Biochemical Aspects of Lignification and Heartwood Formation*

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Abstract——Metabolic differences in the formation of gymnosperm- and angiosperm lignins were explained in terms of the different functions of *O*-methyltransferase and reducting enzymes which participate in methylation and reduction of hydroxycinnamic acid intermediates in biosynthetic pathway of these two types of lignins. Chemical properties of dehydrogenation polymer (DHP) of sinapyl alcohol were characterized and possible occurrence of syringyl lignins in hardwoods was discussed. DHP and dimers of *p*-coumaryl alcohol were also characterized and discussed in relation to the formation of grass lignin. Heartwood formation was explained in terms of metabolic changes in ray parenchyma cells in transition wood and the biosynthetic site of heartwood extractives was elucidated.

Introduction

It has been established that lignification is initiated in the differentiated wood cells from the primary walls adjacent to the cell corner, and then extended to the intercellular layer, primary wall and secondary wall, and that the average lignin concentration in the compound middle lamella of completely lignified cell walls of spruce tracheids is about twice that in the secondary walls[1,2].

Wood cells in the sapwood are generally lignified and are dead except ray parenchyma cells participating in transverse translocation of metabolic products such as sugars and amino acids in leaves and cambium to the inner sapwood. The ray parenchyma cells in the sapwood are dead when trees are of certain ages which may be determined by physiological conditions and tree species, and heartwood formation is initiated from the center of the wood. Both lignification and heartwood formation are characteristic features observed during aging processes in the growing tree. While lignification or lignin biosynthesis generally occurs in all the woody cells, the formation of the heartwood extractives seems to be limited to the ray parenchyma cells, and there are several peculiar differences in both processes from the biochemical point of view.

1. Lignification

It is well known that lignins are polymers which are generally classified to three groups based on their structural monomer units. While gymnosperm lignins

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are composed of guaiacylpropanol and give mainly vanillin (V) in nitrobenzene oxidation, angiosperm lignins which are composed of approximately equal amounts of guaiacyl- and syringylpropanols yield vanillin and syringaldehyde (S), and grass lignins whose structural monomers are guaiacylpropanol, syringylpropanol and *p*-hydroxyphenylpropanol give significant amounts of *p*-hydroxybenzaldehyde (P) together with these two aldehydes. In addition, grass lignins contain about 10 % of *p*-coumaric acid connected through an ester linkage.

It is known[3] that these lignins are synthesized from L-phenylalanine and cinnamic acids which are formed through shikimic and cinnamic acid pathways as shown in Fig. 1. L-Phenylalnine is converted to *trans*-cinnamic acid catalyzed by phenylalanine ammonia-lyase (PAL), which is a key enzyme in the synthesis of phenolic compounds, and widely distributed in higher plants. A taxonomic survey of the distribution of the ammonia-lyase has shown that the enzyme is present only in organisms which can form lignin or some cinnamic acid derivatives[4,5]. The

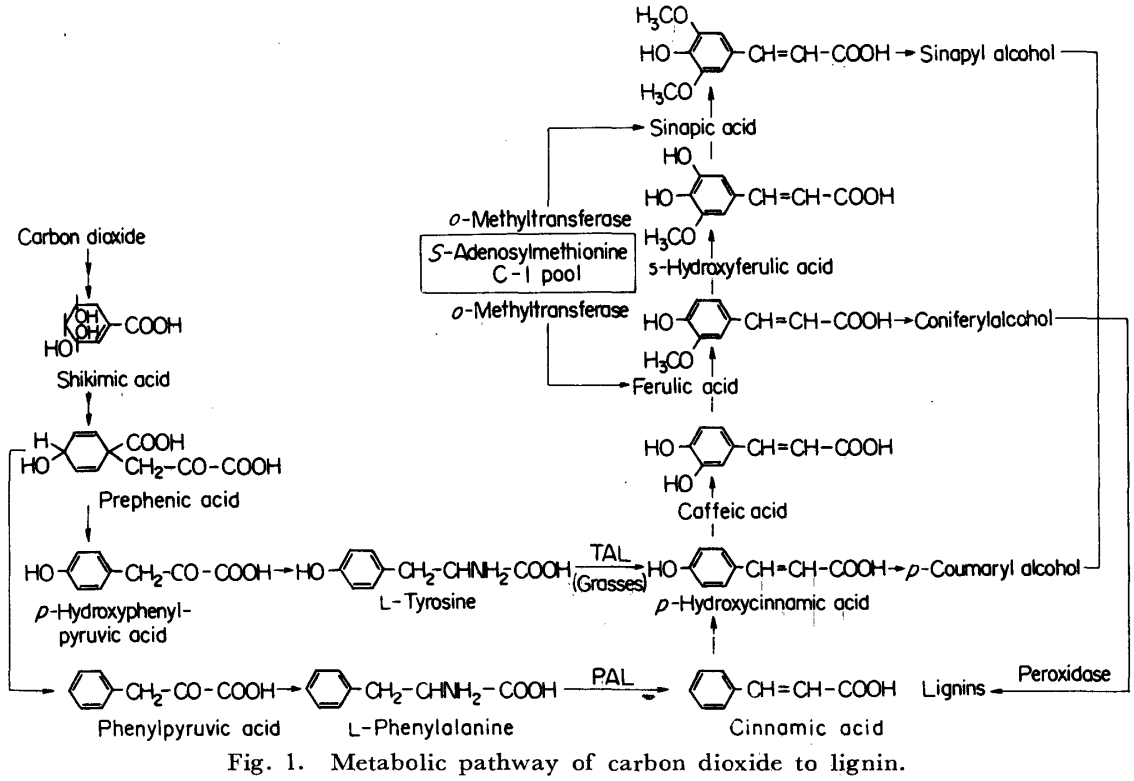


Fig. 1. Metabolic pathway of carbon dioxide to lignin.

enzyme activity is scarcely detected in plant tissues during protein and polysaccharide syntheses in growth but increases drastically during lignification of secondary wall formation[6,7] (Fig. 2).

Cinnamic acid thus formed is hydroxylated to *p*-coumaric acid with cinnamate-4-hydroxylase which was isolated from pea and sorghum seedlings and characterized to be a P-450 type enzyme by RUSSEL and CONN[8]. Tyrosine ammonia-lyase (TAL) which catalyzes the formation of *p*-coumaric acid from L-tyrosine is characteristically found in grasses. The distribution of the enzyme is limited to grasses and L-tyrosine-^{14}C which is not converted to lignins in both gymnosperms and angiosperms is efficiently transformed by grasses to the *p*-hydroxyphenyl, guaiacyl and syringyl moieties of lignin polymer and also to the esterified *p*-coumaric acid of the lignin[9]. *p*-Coumaric acid is subsequently converted to ferulic acid through caffeic acid. The conversion of caffeic acid to ferulic acid is catalyzed by *O*-methyltransferase which was first characterized by FINKLE et al.[10,11] from an apple tree and a pampass grass.

