Lignins in Young Plants

By A. BONDI AND H. MEYER, *Agricultural Research Station, Rehovot, Palestine*

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In contrast to the extensive research into the properties and structure of the wood lignins, little work has been done on the lignins which occur in the cell walls of annual plants in considerable quantities. These amount to as much as 10–20% of the dry matter in the common forage plants, according to the analytical methods usually employed, which use concentrated mineral acids for lignin determinations. Degradation experiments have been carried out only with lignins contained in the more woody tissues of this kind, such as maize cobs or various straws, where a closer similarity to the wood lignins exists (Phillips, 1944). The lignins of young green plants seem to be digestible to a certain extent (Juon, 1934; Crampton, 1939; McAnally, 1942; Bondi & Meyer, 1943), whereas lignins in straw or maize cobs were found to be practically indigestible (König & Fürstenberg, 1906–7; Thomann, 1921; Rogozinski & Starzewska, 1929; Ferguson, 1942).

Lignins from young plants and from animal faeces have, to our knowledge, not been prepared in pure form. This paper presents an attempt to isolate lignins in a state of comparative purity from a number of young plants, and to investigate their chemical properties in order to compare them with the better-known wood lignins. Furthermore, lignins were prepared from the faeces of sheep which had been given the same plants as their sole food. This procedure allowed us to follow the chemical changes undergone by the lignins in the body of a typical ruminant.

EXPERIMENTAL

Materials

All the plants used for lignin extraction (and fed to sheep for obtaining faeces) were annuals, as follows, the age at cutting being given in parentheses:

Eragrostis tef (2.5 months), *Pencillaria* (2.5 months), *Setaria italica* (2.5 months), and *Hordeum murinum*, wild barley (3.5 months) represent the Gramineae.

Trifolium alexandrinum bersem, Bersem clover (1.5 months), *T. alexandrinum fahli*, Fahli clover (2.5 months), *Arachis hypogaea*, peanut hay (4 months), and *Lathyrus ochrus*, Cyprus vetch (2 months) represent the Leguminosae.

Plants belonging to these two families were chosen because they show characteristic differences. Leguminosae remain comparatively soft and non-ligneous until the last stages of their development, while in the Gramineae woodiness sets in early, especially in the stalks. None of the plants used in this investigation was very woody. Peanut hay and wild

barley were the materials in which woodiness had developed most.

The plant material was divided into two parts: one was dried and the lignin subsequently extracted, the other part was fed to sheep. In accordance with the usual procedure in digestibility experiments (see Bondi & Meyer, 1940), the various plants were in each case given to the sheep, kept in metabolism cages, as their only food. After a preliminary period of 6 days, faeces of the sheep were collected during the following experimental period of 10 days. From the dried sheep faeces, lignin was extracted as in the case of the plants.

Extraction methods

The separation of lignin from the other components of the plant can be achieved in two ways. One is to dissolve out all materials other than lignin, and leave it as the only remaining material. In the second method the lignin is dissolved out of the plant tissues leaving the other materials. The first method requires the action of concentrated mineral acids on the plant material. In our experience, however, this method is not suitable for the preparation of larger quantities of lignin from green plants, since the action of strong acids may cause the condensation of soluble carbohydrates contained in them, giving black insoluble substances often erroneously classified as 'lignins' (see Hilpert & Hellwage, 1935).

The second type of methods for the isolation of the lignin includes those in which lignin is extracted by the action of organic solvents such as ethanolic HCl or phenol, or by alkali. We did not use organic solvents for extraction, since these bring about condensation reactions or even degradation of the lignin. Instead, we had recourse to the extraction of lignin by means of alkali. At first we used the method proposed by Phillips & Goss (1934) for the extraction of lignin from maize cobs. They extracted the lignin with 60% ethanolic NaOH (2% w/w) and, after evaporating the ethanol, precipitated it with an excess of ice-cold 0.4N-H₂SO₄. The yields of lignins obtained from green plants by these methods were very small: 0.8–1.6% of lignin were obtained from plants, and 0.9–1.8% from faeces. These figures are much smaller than those obtained in the analytical determination of lignin by treatment with concentrated acids, which gives values of 10–15% in the same forage plants and faecal matter. The lignin obtained by Phillips's method also contains 1.1–3.2% N, according to the type of forage plant. Neither an increase of the

concentration of the ethanol in the extracting solution (up to 96 %), nor an increase in the concentration of NaOH (up to 10 %), brought about either a noteworthy increase in the lignin yield or a decrease in the nitrogen content of the lignin. The raising of the ethanol concentration in the extraction liquid brought about a decrease in the lignin yield.

