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POLYSACCHARIDES AND ASSOCIATED COMPONENTS OF MESOPHYLL CELL-WALLS PREPARED FROM GRASSES*

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ABSTRACT

Mesophyll cells have been prepared from rye-grass leaves by a procedure similar to that used by Edwards and Black[1] for crab-grass. Successive filtrations through nylon cloth of 300-, 80-, 45-, and 15-μm mesh yielded suspensions consisting chiefly of single cells, some broken. Passage through a French pressure cell brought the total breakage to near 100%. Microscopic examination of the walls showed that very little of the cell contents remained. The walls were thin (0.1–0.2 μm) and had numerous pit fields; torn surfaces revealed a randomly oriented, fibrillar structure. Determinations of the polysaccharide content, protein, phenolic esters, lignin, and ash accounted for ~85% of the dry matter. The proportion of glucose in the polysaccharides of these walls was similar to that in the leaf cell-wall fraction as a whole, but the proportions of arabinose, galactose, and uronic acid were higher, and that of xylose lower. The acetyl content was 1.0–1.2%. The walls were readily digested by fungal culture filtrates, by snail digestive juice, and by immersion in the sheep rumen in nylon bags. These findings are discussed in relation to the digestion of forage cell-walls in ruminants.

INTRODUCTION

As forage plants mature, they become less digestible by the ruminant. Maturation involves an increase in the ratio of structural to non-structural components, and changes in morphology, especially increases in the stem-to-leaf ratio, so that there is a change in the proportions of the various types of cell wall present. The cell walls themselves mature through the process of secondary thickening and lignification. Recognising that the digestion of cell walls is the limiting factor in the utilization of forage carbohydrate by the ruminant, many workers have attempted to identify some feature of the cell-wall composition that could be used as an index of digestibility. Useful correlations have been found, based on the insolubility of one or more cell-wall components, namely, crude fibre, lignin, and acid-detergent fibre[2], in suitably chosen reagents.

*Dedicated to the memory of Sir Edmund Hirst, C.B.E., F.R.S.

Measurements of sugar composition and determination of polysaccharide structure have not so far led to much improvement in the prediction of digestibility[3,4].

The interest of plant physiologists in the photosynthetic activity of mesophyll cells has led them to devise methods of separating these cells from leaves, and this creates an opportunity to examine the mesophyll cell-wall, which is recognised to be the type most rapidly digested in the rumen. We now describe some features of the mesophyll cell-walls of perennial rye-grass, in particular their carbohydrate composition. All previous descriptions of their composition have depended upon histochemical examination.

RESULTS

Grass was cut with hand shears and brought immediately to a cold room. The leaves were detached, and rubbed gently in a mortar with water. In this way, a dark-green suspension containing many mesophyll cells was produced in ~10–15 min. This material was passed through muslin, and through nylon cloth of 300- and 80-μm mesh, which removed almost all of the small fragments of other cell types present. The filtrate, containing mesophyll cells singly or in groups, was filtered through nylon cloth of 45-μm mesh, supported on stainless-steel mesh held in a circular frame, which allowed only single cells to pass through. The resulting suspension was washed on nylon cloth of 15-μm mesh, similarly supported, which removed cell debris and small fragments of cell wall. After washing, the suspension consisted almost entirely of mesophyll cells, some of which were broken and had lost their contents. The proportion of broken cells could be very much reduced by freezing the leaves before grinding. It seems that ice, which forms first in the intercellular spaces[5], facilitates the separation of the cells and may actually break the connections between them. No advantages seemed to be gained by the use of aqueous media containing D-glucitol, non-ionic detergent (Triton X-100), or a bactericidal agent (Givgard DXN), either in the yield or integrity of the cells, or in the degree of contamination of the final preparation. A single pass through a French pressure cell produced almost complete breakage. The cell walls were washed in the centrifuge with water, the pellets being slightly yellow, and freeze-dried. The yield was variable: ~150 mg from 100 g of fresh leaves (Table I).

The suspension, consisting of clumps of cells that passed through the 80-μm mesh but were retained on the 45-μm mesh, was also collected and treated similarly. Some analyses are presented below. The yield of walls from this fraction was rather higher (Table I).

Contamination of wall preparations. — Examination by the light microscope during preparation showed that practically all the material present was derived from mesophyll cells. Fragments of other leaf structures could sometimes be seen, and after extensive enzymic digestion, only these could still be recognised. Specimens shadowed and viewed in the electron microscope showed very little contamination with particulate debris inside or outside the walls (Fig. 1*a*).

