Review Article

Biochemical Studies on Bamboo Lignin and Methoxylation in Hardwood and Softwood Lignins

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Contents Page

I. Introduction

Lignin is widely distributed in nature as a cell wall constituent of terrestrial vascular plants. It is a macromolecular substance occurring in the greatest amount next to cellulose. The fact that lignin is absent from aquatic plants but present in the terrestrial ones, particularly plentiful in forest trees, indicates that lignin might have played an important biological role in plant evolution. Because it can be considered that the xylem tissues containing lignin have enabled the vascular plants to develop such tall upright forms as found in forest trees. Again, the fact that hardwood (angiosperm) lignin consists of both guaiacy and syringyl units, whereas softwood (gymnosperm) lignin lacks syringyl units, is chemotaxonomically interesting.

As to the question, "What is lignin?", a great deal of investigations have been performed until today. Gathering from the research works reported so far on lignins, there seem to have bee two academic trends in this field. One is represented by the school of FREUDENBERG[51] who devoted his half life to establishment of chemical structure of spruce lignin. The other is shown by the works of a group of plant biochemists including NEISH[49] who have been trying to elucidate biochemical process of lignification in various higher plants. The former contributed to demonstration of the old KLASONS's and ERDTMAN's[130] hypothesis that lignin is a dehydrogenation polymer (DHP) of conniferyl alcohol or its related alcohols, yielding free radicals on peroxidase-catalyzed dehydrogenation. The "free radical theory" is being established

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as an essential principle of lignin formation in higher plants. The latter, not only contributed to the proposal of the biosynthetic pathways of lignins but also provided a biosynthetic idea which is helpful to understand the chemical structure of lignins. However, the biosynthetic pathways proposed at present are principally based on the findings obtained with tracer techniques using ^{14}C-labeled compounds. Therefore, more of enzyme works (demonstration of the occurrence of the enzymes involved in biosynthesis of lignin) are needed in order to establish the proposed biosynthetic pathways that are still hypothetical.

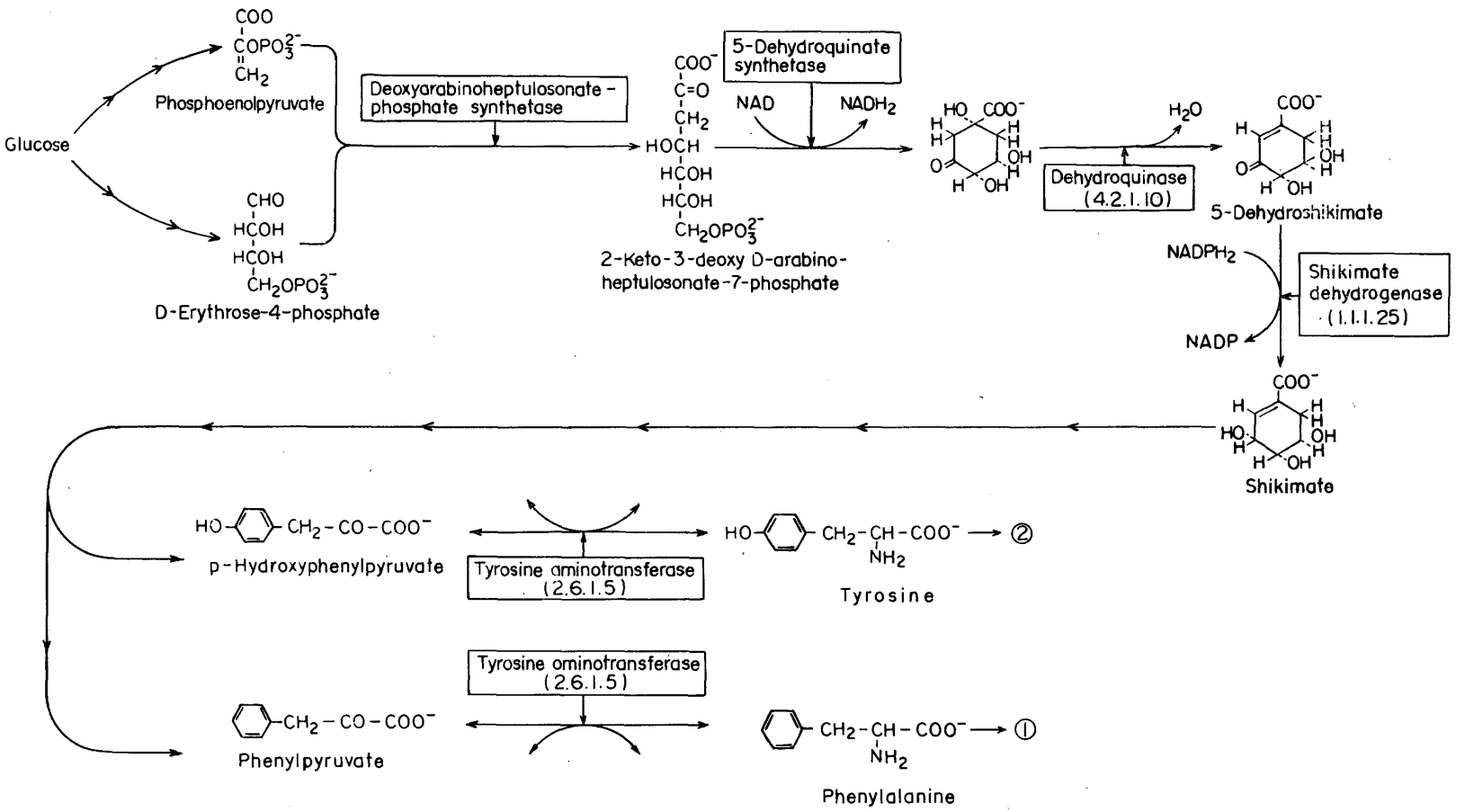
The present investigations have been performed in order to find a clue to the understanding of the mechanism of biochemical formation of lignins in higher plants. As a link in the chain of this research subject, the following problems were taken up to be investigated:

1. How the chemical structure of bamboo lignin is characterized by the presence of *p*-coumaric acid ester linkages.
2. Whether or not ferulic acid, a precursor of guaiacyl lignin, is an obligate intermediate of sinapic acid that is a precursor of syringyl lignin.
3. What biochemical factors influence the formation of guaiacyl and syringyl lignins in hard and softwoods.
4. How the enzymes and the precursors are physiologically related to the lignification of growing bamboos.

Only monocotyledonous plants such as bamboos and grasses contain 5–10 % of *p*-coumaric acid esters in their lignins, whereas these esters are absent in dicotyledonous ones. Such narrow distribution of the lignin containing *p*-coumarate esters is closely related to a unique botanical character of such Graminae plants. Then, it is of interest to elucidate the structural pattern of the ester linkages of *p*-coumaric acid in bamboo lignin with respect to their biochemical formation. However, the chemical structure of the ester linkages has not yet been established. On the basis of the analytical data obtained by methanolysis, thioglycolation, and hydrogenolysis experiments using model compounds and natural lignins, it turned out to be plausible to believe that the majority of *p*-coumaric acid molecules are linked to the γ-carbon atoms of side chains of lignin molecules. Since the occurrence of *p*-coumaric acid esters is regarded as a biological feature depending on plant families, the specific formation of these esters may be biochemically controlled, i.e. by the mediation of an acylating enzyme (Chapter II).

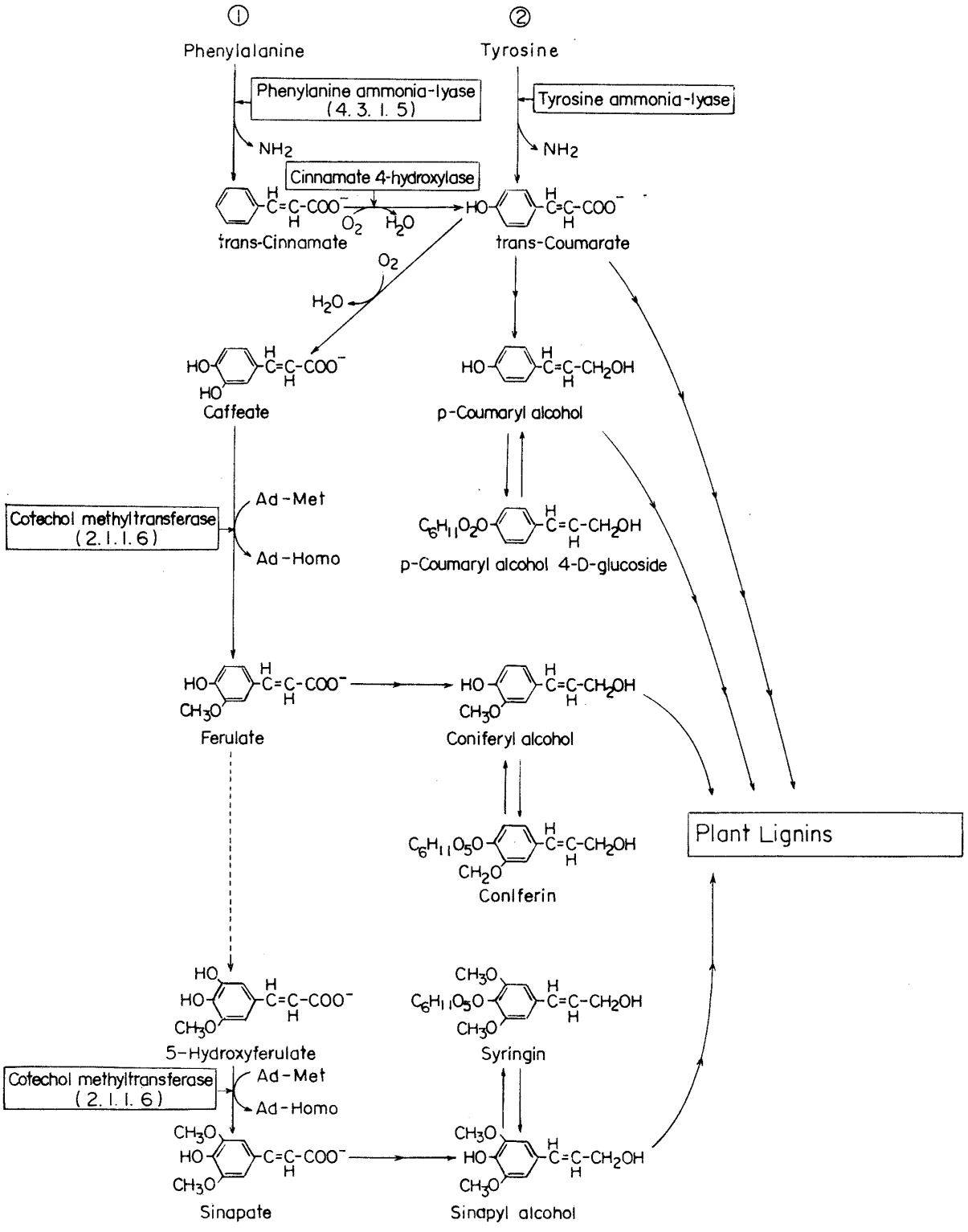
Ferulic acid has hitherto been considered as a natural intermediate of syringyl as well as guaiacyl lignin on the ground that both ferulic acid-2-^{14}C and 5-hydroxyferulic acid-2-^{14}C were incorporated into syringyl units of wheat lignin. However, it must be taken into account that ferulic acid was demethylated or demethoxylated when this compound was administered to plants for the biosynthetic studies. Then, it is impossible to conclude, from the results alone obtained with the compounds labeled at the side chain, that ferulic acid was actually incorporated into the syringyl units without removal of the methyl group of the acid. On this point, it is of importance to examine another possibility that ferulic acid is incorporated into the syringyl units via caffeic acid (3, 4-dihydroxycinnamic acid) and 3, 4, 5-trihydroxycinnamic acid after removal of the methyl group. Therefore, in order to solve this problem, it is necessary to employ ferulic acid-O$^{14}CH_3$ as a tracer which was not tested in the earlier studies on lignin biosynthesis. This labeled compound was successfully prepared from caffeic acid and *S*-adenosylmethionine-$^{14}CH_3$ by the mediation of bamboo *O*-methyltransferase. After feeding this compound to grasses, the analytical data obtained by nitrobenzene oxidation and ethanolysis supported the previous assumption that ferulic acid is a natural precursor of sinapic acid,

(A) Shikimate Pathway :



Scheme 1. Metabolic pathways leading from glucose to lignin.

(B) Phenylalanine-Cinnamate Pathway :



although demethoxylation of ferulic acid to *p*-coumaric acid was observed in bamboo (Chapter III).

"An important indication of an orderly structural principle in lignin is its methoxyl content", said FREUDENBERG[49] in his review article. The methoxyl content of lignin from conifers is fairly constant at about 15% and that of the lignin from broad-leaved trees is about 20%. Such a difference is, as already mentioned above, due to the presence or absence of the syringyl units in lignins, which is also known by the MÄULE color reaction. In order to explain the different methoxyl patterns in biochemical terms, it is necessary to elucidate, at enzyme levels, the mechanism of formation of the methoxyl groups in different plant lignins.

S-Adenosylmethionine-catechol *O*-methyltransferases (OMT, EC. 2. 1. 1. 6) were extracted from shoots of bamboos, poplar, callus tissues, Japanese black pine seedlings, and ginkgo shoots. All OMT's from angiospermous plants are capable of catalyzing the methylation of both caffeic acid and 5-hydroxyferulic acid, yielding ferulic acid (guaiacyl unit) and sinapic acid (syringyl unit), respectively. On the other hand, gymnospermous OMT's methylated caffeic acid in preference to 5-hydroxyferulic acid, scarcely yielding sinapic acid, consequently. Since gymnospermous and angiospermous OMT's utilized one and two substrates, respectively, and in other words the former has one activity and the latter has two, the former was named "monofunction OMT" and the latter, "di-function OMT", respectively. The ratios of the two activities of various OMT's (the ratios of the amounts of sinapic acid and ferulic acid produced) were discussed in relation to the S/V ratios obtained from the lignins. Thus, one of the reasons for the differences in the methoxyl patterns can be universally interpreted in terms of the substrate specificities of plant OMT's. Then, "the orderly structural principle of the methoxyl content" is consistent with the biological principle of the substrate specificities of the enzymes from conifers and broad-leaved trees (Chapters IV, V and VI).

Growing bamboos form an excellent plant material to study the changes in the occurrence of ligninfication intermediates and in the enzymatic system accompanying the successive stages of lignification in the maturing plant tissue. Glucose-6-phosphate: NADP- (G-6-P dehydrogenase; EC. 1. 1. 1. 49) and 6-phosphogluconate: NADP oxidoreductases (6-PG dehydrogenase; EC. 1. 1. 1. 44) involved in pentose phosphate pathway, shikimate: NADP oxidoreductase (dehydroshikimate reductase; EC. 1. 1. 1. 25) and dehydroquinate hydro-lyase (dehydroquinase; EC. 4. 2. 1. 10) in shikimate pathway, and OMT in cinnamate pathway were extracted from the shoots of growing bamboo (*Phyllostachys pubescens*, Mohso) and were characterized. The changes in these enzyme activities and the metabolism of lignin precursors such as shikimic acid, phenylalanine, tyrosine, *p*-coumaric acid and ferulic acid were investigated in relation to the lignin formation of bamboo (Chapter VII). The biosynthetic pathways of lignin in which the enzymes and the precursors are involved are shown in Scheme 1.

II. Ester linkages of *p*-coumaric acid in bamboo and grass lignins[25]

It has been known as a unique feature of bamboo and grass lignins that they contain 5–10% of esters of *p*-coumaric acid (PCA) accompanied by ferulic acid (FA) in small amounts[1]. KUC and NELSON[2] investigated the variation of PCA and FA contents in lignin during growth of maize as part of a genetic and biochemical study of maize hybrids. It is of interest to elucidate the structural pattern of the ester linkages of *p*-coumaric acid in Graminae plant lignins with respect to their biochemical formation.

The occurrence of the esters of PCA in sugar cane and of *p*-hydroxybenzoic acid in aspen

lignins was first demonstrated by SMITH[3,4], indicating that *p*-hydroxybenzoic acid is linked to aliphatic hydroxyl groups.

As for the structural pattern of the ester linkages of *p*-hydroxybenzoic acid in aspen lignin, NAKANO *et al.*[5,6] presumed that part of the acid is esterified with a hydroxyl in the α-position (benzyl carbon) of the side chain of lignin molecules. OKABE and KRATZL[7] also proposed the possibility that *p*-hydroxybenzoic acid might be linked to the α-position. Their supposition is based on the mechanism of the reaction where the conjugate base of the acid attacks nucleophilically the α-carbon atoms of "intermediary quinone methides", and they concluded that some of the ester linkages of *p*-hydroxybenzoic acid in aspen lignin are formed according to this reaction mechanism. SARKANEN *et* al.[8,9] have recently found acetyl groups in lignins of various gymnosperms and angiosperms, indicating that such acetyl groups are formed as suggested by KRATZL et al.[7]

PEW *et al.*[10] proposed another possibility of ester formation on the grounds that a new type of ester compound was isolated from dehydrogenation products derived from propiophenone after a peroxidase-catalyzed reaction.

Since the esters of PCA and *p*-hydroxybenzoic acid found in bamboo and aspen lignins, respectively, do not occur in other species of woody plants, the present author and his associates consider that the formation of these esters is a biological feature depending on plant species and that it is biochemically controlled by the plants. In the present paper a different type of the ester linkage of PCA in bamboo and grass lignins is proposed on the basis of analytical data obtained with model substances and natural lignins.

Spectral Analysis of Ester Linkages

On the basis of the analytical data of hydrolysis experiment and titration curves of aspen

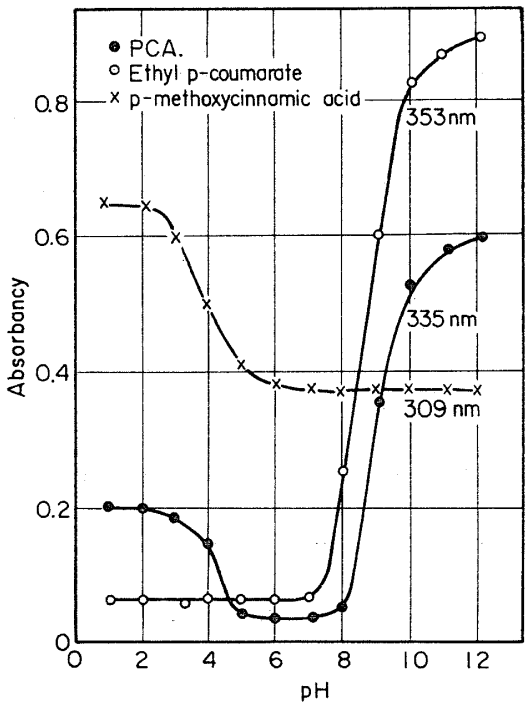


Fig. 1. pH-absorbancy curves for *p*-coumaric acid derivatives.

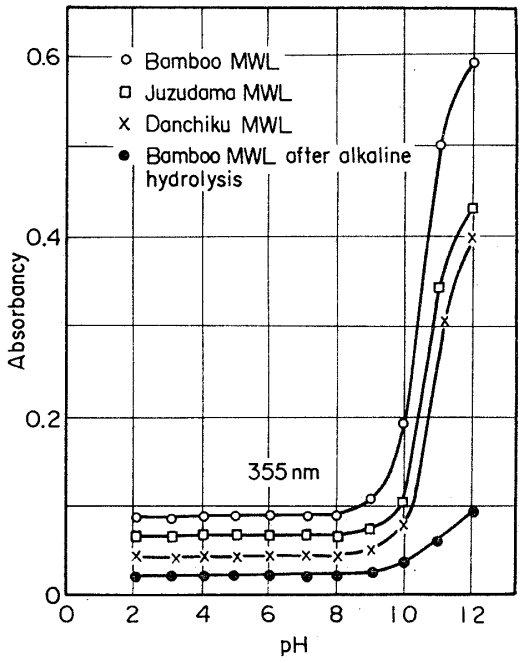


Fig. 2. pH-absorbancy curves for various MWL preparations.

lignin, SMITH[4] concluded that *p*-hydroxybenzoic acid molecules might be linked by ester linkages to aliphatic hydroxyl groups in the lignin molecules. However, definite evidence for the structural patterns of ester linkages of PCA with bamboo and grass lignins have not yet been provided, although the ester carbonyl was observed in IR spectra and the acid is liberated on alkaline hydrolysis[1~3].

