CXL. BIOCHEMICAL AND HISTOLOGICAL STUDIES ON LIGNIFICATION[1].

PART I. THE NATURE OF LIGNIN: ITS PHYSIOLOGICAL SIGNIFICANCE AND ITS ESTIMATION IN TIMBERS.

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(*Received October 21st, 1925.*)

Introductory and General.

Among the secondary chemical changes taking place in the cells of higher plants, lignification has been commonly held to proceed at the expense of cellulose, giving rise to membranes composed of compound cellulose such as lignocellulose. We have no precise knowledge of how lignocellulose is formed, from what substance or substances it originates, and what is its physiological significance in plant economy. Still less do we know concerning its exact chemical nature and its relation to other plant constituents.

It may be resolved by suitable chemical treatments in such a way that the non-cellulose constituent, viz. lignin, is rendered soluble at the least expense, leaving behind a maximum amount of cellulose. Lignocellulose plays an important part in the soil. Thus the processes of decay to which it is subjected vary according to the aerobic or anaerobic conditions to which it is exposed. It is only the cellulose component however that disappears, being broken down into gaseous and other products, whilst the aromatic lignin component remains behind and accumulates in the soil, furnishing that valuable organic constituent—humus—which is largely responsible for the texture of the soil.

Much evidence, *e.g.* Bray and Andrews [1924] and du Toit [1924], exists in favour of the view that the humic matter of the soil is derived from lignin and not from carbohydrates. Closely related to the humic acids of the soil, we find in coal a series of ill-defined substances known as "ulmic acids."

The chemistry of wood substance, designated as wood lignin or wood fibre, and the various microchemical colour reactions, have been the subject of numerous investigations which are well summarised in Czapek's "*Biochemie der Pflanzen.*"

It has generally been considered that the different colour reactions ob-

[1] Part of a Thesis approved for the Degree of D.Sc., University of London.

tained with wood are due to its aromatic constituents, whether free or combined. These colour reactions may be due to coniferyl alcohol or a substance or substances having a similar propenyl or hydroxypropenyl grouping; or they may be due to a substance of the nature of vanillin. There seems no doubt that the active grouping concerned in the coloration is of the nature of an aldehyde, and not of a quinone. This conclusion is supported by the work of Selivanoff [1891] and Nickel [1887, 1889] who first showed that the lignin reaction disappears on treating the wood with sodium bisulphite, or hydroxylamine.

Czapek [1899] obtained a crystalline aldehydic substance which he termed "hadromal," giving all the colour reactions of wood, by treating wood with boiling stannous chloride solution. Hadromal is actually one of the aromatic constituents concerned partly in the lignification process and it is linked chemically with cellulose and other polysaccharides. The yield of hadromal does not exceed 1–2 % of the wood substance by the method employed by Czapek [1899]. On the other hand, the aromatic lignin component according to the methods hitherto proposed for its estimation is obtained in a yield of 10–30 % of the wood substance. The difficulty encountered in settling the hadromal question is due to the fact that so far we are not in possession of any method by which we can isolate this aromatic constituent exactly as it exists in combination with polysaccharides without destroying its reactive grouping. The methods employed for isolating the ligneous aromatic constituent in the above yield (10–30 %) depend on the treatment of lignified cell walls with 72 % sulphuric acid, concentrated hydrochloric acid, or caustic alkali which bring about certain changes. When we take all the properties of lignocellulose into account it appears to the writer that the aromatic constituent just before lignification is not of the nature of hadromal, but that it is produced by a secondary transformation of this aromatic component by processes of oxidation. At any rate the amount of the substance of the nature of hadromal thus produced is very variable even in the fully lignified cell wall.

Phloroglucinol is thus not a stain for the major part of the aromatic constituent which is the precursor of the one present in lignocellulose but rather for an aldehyde of the nature of hadromal. This aldehyde is always associated with lignin into which the major part of the aromatic substance formed first in the plant and not stained by phloroglucinol is converted. A test has been devised by the writer to which aromatic substances utilised in lignification respond readily. It is found that a 1 % solution of vanillin in concentrated sulphuric acid is a very sensitive reagent giving a bright-red coloration with such aromatic substances as are utilised in lignification. Further, the presence of these substances can be detected in parts not shown by the phloroglucinol reaction, and the localisation of the aromatic constituent by this reagent has thrown much light on the mechanism of lignification, for we can actually follow the migration and production of the aromatic substance in certain zones.

On the basis of many observations it was held by Erdmann and Baltzer [1873] and Cross and Bevan [1883–1895, 1] that cellulose is in ester-like combinations in wood. Wislicenus [1909, 1910], on the other hand, held that cellulose is only an adsorption compound with other substances contained in wood. Koenig [1912] also opposed the ester hypothesis since on treating wood with 72 % sulphuric acid and diluting with water he obtained a residue of lignin retaining the cell wall structure, whilst the cellulose was saccharified. Various reasons will be given in the present work in favour of the writer's view that lignin is present in combination with cellulose and certain polysaccharides as a glucoside. Our knowledge of the chemistry of lignocellulose we owe primarily to the valuable researches of Cross and Bevan [1895, 2–1916], where its properties are well summarised.

Lignocellulose does not take part directly in the cellulose xanthate reaction. Cellulose combines with sodium hydroxide, and the alkali cellulose on exposure to carbon disulphide reacts according to the following equation in which one of the hydroxyl groups of the cellulose molecule appears to react with greater facility than the others.

