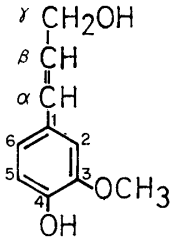
Review Article

Biochemistry of Lignification*

Takayoshi HIGUCHI**

Lignins, which comprise 17–33% of wood are complex aromatic polymers, and are generally classified into three major groups based on their structural "monomer" units. Gymnosperm lignins are dehydrogenation polymers of coniferyl alcohol (1). Angiosperm lignins are mixed dehydrogenation polymers of coniferyl and sinapyl alcohols (2), and grass lignins are composed of mixed dehydrogenation polymers of coniferyl, sinapyl and *p*-coumaryl alchols (3). Grass lignins also contain *p*-coumaric acid esterified to the Cγ-hydroxyl group of the side chains in the lignin polymer.



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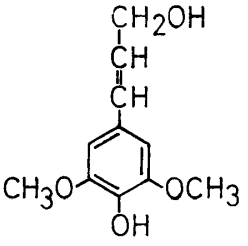

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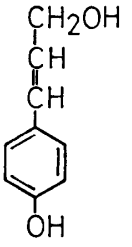

(3)

Tracer experiments[1] and UV-microscopic observations[2] have shown that lignification is initiated in the differentiating wood cells from the primary walls adjacent to the cell corners, and extends to the intercellular areas, primary and secondray walls. Tracer experiments have also established that lignin is synthesized from glucose via the shikimate-cinnamate pathways as shown in Fig. 1.

L-Phenylalanine ammonia-lyase (PAL)[3], which catalyzes deamination of L-phenylalanine to form *trans*-cinnamic acid, is known to be a key enzyme initiating phenolic metabolism in plant cells, and is synthesized during xylem differentiation.[4] L-Tyrosine can be converted to *trans-p*-coumaric acid only by grasses, which characteristically contain tyrosine ammonia-lyase (TAL)[5] in addition to PAL. Accordingly, gymnosperms and angiosperms except grasses can synthesize lignin from L-phenylalanine only, but grasses can do so from both L-phenylalanine and L-tyrosine. Cinnamic acid thus formed is hydroxylated to *p*-coumaric and caffeic acids successively by specific

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** Division of Lignin Chemistry

hydroxylases[6,7].

The conversion of caffeic acid to ferulic acid is catalyzed by *O*-methyltransferases (OMT) which are widely distributed in higher plants[8]. Ferulic acid thus formed may be hydroxylated to 5-hydroxyferulic acid, and 5-hydroxyferulic acid is methylated

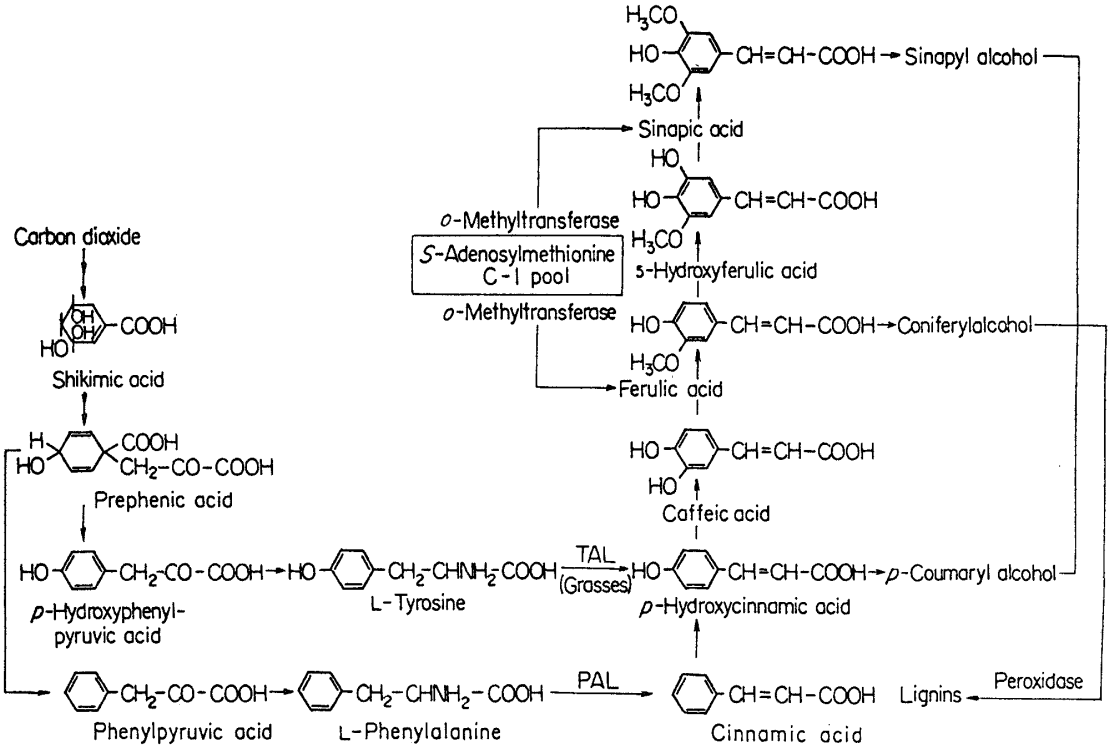

Fig. 1. Metabolic pathway of carbon dioxide to lignin

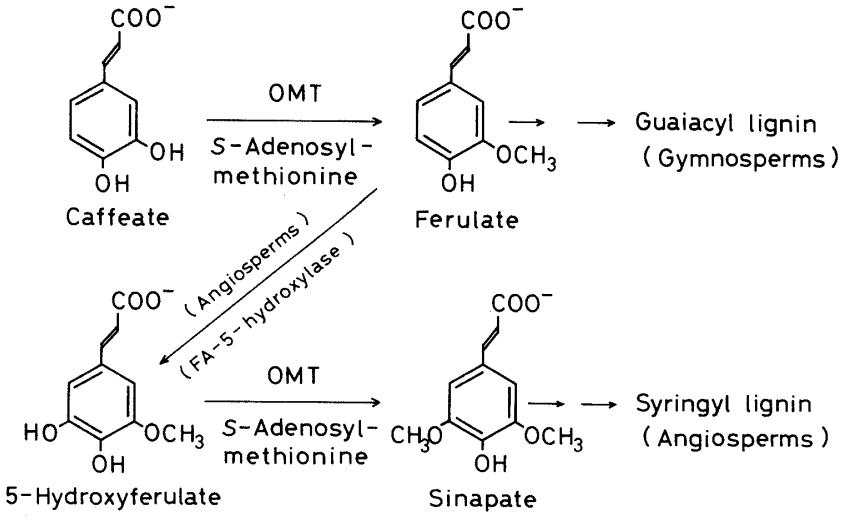

Fig. 2. Formation of sinapate from ferulate in angiosperms

again to sinapic acid[9]. 5-Hydroxyferulic acid is presumed on the basis of tracer experiments[10] to be the intermediate between ferulic and sinapic acids as shown in Fig. 2.

