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CHEMICAL STRUCTURES IN A LIGNIN–CARBOHYDRATE COMPLEX ISOLATED FROM THE BOVINE RUMEN

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ABSTRACT

The soluble, lignin–carbohydrate complex (LCC) from the rumen fluid of steers fed a diet of pure spear grass (*Heteropogon contortus*) has been purified by gel filtration. The purified LCC contained 7.4% of carbohydrate which, on hydrolysis, gave D-glucose, D-xylose, L-arabinose, L-rhamnose, and traces of D-galactose and D-mannose. The structure of the LCC was examined by methylation analysis, using g.l.c.–m.s. for the unequivocal classification of the sugar derivatives. D-Glucose, D-xylose, and L-rhamnose were shown to be glycosidically linked to lignin. Some of the D-glucosyl residues carry other (1→4)-linked D-glucose units, and some of the D-xylosyl residues bear other (1→4)-linked D-xylose units and (1→3)-linked L-arabinofuranosyl groups. The major carbohydrate component is a single D-gluco-pyranosyl group. The LCC was subjected to various chemical treatments in an investigation of the chemical nature of the bonding between lignin and the carbohydrates. D-Glucose could be enzymically hydrolyzed from the LCC, but only with a very high concentration of β-D-glucosidase. The presence of lignin in rumen LCC has been confirmed by nitrobenzene oxidation, vanillin and syringaldehyde being identified by g.l.c.–m.s. as oxidation products from both the original spear grass and the LCC.

INTRODUCTION

The soluble, lignin–carbohydrate complexes (LCC's) originating from several types of starting material have previously been studied. They have been isolated by the mechanical disintegration of plant tissue, followed by solvent extraction[1,2], by the selective extraction of intact, plant cell-walls[3], and from the products resulting from the microbial degradation of plant material[4,5]. Starting materials isolated by modified, Björkman methods[6–8] have been favored by most workers. Koshijima and

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co-workers[1,9-13] studied in detail the Björkman LCC obtained from pine wood. They used extensive purification procedures, including fractional precipitation and gel filtration, and found evidence for the existence of complexes having a range of molecular weights and of lignin:carbohydrate ratios. They also noted, between the complexes and Sephadex[11,12], a pronounced interaction that normally prevented the determination of molecular weight by gel filtration. Similar interaction was also noted between LCC from the bovine rumen and Sephadex[4,5].

The extensive data now available provide convincing evidence that chemical bonds exist between lignin and carbohydrate. There is, however, still considerable speculation[2,12,14-19] as to the nature of the bonds. The "release" of LCC from plant cell-walls by microbial action in the bovine rumen provides a novel approach to study of the complexes. The rumen LCC has been liberated from the plant cell-wall, rendered water-soluble by purely enzymic changes, and not been subjected to the relatively harsh mechanical or chemical treatments required in most of the alternative methods. It is anticipated, therefore, that chemical changes to the lignin–carbohydrate linkages will be minimal. Furthermore, we had already shown[5] that this LCC contains a large proportion of the lignin present in the grass, so that its structure must be very relevant to an understanding of the chemical nature of the lignin–carbohydrate linkages in the cell wall.

In the present study, the "crude" rumen LCC previously isolated was purified, and the structure of the carbohydrate moiety determined. A preliminary investigation of the nature of the various types of bonding between the carbohydrate and lignin by using chemical and enzymic methods is also described.

RESULTS AND DISCUSSION

Initial attempts to methylate crude LCC isolated from rumen fluid centrifuged at 20,000*g* were unsuccessful, and so it was decided to purify the LCC by gel filtration. For comparison, several different types of gel were used. An experiment (see Fig. 1) using Bio-Gel P-30 showed two peaks, in agreement with results published earlier[4] from a similar experiment using Sephadex G-100. The major difference between the two gels is that the Bio-Gel P-30 achieves less resolution of peak 1 from peak 2; however, the carbohydrate and lignin profiles (see Fig. 1) confirmed, on the basis of arguments detailed earlier[4,5], the presence of covalent bonding between the carbohydrate and lignin components in LCC.

Further gel-filtration of crude LCC (100 mg) on Sepharose CL-2B (see Fig. 2) again showed two major peaks. Selected sub-fractions were combined to give three major fractions (I, II, and III). Fraction I, which was opalescent, was concentrated and ultracentrifuged, resulting in a pellet (21 mg, freeze-dried weight) and a supernatant liquor (affording 12 mg of freeze-dried residue). Particulate-free fractions II and III were concentrated and freeze-dried, and yielded 19 and 57 mg, respectively. The pellet from fraction I was found by electron microscopy to consist of cell debris

