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The Present State of the Problem of Lignin-Carbohydrate Bonds in Plant Tissues

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The studies of the last 10-15 years showed that chemical and hydrogen bonds between lignin and carbohydrates and also three-dimensional network structures of lignin immobilising the carbohydrate chains are involved in the formation of a single set of components of the plant cell wall. This review presents data demonstrating the presence of the most likely types of lignin-carbohydrate bonds: ester, ether, acetal, and phenol-glycoside. However, the problems concerning the type, nature, and number of lignin-carbohydrate bonds still remain open. The possible inhomogeneity of the lignin in plant tissues complicates further the solution of these problems.
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I. INTRODUCTION

During the last 10–15 years considerable advances have been achieved in the chemistry of lignin. The Swedish chemist Björkman developed a method for the isolation of lignin, which consists in the mechanical milling of the lignified material in a ball vibromill and does not involve the use of acid or alkali, thereby yielding a product closest to the "natural" material, i.e. lignin not isolated from plant tissue. The German chemist Freudenberg achieved the laboratory synthesis of a "dehydropolymer" in many respects similar to natural lignin from conifer wood. This preparation was obtained by dehydrogenating *in vitro* a very dilute mixture of coniceryl, *p*-coumaryl, and sinapinic alcohols under the action of laccase (a dehydrogenase). The problems of the biosynthesis of lignin in plants have been elucidated to a considerable degree. There have been undoubted advances in the study of the structure of lignin, its functional groups, and the types of its intramolecular bonds, as well as in the study of processes associated with the modification of lignin in the sulphite and sulphate pulping of wood and in its chlorination for the bleaching of cellulose. Some progress in the industrial utilisation of lignin, which is a large-scale by-product of the cellulose, paper, and hydrolytic industries, may also be noted.

However, despite the appreciable and unquestionable advances in the chemistry of lignin, a number of problems still give rise to a lively controversy among investigators in this field, and these are some of the most important problems in both theory and practice. In the first place this applies to the problem of the lignin–carbohydrate bonds in plant tissues and the related problems of the quantitative content of lignin in lignified material.

Indeed, as regards lignin–carbohydrate bonds, not only the problem of the type, nature, and number of chemical bonds of this kind in plant tissues but the very fact of the presence of such bonds is controversial. Nevertheless, the problem of whether lignin is linked to the carbohydrate components of plant tissues by chemical bonds or whether it is present in a free state, i.e. in a mechanical mixture with polysaccharides, is not only of theoretical interest but is also important for the quantitative separation of plant components in order to analyse them and understand various delignification processes in wood. It is noteworthy that in the chemistry of lignin there is still no single standard method for the quantitative determination of the content of lignin in plant materials, which naturally results in an appreciable discrepancy between results obtained using different methods and between the conclusions based on the results.

The problem of the presence or absence of chemical bonds between lignin and carbohydrate components of plant tissues has been the subject of controversy for more than 100 years. At the beginning two opposed views on this problem were in fact put forward: in 1838 Payen put forward the so called "incrustation theory" on the basis of the fact that cellulose in cell walls does not give rise to characteristic reactions until the non-cellulose material has been removed, namely that wood is a physical mixture of components and that cellulose is subjected to mechanical incrustation by lignin, while in 1866 Erdmann [7,8] postulated the existence in plant tissues of "glycolignose" in which cellulose is linked by an ester or ether bond to a non-carbohydrate component (lignin). In the following period a considerable number of data supporting one or other theory were published. However, in the last decade the majority of investigators clearly tend to recognise the presence of a chemical lignin–carbohydrate bond in plant tissues. A comprehensive review of the literature on this problem was carried out by Merewether [9], who examined in detail data on the morphology of the cell wall and microbiological degradation of wood and also data obtained in various chemical reactions of woody tissues and lignin.

During the ten years which have elapsed since the publication of Merewether's review, a considerable number of interesting data concerning the bonds between the components of plant tissues have been obtained and are discussed below. However, although stress has been laid on studies published in the last decade, in some cases earlier publications have been referred to make the exposition more complete.

2. CHEMICAL BONDS BETWEEN LIGNIN AND CARBOHYDRATE COMPONENTS OF PLANT TISSUES

The existence of lignin–carbohydrate complexes (LCC), consisting of soluble fragments of wood containing both lignin and carbohydrates in a form not separable by physical methods, has now been firmly established.

Lignin–carbohydrate complexes have been isolated by heating wood with water [10,11], on chlorination [10,12], alcoholysis [10,13,14], acetylation [15,16] and ozonolysis [17] of wood, in the sulphite [18–21], sulphate [22], soda [23–25], and chlorite [26] pulping of wood, and also as a result of the enzymatic breakdown of wood [27–31] and its treatment with a neutral aqueous solvent [32]; however, the major advances in the isolation and investigation of these complexes are undoubtedly due to Björkman.

In 1954 Björkman [33] proposed a new method for the isolation of lignin from lignified materials. After preliminary extraction, fir wood sawdust was ground in a ball vibromill in toluene or another solvent which does not cause the swelling of the material. Extraction of the ground wood with moist dioxan permitted the isolation of up to 50% of its lignin content in the form of mechanically ground lignin (MGL). [34] Further extraction of the ground wood with dimethylformamide, dimethyl sulphoxide, and water gave an additional amount of material containing, together with lignin, also a considerable amount of carbohydrates, the components being separated only by acid hydrolysis [35]. Fractionation of the extracts yielded a series of lignin–carbohydrate complexes containing between 16 and 34% of lignin. The composition of the carbohydrate component was always the same and corresponded to the composition of wood hemicelluloses (Table 1).

Table 1. Carbohydrate composition (rel. %) of hemicelluloses and lignin–carbohydrate complexes extracted with dimethylformamide from various species of wood (according to the data of Björkman [35]).

Carbohydrates	Fir (*Picea abies*)		Pine (*Pinus silvestris*)	Birch (*Betula verrucosa*)	
	LCC	Hem.	LCC	LCC	Hem.
Galactan	10	8	15	9	4
Glucan	17	18	25		7
Mannan	50	46	32	3	7
Araban	4	4	13	2	7
Xylan	19	24	15	86	75

LCC = lignin–carbohydrate complex; Hem. = hemicellulose (Ed. of Translation).

