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Lignin in Young Plants

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In a previous communication (Bondi & Meyer, 1948) we showed that lignins extracted by alkali ('alkali lignin') from annual plants yield a number of aromatic degradation products similar to those obtained from wood lignins. The preparation of lignin from faeces collected from sheep fed one plant exclusively was used to investigate the changes occurring in lignin during digestion in the ruminant body. It was found that the lignin molecule is not attacked to any appreciable degree during its passage through the animal body. It was found, however, that the alkali-extractable lignins differed considerably both in yield and composition from those obtained by the usual methods using acid (cf. Bondi & Meyer, 1943). The aim of the experiments described in this paper was, therefore, to investigate the influence of various hydrolytic treatments of the starting materials used for the preparation of alkali-extractable lignins and of the isolated lignins on their composition. Furthermore, an attempt was made, by studying methylation of the lignins with diazomethane and dimethyl sulphate, to draw conclusions with regard to the structure of the lignin building unit.

EXPERIMENTAL

Materials

Barley. Barley was harvested at the age of 2·5 months and dried in air. The hay was used for lignin extraction, and part of it was fed to sheep as their only food. Their faeces were collected and lignin was extracted from them by the same methods as from barley.

De-lintered cotton-seed hulls. In this case, lignin could be prepared only from the undigested material, since it proved impossible to maintain sheep on rations containing only cotton-seed hulls.

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Methods

The extraction and purification of lignins extracted by alkali, their methylation with diazomethane and dimethyl sulphate and the method of methoxyl determination have been described previously (Bondi & Meyer, 1948). Acid-processed lignins were prepared by the method of Kalb (1932) by treating the starting materials with a mixture of HCl and H_2SO_4. Samples of 1 g. were used throughout, since lignin prepared by the acid method in larger batches often showed marked inhomogeneity and the batches differed widely in composition.

Hydrolytic treatment of the starting materials

(*a*) *With dilute* H_2SO_4. 300 g. plant or faecal material (finely ground and passing a 0·5 mm. sieve) were suspended in 200 ml. of 0·5 N-H_2SO_4 and heated under reflux, with stirring for 6 hr. The mixture was then filtered through cheesecloth and washed free from acid with hot water. The residue was dried in air and used for the preparation of alkali-extractable lignin.

(*b*) *By heating with water under pressure.* Finely ground plant or faecal material (300 g.) was suspended in 2 l. of water and autoclaved at 210° for 2 hr. The mixture was filtered through cheesecloth, washed with 6 l. of hot water on the filter and the residue was dried and used for the preparation of alkali-extractable lignin. (Attempts to hydrolyse plant and faecal material with N- or 2 N-acid at 210° failed owing to extensive decomposition and charring of the materials together with the formation of ether-soluble degradation products, mostly phenols, presumably from the lignin.)

Hydrolytic treatment of the isolated lignins

(*a*) *By heating with dilute* H_2SO_4. Isolated lignins remain unchanged when refluxed with 0·5 N-acid; they were therefore treated with the acid at higher temperatures in the following manner: lignin (15 g.) was suspended in 200 ml. of 0·5 N-H_2SO_4 and heated for 1 hr. at 150° in a glass-lined autoclave. After cooling the lignin residue was filtered off, washed free from acid and dried in air.

(*b*) *By heating with water.* Lignin (20 g.) was suspended in 200 ml. of water and autoclaved at 210° for 2 hr. The lignin residue was filtered off, washed free from acid and dried.

Degradation reactions

Treatment of the lignin with alkali under pressure. Lignin (20 g.) was dissolved in a mixture of 200 ml. of water and 50 ml. of 12N-NaOH and autoclaved at 155° for 2 hr. The temperature was then raised to 210° and kept there for 1 hr. After that the reaction mixture was allowed to cool. The solution was then acidified with 20% (v/v) H_2SO_4 to precipitate the unreacted lignin. Both the solution and the precipitate were exhaustively extracted with ether, the ether solution was dried with Na_2SO_4 and evaporated. The residue was weighed, redissolved in ether and separated into phenolic, acidic, aldehydic, and neutral fractions by methods previously described (Bondi & Meyer, 1948). The unreacted, ether-extracted lignin was suspended in 100 ml. of water, boiled for 2 min. filtered off, washed with water and dried.