$$ROH + NaOH + CS_2 = CS\begin{matrix} O{-}R \\ S{-}Na \end{matrix} + H_2O.$$

The resulting compound, alkali cellulose xanthate, is perfectly soluble in water giving a very viscous solution. When lignocellulose from jute is submitted to this reaction a partial conversion (20–50 %) of the constituents of the fibre is observed. In this reaction it is the group designated by β-cellulose that takes a part and is in fact dissolved, whilst the undissolved residue possesses all the properties of the original lignocellulose. On the other hand the lignocellulose of wood is completely resistant to the cellulose xanthate reaction. The writer believes that this behaviour points to the fact that in the molecule of lignocellulose from wood both the α- and β-celluloses are in firm chemical union with the lignin component, whilst β-cellulose in those lignocelluloses which participate partially in the xanthate reaction is more probably a mixture of unlignified β-cellulose together with the true lignocellulose. Further, it is well known that of the three free hydroxyl groups in the molecule of cellulose there is one which is much more reactive than the others and is the first to take part in the thiocarbonate reaction. This leads the writer further to conclude that when this reactive hydroxyl group is already occupied or blocked through its linkage with the lignin component there exists a molecule of true lignocellulose composed of celluloses α- and β- linked chemically with the lignin constituent, which, as a whole, although containing free hydroxyl groups, is inert as far as the thiocarbonate reaction is concerned. This behaviour is not surprising as steric hindrance plays an important part in certain reactions in which the hydroxyl groups of polysaccharides are involved. If lignin were merely adsorbed on cellulose as held by Wislicenus and Kleinstuck [1910] we should expect it to be readily

dissolved by the alkali of mercerising strength (17–18 %) used in the thiocarbonate reaction, as it is a powerful solvent for free lignin, and in addition, if the reactive hydroxyl group of the cellulose constituent were free, it should form the cellulose xanthate. As lignocellulose from wood does not take any part directly in the above xanthate reaction, the writer considers she is justified in concluding that the usual reactive hydroxyl group of the normal cellulose is linked chemically with the lignin component in lignocellulose.

Resolution of Lignocellulose.

Amongst the different methods employed for resolving lignocellulose, the chlorination process and the treatment with nitric acid have been employed, but these are too costly for industrial operation. The industrial processes of resolution are chiefly based on treatment with sulphites or bisulphites or alkalis. The recent work of Klason [1917, 1922, 1923], throws much light on the nature of the lignin constituent which is present as a soluble sulphonic derivative in the digested mixture. The sulphite lye after digestion has been shown to contain two ligninsulphonic acids corresponding to lignin-α and lignin-β.

The resolution with alkalis is effected with 7–10 % sodium hydroxide at a pressure of 6–10 atmospheres. The alkaline mode of resolution has many points to commend itself for critical scientific investigation, the results of which are described herein. It is very rapid and complete, and, from the point of view of investigating the lignin component, it has the advantage that this can be isolated in the full yield, without any radical decomposition of the aromatic nucleus, by simply acidifying the alkaline liquid, as it is insoluble in acid solutions or in water. From the crude precipitate, lignin can be isolated quantitatively by suitable purification. This has now been accomplished in a very simple manner, and conditions have been found under which the resolution of lignified plant materials can be accomplished readily, and further, the same conditions have been extended to the preparation and quantitative estimation of lignin.

When lignocellulose is boiled with a 5–10 % solution of caustic soda at the ordinary pressure the resolution does not proceed beyond 15–20 % of the total lignocellulose. On treating with alkali at higher pressures in an autoclave the amount hydrolysed rapidly increases with the pressure. When the pressure is kept constant and the strength of alkali is varied, hydrolysis increases with the concentration up to a certain limit, beyond which decomposition of lignin sets in. The preliminary experiments to establish these conditions were carried out with a homogeneous sample of sawdust from white deal as follows.

Five grams of the wood shavings or sawdust are accurately weighed out and transferred to a 200 cc. "durosil" test tube (2″ × 8″), containing 100 cc. of caustic soda solution of varying strengths and heated at pressures of 2·5 and 3 atmospheres for 1 hour. At the end of this period the contents are

allowed to cool, made up to 250 cc. with distilled water and after thorough mixing, filtered through a filter paper. 50 cc. of the dark brown alkaline filtrate is carefully measured out with a pipette into a 100 cc. cylinder and acidified with 5 cc. of concentrated hydrochloric acid. The precipitated lignin, which is accompanied by traces of other substances, is allowed to settle and filtered through a small Buchner funnel. It is well washed with cold distilled water until the filtrate as tested with silver nitrate is free from chlorides. The lignin is readily freed from the accompanying impurities by dissolving it in boiling 95 % alcohol in which the impurities are insoluble. The precipitate together with the filter paper is transferred carefully to a beaker so that it rests flatly on the bottom. It is treated repeatedly with boiling 95 % alcohol just sufficient to cover the paper and the alcoholic solution carefully filtered into a weighed flask. The extraction is repeated until the alcoholic solution is quite colourless, and any lignin remaining on the filter paper is thoroughly washed out with boiling alcohol. The alcoholic filtrate is evaporated to dryness and weighed to constant weight as lignic acid. On the extraction of the precipitated crude lignin with alcohol a trace of insoluble matter, chiefly composed of cellulose-β giving all its reactions, remains behind [Powell and Whittaker, 1924]. Results of experiments with sawdust from white deal with varying concentrations of alkali at 2·5 and 3·0 atmospheres are given in the following tables.

Concentration of sodium hydroxide	% lignic acid obtained at		% lignocellulose resolved at	
%	2·5 atmos.	3 atmos.	2·5 atmos.	3 atmos.
1·0	5·40	5·90	28·52	31·22
2·5	5·90	7·00	31·22	37·04
4·0	6·10	7·30	32·27	38·62
5·0	5·80	6·90	30·68	36·51
7·5	4·90	5·60	25·93	29·63
10·0	4·20	5·10	22·22	26·98
15·0	3·40	4·40	17·99	23·28

The above results show that the maximum amount of resolution was produced with 4 % sodium hydroxide solution. In the next series therefore concentration was kept constant, and the pressures varied up to 16 atmospheres using 4 % NaOH solution. The results are given in the following table.

Pressure in atmospheres	% lignic acid obtained	% lignocellulose resolved
Normal pressure	2·50	13·23
1	4·60	24·34
2	5·05	26·72
3	7·40	39·15
4	8·67	45·87
5	10·50	55·55
6	12·70	67·19
7	14·30	75·66
8	15·50	82·01
9	17·70	93·65
10	18·90	100·00
12	19·20	—
16	19·20	—

The foregoing results show that the resolution of the lignocellulose is practically complete under the conditions mentioned at 10 atmospheres. As this pressure corresponds with a temperature of 180°, it was considered advisable not to exceed it as ordinary resistant cellulose remains practically unattacked at that temperature. In view of these results a few experiments were carried out to ascertain if it were possible to reduce the pressure below 10 atmospheres and obtain complete resolution by slightly extending the period of digestion from 1 hour to 1·5 hours. The results obtained were as follows.