Since, in spite of the varying of the experimental conditions, the extraction of lignin with ethanolic NaOH did not give practical yields, another method was worked out using hot aqueous 0.5N-NaOH (see also Phillips, 1929). By this method lignins are extracted together with considerable amounts of polysaccharides, probably hemicelluloses. Both materials are precipitated by acidifying the alkaline extract. The lignins are separated from the hemicellulose by redissolving the precipitate in alkali and adding a large excess of slightly acidified ethanol, which dissolves the lignins, leaving the hemicelluloses. The ethanol-soluble lignins still contain about 20 % of hemicellulose, which can, however, be removed easily by a mild acid hydrolysis. The experimental details are as follows:

The plant or faecal material is ground coarsely, extracted thoroughly with ether and dried. 1 kg. of this material and 12 l. of 0.5N-NaOH are put in a vessel of about 16 l. capacity (to avoid foaming over), and heated for 8 hr. to 80–85°, with continuous stirring. The entire contents of the vessel are filtered through cheese cloth, and the residue is washed several times with water (5–6 l. in all). The washings are combined with the filtrate, while the residue is extracted three more times under the same conditions. The extracts are combined and evaporated *in vacuo* to about one third of their original volume. After cooling, the residue is acidified with 5N-H₂SO₄. This precipitates the lignin (together with considerable amounts of hemicellulose) which is allowed

to settle out, after which the supernatant liquid is decanted. The precipitate is washed several times with water, which is decanted off each time, until the supernatant liquid appears only slightly yellow. The precipitate is then separated by centrifuging at 2000 r.p.m. for 15 min., or by filtration.

In order to purify the crude product it is again dissolved in the smallest possible quantity of warm aqueous 1.25N-NaOH. This solution is added to 4 vol. of 96 % ethanol and acidified slightly. The dark-brown supernatant liquid which contains the lignin is separated from the residue (1) by filtration or centrifuging. The greater part of the ethanol is then driven off; this causes precipitation of the lignin. The resulting aqueous suspension of lignin is treated with enough NaOH to dissolve all the lignin, and then 5N-H₂SO₄ is added to make it just acid, causing separation of the lignin in voluminous flakes. The lignin is difficult to filter and contains varying amounts of hemicellulose. In order to remove the latter, the acidified suspension is heated to boiling. At this temperature hydrolysis of the hemicellulose and coagulation of the lignin take place, and the lignin precipitate becomes coarse and easily filterable. The impurities remain in the solution, which is usually deep-brown in colour. During the first precipitation the lignin loses 12–15 % of its weight. A second precipitation causes it to lose 2–3 %; in subsequent reprecipitation no measurable quantities of lignin are lost. After the second reprecipitation, the N, ash, and OCH₃ contents of the lignin remain constant. Lignin obtained in this manner still contains 1–2 % of ash.

The residue (1) remaining after solution of the lignin is again dissolved in warm 1.25N-NaOH. The solution is filtered or centrifuged, acidified with 5N-H₂SO₄, and mixed with 5 vol. of 96 % ethanol, whereupon precipitation of yellow flakes takes place and the lignin still present dissolves in the ethanol. Solution and reprecipitation are repeated, until the supernatant fluid is colourless. The ethanolic solutions are collected and treated for a further yield of lignin. The precipitated flakes are suspended in water and washed by decantation till the complete disappearance of SO₄²⁻. The lignin fraction obtained by separation from the

Table 1. Yields of lignin and nitrogen contents of the different lignins and the original materials

Material		Yields from dried original material (%)	N contents	
			Lignin (%)	Dried original material (%)
<i>Eragrostis tef</i>	Plant	5.4	1.18	1.86
	Faeces	9.0	1.31	1.15
<i>Pencilaria</i>	Plant	4.6	1.21	1.68
	Faeces	7.1	1.40	1.68
<i>Setaria italica</i>	Plant	5.1	1.41	1.08
	Faeces	6.8	1.62	1.20
Wild barley	Plant	5.1	1.63	0.52
	Faeces	6.5	1.70	0.71
Bersem clover	Plant	5.0	2.92	2.99
	Faeces	4.6	3.17	2.16
Fahli clover	Plant	3.0	3.36	1.67
	Faeces	3.4	3.92	1.52
Peanut hay	Plant	5.2	3.24	3.12
	Faeces	6.3	3.45	3.28
Cyprus vetch	Plant	4.1	3.30	2.48
	Faeces	6.5	3.41	1.94

hemicellulose residue was always identical with the principal product (containing the same percentage of N and OCH_3) and, therefore, it is combined with the latter.

The combined precipitates of hemicelluloses are then suspended in acetone, in which they are boiled three times, filtered and dried at 40° *in vacuo*. This material, on hydrolysis with boiling $0.4\text{N-H}_2\text{SO}_4$ left a residue of about 10% consisting of lignin. The rest was split completely into reducing sugars, and is therefore assumed to be a hemicellulose.

Yield and N content of the lignins prepared from different plant and faeces materials are presented in Table 1, and their OCH_3 contents in Table 3. In several cases the lignins obtained in the first to fourth extraction were precipitated and treated separately. The N and OCH_3 contents of the lignins prepared from the different extracts were practically uniform, thus eliminating the need for their separate treatment throughout.