Since tracer studies have shown that ferulic acid as well as intermediate acids in the shikimic and cinnamic acid pathways are efficiently incorporated into not only guaiacyl lignins of gymnosperms but also both guaiacyl- and syringyl compo-

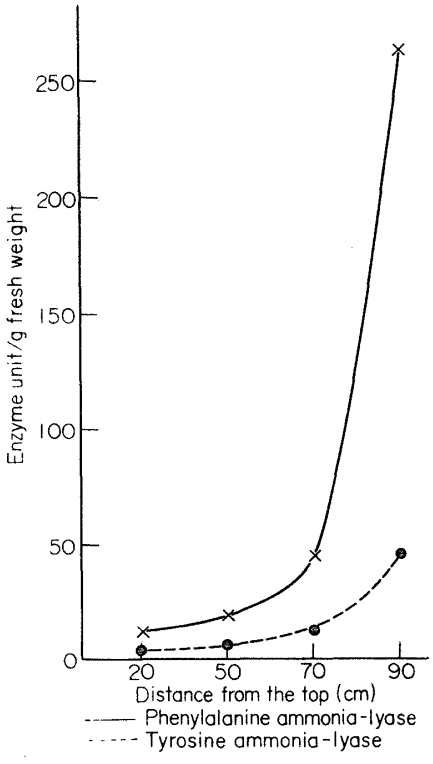


Fig. 2. Activities of phenylalanine- and tyrosine ammonia-lyases of a bamboo shoot (length, 0.9 M).

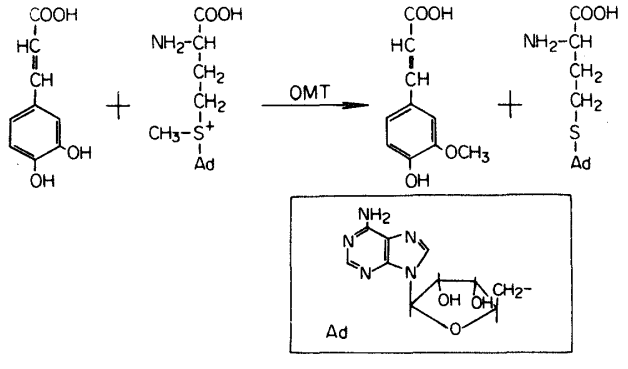


Fig. 3. Methylation of caffeic acid by *O*-methyltransferase.

nents of angiosperm lignins[3], the synthetic pathway up to ferulic acid from sugars is believed to be common in both gymnosperms and angiosperms.

Metabolic differences in the formation of gymnosperm- and angiosperm lignins

If it were the case, why can only angiosperms synthesize syringyl lignin together with guaiacyl lignin? These problems have been elucidated by our group. In the begining, attempts to isolate *O*-methyltransferase (OMT) from bamboo[12,13] and poplar shoots[14] were made and it was found that the enzymes from these angiosperms mediated the formation of both ferulic acid (FA) and sinapic acid (SA) from caffeic and 5-hydroxyferulic acids, respectively, in the presence of *S*-adenosylmethionine as methyl donor as shown in Fig. 3.

The ratio of both acids formed (SA/FA) were found to be constant and greater than 1 which may be determined genetically by tree species. 5-Hydroxyvanillin, protocatechuic aldehyde, 3, 4, 5-trihydroxycinnamic acid and chlorogenic acid were also good substrates. Interestingly, the gymnosperm OMT's which were isolated by our group for the first time from seedlings of Japanese black pine[15] and ginkgo shoots[16] had completely different substrate specificity from those of angiosperm ones, and caffeic acid was the most favorable substrate to any other phenolics tested. Protocatechuic aldehyde and 3, 4-dihydroxyphenylacetic acid served as fairly good substrates but surprisingly 5-hydroxyferulic acid was only slightly converted to sinapic

Table 1. Substrate specificity of various plant OMT's.

Substrate	Relative methylation (%)		
	Pine	Poplar	Bamboo
Caffeic acid	100	100	100
5-Hydroxyferulic acid	10	320	124
3, 4, 5-Trihydroxycinnamic acid	25	60	50
Chlorogenic acid	10	46	3
iso-Ferulic acid	0	0	5
m-Coumaric acid	0	0	0
p-Coumaric acid	0	0	0
3, 4-Dihydroxyphenyl acetic acid	54	0	0
3, 4-Dihydroxymandelic acid	0	0	0
Protocatechuic aldehyde	68	46	45
5-Hydroxyvanillin	0	190	59
Protocatechuic acid	20	0	28
Gallic acid	0	0	0
Pyrocatechol	3	30	0
Pinosylvin	10	—	—
D-Catechin	0	0	0
Catechylglycerol-β-guaiacyl ether	0	—	—

acid by the enzymes. Catechylglycerol-β-guaiacyl ether which was used as a dimeric lignin model compound was not methylated by the black pine OMT suggesting that the methylation of lignin should take place at the stage of hydroxycinnamic acid monomers prior to polymerization of coniferyl and sinapyl alcohols (Table 1).

Further evidence for the differences in *O*-methylation pattern between pine and bamboo OMT's are shown in Fig. 4. In the reaction mixture containing both caffeic and 5-hydroxyferulic acids with *S*-adenosylmethionine, pine OMT gave constant but a very low SA/FA ratio regardless of the length of incubation time but when bamboo OMT was added into the reaction mixture at the time pointed in Fig. 4, sinapic acid was newly formed in parallel with formation of ferulic acid resulting a greater SA/FA ratio. Because gymnosperm OMT's little methylated 5-hydroxyferulic acid but did caffeic acid, we named gymnosperm OMT monofunction OMT[14]. On the other hand, the angiosperm OMT's mediated the formation of both ferulic and sinapic acids, gave greater SA/FA ratios than 1 and was named difunction OMT.

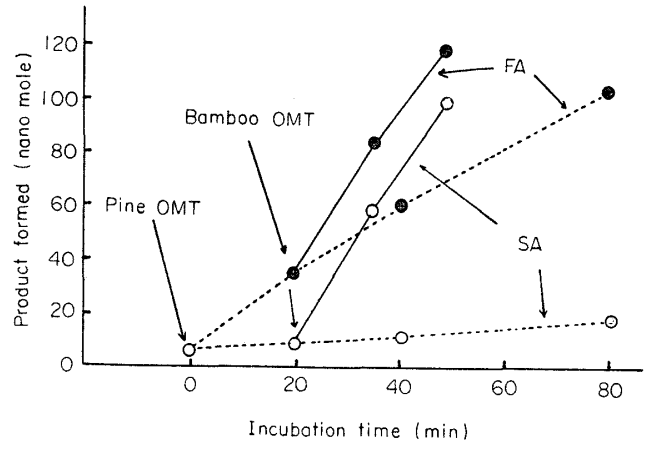


Fig. 4. Difference in *O*-methylation patterns between pine- and bamboo OMT's.