Further grinding of the isolated lignin–carbohydrate complexes in the vibromill yielded 40% of their lignin content in the form of MGL, identical in elemental and functional group composition and also in molecular weight to the MGL isolated in the grinding of wood [36]. Moreover, Björkman [35] carried out special experiments to establish whether the complex is formed during the grinding process. After the usual treatment in the vibromill of a mixture of MGL and hemicelluloses from *Picea abies* in proportions of 1 : 3, about 85% of the initial lignin was isolated. On this basis, it was concluded that the lignin–carbohydrate complexes are present in the wood and are not formed during its grinding in the vibromill.

The latter finding was convincingly confirmed by the data of Lindgren [37], who investigated the electrophoretic mobility of MGL and the lignin–carbohydrate complexes isolated from fir wood by Björkman's method. In the electrophoresis of the fraction designated by Björkman as the lignin–carbohydrate complexes, two subfractions were obtained: a slower carbohydrate fraction and a faster fraction containing both lignin and carbohydrates. The latter give a positive response to the test for lignin and yielded on hydrolysis glucose, mannose, and xylose. The relative amounts of lignin and carbohydrates in the fraction were about 1 : 1. Moreover, Lindgren established that MGL obtained in a preliminary grinding of the lignin–carbohydrate complexes had an electrophoretic mobility approximately equal to that of the MGL isolated initially, which constitutes yet another confirmation of the formation of MGL as a result of the dissociation of the lignin–carbohydrate complexes.

Thus, as a result of grinding in the vibromill, it is possible to isolate up to 50% of the lignin content in the wood in the form of MGL. The difficulty of extracting large amounts of lignin by neutral solvents can be accounted for, according to Björkman [34], either by the existence of strong chemical bonds between the lignin and carbohydrates or by the high molecular weight of the lignin and the possibility of the formation of three-dimensional network structures. Björkman also pointed out the possibility of the effect of weaker forces, namely hydrogen bonds, since the solvents employed for the isolation of MGL and the lignin–carbohydrate complexes should exhibit a high capacity for the formation of hydrogen bonds, which is necessary for the dissociation of the lignin–carbohydrate hydrogen bonds.

Hayashi and Tachi [38] showed that dilute alkali can extract from wheat straw powder a lignin–xylose complex, the so called "xylolignin" which breaks down after prolonged treatment with alkali. A similar complex is extracted with butanol, acetone, or ethanol containing water and sodium hydroxide. It has been established that the carbohydrate component of the complex, consisting mainly of xylose, contains small amounts of glucose and arabinose. Acetylated xylolignin has been extracted from acetylated wheat straw powder with acetone and chloroform [39,40]. The carbohydrate part of this complex has a composition analogous to that of the carbohydrates of xylolignin. Hydrolysis of acetylated xylolignin with a methanol solution of sodium hydroxide results not only in deacetylation but also in the dissociation of the lignin–carbohydrate bonds [41]. In subsequent communications Hayashi and Tachi [42–45] showed that dioxan, 50% acetic acid, and dimethylformamide extract lignin–carbohydrate complexes from wheat straw powder without preliminary grinding in the vibromill. The composition of the carbohydrate component (xylose, glucose, arabinose, and galactose) of the lignin–carbohydrate complexes extracted by these solvents differed from that of xylolignin.

Thus glucose predominated in the lignin–carbohydrate complexes extracted with dimethylformamide and the complexes extracted with dioxan and aqueous acetic acid, in which xylose constituted the major part of the carbohydrates, were enriched in galactose and arabinose compared with xylolignin. On the basis of these investigations, the authors [44] suggest the presence in wheat straw of lignin–carbohydrate complexes of different types.

Brownell and West [46] proposed a comparatively mild procedure for the dissolution of both lignin and wood carbohydrates without the application of strong acids, alkalies, or oxidising agents. After preliminary extrac-

tion with an alcohol–benzene mixture, air-dry sawdust was ground for 21 days in the usual rotary ball mill. As a result of the grinding, the degree of polymerisation of carbohydrates fell to about 40†. Then the ground wood was dissolved by treatment with epoxyethane in dimethylformamide. The dissolved hydroxyethylated wood was subjected to fractional precipitation, separation by paper and column chromatography, electrophoresis, and partition between two immiscible liquids. The investigation established that the main part of lignin cannot be freed from carbohydrates under such conditions. In subsequent publications Brownell [47–50] reported a method for the separation into components of the wood ground in a ball mill. This method consists in the separation of ground wood into components in the system aqueous solution of sodium thiocyanate–benzyl alcohol–dimethylformamide. Lignin and a lignin–carbohydrate complex analogous to the preparations obtained by Björkman's method were obtained, and the stability of the lignin–carbohydrate complex under conditions of various physical and chemical treatments was investigated [47,48]. Although both Brownell [47,48] and Björkman [35] believed it to be established that the lignin–carbohydrate complex is not formed during the grinding by free-radical grafting, we believe that this conclusion requires additional experimental confirmation, since in the course of intense mechanical degradation (grinding in a vibromill or a rotary ball mill) chemical bonds of different types are broken and the resulting macroradicals are capable of recombination, which may lead to the formation of new chemical compounds differing in bond type and composition from the initial polymers.

The fact that the lignin–carbohydrate complexes can be isolated from wood by a wide variety of methods, which include many different types of chemical treatment, grinding in a vibromill or ball mill, and enzymatic breakdown of lignified materials undoubtedly indicates that at least part of the wood exists in the form of lignin–carbohydrate complexes. At the same time the impossibility of isolating the individual components of the lignin–carbohydrate complexes in a pure form without the use of powerful chemical reagents (acids and alkalies) or without the application of mechanical energy, light energy, irradiation, or ultrasonication constitutes one of the most convincing proofs of the existence of chemical bonds between lignin and carbohydrates in plant tissues.

Interesting data concerning the presence of lignin–carbohydrate bonds were obtained in recent years in studies on lignin by physical methods (ESR spectra, infrared spectroscopy, etc.). Rex [51] showed that lignin does not contain free radicals before isolation from plant tissues. At the same time in isolated lignins free radicals persist for a long time during humification and coal formation. Kleinert and Morton [52] observed that free radicals are present in wood subjected to grinding, ground wood giving an ESR signal approximately ten times more intense compared with ground cellulose. On this basis, the authors concluded that the presence of free radicals is due to the presence of lignin which has a more rigid structure than cellulose. It was suggested that during grinding both carbon–carbon and carbon–oxygen ester bonds are broken. Steelink et al. [53] showed that lignins isolated by treating plant tissues with acids, alkalies, and enzymes or as a result of mechanochemical degradation of plant materials give rise to fairly considerable ESR signals.

