Fractionation of cell-wall preparations from grass leaves by centrifuging in non-aqueous density gradients

Alex. H. GORDON and John S. D. BACON

Carbohydrate Biochemistry Department, Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB,
Scotland, U.K.

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1. Dried preparations of cell walls from perennial-ryegrass (*Lolium perenne*) and Italian-ryegrass (*L. multiflorum*) leaves were suspended in mixtures of carbon tetrachloride with light petroleum (b.p. 45–60°C) or alcohols and layered on density gradients formed from the same solvents. 2. On centrifugation, the cell walls become distributed throughout a suitably chosen gradient. Fractions corresponding to various regions of the gradient were separated, examined under the microscope and analysed. 3. Cell-wall preparations made from leaf material ground in liquid N₂, or in a triple roll mill, showed considerable heterogeneity in particle size, and their behaviour in the density gradient was variable, although there was a general indication that walls derived from vascular bundles were less dense than those from sclerenchyma. 4. Treatment in a vibratory ball mill decreased the size of the particles and produced a more uniform material, but made it impossible to distinguish the origins of the particles. This material behaved more reproducibly in the density gradient. 5. Some fractionations were also made by successive centrifugation in media of increasing relative density. 6. Analyses of the fractions obtained by each method indicated that the less dense had a greater proportion of xylose in the polysaccharide components, and higher contents of acetyl groups and lignin, confirming the close relationship between these components in plant cell walls. 7. The results show that there are differences in polysaccharide composition between the cell-wall types in the grass leaf, the vascular tissue being richer in hemicellulose relative to cellulose than the sclerenchyma.

The polysaccharide fraction of plant cell walls comprises pectic substances, hemicelluloses and cellulose, and for many years the proportions of these substances present in various parts of plants have been measured by extraction procedures or inferred from analyses of unfractionated material. In most cases, however, the samples analysed have contained more than one type of cell, so that the amount of each component in a particular cell-wall type was not known. Most discussion of the differences in chemical composition between wall types is based on histochemical techniques, which can give only a rough idea of the presence or absence of polysaccharide components and of their relative proportions (cf. Cutter, 1969; Esau, 1977).

All growing cells form a thin extensible wall, the primary wall, and when growth stops this wall structure is thickened by the deposition of additional material. It is this later deposit that constitutes the bulk of the cell-wall material in mature plant tissues, and one suspects that differences in the chemical composition of this secondary thickening,

rather than its morphological characteristics, account for differences in the susceptibility of cell-wall types to digestibility in the rumen (cf. Akin *et al.*, 1973). As yet there is little firm evidence to support this hypothesis, although where circumstances are especially favourable, some pure cell-wall types have been examined, from e.g. sycamore (*Acer pseudo-platanus*) cells in suspension culture (Albersheim, 1976), endosperm cells of monocotyledons (Mares & Stone, 1973; Smith & Stone, 1973), the aleurone layer of wheat (*Triticum vulgare*) (see Bacic & Stone, 1980), bean (*Phaseolus coccineus*) pod parenchyma (Selvendran, 1975) and mesophyll cells of monocotyledons (Gordon *et al.*, 1977).

We have sought methods by which the cell-wall types in leaves and stems of grasses might be separated for chemical analysis, and we report here some experiments with centrifugation in non-aqueous density gradients, which have had a limited measure of success. Our preliminary experiments were referred to by Bacon & Gordon (1975).

The use of non-aqueous media for the separation

of tissue components has been in intermittent use for at least 50 years. Behrens (1932) used benzene/carbon tetrachloride to prepare nuclei from freeze-dried heart-muscle homogenates, and Bird *et al.* (1965) used hexane/carbon tetrachloride to examine the enzymes associated with chloroplasts. Centrifugation in a succession of media of increasing relative density has been used to fractionate flour (see, e.g., Stevens *et al.*, 1963; Clements, 1979), bran (Stevens, 1973) and bone (cf. Dickson & Kodicek, 1979), and we have performed a few experiments of this type for comparison with the density-gradient procedure.

Materials and methods

Cell walls

A variety of cell walls was used prepared from leaves of perennial ryegrass (*Lolium perenne*) or Italian ryegrass (*L. multiflorum*) broken by grinding in liquid N₂ or in a triple roll mill and dried from the frozen state or by solvent exchange (Gordon *et al.*, 1977).

Solvent mixtures

Laboratory-reagent-grade solvents were used, carbon tetrachloride being taken as the heavy component in each case, in combination with light petroleum (b.p. 45–60°C), methanol, ethanol or butan-1-ol. The densities of the solvent mixtures used to construct the gradients were calculated from those recorded for the pure solvents and selected mixtures in the ranges used made up at room temperature and checked by hydrometers; the gradients used were all in the relative-density range 1.37–1.50.