Figure 1 shows characteristic pH-absorbancy curves of PCA, *p*-methoxycinnamic acid, and ethyl ester of PCA. PCA, having two dissociable groups, shows two inflection points around pH 4 and 9. On the other hand, *p*-methoxycinnamic acid and ethyl ester of PCA, having only one dissociable group, give one inflection point around pH 3 or 9, respectively. By comparison of the absorbancy curves of milled-wood lignin (MWL) shown in Fig. 2 with those of model compounds, it is found that the MWL shows similar inflection to that of ethyl *p*-coumarate in the alkaline pH range. After hydrolysis of the MWL, a significant increase in the absorbance was no longer observed. Therefore, the ester linkages in bamboo and grass lignins are formed between carboxyl groups of the acid and hydroxyl groups of lignin molecules, which is in accordance with the result obtained by SMITH[4].

Effects of Organic Acids on Formation of Esters in DHP

As already mentioned, SARKANEN *et al.*[8,9] reported the presence of acetyl groups in both angiosperm and gymnosperm lignins on the basis of detailed analysis of their IR spectra. It was found that such acetyl esters were formed when coniferyl alcohol was dehydrogenated in acetate buffer solutions of pH 5.5.

As shown in Fig. 3, IR spectra of dehydrogenation polymer (DHP) preparations formed when the reaction mixtures contained a given organic acids show a significant absorption band due to ester carbonyl at 1730 cm^{-1}, except for the spectrum of DHP prepared in the presence of cinnamic acid-2-^{14}C. On the other hand, DHP produced in distilled water without any organic acids gives no significant absorption band around 1730 cm^{-1}.

Table 1. Incorporation of radioactive organic acids into DHP[a].

Radioactive organic acids	Incorporation, %
Acetic acid-1-^{14}C	3.6[c]
Acetic acid-1-^{14}C	0.4-1.6
Cinnamic acid-2-^{14}C	0.4
p-Coumaric acid-2-^{14}C	6.0
Ferulic acid-2-^{14}C	1.7

[a] DHP was prepared as described in the text[25].
[b] The amounts of the organic acids incorporated expressed in percent based on dry weight of DHP.
[c] The high value was obtained only when DHP was formed in 0.1 *M* acetate buffer solution at pH 5.0.

Although these spectra do not directly support the prediction that the aliphatic ester groups are attached exclusively to the α-carbon atoms, it seems reasonable to consider that these carbonyl absorption bands are due to esters formed as a result of addition of aliphatic acids to the α-carbon atoms of "intermediary quinone methides". In order to examine this question further, the IR spectrum of DHP-B was again taken after methanolysis, by which ester groups attached to the α-carbon atoms are replaced by methoxyl groups. Since the absorption band around 1730 cm^{-1} was reduced significantly after the methanolysis, as shown by the dotted line in Fig. 3(a),

acetyl esters were removed by the methanolysis. Therefore, such esters had been linked to the benzyl α-carbon atoms.

However, as far as the esters of PCA in bamboo and grass lignins are concerned, there seem to exist other structural patterns for the ester linkages. IR spectra of bamboo MWL and BRAUNS' native lignin (BNL), and DHP prepared in the presence of PCA and cinnamic acids are shown in Fig. 3(b). After alkaline hydrolysis of bamboo MWL and DHP-I, the former lost the absorption band at 1730 cm^{-1}, whereas the latter showed only partial loss. These results indicate that PCA contained in DHP is involved in linkages different from the ester linkages occurring in bamboo MWL and in DHP preparations formed in the presence of aliphatic acids.

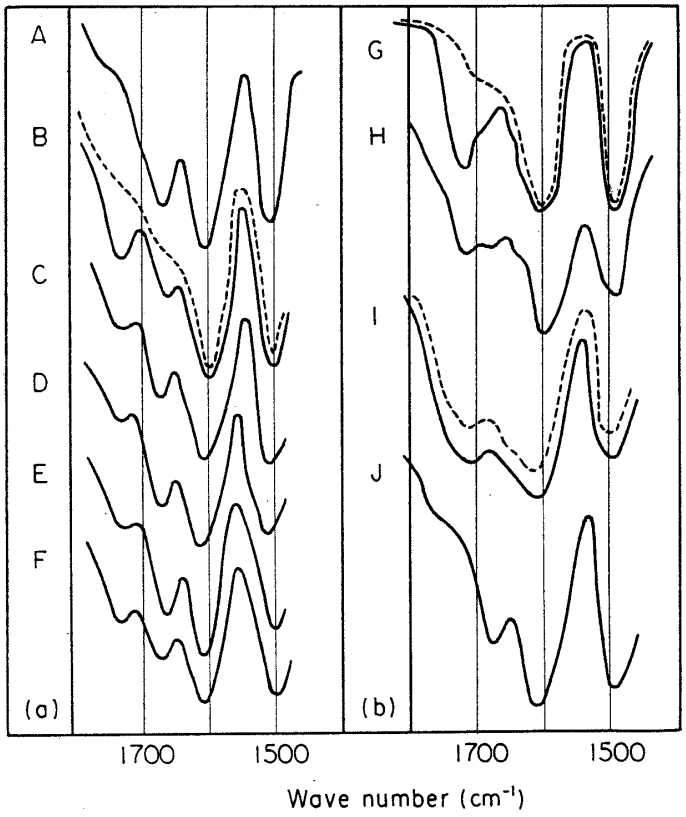


Fig. 3. IR spectra of various DHP preparations and bamboo lignins. Curve A represents the spectrum of the DHP formed in the absence of any organic acids. B, C, D, E and F are the IR spectra of DHP's prepared in the presence of acetic, propionic, *n*-butyric, acrylic, and succinic acids, respectively. The dotted line in part (a) indicates the spectrum taken after methanolysis of DHP-B. G and H are IR spectra of bamboo MWL and BNL, respectively. I and J represent IR spectra of DHPs prepared in the presence of *p*-coumaric and cinnamic acids, respectively. The dotted lines in part (b) indicate the spectra obtained after alkaline hydrolysis.

Incorporation of Labeled Acetic and Cinnamic Acids into DHP

As already described, it is doubtful whether the ester linkages of PCA are formed at only the α-carbon atoms of "intermediary quinone methides", in the same manner as found in the addition of aliphatic acids. Thus, when coniferyl alcohol is dehydrogenated in the presence of PCA, the hydroxyl group of the acid is also affected by peroxidase, so that free radicals are produced which copolymerized with coexisting radicals of dehydrogenated coniferyl alcohol. It

was found that similar copolymerization occurred on preparation of DHP in the presence of *p*-hydroxybenzoic acid[7]. In connection with the IR-spectral data shown in Fig. 3, contents of ^{14}C-labeled acetic and cinnamic acids, FA and PCA incorporated into DHP were calculated from their radioactivities (Table 1). On the basis of repeated experiments, it was found that when these organic acids were added in equimolar or in half-molar concentration, as compared with that of coniferyl alcohol, the amounts of incorporated acids, except for PCA and FA, were relatively small. However, a large amount of acetic acid-1-^{14}C, 3.6%, was incorporated when DHP was formed in 200 ml of 0.1 M acetate buffer solution containing 2 mmoles of coniferyl alcohol. Therefore, the amount of aliphatic acid incorporated into DHP seems to be dependent, to some extent, on the concentration of organic acid added to the reaction mixtures. Among the aromatic and aliphatic acids tested, PCA was incorporated in the greatest amount (6%) in spite of the low concentration, which is in accordance with the copolymerization mechanism just indicated.

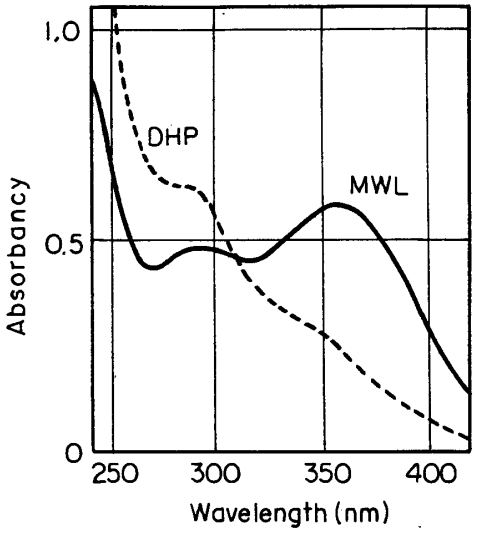


Fig. 4. UV spectra of bamboo MWL and DHP in 0.1*N* NaOH.
DHP contained 6% *p*-coumaric acid-2-^{14}C.

Figure 4 shows the UV spectra of the radioactive DHP containing 6% of PCA and the natural bamboo MWL. The former can clearly be distinguished from the latter. Therefore, PCA incorporated into DHP is not linked by the same ester linkage as that found in bamboo and grass lignins. However, it is likely that cinnamic acid, in spite of the low degree of incorporation, is linked to the α-position in the ester form described above, because cinnamic acid having no phenolic hydroxyl group is not affected by peroxidase. This assumption concerning the linkage formed by cinnamic acid with the α-carbon atoms is examined by the following methanolysis experiment.

Model Experiments for Methanolysis and Alkaline Hydrolysis of Radioactive DHP

In order to determine the position (α or γ) of PCA ester on the side chain of the lignin molecules, it is appropriate to use methanolysis with 0.5% methanolic hydrogen chloride and thioglycolic acid treatments (mercaptolysis) since these reagents attack mainly the α-carbon atoms of the side chain of the lignin molecules, giving the benzyl methylether and thioether[18,19], respectively. The former method was used by FREUDENBERG[20] and ADLER[21] to estimate the

number of benzylalcohol groups and arylether structures in lignin. Model experiments on methanolysis of guaiacylglycerol-β-guaiacyl ether triacetate (GGT), which contains one acetyl ester at the α-carbon atom, were performed. After methanolysis the methoxyl content increased to approximately the theoretical value (theor. 21.1 %, found 19.2 %), providing evidence that the acetyl group at the α-position can be removed by methanolysis. Furthermore, this is in accordance with the fact that the absorption band around 1730 cm^{-1} disappeared after methanolysis of DHP-B, as shown in Fig. 3(a). Therefore, if PCA is located at the α-postion, it will be liberated on methanolysis. On the other hand, it will not be liberated if it is associated with the terminal γ-position. The radioactive DHP's containing cinnamate- and PCA-2-^{14}C can be used as model substances because the incorporated acids should be removed on both alkaline

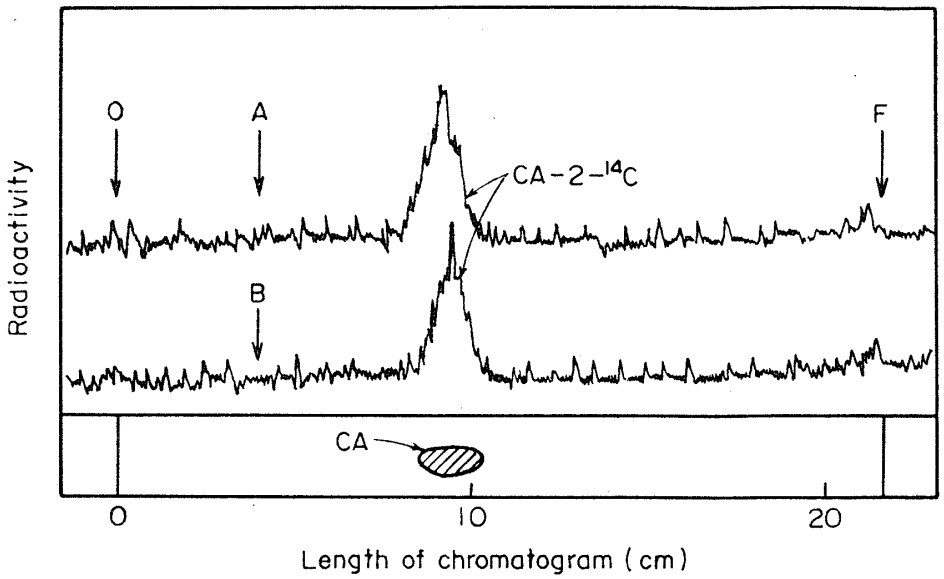


Fig. 5. Radiochromatogram of cinnamic acid-2-^{14}C liberated from radioactive DHP after alkaline hydrolysis and methanolysis. A=radiochromatogram of alkaline hydrolysis product, B=radiochromatogram of methanolysis product, CA=authentic cinnamic acid, O=origin, F=solvent front.

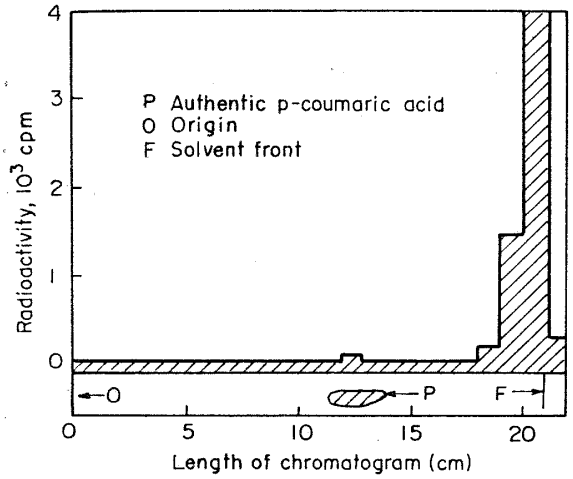


Fig. 6. Radiochromatogram of alkaline hydrolysis products from radioactive DHP.

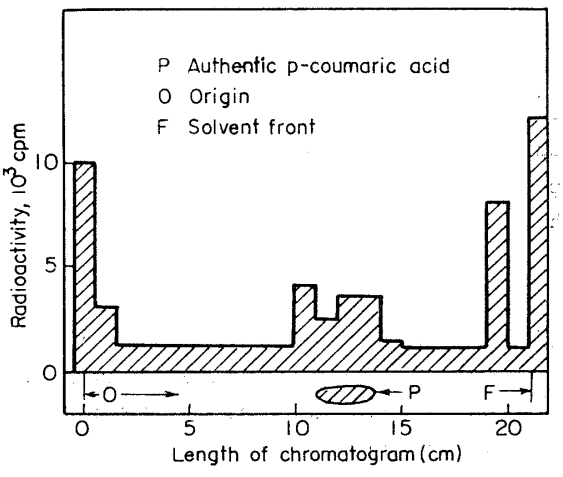


Fig. 7. Radiochromatogram of methanolysis products from radioactive DHP.

hydrolysis and methanolysis, if they are attached as ester groups at the α-position. The incorporated cinnamic acid was actually released by both treatments, as shown in the radiochromatograms of Fig. 5. On the other hand, PCA was not liberated on alkaline hydrolysis (Fig. 6) but only on methanolysis (Fig. 7). The fact that the DHP containing PCA gave several radioactive fragments as well as free PCA after the methanolysis is in accordance with an indication of copolymerization of PCA during the dehydrogenation of coniferyl alcohol. From these results it may be concluded that cinnamic acid is linked to the α-position in the ester form, whereas PCA incorporated into DHP is not bound in the ester form.

UV-Spectra of Variously Treated MWL Preparations

Figure 8 shows UV spectra of variously treated bamboo MWL preparations. A characteristic peak at 355 nm in the spectra did not change after methanolysis and thioglycolation of the MWL, whereas it disappeared after alkaline hydrolysis. Since the peak did not disappear after refluxing the MWL in 0.5% methanolic hydrogen chloride for 48 hr, the ester linkages of PCA are extremely resistant to such methanolysis. This resistance also suggests the presence of the ester linkages at the γ-rather then the α-carbons.

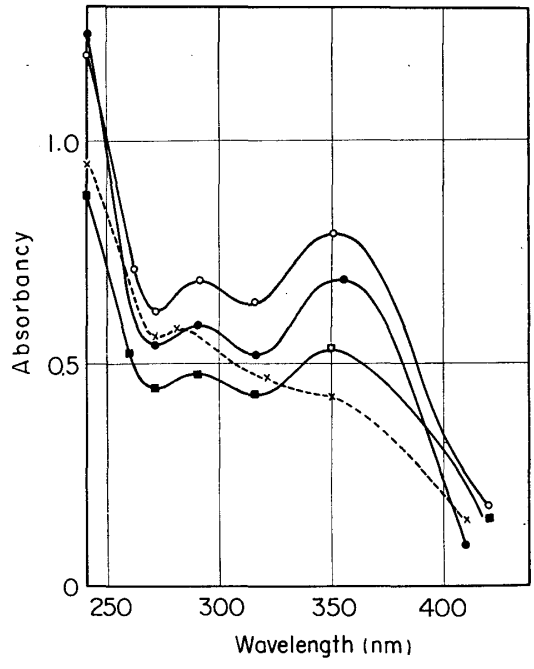


Fig. 8. UV spectra of variously treated bamboo MWL (0.1*N* NaOH).
○, Untreated MWL; ●, Thioglycolic acid treated MWL;
■, Refluxed in methanolic hydrogen chloride for 48 hr;
×, After alkaline hydrolysis.

Methanolysis and Thioglycolation of Various Lignins

Table 2 shows the contents of the ester of PCA and methoxyl groups in bamboo MWL before and after methanolysis of the MWL. The contents of methoxyl groups increased from 14.5 to 21.5%, or from 0.94 to 1.40 OCH_3 per phenylpropane unit. After methanolysis at the refluxing temperature, additional methoxyl groups were introduced into the lignin. On the other hand, the content of PCA did not change after methanolysis. These data are consistent with the UV-spectral data shown in Fig. 8.

Table 2. Content of PCA ester and methoxyl group in bamboo MWL before and after methanolysis of the MWL.

Methanolysis	OCH_3		PCA %[b]
	%[a]	Per C_9	
Before	14.5	0.94	5.5
After[c]	21.5	1.40	5.1
After[d]	25.3	1.64	5.3

[a] Methoxy content is experessed as percent on MWL.
[b] PCA content is expressed as percent on the original or the methylated MWL preparations.
[c] The methanolysis was carried out at room temperature for 3 days.
[d] The methanolysis was carried out by refluxing MWL in methanolic hydrogen chloride (1%) containing anhydrous dioxane for 3 hr.

Table 3. Comparison of PCA content in various lignin preparations before and after thioglycolic acid treatment.

Lignin	Before treatment		After teeatment		
	$(OCH_3)/C_9$[a]	PCA, %[b]	S, %[c]	SCH_2COOH/C_9[d]	PCA, %[e]
Bamboo MWL	0.94	5.5	10.00	0.87	6.1
Bamboo BNL[f]	0.98	8.5	9.60	0.80	7.6
Juzudama MWL	0.90	10.4	8.74	0.74	9.2

[a] Methoxyl content was determined by the conventional method and expressed as number of moles per C_9 unit (14).
[b] Content of *p*-coumaric acid.
[c] Content of sulfur in thioglycolic acid lignin.
[d] Number of moles of thioglycolic acid group combined with lignin which was calculated from analytical data of elementary composition.
[e] Content of *p*-coumaric acid expressed as percentage based on thioglycolic acid lignin.
[f] BRAUNS' native lignin was prepared from sawdust of bamboo by the conventional method[24].

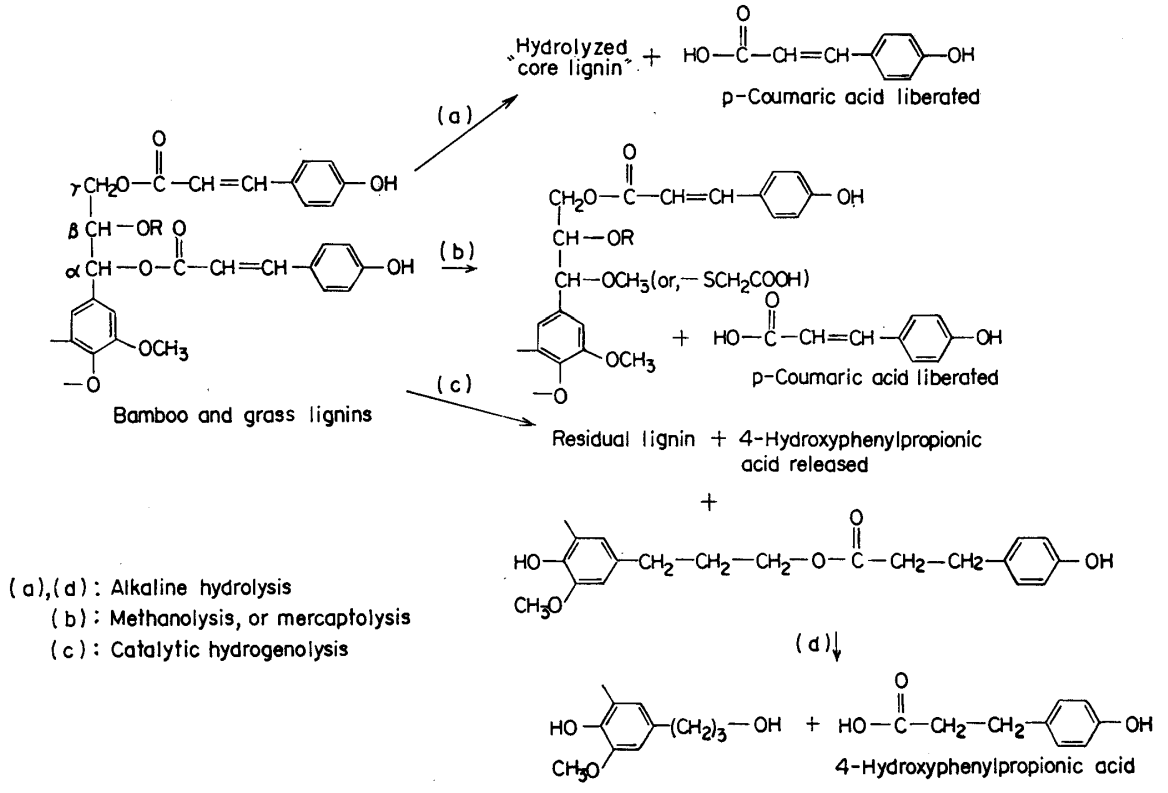
Table 3 gives the contents of PCA in various lignins before and after thioglycolation. Thioglycolic acid reacts more readily with α-carbon atoms than does methanol, because even the phenylcoumarane ring which is stable on methanolysis is cleaved by thioglycolic acid[22]. The α-position was attacked by thioglycolic acid at a ratio of 0.74 to 0.87 mole per C-9 unit, as calculated from the sulfur content. Nevertheless, the contents of PCA retained in the thioglycolated MWL preparations are approximately equal to those in the untreated preparations. These results are quite in accordance with those obtained from methanolysis experiment, indicating that PCA is not esterified at the α-position.

