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Modern Ideas on the Biosynthesis of Lignin

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According to modern ideas, the biosynthesis of lignin consists of two main stages, the first of which includes the formation of aromatic monomeric precursors of lignin synthesised in the plant from carbon dioxide via carbohydrates and the second involves the construction of the lignin macromolecule from the monomeric precursors.
The review deals with studies which led to the determination of the individual stages in the biosynthesis of the aromatic precursors of lignin in plants and also with the main features of the formation of polymeric lignin in its synthesis from coniferyl alcohol *in vitro*. Certain aspects of the biosynthesis of lignin *in vivo* have also been considered.
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I. INTRODUCTION

There is no doubt that the study of the formation of lignin in plants is of considerable theoretical and practical interest. As will be shown below, the elucidation of the main aspects of the biosynthesis of lignin yielded much information about its structure. It is also evident that a knowledge of the characteristics of the formation of lignin in plants is necessary for the solution of important problems in the chemistry of lignin such as the relation between various components of plant tissue, the inhomogeneity of lignin, etc. Moreover, information about the formation of lignin in plants may prove valuable also in the development of rational industrial methods for the delignification of vegetable raw materials. Finally, one must emphasise that the study of the biosynthesis of lignin is of interest not only for the chemistry of wood but also for such branches of biology as plant physiology, plant biochemistry, etc.

The problems of the formation of lignin in plants have for a long time attracted the attention of chemists working in the field of the chemistry of lignin but it was not until 1950 to 1960 following the application of new research techniques (the employment of tracer techniques, the method of tissue culture, various biochemical techniques, etc.) that considerable progress has been achieved in this field. The problems of the biosynthesis of lignin in plants have been frequently considered in a number of reviews and monographs [1-13] written mainly by specialists working in this field and therefore dealing primarily with the investigators' own ideas.

The general biosynthesis of lignin in plants can be represented schematically as follows:

$$\text{carbon dioxide} \xrightarrow{\text{photosynthesis}} \text{carbohydrates}$$
$$\xrightarrow{\text{aromatisation}} \text{primary structural elements}$$
$$\xrightarrow{\text{dimerisation}} \text{secondary structural elements}$$
$$\xrightarrow{\text{formation of polymeric molecules}} \text{lignin.}$$

In the biosynthesis of lignin, it is possible to isolate two main stages, the first of which involves the formation of aromatic monomeric precursors of lignin synthesised in the plant from carbon dioxide via carbohydrates and the second involves the formation of lignin macromolecules from the monomeric precursors. Both stages will be examined in detail below.

However, in considering the biosynthesis of lignin in plants one must not forget that the lignification process always takes place in conjunction with other processes involved in the formation of the cell wall. It is known from plant anatomy that wood cells are formed from live cambial tissue which persists throughout the life of dicotyledonous plants and on the activity of which depends the growth of the thickness of the trunk. Owing to the periodic activity of cambium, the wood elements formed from it are deposited during the vegetative period in a certain sequence which results in the formation of annual layers. Secondary wood consists mainly of conducting and mechanical elements which loose their protoplasmic content at an early stage and are converted into physiologically dead tissues. However, wood includes also the live cells of the wood parenchyma and wood rays, the metabolism of these cells characterising the vital properties of secondary wood. On the basis of the foregoing, it is interesting to examine the lignification of plant cell walls within the framework of the metabolism characteristic of cambial and meristematic tissues, since the lignification process is part of the complex biochemical activity of the young cells of the xylem [14-17]. However, examination of this aspect of the biosynthesis of lignin is evidently a task for the future since at present many biochemical processes occurring in the formation of the cell wall have been inadequately investigated. Nevertheless in the discussion below of the chemical aspects of the biosynthesis of lignin, certain problems of the formation of cell walls will be partly elucidated.

Finally the formation of lignin—a phenolic polymer—cannot be treated in isolation from the biosynthesis of many phenolic compounds widely distributed in plants (essential oils, anthocyanins, lignans, tannins, alkaloids, etc.). A definite chemical relation between the elementary structural units of lignin and the above phenolic compounds is evidently a consequence of similar processes involved in the biosynthesis of aromatic substances in plant tissues [18-20]. For a number of reasons, the consideration of these problems is also omitted from this review.

II. FORMATION OF MONOMERIC INTERMEDIATES IN THE BIOSYNTHESIS OF LIGNIN

1. The Role of Carbon Dioxide in the Lignification Process

In 1950 Hartt and Burr [21] reported for the first time that, after sugar cane plants have been exposed to an atmosphere of $^{14}CO_2$ in the dark, the lignin isolated from them is radioactive. Subsequent experiments by Stone, who exposed wheat plants to both prolonged [22] and brief [23] exposure to an atmosphere of $^{14}CO_2$, demonstrated convincingly the involvement of carbon dioxide in the biosynthesis of lignin. After the nitrobenzene oxidation of lignins isolated from the experimental wheat plants, radioactive vanillin, *p*-hydroxybenzaldehyde, and syringaldehyde were obtained. The participation of carbon dioxide in the biosynthesis of lignin was also confirmed by the data of Kreitsberg and coworkers [24,25], who performed experiments with fast growing poplar trees. At the present time the idea that carbon dioxide is the starting material in the biosynthesis of lignin in higher plants is generally accepted.

2. The Role of Carbohydrates in the Lignification Process

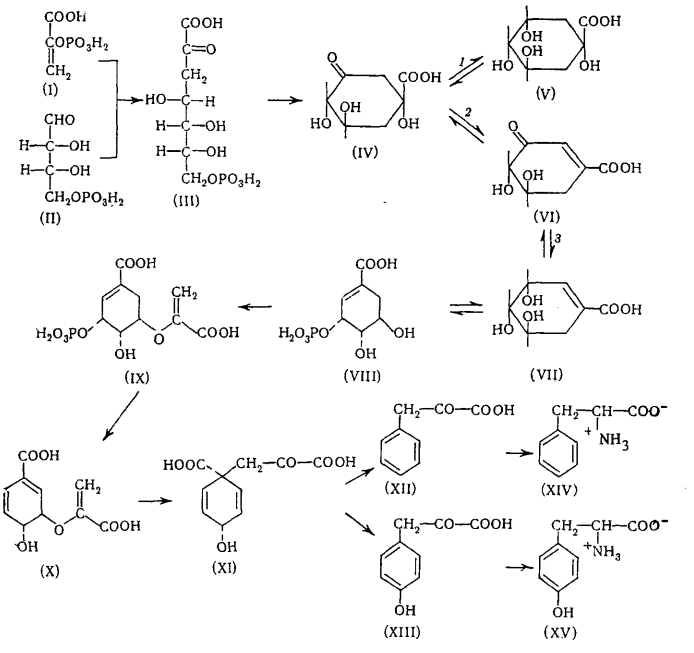
An extensive study on the carbohydrate precursors of lignin was carried out by a group of investigators under the supervision of Nord and Schubert. Schubert and Acerbo [26] demonstrated for the first time an enzymatic mechanism for the conversion of D-glucose into lignin in plants. They introduced labelled D-glucose into the wood of the Norway spruce from the precambial layer of which they isolated radioactive lignin after allowing a period for the metabolism to operate. In a subsequent study by Acerbo et al. [27], a Norway spruce tree was fed [1-^{14}C]-D-glucose and [6-^{14}C]-D-glucose for 28 days, the lignin was oxidised to vanillin, and the latter was decomposed in order to determine the radioactivity of each individual carbon atom. It was shown that in both cases ^{14}C is incorporated preferentially in the 2-, 6-, and 7-positions (carbonyl carbon atoms) and in the 8-position (methyl carbon atom) of the vanillin molecule. These results were confirmed by Kratzl and Faigle [28,29]. Hasegawa and Higuchi also demonstrated the involvement of glucose in the biosynthesis of lignin by introducing labelled glucose into young stems of the eucalyptus [30] and also in tissue culture experiments [31].

However, it has been shown that D-glucose is not the only carbohydrate capable of being converted into lignin. Thus, after injecting radioactive pentoses into the wood of the fast growing poplar, Sergeeva and Kreitsberg [32] isolated radioactive lignin. The involvement of pentoses in the formation of the lignin macromolecule confirmed Klason's hypothesis [33,34] that lignin may be formed from pentosans as a result of intracellular fermentation. Kratzl and Zauner [35] introduced into spruce wood xylitol labelled at all the carbon atoms and observed that the xylitol is incorporated both in polysaccharides and in lignin.

3. The Aromatisation Process

The classical studies by Davis [36-38] and Sprinson [37,39] on mutants of the *Escherichia coli* bacterium established very reliably one of the pathways in the biosynthesis of aromatic aminoacids from carbohydrate precursors. The overall results of these investigations are compiled in Scheme 1.

Scheme 1



According to the sequence presented in Scheme 1, the condensation of phosphoenolpyruvic acid (I), formed in the glycolysis of glucose, and D-erythrose-4-phosphate (II), formed from glucose in the pentose reaction cycle (the "pentose cycle"), leads to the formation of 2-oxo-7-phospho-3-deoxy-D-arabinoheptonic acid (III), which subsequently cyclises into 5-dehydroquinic acid (IV). Subsequent biosynthesis proceeds via the following obligatory intermediates: 5-dehydroshikimic (VI) and shikimic (VII) acids. 5-Phosphoshikimic acid (VIII) combines with a molecule of phosphoenolpyruvic acid (I) to form 3-enol-5-phosphopyruvoylshikimic acid (IX) which is converted into prephenic acid (XI) via the intermediate chorismic acid (X), the presence of which in the reaction cycle was established for the first time by Gibson [40-42]. As shown in Scheme 1, prephenic acid may be decarboxylated with formation of aromatic compounds via two pathways, in only one of which is the ring hydroxyl retained. Thus the first aromatic compounds synthesised by mutants of *E. coli* from carbohydrates are phenylpyruvic acid (XII) and its 4-hydroxy-derivative (XIII), which are converted by transamination into L-phenylalanine (XIV) and L-tyrosine (XV) respectively. Since the above scheme has been considered in detail in all the reviews of the biosynthesis of lignin, we shall not discuss it in depth apart from mentioning that, according to Davis' data [36], shikimic acid (VII) is an obligatory intermediate in the biosynthesis of aromatic compounds in *E. coli*.

At the present time the majority of investigators [3-14] believe that in higher plants the aromatisation of carbohydrate material takes place via shikimic acid (Scheme 1). This hypothesis is based on a number of considerations. Thus Brown and Neish [43] introduced the following ^{14}C-labelled compounds into a solution with wheat and maple cuttings: shikimic acid (VII), L-phenylalanine (XIV), and protocatechuic acid and showed that (VII) and (XIV) were incorporated into the lignin of wheat and maple. Eberhardt

and Schubert[44] converted labelled shikimic acid into the aromatic rings of the lignin macromolecule by implanting the acid in sugar cane plants. The vanillin obtained after the nitrobenzene oxidation of lignin from the experimental plant was decomposed in order to determine the radioactivity of each individual carbon atom. It was found that the vanillin atoms in the 6-, 2-, and 5-positions contained 44, 41, and 0% of the total radioactivity, while the atoms of the initial shikimic acid contained 52, 43, and 0% of the total radioactivity in the same positions. The results of this investigation convincingly demonstrate the conversion of shikimic acid into lignin without any rearrangement of the carbon atoms in its six-membered ring. The studies by Neish and coworkers showed that labelled shikimic acid is incorporated into both phenylalanine (XIV) and tyrosine (XV) by sage[45,46], wheat[47], and buckwheat[47] plants. Since these plants belong to different families, the authors suggested that vascular plants can in general employ shikimic acid for the synthesis of aromatic aminoacids.

Hasegawa and coworkers[48], who studied the distribution of shikimic acid (VII) in plants, established that it is widely distributed in higher plants and particularly in gymnosperms. Among the latter, shikimic acid was detected in greatest amounts in the pine family and the cryptomeria and among angiosperms in the magnolia family. Later the same investigators[49] detected shikimic acid in the leaves of 82 plant species (of the 164 investigated). According to Higuchi's data[50], among 96 woody plant species investigated 70 contained shikimic acid. The wide distribution of shikimic acid in plants is also indicated by Plouvier's results[51-54].

Apart from the data discussed above, the similarity of the biosynthetic pathways to aromatic compounds in micro-organisms and higher plants is also convincingly demonstrated by the observation in plants of enzymes which catalyse some of the reactions shown in Scheme 1. Thus Nandy and Ganguli[55,56] showed that, in the presence of extracts from mung bean seedlings, D-erythrose-4-phosphate (II) and phosphoenolpyruvic acid (I) are converted into 5-dehydroshikimic acid (VI). Mitsuhashi and Davis[57] detected in the pea and in spinach 5-dehydroquinate hydrolyase (5-dehydroquinate dehydratase) (EC 4.2.1.10.)†, which catalyses the (IV) → (VI) conversion (reaction pathway *2*). 5-Dehydroquinate dehydratase was also isolated from the buds of red cabbage[59]. Balinsky and Davis[60,61] isolated shikimate: NADP oxidoreductase‡(shikimate dehydrogenase) (EC 1.1.1.25) from the etiolated epicotyledons of pea seedlings and investigated the effects of various phenolic compounds on them. They found that the enzyme is associated with NADP and does not act on quinic and dehydroquinic acids. Nandy and Ganguli[62] isolated shikimate dehydrogenase from mung bean seedlings and found that in the presence of NADP it determines the position of equilibrium, which is displaced towards the formation of shikimic acid. Higuchi and Shimada[63] investigated the changes in the activity of shikimate: NADP-oxidoreductase in relation to the lignification of bamboo. It is noteworthy that Balinsky and Davis[64] isolated 5-dehydroquinate dehydratase and shikimate dehydrogenase from the extracts of certain higher plants and Gamborg[65] detected these enzymes in suspensions of cell cultures from a number of plants. The above investigators believe that the wide distribution of these enzymes is evidence of their important role in the biosynthesis of aromatic compounds in higher plants.

A few words should be said about the role of quinic acid (V) in the biosynthesis of monomeric lignin precursors. The wide distribution of quinic acid in higher plants[11,48,68,51-54,66-68] evidently indicates that in the biosynthesis of aromatic compounds in plants it can play a more important role than in bacteria. By exposing young rose plants to an atmosphere of $^{14}CO_2$, Weinstein et al.[69] isolated labelled quinic acid, the specific radioactivity of which was higher than that of shikimic acid isolated from the same plants. Subsequent experiments by these investigators[70-72] showed that, when various vascular plants are fed labelled quinic acid, the latter is converted into shikimic acid (VII), phenylalanine (XIV), and tyrosine (XV). Goldschmid and Quimby[73] reported that interconversion of quinic and shikimic acids in hemlock branches. The data obtained by Sergeeva and coworkers[74] in a study of the formation of shikimic and quinic acid in year old poplar shoots fed $^{14}CO_2$ demonstrate that, during the growth of the plant, the acids are genetically related. Gamborg[75] achieved the interconversion of quinic and shikimic acids (reaction pathways *1–3* in Scheme 1) *in vitro* with the aid of three enzymes: 5-dehydroquinate dehydratase, shikimate dehydrogenase, and quinate: NAD oxidoreductase (quinate dehydrogenase) (EC 1.1.1.24) isolated from suspensions of plant cell cultures from mung beans. It is noteworthy that Gamborg was the first to detect quinate dehydrogenase in higher plants[65] and to investigate in detail the properties of this enzyme after isolating it from mung bean cell culture suspension[76].

Among the reactions quoted in Scheme 1, the conversion of shikimic acid (VII) into prephenic acid (XI) has been least thoroughly investigated[77]. So far enzymes catalysing the individual stages of this reaction have not been isolated from micro-organisms in a pure form. In higher plants the enzymes have still not been detected. Therefore there is an evident need for a more detailed study of this reaction at an enzymatic level.

