (Elstner and Heupel, 1976; Halliwell, 1978; Higuchi, 1985). Accordingly, lignifying cell walls of *Populus X euroamericana* were capable of oxidizing syringaldazine without exogenously supplied H_2O_2 (Goldberg *et al.*, 1983). Thus it has been concluded that lignification proceeds through a dehydrogenative polymerization process catalyzed by peroxidase/H_2O_2 (Grisebach 1977).

Research efforts have since been applied to determine the nature of the peroxidase involved in lignification. Characterization of peroxidase isozymes from petunia (van den Berg *et al.*, 1983), peanut (*Arachis hypogaea*) (van den Berg *et al.*, 1983), mungbean (Goldberg *et al.*, 1987), poplar (Imberty *et al.*, 1985) corn (Parish, 1975), lupin (*Lupinus albus*) (Barcelo *et al.*, 1988, 1989) and tobacco (Schloß *et al.*, 1987) have indicated the association of acidic isoperoxidases with cell walls and basic isoperoxidases within the protoplast. It is the acidic peroxidases in the cell wall which are believed to catalyze the lignification process (Imberty *et al.*, 1985). Those peroxidases which are apparently involved in lignification have been shown to be relatively stable to heat treatments (Imberty *et al.*, 1985). Interestingly, analyses of peroxidases in the sapwood and heartwood of angiosperms and gymnosperms showed that peroxidases are detectable in wood left to stand one year after it was cut (Ebermann and Stich, 1982).

Studies on the relationship of peroxidases to stresses such as infection (Bruce and West, 1989; Flott *et al.*, 1989; Lagrimini and Rothstein, 1987) and ozone treatment (Castillo and Greppin, 1986) have resulted in changes in the isozyme patterns shown by electrophoresis. Conversely, over expression of anionic peroxidases in transgenic tobacco plants resulted in chronic wilting (Lagrimini *et*

al., 1990). The exact role of specific peroxidases isozymes to various physiological stress needs to be assessed. In this respect, cationic (Chibbar and van Huystee, 1984; Lambeir *et al.*, 1985) and anionic (Chibbar and van Huystee, 1984) peroxidases excreted from *Arachis hypogaea* suspension cultures showed identical catalytic properties for 4-amino-antipyrine, guaiacol and eugenol as substrates. This is an interesting result since it has been presumed that different peroxidase isozymes possess different catalytic functions and capabilities. Peroxidase isozymes from sapwood of various species have also been shown to have variable substrate specificities with substrates including the following: esterified ferulic acids, eugenol, coniferylaldehyde **31b**, *p*-coumaryl **1** and coniferyl **2** alcohols (Stich and Ebermann, 1988). Although specific isozymes may show less effective conversions of *p*-hydroxycinnamyl alcohols than others, their possible involvement in lignification cannot be ignored (Stich and Ebermann, 1988).

1.7. Lignin Structure Analyses by ^{13}C NMR Spectroscopy.

Efforts to determine the chemical nature of lignin have long relied on the isolation of this rather intractable material through chemically or physically harsh methods. Once isolated, the structural characteristics were deduced from the analyses of the degradation products. The advent of various spectroscopic methods provided scientists with non-destructive alternatives for their attempts to characterize lignin. One spectroscopic technique, ^{13}C nuclear magnetic resonance (NMR) spectroscopy, has found increasing levels of application towards lignin analyses.

Carbon-13 NMR spectroscopy continues to be widely used for the structural characterization of complex organic chemicals. For ^{13}C, greater numbers of

surrounding electrons as compared to ^{1}H results in a ^{13}C NMR spectroscopy chemical shift range of 200 ppm which is 21 times that for ^{1}H NMR spectroscopy (Smith *et al.*, 1975). Through proton noise decoupling, a single line resonance is observed for each carbon in a specific bonding environment. The different functionalities for a given molecule fall within well defined ranges; carbonyl carbons resonate at about 200 ppm, carbons with electronegative substituents between 90-50 ppm and simple aliphatics between 50-10 ppm (Simpson, 1986). Coincident resonances even for complex molecules are relatively rare (Simpson, 1986).

In comparison to ^{1}H NMR spectroscopy, ^{13}C NMR spectroscopy is considerably less sensitive. This lower sensitivity is due to the low intrinsic sensitivity of ^{13}C as compared to ^{1}H along with the low natural abundance of ^{13}C (1.1%) (Smith *et al.*, 1975). Despite the apparent lack of sensitivity, developments in Fourier transformation techniques have facilitated routine application of ^{13}C NMR spectroscopy to compounds with natural abundance ^{13}C in concentrations as low as 10 mM (Smith, 1975).

Studies on the structural characterization of lignin by ^{13}C NMR spectroscopy originally focused on the analyses of isolated lignins or lignin derivatives. Lüdemann and Nimz (1973) analyzed milled wood lignins (MWLs) from beech (*Fagus silvatica*) and spruce (*Picea excelsa*) by solution-state ^{13}C NMR spectroscopy and obtained complex spectra for which peak assignments were made by comparison with ^{13}C NMR spectroscopic analyses of monomeric and dimeric lignin model compounds. Subsequent analyses of synthetic (DHP) lignins yielded ^{13}C NMR spectra which were comparable to those obtained for the MWL preparations (Nimz *et al.*, 1974). From this result, the authors provided further

confirmation that lignin is formed through the dehydrogenative polymerization of *p*-hydroxycinnamyl alcohols. Isolated MWLs from hardwood, softwood and grass species have been analyzed by ^{13}C NMR spectroscopy and have shown differences in monomer composition as well as in structural characteristics (Himmelsbach and Barton, 1980; Nimz *et al.*, 1981, 1984; Obst and Landucci, 1986). Evidence for lignin heterogeneity has not only been shown to occur between species but also within a given species by analyses of different lignin fractions isolated from poplar (*Populus trichocarpa*) (Lapierre *et al.*, 1982). Degradation of spruce (*Picea glauca*) lignin by *Phanerochaete chrysosporium* has also been studied by ^{13}C NMR spectroscopy in an effort to gain a better understanding of the structural changes in lignin brought about by fungal activity (Chua *et al.*, 1982).

As stated previously, compared to other spectroscopic methods, ^{13}C NMR spectroscopy is considerably less sensitive. In part, this lack of sensitivity is the result of the low natural abundance (1.1%) of ^{13}C. In an attempt to improve on this deficiency, poplars were grown from cuttings in air containing CO_2 enriched 20% with carbon-13 (Lapierre *et al.*, 1984). From the resulting ^{13}C labelled tissues, lignin fractions were isolated and analyzed by ^{13}C NMR spectroscopy. Although the ^{13}C labelled lignins did not reveal any new structural bonding patterns with poplar lignin, the authors suggested that ^{13}C enriched lignins could provide more accurate information by reducing the influence of non-lignin resonances resulting from unlabelled sample contaminants.

Up until now the analyses of soluble lignins by ^{13}C NMR spectroscopy has been discussed. It should be noted that the usually harsh physical and chemical methods used to liberate lignin result in changes to the macromolecule. For example, ^{13}C NMR analyses of dioxane lignin, MWL, Cuoxam lignin and periodate lignin

demonstrated that different structural changes which can occur in lignin are dependent on the method of isolation (Bartuska *et al.*, 1980). Since not all lignin preparations are readily soluble the authors analyzed the lignin preparation through a more recent development in ^{13}C NMR spectroscopy that being the analyses of samples in solid form.

Solid-state ^{13}C NMR spectroscopy utilizes cross polarization (CP) to enhance signal intensity and magic angle (θ=54°44') spinning (MAS) which reduces signal broadening resulting from chemical shift anisotropy. The ability to analyze samples in solid form not only allows one to study poorly soluble or insoluble materials but also to probe conformational characteristics. For example, in the solid state the different polymorphs of cellulose (I and II) are readily distinguishable (Atalla *et al.*, 1980, Earl and VanderHart, 1980). Such studies have shown the non-equivalence of the glucose carbons 1 and 4 thereby providing direct evidence that the basic repeating unit of cellulose is cellobiose. Similar analyses of wood have been used to demonstrate the conversion of crystalline cellulose to amorphous cellulose during ball milling (Kolodziejski *et al.*, 1982).

Application of solid-state ^{13}C NMR spectroscopy to lignin studies have involved the analyses of isolated lignins prior to and after derivativization to obtain chemical shift assignments comparable to those in the solution-state (Hatfield *et al.*, 1987). The aromatic resonances of the solid-state ^{13}C NMR spectrum of woody cell walls which are attributed to lignin have been used to measure the lignin content of wood fibers during pulping (Haw *et al.*, 1984). The aliphatic resonances of lignin in such materials are masked by the aliphatic carbohydrate resonances of the other cell wall polymers those being cellulose and the hemicelluloses. Since the majority of the bonding patterns of lignin are thought to involve the aliphatic propyl side chain,

little information about the bonding patterns of lignin was obtained by the analyses of such intact cell walls.

Efforts to determine the aliphatic bonding patterns of lignin *in situ* began with a model system in which coniferyl alcohol **2** specifically labelled with ^{13}C at positions 1, 2 or 3 on the propane side chain were individually dehydrogenatively polymerized to produce synthetic lignins (Lewis *et al.*, 1987b). The resulting ^{13}C enriched DHP lignins were then analyzed by solution and solid-state ^{13}C NMR spectroscopy. Difference spectra were obtained by subtracting the ^{13}C NMR spectrum of a natural abundance ^{13}C DHP lignin from the spectra resulting from the analyses of the ^{13}C enriched DHP lignins. Despite the broad bands observed in the solid-state spectra, tangible information about the bonding patterns (see Figure 18) of specific carbons in the DHP lignins were obtained. For example, the DHP of [2-^{13}C] coniferyl alcohol when analyzed by solid-state ^{13}C NMR spectroscopy showed a broad resonance at 83 ppm that was attributable to the β-O-4 (**C**) substructure of lignin which presumably is the main bonding pattern in native lignin. Combination (w/w) of the specifically labelled DHP (24%) with cellulose (76%) were then analyzed by solid-state ^{13}C NMR spectroscopy and demonstrated that if an enrichment of greater than 4% of ^{13}C in the lignin component of a cell wall could be achieved, the bonding patterns of lignin could be determined *in situ*.

Through the administration of [2-^{14}C] ferulic acid to feland wheat (*Triticum aestivum* L.) under aseptic conditions on agar, incorporation of radioactivity in the root tissues were determined to be approximately 10% (Lewis *et al.*, 1987c). Administration of [1-^{13}C], [2-^{13}C], and [3-^{13}C] ferulic acids was then carried out in the same manner following which the root tissues were analyzed by solid-state ^{13}C NMR. Interestingly, the spectral data did not indicate the presence of the

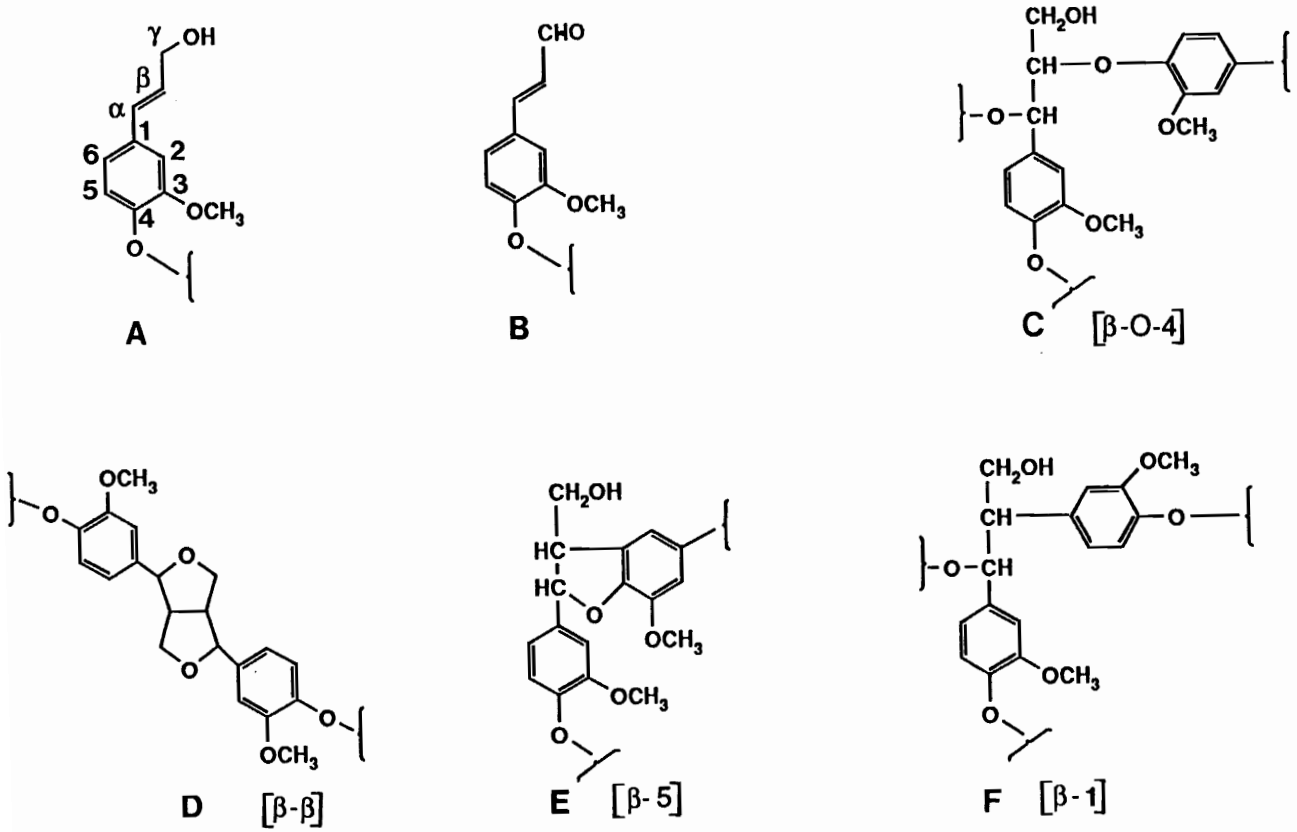


Figure 18. Main DHP Lignin Bonding Patterns.

β-O-4 substructure (**C**) but did show significant hydroxycinnamic acid moieties in various bonding environments. After exhaustive extraction, the ^{13}C enriched tissues were subjected to a lignin isolation procedure (Eberhardt, 1988). The solution-state spectra of the isolated acetal lignins showed resonance enrichment at the same chemical shifts as observed in the solid-state data. Such data suggest that if the lignin preparation was representative of the lignin *in situ*, in wheat, hydroxycinnamic moieties are an important component of that lignin. Subsequent administration of [2-^{13}C] ferulic acid to *Leucaena leucocephala* using a hydroponic system showed the incorporation of the label of this lignin precursor into the β-0-4 substructure (**C**)(Lewis *et al.*, 1988b). As in the case of the wheat experiments, the root tissue showed the greatest incorporation of label. Administration of [1-^{13}C] and [3-^{13}C] ferulic acid were also conducted and resonances corresponding to lignin bonding patterns were also observed (Lewis *et al.*, 1989).

1.8. Lignification in Cell Suspension Cultures.

Cell culture systems possess many qualities which are suitable for biosynthetic studies. In comparison to intact plant systems, cultures are typically simpler because they provide a population of cells with nearly equivalent nutritional and growth conditions. This is especially true for suspension cultures where the cells are suspended in a medium through which nutrients or precursors of specific metabolites can be administered. Since culture systems are maintained under aseptic conditions, complications resulting from microorganisms can be avoided. Ultimately, it is desirable in a culture system that the cells be in complete synchrony with respect to metabolic function and development. Unfortunately, absolute synchrony of cell development is not yet possible to obtain (Bolwell,

1985). Nevertheless, suspension cultures are extremely useful for biosynthetic and developmental studies since they represent the most uniform plant systems available.

Callus and suspension cultures have been used as model systems in attempts to gain a better understanding of the lignification process occurring in plants. Studies have focussed on the induction of lignification as well as the structural characteristics of lignins from these systems. Unfortunately, the efforts to study the induction of lignification in culture systems have rarely provided sufficient evidence that lignin was actually formed. Furthermore, the limited structural characterization of the products often resulted in the reporting of the induction of "lignin-like" substances. Such terminology is extremely misleading since the degree to which the "lignin-like" substances formed are similar to lignin is not normally investigated.

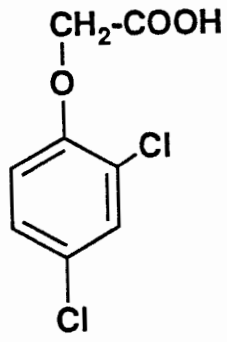
As stated previously, lignin in plants has been suggested to function within the cell wall as a mechanism of resistance to microbiological attack. In this respect, lignification may resist the diffusion of pathogenic enzymes or toxins, prevent dissolution of the cell wall or protect against compressive forces imparted by penetrating fungal hyphae (Vance *et al.*, 1980). Using tissue culture systems, the chemical aspects of fungal attacks have been studied through fungal elicitors which induce a plant's typical response of resistance to certain microbiological insults. Such elicitors are quite variable and include zoospore suspensions (Ward *et al.*, 1989), high-molecular weight fractions from heat-treated mycelial cell walls (Grand *et al.*, 1987) and mycelial cell wall fractions (Kuhn *et al.*, 1984). The actual agent (s) in these fungal elicitor responsible for the induction of lignification is (are) poorly understood.

Through the treatment of bean (*Phaseous vulgaris* L.) suspension cultures with a fungal elicitor, a five-fold increase in extractable cinnamyl alcohol dehydrogenase activity was observed within 10 hours (Grand *et al.*, 1987). Also in bean suspension cultures, phenylalanine ammonia-lyase synthesis is rapidly elicitor induced along with an increase in phenylalanine-ammonia-lyase mRNA (Cramer *et al.*, 1985; Edwards *et al.*, 1985). Phenylalanine ammonia-lyase mRNA has also been shown to be induced with fungal elicitation of parsley (*Petroselinum hortense*) suspension cultures (Kuhn *et al.*, 1984). This rapid induction of enzymes involved in phenylpropanoid metabolism observed in the elicitor treated suspension culture cells closely parallels the rapid induction of phenylalanine ammonia-lyase, 4-coumarate:CoA ligase, cinnamyl alcohol dehydrogenase and peroxidase observed in wheat (*Triticum aestivum* L.) leaves into which a fungal elicitor was injected (Moerschbacher *et al.*, 1989). Curiously, in soybean (Glycine max L.) cinnamyl alcohol dehydrogenase and phenylalanine ammonia-lyase showed lower activities in cells treated with elicitor as compared to those which were untreated (Farmer, 1985). One complication in this result is the induction of "lignification" which occurred through transfers of the cells to the medium without added elicitor. Accordingly, it was concluded that the medium in which the suspension culture is grown can have dramatic effects on the response of a culture to an elicitor (Farmer, 1985).

The levels of medium constituents such as nitrate and phosphate salts as well as sucrose have been shown to have significant effects on the activities of enzymes involved in phenylpropanoid metabolism and the levels of presumed lignin deposition. For example, in soybean (*Glycine max* L.) suspension cultures, phenylalanine ammonia-lyase was observed to reach maximal levels when the

conductance of the medium reached its minimum (Hahlbroch *et al.*, 1980). Through reduction in KNO_3 and $NaH_2PO_4 \cdot H_2O$ in a soybean suspension culture medium, the level of phenylalanine ammonia-lyase activity was greater than that observed in cells grown in full strength medium (Farmer, 1985). Histochemical staining with phloroglucinol suggested that cells grown in this reduced nutrient salt medium were induced to lignify (Farmer, 1985). Apparent lignification did not occur when only one of the salts was reduced thereby suggesting that the reduction of both phosphate and nitrate were needed for lignin induction. Through reduced levels of phosphate and nitrate, along with elevated levels of sucrose, the accumulation of polyphenols in *Catharanthus roseus* suspension cultures was induced (Knobloch and Berlin, 1980). Dilution of the cultures on sucrose (8% w/v) solutions provided optimal polyphenol production; with greater and lower sucrose concentrations, polyphenol production was significantly reduced. Similarly, in black locust (*Robinia pseudoacacia* L.) callus cultures, levels of sucrose greater or less than an optimum level resulted in reduced "lignin" deposition (Fukuda and Sakurai, 1982).

Alterations to the phytohormones in culture medium have also been suggested to result in the induction of lignification. In parsley (*Petroselinum hortense*) and soybean (*Glycine max*) suspension cultures, transfers to fresh medium in which 2,4-D (2,4-dichlorophenoxyacetic acid) **36** (see Figure 19) was substituted with NAA-BAP (naphthaleneacetic acid-benzylaminopurine) induced the cultures to lignify as evidenced by phloroglucinol-HCl staining (Hösel *et al.*, 1982). In the case of the parsley suspension culture, more rapid lignification was observed by histochemical staining when the cells were washed to remove residual 2,4-D **36** at the time of transfer. This suggested that the lignification response was more likely due to the removal of 2,4-D **36** than the addition of NAA-BAP. Along a similar vein,



36

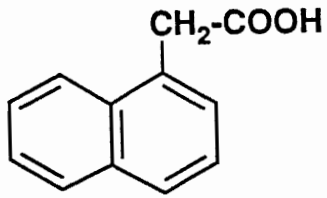


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Figure 19. Structures of 2,4-D and NAA.

in *Vanilla planifolia* suspension cultures it was concluded that 2,4-D **36** suppressed while NAA **37** enhanced the production of extractable phenolics (Funk and Brodelius, 1990). Callus cultures grown on medium in which 2,4-D **36** was substituted with NAA **37** showed a 10-fold increase in extractable phenolics and a 3-fold increase in lignin as evidenced by UV absorbance measurements after digestion of extractive-free tissues (Zagoskina, 1979). Coinciding with the suggested start of lignification in parsley suspension cultures transferred to NAA **37** containing medium was a dramatic but transient increase in cinnamyl alcohol dehydrogenase and phenylalanine ammonia-lyase activities (Hösel *et al.*, 1982). For carrot (*Daucus carota*) suspension cultures the synthesis of phenylalanine ammonia-lyase and phenylalanine ammonia-lyase-mRNA were induced when 2,4-D **36** was omitted from the medium (Ozeki et al., 1990). The levels of phenylalanine ammonia-lyase and phenylalanine ammonia-lyase-mRNA were reduced in cultures grown without 2,4-D **36** after subsequent addition of this growth regulator. Such experiments suggest that in carrot, phenylalanine ammonia-lyase is regulated at the transcriptional level by 2,4-D **36**.

Through manipulations of the phytohormones added to culture medium, cell differentiation can be induced. In *Zinnia elegans*, not only were the phytohormones NAA **37** and BAP important for tracheary element formation but also the levels of extracellular calcium (Fukuda and Komamine, 1980; Roberts and Haigler, 1990). Along with tracheid formation, increases in suggested lignin contents of *Pinus sylvestris* suspension cultures were observed (Ramsden and Northcote, 1987). Coinduced increases in tracheid formation and apparent lignin content were also observed in *Glycine max* callus cultures (Miller and Roberts, 1986). Interestingly though, regression analysis of the number of tracheids per soybean callus against

lignin content per callus showed no correlation. This result was attributed by the authors to lignin not associated with tracheids (Miller and Roberts, 1986). Such a conclusion coincides with observations of soybean suspension cultures which were induced to "lignify" and did not show any differentiation into tracheids (Hösel *et al.*, 1982). Indeed, an inverse correlation of tracheary element formation and lignification has also been observed; callus cultures of *Sedum telephium* when grown in the presence of 8-hydroxyquinoline sulfate (Quinosol) showed increasing tracheid formation, and decreasing apparent lignin contents, with increasing doses of the antimycotic drug (Pissarra *et al.*, 1990). Again, these data suggest that if lignification is occurring in tissue culture systems, it is not dependent on cell differentiation.

Of the descriptions of lignification in suspension or callus cultures up to this point, none have provided sufficient evidence that lignin comparable to that in plants was actually formed. In most cases the experiments have relied on histochemical staining which is no proof of lignification. Likewise, characterization of isolated "lignins" provide only partial evidence that these systems produce lignin as we know in plants.

Isolated milled-wood lignins (MWL) from both intact plants and cultures have been compared by degradative and nondegradative analyses. In loblolly pine (*Pinus taeda* L.), methoxyl contents of a MWL from suspension culture cells was considerably lower than that observed in wood (Fukuda *et al.*, 1988). This result is in agreement with the higher proportion of *p*-hydroxyphenyl **4** units in the suspension culture lignin observed by nitrobenzene oxidation. Since the yield of products by nitrobenzene oxidation of the suspension culture cells was approximately half that obtained from wood, the authors concluded that *P. taeda*

suspension culture lignin possesses considerably more carbon-carbon bonds. Degradative analyses of black locust (*Robinia pseudoacacia* L.) callus MWL has also revealed a significantly lower methoxyl content to that in wood (Fukuda, 1983). Non-degradative analyses of MWLs from *Rosa glauca* and *Glycine max* suspension cultures by solution-state ^{13}C NMR spectroscopy also demonstrated the greater proportion of *p*-hydroxyphenyl **4** moieties (Nimz *et al.*, 1975; Robert *et al.*, 1989). With respect to the *Rosa glauca* cells suspension cultures the authors suggested that the lignin in that system is "intermediary" between that of gymnosperms and angiosperms (Robert *et al.*, 1989).

Unfortunately, characterization of tissue culture lignins are limited by the fact that the lignins studied were isolated through mechanically harsh methods. The changes to the lignins that may have occurred prevent a full understanding of the nature of suspension or callus culture lignin *in situ*. One effort to characterize lignin without harsh chemical and mechanical methods involved the collection of an extracellular "lignin" from the medium of *Picea abies* suspension culture cells (Brunow *et al.*, 1990). This "lignin", which accumulated in the medium as a precipitate, was collected by centrifugation, washed with water and dried. Analyses of this material by solution-state ^{13}C NMR spectroscopy showed that it possessed many of the resonances observed in MWL preparations from *Picea abies* wood. Peaks purported to be pinoresinol substructures and cinnamyl alcohol side chains were observed in the ^{13}C NMR spectrum of the extracellular material but were either absent or present in a lesser extent in the MWL. Since it is not known if the extracellular material was secreted into or polymerized in the medium, it is possible this "lignin" is produced in the medium in a manner analogous to that for synthetic *in vitro* lignins. Although this extracellular "lignin" is easy to procure

and therefore apparently not degraded, it may not be representative of lignin in the cell wall.

2. RESEARCH OBJECTIVES

In addition to improving our understanding of lignin macromolecular structure *in situ* in the tissues of vascular plants, it is of the utmost interest to study the formation of this biopolymer during its temporal and spatial deposition into the developing plant cell wall. Ideally, a comprehensive study of the progression of lignification would require a means to structurally characterize the biopolymer at various stages of cell wall development. In an intact plant system, such as a woody stem, the progression of cell wall formation is easily observable by the increase in cell wall thickness occurring between the cambial zone and the mature xylem. Unfortunately, when working with such an intact system it would be extremely difficult and tedious to isolate sufficient amounts of cell wall layers at specific stages of cellular development. In an effort to circumvent this problem, suspension cultures were used in our research since they can provide significant quantities of a relatively homogeneous population of cells.

Research on plant suspension cultures has suggested that they are capable of producing lignin or "lignin-like" materials. Unfortunately, in each study reviewed, only limited evidence for lignification was obtained and did not meet the criteria for proof of lignification in plants. These criteria require the following: 1) histochemical identification of lignins in the plant cell walls in question, 2) the presence of the "biochemical machinery" for both the production of the monolignols and their subsequent polymerization, 3) proof that they are polymers comprised of monolignols and have bonding environments known to exist in lignins (Lewis and Yamamoto, 1990).

Thus, the overall objective of this research is to develop a suspension culture system to study the onset and continuation of lignification as a function of

cell wall maturation, and to define the lignin structure thereof. To achieve this will require a comprehensive characterization of the culture system before and after lignification is induced. Before lignin induction it is necessary to verify that primary cell wall formation is occurring throughout the culture. Next, the extent of phenylpropanoid metabolism for these cells needs to be assessed especially in relation to lignification as well as other related metabolites. With an understanding of the system at this stage of development, the model can be developed through the induction of cell wall thickening and lignin deposition. As before, the level of cell wall development needs to be correlated with the degree of lignification assessed by the above noted criteria.

In this study using *P. taeda* cell suspension cultures, the specific objectives were:

1) To obtain plant cell suspension cultures containing only primary cell walls, and have not undergone significant lignification.

2) Determine whether other branches of phenylpropanoid metabolism were occurring at this stage of development, and if so, the nature of the products so obtained.

3) Define conditions for induction of secondary cell wall thickening processes and lignification.

4) Determine the nature of extracellular lignins in suspension culture cells.

5) Determine the lignin bonding patterns between the monolignols *in situ* and in solution using solid-state and solution-state ^{13}C NMR spectroscopy, respectively, following administration of specifically labelled carbon-13 precursors.

3. MATERIALS AND METHODS

3.1. Chemicals and Instrumentation.

All chemicals used were either reagent or tissue culture grade unless otherwise stated. Organic solvents were redistilled before use; prior to redistillation, dioxane was refluxed over Na metal with benzophenone as an indicator. Water was purified through a Barnstead NANOpure II system. Universally-labelled [^{14}C]-*L*-phenylalanine **9** (15 GBq mmole^{-1}, 3.7 MBq ml^{-1}) was purchased from ICN Radiochemicals (Irvine, CA) in a 0.01N HCl solution. [1-^{13}C], [2-^{13}C] and [3-^{13}C] *L*-Phenylalanines (99 atom % ^{13}C) were purchased from MSD Isotopes (Merck Chemical Division, St. Louis, MO). Phenylalanine ammonia-lyase (23.3 nkat mg^{-1} protein, 5.0 mg protein ml^{-1}, Grade I) from *Rhodotorula glutinis* and pronase E (88.33 nkat mg^{-1} solid) from *Streptomyces griseus* were obtained from Sigma Chemical Co. (St. Louis, MO); horseradish peroxidase (3.78 μkat mg^{-1} solid, Grade II) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN).

High performance liquid chromatography (HPLC) was accomplished with a Waters Model 600E Multisolvent Delivery System, a 715 Ultra-wisp Sample Processor, a 990^{+} Photodiode Array Detector and a 990 Plotter all integrated with a NEC Powermate 2 Advanced Personal Computer. Reverse-phase HPLC analyses were carried out on Waters Nova-Pak C_{18} (3.9 x 250 mm, stainless steel) and guard (3.9 x 30 mm, stainless steel packed with Waters Bondapak C_{18} Corasil (37-50 microns)) columns with eluate detection at 280 nm unless otherwise stated. HPLC separations of *p*-hydroxybenzaldehyde, vanillin and syringaldehyde was achieved with a gradient solvent system (1 ml min^{-1}) consisting of Solvent A (H_2O:MeOH:AcOH, 95:5:0.1) and Solvent B (MeOH:AcOH, 99:1) as follows: t=0 min

(100% A) to t=25 min (A:B, 60:40). Detection of coniferin **35** utilized an isocratic solvent system (0.7 ml min^{-1}) consisting of $CH_3CN:H_2O$ (10:90) with eluate detection at 259 nm. For matairesinol isolation, HPLC eluent conditions (0.7 ml min^{-1}) were $CH_3CN:H_2O$ (20:80) at t=0 min to 30:70 at t=10 min with this composition being held until t=20 min. The optical purity of matairesinol was determined following chromatography on Daicel Chiralcel OD (4.6 x 250 mm, stainless steel) and guard (4.6 x 50 mm, stainless steel) columns which were eluted for 40 min at 1 ml min^{-1} with EtOH : 1% glacial acetic acid in hexanes (15:85). Ethanol (Pierce anhydrous HPLC/spectrograde) consisted of EtOH (90.5±1%), MeOH (5.0±1%) and 2-propanol (4.5±1%); hexanes (Baker Analyzed HPLC Reagent) contained 95% *n*-hexane. All HPLC samples were prefiltered through an Acrodisc 3 CR PTFE (0.45 μm, Gelman Sciences).

Gas chromatography utilized a Hewlett Packard Model 5890A gas chromatograph, a 7673A automatic injector and a 3396A integrator. Separations of thioacidolysis products were carried out as described by Lapierre and Rolando (1988) using a Chrompack fused silica capillary column (50m x 0.32 mm I.D., 1 μm film thickness, CPSIL 5B).

