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Borohydride-soluble lignin–carbohydrate complex esters of *p*-coumaric acid from the cell walls of a tropical grass

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ABSTRACT

Lignin–carbohydrate complex (LCC), solubilised by treatment of the cell walls of pangola grass (*Digitaria decumbens*) with borohydride, was fractionated from solution in methyl sulphoxide with ethanol into insoluble (LCC-P) and soluble (LCC-S) components. LCC-P had a higher concentration of carbohydrate (mainly pentoses) but less esterified *p*-coumaric acid than LCC-S. Galactose was found only in LCC-P. Treatment of LCC-P and LCC-S with alkali liberated most of the pentose and galactose in water-soluble material which, on gel filtration, gave two polysaccharide fractions from LCC-P and one oligosaccharide from LCC-S, together with *p*-coumaric acid (from LCC-P and LCC-S) and small amounts of *p*-hydroxybenzoic and vanillic acids (only from LCC-P). Of the alkali-degraded LCC-P and LCC-S, ~75% remained insoluble in neutral solution and contained only ~4% of carbohydrate. Methylation analyses established the water-soluble carbohydrates to be mainly branched arabinoxylans. The neutral-insoluble material contained (1→4)-linked glucose residues in addition to a short-chain arabinoxylan. Most of the pentosan was linked in the LCC by alkali-labile bonds that did not involve uronic acid esters. The presence of glycosidic bonds between lignin and a small proportion of arabinoxylan, and also between lignin and a glucan, was indicated.

INTRODUCTION

Treatment with borohydride dissolved ~30% of the cell walls of the stems of pangola grass (*Digitaria decumbens*) which had been degraded with a broad-spectrum commercial cellulase[1]. Of the dissolved material, ~70% was precipitated on neutralisation of the extract and identified as a lignin–carbohydrate complex (LCC). The soluble fraction consisted mainly of carbohydrate from which an arabinoxylan containing esterified ferulic acid has been isolated[2]. LCC comprised mainly polyaromatic molecules, esterified *p*-coumaric acid, and carbohydrate[1], and contained ~50% of the original lignin and 45% of the *p*-coumaric acid. Therefore, this complex represents a significant portion of the non-carbohydrate components in the cell walls. The relatively mild cleavage of the cell walls retained the esterified *p*-coumaric acid residues, and the linkages in the complex may be representative of those in the cell walls. The co-

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precipitation of carbohydrate suggests that it may be bound to lignin. The nature of these linkages is relevant to the nutritional value of the herbage and to the utilisation of the cell-wall polysaccharides in general.

The fractionation of LCC and the elucidation of the structures of associated carbohydrates is now reported.

EXPERIMENTAL

Material. — The lignin–carbohydrate complex (LCC), isolated[1] from cellulase-digested cell walls of pangola grass stem, comprised 19% of the digested cell walls and was a brown powder that was insoluble in water and dilute acid but soluble in methyl sulphoxide and alkali.

General methods. — Solutions were concentrated at $<40°$ under reduced pressure. Carbohydrate in solution was determined colorimetrically[3] and by g.l.c. of the trimethylsilyl ethers[4] after hydrolysis with 2M trifluoroacetic acid. Phenolic acids were determined by g.l.c. of their trimethylsilyl derivatives[5]. G.l.c.–m.s. of trimethylsilyl derivatives was performed on a WCOT fused-silica column (OV-101, 12 m × 0.2 mm)[1]. H.p.l.c. involved a Dextropak "Radial-PAK" cartridge (Millipore Pty) and elution with water at 1.0 mL.min^{-1}. U.v. and i.r. spectra were recorded for aqueous solutions and potassium bromide discs, respectively, unless stated otherwise.

Fractionation of LCC. — In a typical preparation, LCC (109 mg) was dissolved in methyl sulphoxide (5 mL) to give a clear dark-brown solution. Ethanol (25 mL) containing acetic acid (0.1 mL) was then added with shaking. The precipitate (LCC-P) was allowed to settle at 2° before centrifugation at 3000*g*. After washing with ethanol–acetic acid (5 × 3 mL), LCC-P was dispersed in water, freeze-dried, then dried in a vacuum over phosphorus pentaoxide (yield: 58.1 mg, 53.3%). Ethanol was evaporated from the supernatant solution, water (30 mL) containing acetic acid (0.1 mL) was added, and the precipitate was allowed to settle overnight at 2°, then centrifuged (3000*g*), washed free of methyl sulphoxide (4 × 5 mL of water–acetic acid), freeze-dried, and dried as above to give LCC-S (35.2 mg, 32.3%).