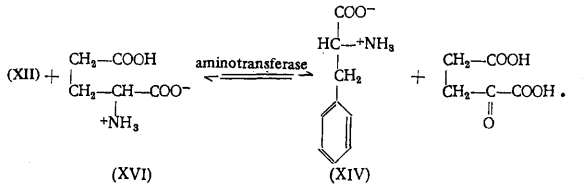
Gamborg and Simpson[78] studied the conversion of prephenic acid (XI) into phenylalanine (XIV) and tyrosine (XV) by enzymes isolated from mung bean plants. They established that mung bean extracts contain aminotransferase (transaminase), prephenate aromatase, and prephenate dehydrogenase, which are responsible for these conversions, and noted that it is impossible to demonstrate the enzymatic formation of phenylpyruvic acid (XII), since it is formed spontaneously when an acid acts upon prephenate. Gamborg and Keeley[79] isolated from the cotyledons of the wax bean prephenate dehydrogenase, which in the presence of NADP catalyses the (XI) → (XIII) conversion. This enzyme was also detected in the cotyledons and shoots of mung bean plants. A study of the changes in the concentration of prephenate dehydrogenase during the development of mung bean plants showed that the maximum concentration is observed in the cotyledons 4–5 days after the start of growth.

The final stage in the formation of phenylalanine and tyrosine, which play significant roles in the biosynthesis not only of lignin but also of other plant phenols[18-20], consists of the transamination of the ketoacids (XII) and (XIII). The transamination reactions were studied in various plants (lupin, maize, barley, pea, oats, and mung beans) by Wilson et al.[80], who detected a number of aminotransferase systems in these plants (α-oxoglutaric

†In these cases where the systematic name of the enzyme is followed by its number (code) in brackets, the name is based on the international nomenclature[58]; in the remaining cases the authors' own designations of the enzymes are retained.

‡ NADP = nicotinamide-adenine dinucleotide phosphate; NAD = nicotinamide-adenine dinucleotide.

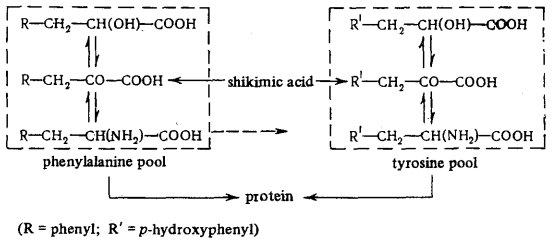
acid–alanine; α-oxoglutaric acid–aspartic acid; α-oxoglutaric acid–glycine; α-oxoglutaric acid–phenylalanine; oxaloacetic acid–alanine; pyruvic acid–methionine). The authors isolated, from etiolated white lupin seedlings and barley seedlings subjected to illumination, aminotransferases which catalyse the transamination of α-oxoglutaric acid with 17 different aminoacids. The authors' experiments with labelled compounds confirmed the presence of aminotransferases in the plants. Kretovich and Uspenskaya [81] investigated the formation of phenylalanine (XIV) as a result of transamination between phenylpyruvic acid (XII) and glutamic (XVI) or aspartic acid in homogenates of pea seedlings:



Gamborg [82,83] isolated from young mung bean plants an aminotransferase catalysing the transamination of a number of cyclic and aromatic aminoacids (including phenylalanine and tyrosine) in the presence of pyruvate or α-oxoglutarate, which behave as amino-acceptors.

In a study of the conversion of labelled shikimic acid into phenylalanine (XIV) and tyrosine (XV) by wheat and buckwheat plants, Gamborg and Neish [47] noted the ease of the interconversion of the α-hydroxy-, α-oxo-, and α-aminophenylpropanoic acids synthesised from shikimic acid and on the basis of their results concluded that there are two different pools of phenyl and *p*-hydroxyphenyl derivatives (the so called "phenylalanine pool" and the "tyrosine pool") (Scheme 2). Within each pool α-hydroxy or α-oxo-acids are readily converted into the corresponding aminoacids. At the same time, no conversion of compounds of the tyrosine pool into those of the phenylalanine pool was observed (the reverse conversion takes place to a significant extent). In addition it was shown that shikimic acid is a better tyrosine precursor than any compound of the phenylalanine pool. The results demonstrate that plants, like bacteria, form tyrosine from prephenic acid as a result of transamination (Scheme 1) but they are also capable of producing it by the hydroxylation of phenylalanine.

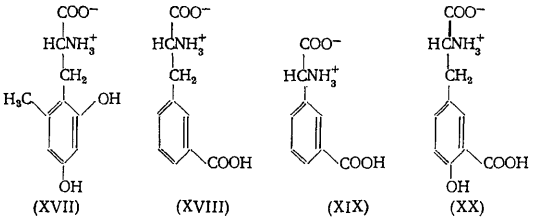
Scheme 2



(R = phenyl; R' = *p*-hydroxyphenyl)

The studies discussed above, which involved both the isolation of plant enzymes and experiments with labelled compounds, suggest that aromatic compounds are formed in plants via a pathway which is identical or very similar to the sequence of reactions presented in Scheme 1. At the same time one should note that many results support the hypothesis that in higher plants there are other synthetic pathways to aromatic lignin precursors than those already discussed. In particular Nandy and Ganguli [55,56] observed that the cell-free extract from mung bean converts glucose-6-phosphate and glucose-1-phosphate into dehydroshikimic acid. Higuchi and Shimada [84] isolated from bamboo shoots D-glucose-6-phosphate: NADP oxidoreductase (EC 1.1.1.49) and 6-phospho-D-gluconate: NADP oxidoreductase (EC 1.1.1.44) and investigated the activities of these enzymes in relation to lignification.

In addition, it is interesting to note that, apart from phenylalanine and tyrosine, certain other aromatic acids have been detected in certain plants. Thus Schneider [85] isolated orcinylalanine (XVII) from the seeds of *Agrostemma githago*. Thompson et al. isolated *m*-carboxy-L-phenylalanine (XVIII) [86] and *m*-carboxy-L-phenylglycine (XIX) [87] from the bulbs of *Iris tingitana*. Larsen and Kjaer [88] detected *m*-carboxy-L-tyrosine (XX) in the seeds of *Reseda odorata*. Unfortunately experiments on the biosynthesis of these aminoacids were not made and it is difficult to say whether they are formed via shikimic acid or in some other way:



According to modern ideas [18–20,89], there exist two main biosynthetic pathways to phenolic compounds: (1) the shikimate pathway, and (2) the acetate–malonate pathway. Studies with labelled compounds, discussed in detail by Neish [90], showed convincingly that the biosynthesis from acetate does not play an appreciable role in the formation of lignin, which is produced from carbohydrates via shikimic acid. It is quite likely that future studies will make it possible to refine and supplement the sequence involved in the formation of aromatic lignin precursors presented in Scheme 1 but there is already no doubt that the scheme correctly reflects the main processes occurring in the biosynthesis of aromatic compounds in plants.

4. Conversion of Aromatic Aminoacids into Monomeric Lignin Precursors

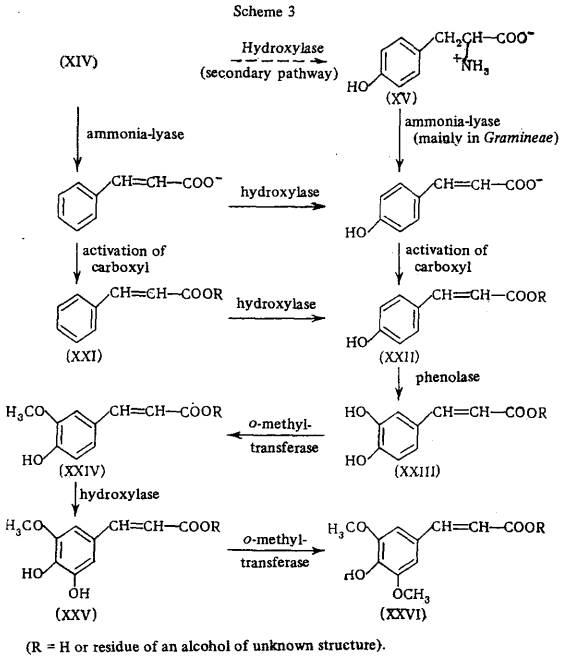
Brown and Neish [91,92] carried out an extensive study on the formation of lignin by introducing into plants labelled phenolic compounds (C_6-C_1 and C_6-C_3), which were regarded as potential intermediates in the biosynthesis of lignin. The results demonstrated that phenylalanine (XIV) is an effective precursor of lignin in many plants, while tyrosine (XV) is used for the biosynthesis of lignin only in a limited number of species. Subsequent studies by Neish [93] and Brown [94], who investigated the formation of lignin from [^{14}C]phenylalanine and [^{14}C]tyrosine in a number of plant species, showed that only species of *Gramineae* employed with equal efficiency both phenylalanine and tyrosine. Although these plant species can incorporate *p*-coumaric acid (XXII, R = H) in lignin, *Gramineae* are distinguished by their capacity to convert tyrosine directly into *p*-coumaric acid, while all other plants form this acid from phenylalanine via cinnamic acid (XXI, R = H).

The above conclusion was confirmed by studies at the enzymatic level. Koukol and Conn [95] demonstrated the presence in six plant species of an enzyme which catalyses the deamination of phenylalanine to cinnamic acid and which was subsequently called phenylalanine ammonia-lyase. At the same time Neish [96] detected in seven grass species an analogous enzyme, tyrosine ammonia-lyase, which deaminates tyrosine to *p*-coumaric acid. Later Young and Neish [97] studied the properties of the above ammonia-lyases which they isolated from wheat plants (*Triticum aestivum*) and bracken (*Pteridium aquilinum*). In addition, Neish and coworkers [98] studied a number of plants belonging to different species and showed that, although tyrosine ammonia-lyase can be detected in other families, only *Gramineae* contain the maximum concentrations of this enzyme.

Thus the results of the above investigations indicate two possible pathways to the formation of *p*-coumaric acid: by direct action of ammonia-lyase on tyrosine, mainly in *Gramineae*, or by the hydroxylation of cinnamic acid formed as a result of the deamination of phenylalanine.

McCalla and Neish [46] studied the sequence of conversions from cinnamic acid (XXI, R = H) to sinapic acid (XXVI, R = H) by feeding labelled phenylalanine to sage cuttings (Scheme 3). They extracted labelled *p*-coumaric (XXII, R = H), caffeic (XXIII, R = H), ferulic (XXIV, R = H), and sinapic (XXVI, R = H) acids, the different rates of incorporation of ^{14}C in each acid agreeing with the sequence shown in Scheme 3. Moreover, the above sequence is confirmed by experiments in which labelled cinnamic and other acids were introduced separately, which led to the observation of rapid conversion of the additive into the final compounds of the sequence and relatively slow conversion into the initial compounds§.

Scheme 3



(R = H or residue of an alcohol of unknown structure).

§ There is convincing evidence for the demethylation of sinapic [99-101] and ferulic [102,103] acids in plants, but the mechanism of this process has not yet been established.

Since it is difficult to suppose that the reactions considered occurred in plants with acids in a free form, the authors suggested that the cinnamic acid derivatives indicated in Scheme 3 are present mainly in alkali-labile bound forms, which are evidently esters. Later El-Basyouni and Neish [104,105], obtained more convincing evidence for the involvement of these bound forms, which they thought to be soluble and insoluble esters, in the formation of lignin. The nature of the bound forms is not entirely clear, but it is believed that they can be bound either to protein fractions [13] or they may be esters of glucose [106-109] or quinic acid [110].

The individual stages of the sequence presented in Scheme 3 have been confirmed by studies at an enzymatic level. Thus Nair and Vining [111] isolated from spinach leaves a phenylalanine hydroxylase, which catalyses the hydroxylation of L-β-phenylalanine to tyrosine at pH 4.2. The same investigators [112] demonstrated the presence in spinach leaves of cinnamic acid 4-hydroxylase, which characterises the hydrolysis of *trans*-cinnamic acid to *p*-coumaric acid. An analogous enzyme was isolated by Russell and Conn [113] from the terminal buds of the pea and Higuchi and coworkers [114] obtained it from bamboo, asparagus, and *Ginkgo biloba* shoots and from Japanese cedar seedlings.

Byerrum et al. [115] showed that in the metabolism of barley and tobacco plants the methyl group of methionine is involved in the formation of the methoxy-groups of lignin. With the aid of labelled compounds, they demonstrated that the reaction consists of the transfer of the methyl group from the sulphur atom of methionine to an oxygen atom in lignin and therefore it can be regarded as an example of transmethylation in higher plants. Later it was shown that in tobacco plants the donors of the methyl group in the formation of methoxy-groups of lignin may be glycine, serine, and formaldehyde [116] and in the poplar glycine may serve this purpose [117]. A number of investigators [118-120] established the presence in grasses and in woody shrubs of enzymes capable of methylating caffeic acid in the presence of *S*-adenosylmethionine. Hess [121,122] detected in petunias and certain other plants an analogous enzyme which methylates 5-hydroxyferulic acid to sinapic acid in the presence of adenosine triphosphate, methionine, or *S*-adenosylmethionine, confirming thereby data obtained earlier with the aid of labelled compounds [101]. Higuchi et al. [123] reported the isolation from bamboo shoots of an *o*-methyltransferase which catalyses the formation of ferulic and sinapic acids. They demonstrated that the activity of the enzyme gradually increases as the bamboo shoots are lignified. Higuchi and coworkers [124] compared the specificities of the *o*-methyltransferases extracted from the bamboo and the poplar (angiosperms) and from *Ginkgo biloba* (gymnosperms). They showed that the methyltransferase from the bamboo and the poplar is *meta*-specific for caffeic, 3,4,5-trihydroxycinnamic, and 5-hydroxyferulic acids. The rate of formation of sinapic acid (XXVI, R = H) from 5-hydroxyferulic acid (XXV, R = H) in the reaction *in vitro* is higher than the rate of formation of ferulic acid (XXIV, R = H) from caffeic acid (XXIII, R = H). On the other hand, the methyltransferase from *Ginkgo biloba* methylates only caffeic acid and not 3,4,5-trihydroxycinnamic and 5-hydroxyferulic acids. The authors suggest that the observed difference between the specificities of the *o*-methyltransferases from gymnosperms and angiosperms is one of the factors responsible for the differences between the metabolism of lignin in these groups of plants. It is noteworthy that, according to the data of Kratzl and Puschmann [125], the methoxylation

of the aromatic ring can occur also in the stage involving the oxidation of coniferyl alcohol.

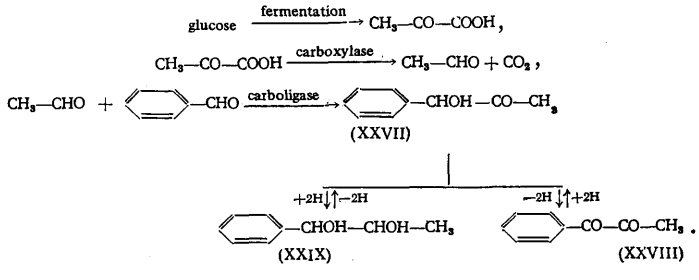
Vaughan and Butt[126-128] isolated from spinach leaves a copper-containing preparation of phenolase which catalysed the hydroxylation of *p*-coumaric acid to caffeic acid. The authors showed that this enzyme behaves like an oxidase with a mixed function (both molecular oxygen and an electron donor, for example ascorbate, NAD.H_2, or 6,7-dimethyl-5,6,7,8-tetrahydropteridine, are essential for the completion of the hydroxylation reaction).