Scintillation counting of radioactive liquid samples, which were dissolved in Ecolume (ICN Biomedicals) cocktail unless otherwise stated, employed a Beckman LS-250 or a Packard Tri-Carb 2000CA liquid scintillation spectrometer. Radioactive tissues were oxidized in either a Packard Model B306 Sample Oxidizer utilizing Carbo-Sorb (Packard) CO_2 absorbing solution and Permafluor V (Packard) liquid scintillation cocktail or a Harvey OX-600 Biological Oxidizer utilizing Carbon-14 Cocktail (Harvey). Dry samples (3-16 mg) were weighed directly into

Combusto-Cone (Packard) paper thimbles or porcelain combustion boats (Harvey). Quenching curves and calibrations utilized either [^{14}C] *n*-hexadecane (14.4 kBq ml^{-1}, Amersham, Arlington Heights, IL) or [^{14}C] poly-(methylmethacrylate) (7.8 Bq mg^{-1}, Dupont, Boston, MA).

UV and visible spectroscopy utilized a Perkin-Elmer Lambda 6 UV-visible spectrometer equipped with a Lambda Accessory Interface and an Epson Equity III+ Computer (PECSS software). Mass spectrometry was conducted with a Hewlett-Packard 5985 GC/MS operating in the EI mode (70eV) using a direct insertion probe. Solution-state ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy utilized a Varian VXR-500 spectrometer using tetramethylsilane (TMS) as an internal standard. The ^{13}C cross polarization-magic angle spinning (CPMAS) NMR spectra were obtained at 50.31 MHz on a Varian XL-200 Spectrometer equipped with a Doty Scientific MAS probe. In the cross polarization experiment, the proton $\pi/2$ pulse was 5.5 μs and the Hartmann-Hahn matched spin lock contact time was 1 ms. A 30 ms acquisition time was employed with a spectral window of 17 kHz and a 200 MHz decoupling field of *ca.* 50 kHz. The chemical shifts were referenced to the methyl resonance of hexamethyl benzene (17.36 ppm) from an independently obtained spectrum recorded under similar spectrometer settings. The samples (50 - 150 mg) were packed into cylindrical sapphire rotors with Kel-F end caps and spun at speeds of 5 kHz. The spinning speed of each sample was carefully matched so that reliable difference spectra could be obtained between different acquisitions. Each spectrum was acquired with a 2 second pulse repetition rate with data acquisition usually continuing for 15-18 h. Transmission electron microscopy of cell culture sections was carried out with a Hitachi H-300 transmission electron microscope (75 kVolts).

3.2. Plant Materials.

P. taeda seedlings were greenhouse-grown from seed (F. W. Schumacher Co., Sandwich, MA) for four months on a 50:50 mixture of vermiculite (Grace Horticultural Products, Cambridge, MA) and sphagnum peat moss (Southern Importers, Inc., Greensboro, NC). Suspension cultures of *P. taeda* were obtained as a gift from R. Mott (Dept. of Botany, North Carolina State University, Raleigh NC).

3.2.1. *P. taeda* Cultures, 2,4-D (11.3 μM) Line: Suspension cultures were grown on a modified Brown and Lawrence (1968) medium freshly prepared each week, in part from stock solutions (A-H) of the following composition (g/l): A. KNO_3 (190), NH_4NO_3 (165); B. $MnSO_4 \cdot H_2O$ (2.1), $ZnSO_4 \cdot 7H_2O$ (4.3), $CuSO_4 \cdot 5H_2O$ (0.05); C. $CaCl_2 \cdot H_2O$ (2.2), KI (0.415), $CoCl_2 \cdot 6H_2O$ (0.013); D. KH_2PO_4 (34), H_3BO_3 (3.1), $NaMoO_4 \cdot 2H_2O$ (0.125); E. $Na_2EDTA \cdot 2H_2O$ (3.73), $FeSO_4 \cdot 7H_2O$ (2.78); F. *myo*-Inositol (2), G. Nicotinic acid (5), Pyridoxine-HCl (1), Thiamine-HCl (1); H. 2,4-Dichlorophenoxy Acetic Acid [2,4-D] **36** (0.221). Stock solutions, except for E and H, were prepared by sequential addition of the constituents to H_2O with the resulting solutions being brought to volume. For stock solution E the two constituents ($Na_2EDTA \cdot 2H_2O$ and $FeSO_4 \cdot 7H_2O$) were individually dissolved in H_2O (400 ml each) to give solutions which were slowly combined and subsequently brought to volume (1 l) with H_2O. For the 1 mM 2,4-D **36** stock solution (H), 1 M NaOH (1 ml) was added to H_2O (80 ml) after which 2,4-D **36** (22.1 mg) was added. After magnetic stirring until the 2,4-D **36** was solubilized, 1N HCl was added (approximately 800 μl) until a pH 7-8 was obtained with the solution then brought to volume (100 ml) with H_2O. All

stock solutions were stored at 4°C in the dark. Stocks F, G and H were replaced every 1-2 months while other stocks were replaced within 6 months.

Medium preparation began with the sequential addition of stock solutions (A through F, 10 ml; G, 1 ml) to 600 ml of H_2O which was stirred magnetically. To the resulting solution, $MgSO_4$ (930 mg) was added. Following dissolution, sucrose (30 g) was added and after its dissolution, the growth regulator (Stock Solution H, 11.3 ml) was added with the medium then brought to volume (1 l) with H_2O. Subsequently, the pH was adjusted to 5.5 by dropwise addition of 1.0 and 0.1 N NaOH. Aliquots (25 ml) of prepared medium were dispensed to Erlenmeyer flasks (125 ml) which were then covered with aluminum foil disks (heavy-duty, two layers, 154 cm diameter) and autoclaved (121°C, 20 min).

Cultures were maintained on a 2,4-D-containing medium by weekly transfers of cells to fresh medium prepared as described above. After autoclaving, flasks containing freshly prepared medium were surface sterilized by spraying with ethanol (70% in water) and allowed to cool (1 hour) in the laminar flow hood (Laminar Flow Inc., Ivyland, PA). Disposable sterile polystyrene serological pipets (10 ml, Falcon 7551, Becton Dickinson & Co.), flasks of 7-day-old culture and an automatic pipet (Pipet-aid, Drummond Scientific) were also surface sterilized and placed in the laminar flow hood. For the serological pipets used, which were individually wrapped, the tapered tip (approximately 3.5 cm in length) was fused to the barrel of the pipet. At this joint, the pipets were carefully bent by hand until broken (special care was taken to prevent breakage of the wrapper seal). With the tip removed, the suspension culture cells could now be drawn up into the pipet. Transfers of cells began with the removal of the aluminum foil cover from a flask containing 7-day-old cells and one containing fresh medium. These covers were

subsequently flamed along with the opening of both flasks. During a cell transfer, a serological pipet, with the tip removed, was inserted into the flask of a 7-day-old culture and cells withdrawn. Then, by depressing the open end of the pipet against the bottom of the same flask, cells could be trapped in the pipet while expelling the used medium. Cell suspension culture was repeatedly drawn into, and used medium expelled from, the pipet until a 2.5 ml packed cell volume (PCV) was achieved. Afterwards, the pipet, containing the appropriate PCV (2.5 ml) of cells, was inserted into the flask (125 ml) containing fresh medium (25 ml). Repeated drawing and expelling of fresh medium released the cells from the pipet. Once the cells were transferred, the flask containing the fresh medium and recently transferred cells was sealed with aluminum foil subsequent to flaming both the lip of the flask and the aluminum foil cover. The above described transfer was repeated for a second flask of fresh medium. Once this was done, the pipet was replaced with a new one and the complete transfer procedure repeated again.

Stock *P. taeda* suspension cultures (2.5 ml PCV) were initiated on the medium (25 ml) described above and cultivated at 24±1°C on a Lab-Line (Melrose Park, IL) Model 3520 Orbital Shaker (100 rpm) under light (25-45 μmol s^{-1} m^{-2}) provided by two fluorescent lamps (40 watt, Philips, cool white). The cultures typically grew to *ca.* 6 ml PCV per flask after seven days while retaining a yellow-green coloration, and were maintained by weekly transfers of 2.5 ml PCV of inocula as before. The cell line only stained faintly with phloroglucinol-HCl, a histochemical test for lignin (Bradbury, 1973). Chemical and biochemical analyses were carried out on freeze-dried tissue; typically, cells constituting 6 ml PCV were removed, recovered by vacuum filtration, briefly rinsed with H_2O, frozen

(liq. N_2) and freeze-dried to give tissue (274 ± 12.3 mg) which was stored at -70°C under N_2.

3.2.2. *P. taeda* Cultures, NAA (11.3 μM) Line: *P. taeda* cells (2.5 ml PCV of 2,4-D line) were transferred to Brown and Lawrence medium containing 11.3 μM α-naphthaleneacetic acid (NAA) **37**, in place of 2,4-D **36**, and cultured under the same conditions of temperature, aeration and light. Subsequent transfers to fresh NAA-containing medium occurred on days 7, 14, 21 and 35 after the initial transfer. Up to this point, the cells grew to *ca.* 6 ml PCV per week and retained their usual yellow-green coloration. Beyond day 35, the cells became aggregated, changed in color to tan then brown and went into a stationary phase. Cells harvested on days 7, 14, 21 and 35 stained faint violet-red with phloroglucinol-HCl, whereas cells on day 49 stained deep violet-red. Freeze-dried tissue from cultures on days 7, 14, 21, 35 and 49 were 276 ± 11.6, 297 ± 19.4, 319 ± 5.7, 274 ± 21.7 and 198 ± 34.8 mg per flask, respectively.

3.2.3. *P. taeda* Cultures, Sucrose Dilution/NAA (11.3 μm) Line: *P. taeda* cells (2.5 ml PCV of 2,4-D line) were transferred to a solution of sucrose in water (8%, w/v, 25 ml) in Erlenmeyer flasks (125 ml) and cultured under the same conditions of temperature, aeration and light. After 2 weeks of culturing, the cells were yellow-tan in color with the sucrose solution having a dense milky appearance. To remove adherent contaminants, cells from 5 flasks of culture were transferred as completely as possible to an Erlenmeyer flask (125 ml) containing fresh medium (25 ml), prepared as described above, and NAA **37** (11.3 μM). These cells were then transferred (3 ml PCV) to fresh NAA-containing medium (25

ml) in each of 5 flasks (125 ml). The milky sucrose solutions were set aside for subsequent manipulations. After 1 week of culturing under the same conditions described above, the resulting tan-brown cells, which stained deep violet-red with phloroglucinol-HCl, were harvested and freeze dried (*ca.* 252 mg per flask).

The milky sucrose solution (900 ml) recovered from the *P. taeda* sucrose dilution / NAA **37** (11.3 μM) line was filtered through a glass wool plug to remove cells and debris, chilled (4°C) and then centrifuged (10,000*g*, 15 min, 10°C). After decanting the light yellow supernatant, the cream-colored precipitate was suspended in water (1L) and centrifuged (12,000*g*, 15 min). The resulting turbid supernatant was decanted and the precipitate resuspended in water (500 ml) and recentrifuged (12,000*g*, 20 min). After decanting this supernatant, the precipitate was suspended in water (30 ml), frozen (liq N_2) and freeze-dried to give a cream-colored powder (560 mg) of which an aliquot (100 mg) was solubilized in $(CD_3)_2SO$ and analyzed by solution-state ^{13}C NMR spectroscopy.

3.3. Cell-Free Extracts.

Into a chilled mortar at 4°C was added acid-washed sea sand (20 mg), insoluble polyvinylpolypyrrolidone (50 mg) and buffer (100 mM Tris-HCl, pH 8.5 at 4°C, 2 ml) containing dithiothreitol (10 mM) and polyethylene glycol (MW 8000, 0.5%, w/v). After mixing the contents of the mortar with a pestle, dried *P. taeda* tissue (125 mg, 7-day-old *P. taeda* (2,4-D line), 7-week-old *P. taeda* (NAA line) or 3-week-old *P. taeda* (sucrose dilution/NAA line) suspension culture cells or 4-month-old stems) was added and the resulting slurry ground until homogeneous (approximately 5 min). The homogenate was transferred to a polycarbonate (10 ml) centrifuge tube using buffer as a wash (2 x 250 μl) and centrifuged (30,000*g*,

30 min, 5°C) to give a clear yellow or yellow-green cell-free extract (*ca.* 1.8 ml). Protein contents were determined by the method of Bradford (1976).

3.4. Ammonium Sulfate Precipitated Cell-Free Extract.

Into a mortar (125 ml) were added acid washed sea sand (400 mg), polyvinylpolypyrrolidone (1 g) and buffer (100 mM Tris-HCl, pH 7.5 at 4°C, 25 ml) containing dithiothreitol (10 mM) and polyethylene glycol (MW 8,000, 0.5%, w/v). The contents of the mortar were mixed with a pestle and then, dried 7-day-old *P. taeda* (2,4-D line) suspension culture cells (2.5 g) were added. The slurry was ground until homogeneous (approx. 10 min.) and filtered through 2 layers of Mira-cloth (Calbiochem-Behring Corp., La Jolla, CA) in 2 layers of cheese cloth (100% cotton, American Fiber and Finishing, Inc., Burlington, MA) of which both were previously rinsed with buffer. The greenish-yellow filtrate was transferred to a polycarbonate centrifuge tube (50 ml) and centrifuged (30,000*g*, 30 min.) to give a clear yellow-green supernatant (23.5 ml). The supernatant was transferred to a chilled beaker (50 ml) and stirred magnetically while $(NH_4)_2SO_4$ (5.71 g) was slowly added to bring the salt concentration to ~40% saturation. After the resulting turbid solution was allowed to stand for 15 minutes, it was centrifuged (30,000*g*, 25 min.). The clear faint yellow supernatant recovered (22 ml) was transferred to a chilled beaker and stirred magnetically while $(NH_4)_2SO_4$ (6.27 g) was added to give a final salt concentration of 80%. After centrifugation (30,000*g*, 25 min, 5-10°C), the ash colored precipitate was solubilized in buffer(100 mM Tris-HCl, pH 7.5 at 4 °C) to which glycerol (30%, v/v) was added. The resulting clear golden-colored solution (2 ml) was loaded onto a Sephadex G-25 (Pharmacia) column (10 ml bed volume) eluted with the buffer (100mM Tris-HCl, pH 7.5 at 4

°C, 30% glycerol, v/v). [The void volume of the column was determined with a Blue Dextran (Parmacia) solution (0.2%, w/v, 2 ml) prepared with buffer (100mM Tris-HCl, pH 7.5 at 4 °C) again containing glycerol (30%, v/v).] This enzyme preparation was immediately assayed. The protein concentration was determined by the method of Bradford (1976).

3.5. Enzyme Assays.

3.5.1. Phenylalanine Ammonia-Lyase (EC 4.3.1.5): Cell-free extracts were assayed for phenylalanine ammonia-lyase activity using a procedure adopted from Knogge and Weissenböck (1986). Into a quartz cuvette (1.5 ml) were added buffer (100 mM Tris-HCl, pH 8.5 at 30°C, 850 µl) and cell-free extract (100 µl). After mixing, 100 mM *L*-phenylalanine **9** in water (50 µl) was added and the reaction was monitored spectrophotometrically at 290 nm. The reference blank was as above, except that no phenylalanine **9** was added. Enzyme activities were calculated from the molar absorption coefficient (10 $M^{-1}\,cm^{-1}$) for cinnamate (Zucker, 1965). The cell-free extracts from the *P. taeda* stem tissue, 2,4-D- and NAA-grown cells had the following average PAL activities: *P. taeda* stem tissue (20 pkat mg^{-1} protein, 0.533 pkat mg^{-1} tissue), *P. taeda* (2,4-D line) (not detected), *P. taeda* (NAA line) (229 pkat mg^{-1} protein, 16.7 pkat mg^{-1} tissue).

3.5.2. Cinnamyl Alcohol Dehydrogenase (EC 1.1.1.195): Cell-free extracts were assayed for cinnamyl alcohol dehydrogenase (CAD) activity by a procedure adapted from Sarni *et al.* (1984). Into a quartz cuvette (1.5 ml) were added buffer (100 mM Tris-HCl, pH 8.8 at 30°C, 945 µl), 4 mM coniferyl alcohol **2** in water (25 µl) and the cell-free extract (10 µl). After mixing, 20 mM $NADP^+$ (mono sodium salt) in water (10 µl) was added and the reaction monitored

spectrophotometrically at 400 nm. The reference blank was as above except that no $NADP^+$ was added. Enzyme activities were calculated from the molar absorption coefficient (21.0 M^{-1} cm^{-1}) for coniferaldehyde (Wyrambik and Grisebach, 1975). The cell-free extracts from the *P. taeda* stem tissue, 2,4-D- and NAA-grown cells had the following average CAD activities: *P. taeda* stem tissue (382 pkat mg^{-1} protein, 9.77 pkat mg^{-1} tissue), *P. taeda* (2,4-D line) (367 pkat mg^{-1} protein, 98.8 pkat mg^{-1} tissue) and *P. taeda* (NAA line) (2800 pkat mg^{-1} protein, 210 pkat mg^{-1} tissue).

3.5.3. Hydroxycinnamate CoA-Ligase (EC 6.2.1.12): Ammonium sulfate precipitated cell-free extracts were assayed for cinnamyl CoA-ligase activity by a procedure modified from that of Grand *et al.* (1983). Into a quartz cuvette (2 ml) were added buffer (100 mM Tris/HCl containing 30% glycerol, pH 7.8 at 30°C, 950 µl), aqueous solutions of 7.5 mM ferulic acid (20 µl), 45 mM ATP (10 µl) and 105 mM $MgCl_2$ (10 µl) along with the protein extract (500 µl). After mixing, 30 µM CoASH in water (10 µl) was added, and the reaction monitored spectrophotometrically at 346 nm. The reference blank was as above except that no CoASH was added. Enzyme activity was calculated using the molar absorption coefficient (19 M^{-1} cm^{-1}) provided by Stöckigt and Zenk (1975) for feruoyl-CoA. The ammonium sulfate precipitated cell-free extract from *P. taeda* (2,4-D line) cells gave an activity for hydroxycinnamyl CoA-ligase of 3.75 pkat mg^{-1} protein and 0.151 pkat mg^{-1} tissue.

3.5.4. Coniferin β–Glucosidase (EC 3.2.1.21): *P. taeda* (2,4-D line) medium and cells were assayed for β–glucosidase activity using procedures derived from Hösel *et al.* (1978) and Marcinowski and Grisebach (1978). Into a microcentrifuge tube were added filtered (Acrodisc 3 CR PTFE, 0.45 µm) or

unfiltered 7-day-old medium (450 µl) and an aqueous solution of coniferin **35** (10mM, 50 µl). After 30 minutes of incubation at 30 °C in a shaking water bath (American Scientific Products, Model YB-521, 150 oscillations per min.), 1 M Na_2CO_3 (500 µl) was added and the absorbance determined at 325 nm. The reference sample contained medium incubated as above except that no coniferin **35** was added. In addition, *P. taeda* cells (0.5 ml PCV) in filtered medium (950 µl) were incubated (30°C, 30 min.) with coniferin **35** (10 mM, 50 µl). Controls for this assay consisted of incubations as follows: (i) filtered medium (950 µl) and H_2O (50 µl); (ii) filtered medium (950 µl) and 10 mM coniferin **35** (50 µl); (iii) *P. taeda* cells (0.5 ml PCV) in filtered medium (950 µl) and water (50 µl). Aliquots of each incubate (500 µl) were combined with Na_2CO_3 (500 µl) and absorbance (325 nm) determined. Unfiltered medium and the suspensions of cells showed β-glucosidase activities of 363 pkat ml^{-1} medium and 2.62 nkat ml^{-1} PCV cells, respectively. No activity for β-glucosidase was detected in the filtered medium.

3.5.5. Peroxidase (EC 1.11.1.7): Peroxidase activity in the medium of *P. taeda* (2,4-D line) cells was determined by an assay adopted from the method of Pütter (1974). Into a polystyrene cuvette (2 ml) was added 100 mM phosphate buffer (pH 7.0 at 30°C, 1.5 ml), guaiacol (20.1 mM, 50 µl) and medium sample (50 µl). After mixing, H_2O_2 (12.3 mM, 30 µl) was added and the change of absorbance at 436 nm was monitored for 2 minutes. The reference blank was as above except that no H_2O_2 was added. Enzyme activity was calculated using the molar absorption coefficient (6.39 M^{-1} cm^{-1}) provided by Pütter (1974).

3.5.6. Assay for Phenylalanine: *L*-Phenylalanine **9** concentrations in the culture media were monitored spectrophotometrically by its conversion to

cinnamate. Phenylalanine ammonia-lyase (10 µl, 0.07 units) was added to buffer (0.1 M Tris-HCl, pH 8.5 at 30°C, 1 ml) in a cuvette (1.5 ml). After mixing an aliquot (25 µl) of medium was added with the change in absorbance at 290 nm determined over time (1 min). The reference blank was as above except for the absence of phenylalanine **9**. For calibration purposes, a standard curve was derived from assays of phenylalanine **9** standard solutions (1.0, 2.5, 5.0, 7.5 and 10.0 mM).

3.6. Peroxidase Activity in Medium of *P. taeda* (2,4-D Line) Cells.

Seven-day-old *P. taeda* (2,4-D line) cells were transferred to fresh modified Brown and Lawrence medium (2.5 ml PCV in 25 ml). Under aseptic conditions, an aliquot (125 µl) of the medium was removed with an Eppendorf pipettor and transferred to an Eppendorf micro-centrifuge at 4°C. Aliquots of the medium from the culture, which was maintained under conditions of light, temperature and aeration described previously, were taken on a daily basis as above. Within 30 minutes after each aliquot was removed, the level of peroxidase activity was determined as described above (section 3.5.5).

3.7. Time Course Monitoring of Phenylanine Ammonia-Lyase and Cinnamyl Alcohol Dehydrogenase Activities in *P. taeda* (NAA Line) Cells.

P. taeda (NAA line) cells were cultured as described previously (section 3.2.2). At the end of each week for the first 5 weeks and every 2 days for the final 2 weeks of the 7 week growth period, cells from 2 flasks were separately harvested, weighed, frozen with liquid N_2 and then stored at -70°C.

In this experiment, cell free extracts were obtained from frozen cells (1 g) which were first added to a chilled (4°C) mortar containing acid washed sea sand (16 mg), insoluble polyvinylpolypyrrolidone (40 mg) and buffer (100 mM Tris-HCl, pH 8.5 at 4°C, 1 ml) containing dithiothreitol (10 mM) and polyethylene glycol (MW 8,000, 0.5%, w/v). Following grinding for approximately 5 min, the homogenate was transferred to Eppendorf micro-centrifuge tubes (1.5 ml) with two aliquots of buffer (250 µl each) used to rinse the mortar subsequently added. The homogenate was centrifuged (16,000*g*, 20 min., 5-10°C) and the supernatant combined (1.5 ml) and assayed immediately. Protein contents were determined by the method of Bradford (1976).

3.8. Cell Wall Preparations.

Two procedures (A and B) were employed: (A) freeze-dried 7-day-old 2,4-D-grown suspension culture cells (33 mg) were transferred to a cellulose extraction thimble (10 x 50 mm, Whatman) and extracted sequentially with benzene (4 h), benzene:EtOH (2:1, 4 h) and EtOH (4 h) in a micro-Soxhlet apparatus under N_2. The extracted residue was dried *in vacuo*, transferred to a test tube and steeped in acetone:H_2O (7:3, 2 ml) for 2 h. After decanting, the steeping procedure was repeated (twice) which, following drying *in vacuo*, afforded an ash-colored residue (19 mg) and organic solubles (13.8 mg). (B) the second procedure was adapted from Talmadge *et al.* (1973) and Robert *et al.* (1989). Dried 2,4-D-grown cells (250 mg) were added to a mortar containing buffer (500 mM phosphate, pH 7 at 4°C, 6 ml) and ground with a pestle (*ca.* 8 min). The resulting slurry and buffer (6 ml) used for rinsing were combined and subjected to centrifugation (1500*g*, 15 min, 5-10°C) in a swinging bucket centrifuge (Beckman Model TJ-6). The

supernatant was decanted, and the precipitate resuspended in buffer (12 ml). After centrifugation and decanting as before, the procedure was repeated using water (3 x 12 ml) and MeOH (1 x 12 ml). The final precipitate was washed into a glass vial (20 ml) with $CHCl_3$:MeOH (1:1, 12 ml) with the resulting suspension stirred magnetically for 18 h at room temperature in the dark. The cell walls were recovered by filtration, washed with $CHCl_3$:MeOH (1:1, 2 x 12 ml), acetone (2x 4 ml) and dried *in vacuo*. The resulting residue was transferred to a cellulose extraction thimble (10 x 50 mm, Whatman) and extracted with toluene:ethanol (2:1, 18 ml) for 20 h in a micro-Soxhlet apparatus (25 ml) under N_2. The extracted tissue and thimble were rinsed with acetone (3 x 5 ml), and dried *in vacuo* to afford a residue (50 mg) which was stored in the dark at 4°C. In an analagous manner, forty-nine day old NAA-grown *P. taeda* cells (250 mg) afforded 161 mg of dry residue.

3.9. Chemical Analyses.

3.9.1. Acetyl Bromide Lignin Determinations: Lignin contents of extractive-free (Procedure B) cell walls were determined by the method of Morrison (1972) using an absorptivity value for lignin of 23.7 l g^{-1} cm^{-1} (Johnson *et al.*, 1961). Into test tubes (14 mm ID x 150 mm), containing dried extractive-free cell walls (10 mg), was added 5 ml of 25% (v/v) acetyl bromide in glacial acetic acid. After capping the test tubes with glass marbles (16 mm diam.), the samples were digested by shaking (150 oscillations per min) for 30 min at 70°C in a shaking water bath (American Scientific Products Model YB-521). During digestion, the samples were briefly removed every 5-10 min from the shaker, vigorously mixed by hand (holding the marble in place) and replaced. After

digestion, the samples were cooled (room temperature) and transferred to volumetric flasks (100 ml) containing 2 N NaOH (1.8 ml) and glacial acetic acid (10 ml). Approximately 3 ml of glacial acetic acid was used to wash the residual contents of each test tube into the respective volumetric flask. After vigorous shaking of the resulting mixture, 0.5 M $NH_2OH \cdot HCl$ (3.2 ml) was added to the contents of each volumetric flask which were brought to volume with glacial acetic acid. The mixture was again vigorously shaken and allowed to stand (1 h). Centrifugation (2,000*g*, 10 min) was done to remove any unsettled particles. Lignin contents were determined by measuring the absorbance of the samples at 280 nm with acetic acid in the reference cell. Control blanks were processed in the same manner differing only in the absence of sample. For 2,4-D-grown cells, the *apparent* acetyl bromide lignin content of extracted cells (Procedure B) was 8.2%, whereas for the forty-nine-day old extractive-free NAA-grown cells it was 15.9%.

3.9.2. Klason Acid-Insoluble Lignin: The acid-insoluble lignin contents of the extractive-free (Procedure B) cell walls were determined by the Klason method (TAPPI standard procedure TS 222 os74). A solution of 72% H_2SO_4 was prepared by adding concentrated H_2SO_4 (66.5 ml) to H_2O (30 ml) in a volumetric flask (100 ml) which was cooled to room temperature, placed on a laboratory balance and then brought to volume using either concentrated H_2SO_4 or H_2O to achieve a specific gravity of 1633.8 g l^{-1}. Extractive-free cell walls (25 mg) were placed into a glass vial (4 ml) to which 72% H_2SO_4 (500 µl) was then slowly added. The resulting suspension was magnetically stirred for 4 h (20°C) and transferred to H_2O (10 ml) in a round-bottom flask (100 ml). The vial was then washed with H_2O (27.5 ml) and the washings combined with the contents of the round-bottom flask. The temperature was raised until refluxing began and maintained for 4 h

following which the resulting suspension was allowed to cool and settle overnight. Without disturbing the settled reddish-brown particles, the solution was vacuum filtered through a weighed Whatman GF/A glass fiber filter supported by a fritted glass funnel (15 ml, medium). Using boiling H_2O, (100 ml), the insoluble lignin was quantitatively transferred to the filter which was subsequently freeze-dried. The acid-insoluble lignin for the extractive-free cell walls from the 7-day-old 2,4-D- and 49-day-old NAA-grown cells averaged 4.6 and 49.2% of the weight of extractive-free cell walls, respectively.

3.9.3. Estimation of Lignin Monomer Composition: This was estimated by two methods: a) *Nitrobenzene Oxidation.* Estimation of the monolignol composition of lignin in extractive-free (Procedure B) cell walls was determined by a nitrobenzene oxidation method described by Scalbert *et al.* (1986). Into a stainless steel reaction bomb (1.5 ml capacity) containing dried cell wall residue (10 mg) was added 2 N NaOH (500 µl) and redistilled nitrobenzene (50 µl). After purging with N_2, the reaction bomb was sealed and placed in a silicone oil bath at 165°C for 2 h. Periodically (approx. every 30 min.), the bomb was shaken by hand with forceps while remaining submerged in the silicone oil. The bomb was removed, cooled to room temperature under running water, wiped clean, opened and the contents removed with a Pasteur pipet and transferred to a glass test tube (13 x 100 mm) with a screw cap. Water (2 x 250 µl) was used to wash the residual contents of the bomb into the test tube. The resulting mixture (approximately 1 ml) was extracted with Et_20 (3 x 3 ml) with the organic solubles then discarded. The extracted aqueous layer was acidified with 6 N HCl (approximately 5 drops) to pH 1 and then extracted with Et_2O (3 ml) containing veratraldehyde (1 mM) as an internal standard. After recovering the Et_2O solubles, the aqueous layer was

further extracted with Et_2O (2 x 3 ml). Ether solubles were combined, dried (Na_2SO_4), evaporated to dryness *in vacuo* and solubilized in methanol (3 ml) with an aliquot (10 µl) subjected to HPLC analysis. Standard solutions of syringaldehyde (1 mM), vanillin (1.2 mM) and 4-hydroxybenzaldehyde (1.0 mM) were prepared in methanol and used for column calibration. Nitrobenzene oxidation products, *p*-hydroxybenzaldehyde:vanillin, were 1:4.3 (5.9% yield) and 1:9.8 (18.6% yield) for 2,4-D- and NAA-grown cells, respectively.

b) *Thioacidolysis.* The monolignol composition in extractive-free cell walls was estimated by thioacidolysis according to the procedures of Lapierre *et al.* (1986) and Lapierre and Rolando (1988). Cell walls (20 mg) were digested with a 10 ml solution of dioxane:ethanethiol (9:1) containing 0.3M BF_3 etherate under N_2 in a teflon lined acid digestion bomb (Parr #4745, Parr Instrument Co.). The reaction bomb was sealed and then immersed in a silicone oil bath (100°C) for 4 hours with occasional shaking. Subsequently, the reaction bomb was removed, cooled in an ice bath (4°C) and to the reaction mixture was added H_2O (10 ml). Using 0.4 M $NaHCO_3$, the pH was then adjusted to 3-4 as determined by Colorphast pH indicator strips (E.M. Science). This solution was extracted with CH_2Cl_2 (3 x 20 ml) with the organic solubles combined, dried over Na_2SO_4, evaporated to dryness *in vacuo.* The sample was reconstituted in CH_2Cl_2 (500 µl) of which and aliquot (10 µl) was added to a conical reaction vial (200 µl) containing tetracosane (4 µg), pyridine (25 µl) and bis(trimethylsilyl)trifluoroacetamide (BSTFA, 50 µl). After sealing, the vial was heated in a boiling water bath for 10 min, cooled to room temperature, opened and the mixture evaporated with N_2 to a volume of 35 µl. The vial was then resealed and the sample analyzed by gas chromatography using an 1 µl injection volume. Thioacidolysis products gave *p*-hydroxyphenyl **4**:guaiacyl **5**

ratios of 1:9.6 (0.23% yield) and 1:6.0 (6.5% yield) for 2,4-D- and NAA-grown cells, respectively.

3.10. Electron Microscopy of *P. taeda* Tissue Sections.

These were prepared following modification of procedures by Hayat (1986). *P. taeda* suspension culture cells (2,4-D line, 7-days-old or NAA line, 7-weeks-old) were rinsed with 50 mM piperazine-*NN*-bis-2-ethanol-2-sulfonic acid-NaOH (PIPES) buffer (pH 7.2 at room temperature), then immersed in the same buffer containing 3% glutaraldehyde (2-3 cell volumes) for 2 h at room temperature. After thorough rinsing with 50 mM PIPES buffer (pH 7.2) as before, the cells were reimmersed in fresh buffer containing 3% glutaraldehyde and kept at 4°C (overnight). After thorough rinsing with 50 mM PIPES buffer (pH 7.2) as before, the cells were postfixed in 2% osmium tetroxide (room temperature, 2-4 h). The cells were again rinsed with buffer (50 mM PIPES, pH 7.2), dehydrated with a graded series of ethanol (15, 30, 50, 70, 80, 90, 95 and 100%) then embedded in L R White resin. Alternatively, dehydration was carried out using a graded acetone series in a manner similar to the alcohol series, in preparation for embedding in Spurr's resin. Sections (90 nm) were cut from blocks of embedded tissues using a Reichert OMU2 ultramicrotome equipped with a diamond knife (Delaware Diamond Knives) and transferred to 100 mesh copper grids coated with Formvar film. Photomicrographs were taken on a Hitachi H-300 TEM after staining with 2% uranyl acetate and then Reynold's lead.