The percentage of lignic acid obtained at 8 atmospheres with 4 % NaOH solution after 1 hour and 1·5 hours' digestion was 15·6 and 17·1 respectively. Similarly the percentage of lignic acid obtained at 10 atmospheres after 1 hour and 1·5 hours was 18·9 and 18·95 % respectively. These preliminary experiments were further sufficient to show that the resolution was complete at 10 atmospheres in 1 hour whilst at 8 atmospheres even after 1·5 hours the percentage resolved did not approach that at 10 atmospheres and 1 hour.

Estimation of Lignin.

Having arrived at these conditions of complete resolution it was thought worth while to investigate if the principle outlined above could not be applied to the elaboration of a reliable method for estimating lignin in plant materials, as the present methods for the determination of lignin are not reliable or suitable for carrying out a large number of determinations. In the first place, it was necessary to ascertain if the lignic acid suffered any decomposition when treated with alkali at 10 atmospheres. For this purpose, varying amounts of lignic acid were dissolved in 20 cc. of 4 % sodium hydroxide and digested at 10 atmospheres for 1 hour. The results were as follows:

Weight of lignic acid taken. g.	Weight of lignic acid obtained after digestion. g.
0·055	0·052
0·110	0·108
0·165	0·162
0·220	0·221

The results show that under the above conditions lignic acid does not suffer appreciable decomposition and the procedure was extended therefore to the determination of lignin in different woods. It will be here unnecessary to review the methods which have hitherto been proposed for the estimation of lignin. An examination of the proposed methods [Konig and Rump, 1914; Konig and Becker, 1918; Willstätter and Zechmeister, 1913; Krull, 1916; Benedikt and Bamberger, 1890; Herzog, 1896; Cross, Bevan and Briggs, 1907; Seider and Hempel, 1907] is sufficient to show that the difficult problem of the estimation of lignin has not yet been solved in a satisfactory manner.

Having now shown that lignocellulose can be resolved quantitatively by heating it with 4 % sodium hydroxide for 1 hour at 10 atmospheres, and that the lignin can be recovered quantitatively after such treatment without any

primary decomposition, a direct gravimetric method has been devised on the basis of these results for estimating lignin.

The procedure is as follows. 5 g. of the wood is accurately weighed out in a thick glass durosil test tube 8″ × 2″ and heated with 100 cc. of 4 % sodium hydroxide solution at 10 atmospheres for 1 hour. The solution after cooling is made up to 250 cc. and filtered. The lignic acid is then estimated in an aliquot volume of the filtrate (50 cc.) according to the process already described. From the amount of lignic acid the percentage of lignin can be readily calculated. The procedure has been applied to the determination of lignin in a large number of woods and has been found to give most consistent results.

When we come to examine the nature of lignin we shall see that, like all other secretory products of constructive metabolism, it is present in varying amounts in different woods partly in the free condition directly extractable by alcohol, the remaining major portion in chemical combination with the cellulose hydrolysable by alkali.

Accordingly, the estimations of lignin have been carried out on unextracted and extracted timbers, giving the total and combined lignin respectively, whilst the difference corresponds with the free lignin. The results of these estimations are given in the following table, and in a few cases (sufficient for the purpose) are also compared with those obtained by the method of Konig and Becker [1918].

It will be seen that the results obtained by the latter method are decidedly higher than those given by the alkali method. Since the estimation of lignin by treatment with concentrated hydrochloric acid (45 %) is well known to give results concordant with those obtained by 72 % sulphuric acid, only the latter method was employed for comparison. The values hitherto recorded for the percentage of lignin obtained by the last two methods are too high for the following reasons. In the first place, it must be admitted that lignin obtained by these two methods is not pure. It is also assumed that no secondary reactions occur and that the carbohydrates are completely removed. Hagglund [1923, Hagglund and Bjorkman, 1924] in a series of papers giving the results of a critical examination of the nature of lignin obtained by the concentrated hydrochloric acid method has shown that the lignin always retains a portion of hexosan and pentosan residues amounting to 10–12 %, which is constant over a very prolonged period of treatment.

The writer also observed on critical examination of the lignin prepared by treatment with 72 % sulphuric acid that it is not only associated with carbohydrate residues amounting to 4·0–6·0 %, but that it suffers sulphonation during the treatment. This is proved by the fact that after carefully washing the lignin free from sulphates it gives, on ignition with pure sodium carbonate, sulphate amounting to 12·0–14·0 % SO_4. The values obtained by these methods can therefore hardly be considered as reliable.

Estimations of total, combined and free lignin in timbers.

Timbers	Total lignin 4 % NaOH %	Total lignin 72 % H_2SO_4 %	Combined lignin 4 % NaOH %	Free lignin 4 % NaOH %
Lignum Vitae	38·39	47·99	15·57	22·82
Teak	31·07	45·80	22·66	8·41
Rose Wood	27·28	41·10	11·89	15·39
Red Deal	26·40	35·70	20·41	5·99
Mahogany	24·22	33·95	19·66	4·56
Pitch Pine	23·77	33·80	17·90	5·87
Oregon Pine	21·11	36·70	18·73	2·38
Larch	21·33	32·80	18·33	3·00
Oak	21·10	31·20	17·33	3·77
Yellow Pine	20·30	32·08	18·70	1·60
White Deal	20·50	29·10	18·70	1·80
Elm	19·32	27·00	17·32	2·00
Ebony	18·97	52·10	15·73	3·24
Box Wood	18·50	26·29	14·44	4·06
Spruce	17·80	30·55	16·98	0·82
Black Wood	17·06	—	7·72	9·34
Parana Eucalyptus	16·30	—	13·03	3·27
Sandal Wood	16·11	—	14·55	1·56
Walnut	16·01	—	11·29	4·72
Canary Wood	15·27	—	11·60	3·67
Plane Wood	14·36	—	9·90	4·46
English Oak	14·14	—	13·17	0·97
Ash	11·93	—	11·05	0·88
Birch	10·48	—	9·66	0·82
Beech	9·32	—	9·00	0·32

Micro-estimation of lignin. There is as yet no reliable micro-method for the estimation of lignin and, being specially interested in the study of its distribution, the writer next directed her attention to elaborating a colorimetric method for its estimation. There was no difficulty in such micro-work as far as the resolution of lignocellulose was concerned and the problem was to estimate the lignin present in the alkaline extract obtained under the conditions described above for its macro-estimation. It was first decided to make use of the marked phenolic properties of lignin in such micro-estimation by coupling it in alkaline solution with an excess of a diazotised aromatic amine. The amount of the dye produced is then under proper conditions proportional to the amount of lignin.