The concluding stage of the biosynthesis of the monomeric precursors of lignin is enzymatic reduction of ring-substituted derivatives of cinnamic acids to the corresponding derivatives of cinnamyl alcohols. A similar conversion of ferulic acid was investigated by Higuchi and Brown[129], who employed a ^{14}C-labelled substrate and showed that ferulic acid is reduced by wheat plants to coniferyl alcohol via the formation of an aldehyde as an intermediate. Gross and Zenk[130-132] confirmed these reactions by studies at an enzymatic level, having isolated the corresponding enzymes from the mycelium of the fungus *Neurospora crassa*. The previously unknown arylaldehyde: NADP oxidoreductase (EC 1.2.1) catalyses the reduction of the carboxy-groups of aromatic acids to the corresponding aldehydes. The second enzyme aryl carbinol: NADP oxidoreductase (EC 1.1.1.2), which catalyses the reversible reduction of aromatic aldehydes to aromatic alcohols, differs from the familiar alcohol dehydrogenases, acting preferentially on primary aromatic alcohols. The authors noted a broad substrate specificity of the enzymes isolated and investigated their properties. Similar enzymes have not so far been isolated from higher plants.

5. The Possibility of Other Biosynthetic Pathways to Monomeric Lignin Precursors

Higuchi[50,133,134] suggested that compounds with the phenylpropane structure, which are monomeric precursors of lignin, may be synthesised in plants via pathways other than those discussed above, namely as a result of a $C_6-C_1 + C_2$ condensation, i.e. by the addition of C_2 compounds to derivatives of phenylmethane formed from 5-dehydroshikimic or shikimic acid.

As early as 1921 Neuberg and Hirsch[135] observed that, in the fermentations of sucrose or pyruvic acid in the presence of benzaldehyde, acetylphenylmethanol (XXVII) is formed. Part of the ketol produced was oxidised to benzoyl methyl ketone (XXVIII), while the remainder was reduced to methylphenylethylene glycol (XXIX). The authors suggested the following sequence of reactions:



The enzyme which catalysed the last reaction was called carboligase. Later Neuberg and coworkers[136,137] reported that, when yeast carboligase acts on aromatic aldehydes (*o*-chlorobenzaldehyde, anisaldehyde, and *o*-toluic aldehyde), the corresponding ketols are formed. Recently the formation of acetylphenylmethanol (XXVII) catalysed by yeast carboligase was noted also by other investigators[138-140].

Since the structures of the side chains of the ketols and diketones thus produced are identical with the products of the ethanolysis of lignin (the so called "Hibbert's ketones"), Higuchi[133,134,141] investigated the possibility of the formation of phenylpropane precursors of lignin as a result of a reaction catalysed by carboligase.

Vanillin, syringaldehyde, and *p*-hydroxybenzaldehyde were separately subjected to fermentation by brewer's yeast in sucrose solution. After fermentation, the resulting solutions were found to contain methyl vanilloyl ketone, methyl syringoyl ketone, and *p*-hydroxybenzoyl methyl ketone, part of these compounds being reduced to the corresponding alcohols and the remainder being oxidised to the corresponding acids. The diketones obtained are identical with certain products of the ethanolysis of lignin. The authors suggested a subsequent formation from the diketones of compounds of the type of acetylphenylmethanol and benzoylmethylmethanol, although the existence of these ketones has not been confirmed experimentally.

The results of a number of investigators[134,141-143] indicate the presence of C_6-C_1 aldehydes in the cambial sap of many woody plants, wheat, and bamboo shoots. Stepanov and Kuzin[144] detected carboligase in the leaves of a number of plants. In addition, Brown and Neish[91,145] reported that, when wheat plants are fed labelled preparations of vanillin and *p*-hydroxybenzoic acid, the latter are incorporated into the wheat lignin. Ishikawa and Takaichi[146] observed that, when adult pea plants are incubated with vanillin and *p*-hydroxybenzaldehyde, their lignin content increases relative to control plants. Freudenberg and coworkers[147,148] showed that labelled [^{14}C]glucovanillin is incorporated into the lignin of young pines. Kratzl[149,150] reported the incorporation of labelled *p*-creosol into lignin.

The results of the above investigations enabled Higuchi[50,133,134] to suggest that C_6-C_1 aldehydes, which may be formed in plants via different pathways[151], are converted into phenylpropanols as a result of a reaction catalysed by carboligase. The phenylpropanols are further transformed by rearrangements and dismutations, as in the mechanism proposed by Hibbert[152,153] and discussed in the next section.

In conclusion of the discussion of questions relating to the formation of monomeric lignin precursors as a result of a $C_6-C_1 + C_2$ condensation, we may note that although such processes may occur in plants, they probably play only a secondary role in the biosynthesis of lignin.

6. The Role of Coniferin in the Lignification Process

As early as 1861, Hartig[154] detected coniferin, which is the β-D-glucoside of coniferyl alcohol, in the cambial sap of conifers. It has now been shown[155] that coniferin is encountered mainly in conifers, comprising six families and 15 species (the syringic analogue of coniferin—syringin—has been detected also in certain deciduous trees). As will be shown below, many investigators regarded coniferin as a precursor of lignin in the wood of conifers

owing to its evident biochemical relation to coniferyl alcohol. Freudenberg and Bittner [156] introduced coniferin synthesised from labelled [^{14}C]coniferyl alcohol under the bark of young spruce stems. Two months after injection, more than 90% of ^{14}C was detected in the lignin fraction. Later Freudenberg and Niedercorn [157] showed that labelled phenylalanine may be converted into both coniferin and into lignin by young spruce stems, and therefore they suggested that coniferin is an intermediate in the formation of lignin. The experiments of Freudenberg and coworkers [147], which are discussed in detail below, showed that coniferin is hydrolysed by β-glucosidase to coniferyl alcohol, from which lignin macromolecules are then constructed. Studies on the role of coniferin by the method of tissue culture [158–165] demonstrated stimulation of lignification by both coniferin and syringin. Kratzl and coworkers [166–168] determined quantitatively the coniferin incorporated in spruce lignin. These studies showed that the aglucone of coniferin is incorporated into lignin without a rearrangement of the side chain.

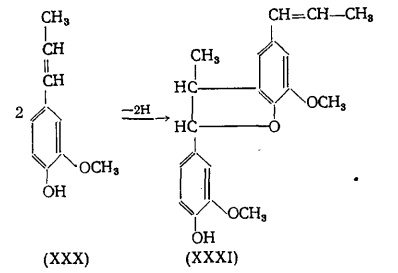
However, despite the above investigations, the question of the role of coniferin in the lignification process still cannot be resolved quite unambiguously. The reason is both the fact that the distribution of coniferin is not universal and the failure to detect β-glucosidase in some lignification processes [150].

III. FORMATION OF LIGNIN FROM MONOMERIC PRECURSORS

The studies discussed in the preceding section concerning the detection in plants of various enzymatic systems catalysing the individual stages of the biosynthesis of monomeric lignin precursors and also experiments with various labelled compounds have been carried mainly *in vivo*, i.e. on plants or tissue cultures. The studies discussed in this section involve mainly experiments *in vitro*. This method has both many advantages and significant disadvantages, which will be discussed below.

1. A Brief Historical Review of Studies on the Biosynthesis of Lignin *in Vitro*

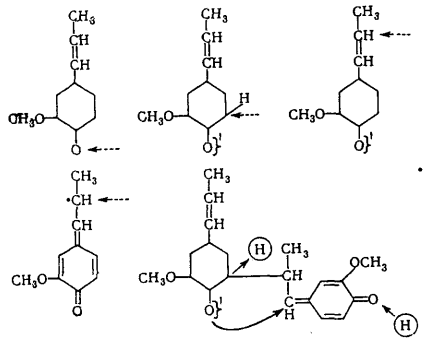
After the determination of the structure of the glucoside coniferin present in the cambial sap of conifers during the vegetative period, Tiemann and Mendelsohn [169] suggested in 1875 that there is a definite genetic relation between coniferin and lignin. In 1897 Klason [170] used this idea in his studies on the chemistry of lignin and suggested that lignin is a product of the condensation polymerisation of coniferyl alcohol formed in the plants on enzymatic hydrolysis of coniferin. Later Klason [171–178] regarded lignin as a condensation product of coniferaldehyde formed by the oxidation of coniferyl alcohol. In 1908 the French chemists Cousin and Hérissey [179,180] observed that, when isoeugenol (XXX) is dehydrogenated, a crystalline product is formed, among others; according to analytical data, it consists of two eugenol molecules. The authors dehydrogenated isoeugenol with an alcoholic solution of $FeCl_3$ or with dehydrogenases from the sap of the fungus *Russula delica* in the presence of oxygen and in both cases obtained the same product. According to the hypothesis of Cousin and Hérissey, the product, which they called dehydrodi-isoeugenol, is a biphenyl derivative. It was not until 1933 that Erdtman [181,182] demonstrated the true structure of dehydrodi-isoeugenol and showed that it is a phenylcoumaran (XXXI):



(XXX) (XXXI)

Erdtman [182] proposed a mechanism for the dehydropolymerisation of isoeugenol to dehydrodi-isoeugenol via the intermediate formation of an oxygen radical¶. The mechanism is presented in Scheme 4 as formulated by the author [182].

Scheme 4



According to Erdtman's hypothesis, the formation of dehydrodi-isoeugenol may be regarded as a model of the biosynthesis of lignin in plants, which is achieved as a result of the dehydropolymerisation of guiacylpropane derivatives containing oxygen in the side chains. Erdtman's hypothesis became the starting point of all subsequent studies on the biosynthesis of lignin, which will be discussed below.

The isolation and identification of various monomeric products of the ethanolysis of wood ("Hibbert ketones") enabled Hibbert to put forward in 1941 the so called dismutation hypothesis [152,153]. According to this hypothesis, the respiration of plant cells is catalysed by substances of the type of coniferyl alcohol, which function as oxygen transferring agents. Hibbert believed that the oxidation-reduction system in plant cells is analogous to the system proposed by Szent-Györgyi [187] for animal cells, which is evident from the Scheme below:

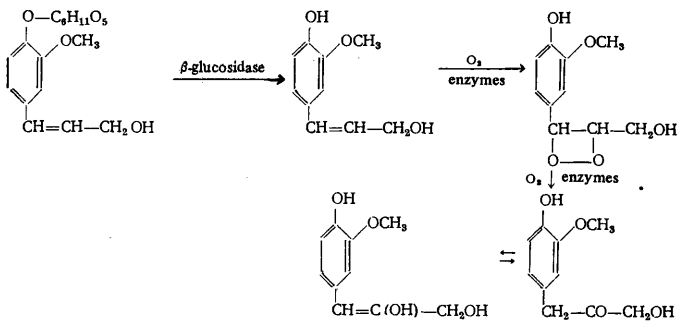
Szent-Györgyi system for animal cells	*Hibert system for plant cells*
HOOC—CO—CH_2—COOH	R—CO—CH_2—CH_2OH
+2H↓ ↑−2H	+2H↓ ↑−2H
HOOC—CH(OH)—CH_2—COOH	R—CH(OH)—CH_2—CH_2OH
↓−H_2O	↓−H_2O
HOOC—CH=CH—COOH	R—CH=CH—CH_2OH
+2H↓ ↑−2H	+2H↓ ↑−2H
HOOC—CH_2—CH_2—COOH	R—CH_2—CH_2—CH_2OH

(R = 4-hydroxy-3-methoxyphenyl)

¶ It is noteworthy that the hypothesis of a radical mechanism of the dehydrogenation of phenols, which subsequently became widely accepted in organic chemistry and biochemistry, was first put forward by Pummerer [183–186].

Thus, according to Hibbert's hypothesis, monomeric phenyl compounds are synthesised by plants as respiration catalysts. In higher plants, in the postmortem state of the cells, these compounds are oxidised and readily undergo various reactions (dehydrogenation, condensation, and polymerisation) leading to the formation of lignin. Hibbert's hypothesis was subsequently developed in Manskaya's studies.

In 1947 Manskaya [188,189] investigated the oxidation processes in wood relating to lignin formation. She showed that the cambial tissue and the wood of the new annual layer in the pine contain oxidising enzymes: peroxidase and polyphenol oxidase, whose substrates are coniferin and its derivatives. Manskaya pointed out the equilibrium in the oxidation-reduction processes in the cambial tissue as a result of which phenol oxidation products do not accumulate in the cells. When live cells die during the wood formation process, oxidation processes begin to predominate markedly over reduction processes and polyphenol oxidation products begin to be deposited in the form of lignin. According to Manskaya's hypothesis, the deposition of lignin is related directly to the breakdown of the respiratory apparatus in plant cells. The hypothesis states that the oxidation of coniferyl alcohol in the lignifying cells of the cambium may be represented by the following scheme:



The products obtained as a result of the enzymatic oxidation are converted by further condensation into lignin, which is deposited in the cell walls of the wood. The results of Manskaya's study [190] of the changes in the activity of peroxidase in cambium and the adjacent woody layer and also of the corresponding changes in the coniferin content showed that in the period of maximum enzymatic activity the coniferin content was lowest and conversely. The results of this investigation served as a basis for the conclusion that coniferin is the substrate of the enzymes. Later Manskaya and Bardinskaya [142] oxidised coniferyl alcohol with peroxidase (from the precambial layer of the pine) or by polyphenol oxidase (from the lignified central part of the root of the sugar beet) and obtained polymeric compounds which were close in their elemental composition, the content of functional groups, the molecular weight, and ultraviolet spectroscopic data to Freudenberg's preparations obtained from coniferyl alcohol by the action of enzymes from the field mushroom.

In considering the questions of the biosynthesis of lignin, attention should be concentrated on the work of Freudenberg and his coworkers the aim of which was to prepare and investigate biosynthetic lignin. The studies of these investigators over a period of many years, which involved highly sophisticated experiments, made a considerable contribution to the chemistry of lignin, having provided information not only about the possible pathways of its biosynthesis but also about the structure of this complex polymeric substance.

In 1943 Freudenberg and Richtzenhain [191] and later Richtzenhain alone [192-194] observed the formation of polymeric lignin-like materials when various monomeric phenolic derivatives of the guiacyl and syringyl series were acted upon by the unpurified enzymes of the mushroom *Psalliota campestris*. The results led the authors to the conclusion that lignin is formed by the enzymatic dehydrogenation of substituted phenol derivatives, confirming thereby Erdtman's hypothesis.

In 1949 Freudenberg [195,196] reported the preparation of biosynthetic lignin, which he called dehydropolymer (DHP). The preparation was obtained by the action of enzymes from the sap of the mushroom (*Psalliota campestris*) on coniferyl alcohol at a neutral pH, 20°C and with aeration over a period of three days. The yield of DHP was 93% relative to the initial coniferyl alcohol. The resulting polymer was very close to lignin isolated from spruce wood in terms of important characteristics, such as solubility, elemental composition, the content of functional groups, the yield of formaldehyde on distillation with 28% sulphuric acid, the yield of aromatic acids on methylation, fusion with alkali, remethylation, and oxidation with $KMnO_4$, colour reactions, ultraviolet spectra, etc. This study began a large cycle of investigation on the enzymatic dehydrogenation of coniferyl alcohol. Later Freudenberg and his coworkers studied the principles of the biosynthesis of lignin *in vitro*, investigated the dehydrogenation of coniferyl alcohol by isolating the intermediates, and compared the properties of DHP and spruce wood lignin.

In 1963 Freudenberg and Harkin [197] reported at during the vegetative period the cambial sap contains, together with coniferin, small amount of *p*-glucocoumaryl alcohol and syringin. Accordingly, Freudenberg and coworkers [198-200] dehydrogenated a mixture of *p*-coumaryl, coniferyl, and sinapyl alcohols in molar proportions of 14 : 80 : 6 in order to obtain biosynthetic lignin closest to the lignin from conifers.