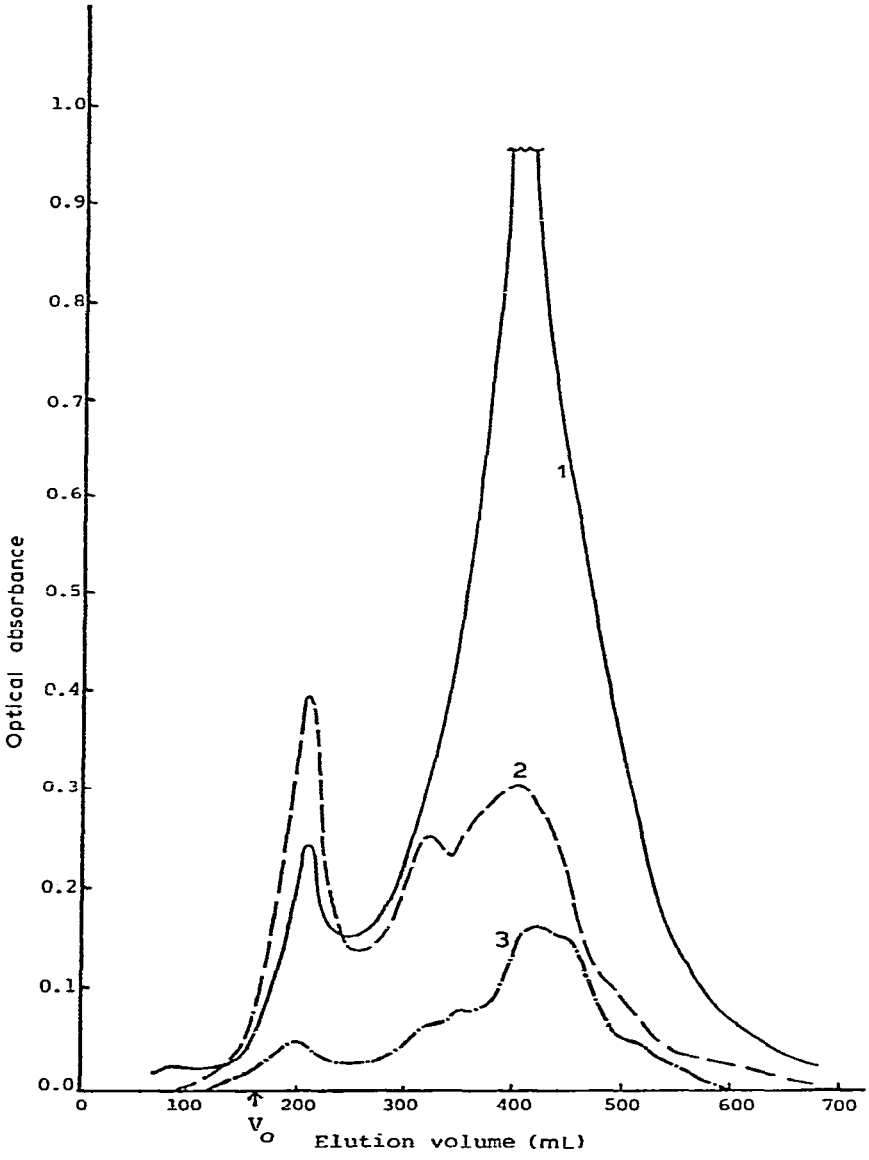

Fig. 1. Gel filtration of crude LCC on Bio-Gel P-30. [Key: 1, A_{280}; 2, phenol–sulfuric acid (480 nm); and 3, A_{455}.]

and virus particles; consequently, in all subsequent repetitions of this procedure, solutions were ultracentrifuged prior to application to columns (see *e.g.*, Fig. 3).

From the separations observed in the fractionation experiments just discussed, the relative absorbances at 280 and 455 nm, and compositional carbohydrate analyses across the peak shown in Fig. 2, it was concluded that further useful resolution within peak 2 would be very difficult, and that the LCC recovered from the second peak

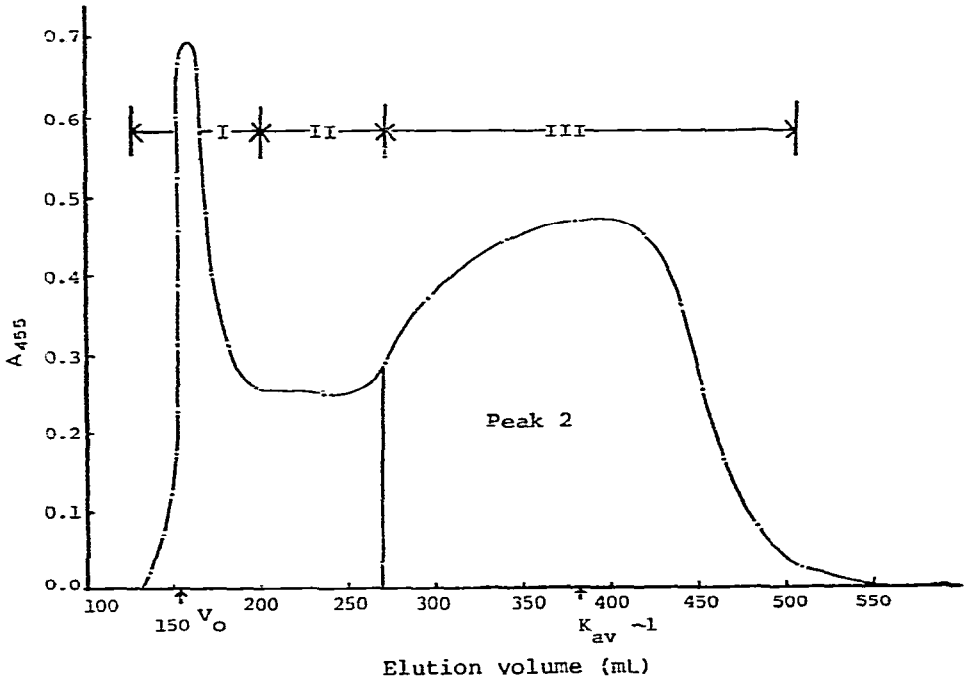

Fig. 2. Gel filtration of crude LCC on Sepharose CL-2B.

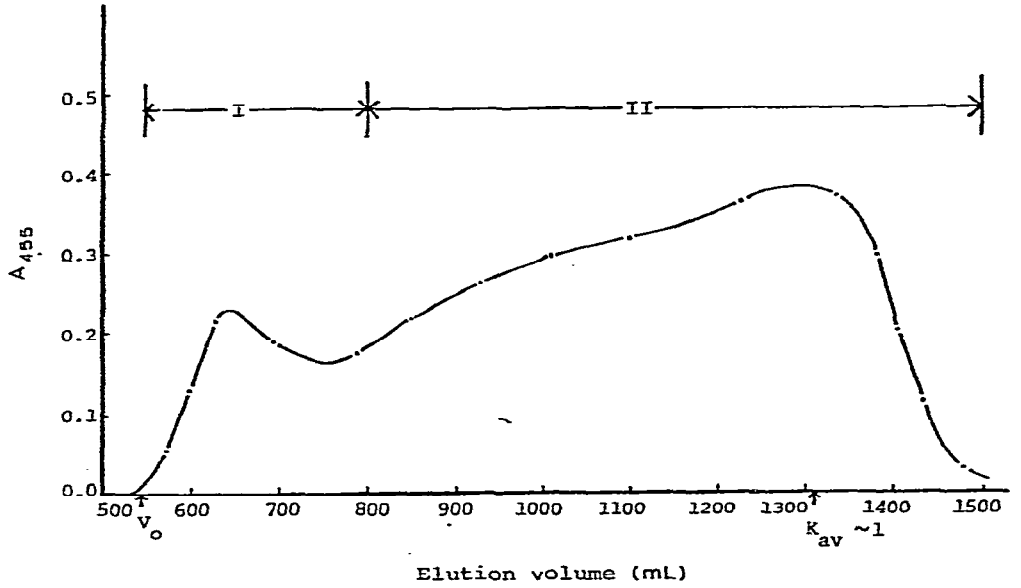

Fig. 3. Preparative, gel filtration of crude LCC on Sepharose CL-2B. [Column K (50–100 mesh); sample ultracentrifuged at 140,000*g* for 3 h at 4°.]