The amine is dissolved in 10 parts of water containing the equivalent quantity of hydrochloric acid. The solution is cooled to 0° and a saturated solution of the calculated quantity of sodium nitrite is added all at once. The solution may be tested after 5 minutes with potassium iodide paper to ensure an excess of nitrite. To carry out the coupling, an alkaline solution of the phenolic substance (*e.g.* lignin) after cooling to 5–10° is treated with an excess of the diazotised base. Care is taken that the phenol solution should contain sufficient alkali and that the mixture does not become acid. On the addition of the diazotised amine, a dye is produced which can be employed for colorimetric estimation. A few preliminary estimations were conducted on the above lines by coupling lignin in alkaline solution with diazotised aniline, *p*-nitraniline, *m*-phenylenediamine, *α*-naphthylamine and benzidine.

Lignin gave rise to dyes having a reddish brown tinge with the first three diazotised bases, whilst α-naphthylamine and benzidine gave deep red colours. Of these two, benzidine was selected as the more suitable as the colour was much more permanent than that obtained with α-naphthylamine. The diazotised benzidine solution was prepared as follows. One g. of benzidine was dissolved in 10 cc. of water containing 2 cc. of concentrated hydrochloric acid and diazotised with a saturated solution containing 0·8 g. sodium nitrite.

Benzidine was particularly suitable compared with other amines because, with these, traces of the corresponding phenols are produced during diazotisation according to the following equation if the temperature is not sufficiently low:

$$R \cdot N_2 \cdot Cl + H_2O = R(OH) + N_2 + HCl.$$

The phenol thus produced itself gives a colour on adding alkali. With benzidine however, this was not observed. A standard solution of lignin was prepared by weighing out 0·1 g. of pure anhydrous lignin, dissolving in a little alkali and making up the solution to 250 cc. To carry out the colorimetric estimation 1 cc. of the above lignin solution = 0·4 mg. lignin was accurately measured out with a 1 cc. pipette, graduated to 0·01 cc., in a 100 cc. Nessler cylinder containing 2 cc. of 4 % sodium hydroxide. To the mixture was added 0·5 cc. of the diazotised 1 % benzidine solution and after diluting to about 50 cc. it was left at the ordinary temperature for half an hour. The volume was then made up to 100 cc. and the tint compared with that obtained in a solution containing an unknown amount of lignin. In order to test the accuracy of the above method, experiments were first conducted with known amounts of lignin, varying from 0·1–1·0 mg. The results of these experiments showed that they were always reliable so long as the tint of the standard or the unknown solution was within 95 % of the other solution. This necessitated the preparation of a large number of standards, and attempts were therefore made in another direction to improve the estimation on the same principle. A solution of phosphotungstic and phosphomolybdic acids in phosphoric acid is the most sensitive reagent we possess for estimating minute amounts of aromatic substances containing hydroxyl groups, which in a solution made alkaline with sodium carbonate give a deep pure blue coloration with the above reagent. The reagent was first introduced by Folin and Denis [1912] for the colorimetric estimation of tyrosine. It is prepared by dissolving 100 g. sodium tungstate, 20 g. phosphomolybdic acid and 50 cc. of 85 % phosphoric acid in 750 cc. of water. The solution is boiled under a reflux condenser for 2 hours, cooled and made up to 1000 cc. A standard solution of lignin in dilute alkali was prepared by weighing out 0·5 g. of pure anhydrous lignin and dissolving it in about 25 cc. of 4 % sodium hydroxide solution; it was then carefully made up to 500 cc. so that 1 cc. = 1 mg. lignin. A few qualitative tests showed that the reagent gave a beautiful blue colour with quantities of lignin varying between 1·0–0·01 mg. The standard conditions employed for the colorimetric estimation are as follows. Known volumes

(1·0–0·1 cc.) of the standard lignin solution are accurately measured out into Nessler cylinders and to each is added 2·5 cc. of the phosphotungstic phosphomolybdic reagent and after 5 minutes 12·5 cc. of a saturated solution of sodium carbonate. The volume is made up to 100 cc. and the colours matched with that obtained under similar conditions from an unknown amount of lignin. Experiments first undertaken with known quantities of lignin showed the method to be thoroughly reliable so long as the depth of the colours under comparison did not differ by more than 20 %. The results were also in close agreement with those obtained by the gravimetric method.

Distribution of Lignin in Wood.

The method was first employed in studying the distribution of lignin in the autumn and spring wood of Oregon pine and spruce. The autumn and spring wood were first separated by cutting a thin shaving of uniform thickness of the wood along the borders of the bands of the autumn and spring tracheides. The relative weights of the autumn and spring tracheides separated in the above manner from Oregon pine were found to be approximately 2 : 1 whilst from spruce they were in the ratio of 1 : 2 respectively. 0·1–0·2 g. of the cuttings containing the autumn or the spring wood was digested with 20 cc. of 4 % NaOH at 10 atmospheres for 1 hour. The digested liquid was then made up to 100 cc. with distilled water and filtered. As the alkaline extracts thus obtained contained substances other than lignin, also giving a coloration with the phosphotungstic-phosphomolybdic reagent, it was necessary to separate the lignin from these.

A known volume (1·0–5·0 cc.) of the digested liquid is accurately measured out into a 15 cc. centrifuge tube and acidified with a few drops of concentrated hydrochloric acid. The precipitated lignin is centrifuged, washed twice with distilled water and dissolved in a few drops of 1 % sodium hydroxide. The alkaline solution is carefully washed into a Nessler cylinder and the colorimetric estimation carried out as already described.

In order to determine whether there was any decomposition of lignin under the conditions employed for the digestion in the micro-estimation, quantities of lignin varying between 5–30 mg. were dissolved in 20 cc. of 4 % sodium hydroxide and heated in an autoclave for 1 hour at 10 atmospheres pressure. The solutions were then made up to 100 cc. and the lignin estimated colorimetrically in an aliquot portion as described, but no diminution in the quantity of lignin was observed.