Properties of lignin

The various lignins prepared by extraction with 0.5N-NaOH are light to dark-brown, fine powders, decomposing without melting on being heated to 400° . They are easily soluble in dilute NaOH, in ethanol, and in acetone. Up to 70% of the material is soluble in 98% acetic acid. It should, however, be noted that completely dry, glacial acetic acid will not dissolve lignin either cold or hot; solution only occurs after the addition of 2–3% of water. Phenol and β -naphthol dissolve up to 15% of their weight of lignin when heated about 20° above their melting points. Lignins are not soluble in other common solvents.

The lignins prepared by us proved to be completely free from carbohydrates. The hydrolysates obtained according to Waksman's method for the determination of hemicellulose and cellulose were always found to be completely free of reducing sugars (cf. Bondi & Meyer, 1943). Nor could furfural be obtained by distillation with 3N-HCl , as in the Tollens pentosan determination.

Nitrogen content of lignin

Wood lignin is practically free from N. Very small quantities of N (0.2–0.3%), found in wood lignin, are considered by Kalb (1923) and Paloheimo (1925) to be accidental impurities caused by protein materials hydrolyzable only with difficulty; and Waksman (1938) assumes that there are lignin proteinates present in humus lignin which are difficult to split. Phillips, Goss, Davis & Stevens (1939) found some N in crude lignin from oats, which decreased in quantity with increasing age of the plant. Our experiments show with certainty that the N content of lignin prepared from green plants is due neither to accidental contamination with protein nor to the presence of lignin-protein complexes. The constancy of the N content of lignin prepared from the same

plant material in consecutive extractions and by different methods shows that there can be no question of fortuitous contaminations.

In order to avoid the formation of lignin-protein complexes during extraction, an attempt was made to remove the protein before extraction by treatment of the plant material with 0.05N-NaOH . It was found that 80–90% of the protein present in the original material was removed by this preliminary treatment, but that at the same time most of the lignin also went into solution. Lignins prepared from material that had undergone this preliminary treatment were practically equal in N content to those obtained without preliminary treatment. On the other hand, the yield of lignin was greatly reduced, so that this preliminary treatment with NaOH was of no practical use.

A trial was also made of treatment of the plant material with a solution of pepsin in dilute HCl in order to secure a diminution of the protein content. By this method, too, 60–75% of the protein present was removed; but the lignin from plants given this preliminary treatment showed approximately the same N content as that from plant material which did not receive it. This equal N content of lignins prepared from protein-rich plants and of those from which most of their protein had been removed, argues against the hypothesis that condensation between lignin and protein takes place during the extraction of lignin.

Finally, experiments were undertaken which were intended to find out whether the N in lignin was protein N or not. The N content of the lignins was not diminished by vigorous acid hydrolysis (refluxing for 24 hr. with $5\text{N-H}_2\text{SO}_4$) or by mild hydrolysis (digestion with 0.5N-HCl for 24 hr. at 38°). Both treatments bring about the dissolution of 10–15% of the lignin, but the solutions do not contain N and the N content of the remaining lignin rises proportionally to the weight loss. Repetition of the hydrolytic treatment leaves the lignin completely unchanged. The same result was obtained when pepsin was added to the HCl solution. The N-containing portion of the lignin was equally resistant to pancreatin at its optimum pH of 8.0. At this pH lignin is soluble, but no degradation of the lignin N could be obtained by this method. The resistance of the lignin N to hydrolysis by mineral acids or by enzymes shows that it cannot be protein N. Additional evidence for the non-protein nature of lignin N is furnished by the fact that the N content of faecal lignin is not lower than that of plant lignin, i.e. the digestive enzymes of the ruminant have no influence whatsoever on lignin N.

Nor can lignin N be primary or secondary amine N, as is shown by the resistance of lignin N to distillation of the lignins with concentrated NaOH and to treatment of the lignins with NaNO_2 (no liberation

of N occurred and the N content of the lignin remained unchanged). Therefore, the only possibility left is that the N is present as tertiary N, possibly as a cyclic compound. In this connexion it may be mentioned that derivatives of pyridine have been found in humus lignin (see Waksman, 1938), and that pyridine and its homologues appear regularly in the dry distillation of wood.

In order to test this assumption of the presence of heterocyclic N compounds in lignin, the following experiments were carried out.

According to Shaw (1937) pyridine decomposes on reduction with Na in absolute ethanol to give NH_3 and glutardialdehyde. Our lignins were, therefore, also subjected to reduction with Na and ethanol, as well as with Zn dust in alkaline solution. These treatments degraded the lignin completely to N-free products of low molecular weight, while all the N is driven off as NH_3 during the reaction with Zn dust, and may be collected in acid and determined by back-titration of the latter. (In one set of experiments, 50–100 hr. were necessary for complete liberation of the lignin N, when 5.0 g. lignin were refluxed with 60 g. Zn dust, 100 g. NaOH and 450 ml. water.)