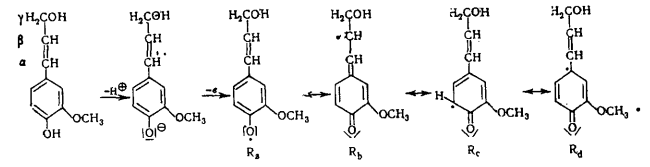
It would hardly be useful to consider the studies of Freudenberg and his coworkers in their historical sequence, which, while giving an idea about the evolution of the authors' views on the structure and biosynthesis of lignin *in vitro*, would make it difficult to describe the present state of this problem. Therefore in subsequent sections we shall consider the main aspects of Freudenbergs investigations and his principal conclusions, which constitute the foundation of present ideas on the biosynthesis of lignin.

2. The Mechanism of the Polymerisation of Coniferyl Alcohol

According to Freudenberg's views [199,201,202]†, coniferyl alcohol is polymerised via a radical mechanism. In the course of the study of the enzymatic dehydrogenation of coniferyl alcohol, Freudenberg and his coworkers [201,203-205] established that, when the action of dehydrogenases from field mushrooms or cambium on an aqueous solution of coniferyl alcohol is terminated as soon as the latter ceases to be detected in the reaction mixture, it is possible to isolate dimeric intermediates in the dehydrogenation reaction. By extracting the reaction mixture with

† Since Freudenberg's studies have served as a basis for his numerous publications dealing with the evolution of his views, in each case the references include both the first publication on the given problem and the latest communications which contain his present views.

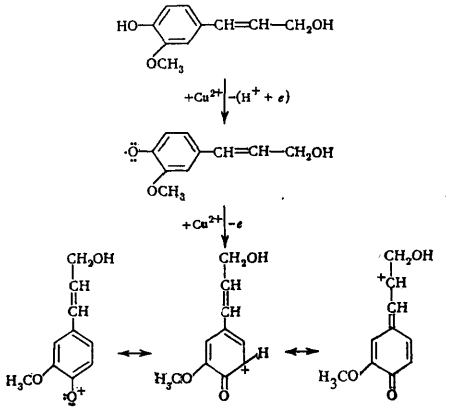
methylene chloride or n-butyl alcohol, dehydrodiconiferyl alcohol [203], DL-pinoresinol [204], and β-coniferyl ether of guiacylglycerol [201] were isolated and then identified. On the basis of these intermediates in the biosynthesis of lignin *in vitro* and also Erdtman's hypothesis that lignin is formed via a radical mechanism, Freudenberg put forward the following scheme for the conversion of coniferyl alcohol in the course of its enzymatic dehydrogenation:



According to this scheme, during enzymatic dehydrogenation, the coniferyl alcohol molecule loses the hydrogen atom from the phenolic hydroxyl to form the aryloxy-radical R_a. This radical can exist in any of the mesomeric forms indicated (R_a–R_d). Freudenberg et al. [206] established that the half-life of these mesomeric forms in a 1 : 1 (by volume) dioxan–water mixture is 45 s. The mesomeric forms of the radical recombine further with one another and undergo various transformations, discussed in detail in the next section, which leads ultimately to the formation of biosynthetic lignin (DHP). One must emphasise that, according to Freudenberg's data [207–209], the dehydrogenation of coniferyl alcohol is not a specific reaction, since it can be initiated by various reactants: laccase isolated from the sap of field mushrooms in the presence of atmospheric oxygen; horseradish peroxidase in the presence of H_2O_2; laccase and peroxidase isolated from the precambial layer of spruce wood; a number of inorganic agents [copper(II) salts, MnO_2, lead peroxide]; radicals such as, for example, 2,4,6-tri-t-butylphenoxyl, etc.

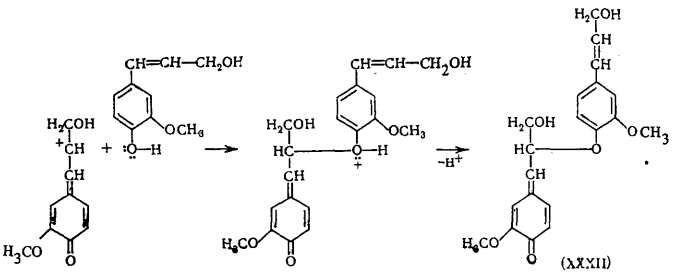
Apart from the radical mechanism of the polymerisation of coniferyl alcohol, discussed above, one should consider the ionic mechanism proposed by Adler [210] (Scheme 5).

Scheme 5



According to Adler's views, the initial reaction has a radical mechanism and takes place without electron transfer. The suggested subsequent "one-electron transfer", as a result of which an unpaired electron is removed from the radicals formed initially, can be achieved either enzymatically or without the involvement of enzymes using Cu^{2+} or Fe^{3+} as catalysts.

The mesomeric cation thus produced can react with coniferyl alcohol which results in the formation of the dimeric quinone methide (XXXII). As will be shown below, the formation of this quinone methide may be represented also as due to the combination of the mesomeric forms R_a and R_b: [201]

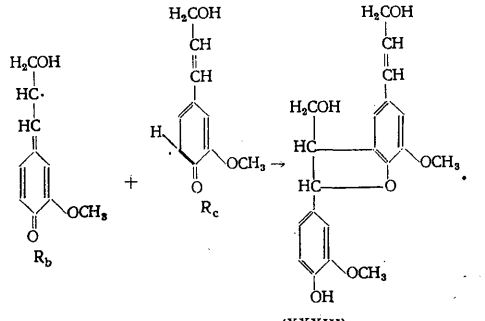


(XXXII)

3. The Principles of the Growth of the DHP Molecule and the Principal Intermediates Isolated in the Course of the Formation of Biosynthetic Lignin

Freudenberg established [211] that the decomposition of the enzymes by NaCN can interrupt the enzymatic dehydrogenation of coniferyl alcohol at any stage with the aim of the subsequent isolation of the intermediate in the biosynthesis of lignin *in vitro*. The best yield of low-molecular-weight intermediates in the dehydrogenation of coniferyl alcohol was obtained after removing approximately one atom of hydrogen from each molecule of the initial carbinol. When large amounts of hydrogen are eliminated, water-insoluble products with a high molecular weight are produced. The intermediates in the biosynthesis of lignin *in vitro* thus produced were separated by paper chromatography. After treating the chromatograms with diazobenzenesulphonic acid, about 40 spots were detected [212]. Freudenberg and coworkers [4,213] succeeded in isolating and then identifying a number of dimeric, trimeric, and oligomeric intermediates. Freudenberg suggested [214] that the intermediates may be referred to as lignols.

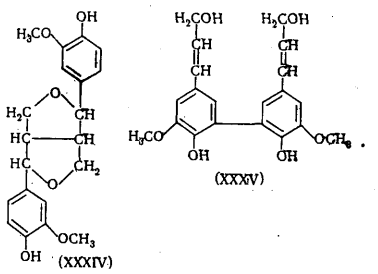
As mentioned in the preceding section, Freudenberg suggested that dilignols are produced as a result of the combination of the mesomeric forms R_a–R_d of the aryloxy-radicals obtained by the enzymatic dehydrogenation of coniferyl alcohol. Thus the combination of the R_b and R_c forms yields dehydrodiconiferyl alcohol (XXXIII): [203]



(XXXIII)

A similar example of the stabilisation of two mesomeric R_b forms owing to dimerisation is provided by the formation of DL-pinoresinol (XXXIV) [204] and also by the formation of dehydrobisconiferyl alcohol (XXXV) [211] as a

result of the combination of two mesomeric forms R_b:

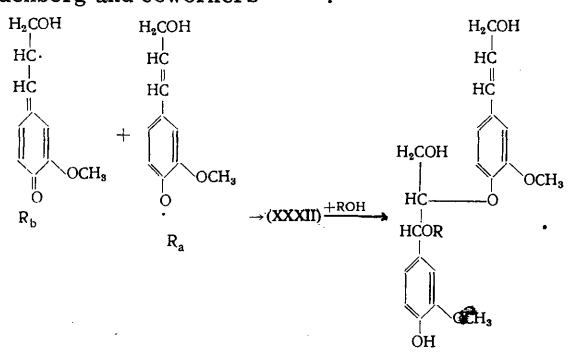


(XXXIV) (XXXV)

According to Freudenberg's views [208,215], the quinone methide (XXXII), formed by the combination of the mesomeric forms R_a and R_b, plays an important role in further biosynthesis of lignin [201]. It is noteworthy that the quinone methide structure proposed initially as hypothetical was subsequently confirmed by ultraviolet spectroscopic measurements [216]. At the same time it was established that the half-life of the quinone methide in a 1 : 1 (by volume) dioxan–water mixture at 20°C is about 60 min. The quinone methide is unstable and cannot be stabilised by an intramolecular rearrangement. Consequently, it is stabilised intramolecularly by combination with the elements of water or other dissociated hydroxyl-containing compounds, provided that steric conditions allow it. The addition of water to quinone methide leads to the formation of the β-coniferyl ether of guiacylglycerol (XXXVI), [201] which constitutes, together with the dilignols (XXXIII) and (XXXIV), the main part of the dimeric intermediates. It is interesting that these three principal dilignols in the biosynthesis of lignin *in vitro* were also detected by Freudenberg and Harkin [197] in the cambial sap of the spruce.

Enzymatic dehydrogenation of coniferyl alcohol in aqueous solution containing 30% of methanol results in the formation of the α-methyl-β-coniferyl ether of guiacylglycerol (XXXVII) [208] and, when the reaction is carried out in an aqueous solution containing 66% of sucrose, the corresponding sugar ester (XXXVIII) is produced [208,217]. Freudenberg regards this benzyl ether linkage as one of the most probable types of lignocarbohydrate linkages in plant tissues.

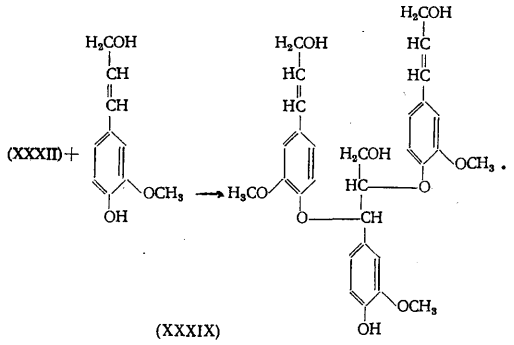
Since the dilignols considered are orthophenols, they can undergo further enzymatic dehydrogenation with reformation of dimeric radicals, which can recombine both with one another and with the mesomeric forms R_a–R_d. Such reactions lead to trilignols, tetralignols, and subsequently oligolignols. A number of the oligomeric compounds were isolated and identified by Freudenberg and coworkers [218–224]:



(XXXVI, R=H)
(XXXVII, R=CH_3)
(XXXVIII, R=$(C_6H_{10}O_5)_nH$)

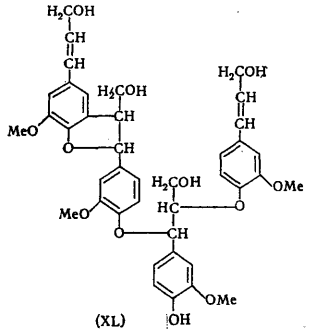
On the basis of the identified intermediates in the biosynthesis of lignin from coniferyl alcohol *in vitro*, Freudenberg [202,212,225,226] formulated the main pathways to the growth of the DHP molecule.

The first pathway consists of continuing dehydrogenation of oligolignols which leads to the formation of new aryloxy-radicals undergoing various recombinations. However, if the growth of the DHP molecule took place exclusively via dehydrogenation, then in this case each structural unit should loose two hydrogen atoms plus a further ~0.4 of a hydrogen atom owing to the formation of the carbonyl groups ($-C\overset{H}{\underset{O}{\diagup\!\diagdown}}$ and $\diagdown C{=}O$), i.e. altogether 2.4 hydrogen atoms. In fact, the maximum loss occurring is approximately two hydrogen atoms [227], which includes the average loss of 1.7–1.8 hydrogen atoms by each DHP structural unit owing to the formation of the bonds and 0.2–0.3 of a hydrogen atom owing to the formation of carbonyl groups. Thus the amount of hydrogen the removal of which leads to condensation corresponds to 1.7–1.8 atoms per structural units, which is insufficient for the formation of large molecules. In other words, a linkage produced as a result of addition or polymerisation and not related to the loss of hydrogen corresponds approximately to each four structural elements associated owing to the loss of hydrogen. This indicates the existence of other pathways to the growth of the lignin macromolecule. In this respect the quinone methide (XXXII), which can combine with phenols without an additional loss of hydrogen, plays a key role. The simplest example of the second pathway to the growth of the DHP macromolecule is the addition of coniferyl alcohol to the dimeric quinone methide (XXXII), which yields a trilignol—the aryl benzyl ether (XXXIX): [218]

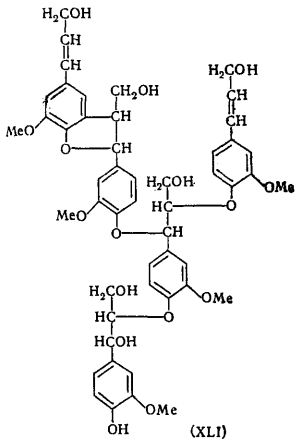


(XXXIX)

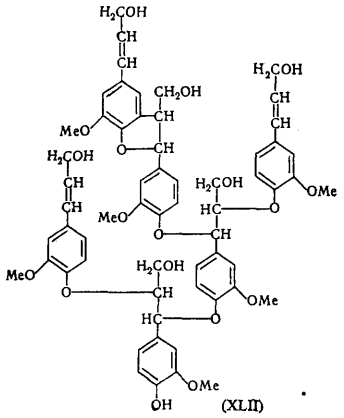
This growth pathway can also be illustrated by the formation of the tetralignol (XL) (the adduct of dehydrodiconiferyl alcohol and the quinone methide [218]), the pentalignol (XLI) (the adduct of the trilignol and the quinomethide) [224], and the hexalignol (XLII) (the adduct of the tetralignol and the quinone methide) [224].



(XL)

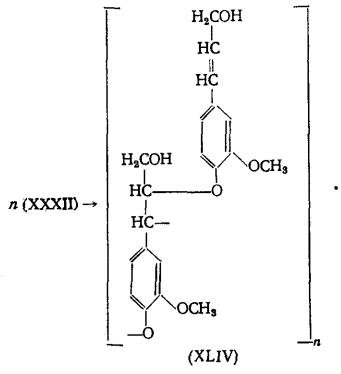


(XLI)



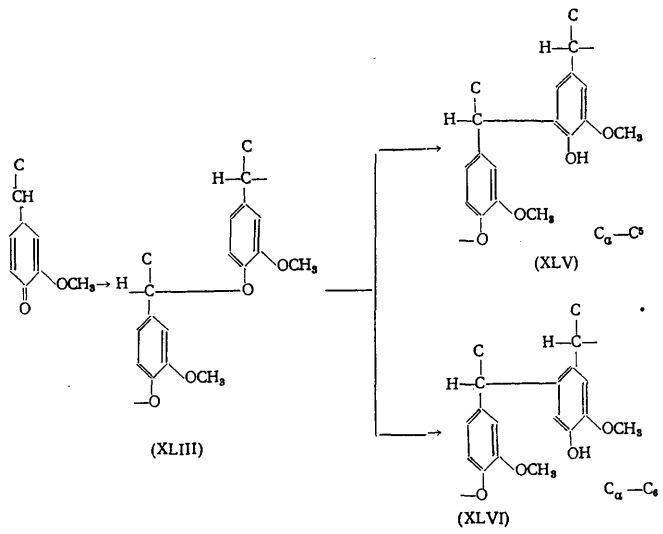
(XLII)

The polymerisation of *p*-quinone methides, leading to the formation of aryl benzyl ethers (XLIII),[228] competes with the addition of phenols to quinone methides. For example, compound (XLIV) is a product of the polymerisation of the quinone methide (XXXII). This process is considered as the third pathway to the growth of the DHP molecule:



(XLIV)

Finally the fourth pathway to the growth to the DHP molecule involves the rearrangement of α-aryl ethers, leading to the formation of diphenylmethane derivatives[222,228]. This process takes place after prolonged treatment with very dilute acids (similar conditions obtain in the cell sap of plants). As a result of the condensation between the α-C atom of the first unit and the fifth carbon atom in the ring of the second unit with simultaneous regeneration of the phenolic hydroxyl of the second unit, the ether (XLIII) is converted into the structure (XLV). A similar condensation of the α-C atom can occur to a lesser extent with the sixth carbon atom of the second unit and even less effectively with the second carbon atom. This rearrangement of α-aryl ethers does not take place solely in polymers but has been observed also with α-aryl guiacylglyceryl ethers (tri- and tetra-lignols)[222,226].