Gradient fractionation

A gradient-forming device was constructed from two 50 ml beakers. They were joined at their bases by a narrow glass tube incorporating a tap. With the tap closed the apparatus was immersed in a thermostatically controlled water bath at 20°C, and solvent mixtures representing the upper and lower limits of density required for the gradient were placed in the beakers. The less dense solvent mixture was stirred with a glass paddle from the top (a compensating addition of glass was made to the other beaker), the tap was opened and solvent pumped from the stirred beaker at 3 ml/min by a Gilson peristaltic pump using Solvaflex tubing through a narrow glass tube reaching to the bottom of a straight-sided glass centrifuge tube (MSE catalogue no. 34411-828A, 100 ml nominal capacity), also held in the bath.

Such a system should produce a linear gradient (cf. Peterson, 1970), and this was checked by incorporating a dye (Sudan Red 4) in the heavier

component and pumping the contents of the centrifuge tube through the flow cell of a spectrophotometer, A_{525} being recorded. The dye gradients remained stable during several hours of centrifuging at 1600 rev./min (700 g) and 20°C (see below).

A sample (50–100 mg) of cell walls was suspended in a mixture of the same composition as the lighter gradient component, sonicated briefly and de-gassed. The suspension (total vol. 5–10 ml) was layered carefully on to the top of the density gradient. The tube was covered with a rubber cap (MSE catalogue no. 34431 4041) and centrifuged for 1–2 h at 1600 rev./min in an MSE Mistral 6L centrifuge, previously adjusted to 20°C.

The tube was carefully withdrawn, the disposition of the cell-wall material noted and successive layers aspirated through a wide-bore glass tube into a series of flasks, each being followed by about an equal amount of ethanol, also aspirated through the collecting tube. The suspensions were centrifuged, washed twice with ethanol and twice with water, suspended in water and a small sample examined under the microscope. The aqueous suspensions were transferred to tared glass vials and freeze-dried.

Chemical analyses. These were performed as described by Gordon *et al.* (1977), except that pivalic acid was used as internal standard in the determination of acetyl-group content. Data from g.l.c. of alditol acetates were processed as described by Bacon (1980).

Results

The following account is based on the results of more than a hundred fractionations carried out on 15 preparations of non-mesophyll cell walls from grass leaves.

Unmilled cell-wall preparations in density gradients

Initially a number of preparations, either freeze-dried or dried by solvent exchange, were centrifuged in gradients made from carbon tetrachloride and light petroleum (b.p. 45–60°C), avoiding the use of benzene, employed by Behrens (1932) and Stevens *et al.* (1963) because of its toxicity. Like Behrens (1932) we found that changes in temperature occurring during centrifugation were a disturbing factor. Despite this, microscopic examination of fractions suggested that the procedure might be affecting some separation of cell-wall types.

Electrostatic effects were also found, which resulted, in extreme cases, in sudden and complete rearrangement of all the suspended particles during handling of the centrifuged tubes. Ethanol was added to the solvent mixture at 1%, and later substituted for the light petroleum; this overcame the problems of electrical charge. Butan-1-ol was also tested, and many separations were made with it; a few were

made with methanol. Centrifuging in a refrigerated centrifuge at temperatures close to ambient decreased the problems of density change with temperature, and the procedure finally adopted is that described in the Materials and methods section.

The 'non-mesophyll' cell-wall preparations we have used for digestibility studies (Morris & Bacon, 1977) have a wide range of particle size, and they can be sorted to some extent under a dissecting microscope. However, the larger particles are rarely composed of a single cell-wall type. Sheets of epidermis residues often have bundles of sclerenchyma cells attached, and the residues of vascular bundles contain several cell types, sometimes having a little adhering mesophyll, although the greater part of the mesophyll is lost during preparation.

Two other cell-wall preparations derived from grass were also tested on the butanol/carbon tetrachloride gradient. These were mesophyll cell walls (Gordon *et al.*, 1977) and cell walls recovered from sheep faeces (Morris & Bacon, 1977). The mesophyll cell walls formed a pellet at the bottom of the tube, whereas the faeces-derived cell walls remained in the uppermost layer of the gradient.