Qualitative Experiments with Catalytic Hydrogenolysis

Benzyl ether and ester groups can be reduced by hydrogenolysis with Pd-charcoal, yielding the corresponding alcohol and acid, respectively[16]. Therefore, GGT was employed for this catalytic hydrogenolysis as a model substance. It turned out to be impossible, however, to split off selectively the acetyl group at the α-position of GGT by this procedure, because all proton signals due to the three acetyl groups were still observed without any changes in the NMR spectrum after hydrogenolysis. Also, MWL was found to change only slightly on hydrogenolysis carried out under the more drastic conditions described under "Materials and Methods"[25]. Such difficulties in the degradation of lignin by this type of hydrogenolysis were also reported by

ADLER[23]. The residual lignin recovered showed the same UV-spectrum as that of untreated lignin. However, the fact that ether-soluble hydrogenolysis products were obtained, although in small amounts (about 10 %), indicates that the products originated as a result of cleavage of ether structures at the α- or β-positions of the lignin polymer. Accordingly, the ethers attached to the α-position could also be split off on hydrogenolysis. From the acid fraction of the products, 4-hydroxyphenylpropionic acid, probably derived exclusively from PCA esters, was isolated by TLC (Rf 0.50). However, it is not clear whether it was liberated by hydrogenolytic cleavage alone or by hydrolytic cleavage of the esters. This compound was also obtained after alkaline hydrolysis of the phenolic fraction and identified with 4-hydroxyphenylpropionic acid by UV-spectrum. At the same time, a strong absorption band due to ester carbonyl was observed in the IR-spectrum of the phenolic fraction. Therefore, 4-hydroxyphenylpropionic acid contained in the phenolic fraction is linked in the ester form to the terminal γ-carbon but not to the α-carbon atoms.

Outlines of the chemical analyses employed for this investigation are given in Scheme 2.



Scheme 2. Chemical analyses of bamboo and grass lignins.

In conclusion, analytical data described so far support the possibility that the majority of PCA molecules in bamboo and grass lignins are linked to the γ-position of the side chain of lignin molecules. However, more appropriate model experiments must be carried out, and the investigation is in progress to obtain definite evidence for the structural pattern of the ester linkages of PCA in lignins. As for the biochemical formation of PCA esters in Graminae plant lignins, two possibilities are considered :

1. *p*-Coumaroyl group may be enzymatically transferred in the hydrophilic system to the terminal γ-carbons of oligomers of DHP or growing lignin polymers as shown in Fig. 9.

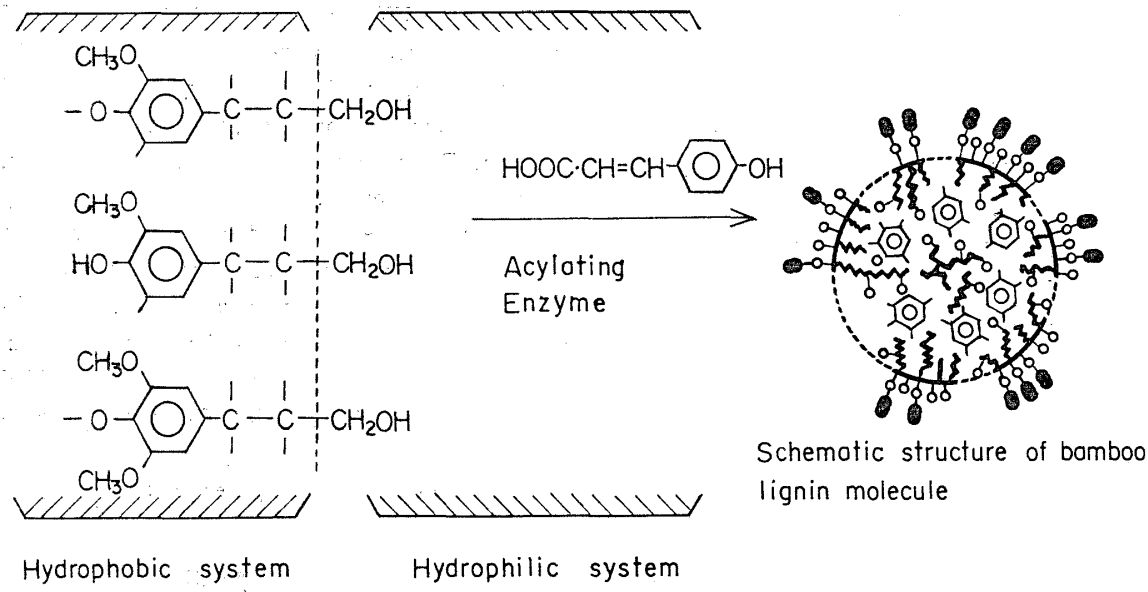


Fig. 9. A schematic model for the formation of PCA ester linkages of bamboo lignin by the mediation of an acylating enzyme.
—●, PCA molecules; —○, -CH_2OH groupings.

2. Coniferyl-γ-*p*-coumarate may participate in lignification. However, the former is considered more likely than the latter on the ground that coniferyl-γ-*p*-coumarate has not yet been found in Graminae plants. If the former possibility is logically extended, lignin-carbohydrate complex (LCC) may be formed by the enzymic transfer of xylose or galactose to the γ-terminal positions via UDP-, ADP- and GDP-derivatives.*

III. Mechanism of formation of syringyl components in lignin[41]

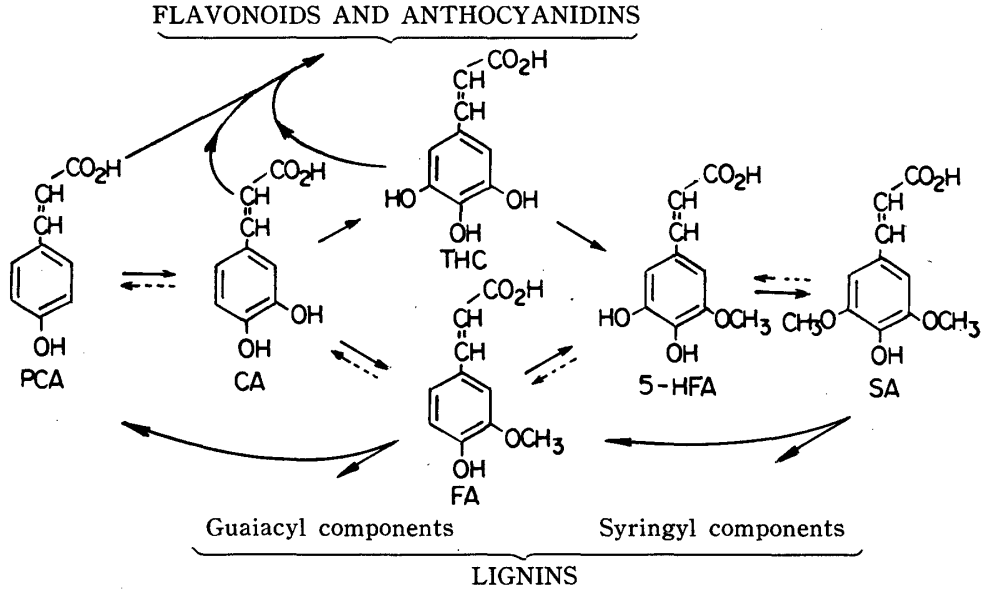
It is presumed that ferulic acid (FA) is a natural intermediate of sinapic acid (SA) since both FA[26~28] and 5-hydroxyferulic acid (5-HFA)[29] were incorporated into syringyl components of lignin molecules. However, a number of problems still remain to be investigated. For example, the evidence for enzymatic conversion of FA to 5-HFA has not yet been provided. Also, it is still uncertain whether FA administered to plants is incorporated via 5-HFA into syringyl components of lignin with the methoxyl group retained intact or via both caffeic acid (CA) and 3, 4, 5-trihydroxycinnamic acid (THC) after demethylation and/or demethoxylation of FA (Scheme 3). In fact, the demethylation and demethoxylation of FA or SA fed to plants has been observed by many investigators. KRATZL and BILLEK[30] reported that radioactive syringin was incorporated into guaiacyl components of spruce lignin. REZNIK and URBAN[31,32] observed the conversion of FA-3-^{14}C to the CA moiety of chlorogenic acid in both wheat and red cabbage. BROWN and NEISH[28] recognized that SA-3-^{14}C was partly incorporated into guaiacyl components, isolated as dihydroconiferyl alcohol. Conversion of SA to FA was reported by HIGUCHI and BROWN[29]. EL-BASYOUNI *et al.*[33] found radioactive vanillin and *p*-hydroxybenzaldehyde after feeding FA-3-^{14}C and SA-3-^{14}C to wheat plants. Recently, STEINER[34] has reported that demethylation of SA occurs in biosynthesis of delphinidin in petunia flowers. He also observed the conversion of SA to FA and to CA. These results show that the methyl ethers, FA and SA are

* UDP, uridine diphosphate; ADP, adenosine diphosphate; GDP, guanosine diphosphate.

easily degraded by plants. Again, THC may serve as a natural precursor for SA. Thus, bamboo *O*-methyltransferase (OMT)[35,36] utilizes THC as a potent substrate, yielding 5-HFA and SA, and MEIER and ZENK[37] reported that THC might be formed from CA, showing that THC was more efficiently incorporated into delphinidin than CA. Then, apart from a biosynthetic relationship between delphinidin and lignins, it is necessary to clarify the mechanism of formation of SA not only with FA labeled at the side chain or on its benzene ring but also with FA labeled at the methyl group. With the former labeled compounds alone, it is impossible to obtain the exact information on the removal of the methyl group of FA. However, FA-O$^{14}CH_3$ has not yet been tested in lignin biosynthesis, although HESS used it for studying the biosynthesis of anthocyanins in petunia.[38]

Demonstration of Demethoxylation of FA to PCA

R_f values of the hydroxycinnamic acids and the location of their radioactivities are shown in Table 4. Radiochromatographic patterns show that FA administered to sliced tissues of bamboo shoot was converted to PCA and probably to 5-HFA. However, no radioactive CA was



Scheme 3. Metabolic pathways of hydroxycinnamic acids in formation of lignins and flavonoids.

Table 4. R_f values of hydroxycinnamic acids and location of their radioactivity.

Solvent	PCA	CA	FA	5-HFA	THC
A	0.60	0.35	0.86	0.50	0.12
B	0.40	0.34	0.54	0.34	0.23
C	0.30	0.07	0.30	0.07	0.02
Radioactivity located on the chromatograms					
A	+	−	+	+	−
B	+	−	+	+	−
C	+	+	+	+	−

A ; TLC, $CHCl_3$-AcOH-H_2O (2 : 1 : 1), B ; TLC, Toluene-ethylformate-HCOOH (5 : 4 : 1), C ; PPC, Xylene-Methyl ethyl ketone-HCO_2NH_2 (25 : 25 : 1).

Table 5. Crystallization of radioactive PCA formed from ferulic acid-2-^{14}C.

Number of crystallization	Specific activity of PCA (cpm/mg)
1	1,000
2	900
3	1,000
Background	60

obtained. Radioactive PCA isolated from the chromatogram was recrystallized from hot water after addition of 20 mg of cold PCA until the specific activity was constant (Table 5). Thus, FA-2-^{14}C was demethoxylated to PCA in bamboo tissues. Similar attempts to crystallize radioactive 5-HFA were unsuccessful.

Figure 10 shows the time courses of formation of PCA and compound X (5-HFA?) during incubation of the bamboo tissue with FA-2-^{14}C. Both compounds were found to be formed rather rapidly, showing that about 20 % of FA fed was converted to them after 100 min incubation. The fact that PCA was obtained from FA is in good agreement with the results of EL-BASYOUNI *et al.*[33] and STEINER[34]. Although the mechanism of this "demethoxylation" is unknown, it seems that hydroxycinnamic acids (Scheme 3) are relatively interconvertible. Therefore, the demethoxylation of FA indicated the importance of using the tracer compound labeled at the methyl group, i.e. FA-O^{14}CH$_3$, for biosynthetic studies on lignins. This labeled compound was

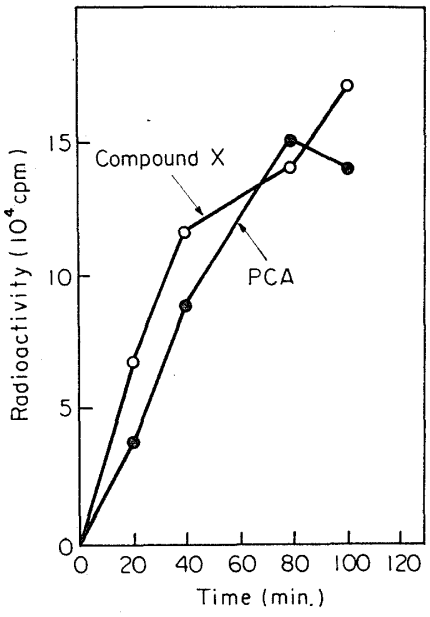


Fig. 10. Time course of formation of PCA and compound X formed from ferulic acid.

Table 6. Crystallization of FA-O-^{14}CH$_3$ prepared with bamboo *O*-methyltransferase.

Number of crystallization	Specific radioactivity of FA-O-^{14}CH$_3$(cpm/mg)
1	48,000
2	48,900
3	49,000
Background	50

prepared from CA and *S*-adenosylmethionine-$^{14}CH_3$ with bamboo OMT. Identification of FA-O$^{14}CH_3$ was achieved by a recrystallization to constant specific activity (Table 6).

Incorporation of FA-O$^{14}CH_3$ into Syringyl Components

After feeding the various labeled compounds to a grass, *Miscanthus sinensis*, vanillin (V) and syringaldehyde (S) were isolated by nitrobenzene oxidation. The yields, specific activities and dilution values for the aldehydes are given in Table 7. The ratios of the specific activities, S/V, obtained with FA-O$^{14}CH_3$ was found to be 0.33, which is nearly equal to the value obtained with phenylalanine (Phe)-U-^{14}C, indicating that the incorporation rate of FA-O$^{14}CH_3$ into syringyl components was as great as that of Phe-U-^{14}C. This result also indicates that the demethoxylation did not take place to such an extent as to lower the specific activity of S derived from FA-O$^{14}CH_3$. This assumption was further supported by the results obtained from an ethanolysis experiment (Table 8). The incorporation rate of FA-O$^{14}CH_3$ into vanilloyl methyl ketone (VMK) and syringoyl methyl ketone (SMK) was found to be slightly greater than any of those of FA-2-^{14}C, CA-2-^{14}C and Phe-U-^{14}C. Yet no distinct differences in the ratio (SMK/VMK) were found, which is in good harmony with the results of nitrobenzene oxidation. The ratios (S/V) and (SMK/VMK) obtained in the present investigation are, as a rule compatible with those (0.2–1.0) re-

Table 7. Incorporation of FA-O-$^{14}CH_3$ and phenylalanie-U-^{14}C into grass lignin.

Compound administered	Yield of aldehydes* (μmole)		Specific activity (μCi/mM)		Dilution	
	V	S	V	S	V	S
FA-O-$^{14}CH_3$ (1)	23.3	8.2	0.96	0.32	192	575
FA-O-$^{14}CH_3$ (2)	29.3	10.5	1.00	0.33	184	551
Phe-U-^{14}C	17.8	6.6	0.98	0.33	337	1,115

* V; Vanillin. S; Syringaldehyde.

Table 8. Incorporation of the various radioactive compounds into grass lignin.

Compound administered	Yield ethanolysis products (μmole)*		Specific activity (μCi/mM)		Dilution	
	VMK	SMK	VMK	SMK	VMK	SMK
FA-O-$^{14}CH_3$	13.6	7.6	1.52	1.16	121	159
FA-2-^{14}C	12.2	8.3	0.61	0.41	320	481
CA-2-^{14}C**	2.1	1.1	0.99	0.98	270	273
Phe-U-^{14}C**	2.5	1.7	0.97	0.58	340	572

* VMK; Vanilloyl methyl ketone. SMK; Syringoyl methyl ketone.

** Most of VMK and SMK were lost by an unexpected accident. However, the values for the specific activity and dilution are valid for comparison with those obtained in other two sets of experiments.

ported in early investigations[26,27,29,39]. It can therefore be concluded that most of FA administered to the two plants was incorporated into syringyl components of lignins without removal of the methyl group, although demethoxylation of FA might occur to some extent. Accordingly, the present results confirm the validity of FA as a natural intermediate of SA in biosynthesis of lignins. Furthermore, perhaps THC is not involved in biosynthesis of lignins, in spite of the methylation of THC to SA by the mediation of bamboo OMT. Therefore, 5-hydroxylation must have occurred at the stage of FA yielding 5-HFA, which is regarded as a diverging step on the biosynthetic pathways to lignins of angiosperms and gymnosperms[36,40].

IV. *O*-Methyltransferase activity from bamboo shoot[35,36]

In plant kingdom are distributed many varieties of methoxylated aromatic compounds such as lignins, lignans, flavonoids and alkaloids. The incorporation of the methyl group of methionine into the methoxyl groups of lignin was first demonstrated *in vivo* by BYERRUM *et al.*[42], using barley plants administered with methionine-$^{14}CH_3$. It was previously reported[35,43] that the methyl group of the amino acid was transferred *in vitro* via *S*-adenosylmethionine (AME) to caffeic acid (CA) in the presence of ATP. Now that AME ; catechol *O*-methyltransferase (OMT, EC. 2. 1. 1. 6), first extracted from plants by FINKLE *et al.*[44,45], has been investigated by several workers[43~48], it has been established that plant OMT's catalyzed in the presence of AME the methylation of CA to ferulic acid (FA)[43~48], 5-hydroxyferulic acid (5-HFA) to sinapic acid (SA)[35,47~48] and 3, 4, 5-trihydroxycinnamic acid (THC) to both 5-HFA and SA[35,36,48]. These findings support the cinnamate pathway for lignin biosynthesis, which has been proposed principally on the basis of tracer studies with ^{14}C-labeled compounds[49].

In this chapter bamboo OMT (angiospermous enzyme) is characterized in relation to its role in the formation of guaiacyl and syringyl units of lignins. "Methionine activating enzyme", ATP : L-methionine *S*-adenosyltransferase (EC. 2. 5. 1. 6) extracted from the shoot is also described briefly.

Extraction of the Enzymes

Bamboo OMT was easily isolated from the shoots on homogenization with buffer (pH 8.0) or 0.1 M $NaHCO_3$ solution. The enzyme activity was determined by measurement of the amount of FA formed from CA in the presence of AME. "Methionine acitivating enzyme" was obtained as acetone powders of the shoots.

Identification of Reaction Products

The reaction products formed after incubation with OMT were identified as FA, 5-HFA, and

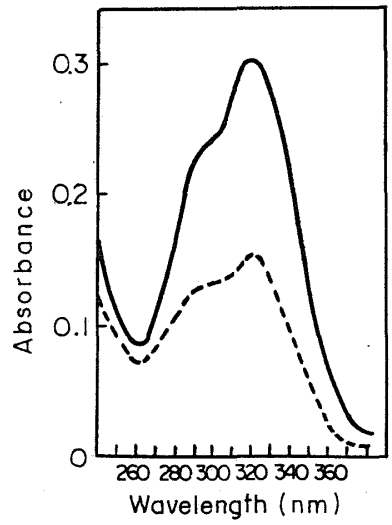


Fig. 11. UV-Spectra of ferulic acid and the reaction product in EtOH.
——; ferulic acid,
……; the reaction product.