Distribution of Lignin in Autumn and Spring Wood of Spruce and Oregon Pine. (Dry.)

	Lignin %	
	Autumn wood	Spring wood
Spruce	17·7	15·1
Oregon pine	14·4	18·6

The above results are the average of a number of estimations.

The same method was next applied to the study of the distribution of lignin in different parts of Scotch fir (*Pinus sylvestris*). The results are given in the following table. The twigs were further subdivided into four concentric rings so as to study the lignin content in separate layers.

Percentage of Lignin in different parts of Scotch Fir.

	Lignin % on green	Lignin % on dry	Moisture %
Leaf	5·10	8·63	40·9
Buds	1·90	9·4	79·7
Thick twig 14 years old: cortex	1·79	5·44	56·5
outer ring	11·11	14·29	24·9
inner ring	15·50	23·08	34·9
centre	8·57	11·69	26·7
Thin twig 4 years old	8·34	15·90	47·6
Thin twig 2 years old: end portion	6·17	9·56	35·5
bottom portion	7·82	12·12	35·5

Percentage of Lignin in different layers of stems of Hawthorn, Ash and Diseased Ash.

	Lignin %			
	Cortex	Outer ring	Inner ring	Centre
Hawthorn	14·07	8·94	11·37	10·24
Diseased ash	12·53	13·71	—	15·30
Normal ash		Average 11·93		
Diseased ash		„ 13·80		

The above results show that there is no marked variation in the lignin content of the autumn and spring wood. The lignin content of the buds is striking in that they contain practically the same amount of lignin as the older parts. The lignin content of the green buds is only 1·9 % owing to the high moisture content. This indicates that lignification commences at a very early stage simultaneously with the thickening of the cell wall.

The woods are known in nature to be susceptible to two types of attack by organisms known as white rot and brown rot. It is believed that in the former decomposition of lignin is induced leaving the cellulose behind, whilst the latter type of rot proceeds at the expense of cellulose leaving lignin undecomposed.

The greater percentage of lignin in different layers of a stem of slightly diseased ash (brown rot) compared with the average percentage in normal ash suggests that a preferential decomposition of cellulose probably takes place.

The Nature and Properties of Lignin.

Lignin as prepared under the conditions described for its estimation is obtained as a light brown powder, insoluble in water but soluble in dilute alkalis, ammonia, alcohol, acetone and acetic acid. It reduces ammoniacal solution of silver nitrate slightly and Fehling's solution still less. When boiled with hydrochloric acid it does not give rise to furfural, hence it is quite free from polysaccharide units, and its characteristics agree with the generally

accepted view that it is wholly an aromatic compound. It has a pleasant aromatic odour distinctly recalling vanillin in some cases. When an alcoholic solution of it is evaporated to dryness in a flat dish, it is obtained in the form of a highly lustrous brown film. It melts at 167–169° to a clear brown liquid without decomposition and with a highly purified product the M.P. did not rise above 170°.

Lignin possesses faintly acidic properties which are more pronounced than those of the polyhydroxy phenols. It dissolves in cold dilute sodium carbonate solution from which it is reprecipitated on acidification. Its acid value—*i.e.* the number of grams of lignin dissolved by a litre of *N* NaOH is 477. Lignin also possesses many of the characteristics of an unsaturated compound. Its iodine value as determined by Wijs' method is 139.

The determination of the acid value of lignin presented unusual difficulties, as, owing to its feeble acidity and the dark colour of its solution, it was impossible to carry out a titration using indicators in the ordinary way. The procedure adopted was as follows: 0·1432 g. of finely powdered anhydrous lignin was accurately weighed out in a small beaker and suspended in about 10 cc. of distilled water. The suspension was heated on a water-bath and 29·1 cc. of *N*/100 sodium hydroxide were gradually run in until a small portion of the lignin remained undissolved. The undissolved lignin was then filtered off, washed thoroughly with distilled water and estimated gravimetrically. The difference between this amount and the quantity of lignin taken, 0·1432–0·0040 = 0·1392 g., corresponds with the lignin dissolved by 29·1 cc. of *N*/100 NaOH. Hence the acid value is 477.

The iodine value which is an indication of the degree of unsaturation of lignin was determined as follows. 0·1 g. of pure anhydrous lignin was dissolved in a little glacial acetic acid in a well stoppered flask together with 25 cc. of Wijs' solution. The stopper was then moistened with potassium iodide solution and the mixture kept in the dark for 45–60 mins. A blank experiment with Wijs' solution was also performed simultaneously under the same conditions. 20 cc. of 10 % potassium iodide solution and 100 cc. of water were then added and the excess of iodine titrated with *N*/10 sodium thiosulphate using starch solution as indicator in the usual way.

Titre of the blank experiment = 49·3, 49·3 cc. of *N*/10 $Na_2S_2O_3$
Titre of the actual ,, = 38·2, 38·4 ,, ,,
Average amount of iodine absorbed by 0·1 g. of lignin; (49·3–38·3) × 0·0127 = 0·1397 g. Iodine value = 139·7.

Lignin prepared by the method described above behaves as a homogeneous compound, because on purification in different ways its properties remain unchanged.

It gives very characteristic almost insoluble normal and basic calcium and barium salts, prepared by adding a solution of barium or calcium chloride to an ammoniacal solution of lignin. The content of calcium or barium of such salts varies according to the strength of the ammonia solution used for

dissolving the lignin. When precipitated from a strongly ammoniacal solution the percentage of calcium or barium is very much higher. The procedure generally followed for the isolation of these salts was as follows. The ammonia solution used for dissolving a known weight of lignin was first carefully freed from carbon dioxide to avoid the subsequent contamination of calcium or barium lignate with the carbonates. To the boiling ammoniacal solution of lignin, calcium or barium chloride was added in excess, when the lignin was precipitated as an amorphous dirty brown precipitate. It was washed free from salts and dried. The salt is absolutely insoluble in water, soluble in acetic acid, dilute sodium hydroxide solution, or alcoholic hydrochloric acid, and the lignin can be recovered unchanged in its properties from such salts. One remarkable fact which will be noticed from the following results is that 95–98 % of the lignin is precipitated as the insoluble alkaline earth salt. This would be very unlikely if the lignin were not of a homogeneous nature. The results of the analysis of calcium, barium and sodium salts are given below. The ammoniacal salt cannot be prepared in the solid form as it dissociates on concentration and the lignin is precipitated as such.