†The degree of polymerisation of wood cellulose is 2500–3000 (by ultracentrifugation).

According to the view of Chudakov et al. [54], these data indicate that free radicals are formed in lignin as a result of the dissociation of the lignin–carbohydrate bonds. Chemical, enzymatic, or mechanochemical treatments of plant tissues can give rise to the formation in lignin of free radicals such as the aroxy-radicals, owing to the dissociation of β-phenyl glycoside bonds, which the authors believe to be the most probable type of bond between lignin and carbohydrates. Another possible explanation [53] is that, as a result of the above treatments, lignin is demethylated and the preparations obtained contain *ortho*-quinonoid and quinomethane structures, which may serve as sources of free radicals.

Hachihama and Takamuku [55] investigated the effects of γ-radiation on wood and lignin and established that irradiation of the powdered wood of the red pine with γ-rays (4×10^6–10^8 r.e.p.) appreciably reduces its content of holocellulose and α-cellulose but has only an insignificant effect on the lignin content. Sulphite pulping of wood subjected to the action of γ-radiation increases the yield of lignosulphonic acid by a factor of three compared with the control specimen (the digestion was carried out under the conditions adopted by the authors). At the same time lignin from irradiated wood has properties analogous to those of lignin from ordinary wood. Irradiation of wood results also in an increased yield of "Braun's lignin", which can be extracted with 95% ethanol, and also an increase in the yield of lignin–carbohydrate complexes [56]. The latter were extracted with dimethylformamide from irradiated wood after the removal of the Braun's lignin. The extraction was carried out over a period of 12 days at room temperature. The yield of lignin–carbohydrate complexes increased from 0.3% (relative to the initial wood) for the non-irradiated specimen to 1.7% (after irradiation with 8×10^7 r.e.p.) and 6.9% (after irradiation with 1.1×10^8 r.e.p.). It was established that the content of carbohydrates in the lignin–carbohydrate complexes varies appreciably in different fractions, while their composition is almost identical and corresponds to that of the wood hemicellulose. The results were interpreted by the authors on the basis that γ-radiation causes partial dissociation of the lignin–carbohydrate bonds.

Information about lignin–carbohydrate bonds was also obtained in a spectroscopic study of wood and its components. Bolker [57,58], who investigated the infrared spectra of different types of wood, wood celluloses containing different amounts of residual lignin, and also isolated lignin, established that all the isolated lignins give rise to an absorption band at 1710 cm^{-1} (±15 cm^{-1}) with a variable intensity, which depends on the method of their isolation, but the band is absent from all the spectra of various types of wood and celluloses. The value of Bolker's work consists primarily in the fact that, by applying differential spectroscopy (an equivalent amount of holocellulose isolated by a standard method was used as a reference), it proved possible to obtain the infrared spectra of lignin contained in wood without modifying it by a chemical reagent. The above band was assigned by Bolker to a non-conjugated β-keto-group formed in the course of the isolation of lignin as a result of the dissociation of lignin–carbohydrate bonds. Bolker's experimental data were confirmed by subsequent studies of Higgins and coworkers [59,60]. Pilipchuk et al. [61] studied the infrared spectra of fir wood, acetylated MGL, holocellulose, MGL, and a mixture of MGL and holocellulose (in proportions of 3 : 7) in the range 1735–1750 cm^{-1} and from the results obtained concluded that there is a chemical bond between lignin and the carbohydrate components of plant tissue.

3. HYDROGEN BONDS AND THE POSSIBILITY OF THE FORMATION OF THREE-DIMENSIONAL NETWORK STRUCTURES

Apart from the chemical bonds between lignin and carbohydrates (the presence of which is regarded as established, as shown in the preceding section), both Björkman [34] and Brownell [48] pointed out the considerable role of hydrogen bonds in the formation of a single complex of plant tissue components.

Lignin is known to contain a large amount of phenolic and alcoholic hydroxy-groups, ester and carbonyl groups, and also a certain amount of carboxy-groups, which may be involved in the formation of intramolecular hydrogen bonds and also hydrogen bonds between lignin and carbohydrates. In a special study of the intramolecular hydrogen bonds in the lignin macromolecule, Lindberg [62,63] noted their influence on important properties of lignin such as solubility and capacity for precipitation. Lindberg established the presence of lignin of hydrogen bonds with an average energy of 2–3 kcal $mole^{-1}$. In order to penetrate the lignin macromolecule and dissolve it, the solvent must overcome this barrier, i.e. must be capable of forming hydrogen bonds with the corresponding lignin group and the energy of these bonds must be equal to or greater than 2–3 kcal $mole^{-1}$. These data were confirmed by the infrared spectra of lignins and model lignin compounds in the region 3600–3000 cm^{-1}, i.e. in the region of absorption by hydroxy-groups, and also by thermodynamic studies on monomeric models.

As regards the hydrogen bonds between lignin and carbohydrates in plant tissues, to which Björkman attributed a great importance, Lindberg concluded that in the complex of bonds between the plant tissue components they play only a secondary role. Indeed, despite the great likelihood of the formation of hydrogen bonds between lignin and carbohydrates in wood, in consequence of the amorphous nature of lignin it is difficult to imagine that these bonds are oriented along the molecules, as happens in cellulose chains, and the effect of irregularly distributed hydrogen bonds is known to be small.

The fact that wood is only very slightly susceptible to the action of enzymes, while the carbohydrate components and lignin are separately readily broken down by microorganisms has been regarded by many investigators [27-31, 64,65] as proof of the presence of a bond between lignin and carbohydrates in plant tissues. The data on the microbiological degradation of wood were interpreted somewhat differently by Pew [66,67]. While noting the undoubted presence of hydrogen and chemical bonds between the plant cell components, Pew emphasised that the immobilisation of the carbohydrate chains by the three-dimensional network structure of lignin, in a manner resembling the "serpent in a cage" system, has a considerable influence on the properties of lignified material and contributes to the formation of a single complex of the components of the plant cell wall. In a study of wood ground in a vibromill and its digestion by cellolytic enzymes, Pew showed that, while ordinary wood is resistant to the action of enzymes, after grinding for 10 min in a vibromill it is possible to remove about two-thirds of the wood carbohydrates and more prolonged preliminary grinding leads to complete digestion of the wood carbohydrate by cellolytic enzymes, leaving lignin as the residue. It is remarkable that the treatment of fir powder with a 1% hydrochloric acid, which should hydrolyse the chemical bonds between lignin and carbohydrates, did not increase its susceptibility to the action of enzymes, while reagents causing the swelling of wood had a very pronounced effect. A similar behaviour in relation to fine grinding and enzymatic digestion was shown by fir holocellulose, which had been impregnated with a water-soluble phenolic resin and then hardened at 110°C. On the basis of the results, Pew concluded that lignification is the factor which prevents the action of cellolytic enzymes on wood carbohydrates. The three-dimensional network structure of lignin, which immobilises the carbohydrate chains, prevents the access of large enzyme molecules to the system. Delignification completely eliminates this barrier, while grinding of wood or its swelling facilitates the contact between enzymes and wood carbohydrates.