(XLIII)

(XLV)

(XLVI)

The non-enzymatic recombination of various mesomeric forms of the aryloxy-group formed on dehydrogenation of coniferyl alcohol results in a lack of optical activity of the polylignols synthesised both *in vivo* and *in vitro* despite the presence of asymmetric centres[205,215].

4. Chemical and Physical Properties of Dehydropolymers and Their Comparison with Those of Lignin Produced by Mechanical Milling

Table 1 presents semiempirical "formulae" for the $C_{(6)}-C_{(3)}$ structural unit of lignin, various DHP preparations, and lignin produced by mechanical milling [milled-wood lignin (MWL)] and isolated from spruce and beech wood by Björkman's method[229,230]. The data presented are evidence of the similarity of spruce wood MWL and DHP obtained by the enzymatic dehydrogenation of a mixture of coniferyl, *p*-coumaryl, and sinapyl alcohols.

In addition to the data in Table 1, we may mention that, in the MWL from spruce wood and DHP obtained by the enzymatic dehydrogenation of coniferyl alcohol, the total content of hydroxy-groups, determined by Verly's method, is in both cases 1.2–1.4 moles per $C_{(6)}-C_{(3)}$ unit[230,231], of which 0.30–0.35 mole comprises phenolic hydroxyls (determined by potentiometric titration in a non-aqueous medium[227,232]); the content of carbonyl groups, determined by the hydroxylamine method, is 0.20 mole per $C_{(6)}-C_{(3)}$ unit for MWL[230] and 0.15 mole for DHP.[231]

It is interesting to note that the molecular weights of preparations of both biosynthetic lignin and lignin isolated from wood or by mechanical grinding are in the range 6000–10 000.[209,230] The results of spectroscopic studies on these preparations are also similar. The infrared spectra of MWL and DHP are identical except for certain

details due to the fact that the preparations differ somewhat in their contents of carbonyl groups (ν = 1710–1740 cm^{-1}) and ethylene double bonds (ν = 970 cm^{-1}).[233–235] The ultraviolet spectra of both preparations are similar as regards the positions of the maxima and minima. At the same time the extinction coefficient of biosynthetic lignin is higher by a factor of 1.2–1.3%. However, after DHP has been kept in 0.1 *N* inorganic acid at 20°C for one week, the extinction coefficient of biosynthetic lignin becomes the same as that of natural lignin[209,236]. Finally, Freudenberg and Harkin showed[237] that both preparations contain small amounts of free radicals. According to ESR data, their concentration is 2.3×10^{16} spin g^{-1} in MWL from spruce wood and 2.15×10^{16} spin g^{-1} in biosynthetic lignin from coniferyl alcohol.

Table 1. Semiempirical "formulae" of certain preparations of biosynthetic and natural lignins (according to the data of Freudenberg et al.[198]).

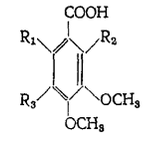
Preparation	Semiempirical "formula"	Loss of hydrogen	Increment of water
DHP from *p*-coumaryl alcohol	$C_9H_{8.75}O_2(H_2O)_{0.30}$	1.25	0.30
DHP from coniferyl alcohol	$C_9H_{7.49}O_2(H_2O)_{0.28}(OCH_3)_{1.01}$	1.51	0.26
DHP from *p*-coumaryl and coniferyl alcohols (1:4)	$C_9H_{7.76}O_2(H_2O)_{0.28}(OCH_3)_{0.83}$	1.44	0.28
DHP from *p*-coumaryl, coniferyl, and sinapyl alcohols (14:80:6)	$C_9H_{7.64}O_2(H_2O)_{0.31}(OCH_3)_{0.92}$	1.44	0.31
MWL from spruce wood	$C_9H_{7.78}O_2(H_2O)_{0.40}(OCH_3)_{0.91}$	1.30	0.40
MWL from beech wood	$C_9H_{6.28}O_2(H_2O)_{0.41}(OCH_3)_{1.36}$	2.36	0.41

*For greater clarity the oxygen content in the "formulae" of the lignins is shown as in the initial alcohol (two atoms per $C_{(6)}$–$C_{(3)}$ unit). The excess oxygen is liberated in the form of water.

The products obtained by the interaction of both DHP and spruce wood lignin with thioglycolic acid had the same elemental composition[196]. This applies also to products of the methylation of DHP and spruce wood lignin with both dimethyl sulphate and alkali and with diazomethane[238], as well as to ammonium lignosulphonates obtained from spruce wood and DHP.[196]

Convincing evidence of the similarity of the compositions of natural and biosynthetic lignins was obtained by cleavage reactions. Thus, when these preparations are treated under identical conditions with hot alkali and then methylated with dimethyl sulphate and the methylated products are oxidised with permanganate. MWL and DHP from a mixture of coniferyl, *p*-coumaryl, and sinapyl alcohols yield more than 20 of the so called degradation acids, mainly methoxybenzoic acids, and the total yield of identified products is about 18% of theoretical[198,214]. The main product of the oxidation of methylated lignins is veratric acid (XLVII), which constitutes about half of the overall yield. The remaining acids were detected in insignificant amounts. Freudenberg and coworkers showed[198,214] that the quantitative ratios and types of acids formed on oxidation of both lignin preparations are identical.

It is interesting to note that in the preparation of biosynthetic lignins from 5-deuterioconiferyl alcohol the loss of deuterium was 45%.[239] On dehydration under the conditions of DHP synthesis, 6-deuterioconiferyl alcohol looses 8–10% of deuterium and 2-deuterioconiferyl alcohol loses 2–4%.[240] These values indicate the relative involvement of various sites in the aromatic ring in the formation of intermolecular C–C bonds of lignin, the reactivity ratios of the 5-, 6-, and 2-positions (45 : 9 : 3) corresponding approximately to the quantitative ratios of the yields of isohemipinic (XLVIII), metahemipinic (XLIX), and hemipinic (L) acids in the products of the oxidative degradation of methylated DHP and MWL:[214]



(XLVII, $R_1=R_2=R_3=H$)
(XLVIII, $R_3=COOH$; $R_1=R_2=H$)
(XLIX, $R_1=COOH$; $R_2=R_3=H$)
(L, $R_2=COOH$; $R_1=R_3=H$)

When biosynthetic lignin obtained from [β-^{14}C]coniferyl alcohol, is cleaved, the isohemipinic acid (XLVIII) formed has 49–52% of the specific radioactivity of the initial alcohol, while metahemipinic acid (XLIX) has 10.5% of the specific radioactivity[241]. The isohemipinic and metahemipinic acids isolated from the oxidative degradation products of lignin, formed in young shoots or after the introduction of similarly labelled phenylalanine[242] or coniferin[206], have the same radioactivities in relation to those of the initial lignins (53 and 12% respectively).

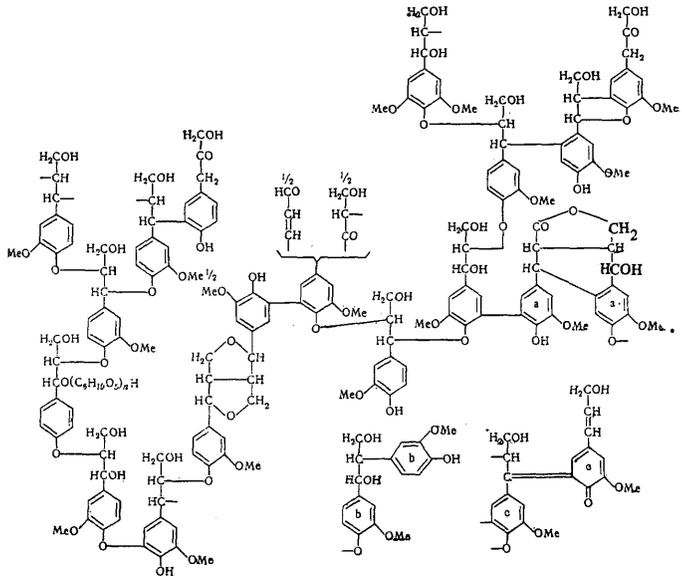
On heating with 28% sulphuric acid, the yield of formaldehyde is 1.1% for MWL and 1.4% for DHP.[209] Finally the yields of vanillin on nitrobenzene oxidation of both preparations are identical and amount to 25% of the initial lignin[209].

Thus the data discussed above demonstrate convincingly the similarity of natural (MWL) and biosynthetic (DHP) lignins as regards many important chemical and physical properties.

5. The Structure of a Lignin Fragment

The data discussed above on the biosynthesis of lignin *in vitro* (intermediates, the ratio of the principal dilignols, the four growth pathways) together with the chemical analyses of spruce wood lignin isolated by Björkman's method (elemental composition, the number and types of hydroxy-groups, the ratio of the three initial monomeric alcohols, the content of carbonyl groups, etc.) and the results of experiments on the polymerisation of labelled [^{14}C]coniferyl alcohol served as a basis of the schematic structural formula of a lignin fragment derived by Freudenberg. The first version[243] proposed in 1961 was subsequently modified several times by the author[220,226,237]. The latest scheme, put forward by Freudenberg at the International Symposium at Grenoble[225], consists of 18 units and corresponds to the composition of the dehydropolymer obtained by the dehydrogenation *in vitro* of a very dilute mixture of 14 moles of *p*-coumaryl alcohol, 80

moles of coniferyl alcohol, and 6 moles of sinapyl alcohol catalysed by laccase.



The detailed characteristics and an assessment of the schematic formula for a lignin molecular fragment as well as its comparison with natural and biosynthetic lignins are given in a number of latest reports by Freudenberg and coworkers [209,215,225,244-247]. In the present review we shall confine ourselves to a Table where certain characteristics of spruce wood lignin are compared with the corresponding data calculated on the basis of the schematic formula of a lignin fragment (Table 2).

Table 2 shows that the characteristics predicted on the basis of Freudenberg's scheme and covering a wide range of the chemical reactions of lignin are satisfactorily consistent with the experimental value. This enabled Freudenberg [225] to assert that at the present time there are no facts in the chemistry of lignin which could not be accounted for by his scheme.

In conclusion of the discussion of Freudenberg's work on the biosynthesis of lignin *in vitro*, we must consider certain methodological questions. Freudenberg [209] correctly suggests that comparison of the biosynthetic and natural lignins is valid only when the following factors are identical in both cases: (1) the initial monomeric products and their ratios; (2) the conditions governing the formation of lignin (enzyme systems, temperature, etc.); (3) the final products.

As shown in the previous sections, in the present stage of the investigation of lignin, all these factors appear to be identical both in the *in vitro* biosynthesis of lignin and in the formation of lignin in the plant. On the basis of the identity of the starting materials and final products in the synthesis of lignin *in vitro* and *in vivo*, Freudenberg [209] concluded that the mechanisms of natural and artificial lignification are the same and that the intermediates formed in both cases are similar. Moreover, Freudenberg [202] postulates that the data obtained in the study of the biosynthesis of DHP from *p*-hydroxycinnamyl alcohols can be used to account for the structure of natural lignin.

The structures of polysaccharides, proteins, and other biopolymers are established by identifying the oligomeric products obtained as a result of their cleavage reactions (mainly by hydrolysis). The determination of the structure of lignin on the basis of those of the oligolignols formed during its biosynthesis *in vitro* constitutes essentially a first attempt to determine by this type of procedure the structure of this complex polymer. One must not forget that the role of lignin in the plant is responsible for a number of the characteristics of its natural synthesis. In contrast to the synthesis of proteins, the molecules of which are constructed with the aid of templets, which ensures their exact reproduction, many stages of the biosynthesis of lignin take place in a random fashion, which is the reason for the wide variety of types of intramolecular bonds in its macromolecule. All this must be taken into account in assessing the structural scheme for a fragment of the lignin molecule devised by Freudenberg.

Table 2. Comparison of certain characteristics of lignin with data calculated on the basis of the schematic representation of a lignin* fragment (according to Freudenberg's data [247]).

Characteristic	Found or calc. for spruce wood lignin	Predicted on the basis of the schematic formula of a lignin fragment
Units capable of reacting with thioglycolic acid (α-ether, α-carbinol, phenylethylene, carbonyl)	0.8	0.8
Formation of lignosulphonic acid**		
Group X } A	0.15	0.17
Group Z } A	0.15	0.20
Group B	0.30	0.28
Total	0.60	0.65
Units capable of splitting off methanol (0.5% HCl, 20°C)	0.62	0.72
Units capable of splitting off methanol after treatment with $NaBH_4$	0.42	0.41
Condensation at 5-C	0.45	0.42
Condensation at 6-C	0.08	0.07
Condensation at 2-C	0.04	
Units capable of involvement in biphenyl linkages	0.25-0.30	0.22
Units capable of forming Hibbert ketones	0.1	0.17
Units capable of forming vanillin and vanillic acid	0.32	0.30-0.35
Aromatic and similar protons } from NMR spectra	2.5	2.4
Aliphatic protons } from NMR spectra	3.8	3.8

*The calculation refers to the average composition of the structural unit.
**Group X = *p*-hydroxybenzyl alcohols and alkyl *p*-hydroxybenzyl ethers; group Z = *p*-alkoxybenzyl alcohol; group B = alkyl *p*-alkoxybenzyl ethers.

On the basis of the foregoing, one must emphasise that the series of Freudenberg's studies on the biosynthesis of lignin has made a major contribution to the chemistry of lignin. Freudenberg's scheme for a lignin macromolecular fragment undoubtedly provides an approximate idea about the structure of lignins. Freudenberg's principal conclusions have been confirmed by the latest studies of Nimz [248-250] on the isolation of dimeric and oligomeric lignin fragments by mild hydrolysis (percolation) of wood. However, Pew's work on the enzymatic dehydrogenation of alkyl-*p*-hydroxyphenylmethanols and *p*-hydroxypropiophenones, discussed in the next section, has revealed a number of new aspects of the biosynthesis of lignin which

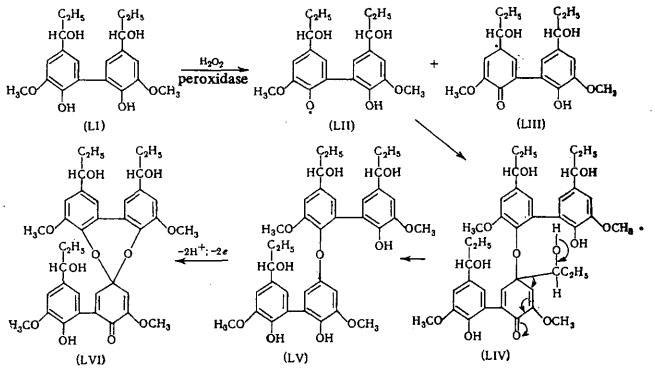
are not accounted for by Freudenberg's theory. Other combinations of groups leading to the formation of hitherto undiscovered structural elements of lignin are quite probable. Thus the question of the formation of lignin in plants and *in vitro* requires further studies.