	Salt	Conc. of ammonia %	g. of lignin taken	g. of salt obtained	g. of sulphate obtained by ignition with H_2SO_4	% of metal in salt	Yield % of theoretical
1	Ca	5	0·4052	0·4215	0·1325	Ca, 9·20	95
2	Ca	1	0·2830	0·2950	0·0840	Ca, 8·40	97
3	Ba	5	0·1400	0·2050	0·1375	Ba, 39·4	104
4	Na*	—	0·1392	0·1498	0·0213	Na, 4·6	102

* Prepared by treating an excess of lignin with a minute quantity of NaOH under conditions described for the determination of acid value.

The alkaline earth salts do not appear to have a definite composition owing to their tendency to form basic salts. An alcoholic solution of lignin when treated with a dilute solution of ferric chloride does not give any coloration but yields a greyish brown precipitate.

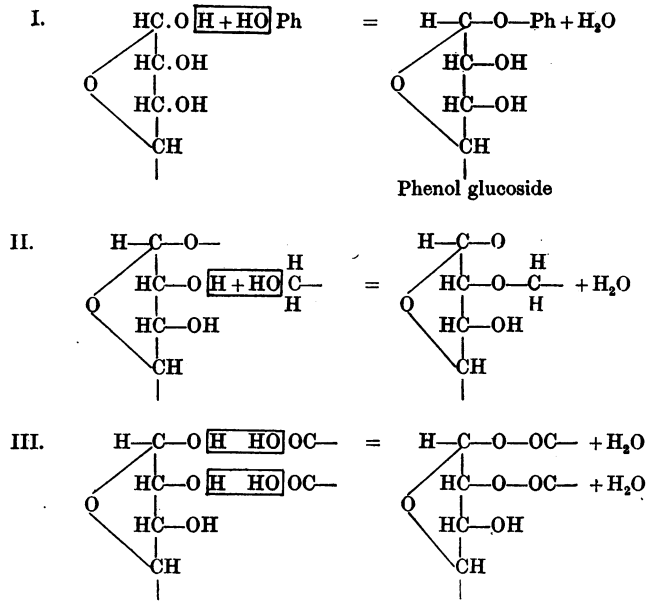
Having found that lignin is capable of forming insoluble compounds with the metals of the alkaline earths, it was of interest to examine some woods in order to ascertain whether any of the lignin was present in this form. This was determined by first removing the free lignin by extraction with alcohol, and, the calcium lignate being easily soluble in cold alcoholic hydrochloric acid, the loss in weight after treatment with the latter corresponded with the amount of lignin present as insoluble lignate. The exact procedure employed is given below.

An accurately weighed amount of wood (2·0–2·5 g.) in the form of thin shavings was extracted with 90 % alcohol in a Soxhlet extractor for 4½ hours and the loss in weight was determined after drying the extracted wood to constant weight. The extracted wood in a glass thimble was dried and then placed overnight at the ordinary temperature in a 1 % solution of hydrochloric acid in 90 % alcohol. It was then well washed free from acid with 95 % alcohol, again submitted to extraction with 90 % alcohol in a Soxhlet

extractor for 4½ hours and the loss in weight corresponding with the amount of insoluble lignate determined by drying the extracted shavings to constant weight. The results showed that lignin is not present in woods to any appreciable extent as insoluble lignate.

Constitution of Lignocellulose.

Having shown that lignocellulose is a compound in which lignin is chemically combined with cellulose, and is not an adsorption mixture of cellulose incrusted with lignin, the experiments next to be described were undertaken with a view of determining the nature of the linkage between the two constituents in lignocellulose. When we take into consideration the nature of the reactive groupings in cellulose and lignin the possibilities narrow down to two only. In the first place we have to remember that celluloses isolated from lignocelluloses, unlike the unlignified celluloses of the cotton type, always show reducing power to some extent. The latter therefore contain only carbinol groups, whilst in the former we have to recognise in addition the presence of reactive carbonyl groups. In the lignin component, on the other hand, we have highly reactive phenolic hydroxyl groups which are partly acetylated and also methylated. Next in importance is the carboxyl group in lignin which as we have seen represents a very small portion of the molecule. In addition, attached to the benzene nucleus, is an aliphatic chain containing carbinol, aldehydic and unsaturated groups, but as these are not of primary importance in determining the nature of the linkage, they will not be considered here. Essentially, therefore, we have to find out the types of possible linkages between the reactive groupings in the two components of lignocellulose. In the first place, the reactive carbonyl group in the cellulose component of lignocellulose can react with the phenolic hydroxyl group of the lignin component giving rise to a type of compound widely disseminated in nature, viz. phenolic glucosides, saponins and glucotannoids. On the other hand, if this reactive carbonyl grouping were absent, as it is from cotton cellulose, glucosidic linkage would not be possible. The hydroxyl groups in polysaccharides, which are relatively not so reactive as the carbonyl groups, can react with aliphatic and aromatic substances containing the primary alcohol group on the one hand, and the carboxyl group on the other. Substances formed by the linkage of the hydroxyl groups of the polysaccharides with the primary alcohol group of aliphatic and aromatic substances are not commonly found at all in nature. This is only what might be expected as the affinity between these two groups is by no means strong. The carboxyl groups of aliphatic and aromatic substances have on the contrary a considerable affinity for hydroxyl and carbonyl groups of polysaccharides and give rise to compounds which are frequently found in nature, *e.g.* tannins. These considerations may be graphically summarised as follows:



It is now necessary to decide between linkages I and III. The earlier workers who were against the adsorption hypothesis of lignocellulose, Erdmann and Baltzer [1873] and Cross and Bevan [1883–1895] suggested that the linkage in lignocellulose is of an ester type as in III. There are however strong reasons against the ester type of linkage in lignocellulose. In the first place a linkage of this type is only possible when one of the constituents possesses marked acidic properties. The writer has already shown that lignin does not possess strongly acidic properties. Further, lignocellulose, when submitted to hydrolytic treatment under conditions by which other esters of the polysaccharides are known to be hydrolysed readily, does not behave in the same manner. Such esters when heated with very dilute mineral acids or even water under pressure are split up readily into their components. Boiling with glacial acetic acid and treating with oxidising agents such as potassium permanganate, potassium dichromate and hydrogen peroxide are also very effective hydrolytic agents.