Treatment with alkali. — Solutions of LCC-P (33.8 mg) and LCC-S (25.3 mg), each in 0.5M sodium hydroxide (0.5 mL), were stored under nitrogen for 20 h at 23°, then neutralised with 7M acetic acid, and centrifuged (3000*g*). Each pellet was washed with 0.2M acetic acid (2 × 2 mL) and freeze-dried to give brown powders P(OH) (i) (25.1 mg, 74.3%) and S(OH) (i) (20.0 mg, 79.1%), respectively. The supernatant solutions, which contained P(OH) (ii) and S(OH) (ii), were concentrated to ~1 mL.

Gel filtration. — Solutions were fractionated on columns (30 × 1.6 cm) of Bio-Gel (Bio-Rad Laboratories) by elution with water at 8–12 mL.h^{-1} (1-mL fractions were monitored for carbohydrate[3] and absorbance at 280 nm). Elution of P(OH) (ii) from Bio-Gel P-2 gave a major carbohydrate fraction that was refractionated on Bio-Gel

P-30 to give P(OH) (ii) (a) (1.2 mg) and P(OH) (ii) (b) (2.4 mg). Elution of S(OH) (ii) from Bio-Gel P-150 gave a major carbohydrate fraction that was refractionated on Bio-Gel P-2 to give S(OH) (ii) (a) (0.7 mg) and S(OH) (ii) (b) (0.8 mg). All isolated fractions were freeze-dried and stored over phosphorus pentaoxide.

Methylation analysis. — P(OH) (ii) (a) and (b), S(OH) (ii) (a) and (b), P(OH) (i) (10 mg), and S(OH) (i) (10 mg) were each dissolved in dry, redistilled methyl sulphoxide in vials with Teflon-coated septa and treated with methanesulphinylmethanide[2] (1 mL) according to the method of Hakomori[6] as described by Jansson *et al.*[7]. The products were isolated as described for oligosaccharides, in order to avoid losses during dialysis. The partially methylated alditol acetates were analysed by g.l.c. on nickel columns (1.8 m × 2 mm) packed with (*a*) 0.4% of OV-225 on surface-modified Chromosorb[8], and (*b*) 3% of ECNSS on Gas-Chrom Q (100–200 mesh) at 150° and 170°, respectively. G.l.c.–m.s. involved a glass column (2.7 m × 6 mm) packed with 3% of OV-225, and the temperature program 5 min at 150° then 1°.min^{-1} to 220°. Mass spectra were recorded in the e.i. mode at 70 eV.

RESULTS AND DISCUSSION

The lignin–carbohydrate complex (LCC) was soluble in methyl sulphoxide, from which it was fractionated into soluble (LCC-S, 33%) and insoluble (LCC-P, 53%) components by the addition of ethanol. LCC-S was recovered by precipitation with water. Of the carbohydrate[3], < 10% was lost in these processes. LCC-S and LCC-P were indistinguishable by i.r. or u.v. spectroscopy. The major i.r. bands were due to hydroxyl groups (3430 cm^{-1}) and aromatic residues (1605, 1515, 1460, and 1425 cm^{-1}). The u.v. spectra contained bands with λ_{max} at 287 and 316 nm of equal intensity. The u.v. spectra were probably largely due to esterified *p*-coumaric acid, which is present in the crude complex[1]. LCC-P had almost twice the carbohydrate content of LCC-S (Table I) and the ratio of pentoses to xylitol was 50% greater in the former. These findings suggested that the avarage molecular size of the carbohydrate was greater in LCC-P, which, together with its higher content of carbohydrate, was primarily responsible for its precipitation with ethanol. LCC-S had higher levels of *p*-coumarate than LCC-P which,

TABLE I

Phenolic acid and monosaccharide composition of lignin-carbohydrate complexes (LCC) fractionated from methyl sulphoxide solution by ethanol precipitation