6. Enzymatic Dehydrogenation of Other Phenylpropane Compounds and "Chemical Synthesis" of Lignin

A considerable number of studies on the preparation of lignin-like polymers by the enzymatic dehydrogenation of phenolic precursors other than coniferyl alcohol have been published. These investigations, which have been quite adequately discussed in the monograph of Brauns and Brauns [2], are of comparatively lesser interest since they deal mainly with the final dehydropolymerisation products. The recently published work of Pew [251-255] on the enzymatic dehydrogenation of a number of phenolic compounds structurally related to lignin is superior in this respect.

The study of the dehydrogenation of model phenols, preferably without C–C bonds in the side chain conjugated with the aromatic ring (eugenol, allylsyringol, isoeugenol, dehydrodieugenol, and others), by the action of peroxidase and hydrogen peroxide, led Pew and coworkers [251] to the conclusion that, apart from the biphenyl linkages, diphenyl ether linkages between monomeric units play a considerable role in the formation of lignin.

In addition, the authors showed that the enzymatic dehydrogenation of the phenols results in the formation of quinone methides of a type different from that described in the literature, which the authors believe can participate in the formation of lignin. Later Pew and Connors [252] published the results of a study of the enzymatic dehydrogenation of a number of alkyl-*p*-hydroxyphenylmethanols. When ethylguiacylmethanol was dehydrogenated in an aqueous solution by peroxidase and hydrogen peroxide, a hitherto unknown dibenzo[*d*,*f*][1,3]dioxepin (LVI) is formed with elimination of the side chain as propionaldehyde:

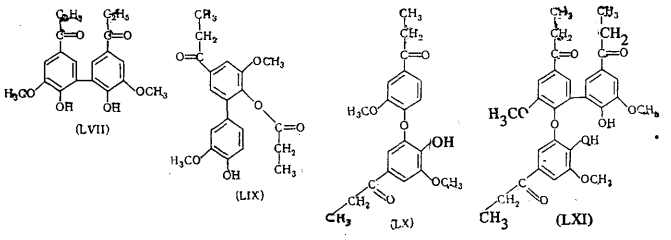


The mechanism of the formation of the dioxepin (VIL) proposed by the authors [252] consists in the initial formation of dehydrobisethylguiacylmethanol (LI) with its subsequent dehydrogenation, which leads to the formation of the biphenylyloxy-radical (LII); this combines with the cyclohexadienone mesomeric form of the same radical (LIII) to yield the dienone tetramer (LIV). The latter is then rearomatised by the elimination of propionaldehyde to form compound (LV), which yields phenoxy- and *p*-cyclohexadienone radicals on further dehydrogenation. The radicals undergo an intramolecular coupling to form the dioxepin (LVI).

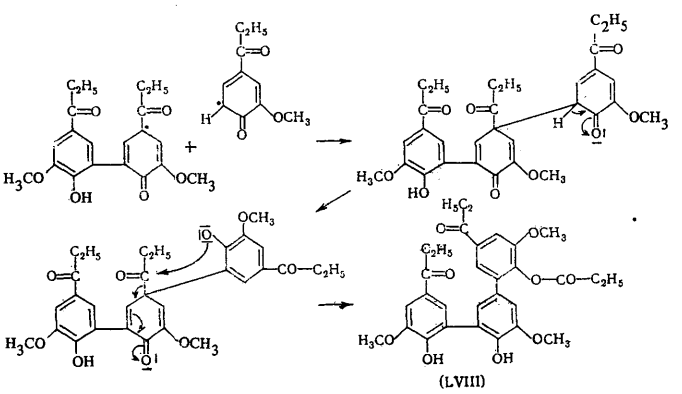
The authors showed [252] that an analogous type of reaction with formation of the corresponding dioxepin is undergone also by β-guaiacyl guaiacylglyceryl ether, the analogue of the extremely important dimeric intermediate in the biosynthesis of lignin *in vitro*. In addition, evidence was obtained showing that other *p*-hydroxybenzyl alcohols and certain ethers reacts similarly.

The results of this investigation enabled Pew and Connors to conclude that cyclohexadienone forms of phenoxy-radicals play an important role in the biosynthesis of lignin. It is particularly true in those cases where the *ortho*-position in relation to the phenolic hydroxyl is blocked, as in the cases where biphenyl and diphenyl ether linkages or a methoxy-group are present in syringic structures. Coupling involving this type of radical is usually accompanied by the removal of the side chain and, when biphenyl groups are present, it leads to the formation of structures of the dioxepin type. The latter can enter into the composition of the lignin macromolecule as such or in the form of quinonoid structures formed by the hydrolysis of the dioxepins.

Pew and Connors [253] also achieved the enzymatic dehydrogenation of a number of *p*-hydroxypropiophenones. When propioguaiacone is dehydrogenated in aqueous solutions by peroxidase and hydrogen peroxide, the product is an *oo'*-dihydroxybiphenyl derivative—dehydrodipropioguaiacone (LVII). In addition to this compound, a triphenyl derivative (LVIII), with an *op'*-biphenyl type of linkage hitherto unknown for lignin and with a propionate ester group, a dimeric ester (LIX), an *o*-diphenyl ether derivative (LX), and a trimer (LXI) containing both the *oo'*-biphenyl and *o*-diphenyl ether linkages were also identified.



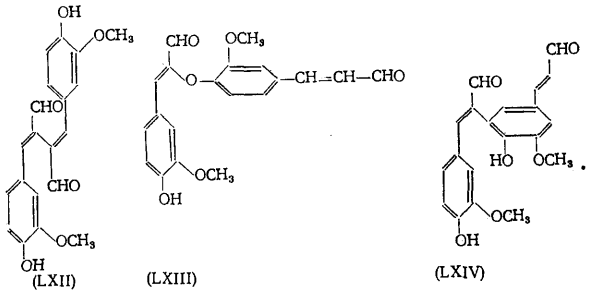
According to Pew and Connors [254], the mechanism of the formation of the trimer (LVIII) consists of the coupling of the cyclohexadienone mesomeric form of the biphenyl radical with the mesomeric *ortho*-radical. The coupling is followed by the rearomatisation of the dienone ring via an intramolecular nucleophilic attack on its carbonyl group in the side chain by the phenoxide ion produced by the deprotonation of the upper unit, which leads to transfer of the side chain and the formation of the ester (LVIII).



The formation of esters via the transfer of the side chain was demonstrated[254] with the aid of spectroscopic methods also on dehydrogenation products of vanillin, acetoguaiacone, and ethyl vanillate and on this basis it was concluded that the reaction is probably general.

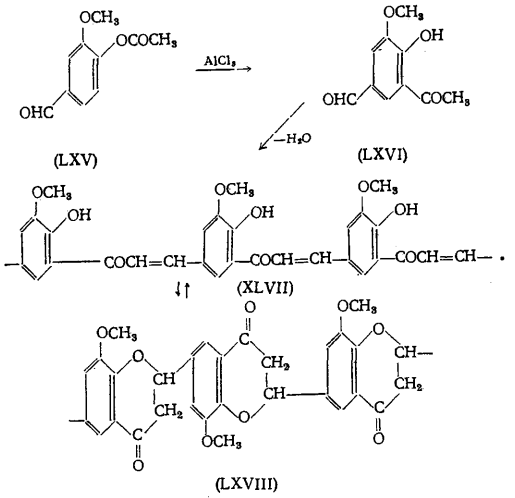
The results led to the hypothesis that lignin contains *op'*-oriented biphenyl linkages—a hitherto unknown type of linkage between monomeric units—and also that aliphatic acid esters are structures characteristic of the lignin macromolecule.

Connors et al.[255] dehydrogenated *trans*-coniferaldehyde in aqueous solution with peroxidase and hydrogen peroxide. They showed that the bulk of the products consists of three hitherto unknown dimers: 2,3-diformyl-1,4-di-(5-guaiacyl)buta-1,3-diene (LXII); α-[4-(β-formylvinyl)-2-methoxyphenoxy]coniferaldehyde (LXIII); α-[5-(β-formylvinyl)-2-hydroxy-3-methoxyphenyl]coniferaldehyde (LXIV):



(LXII) (LXIII) (LXIV)

The results of the above studies on the enzymatic dehydrogenation of various model compounds of lignin, carried out by Pew and coworkers, reveal certain aspects of the biosynthesis of lignin *in vitro* and can be regarded as supplementing Freudenberg's theory. There is no doubt that future studies will broaden current ideas on the biosynthesis of lignin and will lead to the discovery of new structural features of this polymer. Finally, mention should be made of the studies of Russell[256-259] on the chemical synthesis of lignin, which constitutes perhaps the only attempt of this kind. The author employed as the starting material vanillin monoacetate (LXV), which yielded 5-formyl-2-hydroxy-3-methoxyacetophenone (LXVI) as a result of the Fries rearrangement. The acetophenone readily underwent condensation polymerisation to compound (LXVII) which in turn cyclised into poly-8-methoxydihydrobenzopyrone (LXVIII). According to Russell's data, the polymer is in many respects analogous to spruce wood lignin:



(LXV) (LXVI) (XLVII) (LXVIII)

It is noteworthy that, whereas in the biochemical synthesis of lignin the aryloxy-radicals produced react in different forms to yield various recombination products which precludes a strictly defined sequence of linkages between the monomers in the polymer, in the chemical synthesis this type of disorder does not occur. Modern ideas concerning the inhomogeneity of the structure of lignin are inconsistent with Russell's "formula" and therefore his investigations were not continued further and are quoted here only for historical interest.

IV. SOME ASPECTS OF THE BIOSYNTHESIS OF LIGNIN *IN VIVO*

The results of the study of the biosynthesis of lignin *in vitro* led Freudenberg[147,260,261] to a hypothesis concerning the formation of lignin in the plant. During the vegetative period, new cells are actively formed in the cambial layer of wood. Young, still unlignified cells adjoining the cambium are gradually replaced, as they are separated further from the cambial layer, by ripe thick-walled cells, which produce an intense colour on treatment with phloroglucinol and hydrochloric acid. Coniferin has been detected in considerable amounts in the cambial layer and on either side of it. However, the laccase and peroxidase present in the precambial layer are capable of dehydrogenating coniferyl alcohol only. On this basis, one may expect the presence in this part of woody tissue of β-glucosidase which has in fact been detected in cuttings of 2 year old spruce shoots treated with indican (indoxyl glucoside). The β-glucosidase cleaved indican and the free indoxyl formed was rapidly converted in air into indigo, indicating the location of β-glucosidase. It was found that β-glucosidase is absent both from the young cells of the precambial layer and from the ripe lignified cells but is detected in large amounts in an intermediate layer, which is evidently the zone where lignin is formed. On the basis of these results, Freudenberg suggests that the formation of lignin in the plant can be represented as follows. Coniferin diffuses into the tissue where it is cleaved by β-glucosidase to free coniferyl alcohol, which is converted into lignin by the action of peroxidase and laccase. As soon as the tissue, particularly its intracellular parts, is filled by lignin, the cells die and β-glucosidase is no longer detected in them. It is interesting to note that, when the enzyme action continues after the tissue has been filled by lignin, the latter is degraded. Thus, when laccase or peroxidase acts on a suspension of natural or biosynthetic lignin in water, the preparations are degraded in a manner resembling humification and the degradation of phenols in the soil. A similar degradation is observed when wood rot serves as a source of dehydrogenases. Moreover, as mentioned above, the yield of DHP amounts to about 90%, never reaching 100%. According to Freudenberg's views[262], this is because lignin is partly decomposed on dehydrogenation. Thus the same enzymes (laccase and peroxidase) are involved in the formation and degradation of lignin.

As stated in the introduction, the lignification process cannot be regarded in isolation from other processes occurring in the formation of the plant cell. The complete set of these processes includes the biosynthesis of various components of plant tissue, during which different types of chemical linkages may arise between them together with the formation of various morphological components of the cell wall. These problems are undoubtedly of considerable theoretical and practical interest. On the

other hand, the available literature data are very limited, which precludes at the present time the consideration of the problems of the biosynthesis of the complete set of plant cell wall components. Evidently such an attempt is a matter for the future when not only the problems of the biosynthesis of the individual plant components (lignin, celluloses, and hemicelluloses) but also the general relations governing the formation of the plant cells will become adequately elucidated. However, even now many interesting data obtained in the study of the lignification of tissue cultures throw light on certain aspects of the biosynthesis of lignin *in vivo*.

As stated above, the method of tissue culture is widely used [31,99,101,158-165,263-271] in the study of various enzymatic systems involved in the biosynthesis of monomeric precursors of lignin and also in tests on various labelled compounds as lignin precursors. These investigations were considered in individual subsections of section II. Apart from these more of less local problems, experiments on tissue culture permitted an approach to the solution of wider problems of the biochemistry and physiology of plants. These may be formulated as follows: (1) the establishment of the possibility of a local lignification process or of the requirements by this process for compounds entering from the roots or leaves of the plant; (2) the establishment of the identity of the lignin precursors with retention or exclusion of correlations in the plant organism (when the biosynthesis of aromatic lignin precursors is localised, as mentioned above, are obtained); (3) the establishment of the chemical nature of the possible lignin precursors and the discovery on this basis whether the lignification process takes place in isolated tissues via the same pathways as in whole plants; (4) the elucidation of the physiological role of intermediate metabolites of the lignification process in the metabolism of the whole organism.

The studies using the tissue culture method established [264] that the lignin deposited in cell walls of the xylem elements of isolated tissues has a chemical composition similar to that of the lignin of higher plants. Moreover, it has been shown [165] that the biosynthesis of lignin in isolated tissues which have been living for a long time in an *in vitro* culture takes place locally using the carbohydrates of the nutrient medium. Consequently lignification does not require the supply of specific compounds from the roots or plant organs above ground. Since it was observed that inhibition of the growth of tissues does not affect the accumulation of lignin, it has been suggested [162] that the lignification and growth processes are to some extent separate.

The conclusions arrived at as a result of studies on tissue cultures are of great interest and permit an approach to the solution of the problems of the biosynthesis of lignin from a wider standpoint than is allowed by experiments on the biosynthesis of lignin *in vitro*. On the other hand, there is no doubt that these conclusions require certain additions and corrections. Finally a few concluding words may be said concerning the biosynthesis of lignin in connection with the problems of the orthogenesis and phylogenesis of plants.

In 1934 Holmberg [272] studied the lignins isolated from 50 plant species with the aid of thioglycolic acid and observed that the carbon content in the lignins of gymnosperms is higher by 3–4% than in the lignins of angiosperms, while the content of methoxy-groups is higher in the latter than in the former. Later, Hibbert and coworkers [273-275] used Freudenberg's method [276] of nitrobenzene oxidation to investigate plants of different classes and showed that, while the main product of the nitrobenzene oxidation of the lignin of gymnosperms is vanillin, dicotyledonous angiosperms yield a mixture of vanillin and syringaldehyde, while monocotyledonous angiosperms give a mixture of vanillin, syringaldehyde, and *p*-hydroxybenzaldehyde. The results led to the hypothesis that the reaction with alkaline nitrobenzene may be used for a taxonomic classification of plant materials. Subsequent investigations [277,278] by more sophisticated methods for the separation of oxidation products confirmed Hibbert's main conclusion with certain revisions. The results have served as a basis of the concept, generally accepted at the present time, that lignin is made up of phenylpropane derivatives methoxylated to different extents in the aromatic ring. The extensive studies by Manskaya [279-282], Kratzl [283], and others on lignins from different plant groups yielded interesting data concerning the role of lignin in the phylogenesis of plants. Thus Manskaya [279-282] studied living plant forms close to fossil plants in order to establish the formation of lignin in the phylogenesis in various plant groups. The author believes that the distinctive features of the lignins in various plant groups and the nature of the changes in their chemical composition on going from lower to more highly organised plants make it possible to deduce the relative content of lignin and the ratio of its elements methoxylated to different extents in plants existing in the past geological epochs. According to the majority of investigators, lignin which occurred initially in vascular plants of the order *Pteridophyta* (in recent years definite evidence has been obtained for its presence also in mosses [284] and even seaweed [285,286]), probably underwent considerable quantitative as well as qualitative changes during the evolution of plants.