In the summing up remarks at the end of his review, Merewether [9] concluded that the problem of the existence of a chemical bond between lignin and carbohydrates in lignified cellulose materials still remains open. Many facts can be explained on the basis of the incrustation theory, while others may be accounted for only by postulating the presence of chemically bound lignin–carbohydrate complexes. The data obtained during the last decade and discussed above lead to the conclusion that the existence of the chemical bond between at least part of the lignin and the carbohydrate components of plant tissues (mainly hemicelluloses) is established. Hydrogen bonds play a decisive role. Finally, account must be taken of the presence of the three-dimensional network structure of lignin which immobilises the carbohydrate chains.

4. POSSIBLE TYPES OF CHEMICAL LIGNIN–CARBOHYDRATE BONDS

Despite the fact that the majority of studies published in the last ten years hardly cast any doubt on the existence of a chemical bond between carbohydrates and lignin in wood, the problem of the types of the lignin–carbohydrate bond and also the problem whether or not all the lignin macromolecules are chemically bound to carbohydrates remain open.

It is known that after the removal of extractable substances from wood, which are evidently not bound to lignin since they are removed by simple physical methods without the removal of appreciable amounts of lignin, three carbohydrate components remain: cellulose, hemicelluloses, and polyuronides. The reactive groups in the carbohydrate components of plant tissue, which may participate in the formation of lignin–carbohydrate bonds, include primary and secondary hydroxy-groups, carbonyl groups, and finally the carboxy-groups in polyuronic acids. At the same time the reactive groups of lignin include phenolic and alcoholic hydroxyls of various types and also carbonyl groups capable of enolisation. The types of chemical lignin–carbohydrate bonds examined above may in fact arise between these reactive groups of lignin and the carbohydrate components of plant tissue.

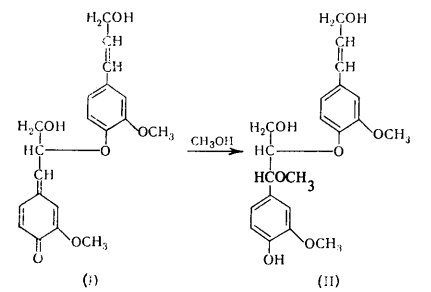
a. Ester and Ether Bonds

The presence of ester and ether bonds between lignin and carbohydrates, postulated at an early stage of Erdmann [7,8], is indicated by the results of a number of investigators. Thus Hoppe-Seyler [68] and Lange [69] concluded on the basis of the results of alkaline digestions of wood that there is an ester bond between the two components. Similar views on the nature of the lignin–carbohydrate bond were maintained by Czapek [70] and Grafe [71].

Klason [72,73] suggested that β-lignin contains a carboxy-group, which is not free in wood and should be bound to cellulose. In a study of the extraction of corn cobs under different conditions, Phillips [74] concluded that lignin is bound to carbohydrates in at least two ways: part of it is weakly bound, probably as ester groups of uronic acids, and the residue is combined via bonds of the ether type. Using the Cross and Beavan method for the determination of cellulose, Hawley and Norman [75] isolated two groups of hemicelluloses, one of which did not contain uronic acid and remained with the cellulose residue, and the other, usually containing uronic acid, was eliminated under the conditions of this determination. The authors believe that lignin is in fact bound to the latter group of hemicelluloses. The concept of an ether or ester bond between lignin and polysaccharides was supported by Lieser and Schwind [76] by experiments on the acetolysis of fir wood. Sarkar and coworkers [77,78] observed that, when jute is treated with 0.25% alkali, the acid number doubles. The authors believe that the additional free acid is formed in the hydrolysis of the ester bonds between lignin and polyuronides. Later Bhattacharjee and Callow [79] also observed an increase in the amount of free carboxyls in jute subjected to both acid (dilute hydrochloric acid) and alkaline (0.25% sodium hydroxide) treatment. Moreover, Sarkar [77] noted for jute and Tachi and Yamamori [80] for beech and elm wood a higher content of carboxy-groups in holocellulose, compared with the initial jute or wood. This observation was also accounted for on the basis of the hypothesis of the presence of an ester lignin–polyuronide bond. Foster et al. [81] believe that the ester bond between the carboxyl of uronic acid and the hydroxyl of lignin constitutes a likely explanation of the different rates of liberation of uronic acid from eucalyptus wood and from the holocellulose of this wood under identical conditions (1 *N* H_2SO_4; 91–92°C; 3 h).

In order to remove hemicelluloses, Harris [82] treated extracted wood powder with cold 17.5% sodium hydroxide and then extracted it four times with boiling methanol. Harris believed that the isolation of the methanol-soluble lignin (24% for fir wood and 70% for maple wood and aspen wood, in relation to Klason's lignin) constitutes proof of the cleavage of ester bonds between lignin and polyuronides. Stewart and coworkers [83,84] carried out a series of methanol extractions of eucalyptus wood at 150°C. The glucuronides found in the methanol extracts of holocellulose or wood pre-treated with alkali were absent from the extracts of the initial wood. The authors suggested that they are formed as a result of the hydrolysis of a lignin–polyuronide complex. Experiments on the pulping of aspen wood with a neutral butanol–water mixture at 158°C led Aaltio [85] to the conclusion that ester bonds are present between the acidic xylan chains and lignin. In a study of the infrared spectra of fir wood, acetylated MGL, holocellulose, MGL, and a mixture of MGL and holocellulose (in proportions of 3 : 7), Pilipchuk et al. [61] established that in the region 1735–1750 cm^{-1} in the infrared spectra of MGL, holocellulose, and their mixture, there are virtually no absorption bands or they are very weak. At the same time the infrared spectra of wood and acetylated MGL show very intense absorption bands in this region. Since it had been shown earlier [86] that the band in the region 1735–1750 cm^{-1} is due to the presence of ester groups, the authors concluded that an ester bond exists between lignin and other components of plant tissue (the amount of acetyl groups in fir wood is very insignificant and therefore their presence alone cannot account for the intense absorption in the above region, which is observed in the spectrum of the wood), without excluding the possibility of the existence also of other types of lignin–carbohydrate bonds.