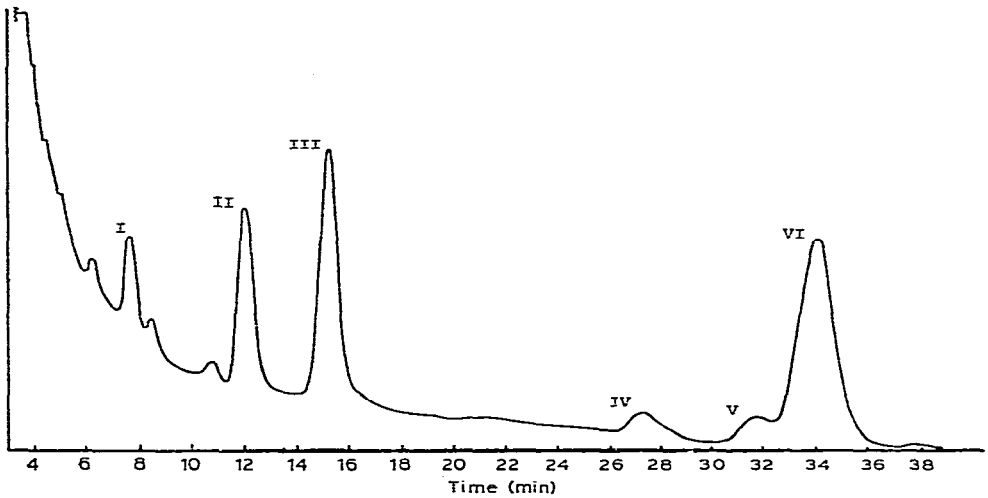

Fig. 4. G.l.c. of the acetylated alditols derived from rumen LCC. [Column 4 at 205°. Key: parent sugar: I, L-rhamnose; II, L-arabinose; III, D-xylose; IV, D-mannose; V, D-galactose; and VI, D-glucose.]

could be regarded as a representative sample of pure, rumen LCC, the broad shape being indicative[5] of the range of molecular types present in the LCC. It is, therefore, important to emphasize that the succeeding, structural data on LCC refer to an average molecular species.

The carbohydrate content of crude LCC, determined by application of the phenol–sulfuric acid method to an aqueous solution, was ~20%, but that value was lowered to 7.4% in purified LCC. The "Klason lignin" content of crude LCC was 52%. However, the true lignin is almost certainly greater than "Klason lignin", as a significant proportion of the lignin may be lost by dissolution during the analysis[17,20].

TABLE I

QUANTITATIVE DETERMINATION OF THE CONSTITUENT SUGARS OF RUMEN LCC, S.G., AND S.G. HOLOCELLULOSE BY G.L.C. OF THE ALDITOL ACETATES

Parent sugar	R_G[a]	*Molar ratios*		
		Rumen LCC	*S.g.*	*S.g. holocellulose*
L-Rhamnose	0.22	0.2[b]	0.1[b]	trace
L-Arabinose	0.35	0.41	0.16	0.17
D-Xylose	0.45	0.72	0.98	0.97
D-Mannose	0.80	0.09	trace	n.d.[c]
D-Galactose	0.93	0.11	trace	trace
D-Glucose	**1.00**	**1.00**	**1.00**	**1.00**

[a]R_G = retention time relative to that of D-glucitol hexaacetate as unity. [b]Lower precision due to overlap and solvent tailing. [c]N.d. not detected.

The material unaccounted for can only be explained by the presence of "acid-soluble", lignin fragments, as we have not been able to find any experimental evidence suggesting that there were significant constituents other than lignin and carbohydrate in the LCC. The 7.4% of carbohydrate determined by the phenol–sulfuric acid method is in approximate agreement with subsequent yields from the methylation analysis.

A typical chromatogram from the compositional, monosaccharide analysis of pure LCC is shown in Fig. 4. The results are given in Table I, and show that the carbohydrate component of LCC consists of the same residues as original spear grass (s.g.) and s.g. holocellulose. The results agree in general with those from earlier work[4,21] on s.g., but, in addition, we now report, for the first time, the presence in this grass of traces of D-mannose and significant proportions of L-rhamnose (unequivocally identified by g.l.c.–m.s.). L-Rhamnose is found mostly in pectic substances, but it has also been reported in xylans isolated from legumes[22,23], and in LCC (7–16% of L-rhamnose) extracted from ball-milled, brome-grass hay and lucerne with 9:1 1,4-dioxane–water[24]. The relative proportions of L-rhamnose in the rumen LCC, compared with those for s.g. and s.g. holocellulose reported here, show that the L-rhamnose residues become concentrated in the LCC as it is formed in the rumen, *i.e.*, it is particularly associated with lignin in the cell wall. Pectic substances would have been removed during formation of the LCC in the rumen.

In the methylation analysis of polysaccharides, a necessary prerequisite for a successful methylation reaction is the complete dissolution of the substrate. Some

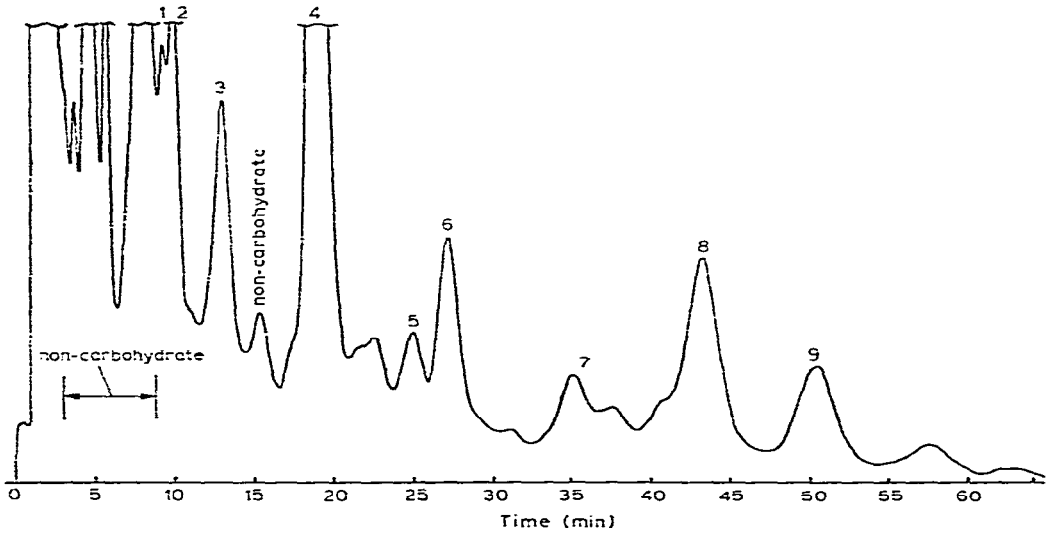

Fig. 5. G.l.c. of the acetates of partially methylated alditols derived from the methylation analysis of rumen LCC. [Column 4 at 184°. Key: 1, 1,5-di-*O*-acetyl-6-deoxy-2,3,4-tri-*O*-methyl-L-mannitol; 2, 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-L-arabinitol; 3, 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-D-xylitol; 4, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol; 5, 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-D-xylitol; 6, 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-D-xylitol; 7, other tri-*O*-methylhexitols; 8, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol; and 9, 1,3,4,5-tetra-*O*-acetyl-2-*O*-methyl-D-xylitol.]