TABLE I

SOURCES OF CELL-WALL PREPARATIONS

Number	*Species*	*Cell-wall fraction*[a]	*Time of year*[c]	*Repetitions of procedure*	*Total amount of fresh material taken (g)*	*Yield (mg)*
1	*L. perenne*	15–45 μm	Late October	4	486	760
2	*L. perenne*	15–45 μm	Mid-November	4	557	680
3	*L. perenne*	15–45 μm	November	9	1140	1179
4	*L. perenne*	45–80 μm	November	8	1015	1400
5	*L. perenne*	15–45 μm	July	1	90	102
6	*L. perenne*	45–80 μm	July	1	90	218
7	*L. perenne*	Non-mesophyll[b]	May	3	60	1470
8	*L. perenne*	Stem	May	1	~20	1170
9	*L. multiflorum* cultivar RVP	15–45 μm	May	13	1450	700
10	*L. multiflorum* cultivar RVP	Non-mesophyll[b]	June	3	60	2470
11	*L. multiflorum* cultivar RVP	Stem	June	3	60	5590
12	*Secale cereale* cultivar Rheidol	15–45 μm	May	6	735	260
13	*Secale cereale* cultivar Rheidol	Non-mesophyll[b]	May	3	60	2260
14	*Secale cereale* cultivar Rheidol	Stem	May	3	60	2750

[a]Mesophyll cell-walls, unless otherwise stated. [b]Almost all of the mesophyll cell-walls were lost during preparation (see Experimental), leaving a mixture of walls derived from epidermis, vascular bundles, and fibre elements. [c]Samples 1–8 were from re-growths of established pasture; samples 9–14 from first Spring-growth after sowing in the previous Autumn.