Similar results were obtained by reducing the lignin with Na in ethanol: 10 g. lignin were refluxed in 300 ml. absolute ethanol, while 50 g. Na were added in small portions. The reaction was terminated in 30–40 hr., lignin N being recovered as NH_3 .

Preliminary experiments showed that oxidation of lignin with KMnO_4 in alkaline solution also produced NH_3 . These experiments were then repeated quantitatively, in accordance with the method used by Charnbury, Eckert, LaTorre & Kinney (1945) in investigating the nature of the N in humic acids. This determination showed that all lignins yield 70–80 % of their N content as NH_3 in the course of

oxidation. For comparison, a derivative of pyridine, nicotinic acid, was subjected by us to the same treatment and gave similar results (see Table 2). The similar behaviour of the N contained in nicotinic acid and in our lignins allows us to infer that the N in lignins and in compounds containing tertiary N is bound similarly.

Table 2. *Liberation of nitrogen of lignins as ammonia by oxidation with KMnO_4*

Lignin from		Percentage N before oxidation	Loss of N as percentage of total N
<i>Eragrostis tef</i>	Plant	1.18	76.2
	Faeces	1.31	75.0
<i>Pencillaria</i>	Plant	1.21	73.9
	Faeces	1.41	76.2
Wild barley	Plant	1.63	77.8
	Faeces	1.70	78.1
Fahli clover	Plant	3.36	74.6
	Faeces	3.91	75.9
Nicotinic acid		9.44	78.4

Hydroxyl and methoxyl groups in lignin

The OCH_3 content of different wood lignins varies from 14 to 20 %, and is higher in hardwood lignins than in softwood lignins. According to Phillips *et al.* (1939) and Bondi & Meyer (1943) the OCH_3 content of lignin isolated from green plants is lower than that of wood lignin, and increases with the age of the plant. In barley, for instance, the OCH_3 content of the lignin increases during growth from 3 to 10 % (Phillips *et al.* 1939).

Table 3 gives the OCH_3 contents of the various lignins isolated by us from plants and faeces. These figures fall naturally into two groups: lignin from the Gramineae contains 9–10 % OCH_3 , while the lignin of the Leguminosae contains approximately

Table 3. *Methoxyl content of lignins*

Lignin from		Percentage OCH_3 in lignin		
		As prepared	After methylation	
			With CH_3N_2	With $(\text{CH}_3)_2\text{SO}_4$
<i>Eragrostis tef</i>	Plant	9.71	19.00	23.48
	Faeces	9.70	19.02	19.34
<i>Pencillaria</i>	Plant	9.65	18.77	22.75
	Faeces	9.65	19.03	19.10
<i>Setaria italica</i>	Plant	9.60	18.94	23.04
	Faeces	9.70	18.81	18.92
Wild barley	Plant	9.85	19.21	21.33
	Faeces	9.77	18.48	18.59
Berseem clover	Plant	5.10	15.29	18.08
	Faeces	5.22	15.01	15.10
Fahli clover	Plant	5.75	15.31	16.70
	Faeces	6.67	15.42	15.51
Peanut hay	Plant	4.28	13.4	15.20
	Faeces	4.40	14.7	14.09

one half of this amount. It is notable that a material like peanut hay, which on inspection appears much more woody than, say, barley, nevertheless shows clearly by the OCH_3 content of its lignin that it belongs to the Leguminosae; this shows that the family to which the plant belongs has, at least in this case, a stronger effect than the age and degree of 'woodiness'.

Table 3 also shows that the OCH_3 content of the lignin isolated from faeces differs hardly at all from that of the corresponding plant lignins; i.e. there is no noteworthy rupture of ether linkages in the alimentary tract.

The determination of OCH_3 in lignin was supplemented by the estimation of OCH_3 introduced into the lignin. All samples of lignin were methylated by two different methods, with dimethyl sulphate and with diazomethane, according to the procedures given by Phillips (1944).

The results of OCH_3 determinations in the methylated lignins are also given in Table 3. The OCH_3 content of lignins from Leguminosae is trebled on methylation with diazomethane, whilst the content of Gramineae lignins is only approximately doubled. We conclude from this that the OCH_3 groups in lignins from Gramineae (which have the higher OCH_3 content before methylation) correspond to unsubstituted hydroxyl groups in the lignins from Leguminosae. Methylation with dimethyl sulphate brings about only slightly larger increases in OCH_3 content than that obtained with diazomethane. Comparison of the results of methylation with dimethyl sulphate and diazomethane therefore shows that in all plant lignins there are many more phenolic (and enolic) hydroxyl groups than aliphatic ones. Since faeces lignins, unlike plant lignins, gain the same amount of OCH_3 by methylation with dimethyl sulphate and with diazomethane, it seems that in faecal lignins non-phenolic hydroxyl groups are completely absent.