TABLE II

METHYLATION ANALYSIS OF RUMEN LCC

Peak No.	*Derivative*	R^{a}_{TMG}		*Molar ratios*
		This work	*Literature*[25]	
1	1,5-di-*O*-acetyl-6-deoxy-2,3,4-tri-*O*-methyl-L-mannitol	0.49	0.46	0.13
2	2,3,5-tri-*O*-methyl-L-arabinitol	0.52	0.48	0.23
3	2,3,4-tri-*O*-methyl-D-xylitol	0.69	0.68	0.23
4	2,3,4,6-tetra-*O*-methyl-D-glucitol	**1.00**	**1.00**	**1.00**
5	2,4-di-*O*-methyl-D-xylitol	1.32	1.34	0.08
6	2,3-di-*O*-methyl-D-xylitol	1.44	1.54	0.21
7	other *tri-O*-methylhexitols	1.86	1.98	0.05
8	2,3,6-tri-*O*-methyl-D-glucitol	2.29	2.50	0.22
9	2-*O*-methyl-D-xylitol	2.66	2.92	0.15

[a]Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol as unity.

difficulty was experienced because of the inherently low solubility of LCC; however, a final concentration of ~0.13% of LCC in dry dimethyl sulfoxide gave consistently reliable methylation results. A typical chromatogram from the completed methylation analysis is shown in Fig. 5, and the results obtained are given in Table II. The partially methylated alditol acetates were tentatively identified by their retention times in g.l.c. and by mixture studies with reference compounds, and were unambiguously classified by g.l.c.–m.s. In addition, g.l.c.–m.s. was used in order to confirm that the leading peaks in the traces were those of non-carbohydrate substances most probably derived from lignin. ECNSS-S was used as the stationary phase (column *4*), and it was noted that the values of the retention time, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (R_{TMG}), were higher than the literature values[25] (using ECNSS-M) when $R_{TMG} < 1$, and lower when $R_{TMG} > 1$ (see Table II). Throughout the analysis, precautions were taken to minimize the loss of the relatively volatile tri-*O*-methylpentoses. A check on the approximate percentage recovery of carbohydrate from the total methylation procedure was made by using samples of authentic polysaccharide and LCC, and the values ranged from 40 to 62%.

Knowing the identity of the methylated sugars and their relative amounts, a proposed, average structure for the carbohydrate components of rumen LCC was formulated (see Fig. 6). (From the known stereochemistry of the source cell-wall polysaccharide constituents, respective D and L forms are assumed.) The attachment of the carbohydrate fragments to the lignin can only be glycosidic, because of the nature of the products of methylation analysis. Our results give no information on the nature of the lignin part of the complex, or on the position of attachment of the sugars to the lignin, but this appears to be the first conclusive evidence of the presence and predominance of glycosidic linkages between lignin and carbohydrate. It has often been postulated that carbohydrate is bound to lignin by ether linkages, and our

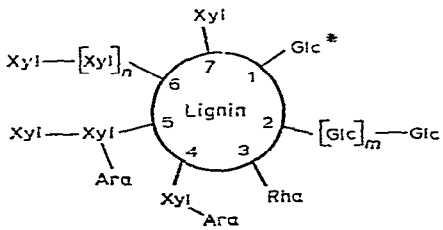

Fig. 6. Proposed, average structure of rumen LCC, showing attached carbohydrate residues. [The asterisk indicates the major component; *m* and *n* are assumed to be low numbers; products identified from methylation analysis: 1, 2,3,4,6-tetra-*O*-methyl-D-glucitol; 2, 2,3,6-tri-*O*-methyl-D-glucitol and 2,3,4,6-tetra-*O*-methyl-D-glucitol; 3, 2,3,4-tri-*O*-methyl-L-rhamnitol; 4, 2,4-di-*O*-methyl-D-xylitol and 2,3,5-tri-*O*-methyl-L-arabinitol; 5, 2-*O*-methyl-D-xylitol, 2,3,5-tri-*O*-methyl-L-arabinitol, and 2,3,4-tri-*O*-methyl-D-xylitol; 6, 2,3-di-*O*-methyl-D-xylitol and 2,3,4-tri-*O*-methyl-D-xylitol; and 7, 2,3,4-tri-*O*-methyl-D-xylitol.]

results do not exclude this additional possibility. Any such linkages would, however, have to survive the hydrolysis step in the methylation analysis, as otherwise, they would have been shown by the results given in Table II. If such ether-linked sugars are present, we would include them within the circle which diagramatically represents lignin in Fig. 6, and such sugars, if present, might carry some of the glycosidically linked sugars that we have detected. On the basis of our results, it is, however, quite certain that the major portion of the acid-hydrolyzable carbohydrate in the LCC is glycosidically linked to lignin, as shown in Fig. 6.

The water-soluble LCC must be released into solution as a consequence of the enzymic degradation of cell-wall polysaccharides in the rumen. It is tempting, therefore, to hypothesize that the LCC in the original cell-wall carried extensive cellulose and hemicellulose chains that "tie" the complex into the wall structure, making it insoluble in water. The microbial endo- and exo-enzymes in the rumen would then degrade the polysaccharide chains, to leave stubs of carbohydrate residues attached to a lignin core as shown in Fig. 6, and resulting in solubility in water. The residue containing one arabinose and two xylose units is[21] a typical component of the hemicellulose of s.g., and thus supports this concept. It is, however, difficult to envisage that such a mechanism would yield single glucose units as the major carbohydrate component of LCC, or that the L-rhamnose in the LCC could have been part of a hemicellulose chain. Furthermore, we have found no evidence for uronic acid (a significant component[21] of s.g. hemicellulose) in the LCC. We prefer to speculate, therefore, that the LCC in the cell wall carries a significant component of single sugar units, especially D-glucosyl, L-rhamnosyl, and D-xylosyl groups, together with some oligo- or poly-saccharide chains. It is also possible that the single and the oligomeric sugar units might function as primers in polysaccharide synthesis in the growing cell-wall.