3.11. Administration of Coniferin to *P. taeda* (2,4-D Line) Cells.

Preliminary labelling experiments involved the administrations of radiolabelled and unlabelled coniferin **35**: a) *[2-^{14}C] Coniferin.* Under aseptic conditions, autoclaved modified Brown and Lawrence medium (5 ml) was dispensed to 3 autoclaved Erlenmeyer flasks (25 ml). Into one of the three Erlenmeyer flasks was then added 500 µl of a filter-sterilized 10 mM [2-^{14}C] coniferin **35** (707 kBq mmole^{-1}) aqueous solution. Suspension culture cells (0.5 ml PCV) from a 7-day-old 2,4-D-grown culture were then transferred to each of the three flasks. With an Eppendorf pipettor equipped with sterile tips, aliquots (25 µl) of the radiolabelled medium were aseptically removed for liquid scintillation counting. Cells incubated under the usual condition of light, aeration and temperature, maintained their yellow-green coloration throughout the 7-day culture period. On day 7, the total radioactivity remaining in the medium (3.5 kBq) was approximately 48% of the total radioactivity administered (7.28 kBq). Cells grown with or without radiolabelled coniferin **35** were harvested as before and gave dry weights as follows (mg): with coniferin 99.6; without coniferin 90.3, 88.7.

Dried radiolabelled cells (25 mg) were transferred to a cellulose extraction thimble (Whatman, 10 x 50 mm) and extracted with benzene:ethanol (2.5:1, 16 ml) in a micro-soxhlet apparatus (max. vol. 25 ml) for 12 hours. Aliquots (25 µl) of the light green extract (11.2 ml) were taken and the total radioactivity of the benzene:ethanol solubles (802 Bq) was determined by liquid scintillation counting. Samples (1.5-4 mg) of the extracted cells (20.5 mg) as well as of the unextracted freeze-dried cells were oxidized and the resulting data were used to calculate the total radioactivities of the cells before (977 Bq) and after (118 Bq) extraction.

Residue collected after freeze-drying the radiolabeled medium was solubilized in water (6.10 mg in 90 µl). An aliquot (10 µl) of a 2 mM solution of coniferin **35** in water, was combined with the 90 µl of the solubilized radiolabeled medium residue. The resulting solution was analyzed by HPLC as before while collecting fractions in scintillation vials (20 ml) every 0.5 minutes after injection (0.5 thru 17 min.). None of the radiolabel injected (83.3 Bq) was coeluted with coniferin **35**.

b) *Unlabelled Coniferin.* In a separate experiment set up in an analagous manner, 7-day-old 2,4-D-grown *P. taeda* cells (0.5 ml packed cell volume) were transferred to 3 Erlenmeyer flasks (25 ml) containing modified Brown and Lawrence medium (5 ml) and 2,4-D **36** (11.3 µM). Two of the flasks also contained unlabeled coniferin **35** (1mM). Two additional flasks contained medium (5ml) with 2,4-D **36** (11.3 µM) and coniferin **35** (1mM) but no cells. Aliquots (100 µl) of medium from each sample as well as the controls were aseptically taken on a daily basis throughout the 7-day growth period. At the end of the experiment (day 7) the green cells were harvested as before and gave dry weights as follows (mg): with coniferin, 71, 72; without coniferin 77. Subsequent HPLC analyses of the medium samples showed that for the controls which contained coniferin **35** but no cells, the concentration of coniferin **35** remained unchanged from the initial level (approx. 1mM). On the other hand, the medium samples for the cells administered coniferin **35** showed a decrease in the concentration of coniferin **35** over time as follows (mM): t=0 day, 0.90, 0.93; t=1 day, 0.87, 0.80; t=2 days, 0.66, 0.60; t=3 days, 0.14, 0.08; t=4 days, 0.01, not detected.

3.12. Administration of [U-^{14}C] Phenylalanine to *P. taeda* Cells.

a)*2,4-D Cell Line.* [U-^{14}C] Phenylalanine **9** (10 µl, 3.7 MBq ml^{-1}) was added to 110 mM phenylalanine **9** (2.25 ml) in H_2O, then filter sterilized (0.22 µm, Millex GV) and subdivided into four (500 µl) aliquots of which three were frozen (-20°C) until required. The remaining aliquot of [U-^{14}C] phenylalanine **9** (500 µl, 149 kBq mmole^{-1}) was then aseptically added to an Erlenmeyer flask (25 ml) containing modified Brown and Lawrence medium (5 ml) and 2,4-D **36** (11.3 µM). To this radiolabelled medium was then added seven-day-old 2,4-D-grown *P. taeda* cells (0.5 ml packed cell volume). After 3 days, the cells (0.5 ml packed cell volume) were transferred to fresh medium containing 11.3 µM 2,4-D **36** and 10 mM [U-^{14}C] phenylalanine **9** (149 kBq mmole^{-1}) as before. This procedure was repeated on days 7 and 10, with the yellowish-green cells harvested after 14 days by filtration, washed with H_2O (*ca.* 2 ml), frozen (liq. N_2) and freeze-dried (80.2 mg, 1913 Bq). A portion of the cells (21.8 mg, 520 Bq) was then extracted (Procedure A) to give an ash-colored extractive-free residue (12.3 mg, 241Bq) and organic solubles (260 Bq). [Note also that on days 3, 7 and 10, cells in excess of 0.5 ml PCV were harvested, and freeze-dried to afford 25.2, 37.7 and 23.3 mg respectively.] Samples of the dried cells from day 3 (9.13 mg, 138 Bq), day 7 (21.0 mg, 536 Bq) and day 10 (12.7 mg, 300 Bq) were extracted as above to give extractive-free residues, day 3 (4.9 mg, 52.1 Bq), day 7 (12.3 mg, 217 Bq) and day 10 (8.0 mg, 120 Bq) and organic solubles, day 3 (69.7 Bq), day 7 (268 Bq) and day 10 (151 Bq).

Parallel experiments were conducted as above, except for the use of 1.0 mM (1.49 MBq mmole^{-1}) and 0.1 mM (14.9 MBq mmole^{-1}) [U-^{14}C] phenylalanine **9**. At day 14, the cells were harvested, freeze-dried, and aliquots (22.5 mg [1.0 mM],

26.3 mg [1.0 mM]) extracted (procedure A) to give cell wall preparations 13.2 mg (637 Bq) and 16.1 mg (1086 Bq).

b) *NAA Cell Line.* To a solution of 110 mM phenylalanine **9** in water (2.75 ml) was added [U-^{14}C] phenylalanine **9** (11.25 µl, 3.7 MBq ml^{-1}). After filter sterilizing as described before, an aliquot of the resulting 110 mM [U-^{14}C] phenylananine solution (2.5 ml, 137.5 kBq mmole^{-1}) was aseptically transferred to an Erlenmeyer flask (125 ml) containing modified Brown and Lawrence medium (25 ml) and NAA **37** (11.3 µM). Thirty-five day old NAA-grown *P. taeda* cells (2.5 ml packed cell volume) were then aseptically transferred to this medium. After 14 days, the resulting brown cells were filtered, rinsed with H_2O (ca. 5 ml), frozen (liq. N_2), freeze-dried (175 mg) of which a portion (69 mg, 2.34 k Bq) was extracted (Procedure B) to give cell walls (32 mg, 1.06 kBq) and extracts (1.12 k Bq).

In a separate experiment, *P. taeda* (NAA line) cells were administered [U-^{14}C] phenylalanine **9** (10 mM, 120.0 kBq mmole^{-1}) as before and harvested after 14 days of culturing to give dried cells (213.3 mg). An aliquot of these cells (80.8 mg, 3.75 kBq) was extracted (Procedure B) to give cell walls (33.5 mg, 1.70 kBq) and extracts (1.48 kBq). Cells in a second flask cultured in parallel with [U-^{14}C] phenylalanine **9** (10 mM, 116.6 kBq mmole^{-1}) were not harvested after 14 days but completely transferred to fresh medium containing NAA **37** (11.3 µM) but not phenylalanine **9**. After 4 days of additional culturing in this medium, the cells were harvested to give dried cells (319.3 mg) of which an aliquot (86.0 mg, 2.33 Bq) was extracted, as before, to give cell walls (28.3 mg, 929 Bq) and extracts (1.24 kBq).

3.13. Administration of [1-^{13}C], [2-^{13}C] and [3-^{13}C] Phenylalanines to *P. taeda* Cells.

a) 2,4-D-Cell Line. A solution of 110 mM [1-^{13}C] phenylalanine **9** (99 atom % ^{13}C) in H_2O (4.25 ml) was filter sterilized and subdivided into four aliquots (1 ml) of which three were frozen and stored at -20°C in the dark until needed. To an Erlenmeyer flask (50 ml) containing Brown and Lawrence medium (10 ml) and 2,4-D **36** (11.3 μM) were added the remaining 1 ml of 110 mM [1-^{13}C] phenylalanine **9** solution and *P. taeda* cells (1 ml packed cell volume). After 3 days, 1 ml packed cell volume was transferred to fresh medium, containing 10 mM [1-^{13}C] phenylalanine **9** and 11.3 μM 2,4-D **36** as before; this procedure was repeated on days 7 and 10. At day 14, the cells were harvested, washed with H_2O (*ca.* 5 ml), frozen (liq. N_2), freeze-dried (133 mg), and extracted (procedure A) to give extractive-free tissue (76.5 mg). In the control experiment, the cells were grown on 2,4-D **36** medium as before, but with no exogenously supplied phenylalanine **9**. After 7 days, the cells were harvested and extracted to give extractive-free tissue (84.9 mg). Each sample was then subjected to solid-state ^{13}C-nuclear magnetic resonance spectroscopic analysis; subtraction of the natural abundance ^{13}C-NMR spectrum (control) from that obtained from the [1-^{13}C] phenylalanine **9** aministered *P. taeda* cells gave a difference spectrum, with a large carbon-13 enriched resonance at 172.4 ppm and much smaller signals at 72.6, 62.8 and 37.5 ppm.

Next, the [1-^{13}C] phenylalanine **9** administered *P. taeda* cell wall preparation (78.3 mg) was suspended in 50 mM phosphate buffer (pH 7.5 at 37°C, 1 ml) containing pronase E (17.6 nkat ml^{-1}) and ground in a mortar with a pestle for 5 min. To this was added 50 mM phosphate buffer (4.5 ml) containing pronase E (17.6 nkat ml^{-1}), and the suspension was then incubated at 37°C in a shaking water

bath (American Scientific Products, Model YB-521, 150 oscillations per minute) for 12 hours. Following centrifugation (1500*g*, 15 minutes) the cell walls were washed with 50 mM phosphate buffer (pH 7.5 at 37°C, 2 x 6 ml) and water (2 x 6 ml), then frozen (liq. N_2) and freeze dried (28.4 mg). The cell wall preparation (36.8 mg) from the control exteriment was treated identically to afford a residue (12.1 mg). The solid-state ^{13}C NMR spectrum of each sample was recorded as before; the difference spectrum so obtained revealed small carbon-13 enriched resonances at 172.9, 72.6, 63.4 and 37.2 ppm.

b) *NAA-Cell Line.* Filter-sterilized 110 mM [1-^{13}C] phenylalanine **9** (99 atom % ^{13}C) in H_2O (2.5 ml) was added to an Erlenmeyer flask (125 ml) containing modified Brown and Lawrence medium (25 ml) and NAA **37** (11.3 μm). Suspension culture cells (2.5 ml PCV), grown on NAA-containing medium for 35 days as described, were then asceptically transferred to this medium. After 14 days, the resulting brown cells were harvested, rinsed with H_2O (*ca.* 5 ml), frozen (liq. N_2), freeze-dried (183 mg), with the solid-state ^{13}C-NMR spectrum of the sample subsequently recorded. A portion of these cells (158 mg) was extracted (procedure B) to afford extractive-free cell walls (76 mg) which were again analyzed by solid-state ^{13}C-NMR spectroscopy. Next, [1-^{13}C]phenylalanine administered extractive-free cell walls (160mg), combined from three experiments, were suspended in 50 mM phosphate buffer (pH 7.5 at 37°C, 2 ml) containing pronase E (17.6 nkat ml^{-1}) and ground in a mortar with a pestle for 5 min. To this was added 50 mM phosphate buffer (8 ml) containing pronase E (17.6 nkat ml^{-1}), and the suspension was incubated at 37°C for 12 h in a shaking water bath. Following centrifugation (1500*g*, 15 min) the cell walls were washed with 50 mM phosphate buffer (pH 7.2, 2 x 10 ml) and H_2O (2 x 10 ml), then frozen

(liq. N2), freeze-dried (119 mg) and analyzed by solid-state ^{13}C-NMR spectroscopy. *P. taeda* cells (NAA-line) either administered unlabelled or no phenylalanine **9** were treated in the same manner and used as controls. Subtraction of the NMR spectrum of the extractive-free pronase E treated cell walls previously administered ^{13}C natural abundance 10 mM phenylalanine **9** from the spectrum of the corresponding [1-^{13}C] phenylalanine **9** administered cell walls gave a difference spectrum with resonances at 180.1, 173.2, 72.7 and 63.4 ppm.

In an analogous manner, parallel experiments were individually conducted using [2-^{13}C] and [3-^{13}C] phenylalanine **9** as precursors. Difference spectra were obtained as above; in the [2-^{13}C] phenylalanine **9** experiment, enriched resonances were observed with cell wall preparations at 127.9, 85.8, 55.4 and 47.7 ppm, whereas those administered [3-^{13}C] phenylalanine **9** afforded resonances at 130.7, 86.4, 74.4 and 38.6 ppm.

3.14. Isolation of Lignin Derivative from *P. taeda* (NAA Line) Cell Walls.

Into a grinding bowl (12 ml) were placed 4 grinding balls (12 mm diam.), dried extractive-free and pronase E treated *P. taeda* (NAA line) cell walls (127 mg) and toluene (2.5 ml). The grinding bowl was purged with N_2, sealed and placed in a "Planetary micromill" (Fritsch GmbH, Germany) and kept in a cold room (10°C). After grinding (60 h, speed level 4), the resulting suspension was transferred to a glass vial (20 ml) using toluene (approx. 2.5 ml) for rinsing the balls and grinding bowl. An aliquot of the suspension was observed by light microscopy and showed the particle sizes to be less than 10 μm. After removing the bulk of the toluene from the cell walls under vacuum using a water aspirator, drying under high vacuum gave cell walls (115 mg) which were suspended in

dioxane:H_2O (9:1, 5 ml) and stirred magnetically for 24 hours under N_2 at room temperature. The suspension was then transferred to a centrifuging tube (15 ml, Corex glass) and spun (7500*g*, 10 min) to obtain a rich amber extract which was dried in vacuo (12.5 mg). The cell walls were resuspended in dioxane:H_2O (9:1, 5 ml) and extracted as before. After centrifugation, the yellow extract was dried *in vacuo* (1.7 mg). The cell walls were placed under vacuum to remove the dioxane, suspended in H_2O (1 ml), frozen (liq. N_2) and freeze-dried (102 mg). Extractive-free and pronase E treated [1-^{13}C], [2-^{13}C] and [3-^{13}C] phenylalanine **9** enriched *P. taeda* (NAA line) cell walls were extracted with dioxane:H_2O (9:1) in the same manner as above. The first dioxane:H_2O extract (crude MWL) for each sample was solubilized in $(CD_3)_2SO$ for analysis by solution-state ^{13}C NMR spectroscopy; the carbon-13 enriched resonances were as follows: δ [1-^{13}C] 194.02, 178.35, 70.90, 70.78, 62.78, 61.58, 60.19, 58.6; [2-^{13}C] 128.04, 125.67, 83.65, 53.5, 45.47, 28.94; [3-^{13}C] 190.95, 153.89, 128.43, 86.80, 84.83, 82.5, 82.3, 81.58, 71.42, 70.83, 36.74, 33.5.

3.15. Isolation of (-)-Matairesinol from *P. taeda* (2,4-D line) Cells.

Freeze-dried *P. taeda* (2,4-D line) cells (5 g) from a seven-day-old culture were suspended in MeOH (50 ml) in an Erlenmeyer flask (125 ml) under an atmosphere of N_2. The temperature was then raised to 50°C and the suspension stirred magnetically for 10 minutes. The resulting green extract was decanted and the extraction procedure repeated (3 times). Extracts were combined, concentrated *in vacuo* to *ca.* 5 ml following which H_2O (50 ml) was added. The resulting cloudy light-green mixture was centrifuged (2700*g*, 10 minutes) and extracted with anhydrous ether (3 x 50 ml). The combined ether solubles were dried (Na_2SO_4)

and evaporated to dryness *in vacuo*. For reverse phase HPLC separations, the sample was reconstituted in MeOH (1 ml), filtered (Acrodisc 3 CR PTFE, 0.45 μm) with 10 μl aliquots subjected to HPLC analyses. Following separation on a Waters Nova-Pak C_{18} column eluted with CH_3CN:H_2O, inspection of the resulting chromatograms revealed the presense of a component (retention volume = 13.8 ml) which coeluted with (±)-matairesinol. After repeated injections (10 x 95 μl), the collected eluates were rotoevaporated to remove CH_3CN and the resulting aqueous solution frozen (liquid N_2) and freeze-dried to afford pure (-)-matairesinol (1 mg) as evidenced by ^1H NMR, mass spectroscopy and chiral chromatographic analyses: ^1H-NMR ($CDCl_3$): δ 2.44-2.63 (4H, m, $C_{7'}H_2$, $C_{8'}H$, C_8H), 2.88 (1H, dd, J_1=14.2Hz, J_2=7.2Hz, C_7H), 2.95 (1H, dd, J_1=13.5Hz, J_2=5.0Hz, C_7H), 3.84 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 3.88 (1H, dd, J_1=8.75Hz, J_2=6.75Hz, $C_{9'}H$), 4.15 (1H, dd, J_1=9.25Hz, J_2=7.25Hz, $C_{9'}H$), 6.41 (1H, s, Ar-H), 6.51 (1H, dd, J_1=7.75, J_2=1.75, Ar-H), 6.60 (2H, m, Ar-H) 6.79-6.83 (2H, m, Ar-H); MS m/z (%): 358 (M^+, 10.8), 137 (100); UV λmax (CH3OH); 281, 229. Chiral Chromatographic separations on a Diacel Chiralcel OD column eluted with EtOH:1% glacial acetic acid in hexanes gave (-)-matairesinol (retention volume = 39.2 ml).

4. RESULTS AND DISCUSSION

4.1. *P. taeda* (2,4-D Line) Suspension Cultures.

Through this research, efforts were applied to develop a model system to study the process of lignification occurring during the early stages of cell wall formation and maturation. As indicated previously, plant suspension cultures were utilized since they provide relatively homogeneous cell populations possessing for the most part only primary cell walls. With suspension culture cells it was envisaged that a plant system would be available that could be induced to undergo cell wall thickening and lignification.

Suspension cultures of *P. taeda* were obtained as a gift from Dr. R. Mott at North Carolina State University, Raleigh, NC. The cultures were initiated using callus tissue derived from the cambial zone of a 3-year-old branch from a tree growing on that campus. The suspension culture was maintained on a modified Brown and Lawrence (1968) medium adapted by Mott and coworkers from the original recipe which was used to maintain the callus of another economically important southern pine species (*Pinus palustris*, Mill.). The growth regulator included in the medium for the maintenance of these *P. taeda* suspension culture cells was 2,4-D **36**.

Routine maintenance of the *P. taeda* (2,4-D line) suspension culture cells necessitated cell transfers to freshly prepared medium every 7 days. At the end of the 7-day growth period, the packed cell volume (PCV) of the cultures was approximately 2-fold the initial inoculum thus providing enough cells to pass one 7-day-old culture to 2 flasks of fresh medium. Culture cells maintained with 2,4-D **36** as the growth regulator retained a yellow-green coloration with the medium remaining relatively clear and colorless throughout the growth cycle.

Seven-day-old *P. taeda* (2,4-D line) suspension culture cells, harvested by filtration, were suspended in H_20 and observed directly by light microscopy. Alternatively, cells harvested were fixed with glutaraldehyde, post-fixed with osmium tetroxide, dehydrated, embedded in resin (L R White or Spurr's), sectioned, stained with uranyl acetate and Reynold's lead, and then observed by transmission electron microscopy. Observations by light microscopy showed the cells to be relatively spherical and tending to aggregate. Note that the mechanism of cell-cell recognition is unknown at present, but may be due to cell surface glycoproteins and homophilic adhesion. Subsequent observations by transmission electron microscopy showed in most instances the cells to have an intact protoplast with organelles and cellular structures such as a nucleus, plastids, mitochondria, vacuoles, endoplasmic reticulum and Golgi bodies to be discernible. By electron microscopy, the overall thickness of cell walls was estimated to be 0.2 μm (see Figure 20); this is consistent with the known dimensions of primary cell walls (Sjöström, 1981). No other distinguishable layers consistent with secondary (*i.e.*, S_1, S_2, or S_3) thickening were observed. These cell walls did not exhibit birefringence under a polarized light microscope indicating that there was no helical organization of the cellulose microfibrils as in secondary cell wall layers. Having established that the *P. taeda* (2,4-D line) suspension cultures only possessed primary cell walls, it was next of great importance to determine whether phenylpropanoid metabolism was occurring.

If phenylpropanoid metabolism was actively occurring in these cells then key metabolic enzymes involved in phenylpropanoid metabolism would likely be detectable. Accordingly, cell-free extracts from 7-day-old *P. taeda* (2,4-D line)

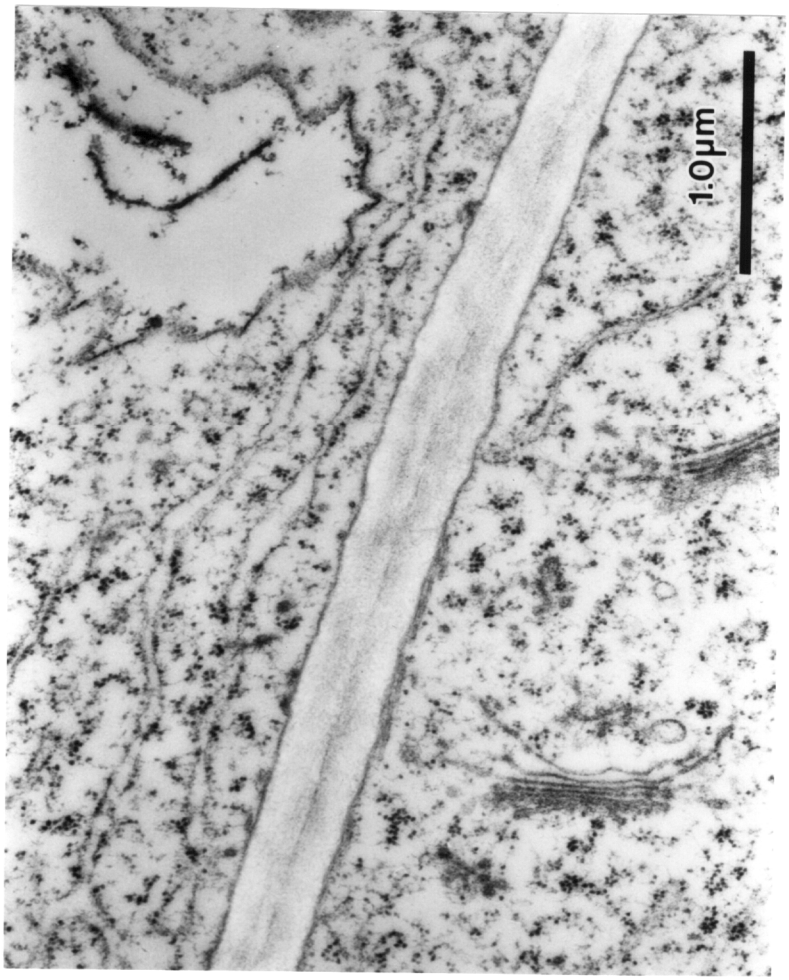

Figure 20. Transmission Electron Micrograph of Cell Walls from *P. taeda* (2,4-D Line) Suspension Culture Cells.

cells were assayed for phenylalanine ammonia-lyase, hydroxycinnamate:CoA ligase and cinnamyl alcohol dehydrogenase.

Phenylalanine ammonia-lyase catalyzes the first committed step in general phenylpropanoid metabolism whereby L-phenylalanine **9** is converted into cinnamic acid **23**. Attempts to detect phenylalanine ammonia-lyase activity in cell-free extracts from fresh and freeze-dried cells showed the activity of this enzyme to be less than the detection limits of the spectrophotometric assay (Knogge and Weissenböck, 1986). On the other hand, in 4-month-old *P. taeda* seedlings, phenylalanine ammonia-lyase activity was readily detectable under similar conditions (0.533 pkat mg^{-1} tissue, 20.7 pkat mg^{-1} protein). This implied that phenylpropanoid metabolism was either not occurring or was occurring at low levels in the *P. taeda* (2,4-D line) cells.

This is not a surprising result since 2,4-D **36** has been suggested to inhibit not only secondary metabolism, but also to partially inhibit the synthesis of phenylalanine ammonia-lyase as well as phenylalanine ammonia-lyase mRNA (Ozeki *et al.*, 1990). Therefore, in our suspension cultures maintained with 2,4-D **36**, it was possible that inhibition of phenylpropanoid metabolism could have very well been occurring as evidenced by the lack of detectable phenylalanine ammonia-lyase activity.

However, in collaboration with Dr. Lanfang He in our laboratory, antibodies, raised against purified phenylalanine ammonia-lyase from *P. taeda* (Whetten and Sederoff, 1992), were used for immunolocalization studies with the *P. taeda* (2,4-D line) suspension culture cells. Through these experiments it was envisaged that the subcellular location of phenylalanine ammonia-lyase (if present) in plant tissues, could be identified. In this respect, ultra-thin sections of *P. taeda* (2,4-D

line) cells prepared for transmission electron microscopy were incubated with rabbit antiserum raised against phenylalanine ammonia-lyase. Localization of the resulting enzyme-antibody complex was achieved by an indirect method whereby sections previously washed with phosphate buffer saline (PBS), were incubated with Protein A complexed with colloidal gold. After washing the sections again with PBS, observations were made by transmission electron microscopy. In parallel, control experiments were conducted by incubations of sections with the following substitutions or alterations to the method: (*i*) omission of phenylalanine ammonia-lyase antiserum, (*ii*) replacement of the phenylalanine ammonia-lyase antiserum with pre-immune serum, (*iii*) pre-incubation of the phenylalanine antiserum with purified phenylalanine ammonia-lyase and (*iv*) incubation of the sections with Protein A before incubation with Protein A complexed with colloidal gold.

Visualization of the gold particles on the sections by transmission electron microscopy indicated the positions where the enzyme (phenylalanine ammonia-lyase) was located. In this study, gold particles were observed in the cytosol, tonoplast, plasma membrane and endoplasmic reticulum thereby providing the first proof that the enzyme was present (see Figure 21). Since no gold particles were observed on sections used as controls, the above results represent the first localization of a key metabolic enzyme in phenylpropanoid metabolism in an intact softwood cell (or culture). These results are in agreement with immunolocalization experiments with potato (*Solanum tuberosum*) discs where phenylalanine ammonia-lyase was localized primarily in the cytosol but appeared to be associated with membranous structures such as the endoplasmic reticulum (Shaw *et al.*, 1990). Accordingly, it appears that phenylalanine ammonia-lyase is located in distinct regions of the cell. To what extent these different locations represent

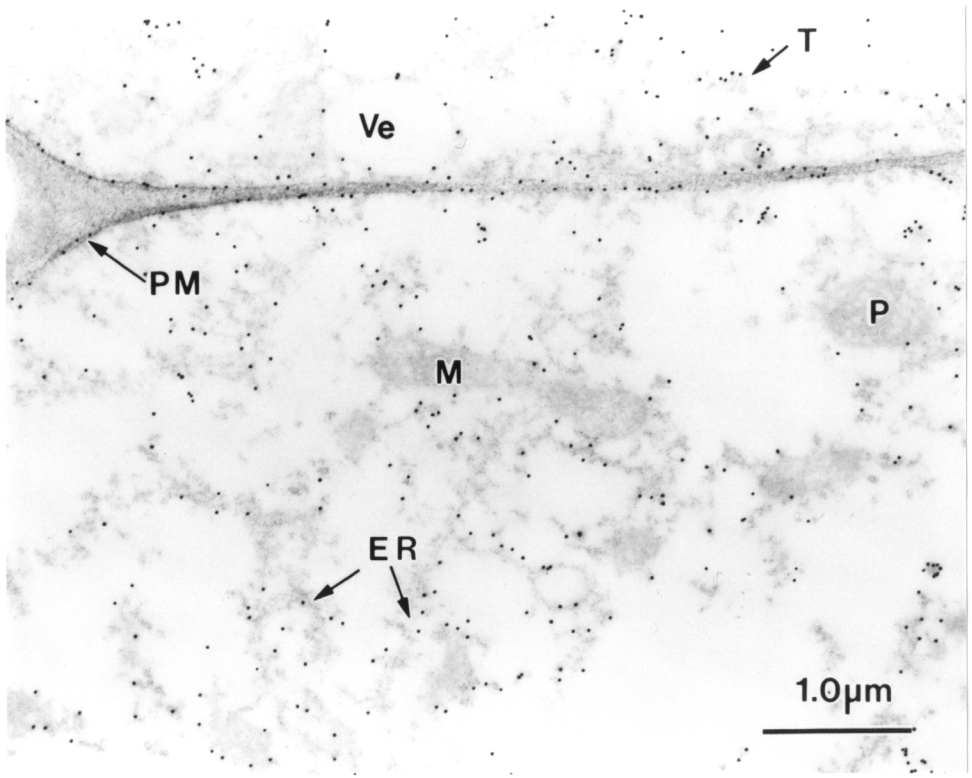

Figure 21. Transmission Electron Micrograph of Immunogold Localization of Phenylalanine Ammonia-lyase in *P. taeda* (2,4-D Line) Suspension Culture Cells [ER = endoplasmic reticulum, M = mitochondria, P = plastid, PM = plasma membrane, T = tonoplast, Ve = vesicle]

different branchpoints of phenylpropanoid metabolism (*i.e.* leading to lignins, lignans, flavonoids, etc.) needs to be determined. Besides the important identification of the sites in the cell where phenylalanine ammonia-lyase resides in a plant cell, these experiments suggest that phenylalanine ammonia-lyase was present in the *P. taeda* suspension culture cells maintained with 2,4-D **36** although activity was undetectable by the spectrophotometric assay.

A branchpoint in the pathway leading to both monolignol and flavonoid biosynthesis is the conversion of the hydroxycinnamic acids to the respective thioesters catalyzed by hydroxycinnamate:CoA ligase. Using crude cell-free extracts, hydroxycinnamate:CoA ligase was also not directly detectable in the *P. taeda* (2,4-D line) cells by the spectrophotometric assay used (Grand *et al.*, 1983). Accordingly, the proteins in the crude cell-free extract were concentrated by ammonium sulfate precipitation thereby possibly improving the ability to detect enzyme activity. Using this procedure, it was demonstrated that the *P. taeda* (2,4-D line) suspension culture cells possessed hydroxycinnamate:CoA ligase activity (3.75 pkat mg^{-1} protein, 0.151 pkat mg^{-1}tissue).

Finally, another enzyme in phenylpropanoid metabolism, cinnamyl alcohol dehydrogenase, catalyzes the reduction of the hydroxycinnamyl aldehydes to the corresponding hydroxycinnamyl alcohols; in turn, these monolignols are subsequently converted into lignans, neolignans and lignins. Using a spectrophotometric assay (Sarni *et al.*, 1984) described in the Material and Methods, cinnamyl alcohol dehydrogenase activity was observed in cell-free extracts from 4-month-old *P. taeda* seedlings (9.77 pkat mg^{-1} tissue, 380 pkat mg^{-1} protein). Similarly, cinnamyl alcohol dehydrogenase was readily detectable in cell-free extracts from the *P. taeda* (2,4-D line) suspension cultures (98.8 pkat

mg^{-1} tissue, 367 pkat mg^{-1} protein). Thus, all three enzymes were present, albeit in the the case of phenylalanine ammonia-lyase only by indirect detection via immunogold subcellular localization. Therefore, to establish if phenylpropanoid metabolism was occurring, we directed our attention to the analysis of both cytosoluble constituents and the composition of the primary cell walls.