The SA/FA ratio of bamboo OMT remained constant during purification by ammonium sulfate precipitation, chromatography on DEAE cellulose, Sephadex gel filtration and analyses by polyacrylamide gel electrophoresis as shown in Table 2. These results definitely show that the two methylating activities for FA and SA formation belong to a single enzyme protein. LINWEAVER BURK plots for the methylation of caffeic acid to FA in the presence and absence of 5-hydroxyferulic acid (10^{-4} M) gave a typical competitive pattern, showing that the FA formation was greatly inhibited by 5-hydroxyferulic acid[17]. Since Km values for caffeic and 5-hydroxyferulic acids were found to be 5×10^{-5} M and 1×10^{-5} M, respectively in excess *S*-adenosyl methionine, the preference of the latter in the formation of an enzyme-substrate complex shows that a feedback control of FA production is possibly regulated by

Table 2. Purification of *O*-methyltransferase from a bamboo shoot.

Purification procedure	Total protein (mg)	Specific activity (unit/mg)		Recovery (%)		SA/FA ratio	Purification
		FA	SA	FA	SA		
0~70% $(NH_4)_2SO_4$	2900	2.06	2.52	100	100	1.22	1
20~55% $(NH_4)_2SO_4$	1300	5.3	6.8	118	118	1.26	3
DEAE-cellulose	118	45.6	58.3	92	93	1.25	23
Sephadex G-200	65	57.5	64.0	63	57	1.11	28
DEAE-cellulose	1.5	200.0	244.0	5	5	1.22	97

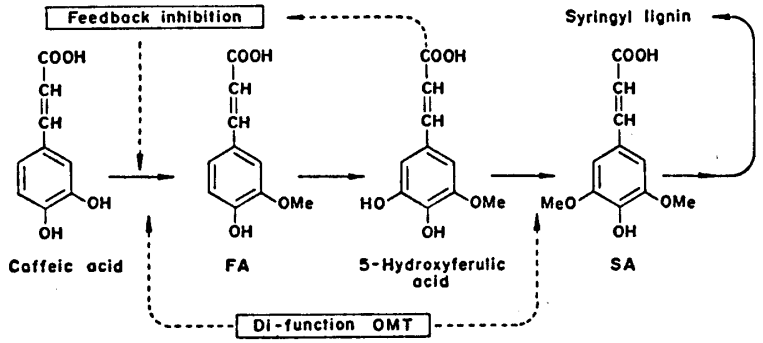


Fig. 5. The possible function of feedback control in biosynthesis of angiosperm lignin.

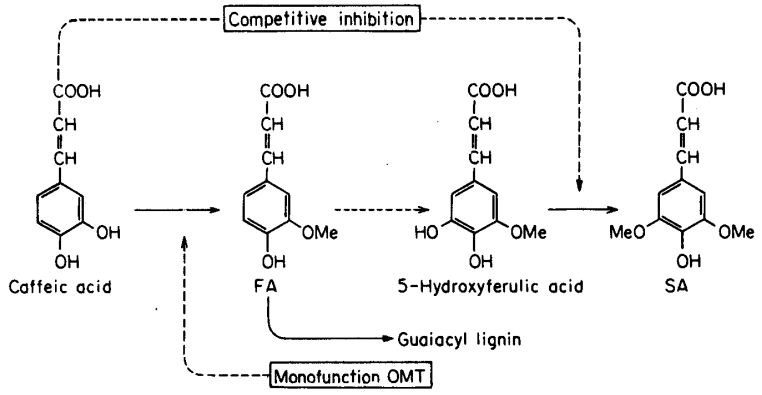


Fig. 6. Preferential formation of guaiacyl lignin in conifers.

varying pool size of 5-hydroxyferulic acid as shown in Fig. 5. The purified OMT from black pine seedlings[18], on the other hand, mainly catalyzed the formation of FA, although SA formation was slightly catalyzed. Km values for FA and SA were 5.1×10^{-5} M and 2.8×10^{-4} M, respectively and the SA formation was competitively inhibited by caffeic acid, showing preferential formation of guaiacyl lignin in conifers as shown in Fig. 6.

Since it has been established that methoxylation of the lignins occurs at the hydroxycinnamic acid stage it seems that the substrate specificity of the OMT gives

a reasonable explanation for the reason why only angiosperms can synthesize syringyl component of the lignins and gymnosperm can not. However, it has been found that some of the gymnosperms such as *Ephedra, Gnetum* and *Podocarpus* give a positive Mäule reaction due to syringyl groups, whereas an angiosperm, *Chloranthus,* which has no vessels in wood and is regarded as a primitive type, gives a negative Mäule reaction. It is suggested that the former plants might have difunction OMT but the latter plant monofunction OMT.

Table 3. Relationship between the SA/FA ratio, the S/V ratio and Mäule reaction.

	SA/FA (OMT)	S/V (lignin)	Mäule reaction
Pteridophyta			
Psilotum nudum	0.2	0	−
Angiopteris lygodifolia	0.3	—	−
Gymnospermae			
Ginkgo biloba	0.1	0	−
Pinus thunbergii	0.1	0	−
Podocarpus macrophylla	0.0	0	−
Taxus cuspidata	0.3	0	−
Angiospermae (Dicotyledoneae)			
Magnolia grandiflora	3.0	2.2	+
Cercidiphyllum japonicum	3.2	2.9	+
Populus nigra	3.0	2.0	+
Pueraria thunbergiana	2.5	—	+
Angiospermae (Monocotyledoneae)			
Oryza sativa	0.9	—	+
Triticum aestivum	1.0	1.0	+
Phyllostachys pubescens	1.3	1.1	+

The relationship between the SA/FA ratio, S/V ratio in nitrobenzene oxidation of the lignins, and the Mäule reaction are shown in Table 3. The SA/FA ratios of bamboo and poplar OMT (angiosperm) are 1.3 and 3.0, respectively, whereas the ratios of both ginkgo and pine OMT (gymnosperm) are less than 0.1.

The finding that the plants having higher SA/FA ratios give the greater S/V ratios or *vice versa* is very indicative of an intimate correlation between the distribution of guaiacyl- and syringyl lignins and that of the two different types of OMT's.

However, 5-hydroxyferulic acid which has been used as a substrate for SA formation is a synthetic compound and the presence of the acid and ferulate-5-hydroxylase in plants have not hitherto been found. So, several problems around sinapic acid metabolism are still remained to be settled.

Reducing enzymes for p-hydroxycinnamic acids

The present author suggested that coniferyl alcohol is formed from ferulic acid through coniferyl aldehyde based on the fact that ferulic acid-2-^{14}C was reduced to the corresponding aldehyde and alcohol in wheat plants and cultured tissues of strob pine[19]. ZENK et al.[20~22] and GRISEBACH group[23,24] recently succeeded in isolation and characterization of three enzymes which participate in the reduction of ferulic acid to coniferyl alcohol through coniferyl aldehyde from cambial zones of young stems of *Salix* and *Forsythia* plants, and cell suspension cultures of soybean, respectively. These enzymes, hydroxycinnamate: CoA ligase, hydroxycinnamate CoA reductase and hydroxycinnamaldehyde reductase were found to be specific for hydroxycinnamate and to distribute widely in differentiating woody tissues. Then it is conceivable that these enzymes participate in the formation of coniferyl, sinapyl and *p*-coumaryl alcohols as lignin precursors. However, it has been found that the hydroxycinnamate: CoA ligase from *Forsythia* does not mediate the formation of sinapyl CoA from sinapic acid although feruloyl- and *p*-coumaryl CoA's are readily formed by the enzyme.