A comparative analysis of total glycoses in the LCC and those recovered from the methylation analysis showed that there is an overall, apparent increase in D-glucose in the methylation products; this may be due to loss of methylated pentoses,

TABLE III

QUANTITATIVE DETERMINATION OF THE SUGARS IN ACID-TREATED AND ALKALI-TREATED LCC

Sugar	R_G[a]	*Molar ratios*		
		Original LCC	*Acid-treated LCC*	*Alkali-treated LCC*
L-Rhamnose	0.24	0.2[b]	0.1[b]	0.2[b]
L-Arabinose	0.36	0.41	0.24	0.24
D-Xylose	0.46	0.72	0.61	0.50
D-Mannose	0.80	0.09	n.d.[c]	n.d.
D-Galactose	0.93	0.11	0.06	0.09
D-Glucose	**1.00**	**1.00**	**1.00**	**1.00**

[a]R_G = retention time relative to that of D-glucitol hexaacetate as unity. [b]Lower precision due to overlap and solvent tailing. [c]N.d. = not detected.

TABLE IV

METHYLATION ANALYSES OF ACID-TREATED AND ALKALI-TREATED LCC

Peak No.	*Derivative*	*Molar ratios*		
		Original LCC	*Acid-treated LCC*	*Alkali-treated LCC*
1	1,5-di-*O*-acetyl-6-deoxy-2,3,4-tri-*O*-methyl-L-mannitol	0.13	n.d.[a]	n.d.
2	2,3,5-tri-*O*-methyl-L-arabinitol	0.23	trace	trace
3	2,3,4-tri-*O*-methyl-D-xylitol	0.23	0.20	0.16
4	2,3,4,6-tetra-*O*-methyl-D-glucitol	**1.00**	**1.00**	**1.00**
5	2,4-di-*O*-methyl-D-xylitol	0.08	trace	trace
6	2,3-di-*O*-methyl-D-xylitol	0.21	0.32	0.39
7	other tri-*O*-methylhexitols	0.05	0.10	0.23
8	2,3,6-tri-*O*-methyl-D-glucitol	0.22	0.40	1.00
9	2-*O*-methyl-D-xylitol	0.15	0.26	0.20

[a]N.d. = not detected.

as they are more volatile than the methylated hexoses. There is, similarly, an increase in the D-xylose:L-arabinose ratio in the methylated products (2.9:1, methylated; *cf.* 1.8:1, unmethylated) as less D-xylose would be lost because of the lower average degree of methylation existing in the D-xylose derivatives than in the L-arabinose derivative.

The nature of the bonding between the carbohydrate and the lignin in rumen LCC was further investigated by using chemical and enzymic methods. An unequivocal classification of bond types was not possible, however, and many of the conclusions presented are general and speculative.

Treatment of unmethylated LCC with acid (see Table III) lowered the L-rhamnose and L-arabinose contents (relative to D-glucose) by ~50%, and the D-

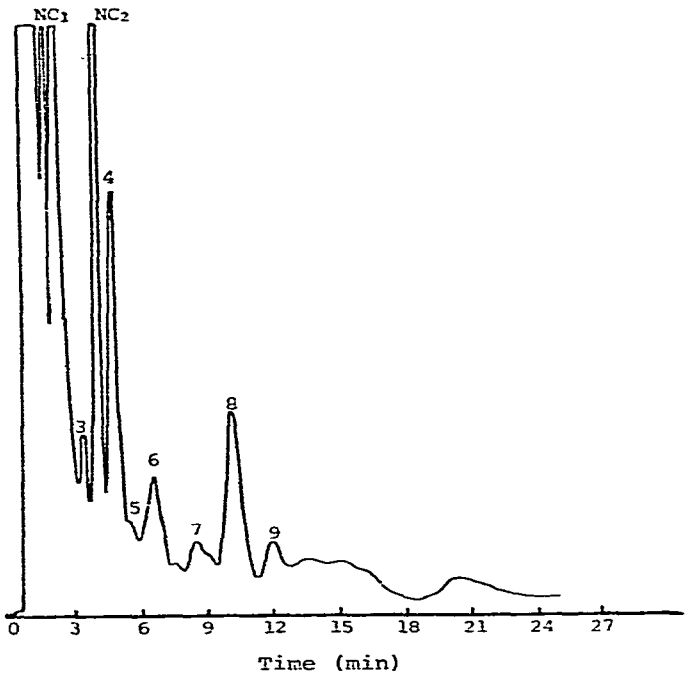

Fig. 7. G.l.c. of the acetates of partially methylated alditols derived from the methylation analysis of alkali-treated, rumen LCC. [Column *4* at 184°. Carbohydrate peak-numbers as in Fig. 5. NC = non-carbohydrate.]

xylose content by ~15%. These results are consistent with the expected, favored hydrolysis of L-arabinofuranosyl and L-rhamnopyranosyl residues by acid. The situation may, however, be complicated by the fact that, under mildly acidic conditions, low-molecular-weight lignin fragments having carbohydrate residues attached could be released, and subsequently lost in dialysis. This could occur, *e.g.*, by cleavage of α-ether bonds, especially in situations where intermediate, "quinone methide" formation is possible, *i.e.*, in those lignin units possessing free *p*-phenolic hydroxyl groups[26]. Also, carbohydrate glycosidically linked to a similar, benzyl carbon atom would be expected to undergo relatively rapid hydrolysis.

The effects of acid treatment in terms of the methylated, residual sugars of the treated LCC are shown in Table IV. In addition to the selective removal of L-arabinose and L-rhamnose already discussed, the acid treatment produced a general, overall increase in the ratios that suggested a decrease in the proportion of 2,3,4,6-tetra-*O*-methyl-D-glucitol. This implies favored hydrolysis of the single D-glucosyl groups from the LCC, and the most likely explanation is that at least some of these D-glucose units are glycosidically attached to lignin units that confer acid lability, such as those already discussed.

A more dramatic effect was produced by alkali (see Fig. 7 and Table IV). These results show a large relative increase in the non-carbohydrate material, presum-

TABLE V

MOLAR RATIOS OF THE RESIDUAL SUGARS IN PERIODATE-TREATED LCC

Sugar	*Molar ratios*			
	Original LCC	*IO_4^-*		
		10 mg	*100 mg*	*300 mg*
L-Rhamnose	0.2[a]	0.3[a]	0.4[a]	0.3[a]
L-Arabinose	0.41	0.34	0.16	0.07
D-Xylose	0.72	0.49	0.53	0.35
D-Mannose	0.09	n.d.[b]	n.d.	n.d.
D-Galactose	0.11	0.08	0.14	n.d.
D-Glucose	**1.00**	**1.00**	**1.00**	**1.00**

[a]Lower precision due to overlap and solvent tailing. [b]N.d. = not detected.

ably lignin-derived (*e.g.*, peak NC_2 in Fig. 7). This material must remain polymeric during alkali treatment and methylation, thus resisting dialysis, and then be degraded during hydrolysis. The carbohydrate content of alkali-treated LCC was 5.6% (compared with the original 7.4%), presumably as a result of alkali scission of covalent bonds, the smaller fragments being subsequently lost in dialysis. There is also a significant change in the ratio of 2,3,6-tri- to 2,3,4,6-tetra-*O*-methyl-D-glucitol. Therefore, some of the single D-glucose units are released by the treatment with aqueous alkali. Presumably, these units were held by some form of aqueous alkali-labile bonding of C-1 of D-glucose to lignin, although it is also necessary to postulate that such a linkage would survive the anhydrous alkaline conditions of methylation. The inflection in peak NC_1 (see Fig. 7) was presumably due to the methylated derivatives of L-rhamnose or L-arabinose, or both; however, attempts to resolve them were unsuccessful.