Complex	*tCa*[a]	*Ara*	*Xyl*	*Xylitol*	*Gal*	*Glc*	*Carbohydrate*	*tCA*
	(Relative molar %)						*(% LCC)*	
LCC-P[b]	17.0	17.6	52.4	3.1	6.0	3.9	18.8	4.1
LCC-S[c]	33.5	14.6	44.4	4.3	—	3.2	11.0	6.0

[a] *trans-p*-Coumaric acid. [b] Precipitated with ethanol. [c] Recovered from the supernatant solution by precipitation with water.

in combination with its lower content of carbohydrate, produced a ratio of phenolic acid to pentoses over twice that of LCC-P. These results suggest that *p*-coumaric acid may be linked to non-carbohydrate residues in LCC rather than to arabinofuranose residues[9,10]. Galactose was present only in LCC-P.

Treatment of LCC-P and LCC-S with alkali cleaved >80% of the carbohydrate and, on neutralisation, 74% and 79%, respectively, of each material was recovered as an insoluble precipitate that contained ~4% of carbohydrate. Gel filtration separated much of the alkali-soluble u.v.-absorbing substances from the carbohydrates, and >90% of the material was recovered.

Most of the carbohydrate in the neutralised solution [P(OH) (ii)] from alkali-treated LCC-P was eluted from Bio-Gel P-2 (Fig. 1) in the exclusion volume (fraction 1, 81% carbohydrate). The remainder (fraction 2, 38% carbohydrate) appeared to comprise a range of oligomers and was not investigated further. U.v.-absorbing material was collected in later fractions 3 and 4 (Fig. 1, fraction 5 not shown). The u.v. spectra of fractions 1 and 3 (Fig. 2) suggested the presence of non-conjugated aromatic residues, whereas fractions 4 and 5 had spectra similar to that of *p*-coumaric acid. G.l.c.–m.s. identified the *cis*- and *trans*-isomers of *p*-coumaric acid as minor components of fraction 3, but the bulk of the phenolic acid (90%) was collected in fraction 5. *p*-Hydroxybenzoic acid and vanillic acid were major components in fraction 4. Elution of fraction 1 (Fig. 1) from Bio-Gel P-30 gave carbohydrate material of high and low molecular weight (Fig. 3). The u.v.-absorbing substances that had co-eluted with fraction 1 (Fig. 1) had a profile that still followed the general pattern of carbohydrate elution from Bio-Gel P-30 (Fig. 3), which may suggest covalent bonds between the two polymers. The u.v. spectra of fractions 1 and 2 (Fig. 3) each had a single maximum (λ_{max} 272 and 274 nm) similar to that of the starting material.

Fractionation of the alkali-labile material from LCC-S on Bio-Gel P-150 separated the major portion of the *p*-coumaric acid (Fig. 4). The bulk of the carbohydrate was

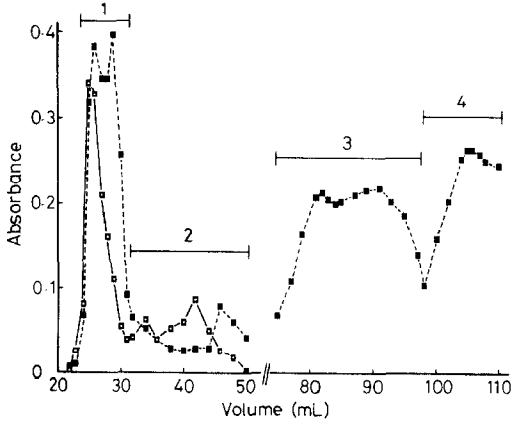

Fig. 1. Fractionation on Bio-Gel P-2 of the water-soluble products liberated with alkali from LCC-P: ——, carbohydrate; ----, A_{280}; V_0 = 27 mL, V_t = 53 mL.

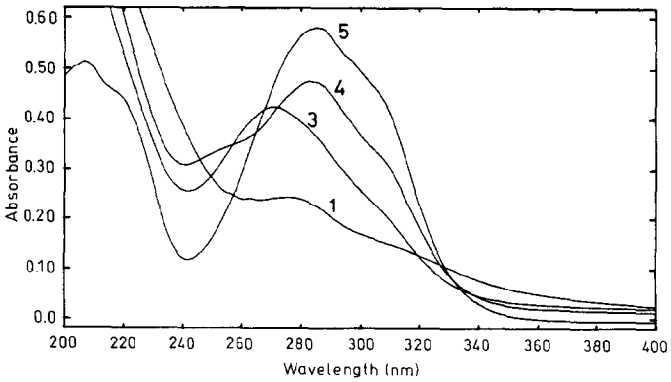

Fig. 2. U.v. spectra of the fractions (Fig. 1) from the degradation of LCC-P with alkali [fraction 5 is the bulked eluate (62 mL) collected after fraction 4].