There are many data demonstrating the changes in lignin during the orthogenesis of plants. Thus Stone et al. [287] showed that the ratio of vanillin and syringaldehyde in wheat lignin oxidation products vary with the age of the plants. Higuchi [134] studied the composition of lignin in the upper, middle, and lower parts of the shoots of three bamboo species. He showed [134], that as lignification progresses, there is a considerable increase in the overall content of aldehydes (calculated as the content of Klason's lignin) obtained on nitrobenzene oxidation and also of the ratio of syringaldehyde and vanillin. In the upper part of the bamboo shoots, where the degree of lignification was lowest, the fraction of syringaldehyde in the nitrobenzene oxidation products was also lowest. As lignification proceeded, the fraction of syringaldehyde increased, reaching a maximum in the adult plant. Freudenberg and Torres-Serres [288] showed that the cambial layer of one- and two-year old spruce shoots contains comparatively more *p*-glucocoumaryl alcohol than older spruces. Correspondingly the content of methoxy-groups in LMG from young spruces is 12–13% compared with 15.6% in MWL from the adult spruce [242,288]. These results suggest that the changes in lignin during onthogenesis corresponds to changes which it undergoes during phylogenesis.

The above data are undoubtedly of considerable interest although in the present stage of investigation it is as yet impossible to explain the changes in the degree of methoxylation of lignin during the onthogenesis and phylogenesis of plants.

REFERENCES

1. F. E. Brauns, "The Chemistry of Lignin", Acad. Press, Inc., New York, 1952, Chapters XXVI–XXVII.

2. F.E.Brauns and D.A.Brauns, "The Chemistry of Lignin" (Translated into Russian), Izd.Lesnaya Prom., Moscow, 1964, Chapter 25.
3. I.A.Pearl, "The Chemistry of Lignin", Marcel Dekker, Inc., New York, 1967, Chapter 4.
4. K.Freudenberg and A.C.Neish, "Constitution and Biosynthesis of Lignin", Springer-Verlag, Berlin–Heidelberg–New York, 1968.
5. F.F.Nord, Tappi, 47, 624 (1964).
6. W.J.Schubert, "The Biochemistry of Lignin" (Translated into Russian), Izd.Lesnaya Prom., Moscow, 1968.
7. W.J.Schubert, Comp.Biochem., 20, 193 (1968).
8. K.Kratzl, in "Cellular Ultrastructure of Woody Plants", Syracuse University Press, 1965, pp.157–180.
9. K.Kratzl, Cell.Chem.Techn., 1, 379 (1967).
10. J.M.Harkin, Fortschr.Chem.Forsch., 6, 101 (1966).
11. S.M.Manskaya, Khimiya Drevesiny, 1, 31 (1968).
12. S.A.Brown, in "Biochemistry of Phenolic Compounds" (Translated into Russian), Izd.Mir, Moscow, 1968, Chapter 9, pp.285–313.
13. S.A.Brown, BioScience, 19, 115 (1969).
14. M.S.Bardinskaya, "Rastitel'nye Kletochnye Stenki i Ikh Obrazovanye" (Plant Cell Walls and Their Formation), Izd.Nauka, Moscow, 1964.
15. S.M.Siegel, in "Biology of the Mouth", Publ.Amer. Ass.Adv.Sci.Washington, D.C., 1968, No.89, p.111.
16. S.M.Siegel, Comp.Biochem., 26A, 1 (1968).
17. D.H.Northcote, Essays Biochem., 5, 89 (1969).
18. M.N.Zaprometov, "Biokhimiya Katekhinov" (The Biochemistry of Catechols), Izd.Nauka, Moscow, 1964.
19. T.A.Geissman, in "Biogenesis of Natural Products" (Translated into Russian), Izd.Mir, Moscow, 1965, Chapter 12, pp.482–529.
20. A.S.Neish, see Ref.12, Chapter 8, pp.234–284.
21. C.E.Hartt and G.O.Burr, "Proceedings of the 7th International Botanical Congress, Stockholm, 1950", 1953, p.748.
22. J.E.Stone, Canad.J.Chem., 31, 207 (1953).
23. S.A.Brown, K.G.Tanner, and J.E.Stone, Canad.J. Chem., 31, 755 (1953).
24. Z.N.Kreitsberg, V.N.Sergeeva, and S.V. Milyutina, Trudy Inst.Lesokhoz.Probl., 12, 235 (1957).
25. Z.N.Kreitsberg and Ya.K.Grabovskii, Izv.Akad. Nauk Latv.SSR, Ser.Khim., No.3, 391 (1963).
26. W.J.Schubert and S.N.Acerbo, Arch.Biochem. Biophys., 83, 178 (1959).
27. S.N.Acerbo, W.J.Schubert, and F.F.Nord, J. Amer.Chem.Soc., 82, 735 (1960).
28. K.Kratzl and H.Faigle, Monatsh., 90, 768 (1959).
29. K.Kratzl and H.Faigle, Z.Naturforsch., 15B, 4 (1960).
30. M.Hasegawa and T.Higuchi, J.Japan.Forest.Soc., 42, 305 (1960).
31. M.Hasegawa, T.Higuchi, and H.Ishikawa, Plant Cell Physiol., 1, 173 (1960).
32. V.N.Sergeeva and Z.N.Kreitsberg, see Ref.24, p.245.
33. P.Klason, Ber., 63B, 1548 (1930).
34. P.Klason, Ber., 69B, 676 (1936).
35. K.Kratzl and J.Zauner, Holzforsch.Holzverwert., 14, 108 (1962).
36. B.D.Davis, Adv.Enzymol., 16, 247 (1955).
37. P.R.Srinivasan, H.T.Shigeura, M.Sprecher, D.B.Sprinson, and B.D.Davis, J.Biol.Chem., 220, 477 (1956).
38. B.D.Davis, Arch.Biochem.Biophys., 78, 497 (1958).
39. D.B.Sprinson, Adv.Carbohydrate Chem., 15, 235 (1960).
40. F.Gibson and L.M.Jackman, Nature, 198, 388 (1963).
41. F.Gibson, Biochem.J., 90, 256 (1964).
42. M.I.Gibson and F.Gibson, Biochem.J., 90, 248 (1964).
43. S.A.Brown and A.C.Neish, Nature, 175, 688 (1955).
44. G.Eberhardt and W.J.Schubert, J.Amer.Chem. Soc., 78, 2835 (1956).
45. D.R.McCalla and A.C.Neish, Canad.J.Biochem. Physiol., 37, 531 (1959).
46. D.R.McCalla and A.C.Neish, Canad.J.Biochem. Physiol., 37, 537 (1959).
47. O.L.Gamborg and A.C.Neish, Canad.J.Biochem. Physiol., 37, 1277 (1959).
48. M.Hasegawa, S.Yoshida, and T.Nakagawa, Kagaku (Science), 24, 421 (1954).
49. M.Hasegawa, T.Nakagawa, and S.Yoshida, J.Japan.Forest.Soc., 39, 159 (1957).
50. T.Higuchi, "Proceedings of the 4th International Congress of Biochemistry, Vienna, 1958", 1959, Vol.2, p.161.
51. V.Plouvier, Compt.rend., 249, 1563 (1959).
52. V.Plouvier, Compt.rend., 250, 1721 (1960).
53. V.Plouvier, Compt.rend., 252, 599 (1961).
54. V.Plouvier, Bull.Soc.France, Physiol.Vegetale, 7, 44 (1961).
55. M.Nandy and N.C.Ganguli, Biochim.Biophys. Acta, 48, 608 (1961).
56. M.Nandy and N.C.Ganguli, J.Sci.Ind.Res., India, 21C, No.2, 34 (1962).
57. S.Mitsuhashi and B.D.Davis, Biochim.Biophys. Acta, 15, 54 (1954).
58. "Nomenklatura Fermentov" (Enzyme Nomenclature), Moscow, 1966.
59. D.Balinsky and D.D.Davies, Biochem.J., 80, 300 (1961).
60. D.Balinsky and D.D.Davies, Biochem.J., 80, 292 (1961).
61. D.Balinsky and D.D.Davies, Biochem.J., 80, 296 (1961).
62. M.Nandy and N.C.Ganguli, Arch.Biochem.Biophys., 92, 399 (1961).
63. T.Higuchi and M.Shimada, Plant Cell Physiol., 8, 61 (1967).
64. D.Balinsky and D.D.Davies, J.Exp.Bot., 13, 414 (1962).
65. O.L.Gamborg, Canad.J.Biochem., 44, 791 (1966).
66. K.Paech, "Biochemie und Physiologie der sekundären Pflanzenstoffe", Springer-Verlag, Berlin–Göttingen–Heidelberg, 1950, p.153–154.
67. S.M.Manskaya and L.A.Kodina, Dokl.Akad.Nauk SSSR, 123, 733 (1958).
68. S.M.Manskaya and L.A.Kodina, Zhur.Prikl.Khim., 32, 2711 (1959).
69. L.H.Weinstein, C.A.Porter, and H.J.Laurencot, Jnr., Contrib.Boyce Thompson Inst., 20, 121 (1959).
70. L.H.Weinstein, C.A.Porter, and H.J.Laurencot, Jnr., Nature, 183, 326 (1959).
71. L.H.Weinstein, C.A.Porter, and H.J.Laurencot, Jnr., Contrib.Boyce Thompson Inst., 21, 201 (1961).

72. L.H.Weinstein, C.A.Porter, and H.J.Laurencot, Jnr., Nature, 194, 205 (1962).
73. O.Goldschmid and G.R.Quimby, Tappi, 47, 528 (1964).
74. Ya.K.Grabovskii, Z.N.Kreitsberg, and V.N. Sergeeva, Izd.Akad.Nauk SSSR, Ser.Khim., No.1, 114 (1967).
75. O.L.Gamborg, Phytochem., 6, 1067 (1967).
76. O.L.Gamborg, Biochim.Biophys.Acta, 128, 483 (1966).
77. E.E.Conn, see Ref.12, Chapter 10, pp.314–339.
78. O.L.Gamborg and F.J.Simpson, Canad.J.Biochem., 42, 583 (1964).
79. O.L.Gamborg and F.W.Keeley, Biochim.Biophys. Acta, 115, 65 (1966).
80. D.G.Wilson, K.W.King, and R.H.Burris, J.Biol. Chem., 208, 863 (1954).
81. V.L.Kretovich and Zh.V.Uspenskaya, Biokhimiya, 23, 248 (1958).
82. O.L.Gamborg and L.R.Wetter, Canad.J.Biochem. Physiol., 41, 1733 (1963).
83. O.L.Gamborg, Canad.J.Biochem., 43, 723 (1965).
84. T.Higuchi and M.Shimada, Plant Cell Physiol., 8, 71 (1967).
85. G.Schneider, Physiol.Plant., 14, 638 (1961).
86. J.F.Thompson, C.J.Morris, S.Asen, and F.Irreverre, J.Biol.Chem., 236, 1183 (1961).
87. C.J.Morris, J.F.Thompson, S.Asen, and F.Irreverre, J.Amer.Chem.Soc., 81, 6069(1959).
88. P.O.Larsen and A.Kjaer, Acta Chem.Scand., 16, 142 (1962).
89. V.L.Kretovich, "Osnovy Biokhimii Rastenii" (Fundamentals of Plant Biochemistry), Izd.Vysshaya Shkola, Moscow, 1971, pp.156–158.
90. A.C.Neish, see Ref.4, pp.17–30.
91. S.A.Brown, A.C.Neish, F.M.Claire, and M.D. Chisholm, Canad.J.Biochem.Physiol., 33, 948 (1955).
92. S.A.Brown and A.C.Neish, Canad.J.Biochem. Physiol., 34, 769 (1956).
93. A.C.Neish, Ann.Rev.Plant Physiol., 11, 55 (1960).
94. S.A.Brown, Canad.J.Bot., 39, 253 (1961).
95. J.Koukol and E.E.Conn, J.Biol.Chem., 236, 2692 (1961).
96. A.C.Neish, Phytochem., 1, 1 (1961).
97. M.R.Young and A.C.Neish, Phytochem., 5, 1121 (1966).
98. M.R.Young, G.H.N. Towers, and A.C.Neish, Canad.J.Bot., 44, 341 (1966).
99. T.Higuchi, Canad.J.Biochem.Physiol., 40, 31(1962).
100. T.Higuchi and S.A.Brown, Canad.J.Biochem. Physiol., 41, 65 (1963).
101. T.Higuchi and S.A.Brown, Canad.J.Biochem. Physiol., 41, 613 (1963).
102. H.Reznik and R.Urban, Naturwiss., 44, 13 (1957).
103. H.Reznik and R.Urban, Naturwiss., 44, 592 (1957).
104. S.Z.El-Basyouni, A.C.Neish, and G.H.N.Towers, Phytochem., 3, 627 (1964).
105. S.Z.El-Basyouni and A.C.Neish, Phytochem., 5, 683 (1966).
106. J.B.Harborne and J.J.Corner, Biochem.J., 81, 242 (1961).
107. J.B.Harborne and J.J.Corner, Arch.Biochem. Biophys., 92, 192 (1961).
108. C.W.Glennie and B.A.Bohm, Canad.J.Biochem., 44, 281 (1966).
109. D.E.Bland and A.F.Logan, Phytochem., 6, 1075 (1967).
110. C.C.Levy and M.Zucker, J.Biol.Chem., 235, 2418 (1960).
111. P.M.Nair and L.C.Vining, Phytochem., 4, 401 (1965).
112. P.M.Nair and L.C.Vining, Phytochem., 4, 161 (1965).
113. D.W.Russell and E.E.Conn, Arch.Biochem.Biophys., 122, 256 (1967).
114. M.Shimada, T.Yamazaki, and T.Higuchi, Phytochem., 9, 1 (1970).
115. R.U.Byerrum, J.H.Flokstra, L.J.Dewey, and C.D.Ball, J.Biol.Chem., 210, 633 (1954).
116. R.L.Hamill, R.U.Byerrum, and C.D.Ball, J. Biol.Chem., 224, 713 (1957).
117. Z.N.Kreitsberg and Ya.K.Grabovskii, Izv.Akad. Nauk Latv.SSR, Ser.Khim., No.1, 118 (1963).
118. B.J.Finkle and R.F.Nelson, Biochim.Biophys. Acta, 78, 747 (1963).
119. B.J.Finkle and M.S.Masri, Biochim.Biophys. Acta, 85, 167 (1964).
120. D.Hess, Z.Naturforsch., 19b, 447 (1964).
121. D.Hess, Z.Pflanzenphysiol., 53, 460 (1965).
122. D.Hess. Z.Pflanzenphysiol., 55, 374 (1966).
123. T.Higuchi, M.Shimada, and H.Ohashi, Agric. Biol.Chem., 31, 1459 (1967).
124. M.Shimada, H.Ohashi, and T.Higuchi, Phytochem., 9, 2463 (1970).
125. K.Kratzl and G.Puschmann, Holzforschung, 14, 1 (1960).
126. P.F.T.Vaughan and V.S.Butt, Biochem.J., 104, 65P (1967).
127. P.F.T.Vaughan and V.S.Butt, Biochem.J., 107, 7P (1968).
128. P.F.T.Vaughan and V.S.Butt, Biochem.J., 111, 32P (1969).
129. T.Higuchi and S.A.Brown, Canad.J.Biochem. Physiol., 41, 621 (1963).
130. G.G.Gross, K.H.Bolkart, and M.H.Zenk, Biochem. Biophys.Res.Comm., 32, 173 (1968).
131. G.G.Gross and M.H.Zenk, Eur.J.Biochem., 8, 413 (1969).
132. G.G.Gross and M.H.Zenk, Eur.J.Biochem., 8, 420 (1969).
133. T.Higuchi, Y.Ito, and I.Kawamura, J.Japan. Forest.Soc., 37, 239 (1955).
134. T.Higuchi, Physiol.Plant., 10, 633 (1957).
135. C.Neuberg and J.Hirsch, Biochem.Z., 115, 282 (1921).
136. C.Neuberg and L.Liebermann, Biochem.Z., 121, 311 (1921).
137. C.Neuberg and E.Reinfurth, Biochem.Z., 143, 553 (1923).
138. Y.Shimazu, J.Chem.Soc.Japan, 71, 503 (1950).
139. P.F.Smith and D.Hendlin, J.Bacteriol., 65, 440 (1953).
140. P.F.Smith and D.Hendlin, Appl.Microbiol., 2, 294 (1954).
141. T.Higuchi and I.Kawamura, J.Japan Wood Res. Soc., 2, 223 (1956).
142. S.M.Manskaya and M.S.Bardinskaya, Biokhimiya, 17, 711 (1952).
143. R.A.Black, A.A.Rosen, and S.L.Adams, J.Amer. Chem.Soc., 75, 5344 (1953).
144. A.Stepanow and A.Kusin, Ber., 64B, 1345 (1931).
145. S.A.Brown and A.C.Neish, J.Amer.Chem.Soc., 81, 2419 (1959).
146. H.Ishikawa and K.Takaichi, J.Japan.Forest.Soc., 37, 244 (1955).

