ACTA CHEMICA SCANDINAVICA 12 (1958) 447—452

The Lignin-Carbohydrate Linkage

BENGT O. LINDGREN

Wood Chemistry Department, Swedish Forest Products Research Laboratory, Stockholm, Sweden

The lignin materials which Björkman obtained by treatment of thoroughly milled spruce wood with neutral solvents, have been studied by electrophoretic methods. That fraction which he called *LCC* ('lignin-carbohydrate complex') could be separated into two subfractions having different electrophoretic mobilities. The slower subfraction was carbohydrate in nature and the other contained both carbohydrate and lignin materials. This latter fraction on electrophoresis in sodium hydroxide solution moved more slowly than did the lignin material which Björkman called *MWL* ('milled wood lignin'). The results obtained give support to Björkman's hypothesis that the lignin in the *LCC* material is chemically linked to a part of the hemicelluloses.

In spite of much work, the question of whether there is a lignin-hemicellulose linkage in wood is still not settled. The arguments for the existence of such a linkage have been summarized by Merewether [1].

A new approach to the study of this question has been made possible by the recent work of Björkman [2–5]. Wood, which had been milled vigorously under non-swelling conditions, was treated firstly with aqueous dioxane and then either with acetic acid-water or with dimethylformamide. By these means Björkman was able to isolate two lignin fractions.

The first, the *A* fraction (called by Björkman *MWL*), accounted for about 30 % of the wood lignin and contained only small amounts of polysaccharides. The second fraction, the *B* fraction (called by Björkman *LCC*), consisted of about one part of lignin to 3—4 parts of carbohydrates. As Björkman was unable to extract its lignin with those solvents which dissolved the *A* fraction, he concluded that the lignin in the *B* fraction was chemically linked to the hemicellulose components.

After milling a sample of a *B* fraction in toluene Björkman [4,5] extracted from it a material which was soluble in aqueous dioxane and consisted of about 90 % of lignin; such materials will be called *C* preparations. As it is known that chemical bonds can be broken during severe milling, Björkman attributed this chemical modification to a splitting of lignin-carbohydrate linkages. This experiment suggests that during the milling of the wood the *A* lignin may be formed in part, or in its entirety, from material similar to,

or identical to, that found in the *B* fraction. Björkman[4,5] also considered the possibility that new chemical linkages might be formed during the milling, but he concluded that this was unlikely in the present case.

It therefore appeared that the *B* fraction would be a most suitable material on which to study the potential existence of a lignin-carbohydrate linkage. An electrophoretic study of these lignin materials was therefore performed and is described below. The electrophoresis was mostly carried out on glass-fibre sheets. This procedure has been successfully used by Smith[6] to fractionate carbohydrate materials. The study was carried out on lignin and hemicellulose materials from Norway spruce (*Picea Abies* Karst.). The relative electrophoretic mobilities of various fractions are listed in Table 1.

Table 1. Relative electrophoretic mobilities of lignin and hemicellulose materials on glass-fibre electrophoretograms.

Material	Lignins				Hemicelluloses	
	A	*B*		*C*	Xylan [a]	Glucomannan[a]
		B_s	B_f			
Buffer	0.05 N sodium hydroxide					
Rel. mobilities	18–20	2–3	7–18	16–18	10	5
Buffer	Phosphate, pH 6, μ about 0.1					
Rel. mobilities	Insoluble	2–3	6–13	Insoluble	6–10	Insoluble

[a] Obtained from a chlorite holocellulose.

The position of lignin spots on the glass-fibre sheets could be located either by reason of their fluorescense when irradiated with UV-light, or by their reaction with *p*-anisidine-sulphuric acid at room temperature, or by use of the sensitive reaction with 5-nitroso-8-hydroxy-quinoline; this last reagent had been suggested previously by Feigl[7] as a spot test for phenols. The carbohydrates did not interfere with those lignin tests.

The positions of the carbohydrate spots were determined be treating the paper either with α-naphthol and sulphuric acid or with *p*-anisidine and sulphuric acid at 110°C. The lignin material *A* also gave a colour reaction with those reagents but its sensitivity was lower than with the carbohydrates. The possibility cannot be excluded that this coloration could be caused by the small amounts of carbohydrates shown to be present in the *A* preparations[3].

The electrophoretic examination showed that *B* contained two subfractions (Fig. 1) having different mobilities. The slower fraction will be called B_s and the other, B_f. The B_s fraction appeared to consist of carbohydrates uncontaminated by lignin. The B_f fraction, on the other hand, gave positive reactions for lignin. It gave also a colour reaction with the naphthol reagent but as lignin may also react with this reagent, this could not be considered as a specific test for carbohydrates. The B_f fraction, therefore, was isolated from an electrophoretogram and was treated with acid. The hydrolysate contained glucose, mannose and xylose. It was therefore apparent that in the B_f fraction there were present both carbohydrates and lignin.