Determination of the molecular weight of lignin

The values in the literature of the molecular weight of lignin vary widely. In earlier publications Beckman & Liesche (1921 *a, b*) found, by cryoscopic determinations in phenol, values of 762 and 784 for

the molecular weight of lignin prepared from wheat straw; later Fuchs (1936) found a value of 900. More recently, Conner (1941) determined a value of 3900 by dielectric measurements. These figures are comparatively low values for high polymers, but become more plausible when we consider that the properties of lignin are different from those of other high-polymer plant materials. The structure of lignin is not fibrous and the viscosity of its solution is low.

Freudenberg (1939) assumes that lignin has a three-dimensional structure created by the polymerization of from 2 to 10 molecules of coniferyl alcohol. This would indicate that the molecular weight was comparatively low. Substances like magnalol and lariciresinol (the so-called lignanes, containing 2 or 3 hydroxyconiferyl alcohol molecules) have an even lower molecular weight (see Haworth, 1942).

The differences between the molecular weight obtained by the cryoscopic method and those obtained by other physical methods appear to be due to the depolymerization of the lignin by the solvents used in the cryoscopic determination, so that the result is the molecular weight, not of the lignin, but of the 'structural units' of which it is composed. Furthermore, the molecular weights given in Table 4 should be regarded as averages only, since there is no evidence that the lignin molecule breaks down to give units identical in molecular weight. The molecular weights given in Table 4 should be accepted with this reservation. It should be noted that the molecular weights of lignins obtained from plants and from faeces are of the same order of magnitude.

The lignins used for cryoscopic determination of their molecular weight were purified by twice dissolving in 98 % acetic acid, reprecipitating by dilution with ice water and by drying in a vacuum over solid NaOH at room temperature. β -Naphthol was employed as a solvent, its molecular freezing point depression was determined by Bruni (1923) as 11.25° . The determinations were carried out in a conventional Beckmann micro-apparatus with an electromagnetic stirring device; glycerol was used in the bath for maintaining temperatures between 115 and 130° .

Table 4. *Molecular weight of lignins determined by depression of freezing point of β -naphthol*

Lignin from	Weight of lignin (g.)	Weight of β -naphthol (g.)	Δt	Molecular weight
<i>Pencillaria</i> Plant	0.1034	2.1272	0.861	635
Faeces	0.1078	2.2964	0.804	657
Wild barley Plant	0.1661	1.9470	1.500	640
Faeces	0.1388	1.8696	1.327	629
Fahli clover Plant	0.2076	1.7250	2.205	614
Faeces	0.1240	2.2095	1.042	606
Peanut hay Plant	0.1002	0.8228	2.203	621
Faeces	0.1228	1.5180	1.497	608

Degradation of the lignins

A number of degradation experiments were undertaken with the purpose of comparing the degradation products obtained from plant and faeces lignins and from wood lignins. The processes used in this investigation were the following: (1) oxidation with nitrobenzene in alkaline solution; (2) fusion with potassium hydroxide; (3) hydrolysis with acid ethanol under pressure.

(1) *Oxidation of lignins with nitrobenzene and alkali.* This reaction, which was successfully used by Freudenberg, Lautsch & Engler (1940) and by Creighton, Gibbs & Hibbert (1944), consists in treatment in NaOH solution with nitrobenzene at 160–200° under pressure for several hours. After removing the reduction products of the nitrobenzene by steam distillation the solution is acidified and extracted with ether. The ether solution is successively extracted with solutions of NaHSO₃, NaHCO₃ and NaOH, to separate aldehydes, acids and phenols, respectively. Four 30 g. samples of each lignin were treated with nitrobenzene and were combined before the ether extraction. The aldehyde extracts were acidified and SO₂ removed *in vacuo*, after which the solution was divided into two parts. The total aldehydes were precipitated from one part with a 2% solution of 2:4-dinitrophenylhydrazine in 2N-HCl. The precipitate was filtered off, washed with water, dried and the OCH₃ content was determined. From the other part of the aldehyde fraction the aldehydes were extracted with ether; this was removed and the aldehydes sublimed in a high vacuum.

A particularly remarkable fact is the total absence from the plant lignins investigated by us of syringaldehyde, a characteristic component of the aldehyde fraction of hardwood lignins. When the aldehydes were distilled at a pressure of 0.1 mm. no fraction could be obtained with a higher b.p. than 150° (syringaldehyde distils at 220–250° at this pressure). In the distillate, vanillin and *p*-hydroxybenzaldehyde could be separated, according to Creighton & Hibbert (1944), by means of their different solubilities in benzene

and identified by mixed melting points. The proportion of the two aldehydes could be calculated in each case from the OCH₃ content of the 2:4-dinitrophenylhydrazones.

The total yields of aldehydes and the proportions of vanillin and *p*-hydroxybenzaldehyde are given in Table 5. It may be mentioned for comparison that Creighton *et al.* (1944) isolated 35–51% of aldehydes (syringaldehyde and vanillin) from hardwood lignins and 15–24% of vanillin from softwood lignins. *p*-Hydroxybenzaldehyde is not present in the aldehyde fraction of wood lignins and only 1.8% was obtained by Creighton & Hibbert (1944) from maize-cob lignin.