Lignocellulose from which free lignin was removed by extraction with alcohol was heated with 20 times its weight of water and 0·5 % sulphuric acid at 10 and 6 atmospheres respectively for 1 hour. The extract was then thoroughly washed out and the residue heated with 4 % sodium hydroxide in a boiling water-bath to dissolve the lignin produced on hydrolysis. The residue from the alkaline extract was washed thoroughly and dried. The unhydrolysed lignin was then estimated in the manner already described. The results were as follows.

The residual lignocellulose after treatment with water at 10 atmospheres for 1 hour contained 49·36 % of the total lignin, the remainder, 50·64 % having been hydrolysed into its components.

After treatment with 0·5 % sulphuric acid at 6 atmospheres for 1 hour, 67·5 % of the total lignin was hydrolysed.

On boiling lignocellulose with five times its weight of glacial acetic acid for three hours only 22·73 % was hydrolysed.

When lignocellulose was treated with small quantities of potassium permanganate there was practically no hydrolysis although with larger quantities a diminution in the lignin content was observed due to its oxidising action.

These treatments are sufficiently drastic and are known to hydrolyse readily and completely the linkage between hydroxyl and carboxyl groups in the different derivatives of polysaccharides. The glucosidic linkage is on the other hand much more resistant. All the evidence available at present points to the presence of a glucosidic linkage in lignocellulose. This is further substantiated by the fact that aromatic substances which take part in lignification migrate from one part to another in the plant as glucosides. The writer has so far searched unsuccessfully for enzymes which would be likely to bring about the hydrolysis of lignocellulose. It is possible that lignin is in combination with the reactive modification of dextrose, the glucosides of which are not split up by the usual glucosidolytic enzymes.

Cellulose as a Component of Lignocellulose.

It has already been mentioned that when lignocellulose is hydrolysed with 4 % sodium hydroxide under the conditions described for the estimation of lignin, a residue of resistant cellulose is obtained of a high degree of purity. Unlike the residue obtained from other methods of resolution of lignocellulose it possesses an exceptionally light colour, being even snow-white in some cases. Chemists and physiologists have for a long time been in doubt as to what is to be understood by pure cellulose, and until we arrive at some definite conception of the purity of cellulose, the problem of its estimation will remain empirical. The writer does not consider that any of the numerous methods which have been proposed for the estimation of cellulose are accurate as will be seen from the considerations set forth below.

Isolation of Cellulose-α. Cellulose has been held by Cross and other workers to exist in raw cellulosic materials in three forms: cellulose-α, -β, and -γ. The distinction is more or less empirical and is based on the relative resistance to the specific solvent action of strong caustic soda. Cellulose-α is not attacked by 17·5 % sodium hydroxide solution, whilst cellulose-β and -γ both pass into solution. Cellulose-β is further differentiated from cellulose-γ in that it is precipitated on acidifying the alkaline solution with acetic acid. The separation is by no means sharp and is determined very largely by the physical condition and penetrability of the material.

The approximate estimation is carried out according to the method described by Cross and Bevan [1916]. To compare the behaviour of different

cellulosic materials when treated with 4 % sodium hydroxide at 10 atmospheres for 1 hour with that of 17·5 % sodium hydroxide at the ordinary temperature as employed by Cross and Bevan, a few preliminary experiments were carried out as a result of which a sharper separation of cellulose-α, uncontaminated with other celluloses has now been effected. Three materials were selected for these experiments, viz. cotton wool, raw untreated cotton fibre and sulphite paper pulp. These were treated with 17·5 % sodium hydroxide. Cotton wool according to this method gave only 2 % of cellulose-β and -γ whilst raw untreated cotton was not found to contain either cellulose-β or -γ. These materials (1·0 g.) were next heated with 4 % sodium hydroxide (25 cc.) at 10 atmospheres for 1 hour. The results are given in the following table.

	Treatment with 17·5 % NaOH		Treatment with 4 % NaOH at 10 atmospheres	
	% cellulose-α	% cellulose-β and -γ	% cellulose-α	% cellulose-β and -γ
Cotton wool	98·01	1·99	83·00	17·00
Raw cotton	100·00	0·00	82·52	17·48
Sulphite pulp	68·50	No cellulose-β	57·20	42·80

The cellulose-α obtained after treating the cotton wool with 4 % sodium hydroxide was treated repeatedly with this reagent for 1 hour periods at 10 atmospheres to ascertain its resistance, the loss in weight being determined after each treatment.

Loss suffered by cellulose-α obtained by treating cotton wool with 4 % *sodium hydroxide at* 10 *atmospheres pressure for* 1 *hour.*

	Loss in weight %	Resistance value %
2nd treatment	5·54	94·46
3rd ,,	6·96	93·04
4th ,,	5·31	94·69
5th ,,	5·62	94·38
Average	5·86	94·14

Experiments were next carried out to ascertain whether the percentage weight lost in the above treatments was affected by varying the relative volume of 4 % sodium hydroxide solution, but no variation was observed in several experiments. These results, taken generally, show that cellulose-α suffers an average loss of 5·86 % on heating at 10 atmospheres for 1 hour with 4 % sodium hydroxide; and, after making due correction for this, the true percentage of cellulose-α in the material can easily be deduced. The results further show that treating the cellulosic material with 17·5 % sodium hydroxide does not effect a sharp separation.

The corrected percentages of cellulose-α are given in the following table.

	% cellulose-α corrected (dry)
Cotton wool	88·17
Raw cotton	87·66
Sulphite pulp	60·76

Estimation of Cellulose-α in Plant Tissues.

Cellulose-α prepared by treatment with 4 % sodium hydroxide under the conditions described is the purest type of cellulose yet isolated.

This conclusion is substantiated by the results of a critical microscopical examination of the stain-taking capacity of celluloses-α, -β and -γ prepared by the method of Cross and Bevan and cellulose-α prepared by the writer's method. It has been found that cellulose-β and -γ are stained by certain dyes which do not stain cellulose-α and yet stain the cellulose-α obtained by the method of Cross and Bevan. This shows that cellulose-α prepared under the conditions employed by Cross and Bevan is associated with cellulose-β and -γ which are not completely removed. Cellulose-α has therefore been estimated by the writer's method.