We[11,12] found that the gymnosperm OMT is almost inactive with 5-hydroxyferulic acid. In contrast, the OMT from angiosperms methylates both caffeic and 5-hydroxyferulic acids at approximately equal rates or at a higher rate with the latter. This result is in good accord with the distribution of guaiacyl lignin in gymnosperms and guaiacyl-syringyl lignins in angiosperms.

Ferulic and sinapic acids are then reduced to the corresponding cinnamyl alcohols by the successive mediation of three enzymes, hydroxycinnamate:CoA ligase, hydroxycinnamoyl-CoA reductase and hydroxycinnamyl alcohol oxidoreductase as shown in Fig. 3. These enzymes were recently isolated from various plants[13,14,15], and their occurrence in lignifying plants was shown to be a common feature. We[16] found, however, that gymnosperms can reduce ferulic acid to coniferyl aldehyde, but not sinapic acid to sinapyl aldehyde, whereas hydroxycinnamyl alcohol dehydrogenases isolated from gymnosperms and angiosperms have similar substrate specificities, and catalyze the reduction of both coniferyl and sinapyl aldehydes. Peroxidases from different plant taxa also have similar substrate specificities[17], and catalyze alcohols to form the lignin polymers.

These results indicate that the formation of guaiacyl lignin and not syringyl lignin in gymnosperms may be attributed to the following factors: absence of ferulate-5-

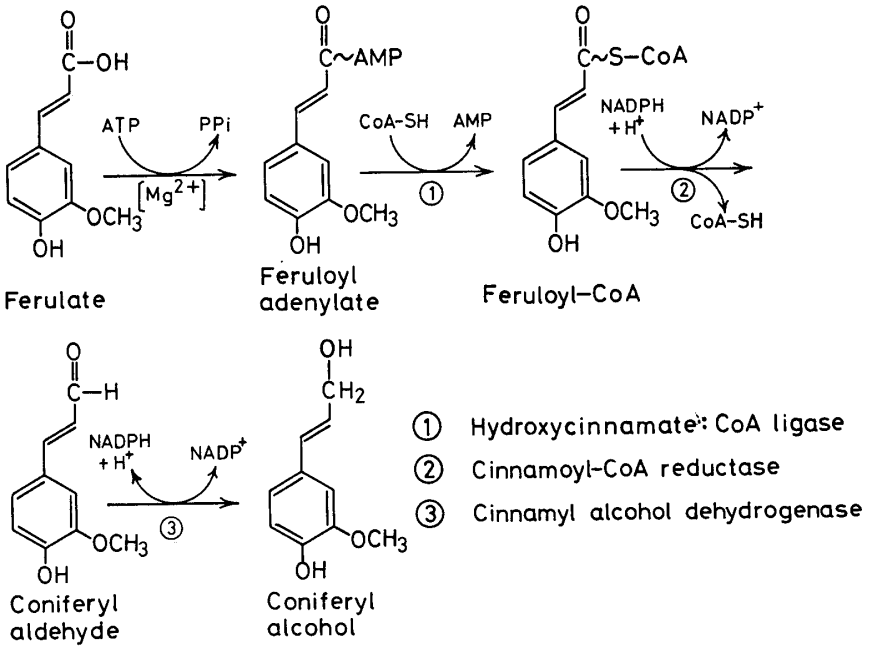

Fig. 3. Enzymic reduction of ferulic acid to coniferyl alcohol

hydroxylase, poor affinity of OMT towards 5-hydroxyferulic acid, and the lack of activation and/or reduction of sinapic acid.

It was shown by Freudenberg *et al.*[18] that conifer lignin is formed by enzymic dehydrogenation of coniferyl alcohol; the radicals of coniferyl alcohol formed by peroxidase or laccase couple, non-enzymically in a random fashion to give dimers ("dilignols"), trimers ("trilignols") and higher oligomers as racemic mixtures as shown in Fig. 4.

It was recently found by Gross[19] that hydrogen peroxide, the electron-accepting substrate for peroxidase in the dehydrogenative polymerization of coniferyl alcohol, is produced by the peroxidase itself, which is bound to cell walls. The reaction involves the superoxide radical, which was suggested to arise via reduction of oxygen by $NAD^{\cdot}$, which would be supplied by radical oxidation of NADH formed by malate dehydrogenase in cell walls.

Further dehydrogenation of the dimers, trimers and oligomers, and subsequent coupling of the resulting radicals, with the formation of a variety of interunit linkages, results in the formation of the lignin polymer. By this mode of formation, lignin would be expected to be optically inactive, as are natural lignins.

Fig. 5 shows a schematic consitutiton of spruce lignin based on dehydrogenation experiments of coniferyl alcohol and on analytical and degradative investigations of spruce lignin.

Freudenberg[20] reported that a mixture of coniferyl and sinapyl alcohols in equal amounts is enzymically dehydrogenated to a polymer similar to angiosperm lignin, and that sinapyl alcohol alone does not give a lignin-like polymer but yields mainly syringaresinol and dimethoxybenzoquinone. He suggested that the steric hindrance of two methoxyl groups of sinapyl alcohol inhibits β-O-4 coupling and results in β-β coupling to produce syringaresinol, and that no formation of syringyl lignin occurs in nature. However, we[21] found that considerable amounts of dehydrogenation polymer, DHP, are formed from sinapyl alcohol alone with peroxidase and H_2O_2. UV, IR, PMR and ^{13}C-NMR spectra, and functional group analysis of the polymer obtained showed characteristic features of syringyl lignin. Acidolysis of the polymer gave syringaresinol, with considerable amounts of typical acidolysis ketols, indicating the occurrence of the β-O-4 linkage, which is the most important structural unit of natural lignin polymers.

The results indicated that the phenoxy radicals of sinapyl alcohol, are coupled not only by the β-β mode to form syringaresinol, but also by the β-O-4 mode to produce a growing, polymeric syringyl lignin as shown in Fig. 6. We[22] further found that various 3,5-disubstitued *p*-coumaryl alcohols such as 3-methoxy-5-iodo-, 3,5-diiodo-, 3-methoxy-5-nitro-, 3,5-dinitro-, and 3,5-dimethyl *p*-coumaryl alcohols are dehydro-

genated to their dimers with ferric chloride; the yields of β-O-4 ethers of sinapyl alcohol and 3,5-diiodo-*p*-coumaryl alcohol were 85% and 86% of the dimers in dioxane (non-polar solvent), and 27% and 80% in acetone-water (polar solvent), respectively, (Table 1).