When LCC was subjected to prolonged treatment with periodate, there was a gradual diminution in the visible and u.v. chromophores. The results in Table V show that L-rhamnose units appear to be rather more resistant than D-glucose units to periodate, perhaps because they are protected by ester groups (*e.g.*, acetoxyl) on C-2, 3, and/or 4. The existence of *O*-acetyl groups is quite probable in grass hemicelluloses, and the groups would be lost during methylation. The L-arabinose and D-xylose residues appear to be more sensitive to periodate than D-glucose, perhaps because there are also ester groups, (*e.g.*, acetoxyl) associated with D-glucose at C-2,3, and/or 4, although less so than on L-rhamnose. It is evident from these experiments that periodate is consumed by lignin, as well as carbohydrate, *e.g.*, free hydroxyl groups of guiacyl units are particularly susceptible to periodate oxidation, and are quantitatively oxidized to *o*-quinones with concomitant liberation of the methoxyl group as methanol[27].

Treatment of LCC with sodium borohydride produced a dialyzable product

that, when acetylated, was shown by g.l.c. to be chromatographically inseparable from authentic L-arabinitol pentaacetate. Therefore, some L-arabinose is attached to lignin by borohydride-labile bonds. If some L-arabinose is attached to the benzyl carbon atom in lignin, such a linkage should be susceptible to scission by aqueous sodium borohydride, and regardless of which hydroxyl group of the L-arabinose was involved in the linkage, L-arabinitol would result.

Enzymic degradation was used in an attempt to confirm the configuration of the D-glucosidic linkages. The results showed that at least 19% of the total D-glucose in LCC is β-glycosidically linked. The true proportion is almost certainly much higher, as a very high concentration of enzyme was needed in order to produce hydrolysis, and the D-glucosidic linkages in LCC are evidently highly hindered, or protected, in order to have survived in the rumen fluid. It might be anticipated that the D-glucosyl groups at the nonreducing end of the $(-[Glc]_m - Glc)$ chains are the most likely to suffer hydrolysis, and this would require that such chains are

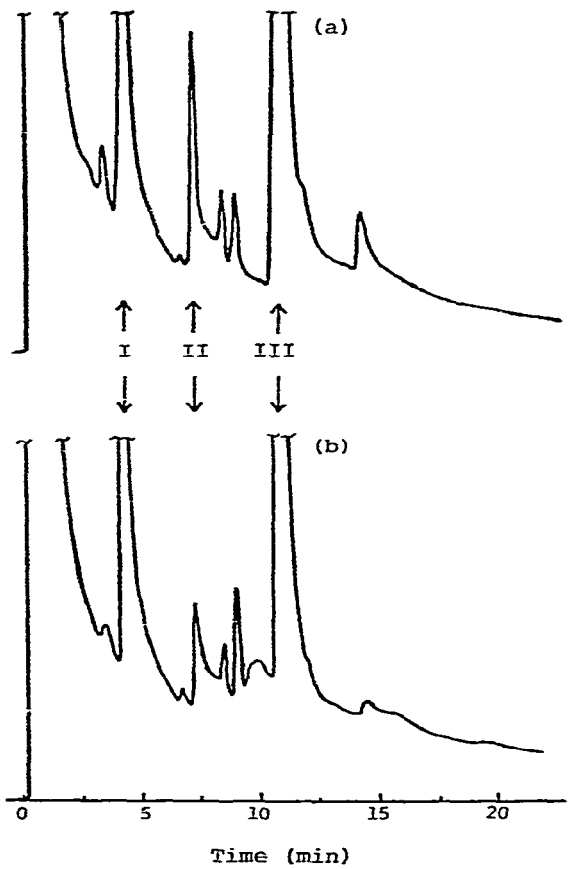

Fig. 8. G.l.c. of the nitrobenzene-oxidation products derived from (a) s.g.; (b) LCC. [Column *3*, at 105° + 10°.min^{-1} to 250°. Key: I, vanillin; II, syringaldehyde; and III, 4-hydroxyazobenzene.]

β-(1→4)-linked, *i.e.*, that they represent either rudimentary, or residual, cellulose chains.

A general conclusion to be reached from the results discussed is that, despite various, and extensive, chemical and enzymic treatments, the rumen LCC, in common with the Björkman LCC that others have extracted from plant material, always retains some residual, unchanged, carbohydrate fragments. The lignin therefore appears to render the carbohydrate inaccessible both to chemical and enzymic attack, either by steric hindrance or by reason of its chemical nature.

Further evidence for presence of lignin in LCC was obtained by oxidation with nitrobenzene, thereby complementing earlier experimental evidence, *e.g.*, u.v. and i.r. results[4,5]. Ferulic acid, *p*-coumaric acid, and vanillin could not be detected in the alkali extracts of LCC (or s.g.), thus providing further proof that the complexes are different from those obtained by Harley[17] by digestion of cell walls of a grass with cellulase.

S.g. and LCC were subjected to oxidation with nitrobenzene, and the resultant gas chromatograms had similar profiles (see Fig. 8). Authentic vanillin and syringaldehyde co-chromatographed exactly with the corresponding peaks, and were unequivocally identified by g.l.c.–m.s. No acetovanillone or acetosyringone could be detected. As there was more vanillin than syringaldehyde, the "lignin core" probably contains more guiacyl than syringyl units. *p*-Hydroxybenzaldehyde was not detected. The profiles also imply that the nature of the lignin moiety in the LCC is similar to that of that in the cell wall. The 4-hydroxyazobenzene (peak III) is a by-product of secondary condensation-reactions between reduction intermediates derived from the nitrobenzene[28,29].

EXPERIMENTAL

General methods. — The collection of rumen fluid (r.f.) from Droughtmaster steers fed a controlled diet of s.g. hay has been described[5]. Particulate matter was removed from the r.f. by centrifugation at 20,000*g* for 30 min at 5°, and the supernatant solution was then dialyzed for 48 h, concentrated, and freeze-dried, to yield a crude LCC product. (Note: all dialysis tubing used was thoroughly prewashed in boiling water for 3 × 30 min). Crude LCC was dissolved in de-ionized water (solution concentration $<1\%$), and purified by gel filtration, or, preferably, by ultracentrifugation at 140,000*g* for 3 h at 4° to remove sub-cellular particulate matter, and then fractionation of the supernatant liquor by gel filtration (see later).