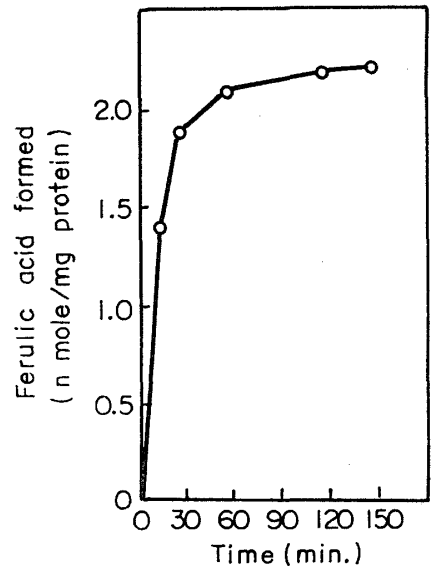


Fig. 12. Time course of ferulic acid formation. Enzyme ; 30 mg protein was used for standard assay as described in the text[35].

SA by paper chromatography with toluene-acetic acid-water (4 : 1 : 5, upper layer), and chloroform acetic acid-water (2 : 1 : 1, lower layer). Alternatively, the enzymic formation of FA was confirmed by measurement of UV-spectrum of the product as shown in Fig. 11.

Time Course of FA Formation

Fig. 12 shows FA was formed almost linearly within 30 min and no significant increase in yield was recognized by further incubation. The amounts of FA enzymatically formed in the complete or control systems are shown in Table 9. No formation of FA was observed in the control systems without either CA, AME, or the enzyme. The reaction system without $MgCl_2$, however, did not exhibit the maximal activity as found in the complete. This indicates that the Mg ion is necessary for this enzymic *O*-methylation.

Table 9. Enzymic formation of FA.

System	Ferulic acid formed (nano mole/mg protein)	Ratio
Complete*	4.1	100
Minus caffeic acid	0	0
Minus AMe	0	0
Minus $MgCl_2$	3.2	78
Minus enzyme	0	0

* Assay conditions were described in the text[35], and 30.8 mg of enzyme protein was used for assay.

Characteristics of Bamboo OMT

Effect of pH on the enzymic methylation rate was studied. Bamboo OMT exhibits maximal activity at pH 8.0 (Fig. 13) which was quite similar to the value of OMT from apple tree (optimum pH 7.0-8.0)[44].

MICHAELIS constants (Km) for CA and AME were found to be 3×10^{-5} M and 2.5×10^{-5} M, respectively, at pH 8.0 (*Phyllostachys reticulata*, Madake).

Effects of PCMB, EDTA and mono-iodoacetic acid on the enzyme activity were examined as

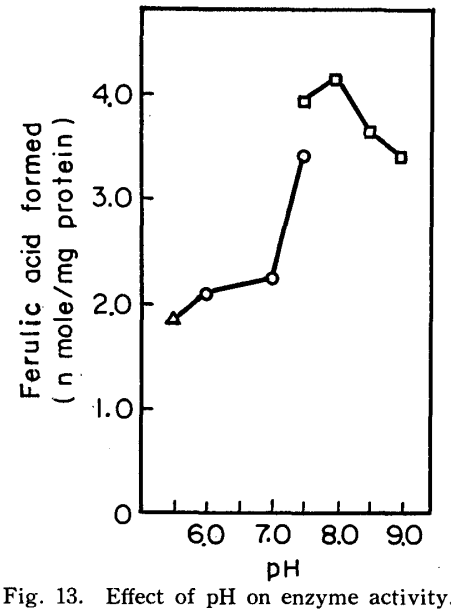


Fig. 13. Effect of pH on enzyme activity.

Table 10. Inhibition experiments.

System	Original activity (%)
Complete without inhibitors	100
Plus PCMB* (3mM)	66
Plus PCMB (0.3 mM)	90
Plus EDTA** (10 mM)	80
Plus iodoacetic acid (3 mM)	106
Plus iodoacetic acid (0.3 mM)	100

* *p*-Chloromercuribenzoate.
** Ethylenediamine tetra acetate

Table 11. R_f values and colour reactions of authentic compounds.

Compounds	R_f values			Colors		
	(1)*	(2)*	(3)*	In u.v.	A**	B**
p-Coumaric acid	0.04	0.53	0.14	dark violet	grey	red-brown
p-Methoxycinnamic acid	0.83	0.71	—	dark grey		
Caffeic acid	0	0.28	0.50	blue	yellow-brown	brown
Ferulic acid	0.35	0.39	0.97	bright blue	blue	red-violet
Iso-ferulic acid	0.15	0.27	—	blue-violet	violet	red-brown
3, 4-Dimethoxycinnamic acid	0.72	0.43	—	faint blue		
3, 4, 5-Trihydroxycinnamic acid	0	0.17	0	blue	yellow-brown	yellow-brown
5-Hydroxyferulic acid	0	0.21	0.21	light blue	grey	dark brown
Sinapic acid	0.25	0.27	0.98	green-blue	grey-brown	pink
	(1)*	(4)*	(5)*	In u.v.	(B)**	(C)**
p-Hydroxybenzaldehyde	0.27	0.35	—			yellow-brown
Anisaldehyde	0.96	0.89	—	dark		brighi yellow
Protocatechualdehyde	0	0.01	0.28			yellow-brown
Vanillin	0.84	0.78	0.84			yellow-brown
Isovanillin	0.59	0.67	0.78	light blue	yellow	yellow-brown
Veratraldehyde	0.80	0.92	0.92	blue	bright yellow	yellow-brown
5-Hydroxyvanillin	0.08	—	0	dark		brown-orange
Gallaldehyde	0	—	0	dark		brown-orange
Syringaldehyde	0.80	—	0.10	dark		brown-orange
	(4)*		(5)*	In u.v.	(B)**	(D)**
p-Hydroxybenzoic acid	0.01		0.28			bright yellow
Anisic acid	0.74		0.84			bright yellow
Protocatechuic acid	0		0.02		red-blue	grey-green
Vanillic acid	0.08		0.42		yellow	yellow-brown
Veratric acid	0.67		0.78			yellow
Gallic acid	0		—		bright yellow	grey-blue
Syringic acid	0.88		—		blue-grey	grey-brown
Pinoylvin	0.27		—	light blue	orange-yellow	
Pinosylvin monomethyl ether	0.90		—	light blue	yellow	

* Solvents used for paper chromatography: (1) toluene-AcOH-H_2O (4 : 1 : 5, organic layer); (2) 2% AcOH; (3) $CHCl_3$-AcOH-H_2O (2 : 1 : 1, organic layer); (4) benzene-HCOOH-H_2O (500 : 1 : 49, organic layer); (5) benzene-MeCOEt-HCOOH-H_2O (450 : 50 : 1 : 49, organic layer).
** Color reagents: (A) diazotized *p*-nitroanilline; (B) diazotized sulphanilic acid; (C) 2, 4-dinitrophenylhydrazine in 2 N HCl solution; (D) 2% $FeCl_3$.

shown in Table 10. PCMB considerably inhibited the enzyme activity and EDTA showed slight inhibition. However, no significant inhibition with mono-iodoacetic acid was observed which is also known as one of the potent SH-inhibitors. This is probably because this enzyme preparation contained a greater amount of other proteins which might protect the enzyme action against the inhibition. Apart from this, bamboo OMT may belong to a class of enzymes containing an SH group at an active center.

Bamboo OMT was found to be quite unstable. More than 70 % of the enzyme activity was lost on ammonium sulphate precipitation. However, this inactivation was effectively prevented by the addition of EDTA and SH-reagents, such as mercaptoethanol, CLERAND's reagent (dithiothreitol), and cysteine, into the buffer solution with which plant tissue was homogenized.

Occurrence and Function of "Methionine Activating Enzyme"

The "methionine activating enzyme" was for the first time extracted from barley plants by MUDD[50], indicating that AME is an important intermediate on the methylation in higher plants. Fig. 14 shows that AME was formed from ATP and methionine-$^{14}CH_3$ by the mediation of this enzyme. Fig. 15 shows that radioactive FA was also enzymatically formed from CA in the presence of ATP and methionine-$^{14}CH_3$. Thus, it can be seen that the methyl group of methionine is transferred via AME to form the methoxyl groups of lignin precursors such as FA, 5-HFA and SA. And methionine of an essential amino acid is necessary as a building stone for lignification of plants.

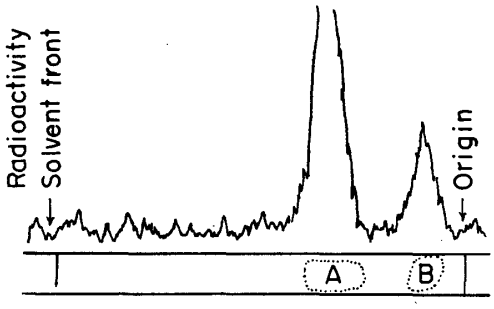


Fig. 14. Radiochromatogram of *S*-adenosylmethionine enzymatically formed.
Spots A and B correspond to methionine and *S*-adenosylmethionine, respectively, detected by ninhydrin color reaction. A solvent system for paper chromatography ; *n*-BuOH-AcOH-H_2O (5 : 1 : 4, upper layer).

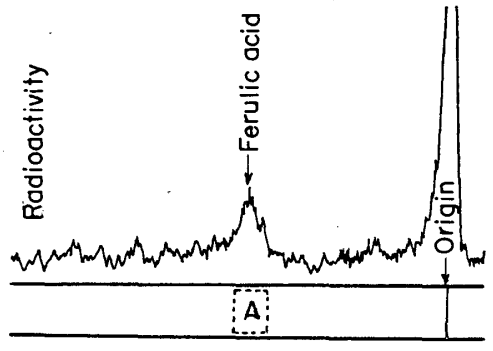


Fig. 15. Radiochromatogram of ferulic acid formed from caffeic acid in the presence of ATP and methionine-$^{14}CH_3$.
A spot A was found to be identical with anthentic FA by color reaction of *p*-nitroaniline reagent after paper chromatography with a solvent of toluene-AcOH-H_2O (4 : 1 : 5, upper layer).

Substrate Specificity of Bamboo OMT

In relation to the mechanism of formation of guaiacyl and syringyl units of lignins, it is quite important to investigate substrate specificity of bamboo OMT. Because this biological specificity is probably correlated with differences in methoxyl patterns between angiospermous and gymnospermous lignins (Chapters V and VI). R_f values and color reactions of the substrates tested and the expected products were listed in Table 11, which was used as criteria for identification of the methylated products. Table 12 shows how the various phenolics were methylated. It is interesting to note that SA (syringyl component) was formed from THC as well

Table 12. Specific methylation by bamboo *O*-methyltranseerase.

Substrate	Expected product	Result	
		(1)	(2)
Caffeic acid	Ferulic acid	+	+
5-Hydroxyferulic acid	Sinapic acid	+	+
3, 4, 5-Trihydroxycinnamic acid	5-Hydroxyferulic acid	+	
3, 4, 5-Trihydroxycinnamic acid	Sinapic acid	+	
Chlorogenic acid*	Feruloyquinic acid	+	
p-Coumaric acid	*p*-Methoxycinnamic acid	−	
Caffeic acid	Iso-ferulic acid	−	
Isoferulic acid	3, 4-Dimethoxycinnamic acid	−	
p-Hydroxybenzoic acid	Anisic acid	−	
Protocatechuic acid	Vanillia acid	−	
Protocatechuic acid	Veratric acid	−	
Gallic acid	Syringic acid	−	
p-Hydroxybenzaldehyde	Anisaldehyde	−	
Protocatechu-aldehyde	Vanillin	−	+
Protocatechu-aldehyde	Isovanillin	−	
Iso-vanillin	Veratraldehyde	−	
5-Hydroxyvanillin	Syringaldehyde	−	+
Gallaldehyde	5-Hydroxyvanillin	−	
Gallaldehyde	Syringaldehyde	−	
Pinosylvin	Pinosylvin monomethylether	−	

(1) The products were identified by the coloring tests given in Table 11.
(2) The products were determined by the measurement of radioactivities transferred from AME-$^{14}CH_3$.

as 5-HFA. Then, THC also acts as a precursor of syringyl lignin if CA is naturally hydroxylated at the 5-position to form THC as discussed in the previous chapter. Chlorogenic acid (caffeoylquinic acid) was also methylated, giving feruloylquinic acid. On the other hand, *p*-hydroxycinnamic acid (PCA) and iso-FA (3-hydroxy-4-methoxycinnamic acid) were not methylated. Similarly, neither iso-FA nor 3, 4-dimethoxycinnamic acid was formed from CA. Other phenolics such as benzoic acids and benzaldehydes were not utilized for the enzymic methylation. However, it was found afterwards that radioactive vanillin and syringaldehyde were formed from protocatechuic aldehyde and 5-hydroxyvanillin, respectively, in the presence of AME-$^{14}CH_3$. This discrepancy was probably due to the different assay methods used. Because the latter method with a radioisotope has much greater sensitivity in determination of the products than the former color tests. HESS[46] reported that protocatechuic aldehyde, protocatechuic and gallic acids, and esculetin were methylated by cell-free extracts from petunia. FINKLE *et al.*[44] observed a similar methylation pattern with OMT from pampas grass. Bamboo OMT also seems to have a rather broad spectrum for substrate specificity. Although the *meta*-orientation specificity is common to all these plant OMT's, it is not clear what factors are involved in such an orientation specificity. One of the factors may be a requirement for a Mg ion because a certain *para*-specific OMT[51] does not require this cation and the Mg ion could affect enzyme-substrate orientation by cross-linking between a *p*-hydroxyl group of catechols and AME as shown in Fig. 16. Consequently, the *meta*-hydroxyl group is substituted by the methyl group of AME yielding *meta*-methylated products. Variations in the pH values of the reaction medium can also influence ratio of *para*- and *meta*-methylated products in some compounds[52]. At any rate, in conclusion,

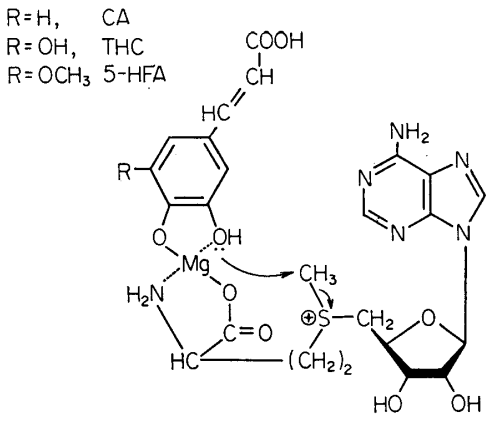


Fig. 16. The possible mechanism of the formation of methoxyl groups of hydroxycinnamic acids by the mediation of plant OMT.

the *meta*-specificity of bamboo OMT is quite compatible with the fact that lignin molecules consist of *meta*-methoxylated components of guaiacyl and syringyl units. Thus, bamboo OMT can be named "di-function OMT" since it has two catalytic functions (FA- and SA-activities) to yield FA (guaiacyl unit) and SA (syringyl unit).

V. *O*-Methyltransferase activities from Japanese black pine and ginkgo[40,53]

In connection with the mechanism of the methoxylation of lignin precursors, as described in the preceding chapter, interest has also been focused on the fact that angiosperm lignin consists of both guaiacyl and syringyl units, whereas gymnosperm lignin lacks syringyl units[54~57]. From a biochemical point of view, gymnospermous plants lack an enzyme or enzyme systems necessary for the formation of the syringyl units. The present author and his associates[41] demonstrated that FA-O$^{14}CH_3$ was efficiently incorporated via 5-HFA into the syringyl units of grass lignin, showing that the hydroxylation of FA at the 5-position is a diverging step in the biosynthesis of syringyl lignin. On this point, it is of importance to determine whether or not gymnosperm OMT acts on both CA and 5-HFA, since these two substrates are efficiently methylated by OMT's from bamboo, poplar[36], and callus tissues of angiosperms[58]. The first cell-free extraction of OMT from gymnosperms has already been reported by the present author and his co-workers[40], showing that gymnosperm OMT should be regarded as a key enzyme for the biosynthesis of softwood lignin (guaiacyl lignin). The present chapter described the studies on OMT from pine seedlings and ginkgo shoots, and the role of gymnospermous enzyme is discussed in relation to biochemical differences in methoxyl patterns between angiosperm and gymnosperm lignins.

Extraction of OMT from Gymnospermous Plants

Roots and hypocotyls from the seedlings of Japanese black pine germinated for 2 weeks were homogenized in a Waring blendor with phosphate buffer (pH 7.5) containing mercaptoethanol. The enzyme protein was precipitated with Am_2SO_4 between 0.25-0.60 saturation. The OMT activity was assayed after passing this enzyme protein through Sephadex G-25.

Many attempts to isolate OMT from *Ginkgo biloba* shoots were unsuccessful unless bovine serum albumine was added in 2% concentration prior to homogenizing the plant tissues. The addition of the serum albumine was found to be indispensable for extraction of active enzyme. It is also necessary to collect the shoots during the period of May to June. The enzyme activity

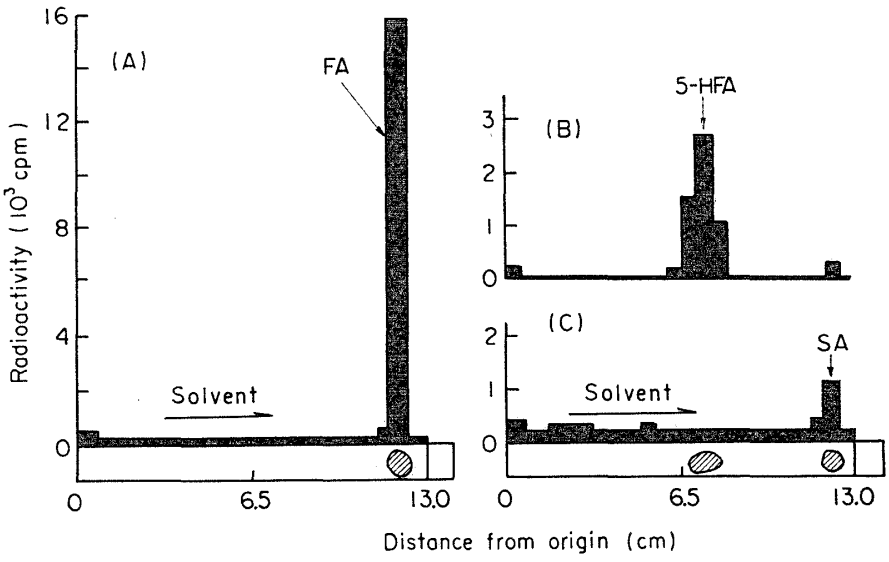


Fig. 17. TLC-Radiochromatography of FA, 5-HFA and SA formed from the corresponding substrates.

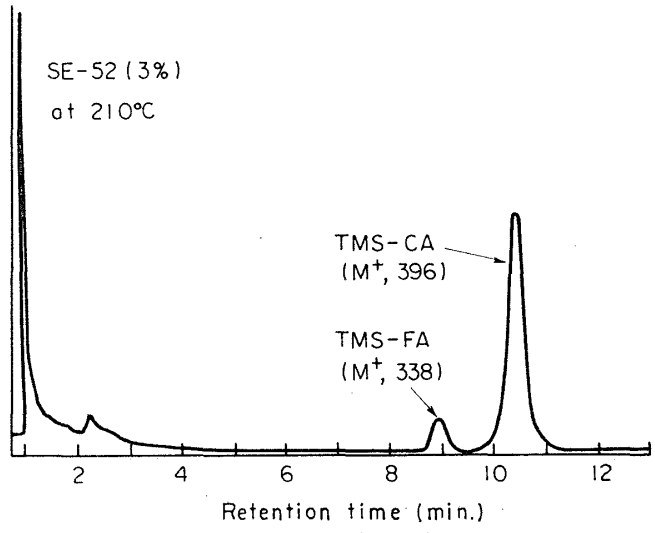


Fig. 18. GC-Mass spectrometry of the TMS-derivatives of FA and CA after enzymic reaction.

from ginkgo was assayed by use of the buffer extracts freshly prepared. The assay of OMT activity is based on the transfer of $^{14}CH_3$ from AME to CA forming FA-O$^{14}CH_3$, the radioactivity of which was determined.