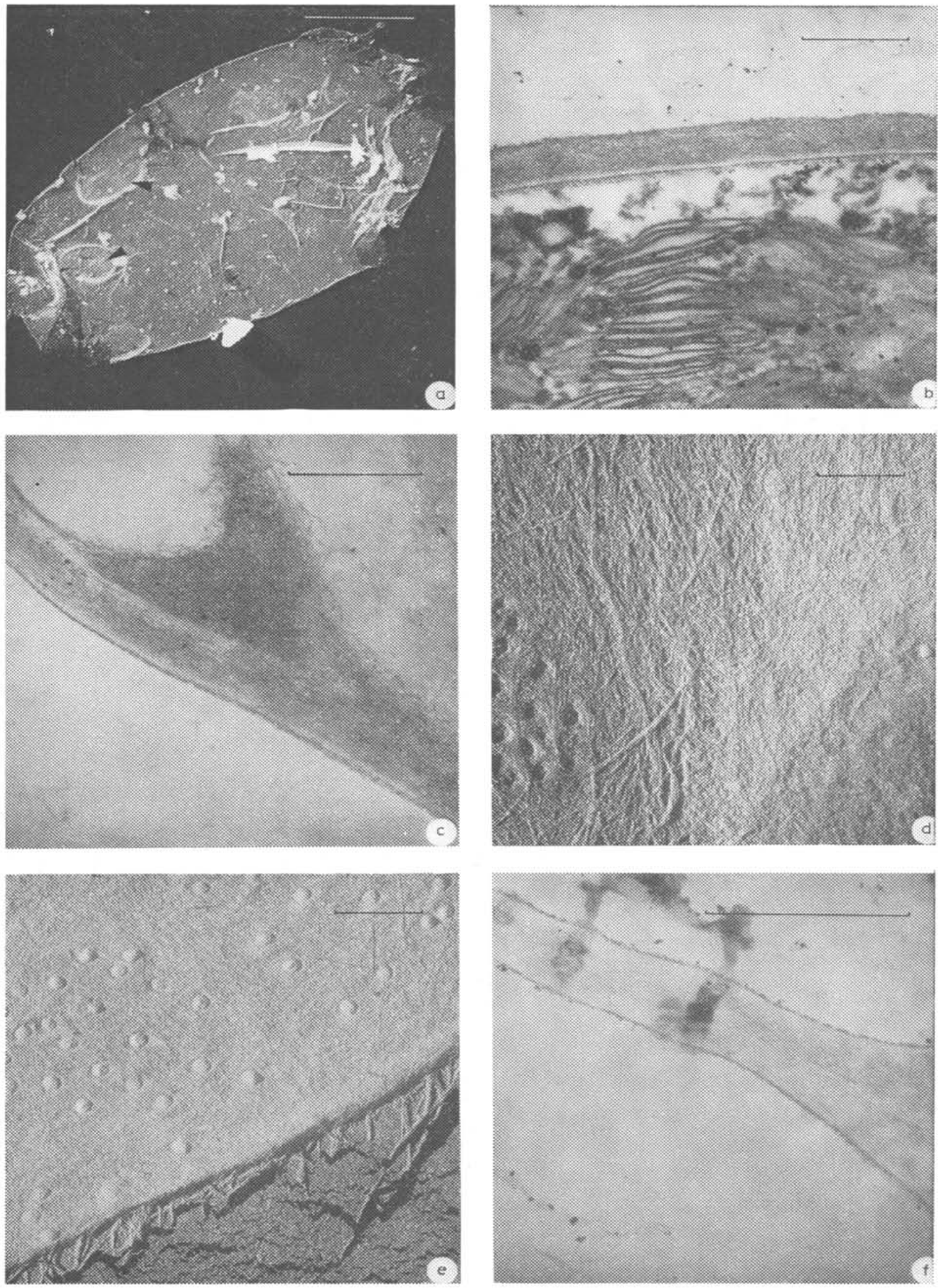

Fig. 1. — See legend p. 239, facing page.

The similarity of chemical analyses of preparations made from grasses from different locations supports the view that a specific cell component is being isolated. Table II shows some variations in the content of total combined sugars, which may have been due to fragments that escaped acid hydrolysis, but the proportions of the neutral sugars were very similar in preparations made at the same time of year (Table III).

The nitrogen content of the walls indicated a protein content of 6.5–7.5%, which fell to 2.9–3.3% after extraction with neutral detergent reagent at boiling point. This might suggest that some cytoplasmic protein was present, although other authors have reported values of 7%, or higher, for cell-wall preparations from monocotyledonous plants[6]. Selvendran[7] found 4.8% of protein in cell walls of bean-pod parenchyma after extractions at room temperature with sodium deoxycholate and phenol–acetic acid–water, which left no protein susceptible to attack by pronase.

Physical properties. — The walls were thin and rigid enough to buckle when dried onto a supporting film for electron microscopy. Their thickness as seen in ultra-thin section was generally close to 0.2 μm, but where plasmodesmata were present, this value fell to 0.1 μm or less (Fig. 1*b*, *c*, *f*). The appearance of sections indicated that the cell walls had been torn apart during separation, leaving loose fibrils (Fig. 1*c*), and in shadowed preparations, the surface so exposed had a randomly arranged fibrillar structure (Fig. 1*d*). Outer surfaces that had not been in contact with other cells, and all the inner surfaces, were generally smooth, with a few short fibrils exposed for short distances (Fig. 1*e*).

In sections, a thin electron-translucent layer could be seen on the inner surface of most regions of the wall, but not around plasmodesmata. Pit fields were numerous and frequently observed in sections. Remnants of plasmodesmata always occupied the holes traversing the wall. In shadowed specimens, this material appeared as projections on the inner surface of the wall (Fig. 1*e*), and in sections, there were indications that the wall was raised around them. Where two walls had been torn apart (Fig. 1*d*), there were depressions in the outer surface at the plasmodesmata.

X-Ray diffraction spectra indicated a low degree of order in the cellulose, despite its fibrillar appearance. Measurements kindly made for us by Dr. M. J. Wilson (Macaulay Institute for Soil Research) indicated a "crystallinity index"[8] of ~1: *cf.* 12 or higher for cotton, and 4–5 for non-mesophyll cell-walls.

The infrared spectra always gave indications of the presence of acetyl groups[9].

Fig. 1. Electron micrographs. (*a*) Metal-shadowed cell-wall showing several points of previous attachment to other cells. (*b*) Section through part of an intact cell; the uniform thickness of the wall and the translucent layer on its inner side are clearly seen. (*c*) Section through wall with translucent layer on inner side and fibrous remnants of another wall still adhering to its outer surface. (*d*) Shadowed outer-surface of wall; on the right, the wall is smooth, but on the left, the fibrillar structure has been revealed where another cell has been torn off; in the latter area, plasmodesmata can be seen as depressions. (*e*) Shadowed inner-surface of wall, smooth with occasional fibrils, and with remnants of plasmodesmata showing as projections. (*f*) Section through two adhering walls of unbroken cells with plasmodesmata; the combined thickness of the two walls at this point is less than 0.2 μm. Magnification is indicated by a bar 10-μm long in (*a*), and 0.5-μm long in all other micrographs.