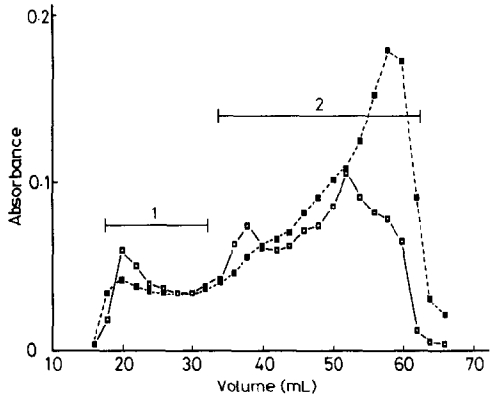

Fig. 3. Refractionation of fraction 1 (Fig. 1) on Bio-Gel P-30: ——, carbohydrate; ----, A_{280}; V_0 = 20 mL, V_1 = 69 mL.

eluted close to the inclusion volume (fraction 1, Fig. 4). The u.v. spectra of fractions 1 and 2 were different (Fig. 5), and g.l.c.–m.s. confirmed that fraction 2 was mainly *trans-p*-coumaric acid. Only a small proportion of *cis*- and *trans-p*-coumaric acid was identified in fraction 1, in agreement with the spectrum which suggested that the u.v. absorbance was largely due to unconjugated aromatic residues. When fraction 1 (Fig. 4) was refractionated on Bio-Gel P-2 (Fig. 6), ~ 70% of the carbohydrate was present in a reasonably narrow peak of low molecular weight close to the inclusion volume (fraction 2, Fig. 6). Only a small amount of u.v.-absorbing material co-eluted with the carbohydrate of higher molecular weight, in contrast to the alkali-soluble products from LCC-P (Fig. 1). The u.v. spectra (Fig. 7) indicated that fractions 1, 2, and 4 (Fig. 6) contained mainly unconjugated aromatic residues, and fraction 5 included the small amount of the phenolic acid which did not separate from fraction 1 (Fig. 4). G.l.c.

supported the spectral data and also indentified sucrose in fraction 4 (Fig. 6). H.p.l.c. (Fig. 8) confirmed the results of gel filtration which indicated that fraction 2 (Fig. 6) comprised oligosaccharides. There was no u.v. absorbance associated with the components detected, of which 75% was eluted as a single peak (T 17.3 min, Fig. 8).

Methylation analysis of the products from the degradation of LCC-P and LCC-S with alkali indicated qualitative and quantitative differences in the carbohydrates (Table II). The neutral-insoluble residues [P(OH) (i) and S(OH) (i)] contained 52 and 37%, respectively, of glucose, which was absent from the alkali-soluble material. Identification of 2,3,4,6-Me_4-Glc and 2,3,6-Me_3-Glc, and negligible 2,4,6-Me_3-Glc, suggested that the glucose originated from a (1→4)-linked glucan. The remainder of the carbohydrate appeared to be a branched arabinoxylan of short average chain-length. Most of the galactose in LCC-P (Table I) was identified as end-groups (Table II) and was a major constituent of the high-molecular-weight fraction P(OH) (ii) (a). This

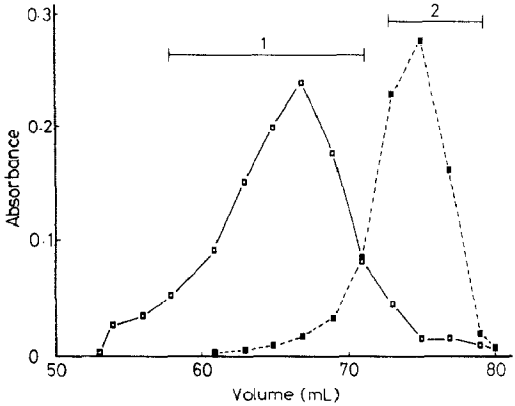

Fig. 4. Fractionation on Bio-Gel P-150 of the water-soluble products liberated with alkali from LCC-S: ——, carbohydrate; ----, A_{280}; V_0 = 16 mL, V_1 = 70 mL.