Enzymatic Formation of Lignin Precursors with Methoxyl Groups

The enzymatic formation of FA, 5-HFA and SA was recognized by the radiochromatographic method (TLC; solvent chloroform-acetic acid-water, 2 : 1 : 1) as shown in Fig, 17. Alternatively, the product formed from CA was identified as FA by analysis of the mass spectrum obtained with the GC-MS spectrometer (Shimadzu-LKB 9000), after transforming the compounds into the TMS-derivatives with TMS-reagents. The two derivatives (CA and FA) were separated by GLC with SE-52 (3 %) at 210°C (Fig. 18). The amount of FA formed by the mediation of OMT increased with the incubation time and the methylation rate was dependent on the enzyme con-

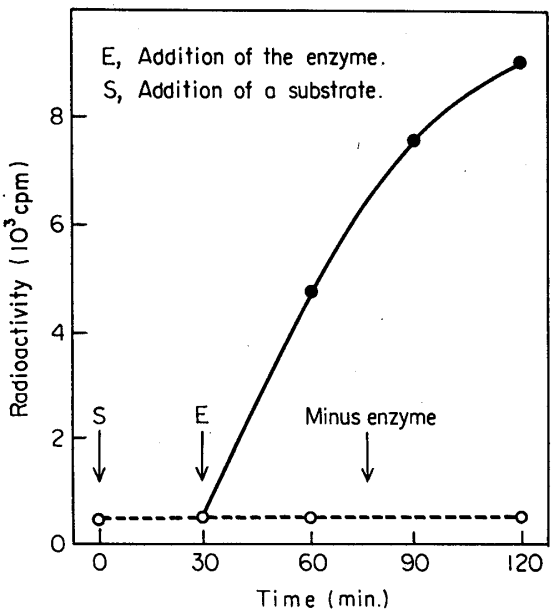


Fig. 19. Time course of enzymatic formation of ferulic acid (*Ginkgo biloba*).

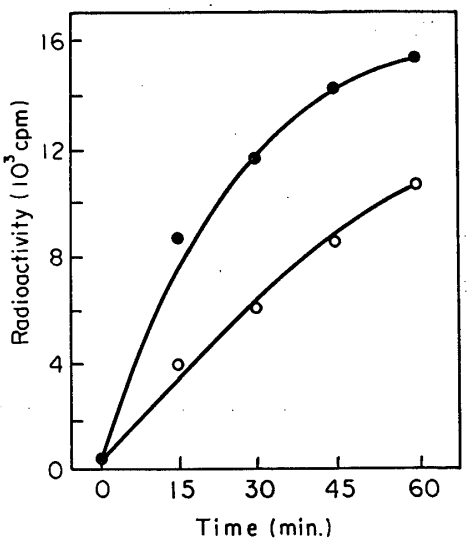


Fig. 20. Time course of enzymatic formation of ferulic acid (*Pinus thunbergii*).
—○—, One ml of the enzyme (3.3 mg protein),
—●—, Two ml of the enzyme (6.6 mg protein).

centrations used (Fig. 19 and 20).

Relationship between OMT Activity and Lignification of Pine Seedlings

A birefringence picture in the cross-section of the hypocotyl of a pine seedling is given in Photo 1, which shows that primary xylem tissues already appeared after 10 days of germination. With a phloroglucinol-HCl reagent, the regions of the primary xylem and epidermis were inten-

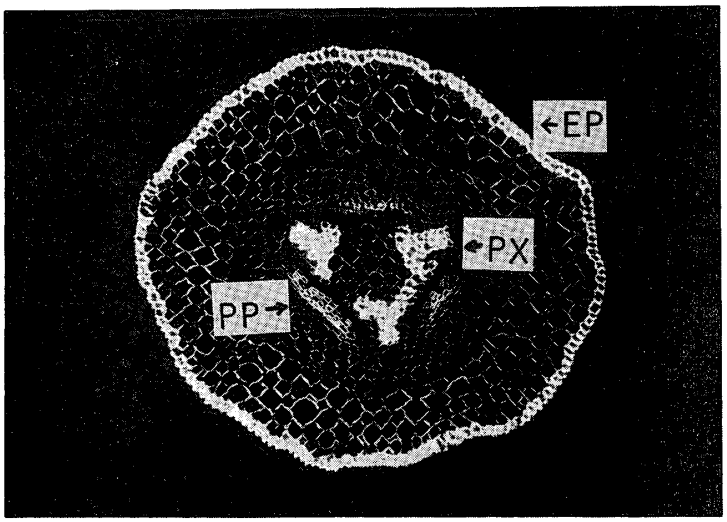


Photo 1. The birefringence in the cross section of a hypocotyl of a pine seedling germinated for 10 days.
PX; primary xylem, PP; primary phloem (130 magnification), EP; epidermis.

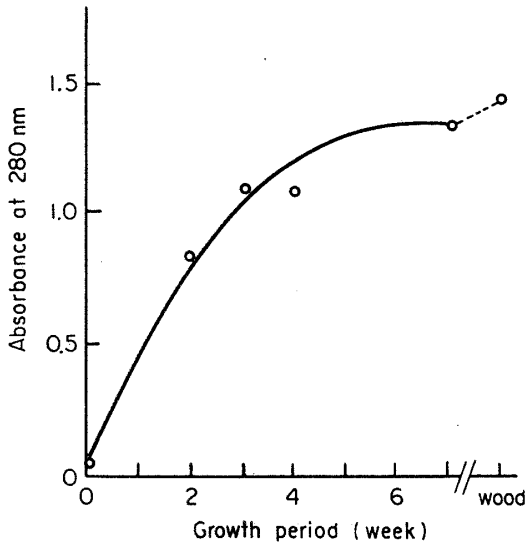


Fig. 21. Lignin content during growth of pine seedlings.

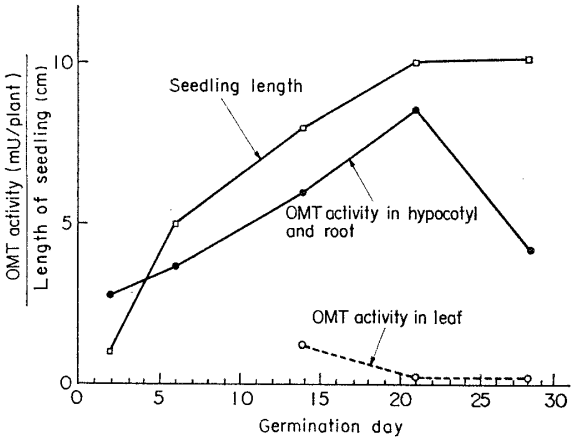


Fig. 22. Changes in activities of OMT during growth of pine seedlings.

sively colored in red. An alternative demonstration of lignin formation is shown in Fig. 21. Nearly 90 % of the lignin content in the wood was already produced in the seedlings grown for 7 weeks. Fig. 22 shows the relationship between pine OMT activity and elongation of the seedlings. The OMT activities in the hypocotyls and roots increased in parallel with their elongation during the period of 21 days of germination. The decrease in the activiy after 28 days may not be due to actual decrease in living plants but due to the loss of the enzyme activity caused by the inactivation with phenolic substances during the extraction procedure[59]. It is of importance to note that a small amount of OMT activity was present in the first leaves but disappeared after 3 weeks. These results are in good harmony with the fact that lignin is present in the hypocotyls and the roots but little in the leaves. The increase in OMT activity during growth of the seedlings is also consistent with the fact that bamboo OMT activity increased during growth of the bamboo shoots[35]. Thus, it can be seen that pine OMT also plays an important role in lignin formation.

Characteristics of Pine OMT

Table 13. Effect of metal ions on methyltransferase activity.

Metal	Experiment 1*		Experiment 2**	
	Methylation rate, cpm	Relative rate, %	Methylation rate, cpm	Relative rate, %
None	22,500	93	8,500	85
$MgCl_2$	24,300	100	10,000	100
$BaCl_2$	22,800	95		
$CoCl_2$	3,400	14	5,700	57
$ZnCl_2$	3,200	13	1,800	18
$CdCl_2$	1,000	4	500	5
$NiCl_2$	1,000	4		

* Metal concentration, 10 mM.
** Metal concentration, 0.1 mM.

Pine OMT was purified 4-fold over the crude extract by ammonium sulfate fractionation. The enzyme activity was retained without any loss for 3 days at 4°C in the presence of mercaptoethanol (5 mM), which served as an enzyme stabilizer, although it was reported that "Nerine OMT" from a plant[51] of the Amaryllidaceae was potently inhibited by mercaptoethanol (10^{-5} M). Pine OMT exhibits maximal activity at pH 7.5 (Fig. 23), which is a little lower than the optimal pH of 8.0 determined for bamboo OMT. Magnesiunm ion seemed to stimulate the enzyme activity. On the other hand, Zn, Cd, Ni and Co ions considerably inhibited the activity (Table 13). These results are quite similar to those obtained with mammalian OMT[60]. It is interesting, however, to notice that "Nerine OMT" catalyzing *para-O*-methylation of norbelladine does not require any divalent metals.

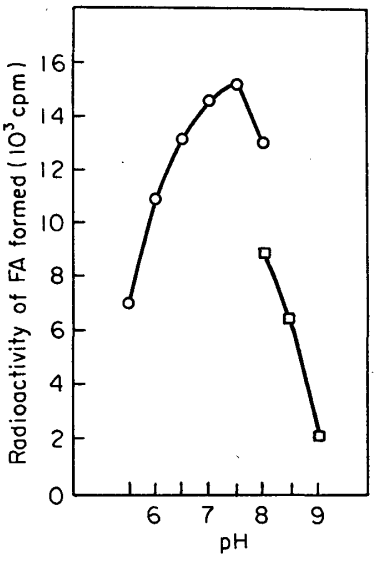


Fig. 23. Optimal pH of pine OMT.

Table 14. Inhibition experiment.

Reagent (concetration)		Methylation rate, cpm	Relative rate, %
None		28,000	100
Thiourea	(5 mM)	27,700	99
α, α-Dipyridyl	(5 mM)	23,600	84
EDTA	(5 mM)	2,300	8
Monoiodoacetate	(5 mM)	900	3
PCMB	(0.5 mM)	8,100	29
NaCN	(0.5 mM)	27,000	97
NaN_3	(10 mM)	28,300	101

The assay conditions are the same as described in the text[53] except that the inhibitors were added into the reaction mixtures.

Effects of chelating agents and SH-inhibitors were examined (Table 14). SH-inhibitors such as monoiodoacetic acid and PCMB also potently inhibited the enzyme reaction, which indicates

a possible function of an SH-group in the active center of the enzyme molecule. The inhibition by EDTA supported partly the requirement for Mg ion as indicated above. No significant inhibition by NaCN and α, α'-dipyridyl or NaN_3 was observed.

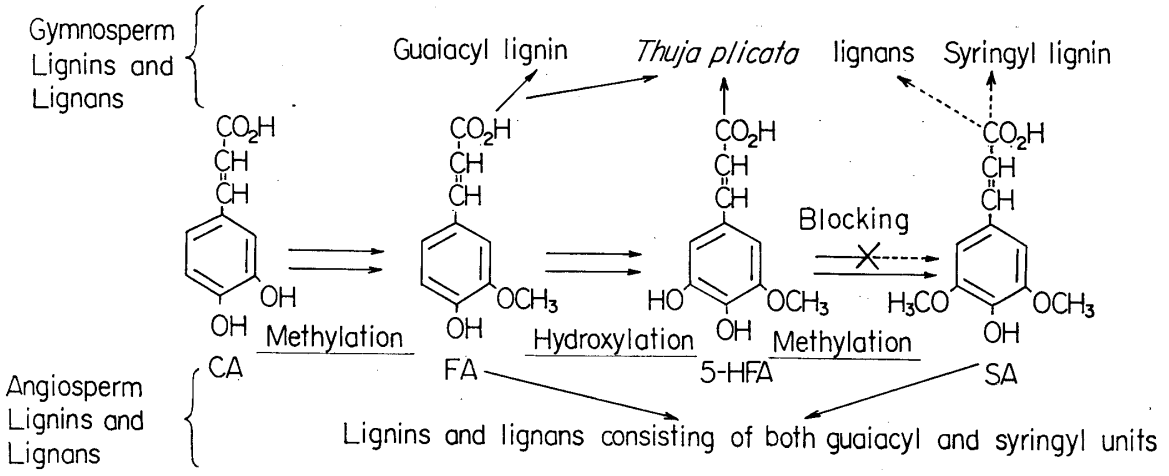
Table 15. Substrate specificity of pine OMT.

Substrate	Methylated product	Relative methylation (%)	
		Pine	Ginkgo
Caffeic acid	Ferulic acid	100	100
Caffeic acid	iso-Ferulic acid	0	0
5-Hydroxy-ferulic acid	Sinapic acid	5	6
3, 4, 5-Trihydroxycinnamic acid	5-Hydroxy-ferulic acid	25	28
Chlorogenic acid	Feruloylquinic acid	10	
iso-Ferulic acid	3, 4-Dimethoxycinnamic acid	0	
p-Coumaric acid	*p*-Methoxycinnamic acid	0	
d-Catechin	"Methylated catechin"	0	
Protocatechuic aldehyde	Vanillin	68	
5-Hydroxyvanillin	Syringaldehyde	0	
Protocatechuic acid	Vanillic acid	20	
Gallic acid	5-Hydroxyvanillic acid	0	
Pyrocatechol	Guaiacol	3	
3, 4-Dihydroxyphenylacetic acid	3-Methoxy-4-hydroxyphenylacetic acid	54	
3, 4-Dihydroxymandelic acid	3-Methoxy-4-hydroxymandelic acid	0	
Pinosylvin	Pinosylvin monomethylether	10	
Catechylglycerol-β-guaiacyl ether	Guaiacylglycerol-β-guaiacyl ether	0	

Table 15 shows how pine and ginkgo OMT's act on a number of phenolic substances. Both gymnospermous enzymes exhibit *meta*-specificity to 3, 4-dihydroxycinnamic acid, which is consistent with bamboo OMT. It is interesting, however, that "Nerine OMT"[51] is strictly *para*-specific in the methylation of norbelladine, a precursor of the Amaryllidaceae alkaloids and also that the *para*-methylation takes place during biosynthesis of anethole[61], and 4'-methoxyflavonoids[62,63] in some plants. In addition to such orientation specificity, these two gymnospermous enzymes have a fairly narrow substrate specificity of utilizing CA in preference to the other phenolics tested. Protocatechuic aldehyde and 3, 4-dihydroxyphenylacetic acid served as fairly good substrates for pine OMT. Pyrocatechol, which is an excellent substrate for rat liver OMT[60,64], was hardly methylated by this enzyme. *p*-Coumaric acid, (+)-catechin, 5-hydroxyvanillin, gallic acid, and catechylglycerol-β-guaiacyl ether (a dimeric lignin model compound) were not methylated. The observation that this lignin model compound did not serve as a methyl acceptor supports the assumption that the methoxylation of lignins must take place at the stage of the hydroxycinnamate monomers prior to polymerization of coniferyl and sinapyl alcohols. Pinosylvin having two *meta*-hydroxyl groups was only slightly methylated, although both pinosylvin and its monomethyl ether occur in heartwoods of *Pinus* species[65].

It is surprising, however, that 5-HFA, which is an important precursor for the syringyl units, was hardly methylated by the gymnospermous enzymes. On this point, both pine and ginkgo enzymes can be named "mono-function OMT", showing FA activity. THC was a more efficient substrate, yielding 5-HFA as a predominant product, but was less effective than CA. Thus, the effectiveness of the hydroxycinnamates for these enzymes is in decreasing order: CA, THC and 5-HFA. Thus, the presence of a hydroxyl or a methoxyl group at the 5-position

of CA causes a steric hindrance to the formation of "ES-complex" and, consequently, slows down the methylation rate, which is also in good accordance with the observation that 5-hydroxyvanillin was not methylated to syringaldehyde, whereas protocatechuic aldehyde was converted to vanillin in a fairly good yield. On the other hand, the angiospermous OMT's (difunction OMT) from bamboo, poplar, and callus tissues do not undergo such a steric hindrance because they are able to methylate both CA and 5-HFA, yielding FA and SA, respectively. Therefore, the lesser ability of gymnosperm OMT (mono-function OMT) to catalyze the methylation of 5-HFA results in the blocking of the biosynthesis of the syringyl lignin, even though the necessary substrate is provided from FA by the 5-hydroxylation[41]. This view is consistent with little occurrence of syringyl units in gymnosperm lignin. Also, the blocking mechanism by "mono-function OMT" may be related to the abundance of lignans such as plicatic acid, plicatin, and thujaplicatins[66] with a guaiacyl and a methyl pyrogallol nucleus (corresponding to 5-HFA) and the scarcity in amount of the lignans, thujaplicatin methyl ethers with a syringyl nucleus, both types of which co-occur in heartwood of western red cedar (*Thuja plicata*, gymnosperm). On the other hand, in angiospermous elm, *Ulmus thomasii*, the lignan with syringyl nuclei[67] is abundant. This can be compared with the situation in angiospermous and gymnospermous lignins. Although the biosynthesis of such lignans has not yet been elucidated, 5-HFA may well play an inportant role as a precursor (see Scheme 4). In conclusion, the differences in the methoxyl patterns between the angisperm and gymnosperm lignins as well as the abovementioned lignans can be universally interpreted in terms of the substrate specificities of the OMT's that markedly differ from family to family, although distribution of gymnospermous "mono-function OMT" must be systematically surveyed further.



Scheme 4. Possible mechanism of biosynthetic differentiation of angiosperm- and gymnosperm-lignins and lignans.

VI. Comparative studies on functions of various plant OMT's in relation to S/V ratios of hardwood and softwood lignins

In the previous chapters, the biosynthetic mechanism on the formation of hardwood and softwood lignins are explained, in principle, in terms of the different functions (substrate specificity) of plant OMT's. Hardwoods, in general, can be distinguished from softwoods not only by their morphological characters but also by the simple color reaction (the MÄULE test)[68], which is probably caused by syringyl units in their lignins. Alternatively, hard- and softwoods are

usually characterized by the ratios of the two lignin aldehydes (syringaldehyde to vanillin, S/V) obtained on alkaline nitrobenzene oxidation. The former woods give greater S/V values ranging from 0.3 to 3.0[54,69,72], whereas the S/V ratios obtained from the latter woods are zero with a few exceptions because of the wellknown scarcity in amount of syringyl lignin in them. It was reported from a chemotaxonomical aspect by several workers[54,69~71] that a correlation exist between the distribution of the MÄULE-positive plants and the greater S/V ratios, or *vice versa*, among gymnospermous and angiospermous species. However, the present author and his collaborators[58] recognized that the distributions of the MÄULE-negative- or its positive plants are probably correlated with the distribution of "mono-" and "di-function OMT", respectively.

A Relationship between the SA/FA Ratios of Various Plant OMT's and the S/V Ratios

As described above, the gymnospermous OMT's hardly methylate 5-HFA to SA but CA to

Table 16. Enzymic formation of FA and SA during different incubation periods (bamboo OMT).

Incubation time (min)	Products (nano mole)		Ratio (SA/FA)
	FA	SA	
0	0	0	—
5	64	75	1.2
10	95	124	1.3
15	133	130	1.0
20	153	186	1.2
30	195	213	1.1
60	213	208	1.0

Table 17. Changes in the ratio of SA to FA formed by bamboo OMT prepared from the shoots at different growth stages.

Samples*		Products formed (nano mole)		Ratio (SA/FA)
		FA	SA	
A	A**	60	55	0.9
	M**	85	74	0.9
	L**	82	78	1.0
B	A	98	104	1.1
	M	84	88	1.0
	L	69	77	1.1
C	A	84	72	0.9
	M	108	96	0.9
	L	92	109	1.2
D	A	60	60	1.0
	M	73	73	1.0
	L	43	43	1.0
E	A	125	119	1.0
	M	230	231	1.0
	L	239	248	1.0

* Samples, A, B, C, D and E, are 15, 20, 50, 50 and 270 cm in length, respectively.
** A, apical part; M, middle part; L, lower part.

FA, thus, giving the low SA/FA ratios, whereas angiospermous enzymes gave greater values of SA/FA. In order to establish these constant values as a biological peculiarity of two types of plants, the following experiments were carried out. The SA/FA ratios of bamboo OMT were found to be constant (1.0–1.1) regardless of the length of incubation time (Table 16). Every OMT preparation from 5 bamboo shoots at different growth stages also show the constant SA/FA ratio of 1.0 in average as given in Table 17, showing that the SA/FA ratio do not change during the plant growth. The observation that bamboo OMT gives the SA/FA ratio of 1.0–1.1 indicates that one enzyme with two OMT activities (FA- and SA-activities) or two different enzymes are involved in the formation of the methoxyl groups of bamboo lignin. However, bamboo OMT consists of one enzyme with two activities, which was also named "di-function OMT", on the ground that only one protein band having both FA and SA activities was observed on the acrylamide gel after electrophoresis[73].