Steric effects of iodine, nitro and methyl groups are evidently larger than that of methoxyl group (Fig. 7), which suggests that the methoxyl group is not so large as to prevent β-O-4 coupling of the phenoxy radical. The racemization rate of the biphenyls *o*-substituted with methoxyl group is known to be faster than of those *o*-

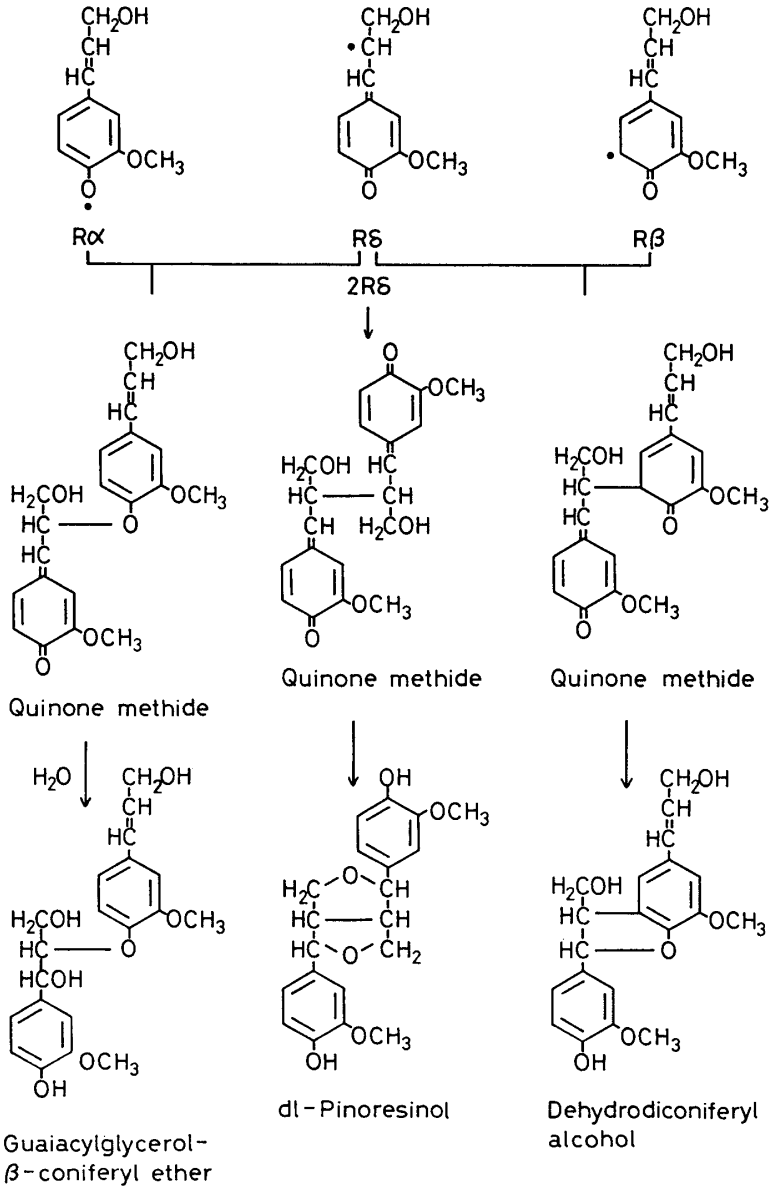

Fig. 4. Formation of dilignols via quinone methides

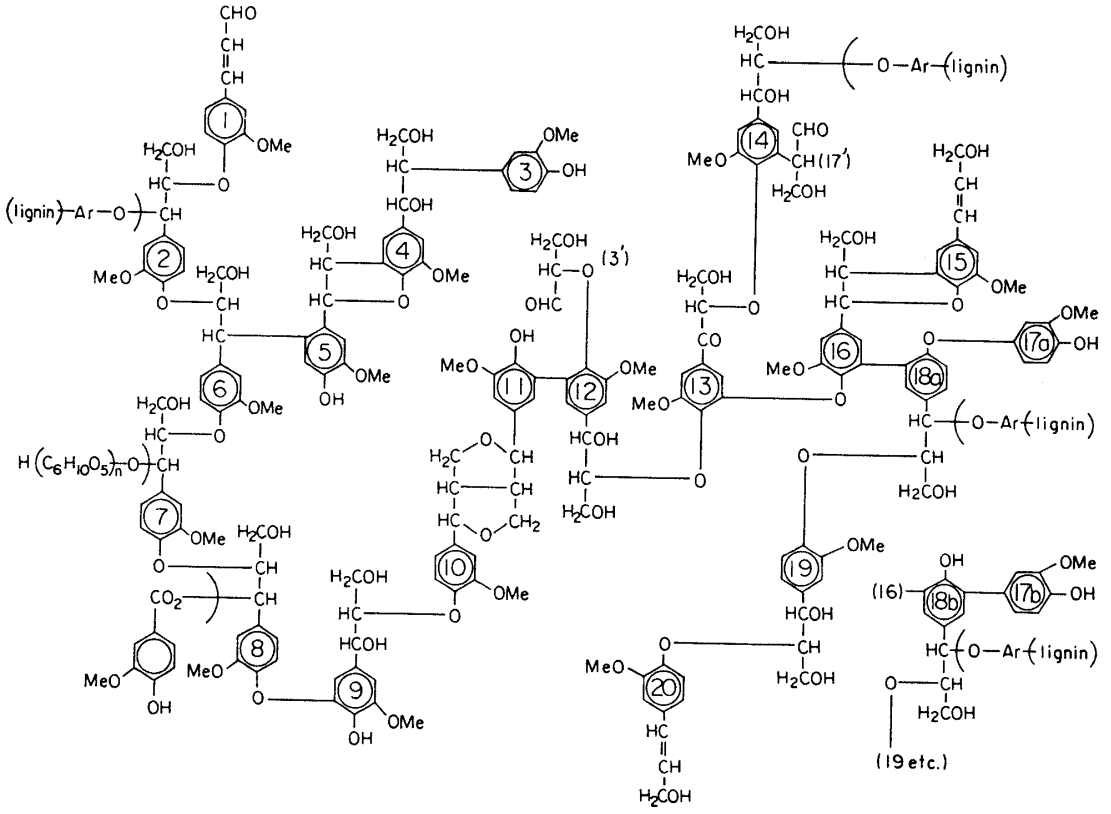

Fig. 5. A schematic constitution of spruce lignin

substituted with iodine, nitro or methyl groups, indicating that the methoxyl group is the least hindered substituent among the four groups. A higher yield of β-O-4 compound from 3,5-diiodo-*p*-coumaryl alcohol than from sinapyl alcohol in the polar solvent could be ascribed to the fact that the methoxyl group has a strong electron releasing effect whereas iodine has an electron attractive effect in the polar solvent.