RESULTS

The original nitrogen contents and the methoxyl contents of the lignins before and after methylation with diazomethane and dimethyl sulphate are given in Table 1.

Lignins from different plant sources. The elementary analyses of the three lignins investigated (see Table 2) do not show any significant differences between them, apart from the lower nitrogen content of the cotton-seed-hull lignin.

Cotton-seed lignin has also a lower initial methoxyl content than either lignin derived from barley (cf. Smith & Purves, 1944). After methylation, however, cotton-seed-hull lignin shows approximately the same values as barley lignin. The conclusion seems therefore justified, that cotton-seed-hull lignin does not differ from the others in its number of hydroxyl groups, but only in the degree of methylation.

Table 1. *Methoxyl and nitrogen content of the lignins as percentage of dry, ash-free material*

		Treatment			OMe*		
No.	Source	Starting material	Isolated lignin	N (%)	(1) (%)	(2) (%)	(3) (%)
1	Barley	Standard 'alkali lignin'	—	1·4	8·7	20·3	24·3
2		Dilute acid	—	1·5	6·3	15·8	18·1
3		Water, 210°	—	1·6	4·7	13·6	16·8
4		—	Dil. acid	1·4	7·0	17·3	21·9
5		—	Water, 210°	1·6	7·2	13·4	16·2
6		—	Alkali, 210°	1·3	7·4	14·6	19·1
7		—	KIO_4	1·9	4·4	13·7	13·9
8		—	Conc. acid	1·5	8·6	20·2	24·0
9		Conc. acid (acid-process lignin)	—	0·6	4·3	7·6	14·9
11	Faeces	Standard 'alkali lignin'	—	1·4	8·5	20·6	21·0
12		Dilute acid	—	1·5	6·6	16·5	19·0
13		Water, 210°	—	1·6	4·4	14·1	16·8
14		—	Dil. acid	1·4	8·0	16·1	20·4
15		—	Water, 210°	1·6	7·8	15·0	17·0
16		—	Alkali, 210°	1·3	6·8	19·3	19·6
17		—	KIO_4	2·6	4·3	14·0	14·3
18		—	Conc. acid	1·4	8·5	20·4	21·0
19		Conc. acid (acid-process lignin)	—	0·9	1·6	7·6	14·0
21	Cotton-seed hulls	Standard 'alkali lignin'	—	0·8	6·0	20·5	24·2
22		Dilute acid	—	0·8	5·1	15·3	19·5
23		Water, 210°	—	1·0	4·3	13·5	15·2
24		—	Dil. acid	0·7	5·2	18·4	21·5
25		—	Water, 210°	0·8	5·8	14·9	19·2
26		—	Alkali, 210°	0·8	5·0	14·8	18·7
27		—	KIO_4	1·2	4·4	13·8	14·0
28		—	Conc. acid	0·8	5·9	20·0	24·1
29		Conc. acid (acid-process lignin)	—	0·2	1·2	5·1	14·3

* Methoxyl contents: (1) initial; (2) after methylation with diazomethane; (3) after methylation with dimethyl sulphate.

The nitrogen content of the lignins. As in the lignins investigated before (Bondi & Meyer, 1948), no treatment could produce nitrogen-free lignins.

Table 2. *Elementary composition of lignins (in percentage of dry, ash-free material)*

Source	N	C	H	O
Barley (1)	1·40	63·3	5·7	29·6
Faeces (11)	1·40	63·8	5·2	29·6
Cotton-seed hulls (21)	0·80	63·6	5·4	30·2