Milled cell-wall preparations in density gradients

The firm adherence between cells of different types puts limits on the attainable homogeneity of fractions, and the large size of some particles, whether sheets or bundles, allows entanglement, with further mixing of cell types. We therefore looked for means of reducing particle size and found the McCrone Micronising mill (McCrone Research

Associates, London NW3 5BG, U.K.), a vibratory ball mill, would decrease the particle size of cell-wall preparations very quickly, either in the dry state or in the presence of an inert solvent such as toluene. The agate cylinders in the mill added very little inorganic material to the preparations in the short periods of time employed. The particles produced after 10 min were no longer recognizable as having originated from particular cell types.

In order to avoid variability that might have arisen from incomplete equilibration between particles and solvent, the suspensions were sonicated and left for at least 1 h before being applied to the gradient. The gradients, being quite shallow, were made up shortly before use.

It was not practicable to pump suspensions of the unmilled cell-wall preparations because the fibrous particles formed a felt across the bore of narrow tubes (hence centrifugation in zonal rotors was not attempted). The fractions were therefore sucked up out of the centrifuge tubes with a wide-mouthed glass tube.

Chemical analyses on fractions from density gradients

A single fractionation in a 100-ml glass tube provided enough material for only one of the various analytical procedures applied, so where several analyses were required the fractions from several similar runs were combined.

In Tables 1–4 below, the amount of each neutral sugar released by acid hydrolysis is expressed as a proportion of the total. For completeness, values are given for rhamnose, fucose, ribose, mannose and

Table 1. *Chemical composition of fractions prepared by centrifuging milled cell walls in an ethanol/carbon tetrachloride gradient*

Non-mesophyll cell walls prepared from field-grown Italian ryegrass (*L. multiflorum*), by using the triple roll mill and drying by solvent exchange, were McCrone-milled for 10 min in toluene and fractionated on gradients made with 24.5 and 21.0% (v/v) ethanol/carbon tetrachloride, of relative densities respectively 1.49 and 1.43. Fractions from five such fractionations were combined for analysis; insufficient material was left for a lignin determination in fraction 1.

	Fraction ...	1	2	3	4	Pellet
Dry matter (as % of total recovered)	7	19	39	18	17	
Sugar composition (as % of total anhydro-sugar)						
Rhamnose/fucose	0.7	0.5	0.6	0.8	0.7	
Ribose	0.2	0.1	0.3	0.3	0.1	
Arabinose	7.1	6.8	6.8	6.8	6.8	
Xylose	31.5	30.9	29.2	27.8	26.6	
Mannose	0.2	0.1	0.1	0.2	0.1	
Galactose	1.8	1.6	1.7	1.7	1.9	
Glucose	58.5	60.1	61.3	62.4	63.8	
Glucose/xylose	1.9	2.0	2.1	2.3	2.4	
Polysaccharide content (total anhydro-sugar as % of dry matter)	56.6	59.6	64.1	63.3	57.1	
Acetyl groups (% of dry matter)	1.63	1.66	1.60	1.48	1.12	
Lignin (% of dry matter)	—	7.7	7.1	6.4	5.2	
Uronic acid (% of dry matter)	4.4	4.2	4.6	4.9	4.3	

Table 2. *Chemical composition of fractions prepared by centrifuging milled cell walls in a butanol-1-ol/carbon tetrachloride gradient*

Non-mesophyll cell walls prepared from field-grown perennial ryegrass (*L. perenne*), by using the triple roll mill and drying by solvent exchange, were McCrone-milled for 10 min in toluene and fractionated on gradients with 19 and 15% (v/v) butan-1-ol/carbon tetrachloride, of relative densities respectively 1.44 and 1.48. Fractions from seven such fractionations were combined; the quantities of fraction 5 and the bottom fraction were insufficient to permit the full series of analyses.

	Fraction ...	1	2	3	4	5	Pellet
Dry matter (as % of total recovered)		16	19	43	14	2	6
Sugar composition (as % of total anhydro-sugar)							
Rhamnose/fucose		0.5	0.4	0.5	0.5	0.8	0.8
Ribose		0.1	0.1	0.1	0.1	0.1	0.3
Arabinose		7.3	6.9	6.9	7.2	7.4	7.2
Xylose		26.9	25.8	24.9	23.6	22.3	21.5
Mannose		0.6	0.6	0.6	0.4	0.4	0.6
Galactose		2.0	1.8	2.1	2.1	2.1	2.3
Glucose		62.5	64.4	65.1	66.1	67.0	67.3
Glucose/xylose		2.3	2.5	2.6	2.8	3.0	3.1
Polysaccharide content (total anhydro-sugar as % of dry matter)		65.1	67.8	66.5	66.0	64.4	62.8
Acetyl groups (% of dry matter)		1.54	1.59	1.55	1.41	—	1.15
Lignin (% of dry matter)		6.5	6.7	6.2	5.7	—	—