4.2. Identification of (-)-Matairesinol in *P. taeda* (2,4-D Line) Cells

To determine if the *P. taeda* (2,4-D line) suspension culture cells were synthesizing phenylpropanoid metabolites, 5 g of freeze-dried cells were extracted (3 X 10 min) with warm (50 °C) methanol. After concentrating the methanol extracts *in vacuo*, H_20 was added to give a cloudy mixture which was then extracted with diethyl ether. The combined ether solubles were dried over Na_2SO_4 and then evaporated to dryness *in vacuo*. The resulting green residue (50.6 mg) was solubilized in methanol (1 ml) and subjected to analysis by reversed-phase high performance liquid chromatography (HPLC).

As can be seen in Figure 22, a large peak at a retention volume of 13.8 ml was observed which was subsequently established to co-elute with authentic matairesinol **38**, whereas the smaller peak at a retention volume of 9.8 ml co-eluted with a pinoresinol standard. Both peaks observed on the original chromatogram displayed UV absorption spectra by the photodiode array detector which were identical to that for authentic samples (see Figure 23). Through multiple injections, these components were separated and individually collected. After removing the acetonitrile from each of the two samples under reduced pressure, the resulting aqueous solutions were individually frozen (liquid N_2) and freeze-dried. The fluffy white residue (200 µg g^{-1} tissue) resulting from the

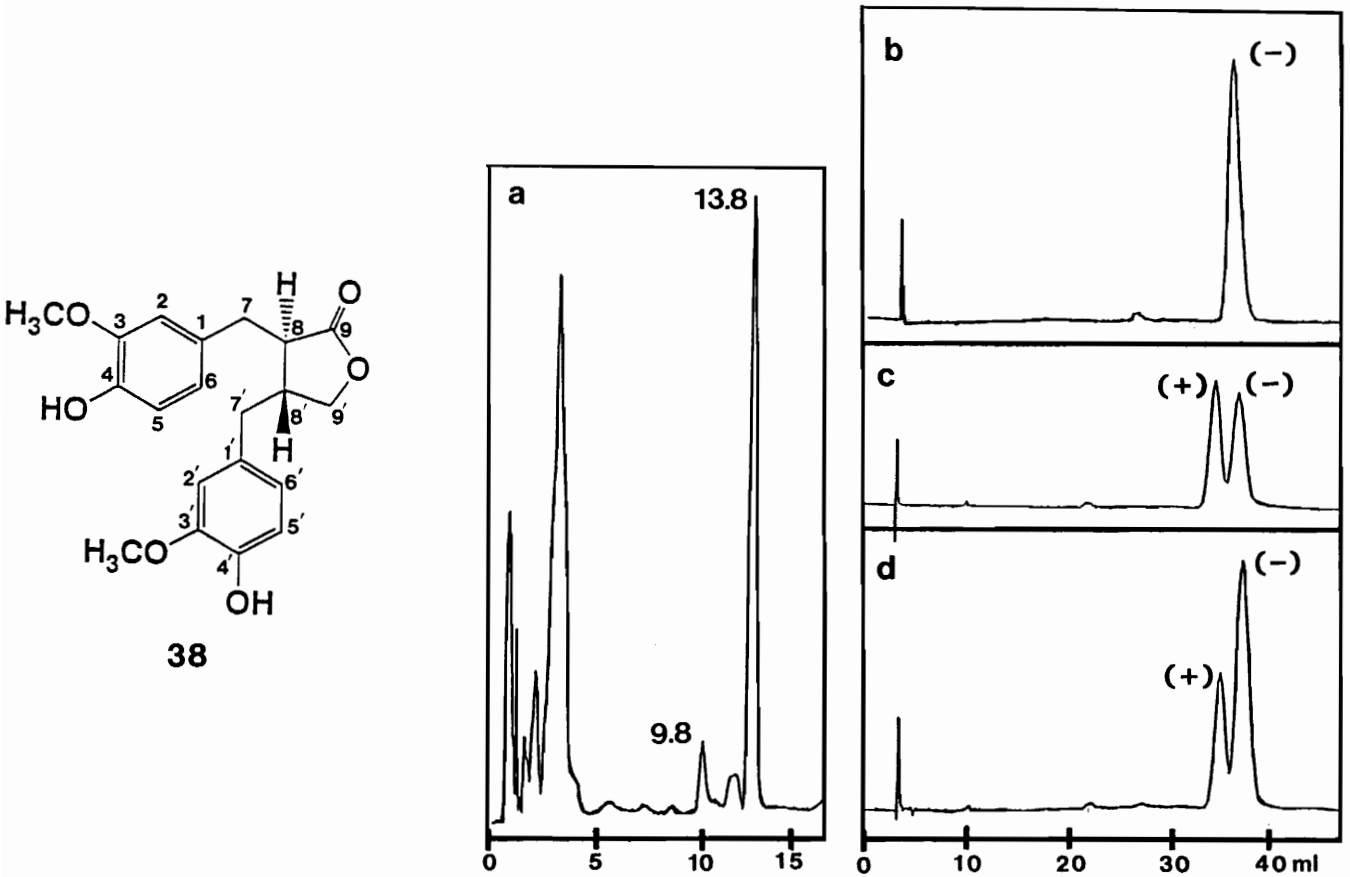

Figure 22. HPLC Separations on Reversed-phase and Chiral Columns: **a**) Methanol Extract of *P. taeda* (2,4-D Line) Suspension Culture [Column = Waters Nova-Pak C_{18}]; **b**) (-)-Matairesinol from *P. taeda* (2,4-D Line) Suspension Culture [Column = Diacel Chiralcel OD]; **c**) Authentic (±)-Matairesinols [Column = Diacel Chiralcel OD]; **d**) Co-Chromatography of **b** and **c** Above.

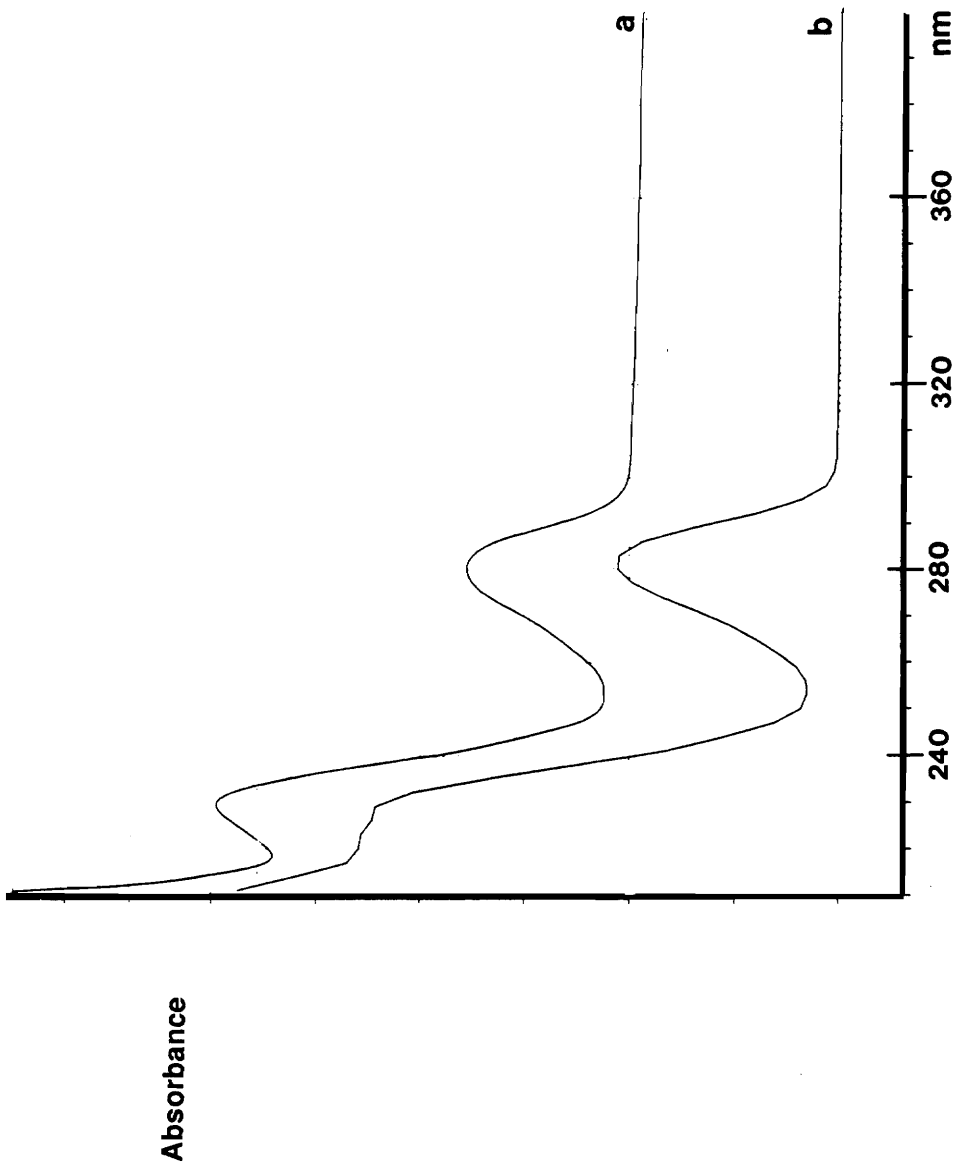

Figure 23. UV Spectra of Eluates from Methanol Extract of *P. taeda* (2,4-D Line) Cells (see Figure 22a) at Retention Volumes of **a**) 9.8 ml [Pinoresinol] and **b**) 13.8 ml [Matairesinol].

eluate which coincided with matairesinol **38** was then subjected to analysis by ^1H NMR spectroscopy and mass spectrometry. The eluate coinciding with pinoresinol (< 20 μg g^{-1} tissue) was set aside for subsequent chromatographic analysis.

^1H NMR analysis of the unknown eluting at a retention volume of 13.8 ml had a spectrum identical to that for authentic (±)-matairesinol (see Figure 24). Mass spectrometry (EI) of the sample using a direct probe gave a molecular ion at m/z 358 and a fragment ion at m/z 137 corresponding to that derived via benzylic cleavage. Thus, matairesinol **38** was present in the methanol extracts of the *P. taeda* (2,4-D line) suspension culture cells thereby establishing that phenylpropanoid metabolism was active. Moreover, the isolation and identification of matairesinol **38** from the suspension culture cells represents the first demonstration of the occurrence of this lignan in *P. taeda*, in culture or whole plants.

The isolated matairesinol **38** was then subjected to further HPLC analyses using a chiral (Chiralcel OD, Diacel, Japan) column under conditions previously developed for the separation of (+) and (-) matairesinols (Umezawa *et al.*, 1990). Chiralcel OD columns are silica-based coated with cellulose tris(3,5-dimethylphenylcarbamate) and they function by forming transient diastereomeric complexes between the solid matrix and the enantiomeric mixture (Davin *et al.*, 1991). The different antipodes can then be separated dependent upon the relative stability of each diastereomeric complex. Analyses of the matairesinol **38** sample from the *P. taeda* (2,4-D line) cells on the chiral column showed that only the (-)-antipode **38** was present; in a similar manner, the pinoresinol obtained was the corresponding (+)-enantiomer.

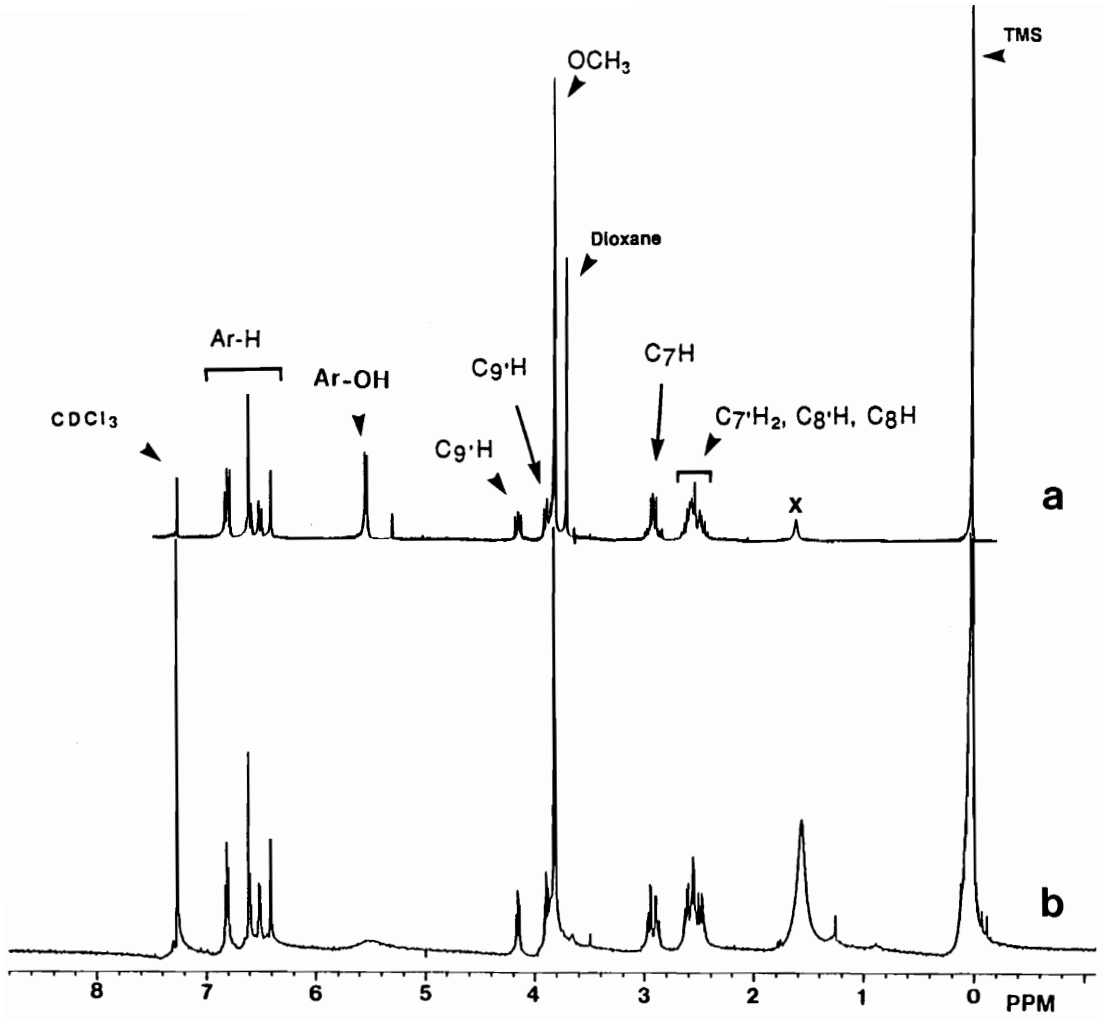

Figure 24. ^{1}H NMR spectra of **a**) Authentic (±)-Matairesinol and **b**) (-)-Matairesinol from *P. taeda* (2,4-D line) Suspension Culture Cells [Solvent = $CDCl_3$, Internal Standard = TMS].

Through the production of enantiomerically pure lignins by the *P. taeda* (2,4-D line) cells it is apparent that this culture system could be most useful to study lignan biosynthesis. Recent work in our laboratory on the enantioselective biosynthesis of *Forsythia* lignans has shown that two molecules of coniferyl alcohol **2** undergo coupling and stereoselective conversion into (-)-secoisolariciresinol which is subsequently converted into (-)-matairesinol **38** (Umezawa *et al.*, 1990). Both sets of enzymatic transformations, which are the first reported in lignan synthesis, are strictly stereoselective. On the other hand, in lignin biosynthesis, the presumed peroxidase catalyzed dehydrogenative polymerization of the monolignols to form the β-β linked quinone methide would result in a racemate formation. The proposed biosynthetic scheme for (-)-matairesinol **38** shown in Figure 25 therefore does not apparently involve a typical peroxidase catalyzed reaction since a specific stereoisomer of the proposed quinone methide intermediate would be necessary to afford (-)-secoisolariciresinol.

In addition to gaining a better understanding of the biosynthesis of enantiomerically pure lignans, many questions remain in regards to their metabolic fate. Upon release to the cell wall, there exists the possibility that lignans could be incorporated into lignin. Evidence for this possibility is not yet available. Typically, lignin is considered to be optically inactive and so it is argued that lignans and lignins are compartmentalized within different parts of the cell for separate functions. If this is the case, the mechanisms by which phenylpropanoid metabolic products are channeled to either lignans or lignin biosynthesis needs to be determined. Accordingly, studies are needed to identify the sub-cellular localization of these lignans as well as their role in metabolically active cells. In addition, research is needed to isolate and characterize the enzymes involved in

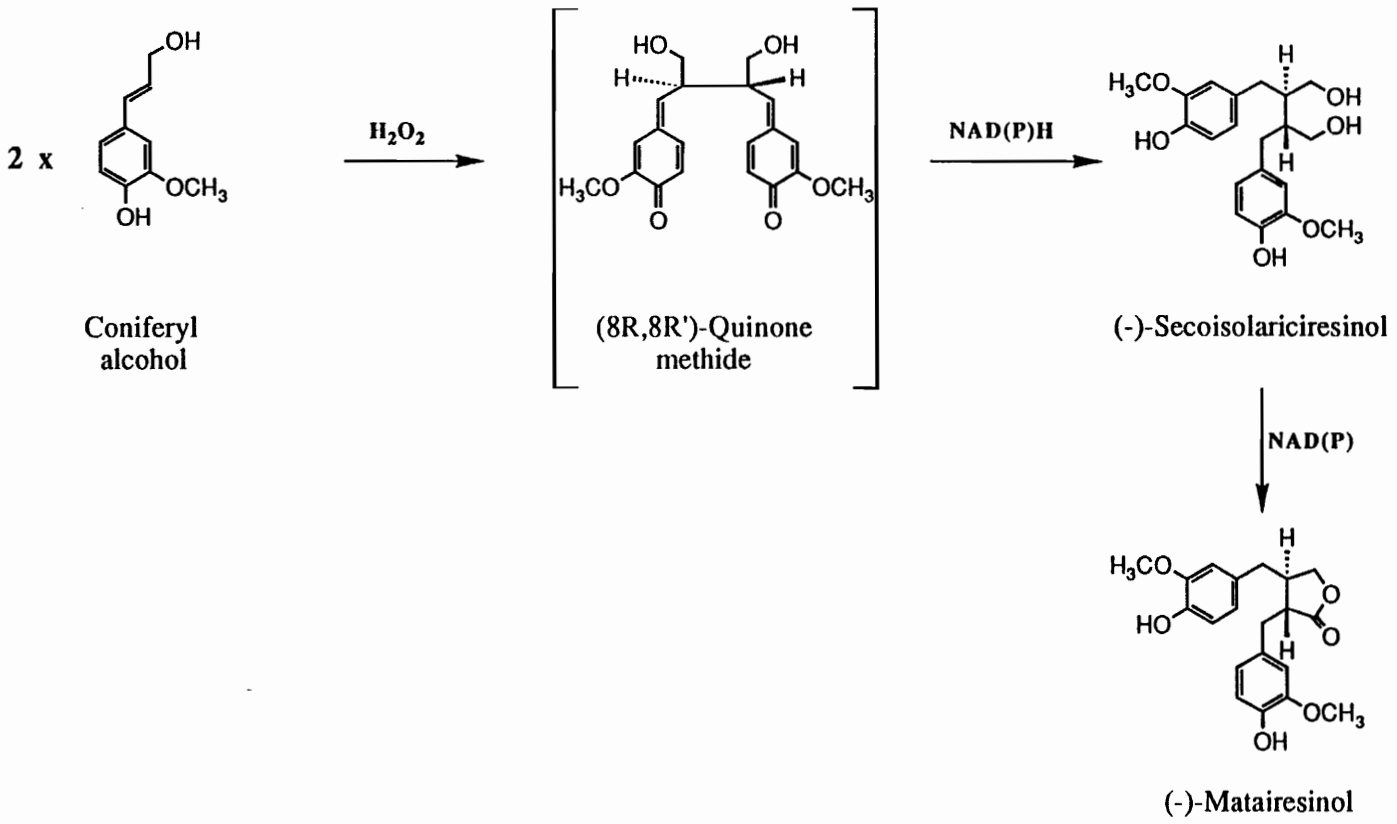

Figure 25. Proposed Biosynthetic Pathway of (-)-Matairesinol in *Forsythia intermedia.*

lignan formation. Through manipulations of the *P. taeda* suspension cultures there is the potential to study the many unanswered questions regarding biosynthesis, metabolism and function of lignans in plants.

4.3. Lignification in *P. taeda* (2,4-D Line) Suspension Cultures.

Knowing that phenylpropanoid metabolism was active in these cells, we then directed our attention to establishing whether or not lignification was occurring. The first indication that the cells maintained in 2,4-D-containing medium were only poorly lignified resulted from the utilization of a histochemical stain commonly used to detect lignin. This method employs a solution of phloroglucinol in ethanol followed by the addition of concentrated HCl to lignified tissues that results in deep violet-red staining (Bradbury, 1973). Positive staining by this histochemical test though indicative, is by no means proof of lignification; in tissue samples known to be lignified, a negative or weak staining has been observed where that lignin component is high in syringyl **6** moieties (Sarkanen and Ludwig, 1971). In the case of the *P. taeda* (2,4-D line) suspension culture cells treated with phloroglucinol-HCl, only scattered aggregates of cells stained faintly violet-red in color. The lack of staining therefore suggested that if these cell were lignifying, they produced considerably less lignin than that observed in woody tissues.

To assess whether the cells were lignifying, various chemical methods commonly applied to the analysis of lignin in woody tissues were applied. Attempts to quantify the amount of lignin in the cells was done by the acetyl bromide (Morrison, 1972) and Klason (TAPPI standard procedure, 1974) methods while the monomer composition of the possible lignin component was determined by nitrobenzene oxidation (Scalbert *et al.*, 1986) and thioacidolysis (Lapierre *et al.*, 1986, 1988).

Note, however, the suspension culture cells may not be comparable to woody tissues since they are comprised of primary cell walls that usually contain more protein than that found in the secondary walls of wood. Therefore, the usefulness of these techniques with the suspension culture cells needed to be determined.

Prior to estimating the lignin content of the suspension cultures by chemical methods, the cells were extracted to remove potentially interfering non-lignin materials. Initially, dried cells were sequentially extracted with benzene, benzene:ethanol (2:1) and then ethanol in a soxhlet apparatus maintained under an atmosphere of N_2 (Procedure A). To remove any tannins, the extracted cells were steeped in acetone:H_2O (7:3) (Laks and Hemingway, 1987). After this final extraction step the ash-colored cells were placed *in vacuo* (water aspirator) to remove the acetone after which they were frozen (liquid N_2) and freeze-dried. On average, as much as 40% of the starting weight of freeze-dried material was removable through this organic solvent extraction procedure.

It should be noted that extraction of the cells as above would not facilitate the removal of proteinaceous materials. Results from the preparation of aqueous cell-free extracts and solid-state ^{13}C NMR spectroscopic analyses of [1-^{13}C] phenylalanine **9** enriched *P. taeda* (2,4-D line) cell walls, to be described later, demonstrated the necessity to remove soluble proteins from the cells prior to extraction with organic solvents. Accordingly, a procedure was adapted from methods used by Talmadge *et al.* (1973) and Robert *et al.* (1989) where soluble proteinaceous materials were removed by an initial aqueous extraction with phosphate buffer (Procedure B). Followed by washing the cells with water, the resulting residue was treated with chloroform:methanol (1:1) to extract any lipid-like materials. The residue was then further extracted with toluene:ethanol (2:1)

by soxhlet extraction. Rinsing of the cells with acetone after the chloroform:methanol and toluene:ethanol extractions was done to facilitate drying of the cell wall residue. After completing this rather comprehensive extraction procedure, approximately 20% of the weight of original freeze-dried material was recovered as an almost white cell wall fraction.

A sample of these cell walls (Procedure B) was analyzed by solid-state ^{13}C NMR spectroscopy and gave the natural abundance ^{13}C NMR spectrum shown in Figure 26. As can be seen, the majority of the resonances are in the aliphatic region of the spectrum. The most prominent resonance at 73 ppm likely corresponds to C-2, C-3 and C-5 in cellulose (as well as corresponding carbons in other polysaccharides, *i.e.* hemicelluloses and pectins). Another resonance at 105 ppm corresponds with anomeric carbons (*i.e.* C-1 of cellulose). Smaller resonances coinciding with C-4 and C-6 in cellulose occur at 83 and 62 ppm, respectively. Downfield, a large resonance at about 175 ppm coincides with carboxyl moieties such as the carboxyl funtionalities of pectin; this resonance could also correspond to peptide linkages of cell wall bound proteins. Methylenic functionalities are observed between 20 and 45 ppm. A small resonance at 56 ppm could correspond to methoxyl moieties of lignin (Lewis *et al.*, 1987c) or the methyl ester moieties of pectins (Sun *et al.*, 1987). In the aromatic region of the spectrum almost no resonances are observed thereby suggesting that if these cells do contain lignin, it makes up only a very small fraction of the cell wall. Therefore, these data suggest that the suspension culture cell walls are comprised primarily of polysaccharides (*i.e.* cellulose, hemicelluloses and pectins).

In an effort to quantify the extent of any lignification in the *P. taeda* (2,4-D line) suspension culture cells, this cell wall fraction (Procedure B) was subjected

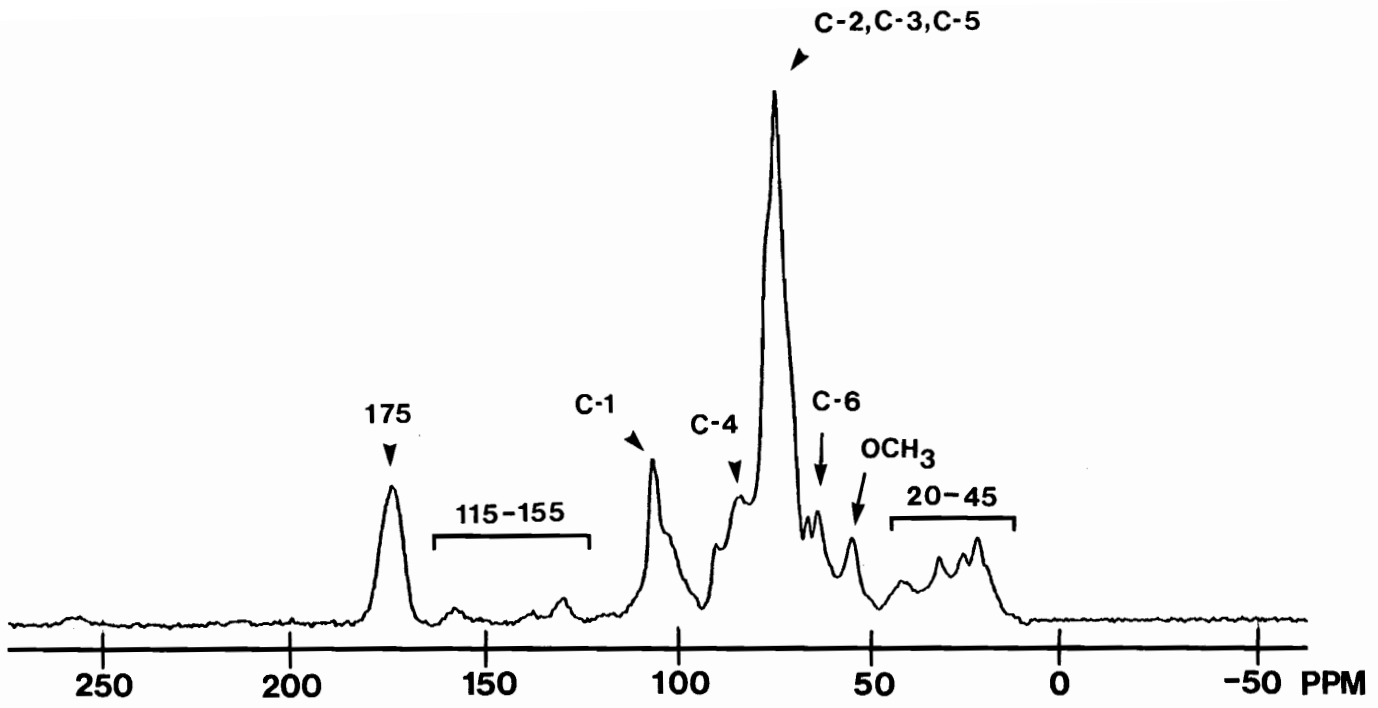

Figure 26. Solid-state ^{13}C NMR Spectrum of *P. taeda* (2,4-D Line) Cell Walls.

to the acetyl bromide lignin determination method. Through this method, the cell walls are solubilized and the absorbance of the resulting solution monitored at 280 nm. Since this method is based only on UV absorbance at a specific wavelength, any chromophores that absorb at 280 nm can contribute to values estimating lignin content. Therefore, as a precautionary measure, cell walls are exhaustively extracted before analysis to remove interfering compounds. The insoluble aromatics remaining are presumed to be lignin although this may be far from true since this method is based on only UV absorbance. Nevertheless, by this method, the quantity of lignin in the extractive-free cell wall fraction of the 2,4-D-grown suspension culture was estimated to be 8.2%. Comparable results had been previously reported in *Rosa glauca* suspension cultures where the lignin content was estimated to be 5% (Robert *et al.*, 1989).

The extractive-free cell wall fraction was also subjected to a Klason acid-insoluble lignin determination. Through this procedure it is assumed that the cell wall carbohydrates are hydrolyzed leaving an insoluble "condensed" lignin which can be collected by filtration and weighed. As in the case of the acetyl bromide method, the Klason method provides no proof that a given tissue is lignified. It can only be assumed that any insoluble residue represents lignin. The digestion of extractive-free cell walls from 7-day-old 2,4-D-grown suspension cultures gave a value for lignin content of 4.6%. This value differs greatly from that obtained by the acetyl bromide method (8.2%). Accordingly, the discrepancy reveals the limitations of one or both methods when applied to determine the lignin contents of suspension culture cell walls.

In a previous study with *P. taeda* suspension cultures, procured from the same source as ours, some of the chemical characteristics of what was believed to be an

isolated lignin from the cells were determined. In this respect, the yield of a milled wood lignin (MWL) from these cells (3.8% of extractive-free material) was considerably lower than that from wood (10.9%) of the same species (Fukuda *et al.*, 1988). Through degradative analyses of the suspension culture MWL by nitrobenzene oxidation, a guaiacyl **5**:*p*-hydroxyphenyl **4** ratio of 16.7:1 (0.64% yield from extractive-free material with 3.8% lignin content) was substantially different from that in the respective wood 100:1 (9.8% yield from extractive-free material assuming 28% lignin content). The yields of MWL and the products obtained by nitrobenzene oxidation suggest that not only do the cultures produce less "lignin" than wood but also that suspension culture "lignin" is significantly different from that in the respective woody tissues. Unfortunately, as typical for previous studies focussing on lignification in suspension cultures, the criteria for lignification, as described previously, were not met. Therefore, although degradation products usually observed by nitrobenzene oxidation of lignin were obtained, it could only be presumed that the products were indeed from lignin.

For the *P. taeda* (2,4-D line) suspension cultures maintained in our laboratory, nitrobenzene oxidation of the extractive-free cell walls were also conducted. Nitrobenzene oxidation of the *P. taeda* (2,4-D line) cell walls showed both guaiacyl **5**:*p*-hydroxyphenyl **4** moieties (4.3:1) with a yield of 0.48% based on the weight of products and the weight of extractive-free tissue; syringaldehyde was not detected. This ratio of products obtained is significantly greater in *p*-hydroxyphenyl units **4** than that observed in the lignin from the intact plant. Moreover, the yield of products from the *P. taeda* (2,4-D line) cells, as compared to *P. taeda* wood meal, is lower by a factor of 20 which further suggests that these cells can only be producing very limited amounts of lignin. If these cells are lignifying,

the data from our cells and that described above suggest that it contains higher levels of *p*-coumaryl alcohol **1** than in the intact plant.