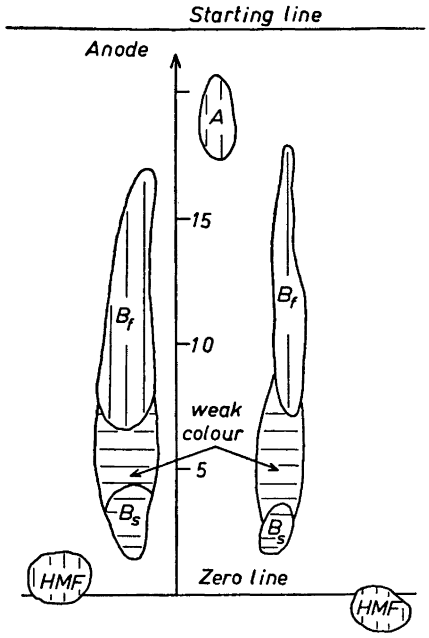

Fig. 1. Sheet electrophoretogram of *A* and *B* materials. Buffer solution, 0.05 N sodium hydroxide. Voltage, 1.8 kV. Time, 3/4 h. Developed with the *p*-anisidine spray. The area marked with vertical lines is that spot which was visible after keeping the sprayed sheet at room temperature. The area marked with horizontal lines is that spot which was visible only after heating the sprayed sheet. HMF is hydroxymethyl-furfural, which was used as reference. Ordinate = distance in cm.

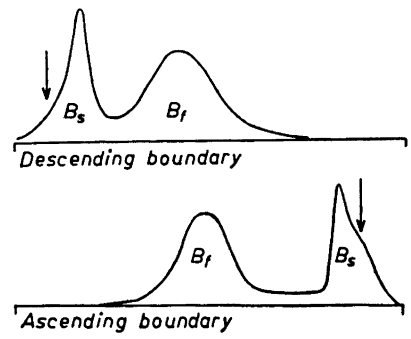

Fig. 2. Zone electrophoretic pattern for *B* material. Buffer, phosphate, pH 7.0. The arrows show the starting points.

The material in the area on the paper between the B_s and the B_f fractions gave a positive reaction for carbohydrates but the reaction was considerably weaker than was that given by the B_s fraction.

B was also fractionated by zone electrophoresis in phosphate buffer at pH 7.0. The refraction pattern (Fig. 2) showed two peaks which were evidently due to the B_s and B_f fractions. From the optical density at 280 mμ it was concluded that essentially all of the lignin was present in the B_f fraction. Assuming as a first approximation that the weights of material in the fractions are directly proportional to the areas of the peaks, the proportion between the lignin and the carbohydrate materials in B_f could be estimated as 1:1.

The fact that the B_f fraction contained both lignin and carbohydrate materials does not, of course, constitute conclusive proof of the existence of a carbohydrate-lignin linkage. It could be that the carbohydrates were free but moved at the same rate as the lignin in the B_f fraction. It was found that a xylan isolated from spruce chlorite holocellulose moved at the same rate as the B_f fraction (Table 1).

The fact that the B_f fraction moved in a neutral solution indicated that it must in some way be associated with groups which were ionised under these conditions. The mobility might be explained if the lignin component of the B_f fraction were chemically linked to a hemicellulose containing uronic acid groups. This cannot be considered as proved, however, as it may be that the ionisable groups are carboxylic groups produced during the severe milling

of the wood. It seems improbable that, during milling, such a large number of these latter groups could be formed to account for the relatively high mobility.

More satisfactory evidence of the existence of a carbohydrate-lignin linkage was obtained by a study of the *A* and *C* lignins which were not associated with carbohydrates. Those lignins moved at about the same rate on glass-fibre electrophoretograms wetted with sodium hydroxide solution (Table 1). This similarity gave further support to the suggestion that the lignin in the *A* fraction is formed during the wood milling from material of the type found in the B_f fraction.

It was also found that the electrophoretic mobility of the *A* and *C* lignin was distinctly higher than that of the B_f fraction (Fig. 1). If the material in the latter fraction is lignin chemically linked to hemicelluloses then its slower movement relative to the other lignins could easily be explained because both glucomannan and xylan move more slowly than do the *A* and *C* lignins (Table 1). It is also possible that the number of ionisable groups was higher in the *A* and *C* lignins than in the lignin in the B_f fraction. This appears to be unlikely as it was found that the proportion of phenolic groups, determined by the spectrophotometric $\Delta\varepsilon$ method [9], was about the same in the *A* lignin as in the lignin in the *B* fraction. As the relative proportion of carboxylic to phenolic groups in the lignins must be low, the former cannot contribute very much to the mobility of the fraction in alkaline solutions. It is, therefore, unlikely that the distinct difference between the lignins was due to a difference in the proportion of carboxylic groups.