Our recent investigation[25] has indicated that sliced xylem tissues from shoots of poplar and cherry reduce ferulic and sinapic acids to the corresponding aldehydes and alcohols, while less differentiated xylems and callus tissues of angiosperms reduce ferulic acid but their ability to reduce sinapic acid is markedly lower than that of the fully differentiated xylem. Gymnosperms such as Japanese red pine and ginkgo, on the other hand, reduced only ferulic acid regardless of differentiation stages and not sinapic acid. This finding seems to indicate that the formation of sinapyl CoA ligase in plant tissues is intimately related to the differentiation and lignification of plant tissues in angiosperms.

We have found[4] that the lignins of angiosperm callus tissues are similar to conifer

Table 4. The relationship between the SA/FA ratio and the S/V ratio in angiosperm callus tissues.

Plant materials	Incubation time (min)	Products (nano mole)		Ratio (SA/FA)	Ratio (S/V)
		FA	SA		
Salix caprea	0	0	0	—	0.10
	30	20	35	1.8	
	60	35	53	1.5	
	90	40	64	1.6	
Morus bombycis	30	17	27	1.6	0.12
	60	27	50	1.9	
	90	35	61	1.7	

lignin in many respects and give very low S/V ratios in nitrobenzene oxidation. However, as shown in Table 4 the OMT's from the callus tissues (*Salix caprea* and *Morus bombycis*) gave greater SA/FA ratios as compared with the gymnosperm ones[26] suggesting that the lowering of the S/V ratio in the callus tissues should not be due to deletion of SA-activity of OMT but to that of other enzymes such as FA-5-hydroxylase and hydroxycinnamate: CoA ligase, which are supposed to be formed in differentiating tissues. If the level of FA-5-hydroxylase and hydroxycinnamate: CoA ligase in the callus tissues were as high as its differentiated xylem tissues, ferulic acid might be efficiently converted to 5-hydroxyferulic acid which might be then incorporated into syringyl lignin after conversion to sinapyl alcohol by the mediations of difunction OMT and then reducing enzymes involved.

Substrate specificities of hydroxycinnamaldehyde reductases are scarcely different between gymnosperms and angiosperms[25], and both classes reduced coniferyl and sinapyl aldehydes to the corresponding alcohols using NADPH as a co-factor, and in agreement with these findings sinapyl aldehyde and sinapyl alcohol, when fed to living plants and tissue cultures of gymnosperms were shown to be readily converted to syringyl lignin which was not originally present, as shown in Fig. 7.

It has been thus established that synthetic pathways of lignin in gymnosperms and angiosperms are distinguished at the stage of methylation and formation of hydroxycinnamaldehydes from the corresponding acids, and that the reduction

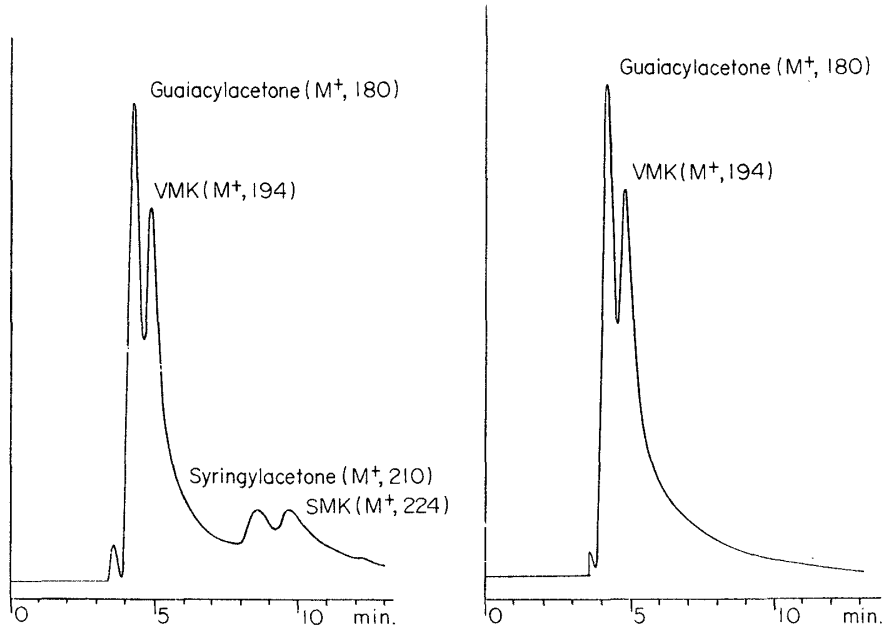


Fig. 7. GC-MS spectrometry of acidolysis products of gymnosperm tissues. right: control, left: sinapyl alcohol fed. (3% SE-52 on chromosorb W, 2 m glass column, 215°C).

stages of hydroxycinnamaldehydes are common between both classes. It seems that in grasses, the pool of *p*-coumaric acid which is formed by mediation of tyrosine ammonia-lyase is relatively large, and the acid is converted to *p*-coumaryl alcohol as an additional lignin monomer and to the esterified *p*-coumaric acid through *p*-coumaryl CoA by mediation of hydroxycinnamate: CoA ligase.

Dehydrogenation of lignin monomers with peroxidase and H_2O_2

Peroxidase is widely distributed in plant tissues and has been characterized in detail. However, physiological role of the enzyme in plants has not been established. Our investigations[27,28] and a recent supplementary investigation by HARKIN[29] have shown that the peroxidase is unequivocally responsible for dehydrogenative polymerization of lignin monomers in plants. According to FREUDENBERG[30] hydroxycinnamyl alcohols are dehydrogenated to their phenoxy radicals by mediation of a fungal laccase, and the radicals are coupled each other without any enzymic control and polymerized to a dehydrogenation polymer (DHP) which is believed to be