Carbohydrate contents of aqueous solutions of purified LCC products were estimated by the phenol–sulfuric acid method[30]. To correct for background absorption (probably associated with the lignin component), the height of the inflexion at 480 nm in the full spectrum of the assay solutions was used, and was related to the total absorbance of a D-glucose standard at the same wavelength.

G.l.c. was used in the study of alditol acetates of neutral sugars, partially methylated alditol acetates, and lignin oxidation-products. Peaks were identified by

co-chromatography with added, authentic compounds, and identities were confirmed by g.l.c.–m.s. The glass columns used were (*a*) 1.5 m × 2 mm, containing 6% of OV-210 on Chromosorb W AW/DMCS (80–100 mesh) for acetylated *O*-methylalditols (column *1*), (*b*) 2.4 m × 2 mm, containing 3% of OV-225 on Chromosorb W HP (80–100 mesh) for acetylated *O*-methylalditols (column *2*), (*c*) 1.6 m × 3 mm, containing 3% of SE-30 on Chromosorb W HP (80–100 mesh) for lignin oxidation-products (column *3*), and (*d*) 2.2 m × 2 mm, containing 3% of ECNSS-S on Gas-Chrom Q (100–120 mesh) for alditol acetates of neutral sugars and acetylated *O*-methylalditols (column *4*). Flame-ionization detection was used, and nitrogen was the carrier gas.

For the g.l.c.–m.s. analyses, column *2* was used for the alditol acetates, with a temperature program of 220° + 5°.min^{-1} to 240°, and an injector–interface temperature of 250°. Column *4* was used for the partially methylated alditol acetates (isothermally at 200°, and an injector–interface temperature of 230°), and column *3*, for the lignin oxidation-products, with a temperature program of 150° + 10°.min^{-1} to 250°, and injector-interface at 250°. The m.s. conditions were: ionization potential 75 eV, accelerating voltage 3 kV, emission current 300 μA, and an ion-source temperature of 130°.

Fractionation of crude LCC by gel filtration. — Glass columns (Pharmacia Fine Chemicals, Sweden) were employed in the ascending mode, using 25mM ammonium acetate, or, more often, water, as the eluant, and were monitored for carbohydrate (phenol–sulfuric acid) and for absorbance at 455 and 280 nm, unless stated otherwise. Crude LCC (50 mg, from r.f. that had been centrifuged at 20,000*g*) was dissolved in buffer (5 mL), and the solution placed on a K 26/100 column of Bio-Gel P-30 (Bio-Rad Laboratories, California). In a similar experiment, an aqueous solution of crude LCC (1%) was placed on a column of Sepharose CL-2B (Pharmacia), with water as the eluant. Preparative gel-filtration was conducted in a K 50/100 column of Sepharose CL-2B.

Analysis of the carbohydrate component of purified, rumen LCC by g.l.c. — LCC (5 to 50 mg) was weighed into a centrifuge tube (10 mL), and triturated with 72% sulfuric acid (50 μL) at 25° into a smooth paste; this was kept in a desiccator, and subsequently stirred several times. After 1 h, the mixture was diluted with water (1.4 mL), heated under reflux for 3 h at 100°, cooled to 60°, the acid neutralized with barium carbonate, the suspension centrifuged, the precipitated salts washed with water (2 × 2 mL), and the supernatant liquors combined, and concentrated to ~2 mL in a 25-mL round-bottomed flask. The subsequent reduction and acetylation procedures were essentially those described by Jansson and co-workers[25]. Column *4* was used in the g.l.c. of the alditol acetates (isothermally, at an oven temperature of 205°, and the injector at 250°, and a nitrogen flow of 45 mL.min^{-1}). As an approximation, the same detector-response per unit weight was assumed for all of the alditols, as any resultant errors would be <5% of the values found[31,32]. Finely ground, s.g. hay and s.g. holocellulose (10 mg each) were analyzed similarly.

A check for uronic acids in LCC was made by using paper chromatography

(p.c.). Following the hydrolysis of a further sample of LCC, the combined supernatant liquors were, after neutralization, passed through a column of Amberlite IR-120 (H^+) ion-exchange resin (0.5 mL), the column washed with de-ionized water (1 mL), and the total eluate (pH 4–5) passed through a column (0.5 mL) of Amberlite IRA-400 (OAc^-) ion-exchange resin that was eluted with 10% acetic acid (8 mL), and the eluate evaporated at 40°, and analyzed by p.c. in the descending mode with Whatman No. 1 paper and 18:3:1:4 (v/v) ethyl acetate–acetic acid–formic acid–water for 22 h (*p*-anisidine hydrochloride spray for detection of the sugars). No uronic acids were detected, and calibration experiments showed that this procedure would have detected >0.4% of uronic acid in the total LCC.

Structure determination of rumen LCC by methylation analysis. — A methylation analysis based on the Hakomori procedure[33], and similar to that detailed by Jansson and co-workers[25], was used. All reagents were freshly distilled, and stored in septum-closed flasks in a desiccator, and samples to be methylated were dried for 4 h at 40°/1 mm Hg over phosphorus pentaoxide.

LCC (20 mg) was weighed into a serum vial (25 mL), containing a small, magnetic stirring bar and closed with a rubber seal. All reagents were introduced *via* a syringe. Dry dimethyl sulfoxide (15 mL) was added, and the vial was flushed with dry nitrogen. Dissolution was facilitated by allowing the mixture to stand overnight at 25° in a desiccator, and then ultrasonicating for 2 h at ~50°. When dissolution was complete, the dimsyl reagent (2.5M; 5 mL) was added dropwise with stirring. The final steps in the procedure, namely, the completion of the methylation reaction, hydrolysis, reduction, and acetylation, were as described by Jansson and coworkers[25]. In the g.l.c. analysis of the partially methylated alditol acetates, columns *1*, *2*, and *4* were used, with column *4* giving the best resolution at an oven temperature of 184° and the injector at 230°. Authentic samples of amylopectin, dextran (Pharmacia T 500), and s.g. hemicellulose B (all 10 mg, in 10 mL of dimethyl sulfoxide) were each subjected to a similar methylation analysis, and the resultant derivatives were used as reference compounds in the identification of the derivatives from LCC. Their identities were confirmed by g.l.c.–m.s.