The possibility of ether bonds of the benzyl ether type between lignin and carbohydrates in plant tissue was postulated by Freudenberg [87,88] on the basis of the preparation and investigation of a synthetic dehydropolymer [89–92]. Enzymatic dehydrogenation of coniferyl alcohol in aqueous methanol solutions results in the formation, together with other dimers and oligomers, of an α-*O*-methyl derivative of guaiacylglyceryl β-coniferyl ether (II, R=CH_3). The formation of this ether takes place via an intermediate quinomethane (I):



(I) (II)

If instead of methanol one uses a fairly concentrated solution of sorbitol or cane sugar, the corresponding α-*O*-derivative of guaiacylglyceryl β-coniferyl ether (II, R=$C_6H_{13}O_5$ or R = $C_{12}H_{21}O_{10}$) is formed. Calculations based on the content of methoxy-groups (10.21%) show that apparently two moles of quinomethane are combined with 1 mole of cane sugar. The sugar ether is readily hydrolysed by inorganic acids to (II, R = H), glucose, and fructose.

In a study of the effect of γ-radiation on wood, Hachihama and Takamuku [55] established that the amount of guaiacylmethanol groups increases in irradiated wood. Since the authors believe that γ-radiation leads to the cleavage of lignin–carbohydrate bonds, one may suppose that these bonds are of the benzyl ether type. In his review, Harkin [93] emphasises the central role of quinomethanes in the formation of the polymeric lignin molecule and of the bond between lignin and carbohydrates.

b. Phenyl Glycosidic Bonds

Hibbert and coworkers [94,95] synthesised a number of compounds simulating glycosides with aliphatic and phenolic aglycones: β-D-xyloside of α-hydroxypropiovanillone, β-o-xyloside of α-hydroxypropiosyringone, β-D-glucoside of α-hydroxypropioveratrone, β-D-xyloside of acetovanillone, β-D-glucoside of acetovanillone, β-D-cellobioside of acetovanillone, phenyl β-D-xyloside, phenyl β-D-glucoside, and guaiacyl β-D-xyloside. The rates of hydrolysis of these compounds and the conditions employed for the isolation of lignin from wood showed that the most likely type of bond between lignin and polysaccharides in plant tissues is the phenyl β-glycosidic bond.

By treating wood with acetic acid containing $MgCl_2$ as a catalyst, Freudenberg and Plankenhorn [96] isolated lignin acetate, which contained 11% of hexosan and after hydrolysis with alkali in an atmosphere of nitrogen yielded a product with a higher content of phenolic hydroxyls. Similar results were obtained by Pauly and coworkers [97,98], who treated wood powder in succession with methanol,

1.5% alkali at room temperature, and 85% acetic acid containing catalytic amounts of sulphuric acid and on the basis of the analysis of the preparations concluded that a phenyl glycoside bond is present between lignin and polysaccharides. Kawamura and Higuchi [99] demonstrated the presence of a lignin–xylose complex in dioxan-lignin isolated from beech wood. The dioxan-lignin obtained was separated by paper chromatography from sugars, exhaustively methylated, and hydrolysed with 5% hydrochloric acid. 2,3,4-Trimethylxylose, and not dimethylxylose, was isolated from the hydrolysis products by paper chromatography, which the authors believe to confirm the presence of glycoside bonds in lignin–carbohydrate complexes.

The work of Odintsov and Shishkova [100] on the cleavage of wood by enzymes, thermophilic cellulose bacteria, soil bacteria, and emulsion showed that the lignin–carbohydrate complex is cleaved, this being accompanied by the formation of new free phenolic hydroxyls. Häggroth and Lindberg [101] made a study whose aim was to explain the differences between sulphite and sulphate celluloses. According to McKinney's hypothesis [102], in sulphate pulping the residual hemicelluloses initially bound to lignin via phenyl glycoside bonds migrate to the cellulose chains of sulphate cellulose. In particular, the enhanced content of pentosans in sulphate cellulose is associated with this fact. Using phenyl β-D-xylopyranoside (III) as a model of the lignin–hemicellulose complex, Häggroth and Lindberg investigated its interaction with various alcohols and polysaccharides (methanol, glycerol, amylose, cotton cellulose). When compound (III) was treated with sodium methoxide in absolute methanol in nitrogen, a good yield of methyl β-D-xylopyranoside was obtained, while the interaction of (III) with sodium monoglyceride in anhydrous glycerol gave a mixture of glyceryl D-xylopyranosides. In the alkaline hydrolysis of compound (III) in the presence of both amylose and cotton cellulose, polysaccharides containing bound xylose were obtained. Thus, after the above treatment, cotton cellulose contained 2.4% of xylose. Hence the treatment of phenyl xylopyranoside in the presence of cellulose involves the cleavage of the phenyl glycosidic bond and xylose migrates to cellulose via transglycosidation, which confirms the McKinney hypothesis.

In the course of studies of lignin–carbohydrate complexes (xylolignin, acetylated xylolignin, complexes extracted with dioxan, 50% acetic acid, and dimethylformamide) isolated from wheat straw, Hayashi and Tachi [103,104] confirmed experimentally the presence of phenyl glycosidic bonds. In the alkaline hydrolysis of the complexes isolated an increase in the content of free phenolic hydroxyls was noted, as determined from the bathochromic shift in the ultraviolet spectrum (in the analogous treatment of lignin the amount of phenolic hydroxyls remains unchanged). Treatment of lignin–carbohydrate complexes with β-glucosidase increased the content of both phenolic hydroxyls of lignin and the reducing groups of sugar. Finally, in the alkaline pulping of lignin–carbohydrate complexes in the presence of cellulose, the migration of the carbohydrates of the complex to cellulose as a result of transglycosidation was observed (in the analogous treatment of a mixture of cellulose and freshly prepared holocellulose, transglycosidation was not observed).