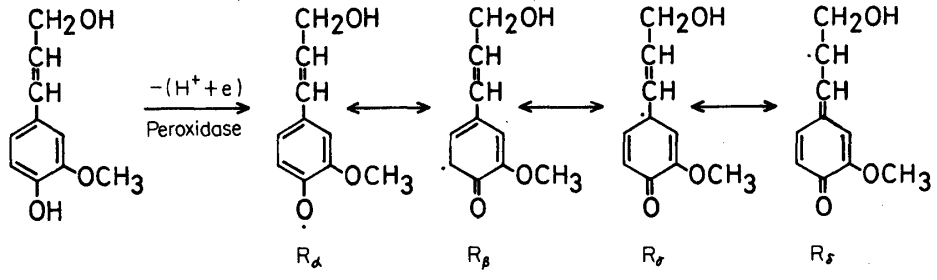
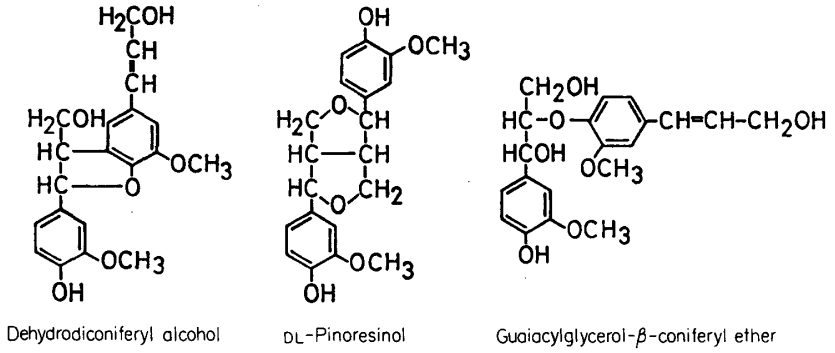
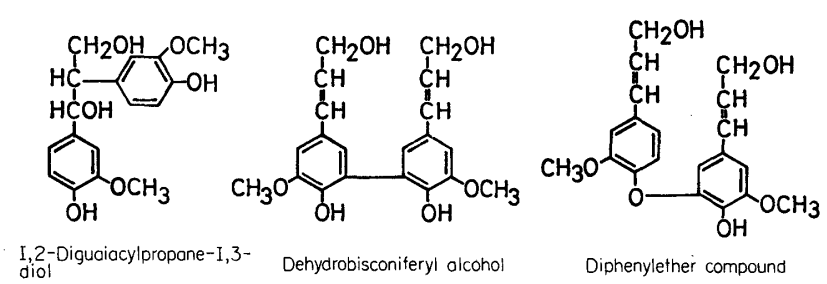


Fig. 8. Dehydrogenation of coniferyl alcohol by peroxidase and H_2O_2.





a lignin as shown in Fig. 8. A similar reaction is mediated with plant peroxidase and H_2O_2 instead of the fungal laccase and oxygen. In this reaction the couplings of $R\beta+R\delta$, $R\delta+R\delta$ and $R\alpha+R\delta$ are preferential, and large amounts of dehydrodiconiferyl alcohol (54 %), DL-pinoresinol (27 %) and guaiacylglycerol-β-coniferyl ether (19 %) were found to be formed. The amounts of 1, 2-diguaiacylpropane-1, 3-diol and dehydrobisconiferyl alcohol were quite small, and diphenyl ether compound was not detected.

However, since considerable amounts of biphenyl- and diphenylether compounds have been isolated from degradation products of lignins, the couplings of $R\beta+R\beta$ and $R\alpha+R\beta$ are supposed to occur in later stages of dehydrogenation, the reactions in which may proceed mainly in oligomers with saturated side chains.

Guaiacyl- and syringylglycerols which have been isolated as lignin degradation products were found to be formed as dehydrogenation products of the corresponding cinnamyl alcohols by our group[31].

FREUDENBERG[30] reported that a mixture of coniferyl- and sinapyl alcohols in equal amounts is dehydrogenated to a polymer similar to angiosperm lignin, but sinapyl alcohol alone does not form a lignin-like polymer but yields mainly syrin-

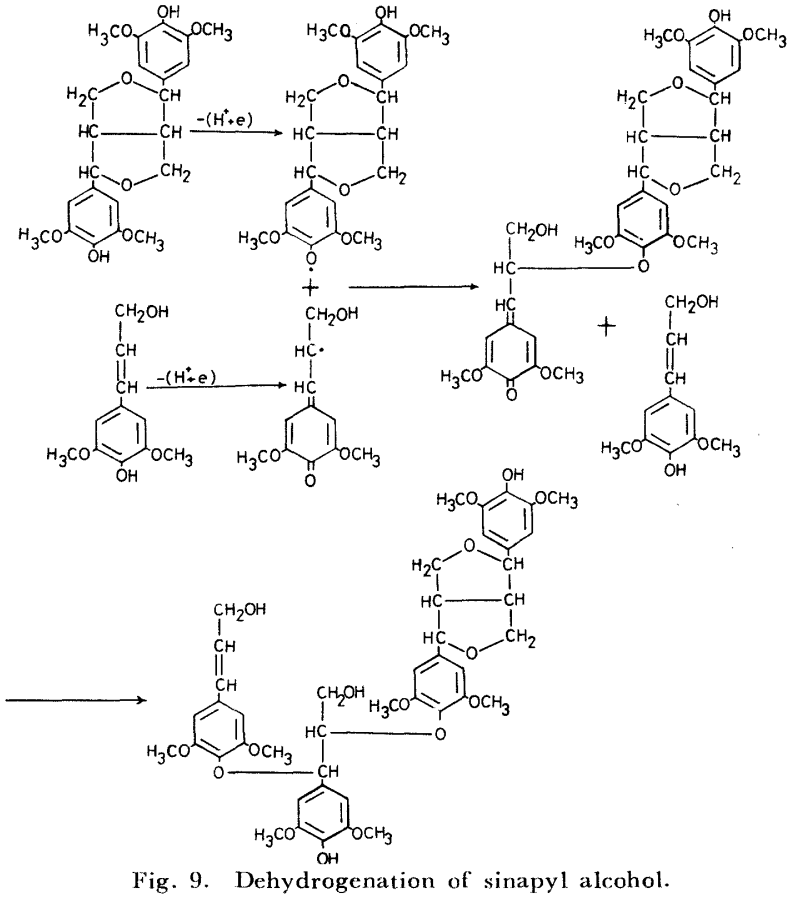


Fig. 9. Dehydrogenation of sinapyl alcohol.

garesinol, and dimethoxybenzoquinone together with other degradation products in prolonged dehydrogenation reaction. From these results FREUDENBERG doubted the occurrence of syringyl lignin in nature. However, FERGUS and GORING[32] proposed on the basis of spectral analysis of lignin in cell walls with UV microscope that birch lignin deposited in the secondary layers of wood fibers and parenchyma cell walls is composed of mostly syringyl component. We[33] recently found that considerable amounts of lignin-like polymers were formed from sinapyl alcohol alone with peroxidase and H_2O_2. UV, IR and ^{13}C-NMR spectra of the polymer showed characteristic features of syringyl lignin, and acidolysis of the polymer gave typical HIBBERT's ketones such as ω-hydroxysyringylacetone in 16 % yields indicating the occurrence of considerable amounts of β-O-4 linkage which is the most important structural unit in growing of lignin polymers. The polymer and its methylated product gave 20 % of syringaldehyde and 16 % of trimethylgallic acid by nitrobenzene oxidation and permanganate-hydrogen peroxide oxidation, respectively. The result therefore clearly shows that the phenoxy radicals formed enzymically are coupled not only by Cβ-Cβ to form syringaresinol but also by Cβ-O-4 to make growth of syringyl lignin through syringylglycerol-β-sinapyl ether as shown in Fig. 9. We[34] have further found that the polymerization pattern of sinapyl alcohol is effected by the solvent used, and when sinapyl alcohol is dehydrogenated with ferric chloride in each of dioxane and acetone-H_2O the alcohol is converted mainly to syringylglycerol-β-sinapyl ether and syringaresinol, respectively. These resutls suggest that the formation of syringyl lignins in hardwoods are effected by physico-chemical factors such as solvent systems which promote the coupling of Rα with Rδ radicals of sinapyl alcohol in the cell walls.