Some treatments, like that with water at 210°, even raised the nitrogen content of the lignins, probably by attacking preferentially the nitrogen-free part

of the molecule. We therefore feel justified in upholding our previous contention that the nitrogen content of the lignins is not due to contamination with proteins and that nitrogen atoms form an integral part of the lignin molecule. Man & Heus (1950) have described lignins containing nitrogen and yielding amino-acids on hydrolysis (cf. also Thomas & Armstrong, 1949). We have often observed this phenomenon in impure lignins which initially contained 2·5–3·0% of nitrogen. When these were hydrolysed under conditions usually employed for protein hydrolysis, amino-acids could be found among the hydrolysis products, and determined quantitatively by the Sørensen and Pope-Stevens methods. When lignins are repeatedly dissolved and reprecipitated from acid, the initially high nitrogen content falls until it attains a constant value which cannot be lowered by any hydrolytic treatment. When this state is reached, no amino-acids can be found among the hydrolysis products. All nitrogen values reported in this paper are such end values. The lignin prepared by Man & Heus, by our method, which contained 8·8% nitrogen, seems to have been a preparation grossly contaminated with protein.

Acid-process lignins

Lignins obtained by the treatment of plant materials with concentrated acid differ considerably both physically and chemically from the alkali-extractable lignins. In contrast to the light brown, alkali- and ethanol-soluble 'alkali lignins', 'acid lignins' are black substances, insoluble in ethanol and only slightly soluble in dilute alkali. Very often 'acid lignins' still show the structure of the plant material from which they have been prepared. 'Alkali lignins' are generally obtained in yields of 3–7%, whereas yields of 'acid lignin' are much higher, in general 9–20%. The methoxyl and nitrogen contents are much lower than those of 'alkali lignins' prepared from the same starting materials, with the exeption of the methoxyl value obtained after methylation with dimethyl sulphate, which remains comparatively high. The reason is probably that 'acid lignins' are condensation products of the lignins proper with carbohydrate materials (Norman & Jenkins, 1934) probably hemicelluloses which contain no nitrogen or methoxyl, but contribute a number of alcoholic hydroxyl groups. Condensation between lignin and hemicellulose affects all the free hydroxyl groups of the lignin, thereby abolishing the solubility in ethanol and alkali. The high lignin yields obtained by treating plant tissues with concentrated acids can therefore give no measure of the true lignin content. The assumption that condensation reactions take place during acid treatment was tested by treating plant materials from which the lignin had already been extracted by alkali with 72% (v/v) sulphuric acid and with hydrochloric-sulphuric acid mixture, according to the method of Kalb (1932). Black substances were obtained which were in appearance indistinguishable from 'acid lignins' but which contained no methoxyl and gave, on degradation with nitrobenzene and alkali, no vanillin, a substance given by the oxidative degradation of all true lignins. Isolated lignins are, however, not affected by treating them with cold concentrated acids. (Compare lignins nos. 8, 18 and 28 in Table 1 with lignins nos. 1, 11 and 21.) Similar results were reported by Kudzin & Nord (1951). It is our opinion that, in order to obtain reliable analytical lignin determinations, carbohydrates, especially hemicelluloses, must be removed as completely as possible from plant tissues before they are subjected to the influence of strong acids.

The influence of the various hydrolytic treatments on the lignins

We have reported previously that dilute alkali extracts a lignin-hemicellulose complex which is easily hydrolysed by dilute acid. This observation, together with the high initial methoxyl values of the 'alkali lignins' (obtained without any treatment of the starting materials prior to the lignin extraction), shows that alkali extraction is one of the mildest methods for lignin isolation, since it leaves even the weak lignin-hemicellulose bonds and acetalic methyl groups intact. We therefore regard the 'alkali lignins' as comparatively unchanged (lignins nos. 1, 11 and 21 in Table 1) and refer to them as 'standard lignins'.

The changes brought about by the various treatments both of the starting materials and of the isolated lignins seem all to affect hydroxyl groups. When plant materials are subjected to acid treatment (or water at high temperatures, which has a similar effect) the lignin-hemicellulose bonds open and hydroxyl groups previously blocked are set free.