TABLE II

COMPOSITION OF MESOPHYLL CELL-WALL PREPARATIONS (% OF AIR-DRY SAMPLE)

Sample	*Total neutral sugar*	*Uronic acids*	*Acetyl*	*Nitrogen ×6.25*	*Lignin*	*Ferulic[b] acid*	*Phenolic[c] ester*	*Ash*	*Moisture*	*Total*
1	56.3	6.5	1.0	6.9	4.1	4.3	1.7	8.9	4.4	88.1
2	52.0	6.5	1.0	7.5	3.8	4.0	1.4	9.5	3.6	83.9
3	56.4	6.5	1.0	6.6	3.9	4.1	1.3	7.7	5.6	87.7
4	57.6	7.0	1.2	6.6	3.6	3.8	1.5	2.8	6.0	84.8
9	56.0	5.5	1.1	4.8	3.5			5.4	5.5	81.8
12	50.5	4.5	1.3	8.1	3.3			10.9	7.3	85.9
Non-mesophyll[a] (*L. multiflorum*)	68.0	4.8	1.4	2.5	5.0			2.7	6.4	90.8
Non-mesophyll[a] (*L. perenne*)	67.5	4.0	1.4	3.1	5.0			1.4	6.2	88.7

[a]For comparison: analyses of non-mesophyll cell-walls by Miss E. J. Morris. [b]U.v. absorption of extract from lignin determination[12] expressed as ferulic acid. [c]Phenolic acid released by hydrolysis with M NaOH at 22° for 24 h, expressed as ferulic acid.

TABLE III

PROPORTIONS OF SUGARS (% OF TOTAL NEUTRAL SUGARS) IN CELL-WALL PREPARATIONS

Species	*Cell-wall type*	*Sample number*	*Rha*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*
L. perenne	Mesophyll	1	0.9	13.8	19.0	—	4.4	62.0
		2	1.1	14.1	19.0	—	5.1	60.7
		3	1.0	14.9	18.7	—	4.3	61.1
		4	1.0	15.1	19.1	—	5.1	59.7
		5	0.8	10.7	15.7	0.3	2.9	68.8
		6	0.9	11.2	15.4	0.4	3.1	69.3
	Leaf non-mesophyll	7	—	7.7	23.8	—	—	68.5
	Stem	8	0.2	5.9	31.0	0.4	1.3	61.3
L. multiflorum	Mesophyll	9	1.1	9.9	18.7	0.5	3.2	66.6
	Leaf non-mesophyll	10	0.5	6.7	29.8	—	1.7	61.3
	Stem	11	0.2	4.9	31.7	—	—	63.3
Secale cereale	Mesophyll	12	0.9	11.6	23.8	0.7	2.3	61.0
	Leaf non-mesophyll	13	—	6.2	35.4	—	1.2	57.3
	Stem	14	—	3.8	32.9	-	—	63.4

Chemical analysis. — A large part of the dry matter could be accounted for as polysaccharide, crude protein (Kjeldahl nitrogen × 6.25), lignin, and ash (Table II). Some of the material unaccounted for could be sugar destroyed during acid hydrolysis (especially xylose), or not measured because of incomplete hydrolysis with sulphuric acid. Hydrolysis with trifluoroacetic acid[10] gave somewhat higher values for arabinose, but lower values for xylose; direct determination of pentose[11] gave higher values for total pentose than either hydrolysis method (Table V).

TABLE IV

PROPORTIONS OF SUGARS (% OF TOTAL NEUTRAL SUGAR) IN RESIDUES FROM EXTRACTION OF *Lolium perenne* MESOPHYLL CELL-WALL PREPARATIONS[a] WITH VARIOUS REAGENTS

Extraction procedure	*Yield of residue* (%)	*Rha*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*	*Uronic acid* (% *sample*)
Untreated[a]	100	1.1	14.9	18.8	tr	4.3	61.2	6.5
Neutral-detergent residue	71.5[b]	0.7	14.0	15.9	0.8	4.1	64.8	3.5
Acid-detergent fibre	40.1	—[c]	tr	4.4	tr	tr	95.2	
Crude fibre	37.5	—	0.65	4.5	tr	—	94.0	
Cellulose	40.2	—	0.4	1.7	tr	—	97.9	
Ammonium oxalate	79.2	0.8	14.1	17.6	0.7	4.3	62.4	2.7
Chlorite holocellulose	73.0	1.1	13.7	17.2	tr	4.2	63.5	3.3
M NaOH (24 h at 22°)	56.7	0.9	10.4	11.6	tr	2.3	73.7	2.5

[a]Sample 3 was used for all extractions, except those for acid-detergent fibre, crude fibre, and cellulose. [b]Values for samples 1 and 4 were 74.0 and 72.0%, respectively. [c]Key: —, not detected; tr, trace.