Kiryushina and Tishchenko [23] fractionated the lignins obtained as a result of brief sodium hydroxide digestion of maple wood with respect to their solubility in acetone. Analysis of the acetone-insoluble fraction showed [24] that it consists of lignin and carbohydrates (mainly pentosans) and behaves similarly as the chemically bound complex. Repeated soda pulping, acid hydrolysis, ozonolysis, and infrared spectroscopy confirmed experimentally [25] the hypothesis of the existence of a phenyl glycoside bond between lignin and carbohydrates in the acetone-insoluble fractions of the soda lignin. Moreover, the presence of a glycosidic bond, formed with a phenolic or aliphatic hydroxyl of lignin and the glycoside hydroxyl of the polysaccharides, has been indicated in the reviews of Richtzenhain [105] and Lindgren [106], who believed that this is one of the most probable types of the lignin–carbohydrate bond.

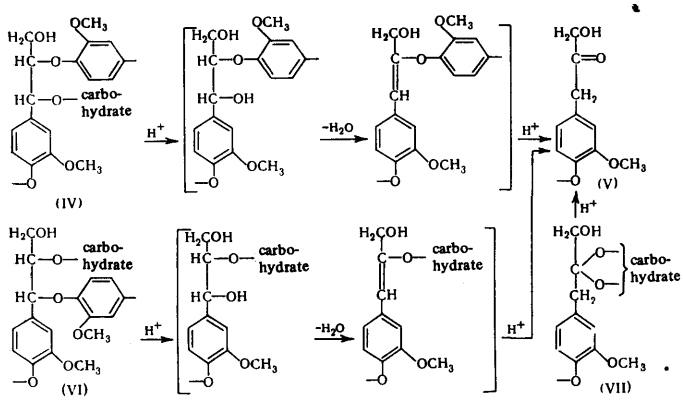
c. Acetal Bonds

The hypothesis of the presence of an acetal (ketal) or hemiacetal (hemiketal) bond between lignin and hydrocarbons, formed by the carbonyl groups of the side chains of the phenylpropane structural elements of lignins and the hydroxy-groups of the polysaccharide chains, was put forward for the first time by Holmberg and Runius [107] on the basis of a study of the alcoholysis of wood. It is known that, when wood is treated with alcohol in the presence of an acid catalyst, the alcohol reacts with lignin to form an alcohol-soluble alcohol lignin. The authors suggested that the reaction consists in transacetalation: in wood lignin is linked to hydrocarbons, forming an acetal, while during alcoholysis the alcohol employed replaces the carbohydrate. To confirm this hypothesis, Maeda and Kobayashi [108] carried out experiments, which showed that before isolation from wood lignin does not contain free carbonyl or acid hydroxy-groups. To test these results, Nokihara et al. [109] subjected wood, holocellulose, and viscose cellulose to ethanolysis in the presence of hydroxylamine. The differences in the rates of reaction of these preparations suggested that the formation in wood of a lignin oxime is preceded by the hydrolysis of other compounds, tentatively identified as a lignin–carbohydrate complex, which incorporates an acetal bond. Berlin [110] carried out a series of experiments on the oxidation of wood with hydrogen peroxide and on the basis of the results concluded that the low-molecular component of natural lignin, localised in the secondary cell wall, is linked to carbohydrates via an acetal or hemiacetal bond, which is readily cleaved by hot water or dilute acids. The hypothesis of the presence of an acetal bond between the components of plant tissues was also supported by Shorygina [111] on the basis of the results obtained in the decomposition of lignin and wood by metallic sodium and liquid ammonia.

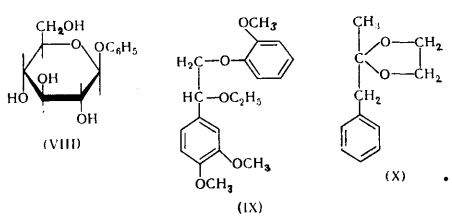
Recently this hypothesis received major support in a number of studies published by Bolker. As stated above, Bolker [57,58] observed that in the infrared spectra of isolated lignins there is an absorption band at 1710 cm^{-1}, which is absent from the spectra of different types of wood and wood celluloses containing residual lignin. This absorption band was assigned to a non-conjugated β-keto-group fromed in the isolation of lignin. Since β-keto-groups appear only in the course of the isolation of lignin, Bolker postulated that in wood and wood celluloses these groups are present as derivatives which do not absorb in the above region, i.e. in the form of acetal or enolic ether groups. Naturally, the spectra obtained do not show whether these groups are involved in intramolecular bonds of lignin (between phenylpropane units) or whether they are incorporated in the link between lignin and carbohydrates. However, the existence of a relation between

the isolation of lignin and the accumulation in the latter of keto-groups and the finding that β-keto-groups are never observed in wood and wood celluloses is evidence, according to Bolker, in favour of the second possibility.

Bolker [112,113] proposed mechanisms for the decomposition of both the acetal (VII) and benzyl ether (IV) bonds between lignin and carbohydrate, resulting in the formation of β-keto-groups (V):



The mechanism of the decomposition of benzyl ether bonds (IV) postulated by Freudenberg [87,88] may, according to Bolker, consist of the following stages: (1) hydrolysis to benzyl alcohol; (2) dehydration to the enolic ether form; (3) further hydrolysis to the β-keto-group (V). An analogous mechanism was also proposed for the case where the carbohydrate is bound to lignin via a β-carbon atom (VI), but the presence of such a structure is much less likely than that of the benzyl ether (IV). As noted by Bolker himself, the postulated dehydration stage, occurring in an aqueous medium under mild conditions, is most vulnerable to criticism. In addition, Bolker put forward also a number of other, albeit indirect proofs, for the presence of an acetal bond between lignin and carbohydrates (the absence of the β-keto-group from lignins isolated by ethanolysis and their appearance after the acid hydrolysis of ethanol lignin [112]; the study of the rate of extraction of lignin from wood by non-aqueous acid mixtures of 2,2-dialkoxypropane and dioxane [113], etc.). In his subsequent communication Bolker [114] presents data on the interaction between two solvent systems: dioxane–2,2-dimethoxypropane–HCl and dioxan–methanol–HCl with the simplest models of the possible lignin–carbohydrate bonds: phenyl β-D-glucoside (VIII; glycoside bond), 1-ethoxy-2-(2″-methoxyphenoxy)-1-(3′,4″-dimethoxyphenyl)ethane (IX; benzyl ether bond), and 2-benzyl-2-methyl-1,3-dioxolane (X; ketal bond):



As was to be expected, the studies of the kinetics of these reactions did not yield any definite conclusions, although Bolker reports that work with more complex compounds simulating various types of lignin–carbohydrate bonds is being continued.