When interpreting this higher content of *p*-hydroxyphenyl units **4** in the *P. taeda* (2,4-D line) cell wall preparation, caution is necessary since this method can oxidize many phenolic moieties, some of which may result in the formation of the same products. For example, nitrobenzene oxidation of tyrosine **8** in its free or protein bound form gives *p*-hydroxybenzaldehyde 15% yield (Borg-Olivier and Monties, 1989); any contribution for such substances would alter the ratios and thereby provide erroneous results. Therefore, in an effort to confirm these results we next utilized another degradative method, thioacidolysis, which is less susceptible to contributions of oxidation products from non-lignin constituents.

During thioacidolysis, aryl glycerol-β-aryl ether linkages in lignins are cleaved to provide the diastereomeric ethane-thiol derivatives therefore still containing the intact "C_6C_3" units and thus overcoming the limitation of the previous method. Thioacidolysis of extractive-free *P. taeda* (2,4-D line) suspension culture cells gave a guaiacyl **5**:*p*-hydroxyphenyl **4** ratio of 9.6: 1, in contrast to that of 4.3:1 for nitrobenzene oxidation. Moreover, the thioacidolysis products totaled 0.019% based on the weight of extractive-free tissue. The guaiacyl **5**:*p*-hydroxyphenyl **4** ratios obtained for both sets of analyses suggest that non-lignin cell wall constituents (*e.g.* tyrosine) could have contributed to the *p*-hydroxyphenyl **4** product (*p*-hydroxybenzaldehyde) obtained by nitrobenzene oxidation. As for an extractive-free wood meal from *Pinus pinaster*, the guaiacyl **5**:*p*-hydroxyphenyl **4** ratio of thioacidolysis products was 4.5:1 with a yield extrapolated to 10.9% for the extractive-free material (assuming a lignin content of 28%). In this respect, not only do the ratios of products differ substantially but

the thioacidolysis yields for wood meal is 500-fold higher than that obtained from *P. taeda* (2,4-D line) cells. Accordingly, these results established that only a minimal amount of lignification had occurred in these suspension culture cells. These results illustrate dramatically the limitations of the acetyl bromide and Klason lignin determinations, as well as the nitrobenzene oxidation method for determining the lignin composition of these cells.

In summary, the histochemical and chemical analyses (*i.e.* thioacidolysis) of the *P. taeda* (2,4-D line) cells and extractive-free cell walls, respectively, demonstrated that minute levels of lignification had occurred in the cultures. Thus the first two objectives of this study were achieved by establishing that although phenylpropanoids (*i.e.* (-)-matairesinol) were being produced by these cells, the primary cell walls had not undergone significant lignification. Accordingly we next directed our efforts to induce the *P. taeda* cells to undergo cell wall thickening and lignification. Since critical evidence for lignification necessitated the identification of lignin bonding patterns *in situ*, we needed to administer carbon-13 specifically labelled lignin precursors to lignifying cells prior to solid-state ^{13}C NMR spectroscopic analyses. To set the foundation for such experiments to be conducted later with lignifying cells, we administered [U-^{14}C] phenylalanine **9** to the *P. taeda* (2,4-D line) cells to determine its incorporation into insoluble cell wall components. Since the *P. taeda* (2,4-D line) cells were primarily unlignified we presumed that uptake of phenylalanine **9** would be incorporated into other aromatic containing cell wall constituents such as proteins. Moreover, it needed to be established if the lignin precursors administered through the medium would be stable in this system prior to incorporation in the cells.

4.4. Administration of [U-^{14}C] Phenylalanine to *P. taeda* (2,4-D Line).

If sufficient labelling of the insoluble phenylalanine **9** derived aromatic constituents of the primary cell wall could be obtained, these materials could possibly be identified *in situ* by subsequent administration of carbon-13 specifically labelled phenylalanine **9**. To determine the most efficient level of precursor incorporation for subsequent carbon-13 experiments, 7-day-old *P. taeda* (2,4-D line) suspension cultures were administered three different concentrations (0.1, 1.0 and 10 mM) of [U-^{14}C] phenylalanine **9** in medium also containing 2,4-D **36** (11.3 μM). On days 3, 7, 10 and 14 of the two week culture period, cells were harvested, frozen (liquid N_2) and freeze-dried.

An aliquot of each sample of dried radiolabelled tissues was then extracted (Procedure A) to obtain ash colored residues of which each weighed approximately 59.9% of that for the starting material. Both the freeze-dried cells and the respective extractive-free residues were then analyzed for their content of radioactivity by using a biological oxidizer which incinerated the samples to release the radiolabel as $^{14}CO_2$ which was collected into a CO_2-absorbing liquid scintillation cocktail. By dividing the radioactivity of a given weight of tissue (Bq mg^{-1}) by the specific activity (Bq mg^{-1}) of the administered [U-^{14}C] phenylalanine **9**, and multiplying by 100, the phenylalanine equivalent (%) could be determined.

The percent [U-^{14}C] phenylalanine equivalent incorporation of the cell wall residues for the cells administered 0.1, 1.0 and 10 mM [U-^{14}C] phenylalanine **9** and harvested on day 14 were 0.0751, 0.506 and 2.19, respectively. Of these values, that for the extractive-free cell walls from the cells administered 10 mM [U-^{14}C] phenylalanine **9** was the only one which was at a sufficient level for

subsequent carbon-13 specifically labelled phenylalanine **9** administrations for *in situ* analyses by solid-state ^{13}C NMR spectroscopy.

For the cells administered 10 mM [U-^{14}C] phenylalanine **9** that were harvested on days 3, 7 and 10 of the two-week culture period, the respective phenylalanine equivalents were 1.19, 1.97 and 1.68%. This level of radioactivity remaining in the insoluble residue represented 37% of that incorporated with the remainder being cytosoluble. Therefore, it was especially of interest to determine the nature of the cell wall constituent(s) which the exogenously supplied [U-^{14}C] phenylalanine **9** was incorporated. The above values for percent phenylalanine equivalents suggested that suitable levels of [U-^{14}C] phenylalanine **9** incorporation could be obtained after one week of precursor administration under these conditions. Nevertheless, to ensure the necessary level of incorporation for the administration of carbon-13 specifically labelled phenylalanine **9**, this precursor was administered at a 10 mM concentration for the two-week culture period used above.

Along with the sufficient incorporation as observed for the cells administered 10 mM [U-^{14}C] phenylalanine **9**, it was of the utmost importance that the radioactivity incorporated into the cells was in the form of [U-^{14}C] phenylalanine **9**. If the [U-^{14}C] phenylalanine **9** in the medium was metabolized prior to incorporation then in subsequent experiments with carbon-13 specifically labelled phenylalanine **9** a scrambling of that specific label would result in erroneous results. To verify that the phenylalanine **9** in the medium remained intact (unmetabolized) prior to incorporation, an assay for phenylalanine **9** was used to determine the concentration of this precursor in the medium.

In this assay, a commercially available phenylalanine ammonia-lyase was used to catalyze the conversion of phenylalanine **9** to cinnamate. By monitoring the rate

of reaction spectrophotometrically (290 nm) for standard stock solutions of phenylalanine **9** (1.0-10 mM), a standard curve (see Figure 27) was obtained. Applying this assay to aliquots of used medium from the cells administered 10 mM [U-^{14}C] phenylalanine **9** and harvested on day 14, the concentration of phenylalanine **9** was determined to be 6.2 mM. This value closely reflected (95% agreement) the concentration of [U-^{14}C] phenylalanine **9** (6.5 mM) calculated from the level of radioactivity remaining in the medium. Thus, it was shown that the specific activity of the [U-^{14}C] phenylalanine **9** in the culture medium remained unchanged and that the precursor had not undergone any metabolic conversion. Accordingly, since sufficient incorporation of [U-^{14}C] phenylalanine **9** could be obtained with the precursor remaining intact in the medium, we then chose to administer [1-^{13}C] phenylalanine **9** to be followed by *in situ* analyses by solid-state ^{13}C NMR.

4.5. Administration of [1-^{13}C] Phenylalanine into *P. taeda* (2,4-D Line).

The results of the radiolabelling experiments with [U-^{14}C] phenylalanine **9** demonstrated that sufficient precursor incorporation was achieved for subsequent administrations of carbon-13 specifically labelled phenylalanine **9** and *in situ* analyses of the cell walls by solid-state ^{13}C NMR spectroscopy. In a similar manner to that described for the administration of [U-^{14}C] phenylalanine **9**, [1-^{13}C] phenylalanine **9** was administered to 7-day-old *P. taeda* (2,4-D line) suspension culture cells through 2,4-D-containing medium. To optimize incorporation, the cells were transferred to fresh medium containing [1-^{13}C] phenylalanine **9** (10 mM) and 2,4-D **36** (11.3 μM) every 3-4 days during the 2 week culture period. As a control, cells not administered phenylalanine **9** were

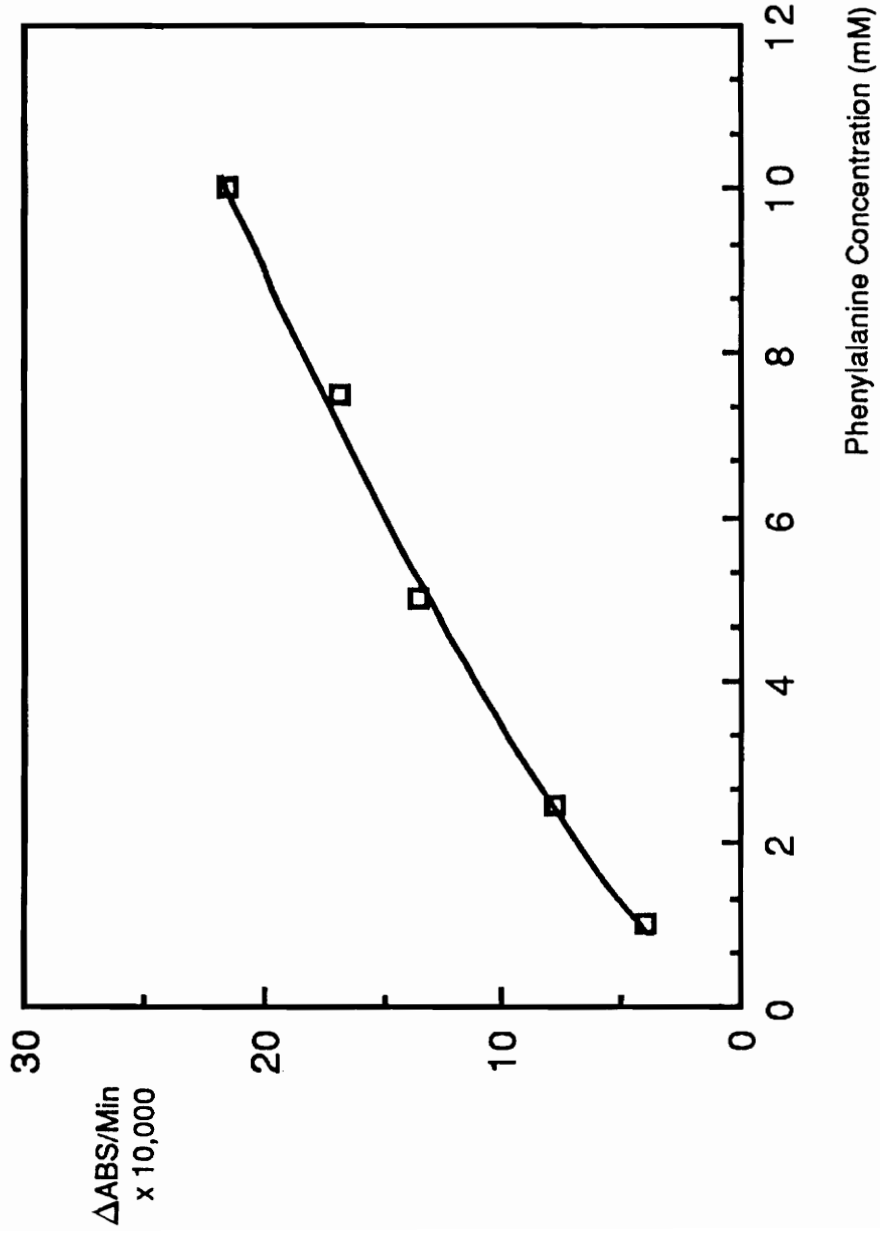

Figure 27. Standard Curve for Determining Phenylalanine Concentration in Culture Media.

cultured in parallel. In both cases, the cells retained their yellow-green color through to the time that they were harvested at the end of the culture period. After freeze-drying, the cells were subsequently extracted (Procedure A) to give ash colored residues which were analyzed by solid-state ^{13}C NMR spectroscopy. Subtraction of the ^{13}C NMR spectrum for the control from that of the ^{13}C enriched sample was done to obtain a difference spectrum (see Figure 28) showing the bonding environments of the incorporated ^{13}C-label. This difference spectrum was dominated by a large resonance at 172.4 ppm which corresponded to phenylalanine **9** in its free or protein bound form. Upfield, small resonances coincided with expected lignin bonding patterns at 72.3 (β-β, **C**) and 62.8 (β-*O*-4, **C**; β-1, **F**; β-5, **E**; allylic alcohol, **A**); equally small resonances coincided with methylenic funtionalities between 30 and 45 ppm. From these results it was apparent that the phenylalanine **9** administered was being incorporated in significant amounts but it was not being metabolized primarily into the lignin component under study. Subsequent to the solid-state ^{13}C NMR spectroscopic analyses, the cell wall residues were treated with pronase E, a non-specific protease. After thorough rinsing with H_2O, the cell wall residues were freeze-dried and analyzed again by solid-state ^{13}C NMR spectroscopy. As can be seen in Figure 29, a dramatic reduction in the resonance at 172.4 ppm resulted from the pronase E treatment, thereby providing supportive evidence that this resonance resulted from the incorporation of the administered [1-^{13}C] phenylalanine **9** into proteinaceous materials.

As stated previously, as much as 10% of the primary cell wall is comprised of protein (Kieliszewski and Lamport, 1988). It is possible that the [1-^{13}C] phenylalanine **9** administered was incorporated into such cell wall proteins. In the cell walls of tomato, 1.3% of the amino acid composition is phenylalanine **9**;

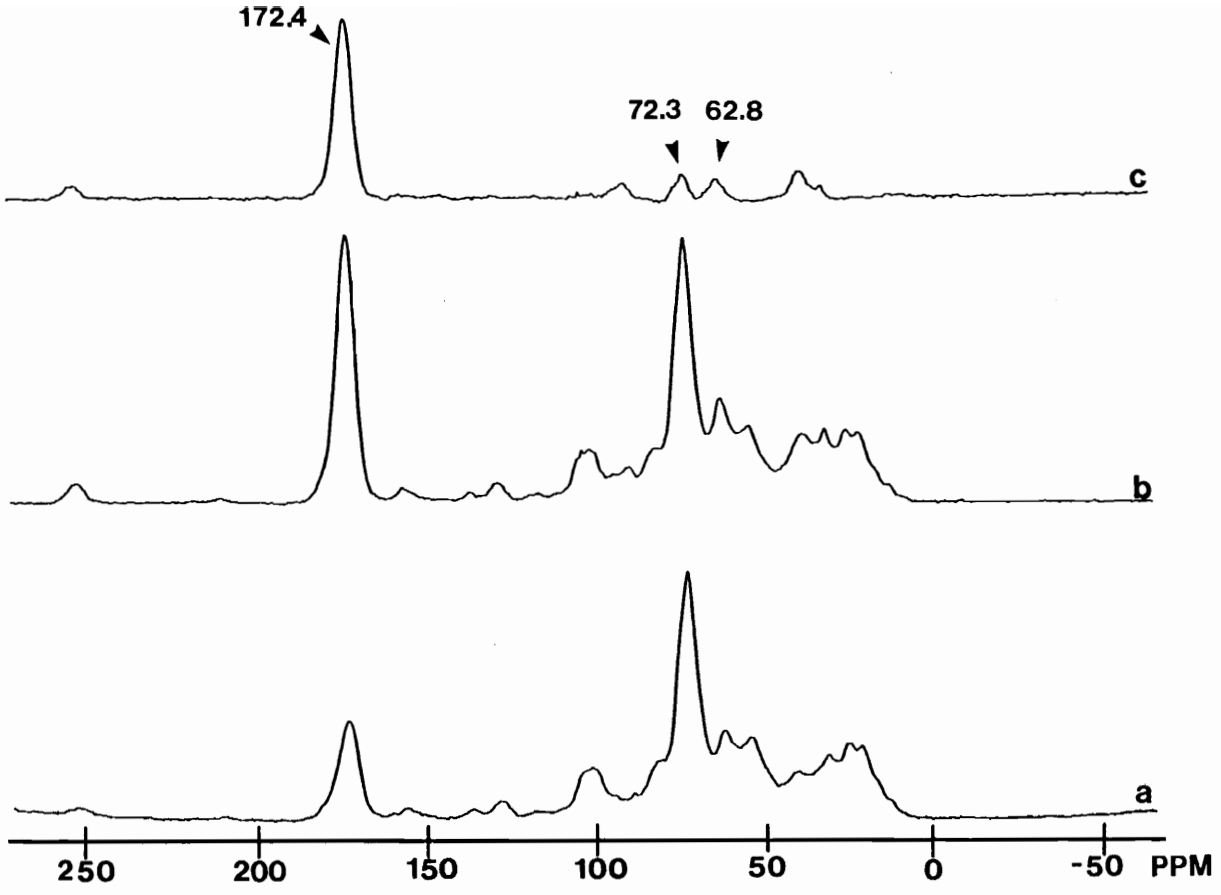

Figure 28. Solid-state ^{13}C NMR Spectra of *P. taeda* (2,4-D Line) Cell Walls: **a)** Natural Abundance Spectrum; **b)** [1-^{13}C] Enriched Spectrum; **c)** Difference Spectrum.

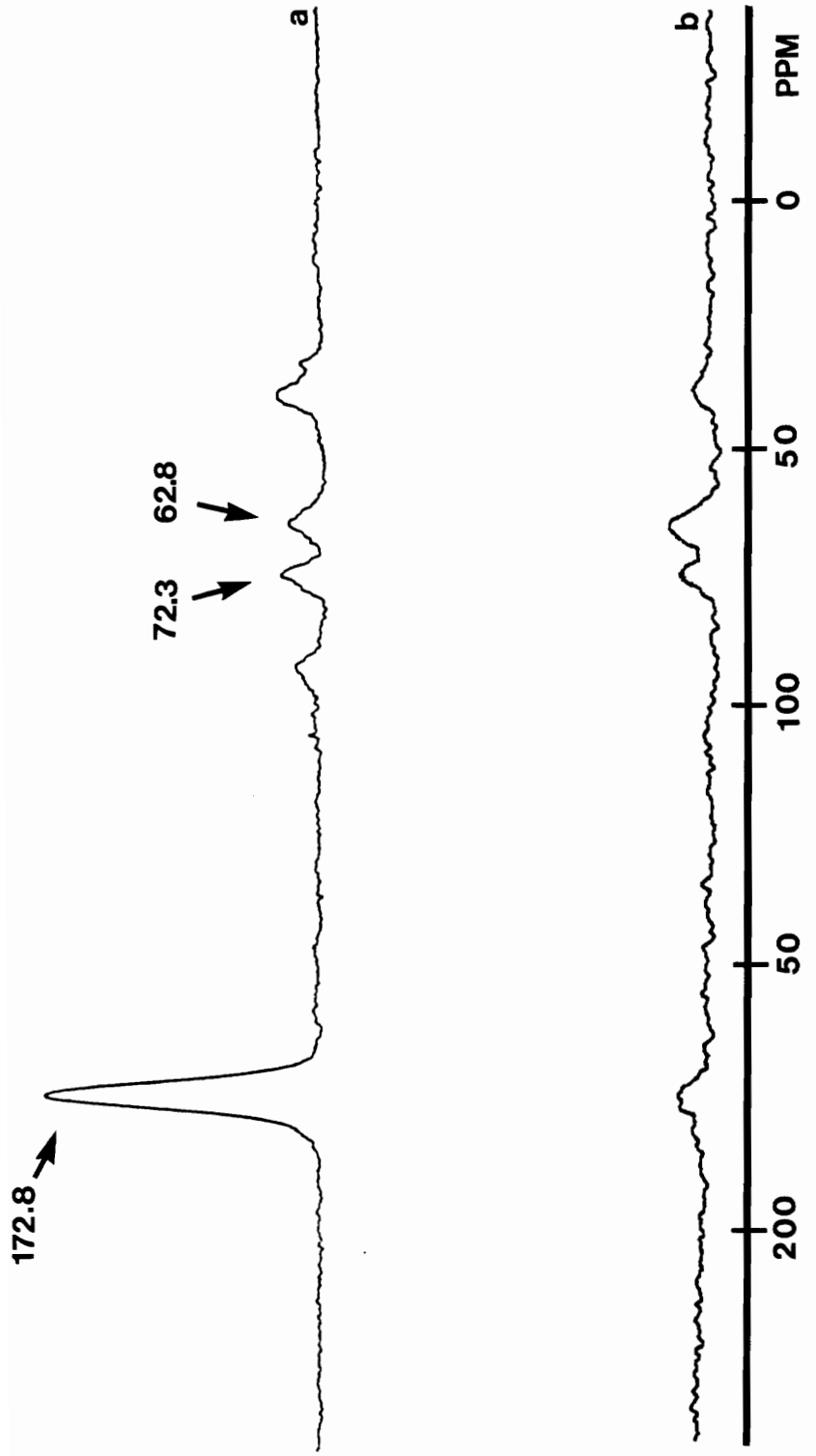

Figure 29. Solid-state ^{13}C NMR Difference Spectra of [1-^{13}C] Phenylalanine Enriched *P. taeda* (2,4-D Line) Cell Walls: **a)** Extractive-free; **b)** Extractive-free/Pronase E Treated.

extensin from the same cell walls does not contain this amino acid (Kieliszewski and Lamport, 1988). Nevertheless, since it is not known if the extensins of *P. taeda* cultures possess a comparable amino acid composition to that from tomato, there remains the possibility that the intense resonance at 172.4 ppm could coincide to the incorporation of [1-^{13}C] phenylalanine **9** into extensin.

Unable to identify lignin bonding patterns *in situ* in the *P. taeda* suspension culture cell maintained with 2,4-D **36** as the growth regulator, it was again apparent that these cultures were relatively unlignified. As discussed in the Introduction, the growth regulator on which the cultures were maintained, 2,4-D **36**, in some cases possesses the potential to inhibit secondary metabolism in culture systems (Hösel *et al.*, 1982). Such an inhibition could explain not only the low lignin content of the cells but also their inability to channel incorporated phenylalanine **9** into the cell wall lignin component. To possibly induce lignification, we next explored the administration of lignin precursors occurring later in the phenylpropanoid pathway than phenylalanine **9**. In this respect the administrations of unlabelled and [2-^{14}C] coniferin **35** are described below.

4.6. Administration of Unlabelled and [2-^{14}C] Coniferin.

The apparently low level of phenylalanine ammonia-lyase activity in the *P. taeda* (2,4-D line) cells resulted in the incorporation of exogenously supplied phenylalanine **9** into cell wall proteins. It was plausible that providing an exogenous supply of lignin precursor later in the phenylpropanoid pathway could result in lignification in the *P. taeda* (2,4-D line) cells. One potential precursor, coniferin (coniferyl alcohol-β-D-glucoside) **35** as well as other cinnamyl alcohol glucosides have long been thought to be involved in the storage of the monolignols and

their transport to the site of lignification. Experiments with *Picea abies* have suggested that coniferin **35** is an intermediate in lignin biosynthesis (Marcinowski and Grisebach, 1977), where the phenolic hydroxyl of the coniferyl alcohol **2** moiety is masked thereby providing a lignin precursor with greater stability. With this advantage, as well as the fact that it is present in *P. taeda* (R. R. Sederoff, personal communication), coniferin **35** was selected as a precursor for possible carbon-13 labelling of lignin *in situ*.

Initial experiments utilized unlabelled coniferin **35** to establish the method by which the precursor would be administered to the *P. taeda* suspension cultures. To medium containing 2,4-D **36** (11.3 μM) and coniferin **35** (1 mM) were added 7-day-old *P. taeda* (2,4-D line) suspension culture cells. Through HPLC analyses of the medium on a daily basis, over the 7-day growth period, the level of coniferin **35** in medium containing cells was rapidly depleted in 3 to 4 days (see Figure 30). Subsequent administration of [2-^{14}C] coniferin **35** in a similar manner showed that 48% of the radioactivity administered remained in the medium after 7 days of culturing. Analysis of this used medium by HPLC showed no peak corresponding to coniferin **35** or any radioactivity eluting with unlabelled coniferin **35** added as carrier. Therefore, it was apparent that the coniferin **35** administered to the *P. taeda* (2,4-D line) cells had been metabolized in the medium and, for this reason, was unsuitable as a precursor.

This was explained as follows: analyses of aliquots of 7-day-old medium from the *P. taeda* (2,4-D line) cells revealed coniferin β-glucosidase activity (363 pkat ml^{-1}) by a spectrophotometric assay. In addition to activity for coniferin β-glucosidase, peroxidase activity was also detectable in the medium. As can be seen in Figure 31, the peroxidase activity in the medium progressively increased until the

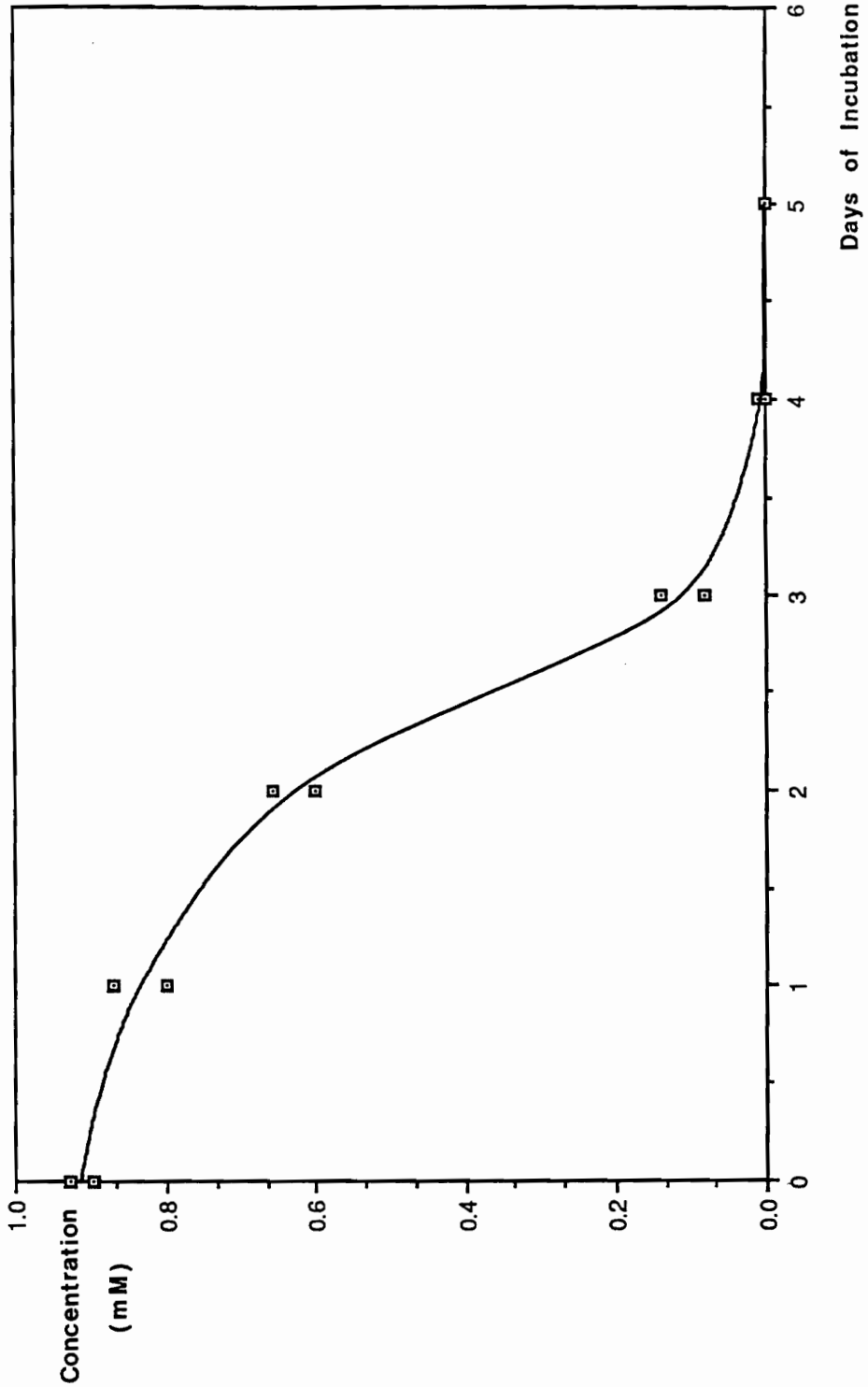

Figure 30. Coniferin Concentration in Culture Medium of *P. taeda* (2,4-D Line) Suspension Culture Cells.

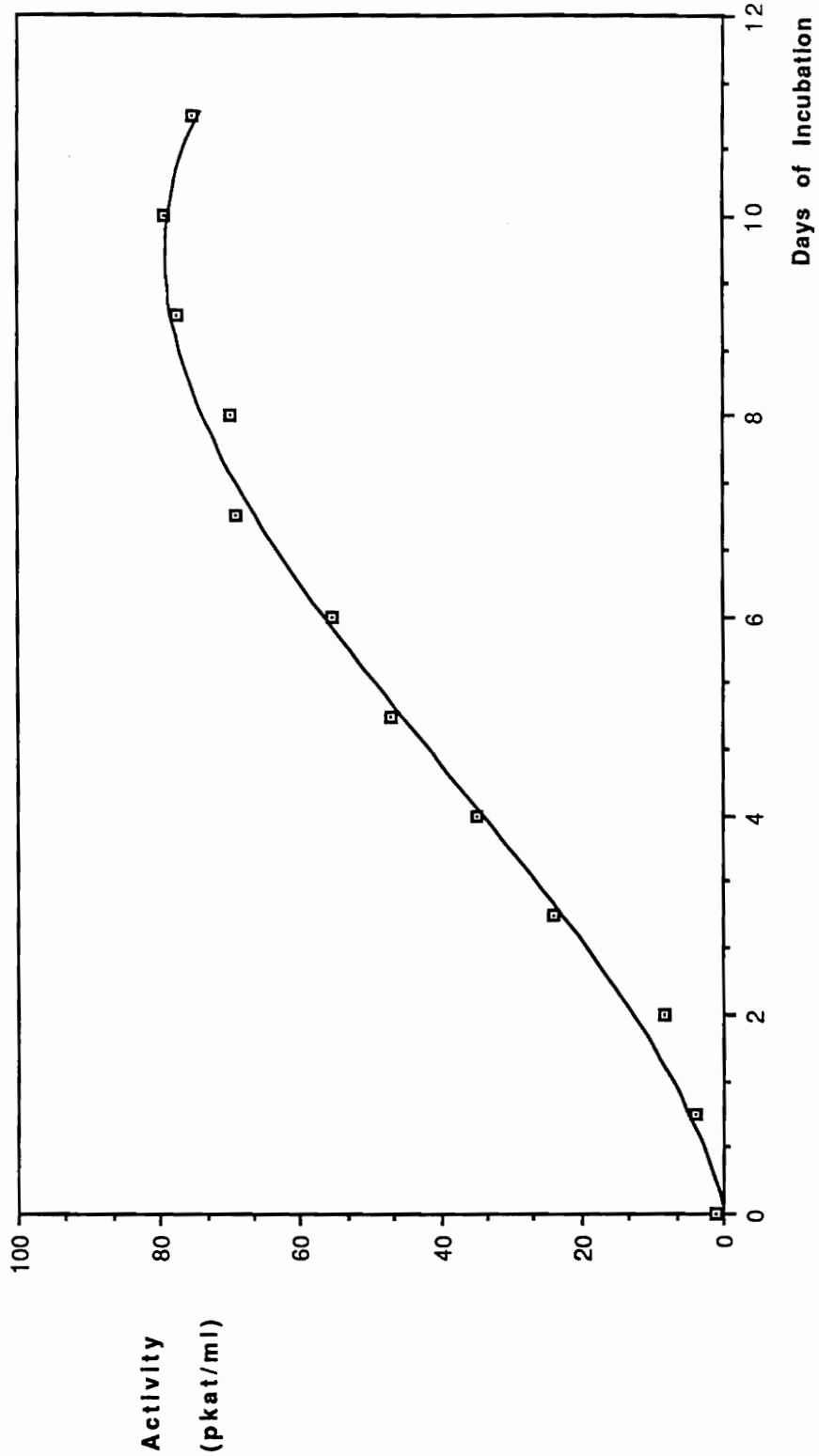

Figure 31. Peroxidase Activity in Culture Medium of *P. taeda* (2,4-D Line) Suspension Culture Cells.

culture went into a stationary growth phase after remaining on the same medium longer than the usual 7-day culture period. It is therefore plausible that the cleavage of coniferin **35** in the medium would result in the release of coniferyl alcohol **2** which could be polymerized via peroxidases if H_2O_2 was also present.