Both acid and high temperatures seem to favour condensation reactions involving the hydroxyl groups previously set free. Consequently all lignins prepared from pre-treated starting materials have fewer free hydroxyl groups than those prepared from untreated starting materials. This is shown by the fact that all methoxyl values obtained after methylation are always lower than the corresponding values in standard lignins. Diminution of the number of free hydroxyl groups in lignin is effected by all treatments, and the differences between the various treatments seem to be in degree rather than in kind. Mere digestion in the animal body influences mainly side-chain (aliphatic) hydroxyl groups (which are methylated by dimethyl sulphate only). In faeces lignin, therefore, the differences between the methoxyl values obtained after methylation with diazomethane and dimethyl

sulphate found in barley lignin (lignin no. 1), have almost entirely disappeared. This effect had previously been observed in a number of plant materials (cf. Bondi & Meyer, 1948). When, however, the starting materials were pretreated with acid or water at high temperature, both aliphatic and aromatic hydroxyl groups take part in condensation reactions, consequently both the diazomethane and dimethyl-sulphate methylation values of all lignins prepared from pretreated starting materials are lowered considerably. In addition, pretreatment of the starting materials removes part of the aliphatic methyl groups originally present, here the influence of water at high temperatures is greater than that of acids. Disappearance of free hydroxyl groups was the main effect caused by the treatment of isolated lignins with the different hydrolysing agents, hydrolysis with water at 210° being the most effective. It lowered both the diazomethane and the dimethyl-sulphate methylation values more than any other treatment.

It has been assumed that condensation reactions are responsible for the lowering of methoxyl values obtainable by methylation of the lignins subjected to the different treatments. This assumption was preferred to the alternative one that hydrolysis splits off both aliphatic and aromatic hydroxyl groups. The splitting off of aromatic hydroxyl is, under the conditions employed by us, a very improbable reaction, and the splitting off of side chains would inevitably lead to the formation of low-molecular degradation products, since it is generally assumed that by condensation reactions between these side chains the peculiar structure of the lignin molecule is achieved.

The assumption of condensation reactions between hydroxyl groups leading to the formation of C—O—C bonds was tested by treating the lignins with hydriodic acid, which, by opening the bonds, would lead to the reappearance of hydroxyl groups. The lignins were refluxed in 2 g. portions with 20 ml. hydriodic acid solution (sp.gr. 1·71) for 4 hr. The residual lignins were filtered off, washed and dried and methylated with dimethyl sulphate. Table 3 shows that in every case products richer in methoxyl were obtained by methylation of lignins treated with hydriodic acid, than by the same methylation of the original lignins. Additional hydroxyl groups have therefore appeared as a result of the hydriodic acid treatment.

No increase in methoxyl could, however, be obtained when lignins were oxidized with potassium periodate and afterwards treated with hydriodic acid. Periodate oxidation apparently leads in the case of the lignin to the oxidative removal of side chains containing both hydroxyl and methoxyl groups (see Wald, Ritchie & Purves, 1947). As a result, lignins so treated have no more condensed hydroxyl groups which can be set free by hydriodic acid treatment. Furthermore, the initial methoxyl content is lowered to approximately one half its former value owing to the loss of side-chain methoxyl and the diazomethane methylation value

Table 3. *Methoxyl values after methylation with dimethyl sulphate of lignins treated with hydriodic acid*

Lignin no.	OMe (after methylation with dimethyl sulphate)	OMe (after treatment with HI and subsequent methylation with dimethyl sulphate)
3	16·8	21·4
13	16·8	21·5
23	15·2	21·3
5	16·2	20·9
15	17·0	21·0
25	19·2	21·8
7	13·9	14·3
17	14·3	14·3
27	14·0	14·4

approaches a limiting value between 13 and 14 %, which is in all probability attributable to aromatic hydroxyl.

Degradation reactions

Potassium periodate oxidation led to the formation of formaldehyde, as was to be expected. Formaldehyde was identified by its reaction with chromotropic acid and its dimedone derivative, but formic acid could not be detected. Degradation of the lignins by heating them with alkali under pressure led to the formation of aromatic degradation products which were separated into an aldehydic, acidic, phenolic and neutral fractions by methods previously described (Bondi & Meyer, 1948). By repeated alkali treatment the yield of aromatic degradation products could be increased to about 25 %. No new degradation products were identified.