We found that syringaresinol was formed from sinapyl alcohol in a 73% yield when acetone-water (3:40, v/v) was used in the dehydrogenation, whereas the alcohol gave syringylglycerol-β-sinapyl ether in 85% yield when dioxane was used as solvent.

Thus it is presumed that the most influential factor in the radical coupling of these alcohols is the electronic effect of the substituent groups on aromatic rings, and not steric hindrance.

Musha and Goring[23] proposed on the basis of spectral analysis of lignin in cell walls that birch lignin deposited in the secondary layers of wood fibers and parenchyma cell walls is composed mostly of the syringyl component. We[24] recently found that the solubility of acetoxymercurated DHP of sinapyl alcohol in acetic acid is considerably higher than that of coniferyl alcohol and that the solubility difference could be applied to the isolation of syrigyl lignin from hardwood lignins. Yamamomo (*Myrica rubra*

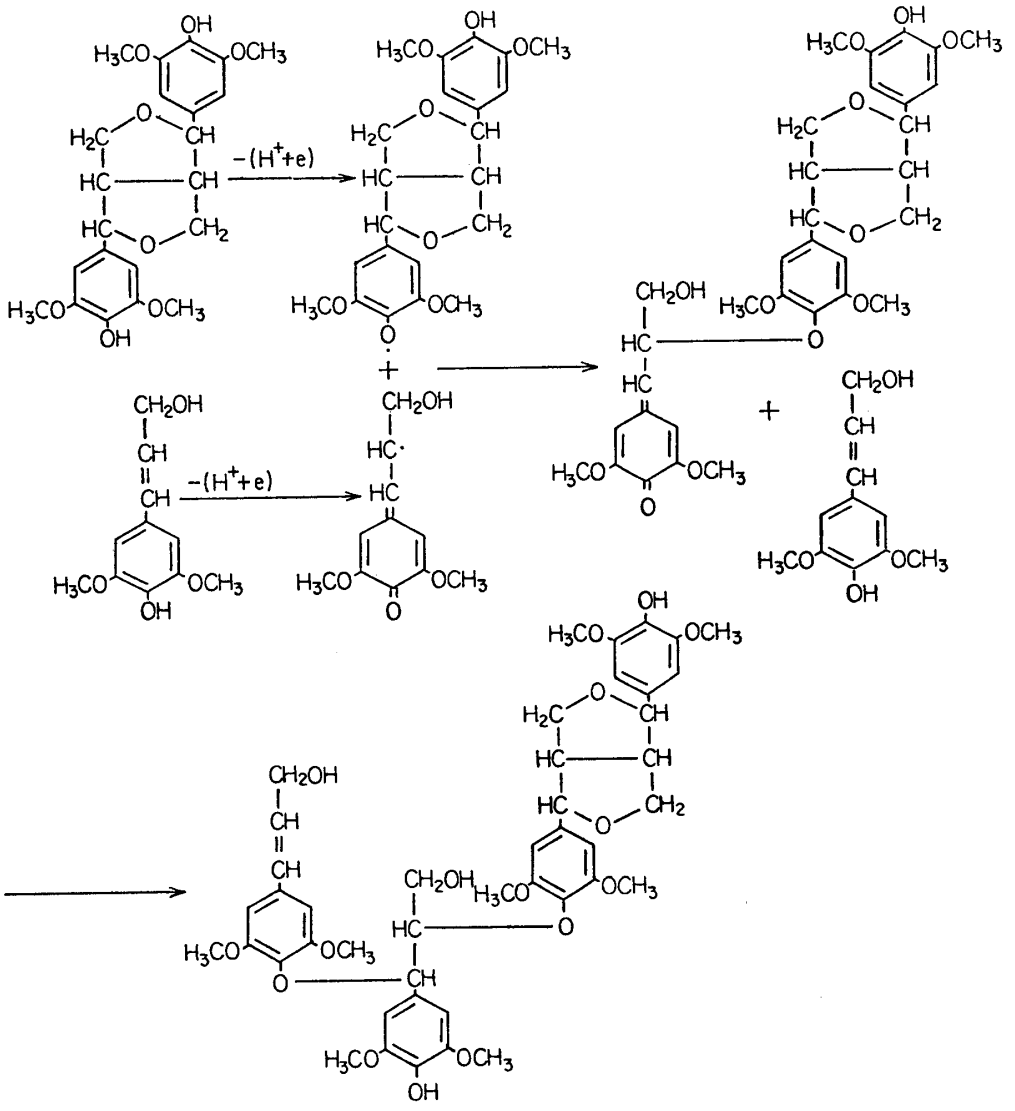

Fig. 6. Formation of dehydrogenation polymer of sinapyl alcohol with peroxidase and H_2O_2

Table 1. Ratio of β-ethers and resinols formed by dehydrogenation of 3,5-disubstituted *p*-coumaryl alcohols.

		β-Ether	Resinol
Peroxidase/H_2O_2	Sinapyl alcohol	9	91
$FeCl_3$ in dioxane-water (5:2)	Sinapyl alcohol	85	15
	3,5-Diiodo *p*-coumaryl alcohol	86	14
	3,5-Dimethyl *p*-coumaryl alcohol	58	42
$FeCl_3$ in acetone-water (3:40)	Sinapyl alcohol	27	73
	3,5-Diiodo *p*-coumaryl alcohol	80	20
	3,5-Dimethyl *p*-coumaryl alcohol	55	45

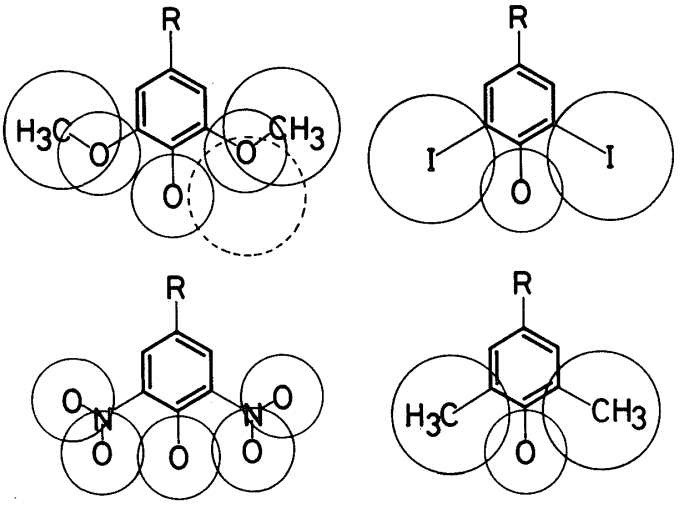

Fig. 7. Diagrams of steric hindrance of dual *o*-substituted phenoxy radicals. Fields of van der Waals of iodine, methyl and nitro groups are fixed because of only one single bond (the 2 formers) and the resonanced π-electrones (the latter). However, methoxyl group has two single bonds which can rotate around the aromatic C–O bond. Dashed circle shows the nearest position of methoxyl group to the phenoxy radical but that position is less possible.