Lignin and related substances. The estimation of lignin in these walls presents some difficulties. The method of Morrison[12] depends upon the measurement of

u.v. absorption in an extract made with acetyl bromide. This absorbance may be expressed as lignin by the use of a regression equation established by Morrison from results with grass samples previously analysed for lignin by a modified Klason procedure[13]; these samples will have included all cell-wall types of both leaf and stem. Harris and Hartley[14] have shown that mesophyll cell-walls contain phenolic esters which absorb at the wavelength used in the Morrison procedure.

We have expressed the u.v. absorbance of the mesophyll cell-walls in Morrison's procedure either as lignin (using his regression equation for grasses) or as ferulic acid; the two values are similar (Table II). We also treated the cell walls with aqueous alkali, and, finding no *p*-coumaric acid to be present, measured the u.v. absorption of the extract in terms of ferulic acid; this corresponded to less than half the figure from the Morrison procedure. After acidification, all the absorbing material could be extracted with ether; values obtained in this way are given in Table II. Either the alkali treatment does not achieve complete extraction of the phenolic acids, or there is another phenolic fraction present. No "true lignin" could be found: 172 mg of walls treated with 72% H_2SO_4 at 25°, and then with boiling 1.2M H_2SO_4 containing cetyltrimethylammonium bromide[15], yielded 6.1 mg of residue. The infrared spectrum gave no indications of the presence of lignin, but was characteristic of opaline silica, which is the form laid down in cell walls of plants[16]. Electron microscopy showed thin sheets and a few hollow spines, which were unchanged after heating to 500° for 2 h.

Nitrogenous constituents. Up to 80% of the nitrogen of wall preparations could be accounted for by amino acids present in acid hydrolysates. The amino acid pattern was very similar for the three 15–45-μm mesh fractions (Samples 1, 2, and 3), but distinctly different for the 45–80-μm fraction (Sample 4). The hydroxyproline contents of samples 1–4 were 0.14, 0.15, 0.15, and 0.26%, respectively. The neutral-detergent residue[17] of mesophyll cell-wall had 0.045% of hydroxyproline, and a non-mesophyll cell-wall preparation less than this. Burke *et al.*[6] found 0.14–0.16% of hydroxyproline in cell walls from several suspension cultures from monocotyledonous species, and $<0.05\%$ in those from cultured cells of rye-grass endosperm. Like them, we conclude that if protein is a structural component of the wall, it must be very different from that found in dicotyledonous plants.

Extraction of cell-wall components. — Although it would clearly be of interest to make a full investigation of the polysaccharide fractions obtainable from these walls, we have confined our attention to two aspects: the question of whether pectic material is present, and the behaviour of the walls when subjected to the various extraction procedures used in chemical assessments of the nutritive value of forages.

Pectic materials. The extraction of a wall preparation (101.8 mg) with 0.5% of ammonium oxalate at 80° for 20 h removed a small fraction (3.6 mg) that was precipitable by ethanol. The insoluble residue (80.6 mg) had a neutral sugar composition closely resembling that of the starting material (Table IV), but the content of uronic acid had fallen from 6.5 to 2.7%. The same effect upon the content of uronic acid was shown by extraction with neutral detergent (Table V). However, in this

case, there was a fall in pentose relative to glucose, and the ratio of arabinose to xylose increased, implying some loss of hemicellulose. Removal of a typical pectic fraction might be expected to give lower contents of rhamnose, arabinose, and galactose, but our analytical methods are not accurate enough to indicate such a small change in composition. Analyses kindly carried out for us by Mr. C. M. Mundie of the Macaulay Institute indicated that 70% of the uronic acid present was galacturonic acid[18].

TABLE V

YIELDS OF NEUTRAL SUGARS (% OF AIR-DRY SAMPLE) ON HYDROLYSIS OF MESOPHYLL CELL-WALL PREPARATIONS

Sample No.[a]	*Rha*	*Ara*	*Xyl*	*Gal*	*Glc*	*Total*	*Pentose*[b]	*Cellulose*
1 (a)	0.5	7.8	10.7	2.5	34.9	56.3	19.5	28
(b)	0.5	7.4	9.1	2.7	1.5	21.3		
2 (a)	0.6	7.4	9.9	2.6	31.6	52.0	20.2	34
(b)	0.6	8.0	9.0	2.2	1.3	21.2		
3 (a)	0.6	8.4	10.6	2.4	34.5	56.4	19.9	36
(b)	0.7	8.5	9.7	2.9	1.4	23.3		
4 (a)	0.6	8.7	11.0	3.0	34.4	57.6	21.2	30
(b)	0.7	9.5	10.9	2.7	1.3	25.2		

[a](a), 12.5M H_2SO_4 at 22° for 1 h, and then 0.5M H_2SO_4 at 100° for 5 h; (b), 2M trifluoroacetic acid at 100° for 5 h. [b]By direct measurement[11] on aqueous suspensions of walls.