Chemical treatment of rumen LCC. — (*a*) *With acid.* LCC (25 mg) was dissolved in water (4.5 mL), and sufficient 0.5M sulfuric acid was added to give a final, acid concentration of 0.05M. The mixture was centrifuged, and measurements showed that, under these conditions, 70% of the carbohydrate material, 80% of the lignin, and 87% of the "color" (absorbance at 455 nm) were precipitated by the addition of acid. (Care was taken to minimize the contact time between the precipitate and the acid to ~15 min.) Water (5 mL) was added to the precipitate, which was then dissolved by the addition of 0.02M sodium hydroxide to pH 6. The solution was dialyzed overnight against running water at 25°, concentrated, freeze-dried, and the residue weighed (18.2 mg). Portions of this material (6% of carbohydrate) were then subjected to compositional, carbohydrate analysis and methylation analysis.

(*b*) *With alkali.* LCC (20 mg) was dissolved in 0.1M sodium hydroxide (5 mL); after 30 min at 25°, the base was neutralized with M acetic acid, and the solution

dialyzed against de-ionized water (25 mL) overnight at 4°. The dialyzate was analyzed qualitatively by t.l.c. for mono- and di-saccharides, but none could be detected. The LCC solution was further dialyzed overnight against running tap-water at 25°, concentrated, freeze-dried, the residue weighed (16 mg), and samples subjected to compositional and methylation analyses. The alkali-treated LCC contained 5.6% of carbohydrate.

(*c*) *With periodate.* LCC (10 mg) was dissolved in de-ionized water (2 mL), and sodium periodate (10 mg) was added. After 20 h at 4° in the dark, ethylene glycol (10 μL) was added, and the solution was dialyzed overnight at 4° against de-ionized water (3 × 500 mL), concentrated, and freeze-dried (8.5 mg). A monosaccharide analysis was performed on this sample. The experiment was repeated with a 0.2% aqueous LCC solution and a ten-fold increase in the amount of periodate.

The results suggested that the reaction might not have reached completion, and so a third experiment was devised in order to monitor the progress of the reaction over several days by periodically measuring the absorbance at 280 and 455 nm and finally analyzing the carbohydrate component of the residual LCC. LCC (10 mg) was dissolved in water (50 mL), and sodium acetate (200 mg; to buffer the reaction) and then sodium periodate (100 mg) were added. The absorbance at 455 nm was measured, additional periodate (100 mg) being added at 18 and 48 h. After a total of 112 h, the pH was unchanged.

The resultant solution was extracted with redistilled hexane (3 × 20 mL), the extract evaporated to dryness, and the residue analyzed by g.l.c. No compounds were detected. Ethylene glycol (300 μL) was added to the aqueous phase, which was then dialyzed overnight against running tap-water, concentrated, and freeze-dried (8 mg). The residual LCC was analyzed for monosaccharide content.

(*d*) *With borohydride.* LCC (10 mg) was dissolved in de-ionized water (10 mL), and the carbohydrate content determined (1-mL aliquot). Sodium borohydride (90 mg) was then added, and, after 24 h at 25°, remeasurement of the carbohydrate content indicated an apparent 64% diminution in the carbohydrate content. However, a blank carbohydrate analysis with a standard D-glucose solution containing a similar concentration of sodium borate showed a 30% diminution in the expected absorbance at 480 nm. Hence, borate ion suppresses the color formation in the phenol–sulfuric acid analysis, and the appropriately corrected value for loss of carbohydrate was 34%.

Amberlite IR-120 (H^+) resin (2 g) was added to the solution, and filtered off after 2 h; the filtrate was then evaporated to dryness, and methanol (5 × 5 mL) added and distilled off. The residue was acetylated in the usual way, and analyzed by g.l.c. One major component was detected, and this was shown to be chromatographically inseparable from authentic arabinitol pentaacetate by g.l.c. retention time/mixture studies.

Enzymic treatment of rumen LCC. — β-D-Glucosidase (*ex* almond emulsin, supplied by P-L Biochemicals Inc., U.S.A.) and α-D-glucosidase (*ex* yeast, supplied by Boehringer–Mannheim Aust. Pty., Ltd.) were used. Free D-glucose was deter-

mined colorimetrically with a kit (Sigma Chemical Co., U.S.A.; 1978 Cat. No. 510) that utilized D-glucose oxidase and peroxidase.

Trial LCC digests (0.5 mL of a 0.25% solution of LCC in buffer[34], pH 6.0) indicated that there was no significant production of D-glucose by either enzyme (0.5 mL of a 0.04% enzyme suspension) after 2 h at 37°. Similar LCC digests containing added cellobiose and maltose showed only 11% conversion of cellobiose, and 8% conversion of maltose.

It was, therefore, decided to increase the enzyme:substrate ratio in digests as follows: *1*, water (0.5 mL) + buffer (0.5 mL); *2*, water (0.5 mL) + β-D-glucosidase (0.5 mL of a 4% suspension); *3* and *4*, LCC (0.35 mL of a 0.25% solution of LCC in buffer, pH 6.0) + buffer (0.15 mL) and β-D-glucosidase (as in tube *2*). Tubes *1*, *2*, and *4* were incubated for 120 min at 37°, and tube *3* for 0 min. Each reaction was terminated by immersing the tube in a boiling-water bath for 5 min. The mixtures were cooled, and the D-glucose determined by adding the enzyme-color reagent (5 mL), incubating for 30 min at 37°, cooling to 25°, and measuring the absorbances at 450 nm. There was no significant, background interference caused by LCC at this wavelength.

The results showed that, in 120 min, 19% of the available D-glucose from the LCC was converted into free D-glucose by the β-D-glucosidase.

The experiment was repeated, but using α-D-glucosidase, and the results showed that, in 120 min, 3% of the D-glucose in LCC was released by the enzyme.

Nitrobenzene oxidation of lignin in rumen LCC. — The general procedures were as described by Hartley[35], using neutral, detergent fiber (n.d.f.) obtained from s.g. (1 g) and the purified LCC. The reaction vessel was a stainless-steel tube (15 mL) fitted with a screw cap and a Teflon seal. The n.d.f. fraction (0.5 g), prepared by the method of Van Soest and Wine[36], was first extracted with 10 mL of M sodium hydroxide, but no saponification products were detected by g.l.c. analysis on column *3* at 101°. The alkali-extracted n.d.f. (0.2 g) was then oxidized with freshly redistilled nitrobenzene (1 mL) and 2M sodium hydroxide (10 mL) for 3 h at 160°. The lignin degradation-products were analyzed by g.l.c. in column *3* with a temperature program of 105° + 10°·min^{-1} to 250°, and an injector temperature of 250°. Peaks were identified by co-chromatography with added, authentic, lignin-derived oxidation-compounds (Pfaltz and Bauer, Inc., U.S.A.). LCC (50 mg) was oxidized in the same way, and the g.l.c. analysis is shown in Fig. 8. The identities of the major peaks were confirmed by g.l.c.–m.s.

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