Sieb. et Zucc.) Milled wood lignin* (MWL) could be separated into syringyl-rich ligin containing 25.2% OCH_3, which corresponds to a syringyl unit content of about 85% in the lignin. Beech dioxane lignin also gave a syringyl-rich fraction whose methoxyl content, 27.8%, corresponded to 95% syringyl units. The results obtained did not show the distribution of the syringyl-rich fraction in the respective layers of cell walls, but indicated that hardwood lignins are not a uniformly copolymerized guaiacyl-syringyl lignin. Clearly they are heterogeneously composed in part of a syringyl-rich fraction and a guaiacyl-rich fraction, in addition to the demonstrated syringyl-guaiacyl copolymer lignin.

Table 2. Methoxyl contents of various demercurated fractions and lignins treated with HCl[1)]

	I	II	IV	V	Lignin
Beech MWL	24. 5	22. 2	20. 1	—	20. 3
Beech dioxane lignin	27. 8	26. 8	22. 3	20. 7	20. 8
Yamamomo MWL	23. 9	25. 2	23. 1	21. 7	21. 8

1) Fractions were separated on basis of solubility in a 2:1 mixture dichloroethane: ethanol containing no acetic acid (Fraction I), and increasing amounts of acetic acid (Fraction II-V) (see Ref. 25).

* MWL is an isolated lignin purified from neutral, aqueous dioxane extracts of finely powdered (ball-milled) wood. It is representative of the lignin in the woody tissue.

p-Coumaric acid and *p*-hydroxybenzoic acid are known to be bound to grass lignin or poplar lignin by ester linkages. They comprise 5–10% of the weight of these lignins, and their presence distinguishes the lignins from that of other angiosperms. Very small amounts of vanillic, ferulic and syringic acids are also found as ester components of several hardwood lignins.

On the basis of alkaline hydrolysis and acidolysis experiments with model compounds such as veratryl *p*-coumarate, 3-(3,4-dimethoxyphenyl)propyl *p*-coumarate, and bamboo and poplar MWLs, we[26] found that about 80% of the *p*-coumaric and

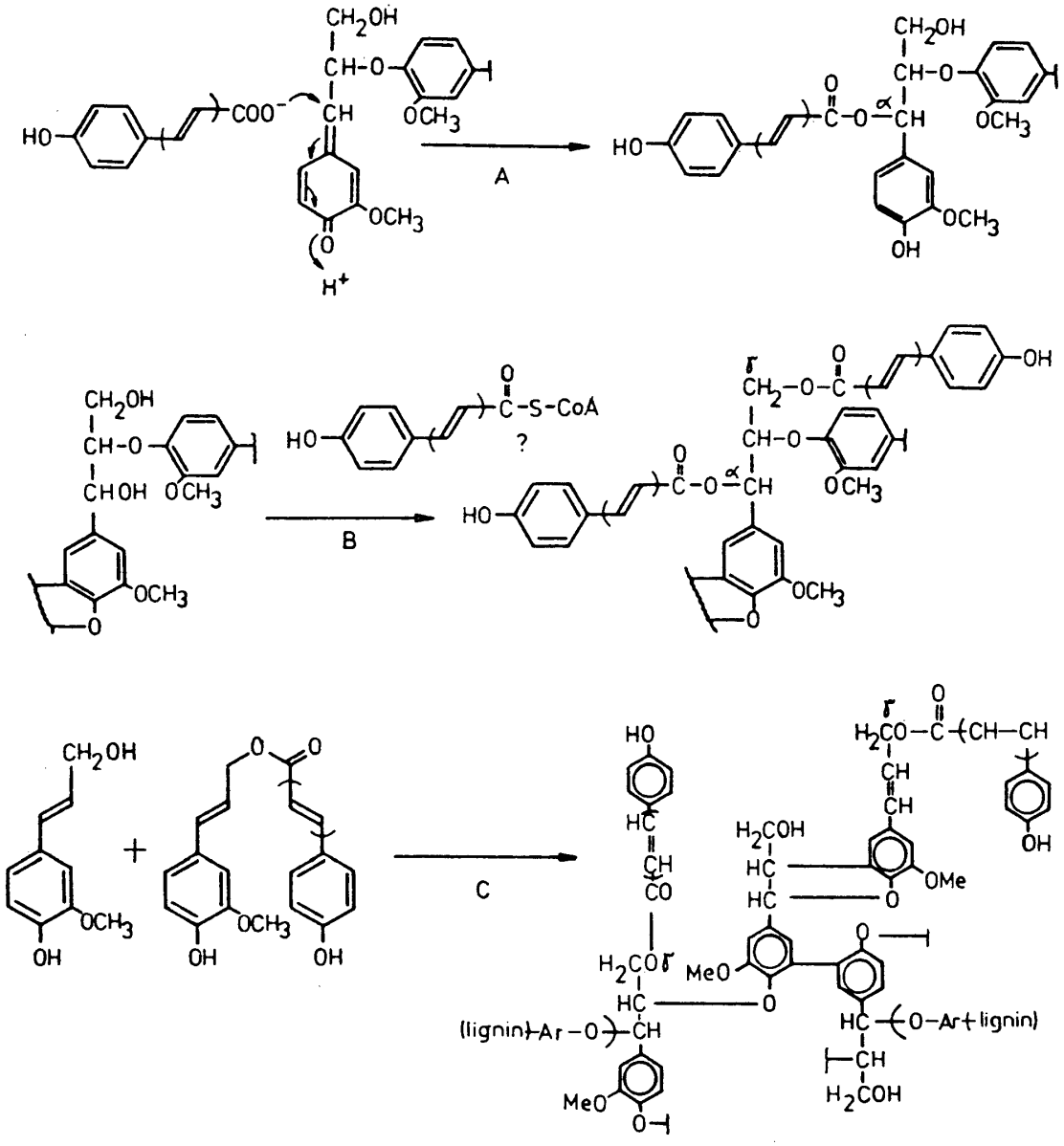

Fig. 8. Three possible mechanisms of formation of the α- and γ-esters of *p*-hydroxybenzoic and *p*-coumaric acids in poplar and bamboo lignins, respectively.

p-hydroxybenzoic acids in bamboo and poplar lignins are esterified via the γ-hydroxyl groups in lignin side chains; the amount of α-linked esters of these acids was estimated to be less than 20%.