In summary, four pieces of evidence may be advanced in support of the theory that a lignin-carbohydrate linkage exists in the B_f fraction *viz.*:

i) The failure to separate the B_f lignin from associated carbohydrates by electrophoresis.

ii) The mobility of the B_f fraction in neutral solution.

iii) The difference in solubility of the *A* and of the *B* fractions.

iv) The higher mobility of the *A* lignin compared to that of the B_f fraction in sodium hydroxide solution.

As mentioned earlier the first two points are not in themselves conclusive evidence proving the existence of a lignin-carbohydrate linkage. The other two points are, however, difficult to explain unless there is such a linkage between the lignin part of the B_f fraction and the hemicelluloses in that fraction.

As Björkman had found it less probable that such linkages are formed during the milling, this study supports the suggestions by Björkman, Pew, Ploetz, Richtzenhain, Traynard and others [1,10] that at least a great part of the wood lignin is linked to hemicelluloses.

EXPERIMENTAL

Lignin materials

A materials. Two samples were isolated and purified according to the normal procedure developed by Björkman [3]. Their methoxyl contents were 15.4 and 15.0 %.

B materials. Two samples were obtained from Björkman. One was isolated by extraction of the dioxane-treated, finely milled wood with acetic acid-water (1:1 v/v)[4],

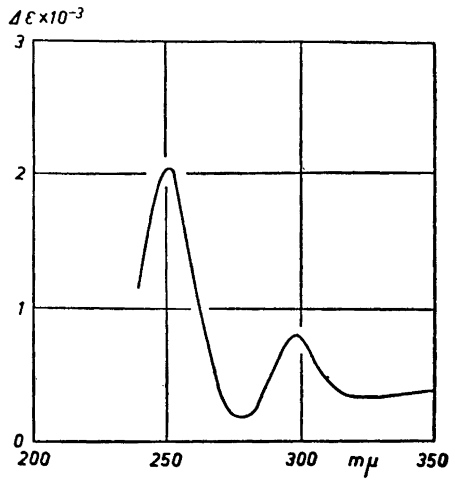

Fig. 3. $\Delta\varepsilon$ curve for *B* material. Solvents: 0.1 N sodium hydroxide and aqueous phosphate buffer, pH 7.

the other, by a corresponding extraction with dimethylformamide [4]. After purification they contained 29.5 and 27.4 % of lignin. No difference was observed in their electrophoretic mobility.

C material. One sample was obtained from Björkman [4,5]. Methoxyl content, 14.3 %.

Solutions of the materials. The lignin materials were dissolved in 0.1 N sodium hydroxide solution. By adding an equal amount of 0.1 M phosphoric acid solution a solution of *B* in a neutral buffer was obtained. By such addition to an alkaline solution of *A* and *C*, the material was precipitated.

The determination of phenolic groups in the B fractions. These determinations were carried out by the $\Delta\varepsilon$ method [9]. The two solvents used were 0.1 N aqueous sodium hydroxide and phosphate buffer (pH 7). The $\Delta\varepsilon$ curve for the *B* fraction extracted from the wood with aqueous acetic acid is shown in the Fig. 3. It shows maxima at 250 mμ, $\Delta\varepsilon$ = 2 100 and at 297 mμ, $\Delta\varepsilon$ = 780. The figures for the $\Delta\varepsilon_{max}$ of the *B* material extracted with dimetylformamide were 1 900 and 690, respectively. Published [3] values for the $\Delta\varepsilon_{max}$ of the *A* fraction are 2 100 and 800, respectively.

Sheet electrophoresis

Glass-fibre sheets. The sheets were made by C. Schleicher & Schüll, Dassel Kr. Einbeck, Germany *(Glasfaserpapier nr. 6)*.

Apparatus. The length of the strips between the electrode vessels was about 50 cm. The strips were pressed between glass plates which were cooled with water.

Procedure. Spots of the solutions (20—40 μl; conc. *ca.* 1 % for the *A* and 2 % for the *B* fractions) were put on the wet strip near the anodic side. It was found that if the solutions were spotted on to dry sheets then the materials were often irreversibly adsorbed. The strips were normally run for 3/4 h at a voltage of 1.8 kV.

The solvent movement caused by the electro-osmotic effect was in all cases stronger than the electrophoretic movement of the materials. Therefore the net effect was a movement against the cathode.