Table 5 shows that the quantity of aldehydes obtained from the oxidation of plant lignins with nitrobenzene varies within wide limits, while the aldehyde fraction is almost completely absent from the nitrobenzene oxidation product of faecal lignins. It is remarkable that Gramineae lignins give a far larger quantity of aldehydes on oxidation than lignins from Leguminosae. Here, too, the botanical family to which the plant belongs has a far greater influence on the composition of the lignin than the degree of 'woodiness', as may be seen in the case of peanut hay, which belongs to the Leguminosae. Though the total percentage of aldehyde obtained varies greatly, the ratio of vanillin to *p*-hydroxybenzaldehyde remains constant throughout and is approximately 2 : 1, without being influenced either by the botanical family or the degree of woodiness of the plant from which the lignin is derived.

The absence of the aldehyde fraction from the oxidation products of faecal lignins shows that in the body the lignin undergoes a change, which renders it incapable of yielding aldehydes on degradation with nitrobenzene.

Table 5. *Formation of aldehydes by oxidation of lignins and nitrobenzene*

Lignin from		Aldehydes (as percentage of the lignin)	Vanillin (as percentage of total aldehydes)	<i>p</i> -Hydroxy- benzaldehyde (as percentage of total aldehydes)
<i>Eragrostis tef</i>	Plant	19.2	64.3	35.7
	Faeces	0	0	0
<i>Pencillaria</i>	Plant	16.3	63.8	36.2
	Faeces	0.31	0	—
<i>Setaria italica</i>	Plant	16.1	66.1	33.9
	Faeces	0.26	—	—
Wild barley	Plant	21.6	66.6	33.4
	Faeces	0.25	—	—
Bersem clover	Plant	4.2	65.4	34.6
	Faeces	—	—	—
Fahli clover	Plant	4.6	66.0	34.0
	Faeces	0.15	—	—
Peanut hay	Plant	8.3	64.7	35.3
	Faeces	0.10	—	—
Cyprus vetch	Plant	6.3	62.6	37.4
	Faeces	0.04	—	—

Benzoic acid and phenol were the main constituents of the acid and phenolic fractions, respectively; no importance can, however, be attributed to them, since they probably originated from the nitrobenzene, as was shown by oxidizing sugars instead of lignins by the same method, when no aldehydes, but considerable quantities of both benzoic acid and phenol, were formed.

(2) *Degradation of the lignins by alkali fusion.* The lignins were fused with KOH at 250–300° according to the method of Heuser & Winsvold (1923). A total of 100 g. of each lignin was treated in batches of 5 g. The melt was diluted with water, acidified and extracted with ether. After evaporation of the ether, catechol and protocatechuic acid could be separated in the residue by their different solubilities in benzene and identified by colour reactions (with FeCl_3 and AgNO_3), and by identification of their methylation products, veratrol and veratric acid (CH_3O contents and mixed melting points).

Lignins from Gramineae yield 1.36–2.25 % of protocatechuic acid and 0.20–0.40 % of catechol on alkali fusion. Lignins from Leguminosae yield 0.20–1.00 % protocatechuic acid and 0.30–0.56 % of catechol. In this case, no difference between plant and faecal lignins could be found.

(3) *Treatment with acid ethanol (ethanolysis).* By 'ethanolysis', i.e. by refluxing the lignins for 40–100 hr. with absolute ethanol containing 3 % by weight of HCl, Cramer, Hunter & Hibbert (1939) obtained a considerable number of well-defined degradation products of wood lignin. We attempted to use this method for the investigation of plant lignins, but obtained only small amounts of degradation products, most of the lignin being converted into a black carbonized mass. For this reason we modified this method by using aqueous ethanol instead of absolute, and by working under pressure, which permitted work at a higher temperature and, therefore, a very considerable reduction of the reaction time.

The detailed procedure was as follows: 10 g. of lignin were dissolved in 40 ml. of 2.5 N-NaOH with gentle heating, and this solution was mixed with 350 ml. of 85 % (v/v) ethanol

to which 10 ml. of conc. H_2SO_4 had been added. Ten batches of each lignin were treated in this manner. The solution of the lignin in NaOH and its reprecipitation with acid yielded a finely divided, reactive product. This mixture was now put in an autoclave with glass lining, and in the course of 2 hr. heated to 180–190°, at which temperature it was kept for 6–7 hr. The black solid residue was filtered off, dried, extracted with ether, and again subjected to the same treatment in 10 g. batches with acidified ethanol. After the ethanolysis had been repeated three times, there were usually only small quantities of unreacted lignin left. All solutions were now combined and 0.25 vol. of water was added. After evaporation of the ethanol *in vacuo*, the residue was extracted with ether, and the ether extracted successively with NaHSO_3 , NaHCO_3 and NaOH solutions, and thus divided into aldehyde, phenol, acid and neutral fractions.