Dietary fibre. Hydrolysis with trifluoroacetic acid released only 4% of the glucose obtained by the sulphuric acid procedure (Table V), which sets an upper limit to the so-called "non-cellulosic glucan"[10] of only 1.4% of the wall. Various extraction procedures confirm that the rest of the glucan present is cellulose (Table IV).

The semi-micro method for determination of cellulose[19] is subject to error through losses of fine particles during the washing of the residue from treatment with acetic acid–nitric acid. The glucose in the residue is determined by the anthrone procedure, which is insensitive to pentose. The results so obtained for cell walls (Table IV) are in reasonable agreement with their contents of glucose residues. If, instead, the walls were treated for 30 min with the acetic acid–nitric acid reagent, at 100° and at boiling point, and the residues washed and recovered by freeze-drying, yields of 47 and 40%, respectively, were obtained. Acid hydrolysis showed the presence of a little xylose in these residues (Table IV). Shadowed preparations examined in the electron microscope showed that the fibrillar network survived this treatment.

The yields of crude fibre (38%) and acid-detergent fibre (40%) are consistent with their consisting chiefly of cellulose and inorganic material. The silica residue obtained in the lignin procedure is presumably also present.

Neutral-detergent residue, suggested by Van Soest and Wine[17] as an approximate measure of cell-wall content of forages, would appear to underestimate mesophyll

cell-walls. Some loss of pectic material is expected with this procedure, but with these cell walls there must be a loss of hemicellulose as well, as 25–30% of the dry matter is dissolved (Table IV). In contrast, we have found that a single extraction of non-mesophyll cell-wall preparations removes only ~10% of their dry matter; repetition of the extraction leads to a progressive loss of much smaller amounts, some of which may be mechanical.

Enzyme degradation. — The walls were rapidly attacked by snail digestive juice, the products being mainly free sugars. A fungal cellulase preparation from *Trichoderma koningii*[20] (provided by Dr. T. M. Wood) also dissolved them, but the products included some polysaccharides rich in xylose and arabinose.

The walls were digested in nylon bags in the sheep rumen more rapidly than preparations of the non-mesophyll cell-walls[21]. Within 2 h, a large part had disappeared, the residue still analysing as if it were mesophyll cell-walls. Electron microscopy of ultra-thin sections showed thin walls present among a mass of bacteria.

DISCUSSION

The mesophyll cells separated in the 15–45-μm fraction can be only a small proportion of the total present in the leaf; some occur in clumps and so are removed by filtration through the 45-μm mesh, others can still be seen attached to the vascular strands.

It is not clear what forces hold these cells together. Kanai and Edwards[22] found that it is necessary to use cellulase preparations in order to get satisfactory enzymic release of viable mesophyll cells from most grasses; in other plants, a pectolytic-enzyme preparation is sufficient[23]. It is perhaps worth pointing out that cells in other structures of the grass leaf are not separated by any of the chemical treatments that we have used, including de-lignification with chlorite, or the sequence of acid and alkali hydrolysis used to prepare crude fibre. It seems that their adhesion cannot be due to pectic substances, nor to hemicellulose. The discussion by MacLeod *et al.*[24] of the adhesion of endosperm cells of grasses is of interest in this connection.

From a careful study of metal-shadowed specimens, we conclude that both surfaces of the wall are relatively smooth, but where cells have been pulled apart, a random network of fibrils is revealed. In section, the walls show at least two layers: a very thin electron-transparent layer on the inner surface, and a thicker, outer layer in which fibrils are discernible.

The sugar composition of the mesophyll cell-walls in each species differs markedly from the aggregate composition of the other cell-wall types in the leaf, and still more from the stem cell-walls (Table III). As a proportion of the total neutral sugars, the content of arabinose is higher and that of xylose lower; the arabinose–xylose ratio in *L. perenne* mesophyll cell-walls reaches 0.76. The stem cell-walls have much less arabinose, down to one third that of mesophyll, and up to twice the xylose; the arabinose–xylose ratio is as little as 0.12 in *S. cereale* (Table III, Sample 14). These observations explain, at least in part, the decrease in the arabinose–

xylose ratio seen as grasses mature, the harvestable parts containing an increasing proportion of stem tissue. It was interesting to see some indications of a change in mesophyll cell-wall composition with season (Table VI), because it is generally believed that the autumn growth of grass is less digestible.