For the formation of α- and γ-ester structures, three possible mechanisms are conceivable (A, B and C, Fig. 8). For the formation of the α-ester structure, routes A and B seem to be important; A—nucleophilic attack of carboxyl groups to the α-carbons of the quinone methides formed during the dehydrogenative polymerization; B—biochemical incorporation of the activated *p*-comarate or *p*-hydroxybenzoate to the α-hydroxyl groups in lignin oligomers or polymers; and C—dehydrogenative polymerization of coniferyl *p*-coumarate or coniferyl *p*-hydroxybenzoate formed coniferyl alcohol and the activated forms of the corresponding acids. Routes B and C could equally be concerned with formation of γ-ester structures, since γ-hydroxyl groups are present at the side chains of lignin monomers and polymers, in contrast to the α-hydroxyl groups, which are not present at the lignin monomers, being formed during the polymerization processes.

We[27] synthesized coniferyl *p*-coumarate and coniferyl *p*-hydroxybenzoate and submitted them to dehydrogenative polymerization with peroxidase and H_2O_2 by "Zulauf" and "Zutropf" methods, respectively[28]. These ester compounds were

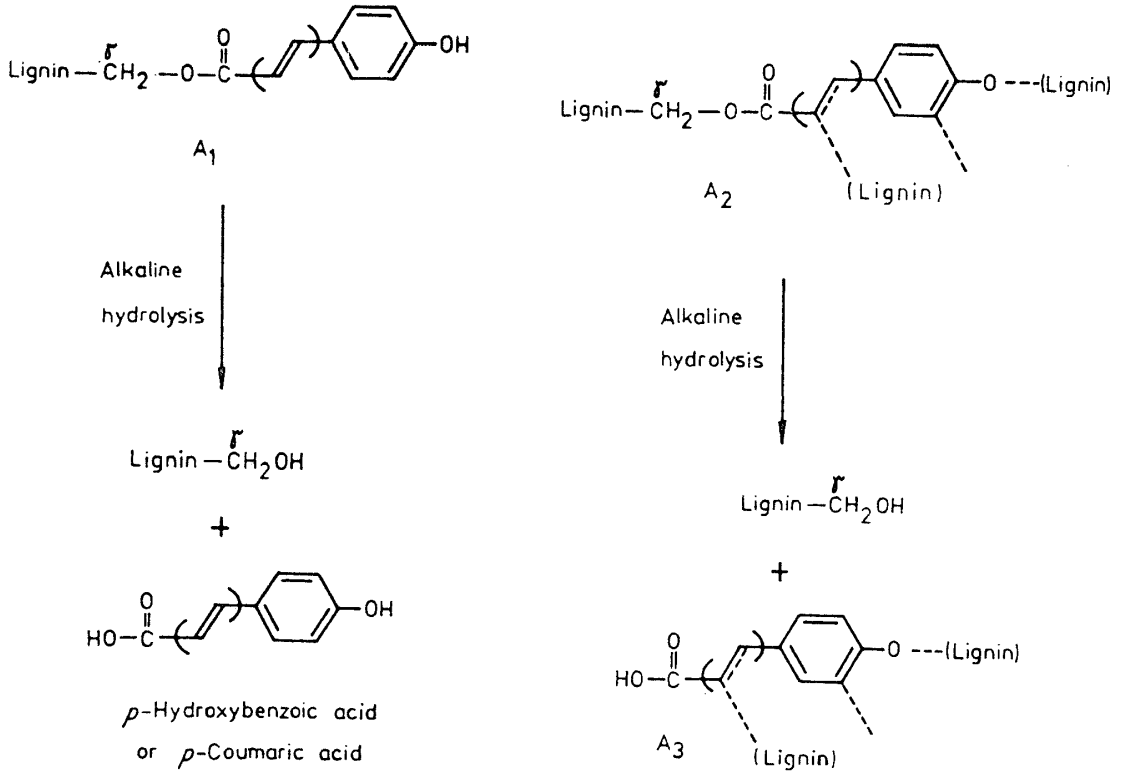

Fig. 9. A_1, A_2, and A_3 structures of *p*-hydroxybenzoic and *p*-coumaric acids.

easily polymerized in the presence or absence of coniferyl alcohol. Large portions of the *p*-hydroxybenzoic acid moiety, and some portions of the *p*-coumaric acid moiety were incorporated into DHPs as the ester in the A-type structure (Fig. 9), in which the acid fraction was found to the polymer only in the γ-ester linkage. In the case of coniferyl *p*-coumarate, the *p*-coumaric acid fragment of the ester, and *p*-coumaric acid itself liberated by hydrolysis of part of the ester compound during the dehydrogenation reaction, were further dehydrogenated, and formed the A2 and/or A3 structures in the DHPs. The acid fragments in A2 were connected to the polymer not only via the γ-ester linkages but also via ether or C-C bonds at phenolic-O, $C_5(C_3)$ in the aromatic rings, or Cβ in the side chains of the *p*-coumaric acid, while acid fragments in A3 were bound to the polymer in the same way as in the A2 structure, but without the γ-ester linkage.

The contents of the A1 structure in DHPs and in bamboo and poplar MWLs as determined by alkaline hydrolysis showed 5–16%. The optical densities of the characteristic peaks at 312–314 and 260–267 nm due to the A1 structure of *p*-coumarate and *p*-hydroxybenzoate corresponded closely to the actual amounts of the A1 structure. DHPs containing more than 10% of the A1 structure gave the well-defined bands of ester carbonyl at 1700 cm^{-1}, while the A2 and/or A3 structure in the DHPs of coniferyl *p*-coumarate did not affect their UV or IR spectra.