We have investigated the dehydrogenation of *p*-coumaryl alcohol in relation to the lignification of bamboo. The DHP of *p*-coumaryl alcohol gave *p*-hydroxybenzaldehyde and acidolysis monomers in about the same yields as vanillin and the corresponding acidolysis monomers from the DHP of coniferyl alcohol, and that the methylated DHP of *p*-coumaryl alcohol gave *p*-anisic-, 4-methoxyisophtalic- and 5, 5′-dehydrodianisic acids in the ratio of 1 : 0.26 : 0.41 which corresponded completely to the ratio of veratric-, isohemipinic- and 5, 5′-dehydrodiveratric acids from that of coniferyl alcohol in permanganate-hydrogen peroxide oxidation[35]. Our recent investigation[36] has indicated that the ratio of dilignols of *p*-coumaryl alcohol such as dehydrodi-*p*-coumaryl alcohol, *p*-coumarylresinol and *p*-hydroxyphenylglycerol-β-*p*-coumaryl ether formed by enzymic dehydrogenation is quite similar to that of dilignols of coniferyl alcohol obtained by a similar experiment as shown in Fig. 10.

We have also found that peroxidases from Japanese black pine seedlings and mulberry callus tissues show a similar substrate specificity for guaiacyl- and syringyl

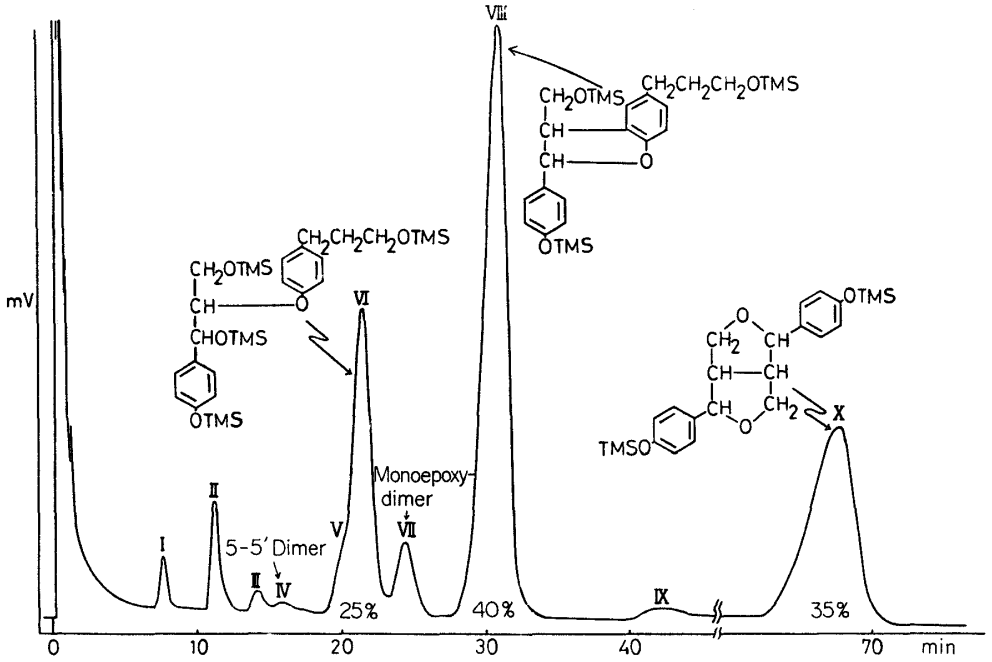


Fig. 10. Gas chromatogram of TMS-derivatives of hydrogenated dimeric compounds obtained by dehydrogenation of *p*-coumaryl alcohol with H_2O_2/peroxidase. (2 % OV-17 on Chromosorb AW, 2 m, glass column, 220°C).

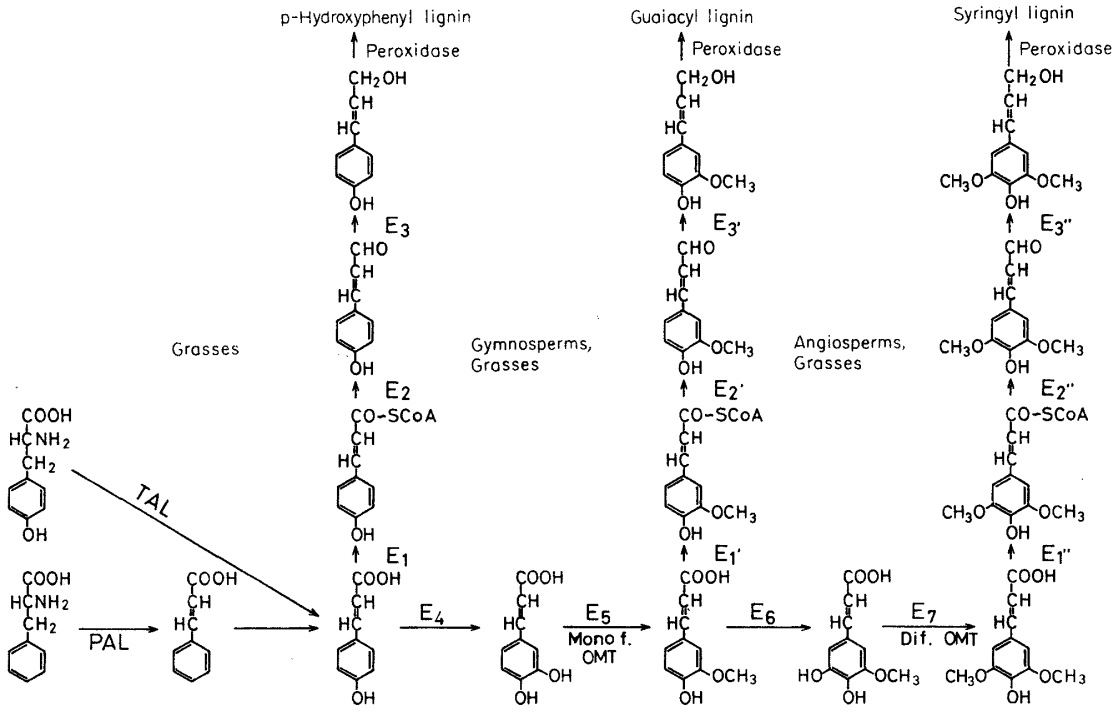


Fig. 11. Differences in lignin metabolism between gymnosperms and angiosperms.

compounds and that the origin of peroxidases is not an important factor controlling preferential formation of syringyl lignin in hardwoods[25]. However, SIEGEL found[37] that the substrate specificities of peroxidases are different in algae and that the enzymes from green algae can catalyze the oxidation of lignin monomers but those from red- and brown algae can not, in harmony with the idea that land plants originated from a progenitor derived from a green alga.