Estimation of Cellulose-α in Timbers.

	% cellulose-α corrected (dry)
White deal	44·71
Spruce	43·34
Red deal	40·80
Plane wood	34·95
Rose wood	34·50
Black wood	29·79
Box wood	28·73

Distribution of Cellulose-α and Lignin in Elm (Ulma campestris).

	Cellulose-α (corrected)		Lignin		
Parts	Moist wood %	Dry wood %	Moist wood %	Dry wood %	Moisture %
Cortex	26·40	30·54	21·28	24·66	13·68
Phloem	27·49	45·44	1·20	1·98	39·46
Sap wood	18·42	22·31	7·90	9·56	17·44
Heart wood	23·11	34·26	7·27	10·69	32·56

Estimation of Cellulose-α and Lignin in Hemp and Flax.

	Cellulose-α corrected (dry) %	Lignin (dry) %
Russian Siretz hemp	57·01	1·47
Italian hemp	50·03	0·26
Russian Rhine hemp	47·80	1·46
Polish hemp	45·47	2·80
Spanish hemp	63·52	0·36
Siberian hemp	56·72	2·50
Kentucky hemp	43·45	0·23
Russian Petchure flax	42·47	0·62
Dew-retted Belgian flax	53·33	0·95
Irish flax	52·05	1·73

Agricultural chemists have long been trying to find the factors which operate in the lodging of crops, and in this connection it was thought of interest to determine the cellulose and lignin content of straws of cereals grown under definite cultural conditions. The results are interesting and show that whenever the true cellulose content of the tissue is low, there is a marked tendency to lodging. Straw from Plot 2, receiving farmyard manure only, is interesting

compared with straws from plots receiving artificial fertilisers in that, although it contains the highest percentage of lignin, it does not make up in stiffness for the lower content of cellulose. Barley straw No. 9 obtained from a water culture contains the lowest percentage of both cellulose and lignin.

The results of these estimations together with the history of the straws are given below. The straws were obtained through the kindness of Sir John Russell from the Rothamsted Experimental Station.

Estimations of Cellulose-α and Lignin in Straws grown under different cultural conditions.

	Cellulose-α (corrected) %	Lignin %
(1) Plot 2 B	24·66	12·40
(2) „ 3	24·68	8·43
(3) „ 6	32·24	7·98
(4) „ 7	27·15	7·31
(5) „ 8	28·04	8·67
(6) „ 12	27·71	8·77
(7) „ 13	34·93	8·09
(8) „ 16	35·37	8·57
(9) „ 9	17·52	4·40
(10) Indian maize	37·06	6·55
(11) „ rice	37·99	4·88
(12) „ savannah grass	36·01	5·88
(13) „ wheat	34·41	7·81

HISTORY.

(1) Broadbalk wheat, plot 2 B, farmyard manure; almost flat in places.
(2) Broadbalk wheat, plot 3, no farmyard manure; individual stacks laid.
(3) Broadbalk wheat, plot 6, minerals plus single S/A (Sulphate of Ammonia); not laid
(4) Broadbalk wheat, plot 7, minerals plus double S/A; somewhat laid.
(5) Broadbalk wheat, plot 8, minerals plus treble S/A; very badly laid.
(6) Broadbalk wheat, plot 12, S/A, super phosphate plus Na_2SO_4; more prone to laying than plot 7; sometimes opposite holds.
(7) Broadbalk wheat, plot 13, S/A, super phosphate plus K_2SO_4; less prone to laying than plot 6; sometimes opposite holds.
(8) Broadbalk wheat, plot 16, minerals plus double N/S (Nitrate of Sodium); becoming laid.
(9) Barley straw from water culture, Serial No. 43, grown with no added SiO_2.
(10) Indian maize.
(11) Indian rice.
(12) Indian savannah grass.
(13) Indian wheat.

SUMMARY.

Lignocellulose is neither an ester-like nor an adsorption compound, as shown by the xanthate reaction. From the considerations of the reactive groupings of the constituents of lignocellulose, lignin seems to occur in chemical combination with cellulose and related polysaccharides as an aromatic glucoside.

The aromatic constituent just before lignification is not of the nature of hadromal, as shown by its negative reaction with phloroglucinol and its positive reaction with vanillin in concentrated sulphuric acid. Vanillin is the most sensitive reagent for localising the aromatic substances which are later

converted by oxidation into substances of the nature of hadromal, which combine directly with cellulose to give lignocellulose and give positive reactions with phloroglucinol and negative with vanillin.

Lignocellulose is resolved completely into its constituents by heating with 4 % sodium hydroxide at 10 atmospheres for 1 hour, without any radical decomposition of the lignin constituent, which can be precipitated by acidifying the alkaline liquid and isolated in the pure condition quantitatively by extraction with alcohol.

Lignin occurs in wood in a condition partly extractable by alcohol, but the major part is in combination with polysaccharides which can be resolved by alkali.

A reagent consisting of phosphotungstic and phosphomolybdic acids in phosphoric acid is a most sensitive reagent for estimating colorimetrically minute quantities of lignin, with which it gives a deep blue coloration in presence of sodium carbonate solution. A study of the distribution of lignin in different parts of Scotch fir by the colorimetric method shows the presence of lignin in young twigs, as well as in leaves and buds.

Lignin as isolated from wood is a brown, amorphous, faintly acidic substance with a pleasant aromatic odour, recalling vanillin in some cases, and melting at 170°. It is insoluble in water, soluble in dilute alkalis, and alcohol. Its iodine value is 139, and acid value 477. It gives insoluble salts with alkaline earth metals which do not possess a definite composition owing to the tendency to form basic salts.

When lignocellulose is resolved by the sodium hydroxide method a residue of pure resistant cellulose-α is obtained which can be estimated accurately.

The writer in conclusion feels it a great pleasure to express her sincere gratitude to Prof. A. R. Ling and Dr D. R. Nanji for their valuable criticism and for their continuous encouragement and interest in the course of these investigations. For a supply of specimens of different timbers her thanks are also due to Mr J. Harris whose practical knowledge has been of great value.

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