Bolker's data are of undoubted interest, although their interpretation gives rise to objections. In the first place the assignment of the band at 1710 cm^{-1} to the β-keto-group is controversial [115–118]. In the second place, according to the data of Adler and coworkers [119–121], β-keto-groups may be formed as a result of the acidolysis of arylglyceryl β-aryl ethers, which may take place in the acid medium most often employed for the isolation of lignin.

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Apart from the types of lignin–carbohydrate bonds discussed above, Chudakov [122] postulates the presence of carbon–carbon bonds between lignin and carbohydrates, assuming that the work of Kratzl and coworkers [123], who showed that, on treating lignin with methanol (at 150°C, without the addition of inorganic catalysts), part of the alcohol alkylates the benzyl alcohol groups of lignin and part forms stable C–C bonds, constitutes indirect proof of this possibility.

It should be noted that the hypothesis of each of the lignin–carbohydrate bond types discussed above gives rise to some criticism based on the study of the stability of lignin–carbohydrate complexes under the conditions of different types of physical and chemical treatment [47,48,103,124]. Therefore, despite the relatively large number of studies concerning the problem of the lignin–carbohydrate bond type, the latter still remains open.

5. LIGNIN–CARBOHYDRATE BONDS AND THE PROBLEM OF THE HOMOGENEITY OF LIGNIN

It can now be regarded as firmly established that lignin is non-uniformly distributed in plant tissue. One of the first attempts to investigate the quantitative distribution of lignin in plant tissue was undertaken by Ritter [125,126], who treated 3 mm wood sections and then separated the lignin residue, which was determined by Klason's standard method. It was found that a large proportion of the lignin (about 75% of the total amount) is concentrated in the middle lamella, while the fraction in the secondary cell wall is much smaller and it should be noted that lignins localised in these two sites in plant tissue differ considerably in their content of methoxy-groups.

Bailey [127] separated by means of a micromanipulator 0.5 mg of the substance in the middle lamella from the wood of the Douglas fir and established that the preparation isolated contained 71% of lignin, 14% of pentosans, and 4% of cellulose. On the basis of data obtained in an ultraviolet microscope study, Lange [128] calculated the weight concentrations of lignin in the secondary cell walls and in the material of the middle lamella, which amounted to 16 and 73% respectively.

The problem of the distribution of lignin in the cell wall of plant tissue has been examined critically by Berlyn and Mark [129], who emphasised that the results of Bailey and Lange are diametrically opposed to those of Ritter. Noting that the volume of the material in the middle lamella in the wood of conifers amounts to only 10 to 12% of the volume of the woody tissue, Berlyn and Mark showed that, even if the middle lamella consisted entirely of lignin, it could not contain more than 40% of the total lignin in wood. This hypothesis was confirmed experimentally in a study by Goring and coworkers [130]. The method employed by the authors consisted of a combination of ultraviolet microscopy and densitometric analysis of the

negatives of the ultraviolet photomicrographs obtained. Some of the results of these investigators are compiled in Table 2, which shows that the material of the middle lamella is more lignified compared with the secondary cell wall but nevertheless contains only a small proportion of the total lignin of wood.

Table 2. Distribution of lignin in tracheides of the fir (according to the data of Fergus et al. [130]).

Wood	Morphological differentiation	Relative u.v. absorption	Vol. of fraction, %	Lignin, % of total amount	Lignin concn., $g\,g^{-1}$
Early	SW	1	87.4	72.1	0.225
	ML (r) (t)	2.21	8.7	15.8	0.497
	ML (cc)	3.77	3.9	12.1	0.848
Late	SW	1	93.7	81.7	0.222
	ML (r) (t)	2.7	4.1	9.7	0.60
	ML (cc)	4.5	2.2	8.6	1.00

SW = secondary wall; ML = middle lamella; r = radial direction; t = tangential direction; cc = cell corners.

Interesting data on the distribution of hemicelluloses in plant tissue were obtained by Asunmaa and Lange [131]. They used a microspectrographic method and investigated the distribution of carboxy-groups in holocellulose isolated from fir wood and birch wood. It was established that the outer portions of the delignified fibres contained the greatest amount of carboxy-groups, while in the vicinity of the lumen (the cellular cavity of the fibre) their content is a minimum. It is well known that polyuronides containing carboxy-groups enter into the composition of hemicelluloses. On this basis, the authors concluded that in plant tissues the relative content of hemicelluloses diminishes almost linearly from the outer layers towards the lumen. Bearing in mind the analogous variation of the concentration of lignin, the results were interpreted in support of the presence of lignin–hemicellulose bonds. Asunmaa and Lange [132] investigated spectroscopically delignified fir and birch fibres esterified with *p*-phenylazobenzoyl chloride. It was shown that holocellulose is distributed approximately uniformly in the cell wall. However, while cellulose is most closely packed around the lumens, hemicelluloses are concentrated in the outer layers of the fibres (hemicelluloses constitute approximately 50% of the entire carbohydrate material in the outer layers of the fibre and only 10–20% around the lumen).

Thus the studies on the morphology of the cell wall demonstrated unambiguously a non-uniform distribution of lignin in plant tissues, which suggests that the composition and structure of the lignin–carbohydrate complexes localised in the middle lamella and in the secondary cell wall are different. This hypothesis was confirmed by the results of Aaltio [85,133], who carried out a series of nine butanol digestions of aspen wood. After preliminary extraction with acetone, the fine sawdust was pulped with a neutral buffered mixture of butanol and water for 4 h at 158°C and each digestive was carried out on the residue from the preceding one. The solubility of wood was followed by analysing the insoluble residue and the pulping solutions. Some of the results of this study are presented in Table 3.

These investigations showed that aspen wood consists partially of homogeneous lignin–uronide complexes. The first of these dissolves digestions readily, passing into solution in the course of the initial five digestions and is localised in the middle lamella. The ratio of lignin to pentosan uronides in this complex is about 0.75. The second complex dissolving in succeeding digestions is localised in the cell wall and is characterised by a lignin : : pentosan uronide ratio of about 0.55. Cellulose is apparently closely associated with this complex. The carbohydrate component of the above complexes includes a homogeneous pentosan uronide in which the weight ratio of pentosans and uronic acids is approximately 7 : 1. Qualitative analysis of the sugar in the acid hydrolysates of the aqueous phases of various digested solutions showed that they contain D-xylose, 4-*O*-methylglucuronosidoxylose, aldobiuronic acid, D-galactose, D-galacturonic acid, L-arabinose, and traces of D-mannose, and L-rhamnose.