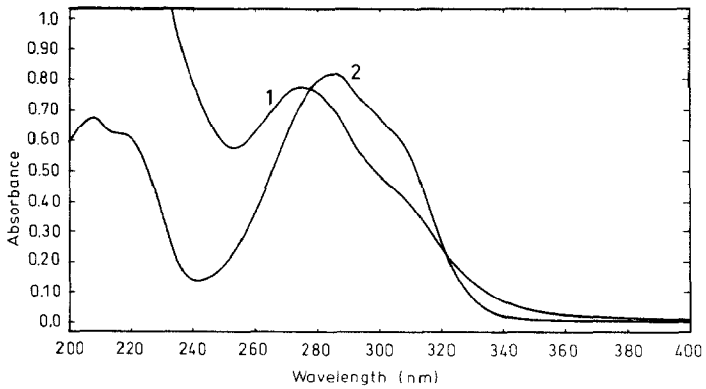

Fig. 5. U.v. spectra of fractions 1 and 2 from Fig.4.

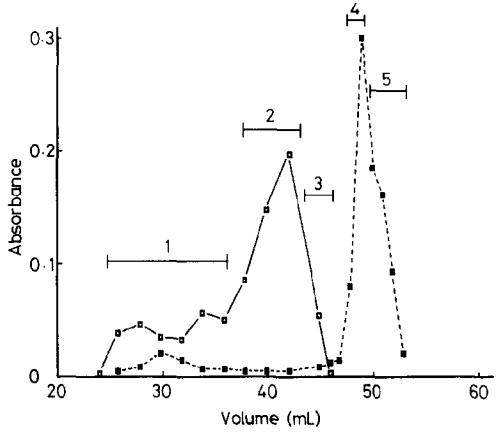

Fig. 6. Refractionation of fraction 1 (Fig. 4) on Bio-Gel P-2: ——, carbohydrate; ----, A_{280}; V_0 = 27 mL, V_1 = 53 mL.

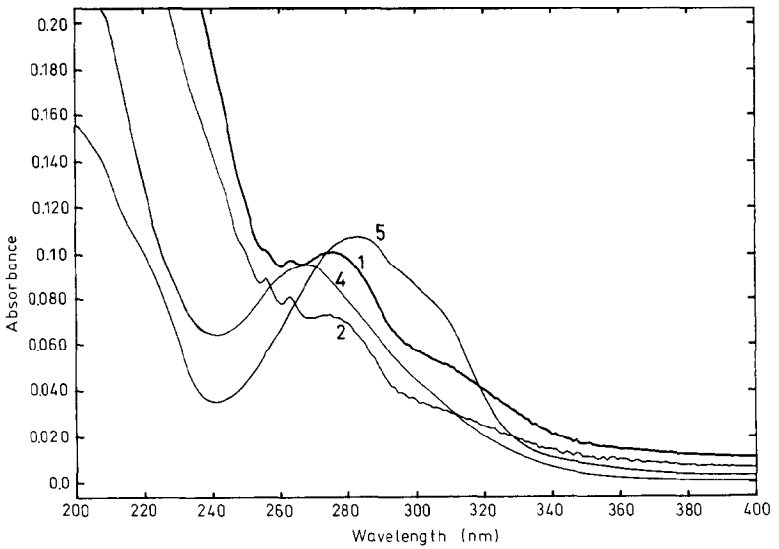

Fig. 7. U.v. spectra of fractions in Fig. 6.

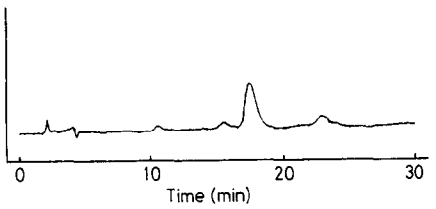

Fig. 8. H.p.l.c. of fraction 2 (Fig. 6). Peaks were detected with a differential refractometer.