These experiments exemplify the necessity of verifying that the exogenously supplied precursor remain intact (unmetabolized) prior to incorporation. Knowing that phenylalanine **9** remained unmetabolized in the culture medium, subsequent labelling experiments utilized [U-^{14}C] and carbon-13 specifically labelled phenylalanines **9**. As stated earlier, the first two objectives of this study were achieved by establishing the avenues of phenylpropanoid metabolism in these cells possessing only primary cell walls. We then proceeded to induce the cultures to undergo cell wall thickening and lignification.

4.7. *P. taeda* (NAA Line) Suspension Cultures.

Experiments with the *P. taeda* (2,4-D line) cells have established minimal levels of lignification occurred in this stage of development, although stereoselective lignan formation was observed. As described previously, this low degree of lignification could possibly result from the partial inhibition of secondary metabolism by the 2,4-D **36** included in the medium as a growth regulator. Indeed, in instances where 2,4-D **36** was substituted with another synthetic auxin, NAA **37**, the production of "lignin-like" materials was observed but was not characterized further (Funk and Brodelius, 1990; Hösel *et al.,* 1982). Since it was our objective to study the early stages of cell wall thickening accompanied by lignification, it seemed plausible that the initiation of lignification could be facilitated by the removal of 2,4-D **36** from the medium and substituting in its

place, NAA **37**. Therefore, with the *P. taeda* cultures we chose to examine the effects of replacing 2,4-D **36** in the medium with an equimolar amount of NAA **37**.

P. taeda (2,4-D line) cells were transferred to medium containing NAA **37** (11.3 μM) and maintained with weekly medium exchanges for 2 months. The cells were routinely histochemically stained for lignin with phloroglucinol:HCl at the time of each medium exchange. Throughout this period, the cells maintained on a weekly basis with NAA **37** showed only faint staining, again indicative of low lignin levels. During subsequent manipulations of cells on NAA-containing medium, a scheduled transfer of cells to fresh medium, 28 days after the initial transfer to NAA-containing medium, was omitted. These cell were then transferred to fresh NAA-containing medium on day 35 of what was to become a total 7-week growth period on this growth regulator; during the final two weeks of the 49-day growth period, the cells became more aggregated and progressively changed in color from green to tan to brown and showed increased staining with phloroglucinol-HCl.

Cells maintained for 49 days on NAA-containing medium exchanged on days 7, 14, 21 and 35 were used for experiments conducted from this point forward. Observation of these 49-day-old *P. taeda* (NAA line) cells by light microscopy showed them to possess the same spherical shape as described for the *P. taeda* (2,4-D line) cells. Therefore, it was apparent that differentiation of the cells into tracheary elements was not induced by the manipulation of the growth regulator. Interestingly though, the cells appeared to be more rigid and cohesive. Although the vast majority of the cells had a similar appearance possessing an intact protoplast within a thin cell wall, a few widely scattered cells appeared dead since they were void of a protoplast. At this point it was of interest to examine these cells by

transmission electron microscopy to determine if any more detailed changes to the cells had occurred.

Transmission electron microscopy of the *P. taeda* (NAA line) cells showed most to have an intact protoplast with cellular organelles and structures such as a nucleus, endoplasmic reticulum, vacuoles, plastids, mitochondria and Golgi bodies. As compared to the 2,4-D-grown cells, those maintained with NAA **37** for the 49-day growth period appeared more vacuolated. Moreover, in these cells it was established that cell wall thickening had been initiated with the average cell wall thickness being 0.4 μm (see Figure 32); this was consistent with deposition of primary and secondary (S_1) cell wall layers (Sjöström, 1981). Despite cell wall thickening, a helicoidal arrangement of the cellulose microfibrils as observed in secondary cell wall layers was not apparent since these cells did not exhibit birefringence under a polarized light microscope. This may be related to the lack of cellular differentiation which normally would provide a cell a longitudinal axis along which the cellulose microfibrils would be oriented.

Thus far, it was established that the manipulation of the cells in medium containing NAA **37** as the growth regulator resulted in an apparent induction of cell wall thickening. The implications of this were considerable since at this point we had two cell lines available, one derived from the other, with different levels of cell wall formation. Accordingly, if lignification was co-induced with cell wall thickening, we then would have a system to study the progression of lignification during the earliest stages of cell wall deposition.

To establish if phenylpropanoid metabolism was active in the *P. taeda* (NAA line) cells, enzymatic assays were carried out as before, but now using cell-free extracts from 49-day-old NAA-grown cells. Activity for phenylalanine ammonia-lyase for

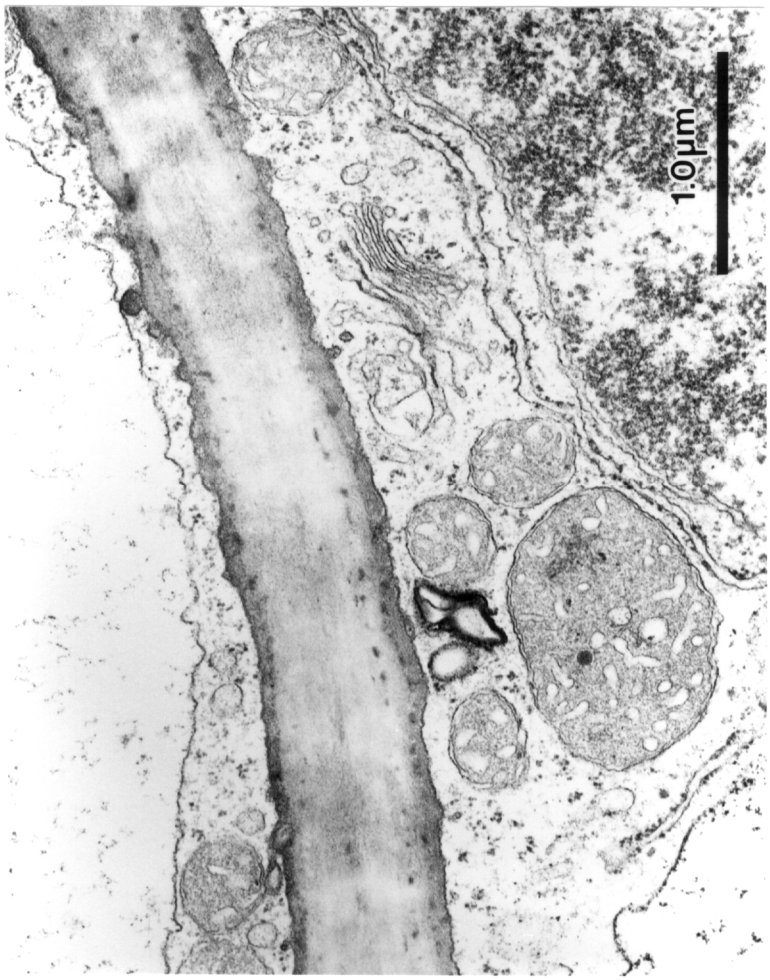

Figure 32. Transmission Electron Micrograph of Cell Walls from *P. taeda* (NAA Line) Suspension Culture Cells.

the *P. taeda* (NAA line) cells (229 pkat mg^{-1} protein) was detected at a level even greater than that for *P. taeda* stem tissues (20 pkat mg^{-1} protein). Such results could coincide with a reversal of the inhibitory effects of 2,4-D **36** on phenylalanine ammonia-lyase synthesis as described for *Daucus carota* (Ozeki *et al.*, 1990). As regards the immunogold localization of phenylalanine ammonia-lyase in these cells, the enzyme was localized in the cytosol, tonoplast, plasma membrane and endoplasmic reticulum as observed before. Accordingly, an alteration of the subcellular localization of phenylalanine ammonia-lyase does not appear to occur during this induction of cell wall thickening.

In addition to detectable levels of phenylalanine ammonia-activity, these NAA-grown cells showed cinnamyl alcohol dehydrogenase activity (2800 pkat mg^{-1} protein) to be enhanced more than 7 times that observed for the 2,4-D-grown cells (367 pkat mg^{-1} protein) and stem tissues (382 pkat mg^{-1} protein). Through this increase in cinnamyl alcohol dehydrogenase activity, further enzymological evidence was provided for active phenylpropanoid metabolism. As described earlier, the NAA-grown cells showed increased staining with phloroglucinol-HCl during the final two weeks of the culture period which suggests that these increased enzyme activities may coincide with lignification. To possibly demonstrate this, a time-course experiment was conducted to monitor phenylalanine ammonia-lyase and cinnamyl alcohol dehydrogenase activities over the 49-day culture period.

Each time during which the cells were transferred to fresh medium for the first 5 weeks of the time-course experiment, cells were harvested by filtration for the preparation of cell-free extracts. During the final two weeks of the total 7-week growth period, cells were harvested every two days. As can be seen in Figure 33, phenylalanine ammonia-lyase activity is only detectable during the last two weeks

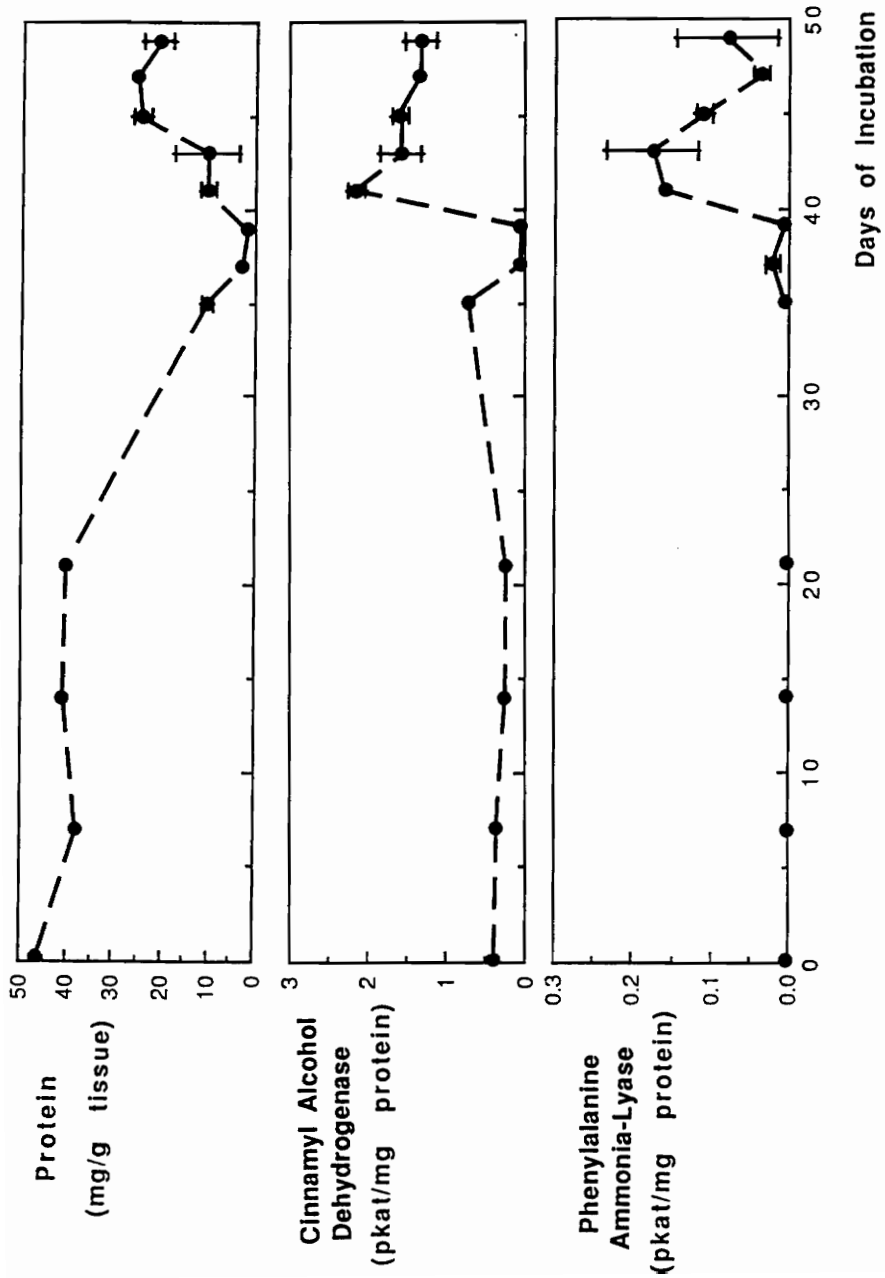

Figure 33. Activity for Phenylalanine Ammonia-lyase and Cinnamyl Alcohol Dehydrogenase in *P. taeda* (NAA Line) Suspension Culture Cells.

of the 7-week growth period; the maximal level of this enzyme detected (0.175 pkat mg^{-1} protein) was obtained mid-way through this two-week period. As for cinnamyl alcohol dehydrogenase, its activity remained fairly constant during the first 5 weeks of culture; during the final 2 week period it dropped and then abruptly rose to its maximal level (2.13 pkat mg^{-1} protein) after which it tapered off. Since these increases in phenylalanine ammonia-lyase and cinnamyl alcohol dehydrogenase activities coincided with increased red-violet staining of the cells with phloroglucinol-HCl, it thereby was apparent that during this stage, phenylpropanoid metabolism was activated.

Between days 21 and 35 of this experiment when the cells were maintained on the same medium for 2 weeks, the level of soluble proteins was greatly reduced from 4.05 $\mu g\ g^{-1}$ to 1.03 $\mu g\ g^{-1}$. After the transfer of cells to fresh medium on day 35, the level of soluble protein continued to decrease and then increased, apparently lagging behind the rapid increase in phenylalanine ammonia-lyase and cinnamyl alcohol dehydrogenase activities. The reduction in protein in the cells may be related to the continuous maintenance of the cells on the same medium for an extended period. Interestingly, the activity for cinnamyl alcohol dehydrogenase on day 35 (0.725 pkat mg^{-1} protein) is not reduced but elevated from that on day 21 (0.255 pkat mg^{-1} protein). Therefore, it appears that during the period in which a depressed level of soluble protein was observed in the cells, metabolic changes likely occurred favoring phenylpropanoid metabolism.

After day 35 of the experiment, the level of cinnamyl alcohol dehydrogenase activity and soluble protein were reduced until 6 days later when both increased. This trend may represent an abrupt alteration of metabolic activity in the cells which subsequently favored phenylpropanoid biosynthesis. Cell maturation through

apparent lignification may be reflected by the progressive reduction in cinnamyl alcohol dehydrogenase and phenylalanine ammonia-lyase activities during the final days of the experiment.

Thus far it appears that during the latter two weeks of the 49-day culture period, the NAA-grown cells undergo active phenylpropanoid metabolism as evidenced by histochemical staining and activities for phenylalanine ammonia-lyase and cinnamyl alcohol dehydrogenase. During their maintenance on NAA-containing medium, the *P. taeda* cells undergo cell wall thickening. To verify that we have achieved our objective of inducing the cells to undergo cell wall thickening and lignification, we then directed our efforts to characterize the phenylpropanoid products.

4.8. Lignification in *P. taeda* (NAA Line) Suspension Cultures.

As stated above, histochemical and enzymological results indicated that phenylpropanoid metabolism was active in the *P. taeda* (NAA line) cells. Nevertheless, lignan biosynthesis was reduced for these cells as compared to those grown with 2,4-D **36**. Analyses of methanol extracts by HPLC revealed very small peaks at similar retention times of pinoresinol and matairesinol **38** which indicated that there was a significantly lower content of each of these lignans (<10 $\mu g\ g^{-1}$ dry tissue) in the 49-day-old *P. taeda* (NAA-line) cells. If these cells are lignifying, this result would suggest that a re-direction of phenylpropanoid metabolism into lignin formation. It remains to be determined whether lignans are incorporated into the lignin component of plant cell walls.

Next, we directed our efforts to determine if these NAA-grown cells were lignifying. Accordingly, dried 49-day-old *P. taeda* (NAA line) cells were extracted

(Procedure B) to give a tan-colored cell wall preparation (weight recovery = 62%) which was then analyzed by solid-state ^{13}C NMR spectroscopy. As can be seen in Figure 34, the ^{13}C NMR spectrum from the *P. taeda* (NAA line) extractive-free cell walls substantially differs from that for the extractive-free cell walls from the 2,4-D-grown cells. Most noticeably, large resonances are observable in the aromatic region of the spectrum (155-115 ppm). Furthermore, a definite increase in the resonance at 56 ppm coinciding with methoxyl functionalities could suggest the deposition of guaiacyl **5** moieties of a possible lignin component. The predominant resonance in the spectrum is that at 73 ppm which corresponds to C-2, C-3 and C-5 of cellulose and other applicable polysaccharides (*i.e.* hemicelluloses, pectins). Resonances at 62 and 83 ppm coincide with C-6 and C-4 of cellulose, respectively. Downfield, a resonance at 105 corresponds to anomeric carbons such as C-1 of cellulose. Even further downfield, a resonance at 175 ppm corresponding to carboxyl moieties showed a greater relative intensity with respect to the resonance at 73 ppm; because of the broad nature of this resonance, it is not possible to distinguish between peptide linkages in protein, carboxyl moieties in pectin or even the carboxyl moieties of hydroxycinnamic acid which may be cell wall bound. Upfield, a relative increase in the methylenic resonances of the spectrum between 20 and 45 ppm are also observed.

The ^{13}C NMR spectrum of cell walls from 2,4-D-grown cells (see Figure 26) showed resonances reflecting cell walls comprised primarily of polysaccharides. The cell walls from cells maintained on NAA **37** differed by also possessing a significant cell wall aromatic component. Thus, in addition to increasing cell wall thickness, tentative evidence suggested that lignification is also occurring in the cells manipulated on NAA-containing medium. In an attempt to further characterize

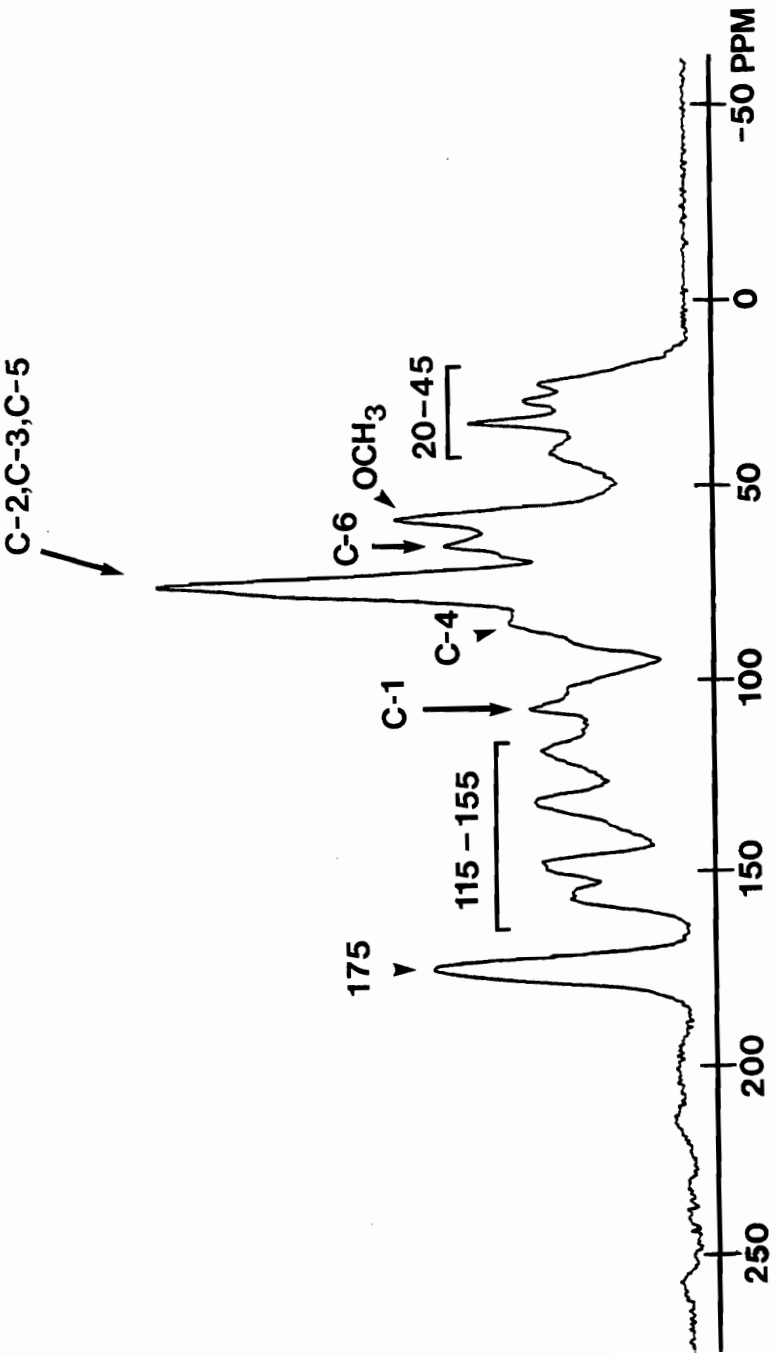

Figure 34. Solid-state ^{13}C NMR Spectrum of *P. taeda* (NAA line) Cell Walls.

this aromatic component, the chemical methods for lignin analysis used to analyze the 2,4-D-grown cells walls were applied to the cell wall fraction from the NAA-grown cells.

Analysis of the extractive-free *P. taeda* (NAA-line) cell walls by the acetyl bromide method gave an apparent lignin content of 15.9% which is approximately 2-fold the value observed for the 2,4-D-grown cells using this method. The Klason lignin determination for these cell walls gave a very unusual value for lignin content of 49.2% based on the weight of recovered acid-insoluble lignin and extractive-free cell walls. Thus, it was especially apparent that the Klason method, which is typically used with woody samples, may be unsuitable for these suspension culture cells. It is likely that incomplete digestion of the non-lignin components of the *P. taeda* suspension culture cell walls would result in non-lignin contaminants in the acid-insoluble lignin thereby overestimating the actual lignin content. The exact reason for the discrepancy between the two lignin determination methods is not known. Nevertheless, these observations exemplify those situations where a chemical analytical method under different conditions can give misleading results which may be dependent upon the characteristics of the sample analyzed.

Next, the monolignol composition of the lignin in the NAA-grown cells was determined by nitrobenzene oxidation and thioacidolysis. In the case of nitrobenzene oxidation, the guaiacyl **5**:*p*-hydroxyphenyl **4** ratio (9.8:1) as well as product yield (1.17 % of extractive-free cell walls) found in the NAA-grown cells reflected somewhat more of that observed in the respective woody sample (100:1, 9.8% yield from extractive-free wood meal with 28% lignin). Similarly, thioacidolytic treatment of the *P. taeda* (NAA line) cell walls gave a guaiacyl **5**: *p*-hydroxyphenyl **4** ratio (6.0:1) and yield (0.41% of extractive-free cell walls) also apparently

more representative of a woody tissue. As described before, extractive-free compression wood from *Pinus pinaster* when analyzed by thioacidolysis gave a guaiacyl **5**:*p*-hydroxyphenyl **4** ratio of 4.5:1 with a yield of products extrapolated to 10.9% for the extractive-free material assuming a 28% lignin content (Lapierre *et al.*, 1988). Therefore, through the definite increase in yield of oxidation products from the NAA-grown cells, as compared to the 2,4-D-grown cells, it is likely that lignification accompanied the induction of cell wall thickening. Final definitive proof that lignification was occurring in the NAA-grown cell would be provided by the identification of lignin bonding patterns *in situ*.

4.9. Administration of [U-^{14}C] Phenylalanine to *P. taeda* (NAA Line).

Results thus far have indicated that the *P. taeda* (NAA line) cells may be lignifying. Final verification of lignification would be provided by the demonstration of lignin bonding patterns *in situ*. Prior to the administration of carbon-13 specifically labelled phenylalanine **9** to the *P. taeda* (NAA line) cells, [U-^{14}C] phenylalanine **9** was administered to determine the conditions necessary for sufficient precursor incorporation.

Taking into consideration that active phenylpropanoid metabolism was occurring during the final two weeks of the 7-week culture period as evidenced by enzyme activities and histochemical staining, this two week period was selected for the administration of the [U-^{14}C] phenylalanine **9**. Since previous experiments showed sufficient radiolabel incorporation with 10 mM [U-^{14}C] phenylalanine **9**, this concentration of precursor was selected for the experiments with the NAA-grown cells. Into medium containing [U-^{14}C] phenylalanine **9** (10 mM) and NAA

37 (11.3 μM) was added 35 day old *P. taeda* (NAA line) cells which were then cultured for 2 weeks before harvesting.

At the beginning and the end of the period of [U-^{14}C] phenylalanine **9** administration, aliquots of medium were removed to verify the level of radiolabel administered and to estimate amounts remaining in the medium. These aliquots of medium were also assayed for their concentration of phenylalanine **9** by the spectrophotometric assay described before. By correlating the radioactivity in the medium with the concentration of the phenylalanine **9** in the medium as determined by the spectrophotometric assay, it was verified that the radiolabelled phenylalanine **9** in the medium was intact (unmetabolized). This is an important result since the precursor administered should remain intact prior to incorporation and metabolism in the cell to avoid such problems as the scrambling of the specific label.

During the administration of the [U-^{14}C] phenylalanine **9**, the cells again became more aggregated as before and changed in color from yellow-green to brown. Cells maintained in parallel in the absence of exogenously supplied phenylalanine **9** behaved in the same manner. After the [U-^{14}C] enriched cells were harvested and dried as before, an aliquot was extracted (Procedure B) to obtain a cell wall fraction (~ 60% weight recovery). The radioactivity of both the freeze-dried cells and the respective cell walls are shown in Table 1 (experiment A). Using the specific activity of the [U-^{14}C] phenylalanine **9** administered, phenylalanine equivalent incorporations of 3.74 and 1.90% were obtained for the freeze-dried cells and extractive-free cell walls, respectively. The latter value for the cell walls is well within the range needed for carbon-13 labelling experiments. Subsequently, this experiment was repeated and gave an even more efficient incorporation as reflected

Table 1

Incorporation of [U-^{14}C] Phenylalanine into
P. taeda (NAA-Line) Suspension Culture Cells

Experiment	Freeze Dried Cells		Cell Walls	
	(Bq/mg)	Phenylalanine Equivalents (%)*1	(Bq/mg)	Phenylalanine Equivalents (%)
A*2	28.2	3.74	15.5	1.90
B*2	46.5	6.40	50.8	6.99
C*3	27.2	3.85	32.8	4.65

*1: Phenylalanine Equivalents $= \frac{\text{Radioactivity in tissue(Bq mg}^{-1})}{\text{Specific Activity of Phenylalanine (Bq mg}^{-1})} \times 100\%$

*2: Two-week growth period as described in Materials and Methods.
*3: Same as *2 except cells were transferred to fresh medium without [U-^{14}C] phenylalanine and cultured for 4 additional days.

by a phenylalanine equivalent incorporation of 6.99% (experiment B) for the cell walls. In this latter experiment, the percent phenylalanine equivalent incorporation is greater for the extractive-free residue (6.99%) than it is for the freeze-dried cells (6.40%). As can be seen in Table 2, this apparently resulted from a greater proportion of the radioactivity being incorporated into the insoluble component (52.7%) as compared to that (35.3%) for the first experiment (experiment A).

To determine if an even greater proportion of the [U-^{14}C] incorporated could be further metabolized into the insoluble residue, a separate experiment was conducted where cells cultured with [U-^{14}C] phenylalanine **9** for 2 weeks, as before, were then transferred to medium without the precursor and cultured for 4 additional days (experiment C). The value for phenylalanine equivalent incorporation for the cell walls (4.65%) was intermediate to that obtained in the first two experiments. As can be seen in Table 2, the value for the proportion of radioactivity in the insoluble component of 42.4% is comparable to the average of the values obtained for the first two experiments (44%).

The distribution of radioactivity during the preparation of extractive-free cell walls for all 3 experiments, shown in Table 2, revealed that between 22.2 and 36.6 % of the incorporated radioactivity was aqueous soluble which could include unmetabolized phenylalanine **9** or even radiolabelled soluble proteins. Extracts with methanol and then chloroform:methanol (**1:1**) removed 27.4 to 34.2% of the incorporated radioactivity. To facilitate drying of the chloroform:methanol (**1:1**) extractive-free residue, acetone was used for rinsing; no significant radioactivity was found in this fraction. A subsequent exhaustive extraction with toluene:ethanol (2:1) resulted in only low levels of radioactivity thereby indicating that the

Table 2

Distribution of Radioactivity in Extracts During Cell Wall Isolation from *P. taeda* (NAA-Line) Suspension Culture Cells

	Percent of Total Radioactivity Recovered		
Extracts	A*1	B	C*2
Phosphate Buffer/H_2O	36.6	23.3	22.2
MeOH/MeOH:$CHCl_3$ (1:1)	26.5	22.4	34.2
Acetone	-*3	-	-
Toluene:EtOH (2:1)	1.6	1.6	1.2
Acetone	-	-	-
Cell Walls	35.3	52.7	42.4
	100	100	100

*1: Two-week growth period as described in Materials and Methods.
*2: Same as *1 except cells were transferred to fresh medium without [U-^{14}C] phenylalanine and cultured for 4 additional days.
*3: Background radioactivity.

extraction process was complete. A final rinse of the extractive-free cell walls with acetone did not contain any significant radioactivity.

In contrast to the first two experiments, the proportion of radioactivity in the aqueous fraction for the third (experiment C) is less than that for the organic solvent soluble fraction. This indicated that during the 4 additional days of culturing for these cells, some of the aqueous soluble radiolabelled metabolites were further metabolized into more organic soluble products. Nevertheless, despite the potential further metabolism of the administered precursor, no improvement of the level of radiolabel in the insoluble component of the cells was observed with this additional 4 days of growth. Therefore, experiments conducted with specifically labelled ^{13}C phenylalanine **9** utilized the final 2-weeks during the 49-day growth period for precursor administration after which the cells were harvested.

4.10. Administration of [Natural Abundance-^{13}C] Phenylalanine to *P. taeda* (NAA Line)

As one of two control experiments for administration of [1-^{13}C], [2-^{13}C] and [3-^{13}C] phenylalanines **9** to *P. taeda* (NAA line) cells, unlabelled phenylalanine **9** was administered to the NAA-grown cells in an analogous manner to that established during the administration of [U-^{14}C] phenylalanine **9**. Accordingly, 35-day-old *P. taeda* (NAA line) cells were transferred to medium containing phenylalanine **9** (10 mM) and NAA **37** (11.3μM), cultured for 2 weeks and then harvested by filtration and freeze-dried.