147. K. Freudenberg, H. Reznik, W. Fuchs, and M. Reichert, Naturwiss., 42, 29 (1955).
148. K. Freudenberg, Angew. Chem., 68, 84 (1956).
149. K. Kratzl, W. Kisser, A. Graf, and G. Hofbauer, Monatsh., 90, 526 (1959).
150. K. Kratzl, Tappi, 43, 650 (1960).
151. M. H. Zenk, "Proceedings of the 2nd Meeting of the Federation of European Biochemical Societies, 1965", 1966, Vol. 3, p. 45.
152. H. Hibbert, Paper Trade J., 113, No. 4, 35 (1941).
153. H. Hibbert, Ann. Rev. Biochem., 11, 183 (1942).
154. T. Hartig, Jahrbuch für Förster, 1, 263 (1861).
155. R. E. Kremers, Tappi, 40, 262 (1957).
156. K. Freudenberg and F. Bittner, Chem. Ber., 86, 155 (1953).
157. K. Freudenberg and P. Niedercorn, Chem. Ber., 91, 591 (1958).
158. A. v. Wacek, O. Härtel, and S. Meralla, Holzforschung, 7, 58 (1953).
159. A. v. Wacek, O. Härtel, and S. Meralla, Holzforschung, 8, 65 (1954).
160. A. v. Wacek, Année biol., 59, 53 (1955).
161. O. Härtel, A. v. Wacek, and S. Meralla, Holzforschung, 12, 33 (1958).
162. R. J. Gautheret, A. v. Wacek, and S. Meralla, Holzforschung, 12, 97 (1958).
163. F. Barnoud, Compt. rend., 243, 1545 (1956).
164. M. S. Bardinskaya, Trudy Inst. Lesokhoz. Probl. i Khim Drevesiny, 19, 25 (1960).
165. M. S. Bardinskaya, Dokl. Akad. Nauk SSSR, 136, 1486 (1961).
166. K. Kratzl, G. Billek, E. Klein, and K. Buchtela, Monatsh., 88, 721 (1957).
167. K. Kratzl and G. Hofbauer, Monatsh., 89, 96 (1958).
168. K. Kratzl and H. Faigle, Monatsh., 89, 708 (1958).
169. F. Tiemann and B. Mendelsohn, Ber., 8, 1136 (1875).
170. P. Klason, Svensk kem. Tidskr., 9, 135 (1897).
171. P. Klason, Ber., 53B, 706 (1920).
172. P. Klason, Ber., 53B, 1864 (1920).
173. P. Klason, Ber., 55B, 448 (1922).
174. P. Klason, Ber., 56B, 300 (1923).
175. P. Klason, Ber., 58B, 375 (1925).
176. P. Klason, Ber., 58B, 1761 (1925).
177. P. Klason, Ber., 62B, 635 (1929).
178. P. Klason, Ber., 62B, 2523 (1929).
179. H. Cousin and H. Hérissey, Compt. rend., 146, 1413 (1908).
180. H. Cousin and H. Hérissey, Compt. rend., 147, 247 (1908).
181. H. Erdtman, Biochem. Z., 258, 172 (1933).
182. H. Erdtman, Annalen, 503, 283 (1933).
183. R. Pummerer and F. Frankfurter, Ber., 47, 1472 (1914).
184. R. Pummerer and E. Cherbuliez, Ber., 47, 2957 (1914).
185. R. Pummerer and F. Frankfurter, Ber., 52B, 1416 (1919).
186. R. Pummerer and A. Rieche, Ber., 59B, 2161 (1926).
187. A. Szent-Györgyi, Ber., 72A, 53 (1939).
188. S. M. Manskaya, Usp. Sovr. Biol., 23, 203 (1947).
189. S. M. Manskaya, "Fermentativnoe Okislenie Fenol'nykh Soedinenii" (Enzymatic Oxidation of Phenolic Compounds), Izd. Akad. Nauk SSSR, Moscow–Leningrad, 1949, pp. 24–25.
190. S. M. Manskaya, Dokl. Akad. Nauk SSSR, 62, 369 (1948).
191. K. Freudenberg and H. Richtzenhain, Ber., 76B, 997 (1943).
192. H. Richtzenhain, Ber., 77B, 409 (1944).
193. H. Richtzenhain, Chem. Ber., 81, 260 (1948).
194. H. Richtzenhain, Chem. Ber., 82, 447 (1949).
195. K. Freudenberg, Angew. Chem., 61, 228 (1949).
196. K. Freudenberg and W. Heimberger, Chem. Ber., 83, 519 (1950).
197. K. Freudenberg and J. M. Harkin, Phytochem., 2, 189 (1963).
198. K. Freudenberg, C.-L. Chen, and G. Cardinale, Chem. Ber., 95, 2814 (1962).
199. K. Freudenberg, Fortschr. Chem. org. Naturstoffe, 20, 41 (1962).
200. K. Freudenberg, Pure Appl. Chem., 5, 9 (1962).
201. K. Freudenberg and H. Schlüter, Chem. Ber., 88, 617 (1955).
202. K. Freudenberg, Science, 148, 595 (1965).
203. K. Freudenberg and H. H. Hübner, Chem. Ber., 85, 1181 (1952).
204. K. Freudenberg and D. Rasenack, Chem. Ber., 86, 755 (1953).
205. K. Freudenberg, Angew. Chem., 68, 508 (1956).
206. K. Freudenberg, C.-L. Chen, J. M. Harkin, H. Nimz, and H. Renner, Chem. Comm., 224 (1965).
207. K. Freudenberg, J. M. Harkin, M. Reichert, and T. Fukuzumi, Chem. Ber., 91, 581 (1958).
208. K. Freudenberg and G. Grion, Chem. Ber., 92, 1355 (1959).
209. K. Freudenberg, J. prakt. Chem., 10, 220 (1960).
210. E. Adler, "Proceedings of the 4th International Congress of Biochemistry, Vienna, 1958", 1959, Vol. 2, p. 137.
211. K. Freudenberg and K.-C. Renner, Chem. Ber., 98, 1879 (1965).
212. K. Freudenberg, J. Polymer Sci., 48, 371 (1960).
213. K. Freudenberg, Adv. Chem. Ser., 59, 1 (1966).
214. K. Freudenberg, Brennstoff-Chem., 44, 328 (1963).
215. K. Freudenberg, Chem. Ber., 92, LXXXIX (1959).
216. K. Freudenberg, G. Grion, and J. M. Harkin, Angew. Chem., 70, 743 (1958).
217. K. Freudenberg and J. M. Harkin, Chem. Ber., 93, 2814 (1960).
218. K. Freudenberg and M. Friedmann, Chem. Ber., 93, 2138 (1960).
219. K. Freudenberg and H. Nimz, Chem. Ber., 95, 2057 (1962).
220. K. Freudenberg and H. Tausend, Chem. Ber., 96, 2081 (1963).
221. H. Nimz, Chem. Ber., 96, 478 (1963).
222. K. Freudenberg, J. M. Harkin, and H.-K. Werner, Chem. Ber., 97, 909 (1964).
223. K. Freudenberg and A. Sakakibara, Annalen, 623, 129 (1959).
224. K. Freudenberg and H. Tausend, Chem. Ber., 97, 3418 (1964).
225. K. Freudenberg, in "The Chemistry and Biochemistry of Lignin, Cellulose, and Hémicelluloses". From Proceedings of the International Symposium in Grenoble (Translated into Russian), Izv. Lesnaya Prom., Moscow, 1969, p. 3.
226. K. Freudenberg, Holzforschung, 18, 3 (1964).
227. K. Freudenberg, K. Seib, and K. Dall, Chem. Ber., 92, 807 (1959).
228. K. Freudenberg and H.-K. Werner, Chem. Ber., 97, 579 (1964).
229. A. Björkman, Nature, 174, 1057 (1954).
230. A. Björkman and B. Person, Svensk Papperstidn., 60, 158 (1957).
231. K. Freudenberg, Fortschr. Chem. org. Naturstoffe, 11, 43 (1954).

232. K. Freudenberg and K. Dall, Naturwiss., 42, 606 (1955).
233. K. Freudenberg, W. Siebert, W. Heimberger, and R. Kraft, Chem. Ber., 83, 533 (1950).
234. K. Freudenberg, Bull. Soc. chim. France, 1748 (1959).
235. K. Freudenberg, "Proceedings of the 4th International Congress of Biochemistry, Vienna, 1958", 1959, Vol. 2, p121.
236. K. Freudenberg and G. Schuhmacher, Sitzungsber. Heidelberg. Akad. Wiss., Math.-naturwiss. Kl. 1953/1955, 1956, p. 127.
237. K. Freudenberg and J. M. Harkin, Holzforschung, 18, 166 (1964).
238. K. Freudenberg and R. Kraft, Chem. Ber., 83, 530 (1950).
239. K. Freudenberg, V. Jovanović, and F. Topfmeier, Chem. Ber., 94, 3227 (1961).
240. K. Freudenberg and V. Jovanović, Chem. Ber., 96, 2178 (1963).
241. K. Freudenberg, K. Jones, and H. Renner, Chem. Ber., 96, 1844 (1963).
242. K. Freudenberg and B. Lehmann, Chem. Ber., 96, 1850 (1963).
243. K. Freudenberg and G. S. Sidhu, Holzforschung, 15, 33 (1961).
244. K. Freudenberg, Ind. Eng. Chem., 49, 1384 (1957).
245. K. Freudenberg, Mitt. Deut. Ges. Holzforsch., 50, 114 (1963).
246. K. Freudenberg and H. Nimz, Chem. Comm., 132 (1966).
247. K. Freudenberg, see Ref. 4, pp. 107–108.
248. H. Nimz, Chem. Ber., 98, 533 (1965).
249. H. Nimz, Chem. Ber., 100, 2633 (1967).
250. H. Nimz, Chem. Ber., 102, 799 (1969).
251. J. C. Pew, W. J. Connors, and A. Kunishi, see Ref. 225, p. 107.
252. J. C. Pew and W. J. Connors, J. Org. Chem., 34, 580 (1969).
253. J. C. Pew and W. J. Connors, J. Org. Chem., 34, 585 (1969).
254. J. C. Pew and W. J. Connors, Nature, 215, 623 (1967).
255. W. J. Connors, C.-L. Chen, and J. C. Pew, J. Org. Chem., 35, 1920 (1970).
256. A. Russell, Science, 106, 372 (1947).
257. A. Russell, J. Amer. Chem. Soc., 70, 1060 (1948).
258. A. Russell, J. Amer. Chem. Soc., 70, 2864 (1948).
259. A. Russell, J. H. Baity, and H. E. Smith, J. Amer. Chem. Soc., 71, 956 (1949).
260. K. Freudenberg, H. Reznik, H. Boesenberg, and D. Rasenack, Chem. Ber., 85, 641 (1952).
261. K. Freudenberg, H. Reznik, W. Fuchs, and M. Reichert, Angew. Chem., 66, 109 (1954).
262. K. Freudenberg, Nature, 183, 1152 (1959).
263. M. S. Bardinskaya and V. I. Safonov, Dokl. Akad. Nauk SSSR, 129, 205 (1959).
264. I. Ya. Barskii and M. S. Bardinskaya, Dokl. Akad. Nauk SSSR, 129, 443 (1959).
265. T. Higuchi, I. Kawamura, I. Morimoto, and N. Kimura, J. Japan. Forest. Soc., 36, 253 (1954).
266. T. Higuchi, Physiol. Plant., 10, 621 (1957).
267. F. Barnoud, T. Higuchi, J.-P. Joseleau, and A. Mollard, Compt. rend., 259, 3589 (1964).
268. F. Barnoud, T. Higuchi, and J.-P. Joseleau, Compt. rend., 259, 4110 (1964).
269. F. Barnoud, T. Higuchi, J.-P. Joseleau, and A. Mollard, Compt. rend., 259, 4339 (1964).
260. T. Higuchi and F. Barnoud, "Chim. Biochim. Lignine. Cellulose, Hémicelluloses", Actes Symp. Inter. Grenoble, France, 1964", p. 255.
271. T. Higuchi and F. Barnoud, J. Japan Wood Res. Soc., 12, 36 (1966).
272. B. Holmberg, Ing. Vetenskaps Akad. Handl., No. 131 (1934).
273. R. H. J. Creighton, J. L. McCarthy, and H. Hibbert, J. Amer. Chem. Soc., 63, 3049 (1941).
274. R. H. J. Creighton, R. D. Gibbs, and H. Hibbert, J. Amer. Chem. Soc., 66, 32 (1944).
275. R. H. J. Creighton and H. Hibbert, J. Amer. Chem. Soc., 66, 37 (1944).
276. K. Freudenberg, W. Lautsch, and K. Engler, Ber., 73B, 167 (1940).
277. B. Leopold and I.-L. Malmström, Acta Chem. Scand., 6, 49 (1952).
278. G. H. N. Towers and R. D. Gibbs, Nature, 172, 25 (1953).
279. S. M. Manskaya, Dokl. Akad. Nauk SSSR, 54, 611 (1946).
280. S. M. Manskaya and M. N. Kochneva, Dokl. Akad. Nauk SSSR, 62, 505 (1948).
281. S. M. Manskaya, Trudy Biogeokhim. Lab., 10, 98 (1954).
282. S. M. Manskaya, "Proceedings of the 4th International Congress of Biochemistry, Vienna, 1958", 1959, Vol. 2, p. 215.
283. K. Kratzl and J. Eibl, Mitt. Österr. Ges. Holzforsch., 3, 77 (1951).
284. V. M. Reznikov and N. F. Sorokina, Zhur. Prikl. Khim., 41, 176 (1968).
285. K. K. Lebedev, O. I. Chernyaeva, M. A. Rakitina, and Z. N. Zheleznyakova, Sbornik Trudov TsNIPI Lesokhim. Prom., No. 16, 259 (1965).
286. R. Ripa and L. M. Borquez, Scientia, 34, No. 133, 36 (1967).
287. J. E. Stone, M. J. Blundell, and K. G. Tanner, Canad. J. Chem., 29, 734 (1951).
288. K. Freudenberg and J. Torres-Serres, Annalen, 703, 225 (1967).

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