Table 3. *Sugar composition of fractions obtained by centrifuging unmilled perennial-ryegrass (*L. perenne*) cell walls in a butan-1-ol/carbon tetrachloride gradient*

Non-mesophyll cell walls prepared from field-grown perennial ryegrass, as used in Table 2, but without McCrone milling, were fractionated in a gradient made from 19 and 14% (v/v) butan-1-ol/carbon tetrachloride, of relative densities respectively 1.44 and 1.48. A single separation in which 73 mg was applied and 63 mg recovered yielded material sufficient for a single determination on each fraction.

	Fraction ...	1	2	3	4	5	6	Pellet
Dry matter (as % of total recovered)		17	22	22	9	15	7	8
Sugar composition (as % of total anhydro-sugar)								
Rhamnose/fucose		—	0.7	0.9	1.0	0.8	1.1	1.3
Arabinose		7.4	6.7	6.6	6.8	6.7	8.8	7.0
Xylose		30.1	27.8	26.8	26.0	23.7	24.0	21.5
Mannose		—	0.8	—	—	0.5	—	0.6
Galactose		—	2.0	1.9	1.8	2.0	1.9	2.4
Glucose		58.2	62.3	63.9	64.6	66.1	64.3	67.6
Glucose/xylose		1.9	2.2	2.4	2.5	2.8	2.7	3.1
Polysaccharide content (total anhydro-sugar as % of dry matter)		59.8	63.6	67.5	70.2	65.6	69.3	61.9

galactose, but we attach no significance to any differences in this respect between fractions. Although the values are reproducible in repeated g.l.c. runs with the same alditol acetate preparation, such small peaks may be incorrectly integrated if they emerge close to a much larger one, and sometimes (cf. Table 3) may not reach the necessary threshold value. The analysis of the constituent sugars by hydrolysis and subsequent separation of the alditol acetates derived from them is further described and discussed by Bacon (1980).

Analysis of the sugar composition of light and heavy fractions from separations of unmilled cell walls on light petroleum/carbon tetrachloride den-

sity gradients showed xylose as a proportion of the total neutral sugar to be higher (34%) in the light fraction than in the heavier (27%), there being a compensating difference in the proportion of glucose. This difference was fully confirmed by separations of milled *L. multiflorum* cell walls on ethanol/carbon tetrachloride gradients (Table 1), of milled *L. perenne* cells walls on butanol/carbon tetrachloride gradients (Table 2), and of unmilled *L. perenne* cell walls on butanol/carbon tetrachloride gradients (Table 3).

The range in the proportions of xylose shown by the fractions in Table 1, 31.5–26.6%, is different from that in Table 2, 26.9–21.5%. This is not due to

the use of ethanol instead of butan-1-ol, but is a species difference. Gordon *et al.* (1977) give some analyses for unfractionated non-mesophyll cell walls of the two species, and the aggregated values calculated from the fractions (Table 1, 29.0 and Table 2, 24.9%) conform with these. Fractionations of *L. multiflorum* in ethanol/carbon tetrachloride in which only the top and bottom fractions were analysed gave xylose proportions of 33.4 and 24.4, 32.6 and 23.8, and 34.6 and 27.0%. Another fractionation of the *L. perenne* sample used in Table 2 in butan-1-ol/carbon tetrachloride gave 27.3 and 22.0% xylose in top and bottom fractions respectively. Fractionations of *L. perenne* by the stepwise procedure using ethanol/carbon tetrachloride (Table 4) show a range of 28.2–21.6% and an aggregate value of 26.6%.

Not enough samples were examined to establish differences due to conditions of growth, but there were indications that cell walls from *L. multiflorum* grown in a glasshouse were less dense than field-grown.

Tables 1–3 also include some analyses for acetyl-group content and for lignin by the method of Morrison (1972). The total neutral-sugar content of each fraction is given and the proportion it represents of the total dry matter recovered from each gradient. Uronic acid determinations were made on the acid hydrolysates of fractions, but these varied little in relation to the total neutral sugar; a typical set of values is that given in Table 1.

Microscopic observations on fractions from density gradients

The nature and degree of separation of cell-wall types may be illustrated by the fractions of Table 3. These were obtained with unmilled *L. perenne*

(non-mesophyll) cell walls under conditions established with milled material. Vascular strands with helical thickening were present in fractions 1 and 2; vascular strands without helical thickening in fraction 3, and particularly fraction 4. There was epidermis in all fractions, particularly fraction 3, and large sheets were present in fraction 2. Fraction 6 consisted mainly of epidermis. Some mesophyll was seen in the pellet. Bundles of fibres were present in fraction 5.