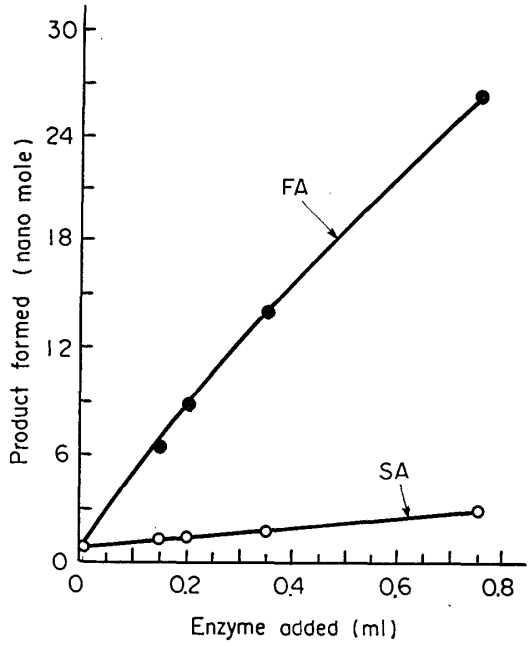


Fig. 24. Effect of substrate concentrations on *O*-methylation rate (Pine OMT).

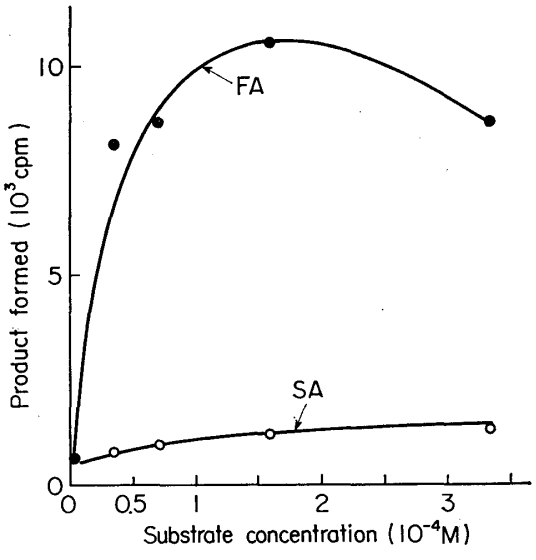


Fig. 25. Effect of enzyme concentrations on *O*-methylation rate (Pine OMT).

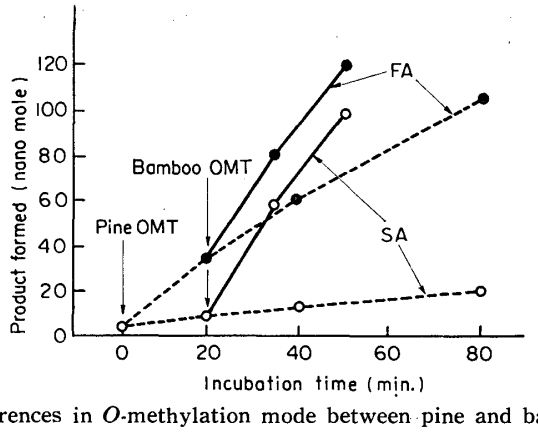


Fig. 26. Differences in *O*-methylation mode between pine and bamboo OMT's.

Pine OMT also gave the constant but low SA/FA ratios regardless of the concentrations of the enzyme (Fig. 24) and the substrates (Fig. 25), and the length of incubation time (Fig. 26). However, the enzymic formation of FA and SA was accelerated by the addition of bamboo OMT to the reaction mixtures containing pine OMT. This sharp contrast in the methylation pattern between pine and bamboo enzymes reveals that plant OMT's evidently differ in methylating functions from family to family (Fig. 26). Furthermore, another marked contrast for the enzymic methylation between bamboo and pine was observed when THC was used as a substrate instead of CA and 5-HFA (Fig. 27). Bamboo OMT methylates two hydroxyl groups stepwisely at the 3- and 5-positions of THC, yielding SA as a predominant product via 5-HFA. On the other hand, pine OMT is able to methylate only one hydroxyl, forming 5-HFA as a major product but SA as a minor. The effects of substituents at the 5-position of CA on the methylation rate was discussed in the preceding chapter.

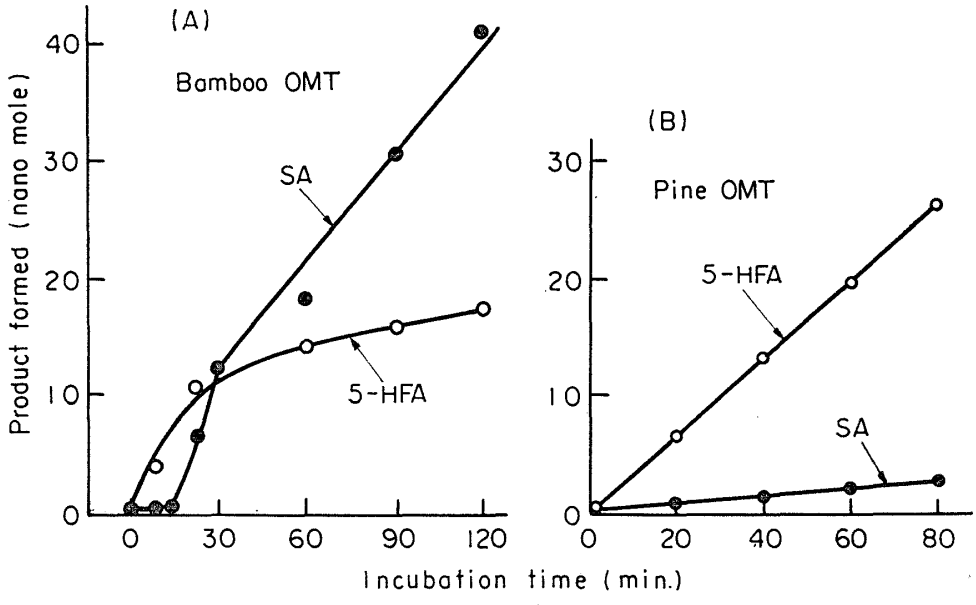


Fig. 27. Comparison of enzymic methylation of THC between pine and bamboo OMT's.

Table 18. The relationships between the ratio of SA to FA formed by various OMT's and the ratio of S to V obtained from the corresponding plant lignins.

Plant materials for OMT and lignin	Products (nano mole)		Ratio (SA/FA)	Lignin aldehyde (S/V)	MÄULE test
	FA	SA			
Bamboo	100	110	1.1	1.1	+
Poplar	100	300	3.0	1.5	+
Pine	100	5~10	0.05~0.1	≈0	−
Ginkgo	100	5~10	0.05~0.1	≈0	−

The relationship between the SA/FA ratios of four plant OMT's, the S/V ratios, and the MÄULE test is shown in Table 18. The SA/FA ratios of bamboo and poplar OMT's are 1.1 and 3.0, respectively, whereas the ratios of ginkgo and pine OMT's are less than 0.1. The finding that the plants showing the higher SA/FA ratios have the higher S/V ratios or *vice versa*, is very indicative of a correlation between the distribution of guaiacyl and syringyl lignins and

that of "mono-" and "di-function OMT", respectively.

In addition to such different distribution of softwood (guaiacyl) and hardwood (syringyl and guaiacyl) lignin among different families of woods, it has been reported that these two types of lignins are differently located in a particular morphological region of even a single hardwood. KAWAMURA and HIGUCHI[74] found the lignin of the first growth ring of *Robinia pseudoacacia* resembled the softwood lignin. Similar findings on growing poplar were reported by VENVERLOO[75], showing that syringyl lignin predominates in the older xylem tissues rather than the younger ones. HIGUCHI[76] also noted that the methoxyl contents in different species of bamboos increased with proceeding of their lignification. BLAND[77] observed that the lignin of midrib and petioles of *Eucalyptus botryoides* was low in the methoxyl content and a low S/V ratio. NAKANO *et al.*[78] found the S/V ratio for the lignin of lateral veins of hardwoods to be very low. Recently, FERGUS and GORING[79,80] have reported that guaiacyl and syringyl lignins are rather heterogeneously distributed within a cell wall of birch xylem. It is also of interest to note that cultured callus tissues of angiospermous species scarcely give rise to syringaldehyde on the oxidation[75,81,82].

Is it also possible to explain in terms of "di-function OMT" the above-described S/V ratios varying within angiospermous species or the heterogeneous distribution of syringyl lignin within a single hardwood? In this case, the author is considering that the level of "FA-5-hydroxylase" is a rather important factor determining the production of syringyl lignin.

Table 19. Enzymic formation of FA and SA during different incubation periods (OMT's from callus tissues of angiosperms).

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

For example, in spite of the low S/V ratios, the OMT's from angiospermous callus tissues (*Salix caprea* and *Morus bombycis*) gave much greater SA/FA ratio (Table 19) as compared with gymnospermous enzymes. Then, the callus OMT's also belong to a class of "di-function OMT". And, this discrepancy can not be explained in terms of the functions of OMT although the author expected that the lowering of the S/V ratios of angiospermous callus might be due to disappearance (deletion) of SA-activity or transformation of "di-function OMT" to "mono-function OMT" during cultivation of the tissues. However, it seems that there exists a correlation between the degree of xylem differentiation and the S/V ratios because older xylem tissues (well-differentiated) gave greater S/V ratios, whereas younger xylem tissues or the callus (un- or de-differentiated) yielded the lower S/V values. Therefore, an enzyme or enzyme systems except OMT must be involved in the deviations of the S/V ratios within angispermous species. Although the occurrence of "FA-5-hydroxylase" has not yet been demonstrated in cell-free extracts, it is presumed that the level of "FA-5-hydroxylase" activity is a rate determining step

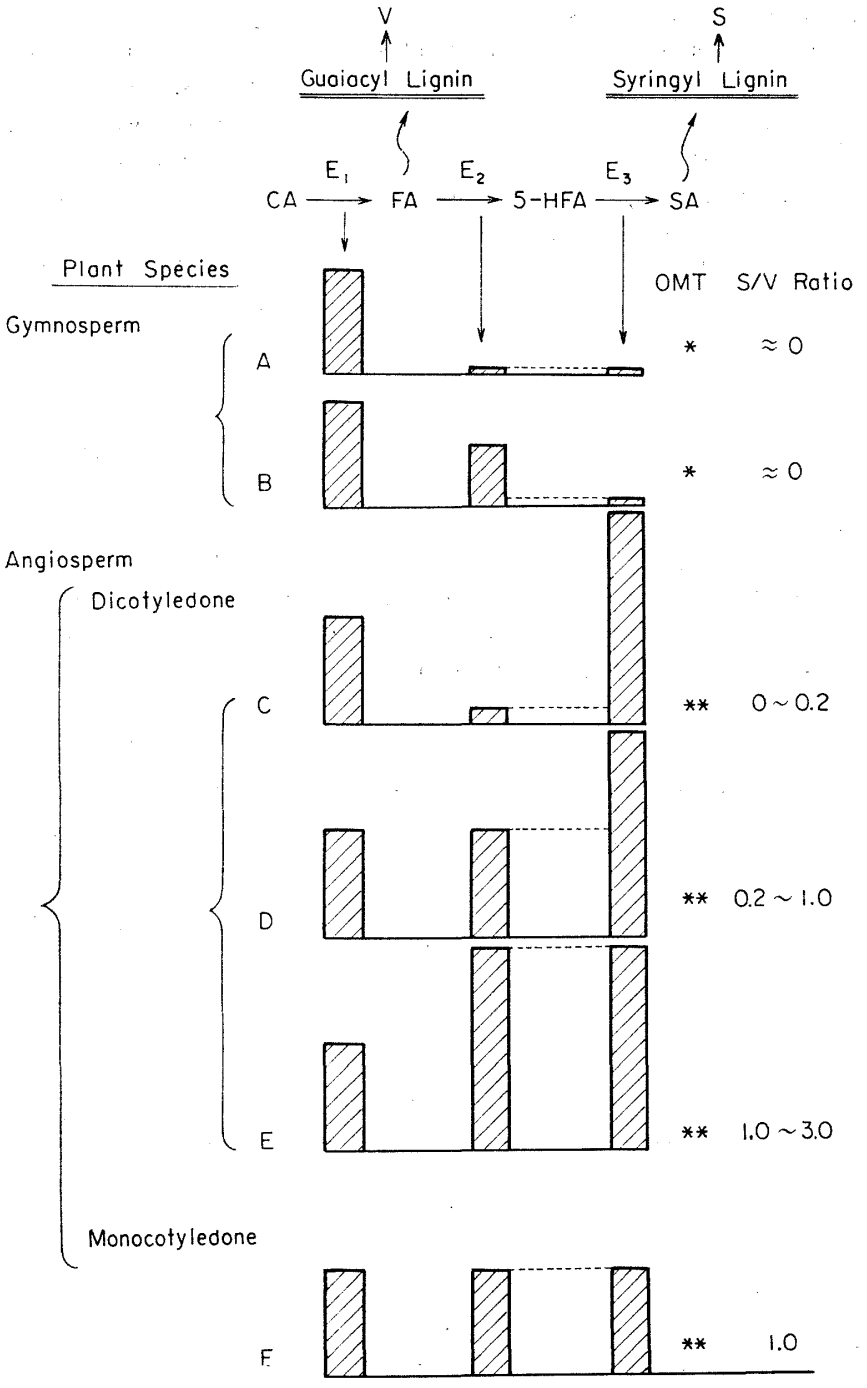


Fig. 28. Classification of plants on the basis of the enzyme levels and the S/V ratios from lignins.

in biosynthesis of syringyl lignins, as shown in Fig. 28. Thus, the subsequent activation of this hydroxylase at a later growth stage results in rather localized distribution of syringyl lignin in the older xylem tissues, which give the larger S/V ratios. This type of activation of hydroxylation systems is more likely since cinnamic acid-4-hydroxylase shows a tendency to increase in the activity during the growth of a bamboo shoot (Chapter VII, Fig. 39)[83].

In conclusion, the contents of guaiacyl and syringyl components in lignin polymers of various

plants may be universally explained in terms of the different levels of the enzymes, such as "mono-" and "di-function OMT", and "FA-5-hydroxylase", although the varying S/V ratios do not always exactly reflect the original ratios of syringyl to guaiacyl components in lignins on the ground that V is not produced from the guaiacyl components condensed at the 5-position. Then, a number of plant species forming lignins can be classified, for example, into the 6 categories (A to F) on the basis of the dynamic variations in the enzyme levels as shown in Fig. 28. Because it is evident from enzymology that the production of FA, 5-HFA and SA is limited by the levels of the respective enzymes under the steady state conditions.

VII. Changes in activities of the enzymes and in metabolism of lignin precursors during lignification of bamboo shoot

Several enzymes involved in biosynthesis of lignins were demonstrated to occur in growing bamboo shoots[84~88] and their properties were described in the original papers.

Concerning control mechanism in lignin formation, it is interesting to know how those enzymes work and how the lignin precursors are metabolized during lignification of plants. However, such investigations on the enzymes in woody plants are very few and systematic investigations have been expected in relation to lignin formation. For such studies growing bamboo shoots are excellent plant materials because during growth of bamboo shoots the changes in activities of the enzymes and in metabolism of intermediates accompany the successive stage of lignification in a single maturing plant. In addition, the shoots are very suitable as an enzyme source, since these monocotyledonous plants yield no browning matters on homogenization, whereas other woody plants give intensive browning which often denatures enzymes[59].

The present chapter describes the changes in the activities of the enzymes involved in pentose phosphate, shikimate, and cinnamate pathways during growth of bamboo shoots,

The Enzymes in Pentose Phosphate Pathway

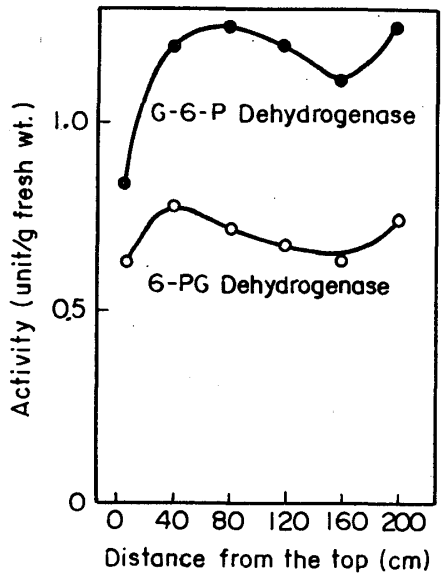


Fig. 29. Changes in activities of G-6-P and 6-PG dehydrogenases in different parts of a bamboo shoot.

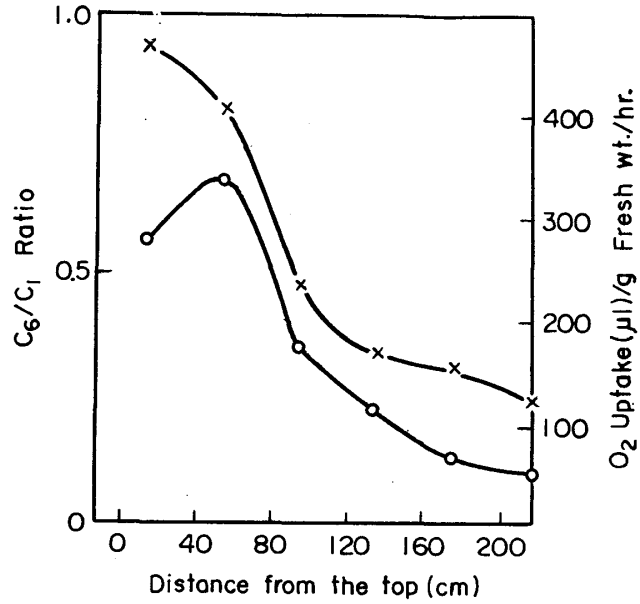


Fig. 30. Pattern of respiratory breakdown of labeled glucose.
—○—, C_6/C_1 ratio; —×—, O_2-uptake (μl).

EMBDEN-MEYERHOF-PARNAS and pentose phosphate pathways are wellknown as the two main pathways of sugar metabolism in higher plants. Pentose phosphate pathway contributes not only to the formation of $NADPH_2$ but also to the formation of shikimic acid which is synthesized by condensation of erythrose-4-phosphate and phosphoenolpyruvate.

As shown in Fig. 29, the activities of G-6-P- and 6-PG dehydrogenases seem to be activated and maintained to a certain level toward the basal parts of the bamboo shoots. These patterns of the activities indicate that both enzymes are correlated with the lignification. However, with larger bamboo shoots (4–5 m in height) sampled before the twigs sprouted, the enzyme activities were highest in the apex and decreased toward the lower parts of the shoots.

In order to obtain the information about the relative extents of utilization of glucose in the two pathways in growing bamboo, the "C_6/C_1 ratio" was measued by administering glucose-6-^{14}C and glucose-1-^{14}C to the tissues. Fig. 30 shows that total amount of O_2-uptake by the tissues decreased and the C_6/C_1 ratio also declined toward the lower parts. In view of the finding that the C_6/C_1 ratio obtained with mature tissues was lower than that with young meristematic tissues[89~91], the above-described results may be taken as suggestive of the domination of the pentose phosphate pathway in the respiratory process of lignifying tissues. Similar results were obtained with tissues of outermost sapwood adjacent to cambial zones of trees[92].

Shikimic Acid and the Enzymes in Shikimate Pathway

Since the first evidence for the operation of the shikimic acid pathway in lignin formation was provided by BROWN and NEISH[93], it is established that shikimic acid plays an important role as a precursor of naturally occurring aromatic amino acids, flavonoids[94] and lignins[95,96] in higher plants. Therefore, variation of shikimic acid content and changes in activities of 5-dehydroquinate hydro-lyase and 5-dehydroshikimate reductase (shikimate : NADP oxidoreductase) were examined with growing bamboo shoots[86].

As shown in Figs. 31 and 32, 5-dehydroquinate hydro-lyase[87] and 5-dehydroshikimate reductase exhibited maximum activity at the locus immediately below the apex and a quite gradual decline in activity toward the basal parts of the shoots. The shikimic acid content of the bamboo tissue had a pronounced maximum below the apex as shown in Fig. 33. It appears that young tissues synthesize shikimic acid vigorously as a precursor for phenylalanine and tyrosine that are incorporated into protein. Consequently, the increase in the acid content just below the apex and the decrease in the basal parts show that shikimic acid is, in fact, a metabolically

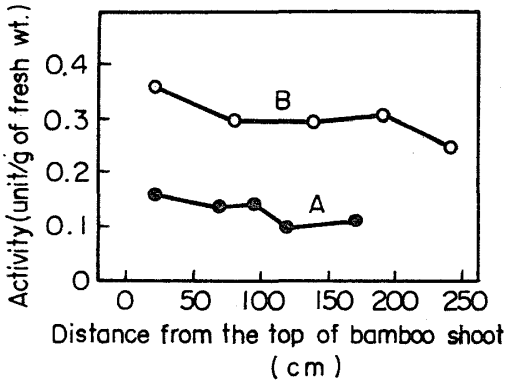


Fig. 31. Activities of 5-dehydroquinate hydro-lyase in different parts of a bamboo.
A ; Sampled on May 25th, 2.5 m in height,
B ; Sampled on May 29th, 4.0 m in height.