In conclusion, the differences in lignin metabolism between gymnosperms and angiosperms including grasses should be explained in terms of the different levels of the key enzymes such as mono- and difunction OMT's, FA-5-hydroxylase and hydroxycinnamate CoA ligase as shown in Fig. 11. Activities of all enzymes in the figure are known to be increased during differentiation of tissues. The activities of E6 and E1'' which are both specific for angiosperms and not found in gymnosperms are believed to especially depend on differentiation stages of the tissues. The activities of E6 and E1' are supposed to be equilibrated in differentiating angiosperm tissues resulting in the formation of almost equal amounts of guaiacyl- and syringyl lignins. While in grasses the pool of *p*-coumaric acid is considerably large, the reaction mediated by E4 might be saturated and parts of the acid are converted to *p*-coumaryl alcohol by E1 and also to the esterified *p*-coumaric acid which is a characteristic of grass lignins.

2. Heartwood formation

Changes in the metabolism in ray parenchyma in transition wood

Ray cells in the sapwood are alive and they contain generally starch granules and/or oil droplets. It is conceivable that these compounds together with sugars translocated from leaves are converted to heartwood extractives such as stilbene, flavonoids and terpenoids via intermediate compounds in glycolysis, TCA cycle and pentosephosphate pathway as shown in Fig. 12. Acetyl CoA (malonyl CoA) formed from pyruvic acid or fatty acid by β-oxidation is used for the synthesis of both terpenoids via mevalonic acid and A ring of flavonoids. Alternatively, glucose-6-phosphate is metabolized to the B rings of both stilbenes and flavonoids as heartwood extractives via shikimic and cinnamic acid pathways. Thus it seems essential to characterize the enzyme systems responsible for the synthesis of heartwood extractives in ray cells and regulating mechanism for the formation of these enzymes during aging processes[38].

Woody cells differentiated from cambium initiate lignin synthesis at the stage of secondary layer formation of the cell walls regardless of heartwood formation. Since cinnamic acid is a precursor of both lignin and main heartwood extractives such as flavonoids, stilbenes and tannins, it is conceivable that the enzyme systems

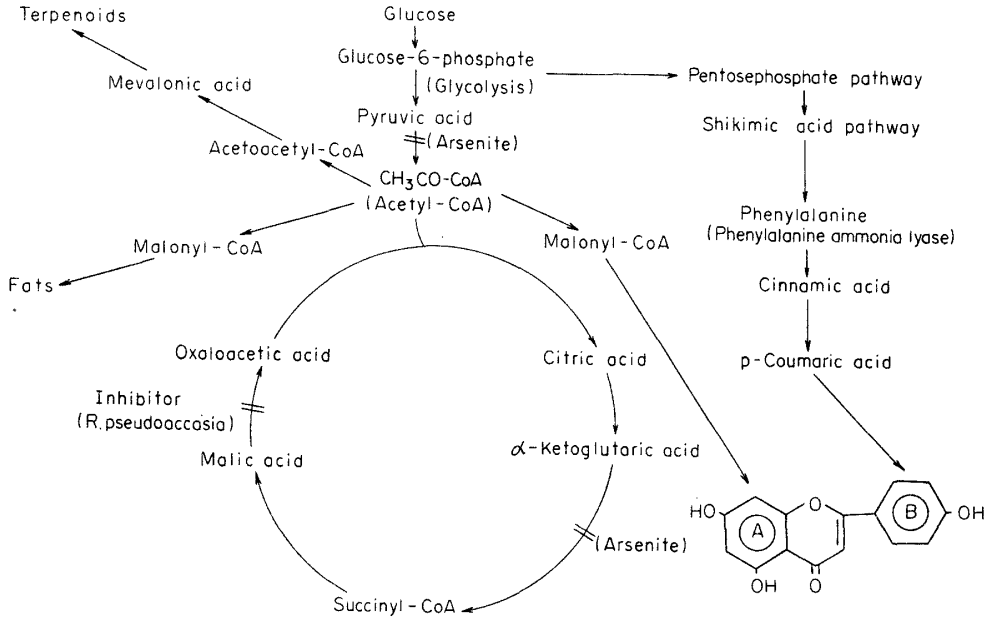


Fig. 12. Synthetic pathways of heartwood extractives.

responsible for the synthesis of the B ring occur in outer sapwood near cambium where lignification is progressing. Nevertheless, the cells without ray cells do not seem to produce heartwood extractives during aging processes after or during lignification. Ray cells, on the other hand, gain the enzyme systems for the synthesis of the A rings of the flavonoids, and stilbenes as well as terpenoids at a certain stage which may depend on physiological conditions. Then, it seems the most important problem to elucidate that what factors induce the formation of the enzyme systems for heartwood extractives, especially the A rings of both flavonoids and stilbenes in addition to the preexisted enzyme systems for cinnamic acid as a precursor of lignin and the B ring. Investigation on the metabolic pattern of ray parenchyma cells in transition wood should therefore give a clue to the elucidation of this problem. In this connection HILLIS[39] has recently found that in radiata pine, ethylene was liberated in significant amounts by transition wood during the dormant season of the year and that the ethylene stimulates the formation of stilbenes in pine sapwood blocks. Our cytological and metabolic studies on ray parenchyma[40] indicated that the metabolic activity such as respiration gradually decreases from cambial region towards transition wood. However, ZIEGLER[41] fractionated the nitrogen compounds in woods to trichloroacetic acid-soluble fraction and proteins in the individual annual rings in trunks of conifers and deciduous trees, and found a rise in protein contents in the inner parts of the sapwood in all the test species. He has also investigated the concentrations of a series of water-soluble vitamins as coenzymes in the individual annual rings of the same species and frequently found an increase in the area near

the sapwood-heartwood boundary. From these findings he concluded that the physiological activity for heartwood formation was increased in the inner zones of the sapwood. Increased activity of peroxidase, phenol oxidase and invertase in these area has also been reported by other investigators[42].

These appearantly contradicted findings reported seem to have resulted from the technical difficulties in separating living ray cells alone from the respective wood regions. The results on the physiological activities obtained so far, are based on certain parts of sapwood or transition wood which contain very small amounts of living ray cells with a large amount of dead cells. So, for quantitative investigation of physiological activity of these tissues it is especially necessary to use a minutely precise sample to reduce the influence of the dead cells as possible.