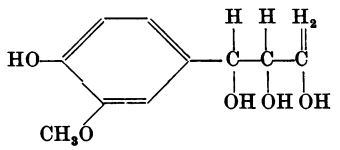
The structure of the lignins

That the phenylpropane unit (C_6–C_3) forms the backbone of the wood-lignin building units seems now to be well established by the investigations of Hibbert (1942) and Freudenberg (cf. Freudenberg, Sohns & Janson, 1935). Support is also given by the work of Phillips & Goss (1932) and of ourselves (Bondi & Meyer, 1948). The experimental results presented in this paper will therefore be discussed under the assumption that the basic lignin structural unit is a phenylpropane derivative, substituted in the ring by OH and/or CH_3 groups and containing in its side chain alcoholic and enolic OH groups and probably also aldehyde or keto groups (cf. Brauns, 1939).

One half of the initial methoxyl content of the lignins is apparently contained in its side chains, since it is removed by potassium periodate oxida-

tion, the remainder is aromatic, as appears from the presence of vanillin among the lignin degradation products. The side-chain methoxyl is probably acetalic, since it is resistant to alkaline hydrolysis, but it is easily split off by acid. Since it was previously found (Bondi & Meyer, 1948) that every lignin building unit contains two methoxyl groups and yields vanillin on degradation, the total number of methoxyl groups initially present would be four, two aromatic and two aliphatic. (In cotton-seed lignin only three methoxyl groups are present, the fourth being unsubstituted OH.) The average percentage of initial methoxyl is 8·6 (lignins nos. 1 and 11) thus making the molecular weight of a building unit 1430, and the empirical formula $C_{76}H_{78}O_{26}N$. Deducting the four carbon atoms of the methoxyl groups leaves 72 carbon atoms, or eight C_6–C_3 units. The average value of 20·4 % methoxyl after methylation with diazomethane accounts for six additional (aromatic and enolic) hydroxyl groups. The increase in methoxyl after methylation with dimethyl sulphate to 24·25 % (average value of lignins nos. 1 and 21) corresponds to the presence of two additional (aliphatic) hydroxyl groups. The eight phenylpropane units forming the lignin building unit are supposed to be subdivided into units of four, connected by a nitrogen instead of an oxygen bridge, but not regularly, since the nitrogen content of the various lignins is more variable than that of the other constituents. Depolymerization of the lignin molecule seems to take place preferably at the nitrogen bridges, which explains the lower molecular weights found by us by cryoscopic determination in naphthol, which is a depolymerizing agent.

If the phenylpropane unit has the structure



according to Freudenberg's theory, it will follow that every such unit must be connected to the others by at least three oxygen bridges in order to correspond to the formula proposed in this paper. This would explain the extraordinary stability of the lignin molecule, which can be destroyed only by very strong reducing agents at high temperatures, such as hydriodic acid and phosphorus under pressure (cf. Willstaetter & Kalb, 1922). The exact mode of connexion between the phenylpropane units must, however, await further experimental elucidation.

SUMMARY

1. Alkali-extractable lignins ('alkali lignins') were prepared from barley, from faeces collected from sheep fed exclusively with barley, and from cotton-seed hulls.

2. The materials used for lignin extraction and the isolated lignins were subjected to hydrolysis with dilute acid, water at 210° and alkali at 210°.

3. From determinations of the initial methoxyl content and of the methoxyl content after methylation with diazomethane and with dimethyl sulphate, of all the lignins, the following conclusions were drawn:

(*a*) All treatments with acid, including the conventional lignin determination methods, and treatment involving high temperatures, lead to extensive condensation reactions within the lignin molecule, through the aliphatic and aromatic hydroxyl groups.

(*b*) Treatment with dilute alkali leaves lignin comparatively unchanged, treatment with stronger alkali and at temperatures over 100° leads to their gradual degradation.

4. The nitrogen content of the lignins is not due to their contamination with protein.

5. The lignins contained in the plants investigated are very similar, probably all containing the same phenylpropane basic structural unit. The structural details of the lignin molecule have been discussed in the light of the results obtained.

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