TABLE II

Methylation analyses of the carbohydrates obtained after treatment of LCC-P and LCC-S with alkali and fractionation by gel filtration (peak area %)

Compound[a]	*P(OH) (i)*[b]	*P(OH) (ii)*		*S(OH) (i)*	*S(OH) (ii)*
		(a)[c]	*(b)*[c]		*(b)*[d]
2,3,5-Me_3-Ara	6.1	10.8	9.7	10.0	26.8
2,3,4-Me_3-Xyl	3.1	4.1	10.2	8.4	21.9
3,5-Me_2-Ara	1.8	7.5	2.3	–	3.5
2,5-Me_2-Ara	–	9.0	1.9	–	–
2,3,4,6-Me_4-Glc	6.7	–	–	9.7	–
2,3,4,6-Me_4-Gal	8.3	20.5	2.7	–	–
2,4-Me_2-Xyl	–	–	–	–	6.4
2,3-Me_2-Xyl[e]	15.4	22.9	44.6	17.5	12.1
2,3,6-Me_3-Glc	45.3	–	–	26.9	–
2-Me-Xyl	13.3[f]	25.2[f]	28.6[f]	27.6[f]	29.1
Xylitol	–	+	+	–	–

[a] 2,3,5-Me_3-Ara = 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-L-arabinitol, *etc.* [b] See Experimental. [c] (a) is fraction 1 and (b) is fraction 2 from Bio-Gel P-30 (Fig. 3). [d] (b) is fraction 2 from Bio-Gel P-2 (Fig. 6). [e] 2,3-Me_2-Xyl, rather than 3,4-Me_2-Xyl, is assumed because the main structural feature of plant xylan is known to be a (1→4) linkage. [f] Includes 3-Me-Xyl as a minor component.

polysaccharide has structural similarities to a neutral, water-soluble feruloylated arabinoxylan isolated from the same cell walls[2]. The lower-molecular-weight fraction P(OH) (ii) (b) was an arabinoxylan with a lower degree of branching. A small proportion (not measured) of xylitol was identified among the products of methylation of P(OH) (ii) (a) and (b), but not in those of P(OH) (i) or any fraction from LCC-S. Methylation revealed fraction S(OH) (ii) (b) to consist largely of a highly branched pentose oligomer. However, an additional component in the methylation products was detected in a proportion slightly greater than that of 2,3-Me_3-Xyl, and with a retention time in g.l.c. much shorter than that of 2,3,5-Me_3-Ara. The compound was tentatively identified from the mass spectrum as 2-*O*-acetyl-1,3,4,5-tetra-*O*-methyl-D-xylitol. Based on the proportions of methylated fragments (Table II), the principal constituent oligomer could be a nonasaccharide that comprised five (1→4)-linked xylose residues with single branches of one xylose and two arabinose residues and a 4-linked xylitol terminus.

Previous studies of LCCs in herbage have generally used water-soluble preparations that were extracted from whole[11,12] or enzymically degraded[5] plant cell walls with selected solvents, or isolated from bovine rumen fluid[13]. However, the LCC in the present work was water-insoluble, probably as a result of the low ratio of carbohydrate to hydrophobic residues brought about by treatment of the cell walls with "cellulase"[1]. The presence of lignin-like aromatic residues as major components of LCC-P, LCC-S, P(OH) (i), and S(OH) (i) has been established by pyrolysis–mass spectrometry[1,14]. The average molecular size of the carbohydrates associated with these residues, as judged by gel filtration, was rather small. In particular, the arabinoxylan oligomer of S(OH) (ii) (b) and the glucans (assuming a hexosan origin for the glucose residues) in P(OH) (i) and