Dried *P. taeda* (NAA line) cells previously administered [natural abundance-^{13}C] phenylalanine **9** were extracted (Procedure B) to give an extractive-free cell wall fraction which was analyzed by solid-state ^{13}C NMR spectroscopy (see Figure 35). Comparison of the ^{13}C NMR spectrum of the *P. taeda* (NAA line) cell walls

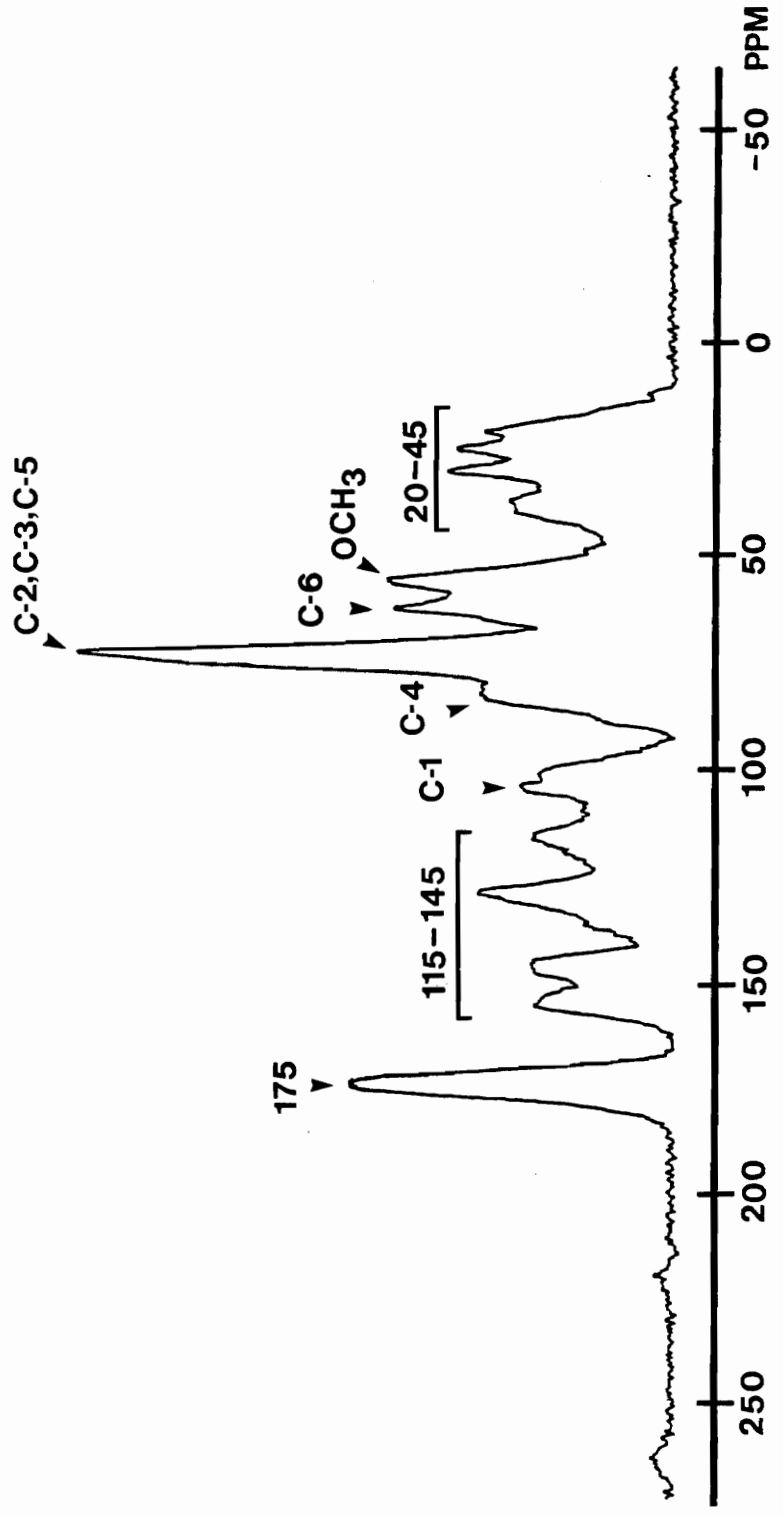

Figure 35. Solid-state ^{13}C NMR Spectrum of *P. taeda* (NAA Line) Cell Walls from Cells Previously Administered [Natural-abundance^{13}C] Phenylalanine.

with the cells grown with or without an exogenous supply of phenylalanine **9** (see Figure 34) showed both spectra to be essentially identical. Therefore, it was apparent that the exogenous supply of phenylalanine **9** in the medium does not have a detectable effect on the growth and development of these cells as evidenced by ^{13}C NMR spectroscopic analyses. The analyses of ^{13}C enriched *P. taeda* (NAA line) cells and cell walls by solid-state ^{13}C NMR spectroscopy, to be discussed, was used to analyze and determine lignin bonding patterns *in situ*. Although important information is revealed by solid-state ^{13}C NMR spectra, significant line broadening prevents the ambiguous assignment of certain bonding patterns. Therefore, to confirm the assignments of certain resonances to specific lignin bonding patterns the presumed lignin-derived component was isolated and analyzed by solution-state ^{13}C NMR spectroscopy.

For the isolation of the presumed lignin component of the *P. taeda* (NAA line) cells, extractive-free cell walls from three experiments were combined in an effort to provide sufficient sample for solution-state ^{13}C NMR spectroscopic analyses. Prior to the isolating the lignin derivative, these cell walls were first treated with pronase E to possibly facilitate the removal of any insoluble proteins. Extractive-free cell walls were suspended in buffer containing pronase E and ground with a mortar and pestle after which additional buffer also containing pronase E was added. After incubation, the suspension was centrifuged to give a cell wall residue which was repeatedly rinsed with H_20, frozen with liquid N_2 and freeze-dried. Following analyses of the cell walls by solid-state ^{13}C NMR spectroscopy, the cell walls were suspended in toluene and ground in a "planetary micromill" to a particle size of less than 10 μm in diameter as determined by light microscopy. Dried cell walls were then suspended in dioxane:H_2O (9:1) and stirred for 24 hours under an atmosphere

of N_2. The rich amber extract obtained after centrifugation was dried *in vacuo* and then dissolved in $(CD_3)_2SO$ for analysis by solution-state ^{13}C NMR spectroscopy. To avoid losses of the limited amount of sample, steps to further purify this lignin derivative were not undertaken.

Through overnight accumulation, a solution-state ^{13}C NMR spectrum was acquired for the natural abundance ^{13}C lignin derivative. Typically, analyses of similar lignin preparations utilize greater sample size and extended instrument times. Despite the limited signal to noise ratio, resonances are readily discernible in the aliphatic and aromatic regions of the spectrum as usually observed for MWL samples (see Figure 36) (Lapierre *et al.*, 1984; Robert *et al.*, 1989). Assignments for the discernible signals are shown in Table 3. The predominant resonance occurs at 55.6 ppm and corresponds to the methoxyl functionality of guaiacyl **5** moieties in the lignin preparation. As expected, resonances between 110.4 and 147.5 ppm are attributable to aromatic carbons in *p*-hydroxyphenyl **4** and guaiacyl **5** moieties; no resonances between 103 and 107 ppm corresponding to C-2 and C-6 of syringyl **6** moieties were observed. Since no resonances were observed at 105 ppm corresponding to C-1 of cellulose (and respective carbons of other polysaccharides), it was presumed that carbohydrate contamination of this sample was minimal. Upfield, aliphatic resonances at 85.0, 83.4, 62.7, 61.6 and 60.1 coincided with C-β and C-γ in the β-O-4 bonding pattern of lignins. The resonances at 71.0 and 53.6 ppm corresponding to the β-β linkage suggested a significant role for this bonding pattern in *P. taeda* lignin. The resonance at 81.7 ppm is yet unassigned. Finally, CH_3 and CH_2 saturated chains are represented by the resonance at 28.9 ppm.

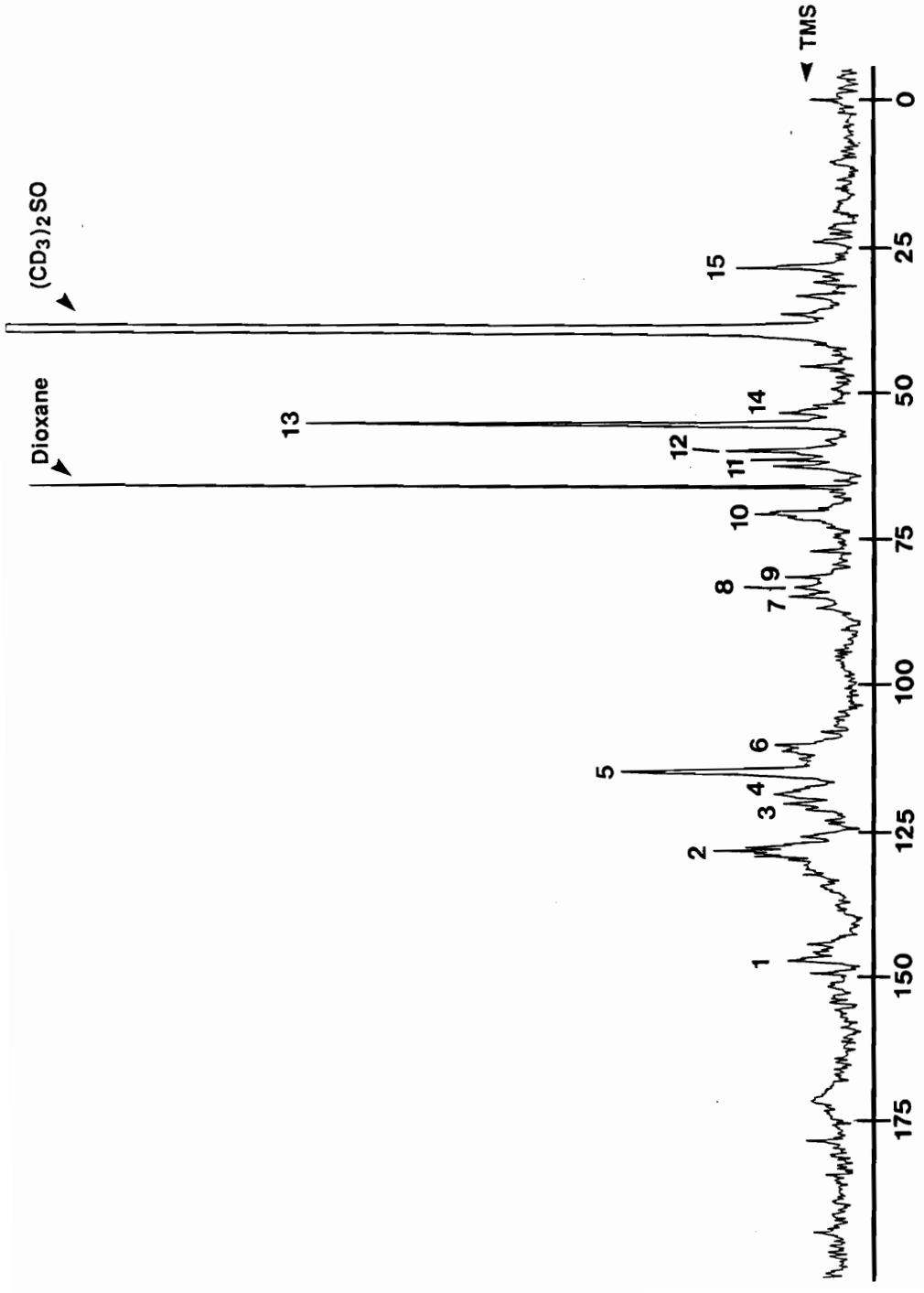

Figure 36. Solution-state ^{13}C NMR Spectrum of Lignin Derivative Isolated from [Natural-abundance ^{13}C] Phenylalanine Enriched Extractive-free/Pronase E Treated *P. taeda* (NAA Line) Cell Walls [Solvent = $(CD_3)_2SO$, Internal Standard = TMS].

Table 3

Assignment of ^{13}C NMR Signals of Lignin Derivative from Cell Walls from *P. taeda* Cells Previously Administered [Natural-abundance ^{13}C] Phenylalanine

Signal Number	ppm/TMS	Assignments*1
1	147.5	C-4 in G etherified
2	128.6	CH=CH in ArCH=CH-
3	120.5	C-1 in H
4	118.9	C-6 in G
5	115.1	C-5 in G, C-3 and C-5 in H
6	110.4	C-2 in G
7	85.0	C-β in β-O-4
8	83.4	C-β in β-O-4 with α-CO
9	81.7	-
10	71.0	C-γ in β-β
11	62.7, 61.6	C-γ in β-O-4 with α-C=O, C-γ in β-5
12	60.1	C-γ in β-O-4
13	55.6	OCH_3
14	53.6	C-β in β-β
15	28.9	CH_3 and CH_2 in saturated chain

*1: See Figure 18 in Introduction.

Despite the improved resolution of the solution-state ^{13}C NMR spectrum, it is still difficult to make definitive assignments because of the complexity of the spectrum. The uncertainty of the assignments is compounded because perhaps not all of the resonances are due to the lignin derivative but also potential contaminants. As will be discussed later, through the labelling of specific carbons in the propane side chain of lignin it is possible to identify the bonding environments of specific substructures. Such experiments provide not only the ability to distinguish between lignin and non-lignin cell wall components during *in situ* analyses but also provide the ability to make more definitive assignments for isolated lignin derivatives.

4.11. Administration of [1-^{13}C], [2-^{13}C] and [3-^{13}C] Phenylalanines to *P. taeda* (NAA Line).

From the natural abundance solid-state ^{13}C NMR spectra observed thus far, it is quite apparent that important aliphatic resonances corresponding to lignin bonding patterns of the phenylpropanoid side chain are masked by resonances corresponding to other major cell wall constituents such as cellulose and hemicelluloses. As described previously, efforts to study lignin *in situ* have involved the administration of lignin precursors specifically labelled on positions 1, 2 and 3 of the propane side chain, to intact plants. The resulting ^{13}C enriched tissues were then analyzed by solid-state ^{13}C NMR spectroscopy. Subtraction of the spectra for each of the ^{13}C specifically labelled tissues from a control, which was not ^{13}C enriched, yielded difference spectra. These spectra were then used to identify the bonding environments of specific carbons within lignin in the cell walls of grass and hardwood species (Lewis *et al.*, 1989).

Prior to the development of this technique, ^{13}C specifically enriched dehydrogenatively polymerized lignins (DHPs) were prepared from coniferyl alcohol **2** enriched with ^{13}C (11 atom%) at positions 1, 2 and 3 of the propane side chain (Lewis *et al.*, 1987b). As a control, a DHP was prepared with unlabelled coniferyl alcohol **2**. Each sample was then analyzed by solid- and solution-state ^{13}C NMR spectroscopy. By subtracting the ^{13}C NMR spectra for the ^{13}C enriched samples from that of the control, difference spectra were obtained. The solid-state ^{13}C NMR difference spectra are shown in Figure 37. The assignments of the resonances to the main bonding pattern identified in lignin correspond to the substructures shown in Figure 18 (see Introduction). Using the assignments from these experiments, resonances in the solution and solid-state ^{13}C NMR obtained from the *P. taeda* (NAA line) cells were assigned to specific lignin bonding patterns.

Phenylalanine **9** specifically labelled with ^{13}C at positions 1, 2 or 3 of the propane side chain was administered to *P. taeda* suspension culture cells in the same manner as described for [U-^{14}C] and [natural abundance ^{13}C] phenylalanine **9**. Harvested cells obtained were analyzed by solid-state ^{13}C NMR spectroscopy, extracted as described and reanalyzed. Results presented are from three experiments for each label and the controls.

The solid-state ^{13}C NMR difference spectrum of *P. taeda* (NAA line) cells previously administered [1-^{13}C] phenylalanine **9** is shown in Figure 38. Based on peak assignments from the analyses of the [1-^{13}C]-labelled DHP, a large enhancement of a resonance at 63.4 ppm consistent with expected chemical shifts for β-*O*-4 (**C**) and β-1 (**F**) and β-5 (**E**) and allylic alcohol (**A**) lignin bonding patterns in the *P. taeda* (NAA line) cells. In the solid-state experiment, considerable line broadening prevents us from distinguishing between these

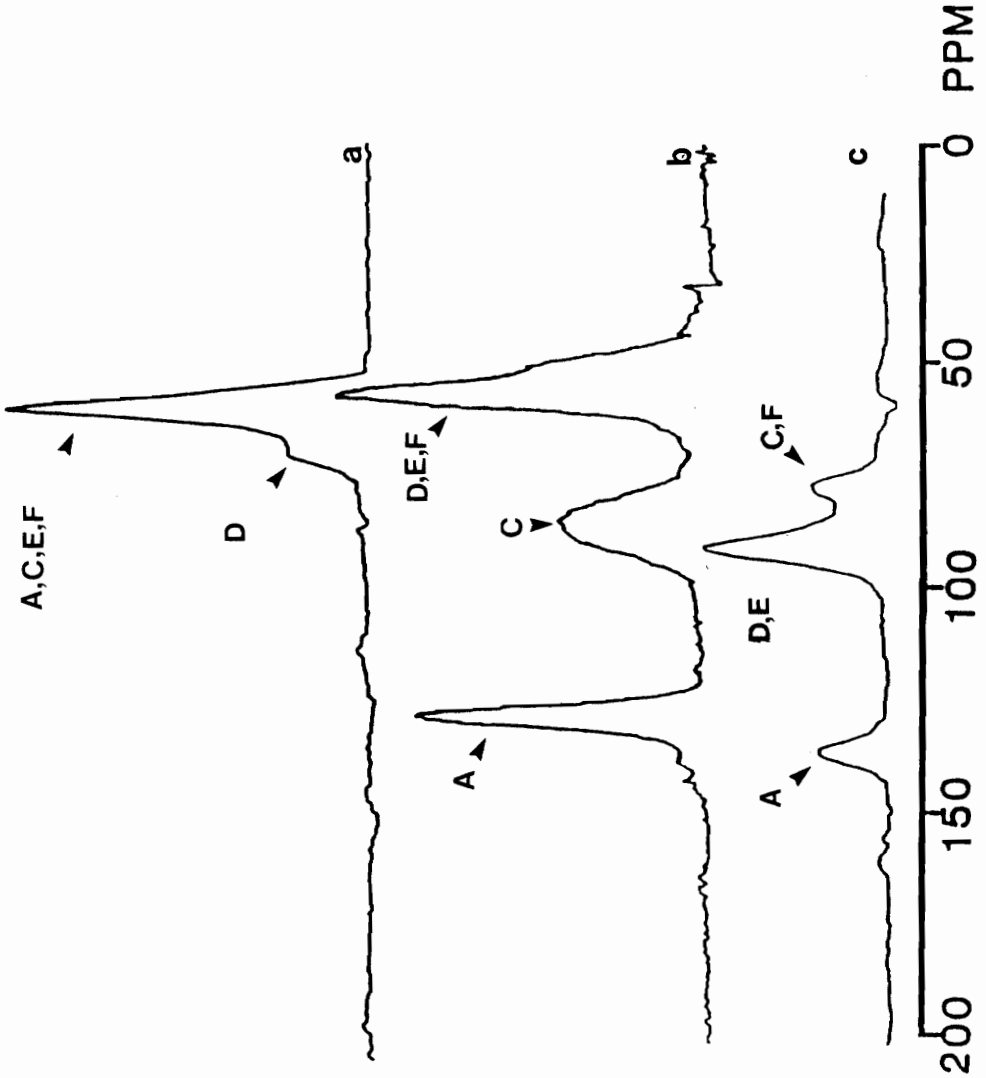

Figure 37. Solid-state ^{13}C NMR Difference Spectra of DHP Lignins Synthesized from **a)** [1-^{13}C], **b)** [2-^{13}C] and **c)** [3-^{13}C] Coniferyl Alcohols.

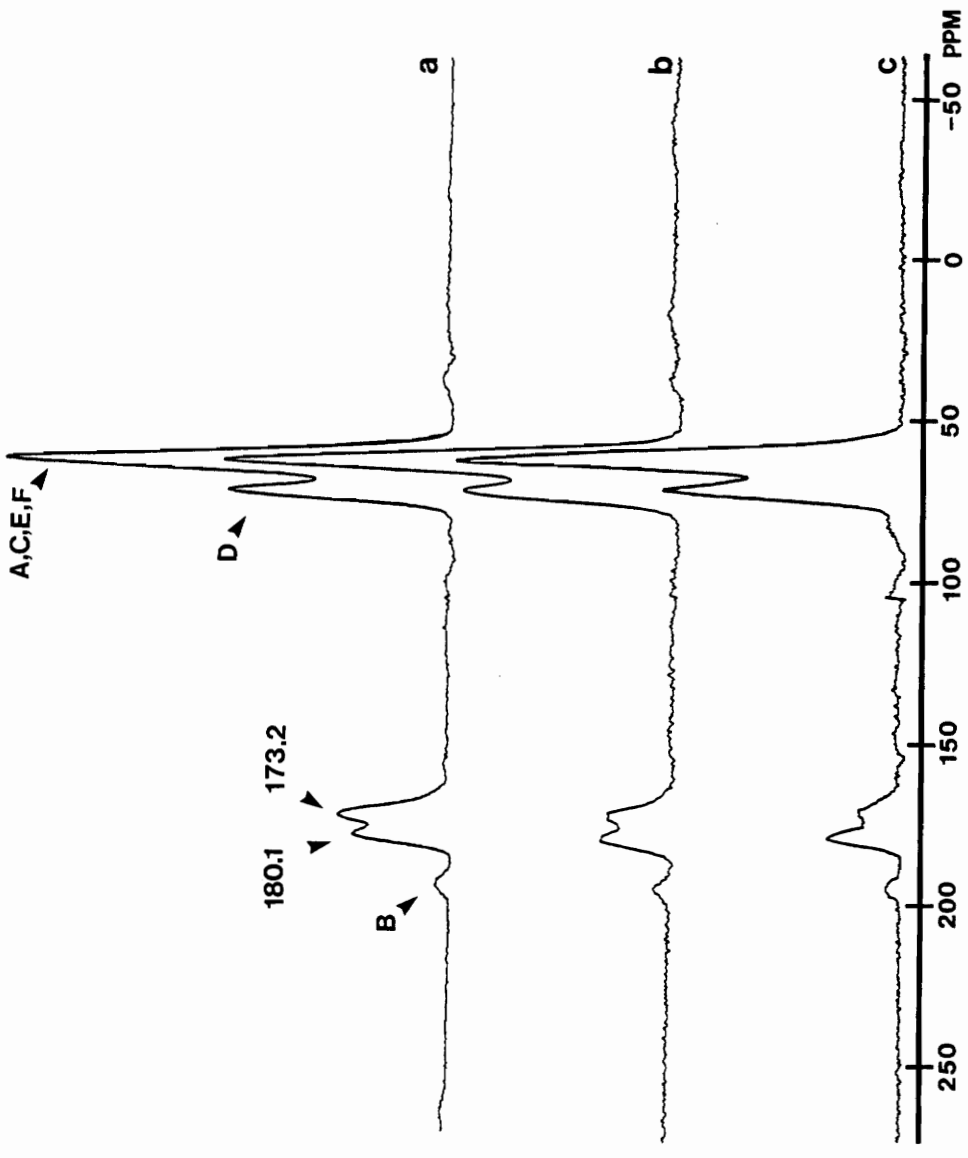

Figure 38. Solid-state ^{13}C NMR Spectra of *P. taeda* (NAA Line) Cells Previously Administered [1-^{13}C] Phenylalanine: **a)** Not Extracted; **b)** Extractive-free; **c)** Extractive-free and Pronase E Treated.

possibilities. Downfield, a resonance of 72.7 ppm is solely attributed to an apparent significant presence of the pinoresinol (β-β, **D**) substructure in the *P. taeda* (NAA line) lignin component. Indications that the phenylalanine **9** administered was not completely metabolized in the lignin component of the cell walls was shown by the resonance corresponding to phenylalanine **9** at 173.2 ppm. Of this phenylalanine **9** incorporated, but apparently not metabolized into lignin, a significant amount is removed by the extraction of the cells (Procedure B) to produce a cell wall fraction. Treatment of the cell walls with pronase E resulted in a further, but not quite complete, reduction in the resonance at 173.2 ppm. Accordingly, it appears that a considerable amount of phenylalanine **9** in its free or protein bound form accounts for this resonance in the solid-state ^{13}C NMR spectrum of the [1-^{13}C] enriched freeze-dried cells.

In addition to the above described resonances, downfield resonances occurring at 180.1 and 195 ppm could likely correspond to carboxyl and aldehydic moieties, respectively. These resonances appear to be relatively unaffected by the extraction and pronase E treatment. The exact identification of these resonances, especially that at 180.1 ppm, warrants further investigation.

Using the *P. taeda* (NAA line) extractive-free/pronase E treated cell walls, a lignin derivative was isolated as described previously. It should be noted that it is possible to obtain difference spectra by subtraction of the solution-state spectra of the ^{13}C enriched lignin derivatives from that of the control. Nevertheless, since the ^{13}C enriched spectra (not subtracted) proved to be most informative they are presented here. As can be seen in Figure 39, resonances at 70.78 (β-β, **D**), 62.78 (β-5, **E**; β-1, **F**), 61.58 (-CH=CH-CH_2OH, **A**) and 60.19 ppm (β-*O*-4, **C**), corresponding to lignin bonding patterns, are readily distinguishable. This

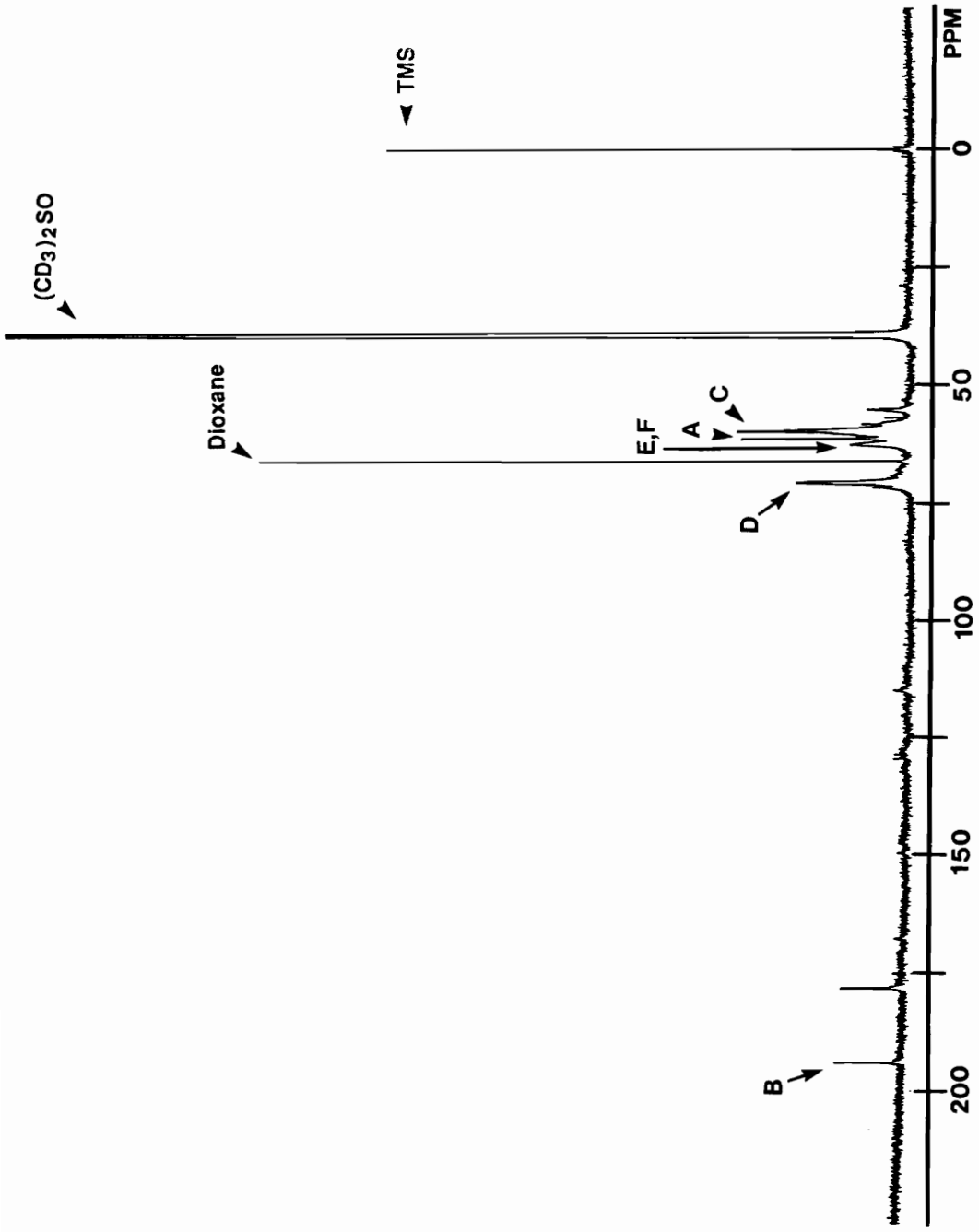

Figure 39. Solution-state ^{13}C NMR Spectrum of Lignin Derivative Isolated from [1-^{13}C] Enriched *P. taeda* Extractive-free/Pronase E Treated Cell Walls [Solvent = $(CD_3)_2SO$, Internal Standard = TMS].

spectrum, along with the solid state spectra described above, allowed the identification of lignin bonding patterns, thereby providing confirmation that the *P. taeda* (NAA line) cells are capable of lignifying.

In addition to the resonances observed in the solution-state ^{13}C NMR spectrum that coincided with known lignin resonances in the solid-state ^{13}C NMR spectra, a sharp resonance at 66.5 ppm corresponded to residual dioxane in the lignin preparation. In the solid-state ^{13}C NMR spectra, a very small resonance at 195 ppm was tentatively assigned to an aldehydic functionality. This resonance also appears in the solution-state spectrum (194.02 ppm) and coincides with the aldehydic resonance observed in the solution state spectrum of the [1-^{13}C] DHP. In the case of this resonance in the DHP it was suggested that it resulted from the allylic oxidation of the DHP by the solvent $(CD_3)_2SO$ (Lewis *et al.*, 1987b). For the [1-^{13}C] enriched *P. taeda* (NAA line) cells, this resonance was observed, although small, in the solid-state spectra thereby suggesting the possibility of an aldehydic moiety in the lignin component.

Other resonances in the solution-state ^{13}C NMR spectrum of the [1-^{13}C] lignin derivative includes the resonance at 178.35 ppm. Again, further studies are needed to identify the exact nature of the bonding environment this resonance represents. Another resonance at 56.0 ppm in the solution state spectrum appears to coincide with an unlabelled methoxyl functionality. Through subtraction of the solution-state spectrum of the [natural abundance ^{13}C] lignin derivative from the [1-^{13}C] lignin derivative, this resonance was not observed in the resulting difference spectrum (data not shown). Finally, it should be noted that in the solution-state spectrum of the lignin derivative, no peak corresponding to the carboxyl

functionality of phenylalanine **9** was observed; this further confirms the assignment of this resonance in the solid-state spectra.

Further verification that the *P. taeda* (NAA line) suspension cultures were capable of lignifying was shown by the solid-state ^{13}C NMR spectra of the cells previously administered [2-^{13}C] phenylalanine **9**. For these cells, metabolism of [2-^{13}C] phenylalanine **9** resulted in a strong resonance at 85.8 ppm corresponding to the β-*O*-4 (**C**) bonding pattern in the solid-state ^{13}C NMR difference spectrum of the respective freeze-dried cells (see Figure 40). Additional evidence that the culture produces lignin comparable to plant tissues is shown by the other enhanced resonances such as that observed at 127.9 ppm corresponding to lignin allylic alcohol group (**A**). Upfield, large resonance enhancements between 47 and 57 ppm are assigned, in part, to β-β (**D**), β-5 (**E**) and β-1(**F**) linkages. It should be noted that the intensity of the signal at 55.4 ppm cannot be solely attributed to the above mentioned lignin bonding patterns since the resonance corresponding to C-3 of phenylalanine **9** also occurs near 56 ppm. The relative intensity of this resonance was somewhat reduced in the spectrum from the respective cell wall preparation (see Figure 40) thus suggesting the removal of phenylalanine **9** not metabolized into the insoluble or lignin component of the cell wall. Subsequent treatment of the extractive-free cell walls with pronase E to possibly remove protein bound phenylalanine **9** resulted in a further relative reduction of this resonance. Since it was not known to what extent the removal of protein bound phenylalanine **9** was complete, the resonance at 55.4 ppm could still not be solely attributed to lignin.