TABLE VI

PROPORTIONS OF SUGARS (% OF TOTAL NEUTRAL SUGAR) IN MESOPHYLL CELL-WALL PREPARATIONS MADE FROM SUCCESSIVE CUTTINGS OF *L. perenne*[a]

Sample	*Date*	*Nature of sample*	*Rha*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*
(a)	6 May	Spring growth	0.8	9.7	16.9	0.7	2.4	69.4
(b)	21 May	Spring growth	1.0	10.1	17.0	0.6	2.7	68.3
(c)	12 June	Re-growth of (a)	1.0	10.2	16.8	0.8	3.2	67.3
(d)	2 July	Full season's growth	1.0	11.4	16.1	0.9	6.8	64.3
(e)	17 September	Full season's growth	0.7	11.4	17.0	—	4.1	66.7
(f)	19 September	Re-growth of (b)	0.6	12.3	20.3	—	4.7	62.4
(g)	23 September	Re-growth of (d)	0.7	12.3	18.9	0.7	5.5	62.4

[a]Taken in 1975 from field used for Sample 1 (Table I) in 1974.

The sugar composition of walls from cultures of endosperm cells of Italian rye-grass has been reported by Smith and Stone[25]. These walls also have a high arabinose–xylose ratio (0.73), and a substantial part of the wall glucan is probably cellulose. Mares and Stone[26] noted that the walls from the cultured cells were thicker than those in the intact seed, but still regarded them as "similar to typical primary plant cell walls". There is considerable attraction in the idea that the walls of parenchymatous cells have the same composition as all primary walls, and that the secondary thickenings which are laid down in more specialised cells consist of different, less digestible, material. However, it seems to us that the primary cell-wall is essentially a biological concept, defined in the first place as the wall round a cell that is still growing, and secondarily as a thin wall having a randomly arranged (multinet), fibrillar structure. There seems to be no reason why there should be a corresponding chemical concept, as a number of polysaccharides can assume fibrillar forms, and many others, or for that matter substances other than carbohydrates, could form the wall matrix.

It seems unlikely that many of the mesophyll cells that we have used were still growing, and it seems plausible that they should be specialised, to some extent, to serve their particular function. Their interest to us is that they are very quickly digested in the rumen, and a comparison of their composition with that of less-digestible cell-walls may help to throw light upon the factors that impede digestion. Inevitably, attention is focused on the absence of true lignin. In the few instances in which grasses have been analysed for both xylan and lignin at successive stages of growth, a linear relationship, though not direct proportionality, between them can be discerned (A. W. Boyne and J. S. D. Bacon, unpublished results). This relationship could be explained by the presence of two fractions of xylan, one laid down in

association with lignin, the other without. The xylan of the mesophyll cell-wall would then be an example of the latter.

EXPERIMENTAL

Materials. — Perennial rye-grass was taken from established pastures originally sown with commercial mixture. The samples were sorted at 4°, foreign species being rejected, and the stems, sheaths, and yellow or withered tips of leaves were rejected. Italian rye-grass (R v P) and cereal rye (Rheidol) were taken in May 1976 from pure stands sown in 1975, and were subjected to the same sorting and dissection procedure.

Nylon cloth with 300-, 80-, and 45-μm mesh was obtained from Henry Simon Ltd. (Stockport, Cheshire, U.K.), and with 15- and 5-μm mesh from A. J. Polak Ltd. (439–443 North Circular Road, London, NW10 OHR).

Preparation of cell walls. — (*a*) Walls from mesophyll cells were prepared by a procedure based on that of Edwards and Black[1]. Leaves (15 g) were placed in a mortar (17-cm diameter) with water (50 ml), and rubbed with a pestle. After 10–15 min, a dark-green suspension was produced and this was decanted through muslin; the fibrous residue was extracted twice more. Suspension equivalent to 100–120 g of fresh leaf was collected, passed through nylon cloth of 300-μm mesh, and centrifuged at 500 *g* for 15 min at 5°. The pellet was examined with a stereo-zoom microscope at magnifications up to 160.

In the earliest experiments in 1974, the suspension was found to consist chiefly of unbroken mesophyll-cells, but in 1975, they contained many empty cell-walls. In 1976, cereal rye yielded hardly any intact cells. Recalling that the 1974 samples had often been taken from the field with frost on them, we froze leaves at −20° for a few hours. After this treatment, suspensions of intact cells were obtained, and this effect was confirmed with Italian rye-grass harvested in May 1976 and perennial rye-grass harvested in July; unfrozen leaves gave many broken cells, but frozen leaves gave practically none. The freezing treatment was severe enough to kill the leaves, but since it made it possible to establish the origin and homogeneity of the wall preparations, it was thereafter used routinely.