S(OH) (i) (average d.p. 6.8 and 2.8, respectively) would have been expected to be highly water-soluble. The lack of solubility of these oligosaccharides may reflect covalent lignin–carbohydrate bonds, which seems more likely than formation of a gel matrix by molecular entanglement[15]. Most of the arabinoxylan was liberated during treatment of LCC-P and LCC-S with dilute alkali, whereas all the glucose appeared to be linked by alkali-stable bonds. Since LCC-P and LCC-S were fractionated from material which had been liberated from the cell walls with borohydride[1], esterified uronic acid would be absent. Therefore, the alkali-labile linkages to the pentosan possibly involved esterification of a carboxyl group bound to, or conjugated with, an aromatic residue. Such linkages would survive treatment with borohydride[1,2,16]. Other postulated lignin–carbohydrate bonds[17], such as benzyl ether linkages (with no free *p*-hydroxyl group)[18,19], free-radical coupling, or phenyl glycosidic bonds, would have survived the treatment with alkali. The location of the pentose hydoxyl groups involved in the alkali-labile linkages cannot be specified. However, for S(OH) (ii) (b) (Table II), C-1 cannot be involved since the reducing terminal residue was xylitol. Conversely, the results of methylation suggest that the alkali-stable linkages binding the carbohydrate in P(OH) (i) and S(OH) (i) (Table II) are probably glycosidic. Sugar benzyl ethers or linkages formed by radical coupling have been proposed as the most likely lignin–carbohydrate bonds in terms of phenol oxidation theory, whereas glycosidic linkages were considered to be improbable[19]. However, glycosidic linkages between carbohydrate and lignin have been proposed for LCC material isolated from wheat straw[17] and rumen fluid[20], although direct glycosidation to a phenyl hydroxyl group in macromolecular lignin has yet to be proved.

The detection of sucrose in the alkali-soluble products from LCC-S may reflect contamination; sucrose has been identified in solutions obtained after the degradation with alkali of cell walls of two tropical grasses, but was not detected in those from temperate species rye grass or barley[21]. The identification of *p*-hydroxybenzoic and vanillic acids (0.02 and 0.04%, respectively, of LCC-P) only in fraction 4 (Fig. 1) suggested that some fractionation of the aromatic components had occurred during the precipitation with ethanol. Previously, only traces of these acids have been reported among the products of degradation with alkali of graminaceous cell walls[22,23]. Their location found here and their alkali lability suggest a cross-linking function in particular lignified tissues. The galactose, found only in LCC-P and mainly as end groups, may have a specific location in the cell-wall structure.

The LCCs studied may be analogous to "milled wood lignins" (MWL)[24] isolated by Higuchi *et al.*[25] from several grasses. They recorded u.v. spectra and levels of esterified *p*-coumaric acid similar to those found in the present work. The i.r. spectra of the LCCs, as illustrated for LCC-S (Fig. 9), were also similar to those of MWL[26], with characteristics of both hard and soft woods. The absorbance at 1700 cm^{-1} was attributed to esterified *trans-p*-coumaric acid, since it had survived the extraction with borohydride but was eliminated by the treatment with alkali. MWLs from conifers are considered to originate largely from the middle lamella[17], as are hemicelluloses that contain galactose[17]. If this is true for the cell walls of grass stems, borohydride will be a

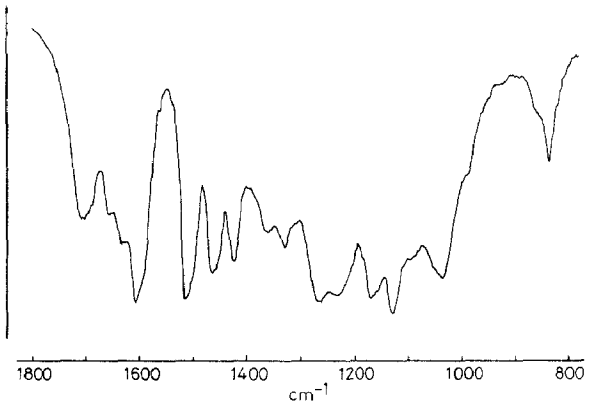

Fig. 9. I.r. spectrum of LCC-S (1.1 mg in 128 mg of KBr) (see Table I).

useful reagent for the isolation of the carbohydrate–aromatic complexes responsible for intercellular binding. Galactose-rich fractions were found in LCC P(OH) (i) and P(OH) (ii) (a) (Table II); a water-soluble polysaccharide rich in galactose has been extracted from pangola cell walls with borohydride[2].

The high ratio of *p*-coumaric acid to arabinoxylan in the LCCs suggests esterification of the phenolic acid to non-carbohydrate hydroxyl groups, as proposed for MWL[25] and native lignins[27] of several grasses. However, the mode of linkage has not been proved. It is possible that some of the *p*-coumaric acid is esterified to carbohydrate[9,10], and that the phenolic hydroxyl group of some molecules may also participate in ether linkages [28].

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