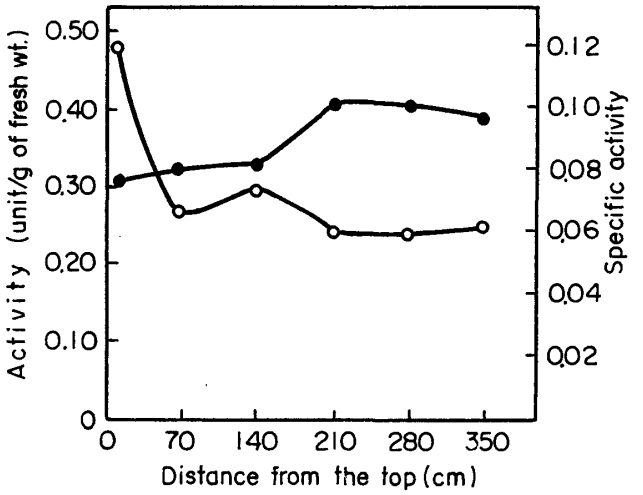


Fig. 32. Activities of 5-dehydroshikimate reductase in different parts of a bamboo shoot.
—○—, Activity (unit/fr, wt.)
—●—, Specific activity (unit/mg protein)

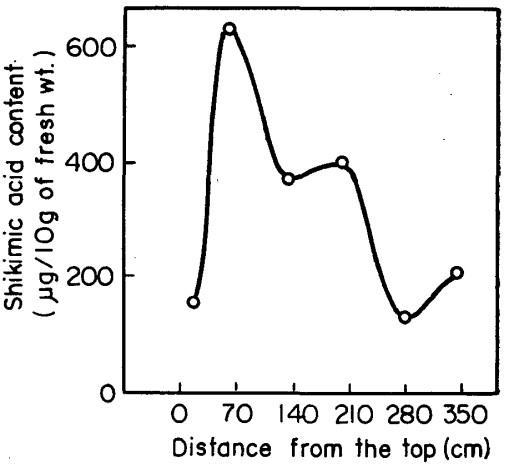


Fig. 33. Variation of shikimic acid content in different parts of a bamboo shoot.

active lignin precursor in a growing bamboo, which is in good agreement with the early findings[93,95,96].

The activities of 5-dehydroquinate hydro-lyase and dehydroshikimate reductase were found to increase by wounding[97] or light irradiation of plant tissues[98]. However, such a phenomenon was not observed in the lignifying process of bamboo shoots.

The Precursors and the Enzymes in Phenylalanine-cinnamate Pathway

Phenylalanine has been known to be a natural intermedate of phenylpropane constituents of lignins in higher plants[99]. Tyrosine has also been shown to be incorporated into lignins of a few families of higher plants such as Gramineae and the Compositae[100,101]. These two aromatic amino acids are formed by mediation of transaminase[102] from phenylpyruvic and *p*-hydroxy phenylpyruvic acids, respectively, and converted to cinnamic acid and PCA by phenylalanine[103]- and tyrosine ammonia-lyases[104], respectively.

The free amino acids contained in a young bamboo were examined by paper chromatography and the following were detected: phenylalanine, tyrosine, alanine, leucine, methionine, valine, proline, lysine, serine, glutamic acid, tryptophan, aspartic acid etc. in good accordance with results already reported[105].

Among these amino acids, phenylalanine, tyrosine, and methionine, which are necessary building stones for the formation of lignin, were examined further. Both phenylalanine and tyrosine are efficiently utilized as precursors for lignins in grasses; methionine is known to be a methyl donor for the methoxyl groups of lignins[42], glutamic acid, which is a main amino donor in transamination reactions in plant tissues, was determined as a metabolic indicator, although it may not be directly related to lignin formation.

Figure 34 shows the changes in contents of those four amino acids during the growth of a bamboo. Tyrosine was present in the highest amount at any stage of growth, and was highest in the tissue at the apex of the shoot, decreasing rapidly toward lower parts of the shoot. The other acids were present in much smaller amounts and showed less drastic decreases during the growth. The decrease in tyrosine content is very indicative of the presence of a system meta-

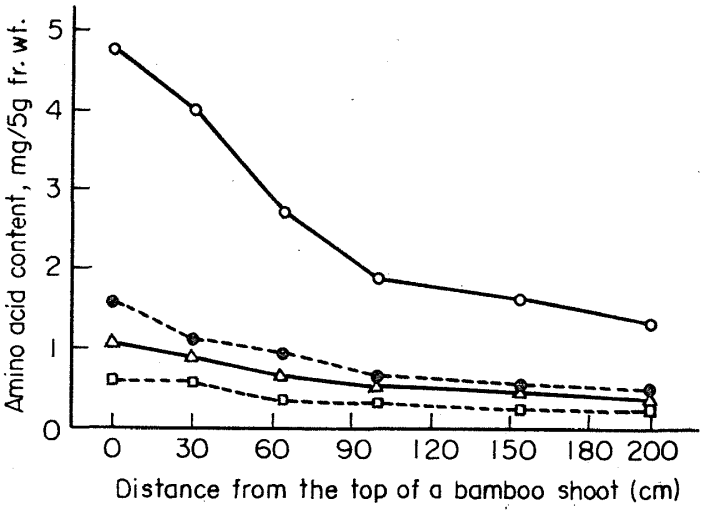


Fig. 34. Changes in amino acid content with growth of a bamboo.
—○—, tyrosine; ···●···, phenylalanine;
—△—, methionine; ···□···, glutamic acid.

bolizing tyrosine efficiently, which is in good agreement with the increase in tyrosine ammonia-lyase during lignification of a growing bamboo[106].

Attempts were made to isolate a transaminase from bamboo shoots, but reproducible results could not be obtained. Sliced tissue of the bamboo shoot was therefore used for the estimation of transaminase activity with phenylpyruvate and *p*-hydroxyphenylpyruvate as substrates. It was found that phenylalnine and tyrosine were formed in substantial amounts by incubation of the substrates with the sliced tissue. *p*-Coumaric acid (PCA) was also found to be formed and isolated from both incubated reaction mixtures[88]. Since it is impossible to express the transaminase activities in the amounts of the two aromatic amino acids formed, the activities were, for the time being, determined as the recovered amounts of the substrates after the incubation. The changes in transaminate activities during the growth of a bamboo are given in Fig. 35. The results indicate that more phenyl pyruvate and *p*-hydroxyphenylpyruvate was recovered from the reaction mixtures when tissue slices from the upper part of the plant were used, suggesting that the tissue at the apex has a lower metabolic activity than the tissue from lower

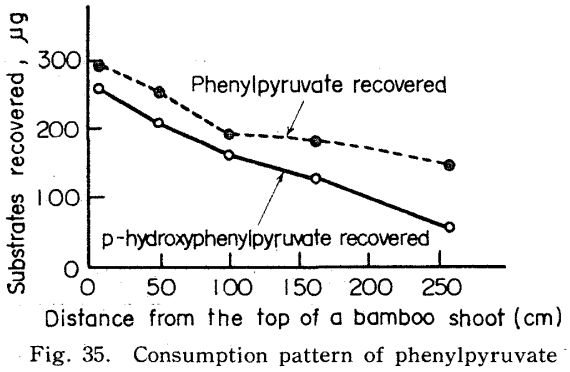


Fig. 35. Consumption pattern of phenylpyruvate and *p*-hydroxyphenylpyruvate incubated with sliced tissue.

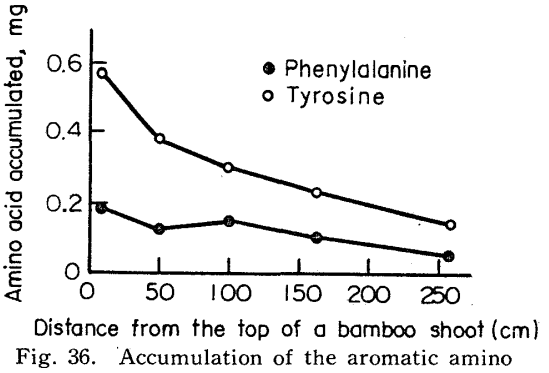


Fig. 36. Accumulation of the aromatic amino acids by the tissue.

parts of the shoot.

The patterns of the formation of phenylalanine and tyrosine were determined in the same series of experiments (Fig. 36). The results obtained appear somewhat similar to those shown in Fig. 34. The fact that higher amounts of phenylalanine and tyrosine were obtained from the upper tissues indicates that these tissues cannot convert phenylalanine and tyrosine to other substances, such as cinnamic acids, as rapidly as can lower tissue. The changes in the formation of PCA from phenylpyruvate and *p*-hydroxyphenylpyruvate seem to support this explanation (Fig. 37). Because PCA, formed via the corresponding aromatic amino acids, was accumulated in higher amounts by the tissues from the lower parts.

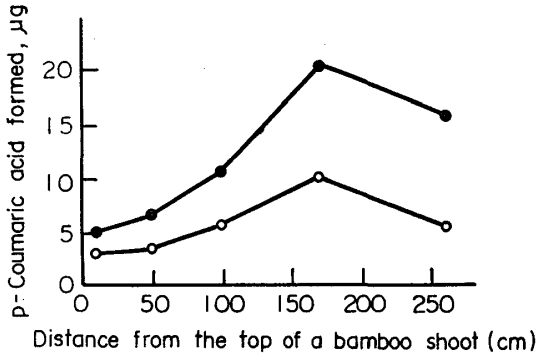


Fig. 37. Formation of PCA by sliced tissue.
—●—, PCA formed from phenylpyruvate;
—○—, PCA formed from *p*-hydroxyphenylpyruvate.

Metabolic studies with the tissue samples from various parts of the shoot showed that the activity of transaminase increased toward the lower parts of the shoot. The results further suggest that the *para*-hydroxylation of cinnamic acid to PCA is mediated in the tissues. Cinnamic acid-4-hydroxylase[107~110] is presumed to be functioning intensively because higher amounts of PCA were obtained from the reaction mixtures with phenylpyruvate as a substrate. The possibility of hydroxylation of phenylalanine to tyrosine was ruled out in the present experiment by the negative results obtained from tracer experiments with labeled phenylalnine-U-^{14}C, although NAIR and VINING[107] demonstrated phenylalanine hydroxylase in spinach leaves.

From the results of present experiments and the results reported by GAMBORG and WETTER[102], aromatic amino acid transaminase may contribute to the formation of lignin precursors by supplying phenylalanine as a substrate.

Furthermore, the fact that *p*-hydroxyphenylpyruvate was converted to both tyrosine and PCA, acting as lignin precursors, in bamboo shoots is in good agreement with the results obtained by ACERBO, SCHUBERT and NORD[111] and by WRIGHT and NEISH[112]. Therefore, the conversion of the phenylalanine and tyrosine to lignins is of specific interest from the point of view of lignin biosynthesis. Phenylalanine ammonia-lyase (phenylalanine deaminase, PAL) was first isolated from acetone powders of barley by KOUKOL and CONN[103]. NEISH[104] found tyrosine ammonia-lyase (tyrase, TAL) in all members of Graminaceae that were studied such as sorghum, wheat, corn, barley, oats, rice and sugar cane. The enzyme could not be detected in peas, lupine or sweet clover[104] or in tissue cultures of conifers and woody angiosperms[113,114].

Bamboo and grass lignins differ from lignins in other higher plants by virtue of their high content of PCA ester groups[115] and there exists a probable connection between these groups and the presence of TAL. It is possible as proposed by HIGUCHI[106] that a large portion of PCA

generated through the deamination of tyrosine becomes channeled to these ester groups.

The changes in activities of the two ammonia-lyases (Fig. 38), cinnamic acid-4-hydroxylase (Fig. 39) and OMT (Fig. 40) were investigated in relation to lignin formation of bamboo shoots. As shown in Fig. 10, the fact that the activities of PAL and TAL increased from the apex toward the basal parts of the shoot, i.e. with proceeding of lignification, coincides well with the

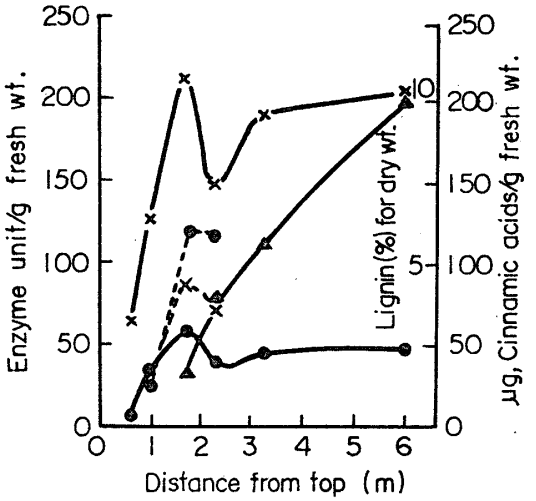


Fig. 38. Changes in activities of the enzymes, cinnamic acid contents, and lignin content of different parts of an immature bamboo (*P. pubescens*, 8.8 m in length)[106].
—×—, phenylalanine deaminase; —●—, tyrase; ···×···, cinnamic acid; ···●···, PCA; —▲—, lignin.

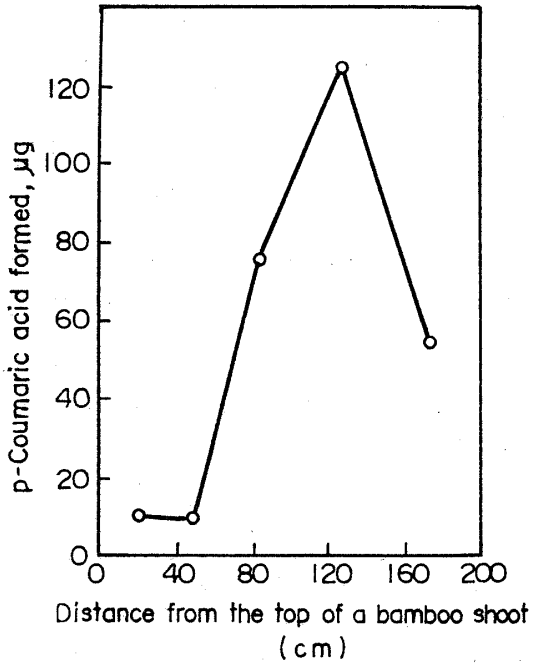


Fig. 39. Pattern of cinnamic acid hydroxylase activity of different parts of the shoot (*P. pubescens*).

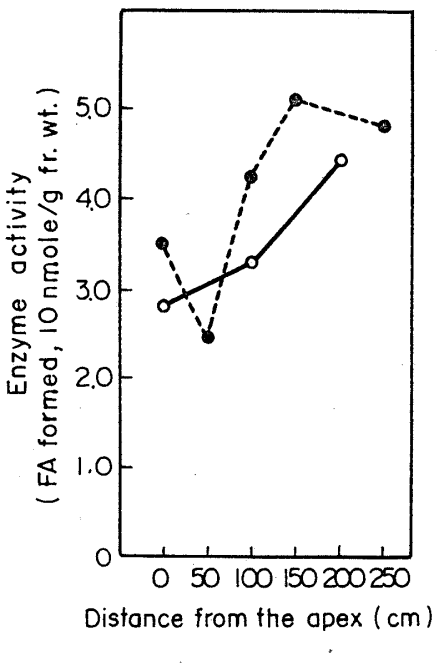


Fig. 40. Changes in activity of OMT. *P. reticulate* was used for the enzyme assay.
—○—, bamboo shoot, 220 cm in length;
—●—, bamboo shoot, 280 cm in length.

observation that the pool sizes of the aromatic amino acids became smaller toward the lower parts of the shoots (Fig. 36), indicating that the two enzymes are intimately correlated with lignin formation. The same results were already reported with buckwheat by YOSHIDA and SHIMOKORIYAMA[116]. A similar relationship between the rate of lignification and PAL was established for *Sequoia* by RUBERY and NORTHCOTE[117].

Cinnamic acid-4-hydroxylase also increased toward the lower parts of the shoot. Therefore, this enzyme may be related with lignin formation. Also it is playing an important role in formation of PCA (Tables 20 and 21) and its esters associated with lignin (Fig. 41 and Fig. 9 in

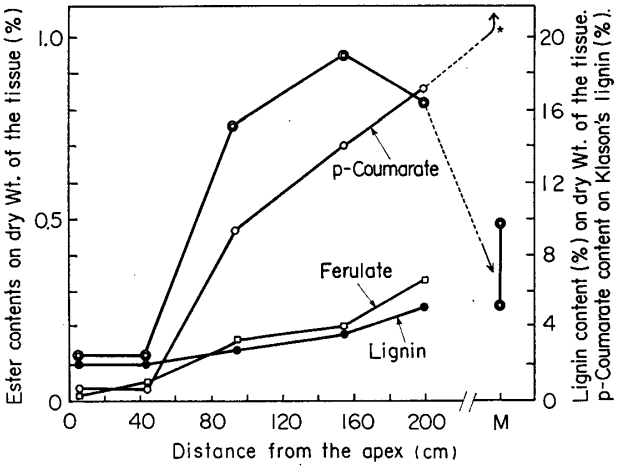


Fig. 41. Changes in contents of hydroxycinnamic acid esters and lignin (*Phyllostachys reticulata*). M; Mature bamboo, *; PCA ester content (2~3%) on dry Wt. of mature bamboo; —◎—, PCA content based on lignin.

Table 20. Variations in the content of free PCA and FA in bamboo (*Phyllostachys pubescens*) with growth.

Distance from the top (cm)	PCA (μg/10 g fresh weight)	FA (μg/10 g fresh weight)
0- 20	2.6	0.2
80- 90	2.9	1.1
140-150	10.6	3.1
190-200	55.6	3.8
260-270	248.8	4.5
340-350	398.7	1.7

Table 21. Variations in content of free PCA and FA acids with growth of a bamboo (*Phyllostachys reticulate*).

Distance from the top (cm)	PCA (μg/10 g fresh weight)	FA (μg/10 g fresh weight)
0- 10	2.7	2.0
40- 50	5.0	2.1
90-100	55.5	3.9
120-130	152.8	5.9
170-180	116.8	5.5
210-220	161.6	7.3

Chapter II).

OMT also showed the patterns of increase in activity during the growth of bamboo shoots (Fig. 40), which means that the enzyme contributes to the formation of guaiacyl and syringyl lignin components by methylating caffeic and 5-hydroxyferulic acids, respectively, during the lignification.

As described above, the activities of the enzymes involved in the transamination, deamination, hydroxylation and *O*-methylation steps were found to be intimately correlated with the lignification of bamboo shoots, whereas the enzymes in pentose phosphate and shikimate pathways did not show such significant increasing patterns. Similarly, the enzymes in TCA cycle did not increase but rather decreased during lignification[88]. From these results it is reasonable to consider that enzymes in the phenylalanine-cinnamate pathway situated at the locus quite near to the end product of lignin are activated rather than the enzymes in pentose phosphate and shikimate pathways situated at the early stages in the metabolic pathway leading to lignin.

In connection with the changes in enzyme activities, it is of interest to investigate behaviors of the esters of PCA and FA that are demonstrated to be natural precursors of lignins[118,121]. The intensive accumulation of free PCA and FA are already shown in Tables 20 and 21. Similar accumulation patterns for PCA and FA esters bound to cell wall constituents are given in Fig. 41. Fig. 42 shows relationships between PCA ester and lignin, and between FA ester and carbohydrate, indicating that PCA esters are exclusively bound to lignin molecules, whereas FA esters are to carbohydrate molecules. Therefore, PCA ester formed during the growth of the shoots is more correlated with lignin formation than FA ester.

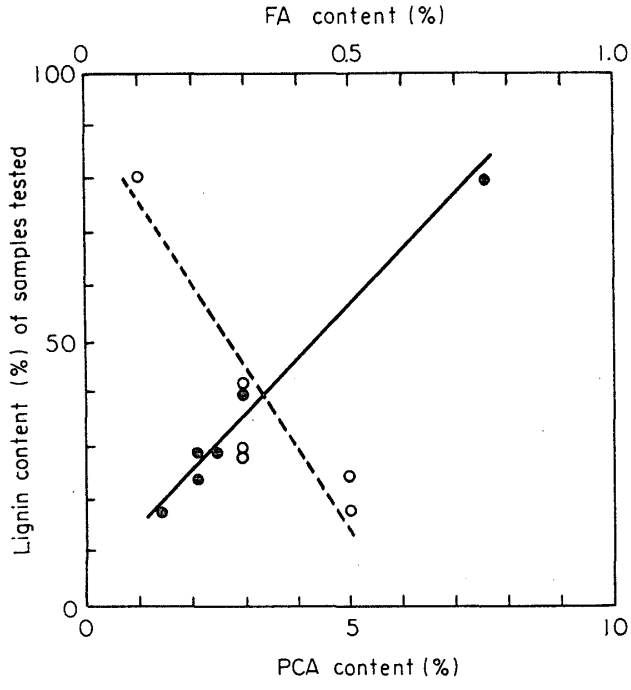


Fig. 42. The relationships between lignin content and PCA and FA esters contained in MWL and LCC fractions from bamboo meal (*P. pubescens*).
—●—, PCA; ···○···, FA.
Lignin content was determined by the acetyl bromide method and expressed as KLASON's lignin. The method of preparation of MWL and various LCC fraction is described in the text.