The suspension was resuspended in water (100 ml) and passed through nylon cloth of 80-μm mesh, which removed most of the fragments of epidermis, vascular bundles, and fibres, leaving only the smallest pieces of these cell types; and then through cloth of 45-μm mesh, which allowed only single cells to pass. The fraction retained was kept, and the filtrate was placed on a filter of 15-μm mesh, which held back the mesophyll cells, both broken and unbroken. These two cell fractions, 45–80 μm and 15–45 μm, were collected by centrifugation for 5 min at 500 *g* and 5°, and each was resuspended in water (40 ml) and passed through a French pressure cell (American Instrument Co., Maryland, U.S.A.) at 15,000 p.s.i., which took the breakage to almost 100%. The resulting wall preparations were washed on the centrifuge repeatedly with water (500 *g* for 5 min at 5°), being resuspended each time with the help of a weak ultrasonic bath (Dawe Sonicleaner, Type 6441A), and freeze-dried.

(*b*) Walls representative of other cell types in the leaf (non-mesophyll cell-walls) were made as already described[9], or by using a triple roll mill (Pascall Engineering Co., Crawley, Sussex, U.K.), first used for extraction of leaves by Bawden and Pirie[27]. With the mill running, leaves were allowed to wrap themselves round the slowest roll to form a felt-like layer. This layer could be squeezed by bringing the roll closer to the middle roll, and judicious additions of a 0.1% solution of Triton X-100 then washed out most of the green colour; the resulting extract contained many intact mesophyll-cells. After being cleaned in this way, the fibrous material could be torn off the slow roll by shearing action, when it passed through the mill and was collected as a suspension of fragments of epidermis, vascular bundles, and fibre elements, similar to that obtained by grinding leaves successively in liquid nitrogen and water[9]. Yields by this method were ~5 g per 100 g of fresh leaf.

Freeze-drying. — Aqueous suspensions were shell-frozen in an ethanol bath held at −60°, and dried by attachment to an evacuated system (giving ~50 mtorr) with a condenser temperature of −50°.

Microscopy. — Aqueous suspensions were examined unstained with phase-contrast, or with an Olympus Model Sz-III stereo-zoom microscope. For electron microscopy, suspensions were dried on Formvar film and shadowed with gold palladium at $\tan^{-1}\frac{1}{3}$; or fixed in 5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.3), and in 1% osmium tetroxide embedded in Araldite, sectioned with glass knives with a LKB Ultrotome III, stained with uranyl formate and lead acetate, and examined in an AEI 801 electron microscope.

Sugar analysis. — (*a*) Acid hydrolysis was performed as described by Sloneker[28]. Determinations of reducing sugar were performed on neutral solutions by using the neocuproine reagent in a Technicon Autoanalyser II, and other colorimetric determinations were made with the anthrone reagent for hexose[29], orcinol–ferric chloride for pentose[11], and *m*-phenylphenol for uronic acid[30].

(*b*) After addition of inositol as internal standard, the neutral sugars in hydrolysates were determined by g.l.c. as their alditol acetates[28], on a Pye Unicam Model 104 gas chromatograph, the peak responses being integrated with an Infotronics CRS 309 microprocessor. Some analyses were made with 5% of SP 2340 as stationary phase (*cf.* Supelco Catalogue 9, 1975) (Ref. 31).

Other analyses. — Nitrogen was determined by a micro-Kjeldahl method, or by a Hewlett–Packard 10185 C,H,N-analyser; lignin by the method of Morrison[12]; and acetyl groups by the g.l.c. method of Bethge and Lindstrom[32]. The Chemical and Physical Analysis Department of this Institute carried out amino acid analyses on hydrolysates made by the action of 6M HCl for 24 h at 110°.

Extraction procedures. — Neutral-detergent residue[17], acid-detergent fibre[2], crude fibre[33], cellulose[19], and holocellulose[34], were obtained from small samples (100–200 mg); centrifugation was used to collect and wash the residues. No corrections were made for ash content. In the chlorite-oxidation procedure[34], lactic acid was substituted for acetic acid.

Nylon-bag technique. — Bags (200 × 90 mm) made from nylon cloth (5-μm mesh)

were suspended in the rumen of a fistulated sheep. After removal, they were rinsed well under the cold tap, and sampled for electron microscopy, and the contents were washed with water on a filter of 5-μm cloth before being freeze-dried from aqueous suspension. (These experiments were carried out by Miss E. J. Morris.)

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