Analyses of the [2-^{13}C] enriched lignin derivative by solution-state ^{13}C NMR also showed resonances corresponding to lignin bonding patterns (see Figure 41). These resonances at 128.04 (-C=*C*-CH_2OH, **A**), 125.67 (-*C*=CH-CHO, **B**), 83.65

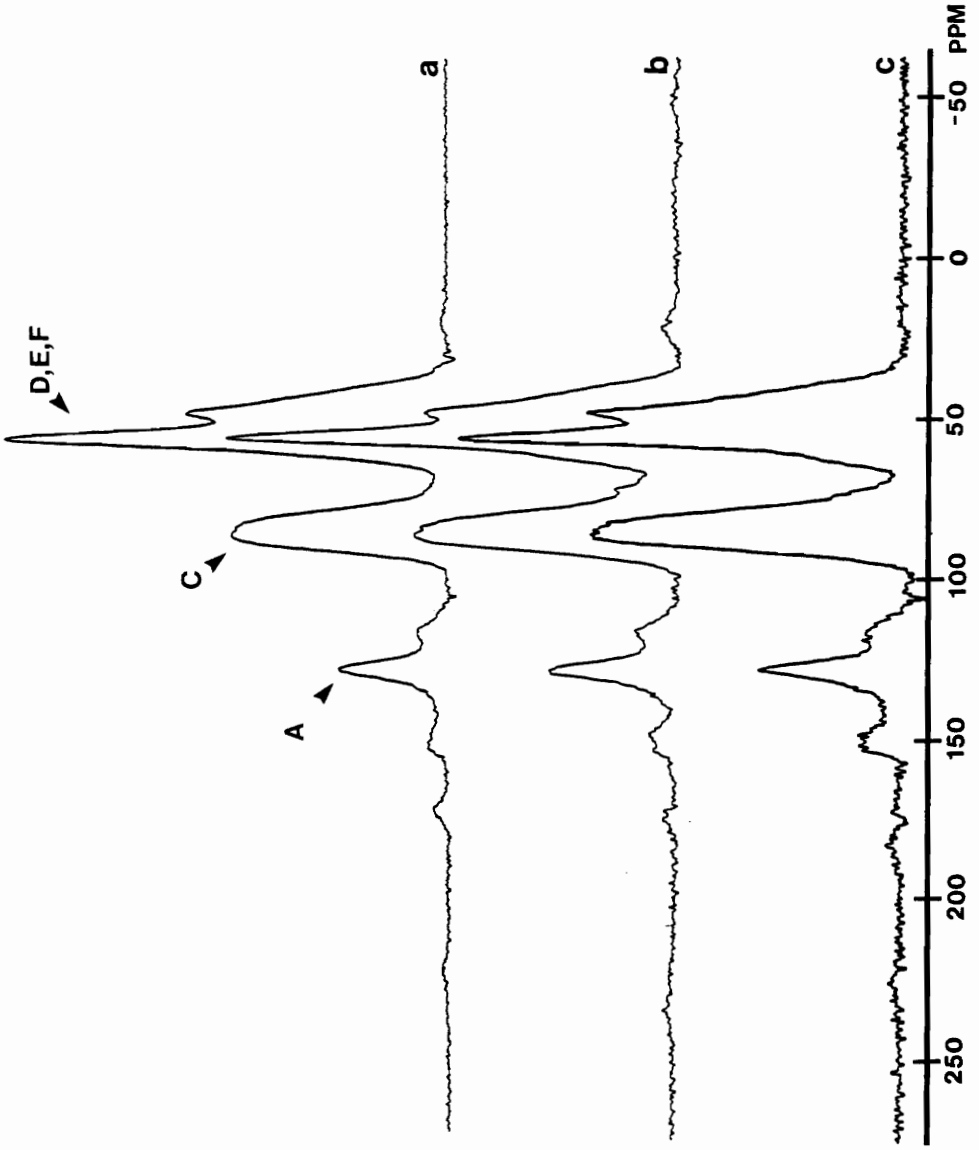

Figure 40. Solid-state ^{13}C NMR Spectra of *P. taeda* (NAA Line) Cells Previously Administered [2-^{13}C] Phenylalanine: **a)** Not Extracted; **b)** Extractive-free; **c)** Extractive-free and Pronase E Treated.

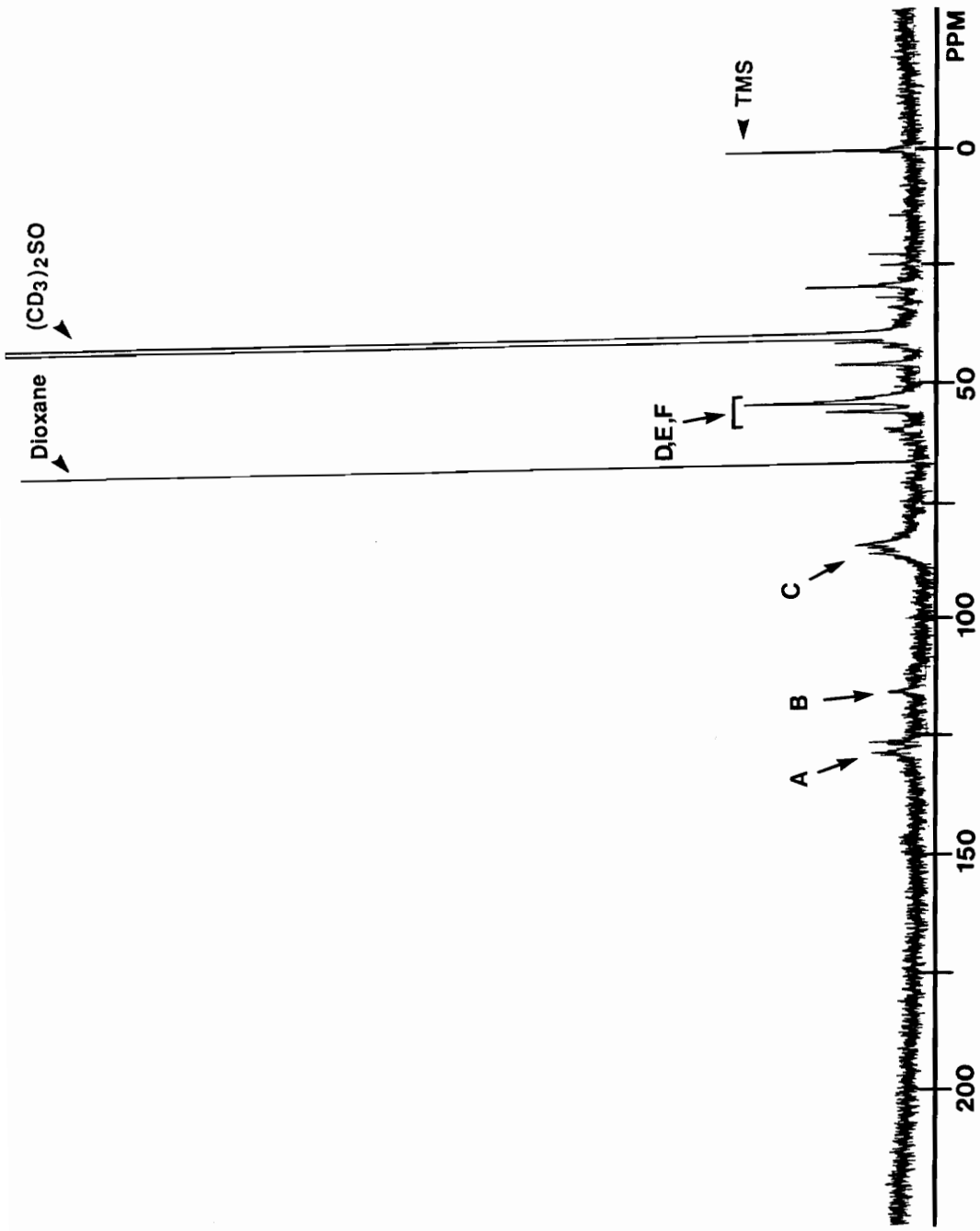

Figure 41. Solution-state ^{13}C NMR Spectrum of Lignin Derivative Isolated from [2-^{13}C] Enriched *P. taeda* Extractive-free/Pronase E Treated Cell Walls [Solvent = $(CD_3)_2SO$, Internal Standard = TMS].

(β-*O*-4, **C**) and 53.5 (β-5, **E**; β-1, **F**; β-β, **D**) ppm verified the assignments of the resonances observed in the solid-state spectra. Again, resonances at 65.5 and 55.46 ppm corresponded to dioxane and the unlabeled methoxyl functionality. Interestingly, at 45.47 and 28.94 ppm, resonances were observed which either represent additional lignin bonding patterns or artifacts of the lignin derivative isolation procedure. It is more likely that the latter reason for these resonances applies since there is no evidence for the resonance at 28.94 ppm in any of the solid-state ^{13}C NMR spectra.

Further evidence of the incomplete metabolism of administered phenylalanine **9** into the lignin component is shown by the solid-state difference spectrum for the cells previously administered 3-^{13}C phenylalanine **9** (see Figure 42). In this spectrum, a large resonance at 38.6 ppm is assigned to phenylalanine **9** incorporated, but not metabolized, into lignin. Resonances corresponding to lignin bonding patterns occur downfield at 86.4 and 74.4 ppm and are attributable to β-β (**D**) and β-5 (**E**) substructures and β-*O*-4 (**C**) and β-1 (**F**) substructures, respectively. The allylic alcohol moiety (**A**) is again indicated by a resonance which in this case appears at 130.7 ppm. As seen previously, the ^{13}C NMR spectrum of the respective cell wall fraction isolated showed a considerable reduction in the relative intensity for the resonance corresponding to phenylalanine **9**. Further reduction of this resonance was observed after treatment with pronase E. Nevertheless, the size of the residual resonance indicated that the pronase E treatment may not be complete. Possibly, the incomplete removal of phenylalanine **9** in the NAA-grown cells may be related to their higher degree of lignification which may have rendered cell wall structural proteins inaccessible to the protease.

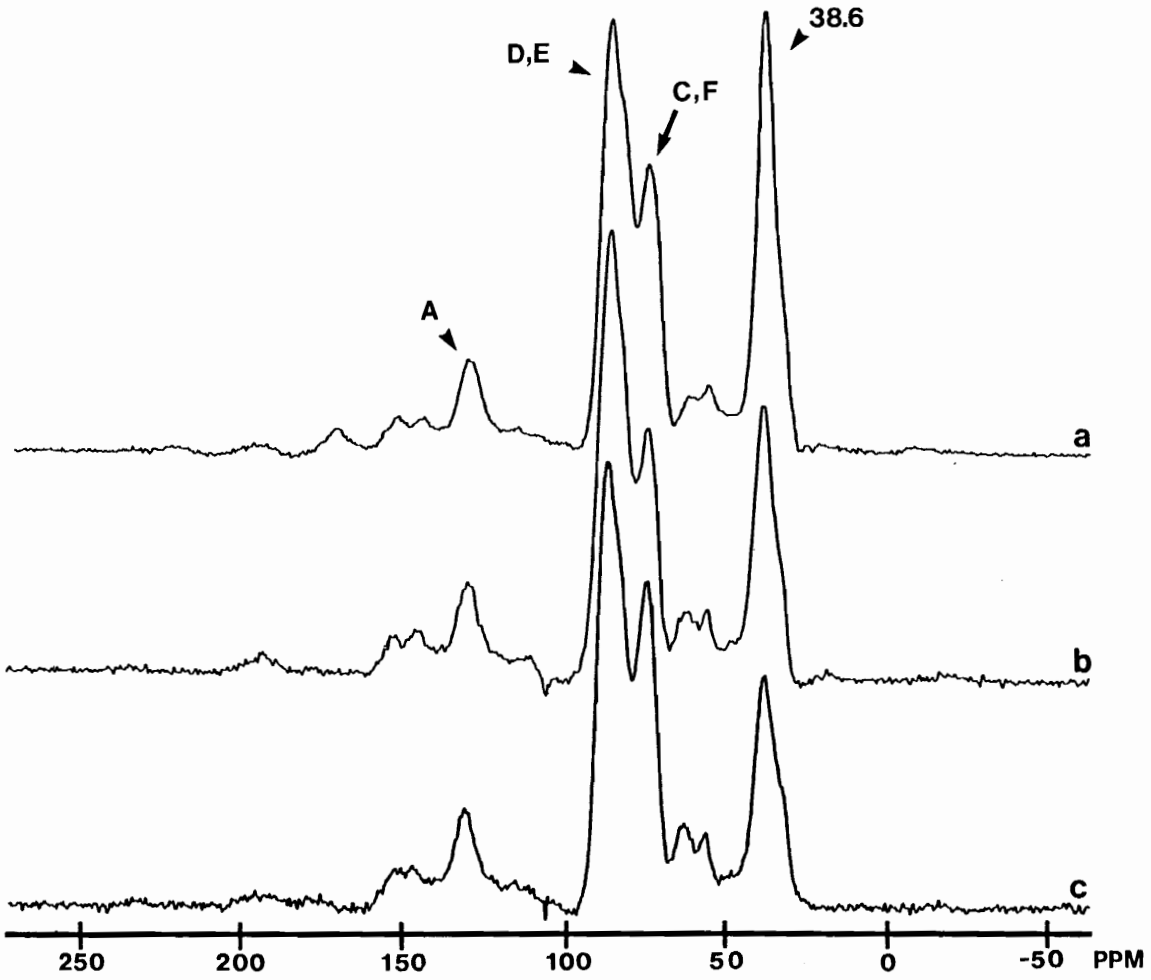

Figure 42. Solid-state ^{13}C NMR Spectra of *P. taeda* (NAA Line) Cells Previously Administered [3-^{13}C] Phenylalanine: **a**) Not Extracted; **b**) Extractive-free; **c**) Extractive-free and Pronase E Treated.

Similar to the results above, analyses of the [3-^{13}C] enriched lignin derivative showed sharp resonances which verified assignments made in the solid-state NMR spectra (see Figure 43). Resonances corresponding to the β-*O*-4 (**C**) and β-1(**F**) substructures possessing C-3 hydroxyl moiety are readily distinguishable at 70.83 and 71.42 ppm, respectively. Downfield, resonances at 84.83 (β-β, **D**) and 86.80 (β-5, **E**) were not distinguishable in the solid state and were assigned to a broad resonance at 86.3 ppm. Allylic alcohol (**A**) and aldehyde (**B**) moieties are observable by resonances centered at 128.43 and 153.89 ppm, respectively. Resonances at 81.58, 82.5 and 82.3 ppm likely correspond to aryl ether bonds such as in diaryl glycerol 2,3-diaryl ether substructures; model compounds of aryl glycerol 2,3-diaryl ethers in $CDCl_3$ show resonances by solution-state ^{13}C NMR between 81.59 and 82.25 ppm for which the specific resonances depend upon whether the actual model compound used is in its erythro or threo stereochemical form (Katayama, 1989). Cleavage of the propane side chain between carbons 2 and 3 is evidenced by the resonance at 190.95 ppm which corresponds to a benzyl aldehyde moiety; how this occurs is unknown. In some of the aryl glycerol 2,3-diaryl ether model compounds referred to above, solution-state ^{13}C NMR spectra show the chemical shifts of the benzaldehyde moiety to be between 190.47 and 190.62 ppm. Since the resonance at 190.95 in the solution state ^{13}C NMR spectrum is not observed in the solid-state spectra, it appears to be an artifact of the lignin isolation procedure. Another resonance at 33.5 ppm is unassigned and may represent either alternative lignin bonding patterns or artifacts of the isolation procedure. As before, resonances at 55.5 and 66.5 ppm correspond to unlabelled methoxyl functionalities and residual dioxane, respectively. Interestingly, a resonance at 36.74 ppm was observed which suggested that in this instance the

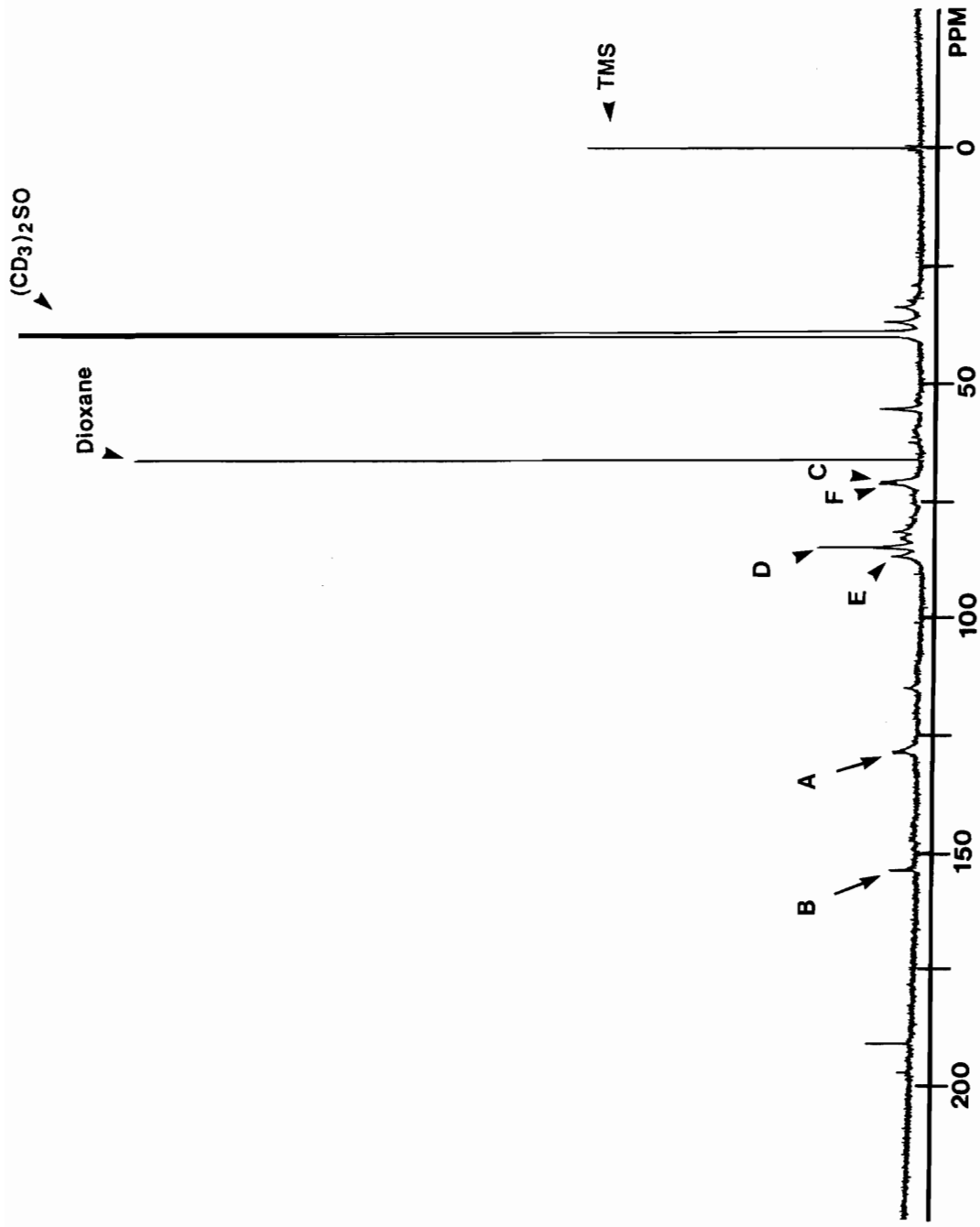

Figure 43. Solution-state ^{13}C NMR Spectrum of Lignin Derivative Isolated from [3-^{13}C] Enriched *P. taeda* Extractive-free/Pronase E Treated Cell Walls [Solvent = $(CD_3)_2SO$, Internal Standard = TMS].

lignin derivative could contain C-3 methylene functionalities such as that in phenylalanine **9**.

Through the administration of [1-^{13}C], [2-^{13}C] and [3-^{13}C] phenylalanine **9** to the *P. taeda* (NAA line) suspension culture cells it has been possible to identify specific lignin bonding environments *in situ* for the first time in a softwood species. Through analyses of the respective ^{13}C enriched lignin derivatives, specific lignin bonding patterns undistinguishable in the solid-state ^{13}C NMR spectra because of line broadening were readily discernible in the solution-state. These results, in contribution to the histochemical, biochemical and chemical evidence described previously, demonstrate that the *P. taeda* suspension culture system developed does lignify. Taking into consideration that the suspension cultures show only limited cell wall thickening leads to the conclusion that this culture system provides an excellent model for the study of lignin at the early stages of its deposition.

4.12. Isolation of Extracellular Precipitate from *P. taeda* (Sucrose Dilution/NAA Line) Medium.

Recently, suspension cultures of *Picea abies* have been suggested to release "lignin" into the nutrient medium (Brunow *et al.*, 1990). This "lignin", collected by centrifugation of the medium, was acetylated and analyzed by solution-state ^{13}C NMR. Comparison of the resulting spectrum with that of *Picea abies* MWL showed the two to be quite similar. With some exceptions, resonances were observed at the same positions in the spectrum although relative intensities were variable. Molecular weight determinations indicated that the isolated material was polymeric. It was concluded by these authors that this extracellular precipitate represented the first conifer lignin that could be characterized without mechanically or chemically harsh isolation methods.

Interestingly, we had observed a similar phenomenon in the *P. taeda* suspension cultures grown on an 8% sucrose solution. After 2 weeks of culturing of the cells on the 8% sucrose solution, the cells became more aggregated and changed color from yellow-green to yellow-tan. At this time, the medium had a dense milky appearance. After filtration through a glass wool plug to remove cellular debris, the medium was centrifuged. The precipitate collected was washed with water (twice) and freeze-dried to afford a creamy white powder which was solubilized in $(CD_3)_2SO$ and analyzed by solution-state ^{13}C NMR spectroscopy.

The solution-state ^{13}C NMR spectrum of the extracellular precipitate is shown in Figure 44. Interestingly, a vast majority of the resonances observed are coincident with resonances observed in guaiacyl MWLs (Lapierre *et al.*, 1984; Robert *et al.*,1989). Accordingly, assignments of the resonances observed to lignin bonding environments are shown in Table 4. As in a guaiacyl lignin, resonances are observed in the aromatic region of the spectrum between 110.8 and 149.5 ppm; resonances between 103 and 107 ppm indicative of syringyl **6** moieties are not observed. Downfield, a resonance at 194.1 ppm indicated α-ketonic bonding environments. Upfield, resonances include those at 55.4 and 28.9 corresponding to methoxyl as well as CH_3 and CH_2 in saturated chains, respectively. Resonances corresponding to C-β and C-γ in the β-O-4 bonding pattern occur at 85.0, 84.3, 83.6, 62.8, 61.6 and 55.9 ppm. The β-β bonding pattern of lignin is represented by resonances at 70.8 , 53.1 and 52.9 ppm correponding to C-β and C-γ. Finally, a resonance at 71.5 ppm is attributed to C-α in the guaiacyl **5** moiety.

Resonances that are yet unassigned occur at 178.4, 121.5, 113.3, 112.2, 86.9 and 45.5 ppm. The unassigned resonance at 178.4 is especially interesting since administration of [1-^{13}C] phenylalanine **9** to *P. taeda* (NAA line) cells resulted in

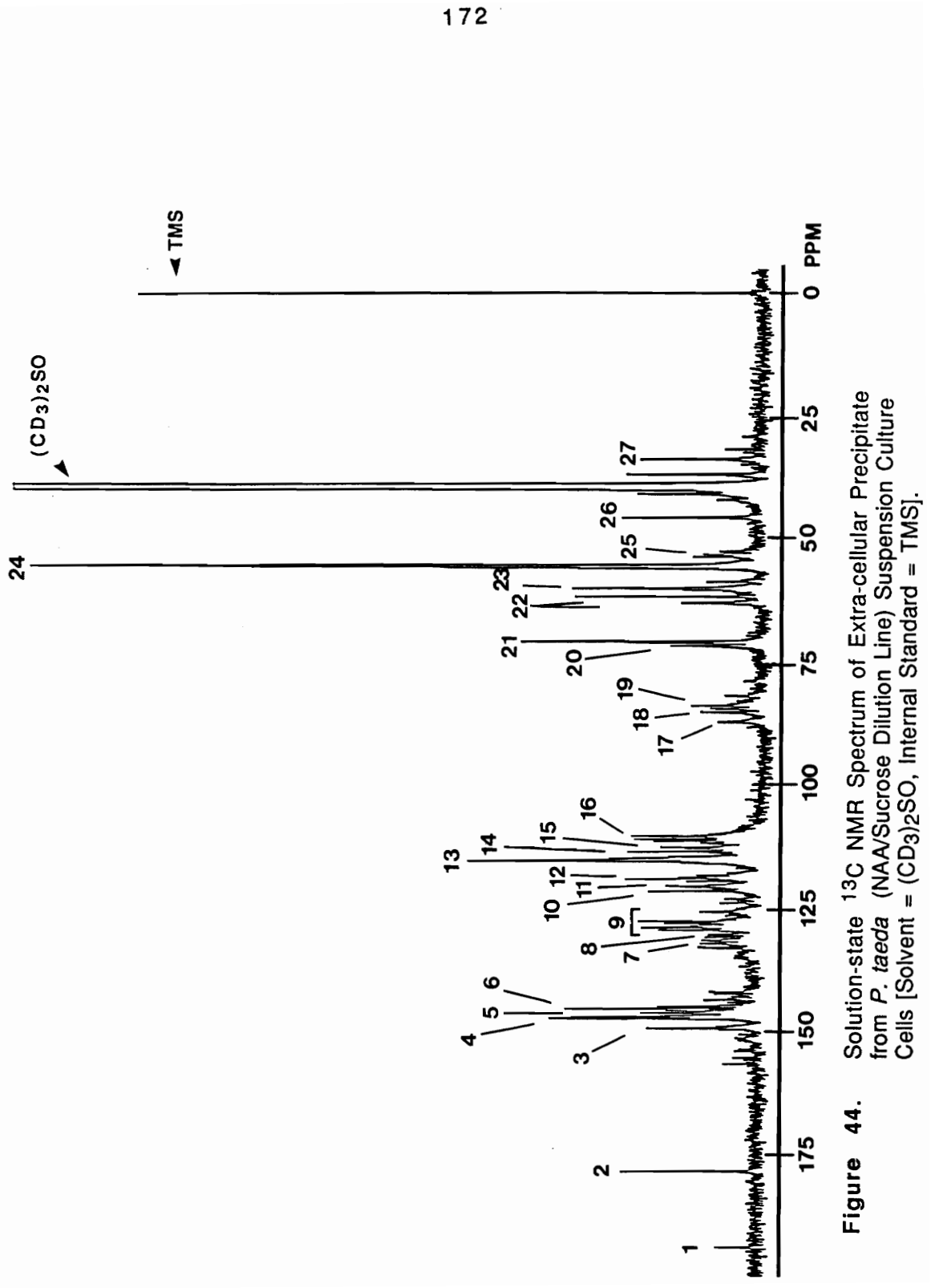

Figure 44. Solution-state ^{13}C NMR Spectrum of Extra-cellular Precipitate from *P. taeda* (NAA/Sucrose Dilution Line) Suspension Culture Cells [Solvent = $(CD_3)_2SO$, Internal Standard = TMS].

Table 4

Assignment of ^{13}C NMR Signals of Extracellular Precipitate from *P. taeda* (Sucrose Dilution/NAA Line) Suspension Culture Cells

Signal Number	ppm/TMS	Assignments*1
1	194.1	ketonic α-CO
2	178.4	-
3	149.5	C-3 in G etherified
4	147.4	C-4 in G etherified
5	146.3	C-3 in G non-eterified
6	145.4	C-4 in G non-eterified
7	133.3, 133.0, 132.5	C-1 in G with C-4 OH
8	131.4	C-2 and C-6 in H
9	127.5-129.4	CH=CH in ArCH=CH-
10	121.5	-
11	120.4	C-1 in H
12	118.9	C-6 in G
13	115.1	C-5 in G, C-3 and C-5 in H
14	113.3	-
15	112.2	-
16	110.8-111.2	C-2 in G
17	86.9	-
18	85.0, 84.3	C-β in β-O-4
19	83.6	C-β in β-O-4 with α-CO
20	71.5	C-α in G
21	70.8	C-γ in β-β
22	62.8, 61.6	C-γ in β-O-4 with α-C=O, C-γ in β-5
23	59.9	C-γ in β-O-4
24	55.4	OCH_3
25	53.1,52.9	C-β in β-β
26	45.5	-
27	28.9	CH_3 and CH_2 in saturated chain

*1: See Figure 18 in Introduction.

the subsequent observation of a resonance at 178 ppm in the solid and solution-state ^{13}C NMR spectra of the [1-^{13}C] enriched cell walls and lignin derivative, respectively. Therefore, not only does the extracellular precipitate show a strong similarity to a milled wood lignin but also an interesting feature of the lignin component in the cell walls of *P. taeda* suspension cultures. Accordingly, it is possible that this extracellular precipitate may be formed within or in close association with the cell walls and then released into the medium. The exact process by which this precipitate appears warrants further investigation. The possibility that this material is formed within the medium itself cannot be excluded; as described earlier, *P. taeda* (2,4-D line) cells release peroxidases into the medium which may be capable of polymerizing low molecular weight phenolics presuming H_2O_2 is also present.

Despite its similarity to isolated lignins, this material is not an insoluble component of the cell wall. Since lignin is a cell wall polymer, the use of the term lignin to describe this material is incorrect. Analyses of such precipitates do not provide any greater insight into the process of lignin deposition occurring along with cell wall thickening. Under harsh culture conditions the *P. taeda* suspension culture possessed a similar capability to produce an extracellular precipitate comparable to that reported for *Picea abies* suspension cultures. In no way does the analysis of this material provide insight into the nature of the lignin *in situ* as well as described previously. It remains to be seen if *Picea abies* cultures could be induced to undergo cell wall thickening and lignification under the culture conditions developed for the *P. taeda* (NAA line) suspension culture cells.

5. CONCLUDING REMARKS

In an effort to gain a better understanding of the process of lignification during the early stages of cell wall formation, a model system was developed. In this model, *Pinus taeda* L. suspension cultures, maintained in medium containing 2,4-D as a growth regulator, were employed since they provided a large population of relatively homogeneous cells with primary cell walls. These cells were capable of only minimal lignification as demonstrated by biochemical and chemical analyses. Significant amounts of cell wall proteins present presumably served as structural components. Proof of phenylpropanoid metabolism was provided by the isolation and identification of a lignan, (-)-matairesinol **38,** in methanol extracts; this represents the first demonstration of the occurrence of this enantiomerically pure lignan in *P. taeda.* These results are especially interesting in relation to the immunogold localization of phenylalanine ammonia-lyase in specific sites in the cell. The activity for this enzyme at such sites is likely involved in the ability of the plant cells to regulate the flow of carbon into proteins, lignins and lignans.

Through the maintenance of the suspension culture cells on an alternative growth regulator, NAA **37**, cell wall thickening and lignification were induced. Observations by transmission electron microscopy indicated cell wall thicknesses consistent with primary and secondary (S_1) cell wall layers. Tentative evidence of lignification was shown by histochemical staining of these cells with phloroglucinol-HCl. Active phenylpropanoid metabolism was suggested by easily detectable levels of phenylalanine ammonia-lyase and cinnamyl alcohol dehydrogenase activities. Further evidence for lignification in the *P. taeda* (NAA line) cells was provided by solid-state ^{13}C NMR spectroscopy of extractive-free cell walls. This was supported by chemical analyses where nitrobenzene oxidations

and thioacidolyses gave guaiacyl:*p*-hydroxyphenyl ratios and yields more consistent with that observed in softwood extractive-free wood meal. Efforts to quantify the presumed lignin component by the acetyl bromide and Klason methods were proven to be erroneous for these tissues when considering the results obtained by solid-state ^{13}C NMR spectroscopy, nitrobenzene oxidation and thioacidolysis. Critical evidence, needed to demonstrate that these cells were lignifying, was provided by the identification of lignin bonding patterns *in situ* by solid-state ^{13}C NMR spectroscopy. This was accomplished by administration of [1-^{13}C], [2-^{13}C] and [3-^{13}C] phenylalanines **9** to *P. taeda* (NAA line) cells thereby allowing the specific labelling of carbons in the propane side chains of lignin. These experiments represent the first demonstration of lignin bonding patterns *in situ* for a softwood species as well as a plant culture. Assignments for resonances in the solid-state were confirmed in the solution-state ^{13}C NMR spectra of lignin derivatives isolated from each sample of ^{13}C specifically labelled cell walls. Although comparable to guaiacyl milled wood lignin, an extracellular "lignin" from the *P. taeda* (sucrose dilution/NAA line) cells was not a component of the cell wall and therefore could not provide insight into the nature of lignin during cell wall maturation processes.

Through these experiments, the demonstration of active phenylpropanoid metabolism in the *P. taeda* (2,4-D line) suspension cultures resulting in (-)-matairesinol **38** biosynthesis provides a potentially useful system to explore lignan biosynthesis and metabolism. Moreover, having met all the objectives of this study, a model system is now available to explore the early stages of lignification during cell wall thickening. Future work with this culture system can undoubtedly provide insight into the signals involved in the induction of lignification as well as changes to the macromolecule which may occur during its deposition in the developing plant cell wall.

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VITA

Thomas Leonard Eberhardt was born in Madison, Wisconsin on July 19, 1963. He enrolled in the forestry program at the University of Wisconsin and earned his B.S. degree in 1985. While at the university his expenses were covered, in part, by four Vicky Lee Hirsh Scholarships for Conservation and a Daniel Boone Conservation League Scholarship. During the summer between his 3rd and 4th years at the university he interned as an assistant forester with the Wisconsin Department of Natural Resources. In 1985, he enrolled in the Master of Science Program in the Department of Wood Science and Forest Products at VPI & SU in Blacksburg, VA. After completing his M.S. degree requirements in 1988 he continued in the department towards his Ph.D. In 1990, he assisted his advisor's transfer to Washington State University. While in Washington, he found great fulfillment in his marriage to Dawn Elizabeth Spencer. After completing his research at WSU, he returned to VPI & SU to defend his dissertation on March 27, 1992.