The yields of the various fractions are given in Table 6. As in the case of nitrobenzene oxidation, treatment by acidified ethanol yields much larger quantities of carbonyl compounds from Gramineae than from Leguminosae lignins. In both cases, practically no carbonyl compounds were formed from faecal lignins. The composition of the aldehyde fraction obtained by ethanolysis is, however, completely different from that obtained by nitrobenzene oxidation. The main part of this fraction was a light yellow oil which, after distillation under 0.05 mm., partially crystallized on cooling. It was assumed that this was a benzene derivative containing a side chain with an aldehyde or ketone group in it; many such compounds have been found by Hibbert (1942) and Freudenberg (1939). By addition of a Ni salt and hydroxylamine a brown complex Ni salt could be prepared, whose Ni content (determined by dimethylglyoxime after treatment with H_2SO_4) agreed with that calculated for 1-(4'-hydroxy-3'-methoxyphenyl)propane-1:2-dione dioxime (11.63 % Ni). This Ni salt was prepared according to Kulka, Hawkins & Hibbert (1941). 1-(4'-Hydroxy-3'-methoxyphenyl)propane-1:2-dione is the only material obtained in this investigation with a side chain containing three carbon atoms.

The phenol fraction was separated by fractional distillation into two portions, b.p. 180–190° and 200–210°. After redistillation of each fraction, phenol and guaiacol were identified as their principal components by colour reactions (with FeCl_3 , and with FeCl_3 and AgNO_3 , respectively) and by the mixed melting point of their 3:5-dinitrobenzoates.

Table 6. Amounts of degradation products obtained by ethanolysis of lignins

(As percentage of the starting material.)

Lignin from		Aldehydes	Phenol	Acids and neutral fraction	Total yield
<i>Eragrostis tef</i>	Plant	2.31	6.40	4.28	12.99
	Faeces	0.22	5.77	5.22	11.20
<i>Setaria italica</i>	Plant	1.98	6.24	2.32	10.54
	Faeces	0.22	4.00	3.45	7.67
Wild barley	Plant	2.56	7.83	3.22	13.61
	Faeces	0.18	2.20	3.18	5.56
Bersem clover	Plant	0.61	1.28	0.42	2.31
	Faeces	—	0.96	1.75	2.71
Fahli clover	Plant	0.84	2.65	0.33	3.82
	Faeces	0.04	1.21	2.02	3.27
Peanut hay	Plant	0.98	4.50	2.07	6.55
	Faeces	0.26	1.80	3.74	5.80

Vanillic acid was found along with *p*-hydroxybenzoic acid, in the combined acid and 'neutral' fractions (the latter being the compounds remaining after aldehydes, acids and phenols were removed). The 'neutral' and acid fractions were worked up together, because the acid fraction was always obtained in very small quantities only. The two fractions were subjected to oxidation with KMnO_4 by the following method:

The residue remaining after evaporation of the ether was dissolved in a little ethanol, 50 ml. of water were added and the mixture heated on the water bath. An excess of 4% KMnO_4 solution was then added. The mixture was heated on the steam bath for 1 hr. and filtered. The filtrate was acidified with H_2SO_4 and extracted with ether. The ether was evaporated and the residue distilled under 0.05 mm. Two fractions were obtained. One had a very low OCH_3 content (0.2%), and could be purified by digestion with ice-cold benzene and a second sublimation under high vacuum. Its identity with *p*-hydroxybenzoic acid was proved by the m.p. and mixed m.p., 213–215°, with an authentic specimen, m.p. 215°. The fraction obtained at higher temperature could be recrystallized from water and was afterwards re-sublimed in a high vacuum. Its m.p., 205–206°, and OCH_3 content proved its identity as vanillic acid.

DISCUSSION

The structure of the lignins

The degradation products obtained by us correspond to those obtained from wood lignins. It seems, therefore, that the unit:

assumed by Klason (1936) and by Hibbert (1942) and Freudenberg (1939) to be present in wood lignins is also an essential constituent of the lignins in young plants (see also Percival, 1942). The occurrence of vanillin and *p*-hydroxybenzaldehyde can easily be explained by the oxidative fission of the side-chain double bond. From model experiments carried out recently by Wacek & Kratzl (1947) it is apparent that only those aromatic compounds which contain an olefinic double bond between carbon atoms 1 and 2 in the side chain, e.g. 4-hydroxy-3-methoxycinnamic acid, give, on oxidation with nitrobenzene, vanillin in good yield, while compounds like 3:4-dimethoxybenzylacetone do not. The assumption that the aldehydes formed by nitrobenzene oxida-

tion are derived from compounds containing a three-carbon side chain is supported by the finding of 1-(4'-hydroxy-3'-methoxyphenyl)propane-1:2-dione. However, the side chain is not split by ethanolysis and, therefore, no aldehydes can be found. Vanillic, *p*-hydroxybenzoic and protocatechuic acids result from a complete oxidation of the side chain to COOH , which is followed in some cases by the loss of CO_2 , thus leading to the formation of catechol, guaiacol and phenol. The constant proportion of vanillin to *p*-hydroxybenzaldehyde (2:1) observed by us seems to point to the fact that a lignin structural unit contains three aromatic nuclei, two yielding vanillin on oxidation and one *p*-hydroxybenzaldehyde (the loss of a OCH_3 group by oxidation with nitrobenzene has never been observed and seems improbable).