More interesting is the fact that the relative content of PCA ester based on KLASON's lignin increased from the apex, reaching a maximum at the locus around 150 cm and finally decreasing to the content (5–10 %) for mature bamboo as shown by a dotted line in Fig. 41. On the other hand, the, the ester content on dry weight of the shoot increased intensively from the apex to the basal parts, finally reaching the greatest amount (2–3 %) as shown by another dotted line (Fig. 41). This indicates that the greater majority of PCA esters are formed at the early stages of lignification, since the content of the ester in the young bamboo shoot was 2 to 3 times greater than that of the ester in the grown-up one. Accordingly, it may be assumed that PCA esters bound to lignin are heterogeneously distributed within cells wall, although merely average content of the ester was obtained by the conventional method to isolate milled wood lignin (MWL). The ester linkages of PCA is discussed in Chapter II[25].

Metabolic Regulation of Lignification

There are many factors influencing lignification. SIEGEL and his coworkers reported that IAA repressed lignin formation, indicating that IAA acts as an antioxidant to inhibit the oxidation of phenolic compounds catalyzed by peroxidase[122]. KOBLITZ recognized that kinetin increased lignin content from 20 to 30 % in carrot tissues[123]. BERGMAN[124] also obtained the similar results that lignin content in tobacco tissues grown under the presence of kinetin increased from 4 to 22 %, suggesting that this hormone could activate the enzymes in the pentose phosphate, the shikimate and the cinnamate pathways. Gibberellins are also considered to promote the lignin formation[125,126]. However, the physiological nature of the trigger for lignification is still obscure. The results described above on the variations of the enzyme activities point out that from a physiological aspect the activation or synthesis of the enzymes in phenylalanine-cinnamate pathway, e.g. the activation of PAL, is one of the most important factors to induce lignin formation. Because PAL is located at a diverging point on the metabolic pathways for "primary metabolites" (amino acid and protein) and "secondary metabolites" (phenolics including lignin). ZUCKER[127] reported that PAL in the potato tuber was induced by light irradiation although no PAL activity could be detected in the fresh tissue before the irradiation. YOSHIDA[128] also reported that the PAL synthesis was greatly enhanced by light in germinating pea seedlings, indicating that phytochrome and the related pigments are involved as an inducer in the enzyme appearance. A similar inducer may participate in the formation of PAL in growing bamboo shoots.

As another regulating factor for lignification, NADP level in tissues should be considered. As described above, the enzymes in pentose phosphate and shikimate pathways are NADP-specific and accordingly low level of NADP should repress the function of these enzymes. As factors to increase NADP level in plant tissues, oxygen, red light and kinetin have been known[129]. Although such many factors are involved in initiation of lignification, further investigations are needed on regulating mechanism for induction of enzyme synthesis during plant development.

VIII. Concluding remarks

The presence of *p*-coumaric acid esters in bamboo and grass lignins are characterized as a biological feature of Graminae plants. Methanolysis, thioglycolysis, and mild hydrogenolysis were found to be useful analytical methods for the determination of the position (α or γ) of *p*-coumarate esters in the side chains of lignin molecules. The analytical data obtained with model substances and natural lignins led the author to a conclusion that the majority of *p*-coumaric acid molecules are linked to the terminal γ-carbon atoms in the lignin. A possible model for the

formation of such esters were schematically shown in relation to the formation of lignin carbohydrate complex (LCC) (Chapter II).

Various labeled compounds including ferulic acid-O-$^{14}CH_3$ were administered to a bamboo and a grass to study the biosynthesis of syringyl lignin. It was found that ferulic acid was demethoxylated to *p*-coumaric acid in sliced bamboo tissues. However, the analytical data obtained by nitrobenzene oxidation and ethanolysis of the fed plant showed that ferulic acid-O$^{14}CH_3$ was incorporated into syringyl units as well as into guaiacyl units without randomization of the labeled methoxyl group. This finding supports the early indication that ferulic acid can serve as a precursor of syringyl lignin. Therefore, the hydroxylation whereby 5-hydroxyferulic acid is formed from ferulic acid can be regarded as a diverging step on the biosynthetic pathways to the lignins of angiosperms and gymnosperms (Chapter III).

O-Methyltransferases (OMT) were extracted from bamboo, poplar, callus tissues (angiosperm), pine and gikgo (gymnosperm) and general enzymic properties were studied. Particularly, the functions of those plant OMT's (substrate specificities) were discussed in relation to the formation of guaiacyl and syringyl lignins. The gymnospermous and angiospermous enzymes were named "mono-" and "di-function OMT", respectively. Because the former enzymes catalyze the methylation of caffeic acid to ferulic acid (guaicyl lignin precursor) but scarcely methylate 5-hydroxyferulic acid to sinapic acid (syringyl lignin precursor), whereas all the latters methylate the two natural substrates yielding both guaiacyl and syringyl lignin precursors. Thus, gymnospermous enzymes markedly differ in substrate specificity from comparable enzymes from angiospermous species. Consequently, the differences in the methoxyl patterns between the angiosperm (hardwood) and gymnosperm (softwood) lignins can be universally interpreted in terms of substrate specificities of "mono-" and "di-function OMT" (Chapters IV and V).

"Di-function OMT" has two activities, FA- and SA activities, consequently, yielding greater SA/FA ratio. On the other hand, "mono-function OMT" gives a very low SA/FA ratio. Relationships between the SA/FA ratios of various plant OMT's, the S/V ratios obtained from lignins, and distribution of MÄULE-positive or the negative plants were examined. The variations in the contents of guaiacyl and syringyl components of lignins within a single hardwood and between hard- and softwoods were reasonably explained in terms of the different levels of the enzymes such as "mono-" and "di-function OMT", and "FA 5-hydroxylase" that are a biochemcal factor determining the S/V ratios of various plant lignins. An attempt was made to classify a number of plant species forming lignins into 6 categories on the basis of dynamic changes in such enzyme levels (Chapter VI).

Changes in activities of the enzymes such as glucose-6-phosphate- and 6-phosphogluconate dehydrogenases involved in pentose phosphate pathway, dehydroquinate hydro-lyase and dehydroshikimate reductase in shikimate pathway, and OMT in cinnamate pathway were investigated in relation to lignification of growing bamboo shoots. It was found that OMT and phenylalanine ammonia-lyase were activated during the lignification rather than the enzymes in pentose phosphate and shikimate pathways that are situated at the early stages on the metabolic pathways to lignin. The variations in contents of lignin precursors such as shikimic acid, phenylalanine, tyrosine, *p*-coumaric acid, and ferulic acid were also investigated.

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References

1) T. HIGUCHI, Y. ITO, M. SHIMADA and I. KAWAMURA, Phytochem., **6**, 1551 (1967).
2) J. KUC and O. NELSON, Arch. Biochem. Biophys., **105**, 103 (1964).
3) D. C. C. SMITH, J. Chem. Soc., 2347 (1955).
4) D. C. C. SMITH, Nature, **176**, 267 (1955).
5) J. NAKANO, A. ISHIZU and N. MIGITA, J. Japan Wood Res. Soc., **4**, 1 (1958).
6) J. NAKANO, A. ISHIZU and N. MIGITA, Tappi, **44**, 30 (1961).
7) J. OKABE and K. KRATZL, Tappi, **48**, 374 (1965).
8) K. V. SARKANEN, HOU-MIN. CHANG and G. G. ALLAN, Tappi, **50**, 587 (1967).
9) K. V. SARKANEN, HOU-MIN. CHANG and G. G. ALLAN, Tappi, **50**, 583 (1967).
10) J. C. PEW and W. J. CONNER, J. Org. Chem., **34**, 585 (1969).
11) I. KAWAMURA and D. E. BLAND, Holzforsch., **21**, 65 (1967).
12) A. BJORKMAN, Svensk Papperstid., **59**, 477 (1956).
13) E. ADLER, in "Biochemistry of Wood", Proceedings of VIth internationai Congress of Biochemistry, Vienna (K. KRATZL and G. BILLEK, eds.), Pergamon Press (1958).
14) S. ZEISEL, in "Methods of Wood Chemistry" (B. L. BROWNING, ed.), Interscience Publishers, 1967. Vol. II, p. 660.
15) K. FREUDENBERG, K. SIEB and K. DALL, Chem. Ber., **62**, 1814 (1959).
16) W. H. HARTUNG, "Organic Reactions", N. Y. and London, John Wiley & Sons, 1960. Vol. VII, p. 660.
17) J. M. PEPPER, W. STECK, R. SWOBODA and J. C. KARAPALLY, "Lignin Structure and Reactions", ACS, 1966, p. 238.
18) H. MIKAWA and K. SATO, Kogyokagaku Zasshi, **57**, 605 (1954).
19) H. MIKAWA, K. SATO, C. TAKASAKI and K. EBIZAWA, Bull. Chem. Soc. Japan, **28**, 649 (1955).
20) K. FREUDENBERG, J. M. HARKIN and H. K. WERNER, Chem. Ber., **97**, 909 (1964).
21) E. ADLER, H. D. BECKER, T. ISHIHARA and A. STAMVIK, Holzforsch., **20**, 3 (1966).
22) B. O. LINDGREN and H. MIKAWA, Acta Chem. Scand., 8, 954 (1954).
23) E. ADLER, Paper and Timber, **43**, 634 (1961).
24) F. E. BRAUNS, in "Methods of Wood Chemistry" (B. L. BROWNING, ed.), Interscience Publishers, 1967. Vol. II, p. 720.
25) M. SHIMADA, T. FUKUZUKA and T. HIGUCHI, Tappi., **54**, 72 (1971).
26) S. A. BROWN and A. C. NEISH, Can. J. Biochem. Physiol., **33**, 948 (1955).
27) S. A. BROWN and A. C. NEISH, Can. J. Biochem. Physiol., **34**, 769 (1956).
28) S. A. BROWN and A. C. NEISH, J. Am. Chem. Soc., **81**, 2419 (1959).
29) T. HIGUCHI and S. A. BROWN, Can. J. Biochem. Physiol., **41**, 613 (1963).
30) K. KRATZL and G. BILLEK, Holzforschung, **7**, 66 (1953).
31) H. REZNIK and R. URBAN, Naturwissenschaften, **44**, 13 (1957).
32) H. REZNIK and R. URBAN, Naturwisaenschaften, **44**, 592 (1957).
33) S. Z. EL-BASYOUNI, A. C. NEISH and G. H. N. TOWERS, Phytochem., **3**, 627 (1964).
34) A. M. STEINER, Z. Pflanzenphysiol., **63**, 370 (1970).
35) T. HIGUCHI, M. SHIMADA and H. OHASHI, Agri. Biol. Chem., **31**, 1459 (1967).
36) M. SHIMADA, H. OHASHI, Phytochem., **9**, 2463 (1970).
37) H. MEIER and M. H. ZENK, Z. Pflanzenphysiol., **53**, 415 (1965).
38) D. HESS, Planta, **60**, 568 (1964).
39) T. HIGUCHI and S. A. BROWN, Can. J. Biochem. Physiol., **41**, 65 (1963).
40) M. SHIMADA, H. FUSHIKI and T. HIGUCHI, J. Japan Wood Rearch. Soc. (Mokuzai Gakkaishi), **18**, 43 (1972).
41) M. SHIMADA, H. FUSHIKI and T. HIGUCHI, Phytochem., **11**, 2247 (1972).
42) R. U. BYERRUM, J. H. FLOKSTRA, L. J. DEWEY and C. D. BALL, J. Biol. Chem., **210**, 633 (1954).

43) D. Hess, Z. Naturforschung, **19b**, 447 (1964).
44) B. J. Finkle and R. F. Nelson, Biochem. Biophys. Acta, **78**, 747 (1963).
45) B. J. Finkle and M. S. Masri, Biochim. Biophys. Acta, **85**, 167 (1964).
46) D. Hess, Z. Pflanzenphysiol, **53**, 1 (1965).
47) D. Hess, Z. Pflanzenphysiol, **53**, 460 (1965).
48) M. Shimada and T. Higuchi, Wood Research (Bulletin of the Wood Research Institute of Kyoto University), No. 50, 19 (1970).
49) K. Freudenberg and A. C. Neish, Molecular Biology Biochemistry and Biophysics II (Constitution and Biosynthesis of Lignins), Springer-Verlag (1968).
50) S. H. Mudd, Biochim. Biophys. Acta, **38**, 354 (1960).
51) J. D. Mann, H. M. Fales and S. H. Mudd, J. Biol. Chem., **238**, 3820 (1963).
52) S. Senoh, J. W. Daly, J. Axelrod and B. Witkop, J. Am. Chem. Soc., **81**, 6240 (1959).
53) M. Shimada, F. Fushiki and T. Higuchi, Phytochem., **11**, 2657 (1972).
54) R. H. J. Creighton, R. D. Gibbs and H. Hibbert, J. Am. Chem. Soc., **66**, 32 (1944).
55) B. Leopold, Acta Chem. Scand., **6**, 38 (1952).
56) I. Kawamura and T. Higuchi, Mokuzai Gakkai, **11**, 19 (1965).
57) W. Shweers, Holzforschung, **23**, 5 (1969).
58) M. Shimada, H. Fushiki and T. Higuchi, Mokuzai Gakkaishi, **18** (1972), in press.
59) W. D. Loomis and J. Battaile, Phytochem, **5**, 423 (1966).
60) J. Axelrod and R. Tomchic, J. Biol. Chem., **233**, 702 (1968).
61) K. Kaneko, Chem. Pharm. Bull., **10**, 1085 (1962).
62) J. Ebel, H. V. Achenvach, W. Barz and H. Grisebach, Biochim. Biophys. Acta, **215**, 203 (1970).
63) J. Ebel, W. Barz and H. Grisebach, Phytochem., **9**, 1529 (1970).
64) P. J. Anderson and A. D'ioro, Biochem. Pharmacol., **17**, 1943 (1968).
65) W. E. Hillis, Wood Extractives, p. 382, Pergamon Press, Oxford (1963).
66) E. P. Swan, K. S. Jiang and A. F. Gardner, Phytochem, **8**, 345 (1969).
67) M. K. Seikel and F. D. Hostettler, Tetrahedron, **24**, 1475 (1968).
68) F. E. Brauns and D. A. Brauns, The Chemistry of Lignin, Academic Press, N. Y. and London, p. 51 (1960).
69) G. H. N. Towers and R. D. Gibbs, Nature, **172**, 25 (1953).
70) K. Nishio, J. Japanese Bot., **37**, 243 (1962).
71) I. Kawamura and T. Higuchi, Mokuzai Gakkaishi, **11**, 19 (1965).
72) K. Kitao, Wood Research (Bulletin of the Wood Research Institute, Kyoto University), No. 34, 54 (1965).
73) H. Fushiki, T. Higuchi and M. Shimada, Unpublished data.
74) I. Kawamura and T. Higuchi, Mokuzai Gakkaishi, **8**, 148 (1962).
75) C. J. Venverloo, Holzforschung, **25**, 18 (1971).
76) T. Higuchi, Physiologia Plantarum, **10**, 633 (1957).
77) D. E. Bland, Holzforschung, **20**, 12 (1966).
78) J. Nakano, C. Takatsuka, Y. Taniguchi and N. Migita, Mokuzai Gakkaishi, **13**, 28 (1967).
79) B. J. Fergus and D. A. I. Gnring, Holzforschung, **24**, 113 (1970).
80) B. J. Fergus and D. A. I. Goring, ibid, **24**, 118 (1970).
81) T. Higuchi and F. Barnoud, Mokuzai Gakkaishi, **12**, 36 (1966).
82) J. P. Joseleau, Ph. D. Thesis, Univ. Grenoble, p. 52 (1965).
83) M. Shimada, T. Yamazaki and T. Higuchi, Phytochem., **9**, 1 (1970).
84) T. Higuchi and M. Shimada, Plant & Cell Physiol., **8**, 71 (1967).
85) T. Higuchi and M. Shimada, Agr. Biol. Chem., **31**, 1179 (1967).
86) T. Higuchi and M. Shimada, Plant & Cell Physiol., **8**, 61 (1967).
87) T. Higuchi and M. Shimada, Agr. Biol. Chem., **31**, 1459 (1967).
88) T. Higuchi and M. Shimada, Phytochem., **8**, 1185 (1969).
89) H. Beevers and M. Gibbs, Plant Physiol., **29**, 322 (1954).
90) M. Gibbs and H. Beevers, Plant Physiol., **30**, 343 (1954).
91) M. Gibbs and J. M. Earl, Plant Physiol., **34**, 529 (1959).
92) T. Higuchi, M. Shimada and K. Watanabe, Japan Wood Res. Soc., **13**, 269 (1967).
93) S. A. Brown and A. C. Neish, Nature, **175**, 688 (1955).
94) T. A. Geissman and T. Swain, Chem. & Ind., 984 (1957).

95) G. EBERHARDT and W. SCHUBERT, J. Am. Chem. Soc., **78**, 2835 (1956).
96) M. HASEGAWA, T. HIGUCHI and H. IHIKAWA, Plant & Cell Physiol., **1**, 173 (1960).
97) T. MINAMIKAWA, M. KOJIMA and I. URITANI, Plant & Cell Physiol., **7**, 583 (1966).
98) S. I. AHMED and T. SAWAIN, Phytochem., **9**, 2287 (1970).
99) S. A. BROWN, Science, **134**, 305 (1961).
100) S. A. BROWN, Can. J. Botany, **39**, 253 (1961).
101) M. R. YOUNG, G. H. N. TOWERS and A. C. NEISH, Can. J. Botany, **44**, 341 (1966).
102) O. L. GAMBORG and L. R. WETTER, Can. J. Biochem. Physiol., **41**, 1733 (1963).
103) J. KOUKOL and E. E. CONN, J. Biol. Chem., **236**, 2692 (1961).
104) A. C. NEISH, Phytochem., **1**, 1 (1961).
105) K. KOSHIMIZU and T. MITSUI, J. Agr. Soc. Japan, **30**, 63 (1956).
106) T. HIGUCHI, Agr. Biol. Chem., **30**, 667 (1966).
107) P. M. NAIR and L. C. VINING, Phytochem., **4**, 161 (1965).
108) P. M. NAIR and L. C. VINING, Phytochem., **4**, 401 (1965).
109) D. W. RUSSEL and E. E. CONN, Arch. Biochem. Biophys, **122**, 257 (1967).
110) N. AMRHEIN and M. H. ZENK, Z. Pflanzenphysiol., **64**, 145 (1971).
111) S. N. ACERBO, W. J. SCHUBERT and F. F. NORD, J. Am. Chem. Soc., **80**, 1990 (1958).
112) D. WRIGHT and A. C. NEISH, Can. J. Biochem. Physiol., **37**, 25 (1959).
113) T. HIGUCHI and F. BARNOUD, Chim. Biochim., Lignine Cellulose Hemicellulose, Grenoble, p. 255 (1964).
114) F. BARNOUD, T. HIGUCHI, J. P. JOSELEAU and A. MOLLARD, Compt. Rend., **259**, 4339 (1960).
115) T. HIGUCHI, Y. ITO and I. KAWAMURA, Phytochem., **6**, 875 (1967).
116) S. YOSHIDA and M. SHIMOKORIYAMA, Bot. Mag. (Tokyo), **78**, 14 (1965).
117) P. H. RUBERY and D. H. NORTHCOTE, Nature, **219**, 1230 (1968).
118) D. R. MCCALLA and A. C. NEISH, Can. J. Biochem. Physiol., **37**, 531 (1959).
119) S. A. BROWN and A. C. NEISH, J. Am. Chem. Soc., **81**, 2419 (1959).
120) T. HIGUCHI, Can. J. Biochem. Physiol., **40**, 31 (1962).
121) T. HIGUCHI and S. A. BROWN, Can. J. Biochem. Physiol., **41**, 65 (1963).
122) S. M. SIEGEL, P. FROST and F. PORTO, Plant Physiol., **35**, 163 (1960).
123) H. KOBLITZ, Flora (Jena), **154**, 511 (1964).
124) L. BERGMAN, Planta, **62**, 221 (1964).
125) R. GAUTHERET, Compt. Rend., **256**, 2071 (1963).
126) E. V. PARUPS, Can. J. Plant Science, **44**, 253 (1964).
127) M. ZUCKER, Plant Physiol., **40**, 779 (1965).
128) S. YOSHIDA, Shokubutsu Seiri, **7**, 33 (1968).
129) Y. YAMAMOTO, Shokubutsu Seiri, **7**, 47 (1968).
130) H. ERDTMAN, Research, **3**, 63 (1950).