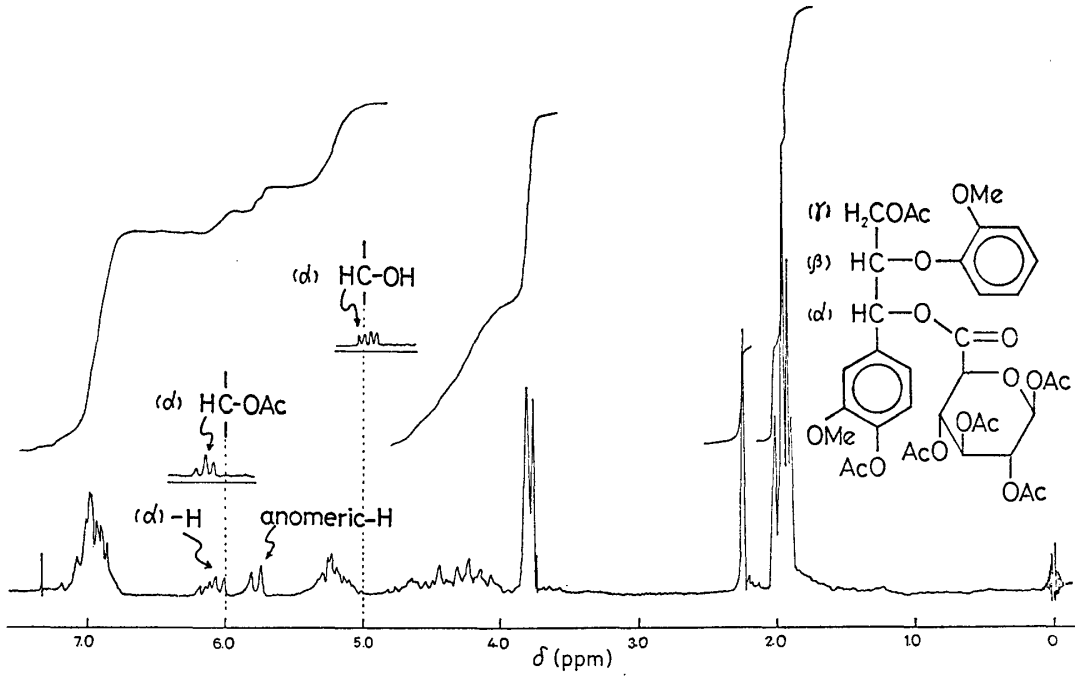

Fig. 10. NMR spectrum of guaiacylglycerol-α-[tetra-*O*-acetyl-β-D-glucuronate]-β-guaiacyl ether diacetate

The analytical and spectral data of the DHPs containing the γ-ester structures of *p*-hydroxybenzoic and *p*-coumaric acids are in good agreement with those of poplar or bamboo MWL, which suggests a possibility of the formation of these lignins via rout C (Fig. 8).

Freudenberg[29] found that sucrose is connected to Cα of quinone methide intermediates of DHP when coniferyl alcohol is dehydrogenated with manganese dioxide in sucrose DMF solution, and proposed that this type of ether linkage between carbohydrates and lignols may be the main pathway in which lignin is grafted onto carbohydrates of the cell walls.

We[30] recently found that when 1,2,3,4-tetra-*O*-acetyl-D-glucuronic acid and/or D-glucuronic acid was added to a solution of the quinone methide of guaiacylglycerol-β-guaiacyl ether (GGE) the product in which carboxyl groups of the uronic acids are linked by an ester linkage to Cα of GGE is formed (Fig. 10).

This result suggested that the C6 carboxyl group of glucuronic acid, which is acidic and which has the least steric hindrance of all the functional groups (hydroxyl), attacks preferentially the Cα of the quinone methide. It was also found that the quinone methide reacts with D-glucose and methyl-D-glycoside with almost equal rates but very slowly with 2,3,4,6-tetra-*O*-methyl-D-glucose, which suggests that the C1 hydroxyl

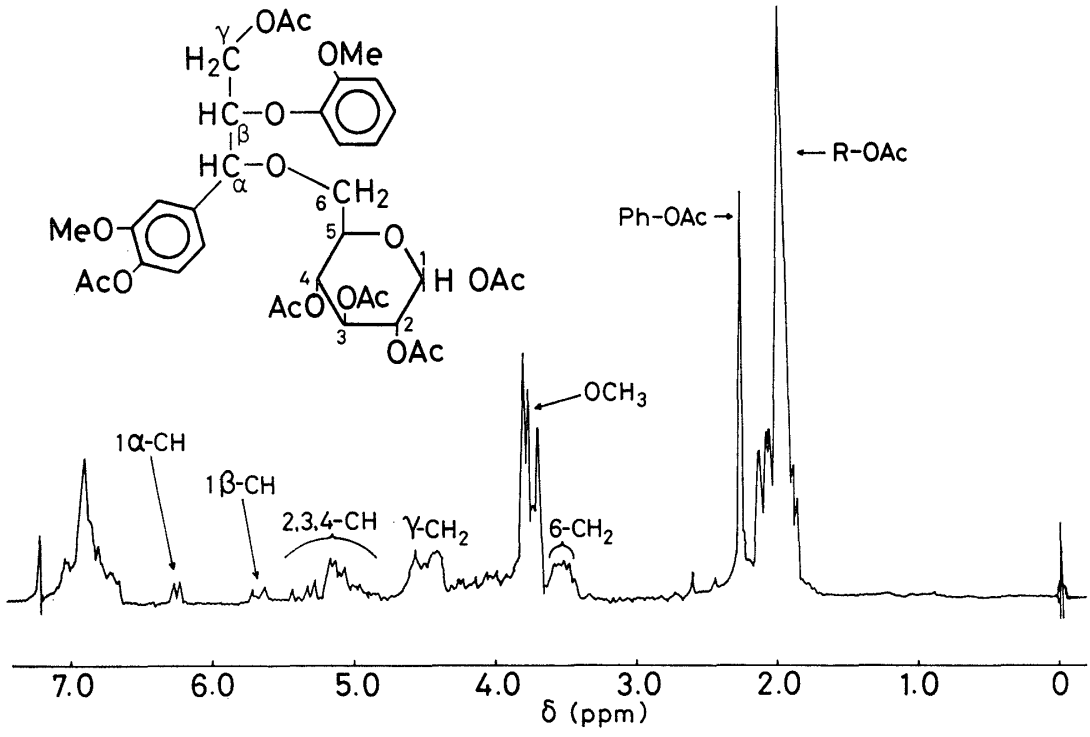

Fig. 11. NMR spectrum of guaiacylglycerol-α-[6′-*O*-(1′,2′,3′,4′-tetra-*O*-acetyl-*D*-glucose)]-β-guaiacyl ether diacetate in $CDCl_3$

group of glucose does not function as a major reactive site. Analyses of the reaction products proved that primary hydroxyl groups at C6 of the sugars are predominantly connected to Cα of the quinone methide via in ether linkage, and that no glycosidic linkages are formed; the NMR spectrum of the product, after acetylation, is given in Fig. 11.

The predominant formation of an ether linkage between the C6 hydroxyl of glucose and the quinone methide can be explained by the primary hydroxyl group at C6 has the least steric hindrance.

It was further found[31] that the quinone methide reacts with glucuronic acid remarkably well, and with glucose to a small extent, even in the presence of water. The water did, however, with the sugars in the reaction with the quinone methide, and the yields of the expected products, guaiacylglycerol-α-(D-glucuronate)-β-guaiacyl ether and guaiacylglycerol-α-(6′-*O*-D-glucose)-β-guaiacyl diether, decreased with increasing amounts of water in the reaction mixtures (Fig. 12, 13).