As already pointed out on p. 253, wood lignins yield, on oxidation with nitrobenzene, considerable quantities of aldehydes rich in OCH_3 , a fact which is in accordance with the larger OCH_3 contents of wood lignins in general.

The OCH_3 content of the Gramineae lignins shows that each structural unit (assuming its molecular weight to be 625) must contain two OCH_3 groups (theory, 9.9% OCH_3); see Table 3. Methylation with diazomethane raises the OCH_3 content to 19%, which shows that two phenolic OH groups are methylated. Since the experimental evidence points to the presence of three aromatic rings, two of them substituted with OCH_3 and OH and the third with OH only, one must necessarily assume that the remaining phenolic OH group is attached in such a position as to make it incapable of being methylated with diazomethane.

Leguminosae lignins contain only one OCH_3 group per structural unit (theory, 5.1%) and show on methylation with diazomethane an increase in OCH_3 content which corresponds to the presence of two phenolic OH groups. Since, on oxidation with nitrobenzene of the Leguminosae lignins, vanillin and *p*-hydroxybenzaldehyde are also formed in the proportion 2:1 (although in smaller yields), one must assume that part of the units still contains a system of two rings, each of them substituted with OH and OCH_3 , and one ring substituted with OH only. This would mean that half of the Leguminosae lignin is methylated in the usual manner, i.e. it contains two OCH_3 groups in one structural unit. The other half is not methylated at all. A large part of the free phenolic OH groups in Leguminosae lignins is apparently incapable of being methylated.

Plant lignins take up one OCH_3 group more on methylation with dimethyl sulphate than on methylation with diazomethane indicating the presence of one non-phenolic OH group.

Plant lignins contain, in contrast to wood lignins, nitrogen as a characteristic component. All lignins

can be classified by their increasing N and decreasing OCH_3 content as follows:

- Hardwood lignins, N, nil; OCH_3 , 20 %.
- Softwood lignins, N, 0.2–0.3 %; OCH_3 , 15 %.
- Gramineae lignins, N, 1.2–1.6 %; OCH_3 , 10 %.
- Leguminosae lignins, N, 2.9–3.4 %; OCH_3 , 5 %.

Digestion of lignins

The solubility of the lignins, the apparent molecular weight of the building units, and the OCH_3 and N content remain unchanged by digestion. On the other hand, the disappearance of the aldehyde fraction from among the degradation products of faecal lignins shows that changes in the side chains of the aromatic rings have occurred, probably by hydrogenation of the side-chain double bond, preventing their oxidative fission and the formation of aldehyde groups. The other significant change is the complete disappearance of the non-phenolic OH group indicated by the fact that the increase in OCH_3 content, obtained by methylation with dimethyl sulphate, is identical with that obtained with diazomethane.

SUMMARY

1. Lignins were prepared by extraction with dilute alkali from a number of annual forage plants and from faeces collected from sheep fed exclusively with the same plants. The plants used were *Eragrostis tef*, *Pennisetum purpureum*, *Setaria italica*, *Hordeum murinum* (wild barley), *Trifolium alexandrinum* bersem, *T. alexandrinum* fahli, *Lathyrus ochrus* and hay of *Arachis hypogaea* (peanut).

2. All lignins contained nitrogen whose presence is not due to accidental contamination with proteins, but seems to be a characteristic component of the lignin molecule itself. The methoxyl content of the lignins ranges from about 5 % in Leguminosae lignins to about 10 % in Gramineae lignins.

3. On methylation with diazomethane, the original methoxyl content of the Leguminosae lignins is trebled, that of the Gramineae lignins doubled. Methylation with dimethyl sulphate brings about a small increase in methoxyl uptake over that obtained with diazomethane. In faecal lignins no additional methylation with dimethyl sulphate could be obtained.

4. Cryoscopic molecular weight determinations in β -naphthol showed values of about 600 both for plant and faecal lignins.

5. Oxidation of the plant lignins with nitrobenzene in alkali yielded vanillin and *p*-hydroxybenzaldehyde. Faecal lignins did not yield any aldehydes. The ratio of vanillin to *p*-hydroxybenzaldehyde was always 2 : 1.

6. On fusion with potassium hydroxide all lignins gave catechol and protocatechuic acid.

7. On heating the lignins with ethanol containing 3 % sulphuric acid to 170–180° under pressure, 1-(4'-hydroxy-3'-methoxyphenyl)propane-1:2-dione was obtained only from plant lignins; both plant and faecal lignins yielded phenol, guaiacol and an acid fraction, which, after further oxidation with KMnO_4 , yielded vanillic and *p*-hydroxybenzoic acids.

8. The experimental results are discussed in relation to the structure of the lignins.

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