The electro-osmosis was determined by running spots of hydroxy-methyl-furfural together with the samples. This compound does not move in either alkaline or in neutral solutions and is strongly coloured by the anisidine and the naphthol reagents.

Proof of the absence of adsorption. The difference in the electrophoretic mobilities of the materials in the fractions was not due to their having been adsorbed on the glass-fibre sheets as it was shown that they followed the solvent front when they were "chromatographed" on the sheets using 0.05 N sodium hydroxide as developing solvent.

Added in proof. In later experiments when using a new lot of sheets the lignin material *A* moved considerably slower than did the front on the glass-fibre chromatograms; the

hemicelluloses (xylan, glucomannan) on the other hand followed close to the front. The *B* materials gave two spots. One moved slower than did the front and it contained both lignin and polysaccharide materials; the other followed the front and contained only polysaccharides. The lignin materials were therefore to some extent absorbed on these sheets, but if the sheets were first washed with a phosphate buffer solution (pH 6) and then with water no absorption took place.

Zone electrophoresis

Apparatus, Spinco model H. Current: 155 V, 12 mA. Time, 237 min. Buffer, phosphate, pH 7.0, $\mu = 0.1$.

From the front of the descending boundary zone 0.7 ml of the solution containing only the B_s fraction were collected. Similarly, a solution (1.5 ml) containing only the B_f fraction was obtained from the descending boundary. Both solutions gave a normal UV absorption curve for lignin. Log I_0/I were 0.78 and 18.1, respectively and the weight of lignin in those solutions was estimated as 0.01 and 0.57 mg, respectively (log ε was assumed to be 3.50 calculated on the methoxyl basis).

The ratio of the areas under the B_f peak to that under the B_s peak was about 2:1 and the B_f fraction was, therefore, calculated to contain about 2/3 of the material in the *B* fraction. As all of the lignin (1/3 of the *B* material) is present in this fraction it follows that the ratio of the weight of lignin to that of the carbohydrates is *ca.* 1:1.

Spray methods

Sensitivity. 10 µl of 0.1 N sodium hydroxide solution containing different concentrations of the lignin or of the carbohydrate materials were put on a sheet. The percentage figures reported below were the lowest concentrations at which a coloration was clearly observed after spraying.

Method for carbohydrates. Spray solution: α-naphthol (1 g) and concentrated sulphuric acid (5 ml) in *n*-butanol (100 ml). After the spraying the sheets were heated for about 2—5 min at 110°C. The carbohydrates gave blue or purple spots (sensitivity, 0.04 % for spruce xylan). The lignin gave a dark spot (sensitivity, 0.2 % for *A*).

Methods for lignin. 1) Spray solution: 5-nitroso-8-hydroxy-quinoline (1 g) in concentrated sulphuric acid (100 ml). After the spraying the sheets were kept at room temperature for some minutes. Lignin gave dark spots (sensitivity for *A*, 0.01 %). Xylan gave no coloration (the most concentrated solution tested was 1 %).

2) Spray solution: *p*-anisidine (3 g) and concentrated sulphuric acid (8 ml) in ethanol (100 ml). After the spraying the sheets were left at room temperature for 5 min. The lignin gave a yellow spot (sensitivity, 0.04 % for *A*). Xylan gave no colour (the most concentrated solution tested was 1 %). But if the sprayed sheets were heated at 110°C for 30 min the carbohydrate spots were also coloured (sensitivity 0.04 % for xylan).

Acknowledgement. The author wishes to thank Mr. B. Lindquist, Mjölkcentralen, Stockholm, for his kindness in carrying out the zone electrophoresis experiment. He is also grateful to Dr. A. Björkman for his gift of the *B* preparations used in this investigation and to Miss Marit Goliath for skilful assistence.

REFERENCES

1. Merewether, J. W. T. *Holzforschung* **11** (1957) 65.
2. Björkman, A. *Svensk Papperstidn.* **59** (1956) 477.
3. Björkman, A. and Person, B. *Svensk Papperstidn.* **60** (1957) 158.
4. Björkman, A. *Svensk Papperstidn.* **60** (1957) 243.
5. Björkman, A. *Svensk Papperstidn.* **60** (1957) 329.
6. Lewis, Bertha A. and Smith, F. *J. Am. Chem. Soc.* **79** (1957) 3929.
7. Feigl, F. *Qualitative Analysis by Spot Tests*, 3rd English Ed., New York 1947, p. 329.
8. Adler, E., Björkquist, K. J. and Häggroth, S. *Acta Chem. Scand.* **2** (1948) 93.
9. Aulin-Erdtman, G. *Svensk Papperstidn.* **57** (1954) 745.
10. Pew, J. C. *Tappi* **40** (1957) 553.

Received December 9, 1957.