The difference in reactivity between D-glucose and D-glucronic acid and the quinone methide is probably ascribable to the degree of acidity of the hydroxymethyl and

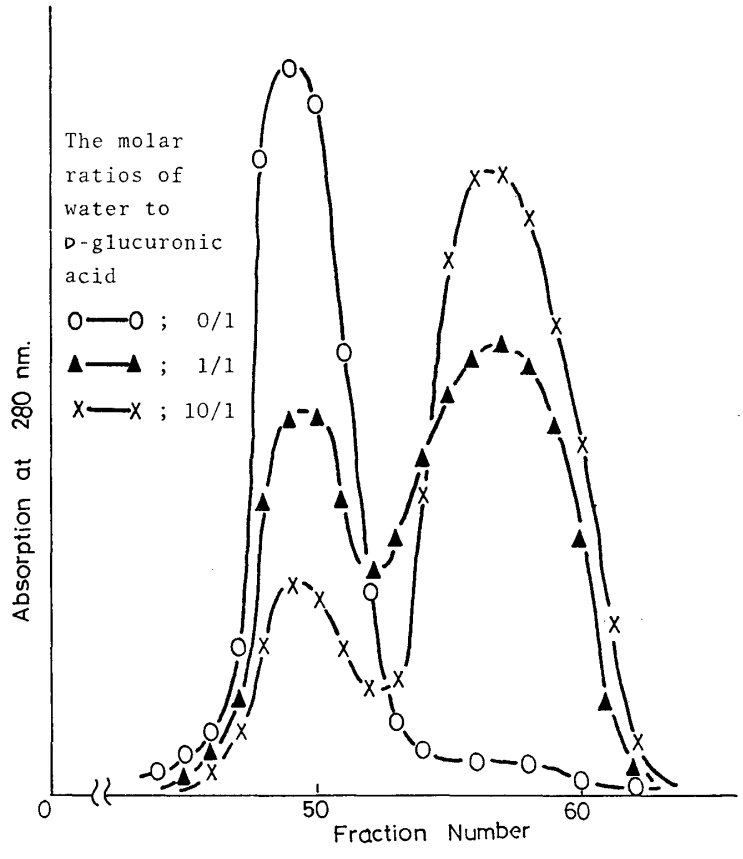

Fig. 12. Gel filtration curves of the reaction products of quinone methide with D-glucuronic acid.

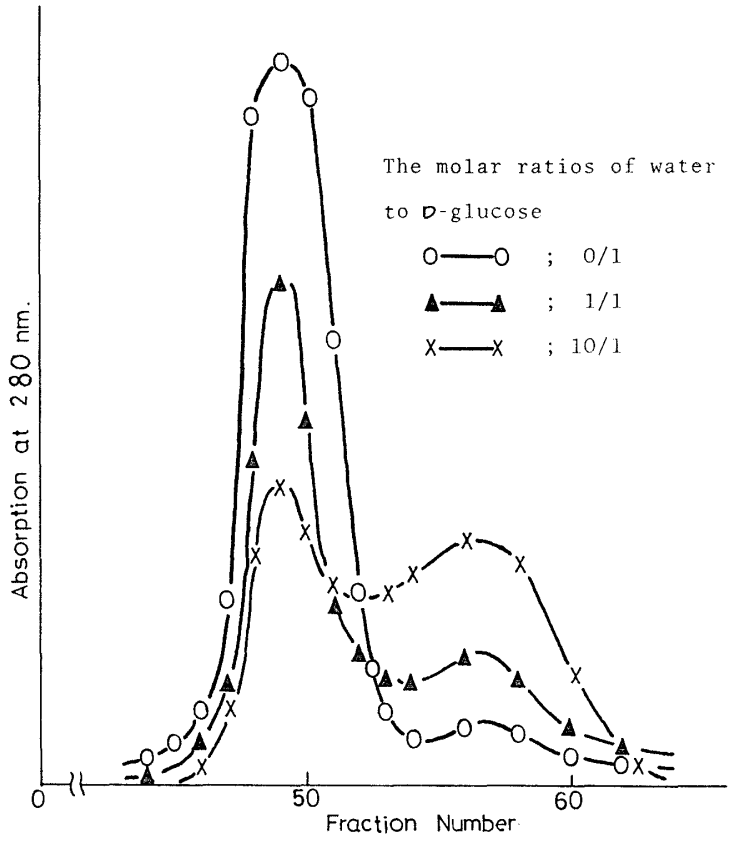

Fig. 13. Gel filtration curves of the reaction products of quinone methide with D-glucose.

carboxyl groups dissociated in water.

The ester and ether linkages of the sugars and lignol moieties of guaiacylglycerol-α-(tetra-*O*-acetyl-D-gluconate)-β-guaiacyl ether diacetate and guaiacylglycerol-α-(tetra-*O*-acetyl-D-glucose)-β-guaiacyl ether diacetate were found to be cleaved completely by mild acidolysis (50°C), acidolysis (100°C) and alkaline hydrolysis. Veratrylglycerol-α-(6′-*O*-methyl-2′,3′,4′-tri-*O*-methyl-D-glucoside)-β-guaiacyl-γ-methyl triether, however, was not cleaved by mild acidolysis, although it was cleaved completely by acidolysis, which seems to be related to the stability of the lignin-carbohydrate complex in the isolation process from cell wall materials[32].

It has been shown that Golgi bodies are involved in the biosynthesis of cell wall polysaccharides, but in lignin biosynthesis it is still unknown whether or not any organelles are involved in the transportation of lignin monomers to the cell walls, or where the lignin-carbohydrate linkages are formed.

However, it is conceivable that the addition reaction of sugars to quinone methide intermediates occurs in the same area as the dehydrogenative polymerization of lignin monomers. Acidic polysaccharides such as pectic substances, composed of galacturonic acid, would possibly react with quinone methide intermediates even in the presence of

water. Even the reaction between quinone methides and neutral sugars to give lignin-carbohydrate complex cannot be ruled out. This is because it is thought that the dehydrogenative polymerization of monolignols occurs on the outer cell membrane, which is closely overlaid with cell wall polysaccharides containing comparatively small amounts of free water.

It is plausible, alternatively, that lignin-carbohydrate linkages are formed by transglycosylation from nucleoside diphosphate sugars such as UDPG to the primary hydroxyl group at Cγ of the lignin side chain in plant cell wall; several sugar ethers of dilignols in which sugars are connected to the primary hydroxyl group of the diligonls by a glycosidic linkage have recently been identified among the extractives of conifers[33].

This paper is based on recent investigations related to the biochemistry of lignification by members of the Division of Lignin Chemistry, Wood Research Institute. The author is indebted to Dr. Y. Nakamura, Sanyo-Kokusaku Pulp Co., Ltd; to Dr. T. Yamasaki, Faculty of Agriculture, Kagawa University, and to all members of the Division of Lignin Chemistry, for their cooperation in the course of these investigations.

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