We[43] found that the amount of oxygen-uptake per gram weight of tissue slices from sapwood markedly decreased as compared with that in cambial zone, but that the respiratory pattern is considerably different between them. In the cambial zone glycolysis and TCA cycle are predominant in respiration but in inner sapwoods relative contribution of pentosephosphate pathway increased, and this is favor with the formation of cinnamic acid as a precursor of heartwood extractives. In this connection, HILLIS and INOUE[44] found that arsenite, as an inhibitor for TCA cycle administered to the xylem tissues of *Rhus succelanea* increased the formation of flavonoids. They explained that the arsenite inhibited the respiratory consumption of acetate and then the excess unconsumed acetate was incorporated into A ring of the flavonoids via malonic acid pathway. Our investigation[43] showed that arsenite and sodium fluoride not only inhibited TCA cycle but also effected to increase the pentose phosphate pathway which is favorable for the formation of NADPH and erythrose-4-phosphate. HÖLL[45] found an inhibitor for malate dehydrogenase in the transition wood of *Robinia pseudoaccacia,* and HULME and JONES[46] showed that polyphenols inhibited the mitochondria activity.

The following indications are obtained from these findings: 1) lignification of ray parenchyma cell walls is initiated by the formation of phenylalanine ammonia lyase during aging process. In progress of lignification the unused residual phenolic compounds in the cells induce disorganisation of mitochondria containing the enzymes of TCA cycle and related phosphorylation systems, and then pyruvic acid which had been metabolized normally in the mitochondria, was used for the material of heartwood extractives such as terpenoids and A ring of flavonoids. 2) concomitantly, the sugar metabolism in ray parenchyma cells altered gradually from glycolysis and TCA cycle to pentosephosphate pathway favored for the synthesis of B ring of flavonoids and stilbenes.

ZIEGLER[41] presented a similar hypothesis and pointed out that phenolic inhibitors

accumulated in the ray parenchyma cells initiated disorganisation of mitochondria to induce the biosynthesis of heartwood extractives. Phenolic compounds formed in ray parenchyma are more or less toxic and must be translocated or accumulated in the vacuole separated with tonoplast membrane[47]. However, when the concentration of the phenolic inhibitor increases at a certain level, the tonoplast is destroyed and the compounds diffuse to the whole cell and to the neighboring tracheids or vessels[38]. Thus, phenolic compounds which was isolated by the tonoplast membrane contact with hydrolase, phenol oxidase and peroxidase in the cytoplasm and associated with cell walls to lead hydrolysis of phenolic glucosides and oxidative polymerization. This pattern of the metabolism occurs in the ray parenchyma of transition wood by the influence of ethylene for example, and the enzymes related to this metabolic changes such as hydrolases, phenol oxidases and peroxidases must be activated. Therefore, partial increase in protein contents in inner sap-and transition woods found by ZIEGLER[41] might be ascribed to the increase in protein of enzymes involved in the formation of heartwood extractives. However, tolerability of parenchyma cells for the concentration of respective phenols is different and then heartwood is formed rather at an early stage in some species and delayed in the other.

Site of biosynthesis of heartwood extractives

It has been regarded that the heartwood extractives are formed not only from sugars translocated but also from starch and fat stored in the ray parenchyma cells. HASEGAWA[48] found that both sucrose-^{14}C and glucose-^{14}C administered into a living cherry log are converted to shikimic acid, quininc acid and phenols and that the heartwood phenols may be synthesized in the transition wood. HILLIS[49] observed quantitative differences in the phenols in sap- and heartwoods of *Pinus radiata* and *Eucalyptus astringens* and concluded that the respective phenols are synthesized in the respective wood tissues *in situ*.

However, distinctive evidence for the site of biosynthesis of heartwood extractives has not yet been obtained. Because both sugars and phenols are translocated through ray parenchyma, and the labeled sugars administered into a tree trunk are translocated partly as sugar itself to transition woods, but some other parts of the sugars are converted to phenols in the ray cells in sapwood and then translocated to the transition wood. To obtain distinct evidence for the synthetic site of heartwood extractives, we[50] made the following two sets of experiments.

1) Sections of cambial zone, sapwood and transition wood of *Cryptomeria, Chamaecyparis, Fagus* and *Quercus* were administered with acetic acid-2-^{14}C, mevalonic acid-2-^{14}C, glucose-^{14}C and phenylalanine-^{14}C, respectively and allowed to metabolize for 5 hr at 30°C.

2) Wood discs were cut out from freshly feld tree trunks of the same species, small

holes were drilled at the outer sapwood, inner sapwood and transition wood of the discs and steel baricades were driven into 2 cm inside the holes to interrupt the translocation of the compounds to centripetal direction by the ray cells. Then, the sterilized cotton was filled in the holes, the radioactive compounds were injected, respectively and the discs were kept at room temperature for 14 days.

It was found that the compounds administered or injected in both experiments were transformed to the wood extractives not only in the cambial zone but also in

Table 5. Incorporation of radioactive compounds into various fractions of wood extractives of *Cryptomeria japonica*.

	Radio activity (%)		
	Hexane-soluble	Ether-soluble	Acetone-soluble
Acetate-2-^{14}C			
Cambial zone	44.5	22.5	33.0
Outer sapwood	27.0	24.5	48.5
Inner sapwood	27.3	25.2	47.5
Transition wood	26.5	24.8	48.7
Glucose-G-^{14}C			
Cambial zone	18.5	28.2	53.3
Outer sapwood	22.1	28.3	49.6
Inner sapwood	27.2	25.6	47.2
Transition wood	20.6	30.5	48.9
Mevalonate-2-^{14}C			
Cambial zone	60.0	20.8	19.2
Outer sapwood	65.0	28.0	7.0
Inner sapwood	70.6	25.3	14.2
Transition wood	48.4	32.8	18.8
Phenylalanine-G-^{14}C			
Cambial zone	4.4	43.7	51.9
Outer sapwood	2.2	32.5	65.3
Inner sapwood	3.9	41.2	54.9
Transition wood	2.1	67.6	30.3

sap- and transition woods. The results are shown in Table 5. While mevalonic acid was incorporated mainly into the hexane-soluble fraction with terpenoids, phenylalanine was converted to both ether-soluble and acetone-soluble fractions containing mainly phenolics. Acetic acid and glucose, on the other hand, were incorporated into every fraction rather consistently in agreement with the role of these compounds as precursors of fatty acids, terpenoids and phenolic compounds. Thus, the results seem to explain that the precursors of heartwood extractives are synthesized

in the tissues through sap-to transition woods as well in the cambial tissues and could be converted to heartwood extractives in the transition wood. Although the number of living cells is quite small in the sap- and transition woods and apparent metabolic activity of both tissues is considerably lower than that of cambial tissues, the metabolic activity per living cell must be high enough to synthesize the heartwood extractives and their precursors. Thus, it is concluded that 1) the heartwood extractives are synthesized by ray parenchyma cells both in sap- and transition woods from sugars and fats which are translocated from leaves and/or stored, respectively. 2) since the compounds synthesized are more or less toxic for the cells, they must be translocated to the less active part of the wood. 3) when their concentrations rise to lethal level, the parenchyma cells are disorganized and the accumulation of the diffused heartwood compounds gives larger heartwood gradually. 4) hydrolysis of glycosides and oxidative polymerization of phenolics should occur in ray parenchyma in transition wood.

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