Table 3. Some results of a study on consecutive pulping of aspen wood by buffered aqueous butanol (according to the data of Aaltio [85]).

No. of digestions	Ratio of dissolved lignin to dissolved pentosan uronides	Content of CH_3O groups in dissolved lignin, %	
		found	calculated
1	1.05	21.4	21.7
2	0.75	—	21.3
3	0.77	20.1	20.2
4	0.73	—	21.4
5	0.74	17.9	17.9
6	0.59	—	15.3
7	0.55	15.0	15.0
8	0.54	—	15.0
9	0.55	14.9	12.0

The lignin fractions in both complexes are structurally different. While lignin in the readily soluble complex contains approximately 20–21% of CH_3O groups, the lignin in the more stable complex has a much lower methoxy-group content (about 15%). According to Aaltio's data, this difference is due to the different distribution of vanillin and syringin structures in the molecules of these lignin fractions. Aaltio interpreted his results on the basis that in the course of the first five digestions the pulping solution gradually acts upon the middle lamella, which leads to the isolation of the highly methoxylated lignin and pentosan uronides in proportions of 0.75 : 1. After the dissolution of the entire middle lamella, the material is freed from fibrous tissue and the secondary cell wall is acted upon in subsequent digestions. This leads to the isolation of lignin with a low methoxy-group content and pentosan uronides, again in constant proportions (0.55 : 1), which are however different from those found in the dissolution of the middle lamella.

It is noteworthy that, according to Aaltio, the greater part of lignin in the complex isolated from the middle lamella is in a free state and is not linked chemically to the carbohydrate components. This is consistent with the findings of Bailey [134], who concluded, on the basis of a study of the digestion of wood with a neutral aqueous butanol at 158°C, that in aspen wood the entire lignin is present in a free state, while in pine wood about 20% of the lignin is bound chemically to carbohydrates.

According to Björkman's hypotheses [36], lignin present in plant tissue may be divided into two fractions as regards

its bonds with hemicelluloses. One fraction forms a very insignificant number of bonds with hemicelluloses or not at all and is probably localised in the middle lamella, where the concentration of lignin is a maximum. This fraction is isolated under mild conditions and corresponds to MGL obtained by Björkman's method. The second fraction is firmly bound to hemicelluloses, and may be isolated by more severe methods. This fraction corresponds to the lignin–carbohydrate complexes obtained by Björkman's method. The proportions of the fractions are approximately 1 : 1.

Brownell and West[46] also concluded, as a result of the experiments, discussed above, on the fractionation of hydroxyethylated wood dissolved in dimethylformamide and subjected to preliminary grinding in a ball mill, that in wood approximately three-quarters of the total amount of lignin is bound chemically to carbohydrates. About one-quarter of the lignin can be readily separated from carbohydrates. The authors suggest that this part of the lignin is not bound chemically to carbohydrates, although it is quite possible to postulate that the bonds are broken during the treatment of the wood in the ball mill.

As a result of his studies on the oxidation of wood with hydrogen peroxides, Berlin[110] concluded that the lignin in the intercellular material (i.e. localised in the middle lamella) has a higher molecular weight and is not bound to polysaccharides. Tishchenko[17] noted, on the basis of the results of a stepwise ozonolysis of fir wood in ethyl acetate, that the wood contains both free lignin (not less than 43% of the total lignin) and lignin bound chemically to carbohydrates.

All these data confirm partly the hypothesis of Purves[135] who suggested, as early as 1943, that the molecular weight and the relative amounts of "free" and "bound" lignin in plants can vary not only as a function of the plant species but also as a function of its age and conditions, and the even earlier hypothesis of Klason[72] of the presence in wood of two types of lignin: α- and β-lignins.

The data concerning the existence of various lignin–carbohydrate complexes localised in the middle lamella and in the secondary cell wall can explain to some extent why by pulping wood it is impossible to achieve complete delignification of cellulose and additional treatment, in particular bleaching, is necessary.

To some extent, the inhomogeneity of the lignin contained in plant tissue is confirmed by the determination of the molecular weights of preparations obtained by the fractional isolation of lignin using different methods: by sulphite pulping[136–138], by extraction with a dioxane–hydrochloric acid mixture[139], by sulphate pulping[140], and by nitrating with nitric acid in ethanol[141]. These data led to the hypothesis of the presence in plant tissue of two types of lignin: with low and high molecular weights, localised in different sites of the plant cell walls.

Summarising the foregoing, we may note that the "biological and structural role of lignin, the conditions of its formation, and the nature of its interaction with other components of the cell wall in the primary layer and in general in the middle lamella can, of course, differ from the role of lignin and the nature of its bond with other substances in the secondary cell wall. Therefore one must recognise that the attempts to establish a single relation governing the nature of the bond between cellulose and other polysaccharides on the one hand and lignin on the other in all parts of the cell wall without taking into account the characteristics of its structure and the mutual bonds between the components in its various parts are not justified".[142] In addition, one should bear in mind the irregularity of the lignin structure, which follows from its role in the plant and also from the characteristics of its biosynthesis. According to Freudenberg's data[89–92], only dehydrogenases which remove hydrogen from the phenolic hydroxyl of the monomer participate in the *in vitro* biosynthesis of lignin from coniferyl alcohol. The subsequent stages take place, without the participation of the enzyme, in accordance with stochastic laws, which give rise to a wide variety of intramolecular bonds in lignin as well as bonds between lignin and carbohydrates. Thus it is quite reasonable to suppose that the contribution of chemical bonds to the formation of a single complex of components of the plant tissue is different in the middle lamella and in the secondary cell wall. In addition, as stated above, one cannot exclude from consideration the presence of hydrogen bonds between lignin and carbohydrates and also the possibility of the formation of three-dimensional network structures of lignin immobilising the carbohydrate chains.

The problem of the type of chemical bond between lignin and carbohydrates in plant tissues can be solved most probably only as a result of parallel experiments on lignin–carbohydrate complex preparations isolated from wood and compounds simulating the above possible types of chemical lignin–carbohydrate bonds. The behaviour of compounds of these types in different kinds of treatment under otherwise identical conditions will undoubtedly yield information which will permit a first approximation solution of this problem, which is one of the most interesting in the chemistry of wood.

Finally, returning to the problem of the homogeneity of lignin, it is noteworthy that, according to the valid comment by Freudenberg[143], when only part of the lignin in plant tissues is bound to carbohydrates both the incrustation theory and the theory postulating the presence of lignin–carbohydrate bonds are correct and not mutually exclusive as appeared previously.

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