Helical thickening is the most readily identifiable feature of the cell-wall preparations, and small pieces of helix were often seen free in the lightest fractions. Sclerenchyma tended to be present in the heavier fractions, but both it and vascular bundles were often attached to sheets of epidermal walls. The distribution of epidermis was difficult to quantify, but there seemed to be a tendency for the upper (adaxial) epidermis to be in the lighter fractions and the lower epidermis to be at the bottom of the gradient. Occasionally, through a fortunate chance, the fragmentation was such that relatively pure fractions of vascular bundles, fibres and epidermis were obtained.

Successive centrifugations in media of increasing relative density

Because the cell-wall suspensions could not be pumped, the final separation in the gradients had to be visible, and, of the transparent materials available, only glass completely resists the solvent mixture. The size of the glass tubes available thus restricted the quantities that could be handled, and we sought methods of fractionation that could be used with opaque tubes. Successive centrifugations in media of increasing density were tested by using first the same glass centrifuge tubes in the swing-out

Table 4. *Chemical composition of fractions prepared by successive centrifugations in media of increasing relative density*

Non-mesophyll cell walls were prepared from field-grown perennial ryegrass by using the triple roll mill and drying by solvent exchange and then McCrone-milled for 10 min in toluene. Duplicate samples were suspended in 26% (v/v) ethanol/carbon tetrachloride and centrifuged at 20000 rev./min for 1 h at 20°C; the suspended matter was collected and the pellet re-suspended in the same medium and again centrifuged. This was done once more, and then the whole process was repeated by using 25, 24 and 23% (v/v) ethanol/carbon tetrachloride in succession. The duplicate recoveries of dry matter at each stage agreed well and the total recoveries were 94 and 95%.

	Ethanol (% v/v) ...	Suspended				Sedimented
		26	25	24	23	23
Dry matter (as % of total recovered)		34	27	16	11	6
Sugar composition (as % of total anhydro-sugar)						
Rhamnose		0.5	0.5	0.4	0.5	0.5
Arabinose		8.5	7.6	7.1	7.3	7.5
Xylose		28.2	27.1	26.7	23.1	21.6
Galactose		2.3	2.2	2.1	2.4	2.6
Glucose		60.5	62.6	63.5	66.8	67.8
Glucose/xylose		2.2	2.3	2.4	2.9	3.2
Polysaccharide content (total anhydro-sugar as % of dry matter)		57.7	60.4	59.8	61.5	54.6

rotor. Four such separations yielded fractions showing the same gradient of chemical composition.

However, when polypropylene tubes (diameter 40mm, height 120mm) were used in an angle rotor, the material did not pack satisfactorily unless much higher relative centrifugal forces (about 50000g) were used. Difficulties also arose with slight swelling of the threaded plastic tubes. The results of such a fractionation are shown in Table 4. An unexpected finding was that the fractions were distributed through a range of densities distinctly lower than that in the density gradients; thus a substantial fraction, with both glass and plastic tubes, apparently remained in suspension in 26% (v/v) ethanol/carbon tetrachloride; this may have been the result of convection currents disturbing the very lightly held top layer of the pellet.

Discussion

The structure of the grass leaf is such that the mesophyll cells may be separated fairly easily from the other cell types (cf. Edwards & Black, 1971), but the latter cannot be separated from one another, even by such drastic procedures as sequential treatment with acid and alkali, or by delignification. A comparison of mesophyll cell walls with this mixture of 'non-mesophyll' cell walls has shown that there are considerable differences in chemical composition, relating particularly to the proportions of arabinose, xylose and glucose. The present results show that differences exist within the non-mesophyll cell-wall mixture, in this case involving only xylose and glucose. Although most of the chemical analyses were made on particles of cell wall too small to be recognized as originating in particular cell-wall types, enough evidence was obtained with unmilled preparations to associate the lighter fractions with vascular strands, and with the annular and helical secondary thickenings from these structures that could sometimes be seen in the free state accompanying them. Fibre cells outside the vascular strands were often associated with sheets of epidermis residues, but where they had been detached during the preparation of the walls, they constituted a heavy fraction. Sheets of epidermal cell walls were found at all levels in the gradients.

There is here a correlation between density and what is known about the relative digestibility of cell-wall types (cf. Mason, 1969, and references therein; Selim *et al.*, 1975). The less dense the cell-wall type, the more resistant it seems to be to digestion. The observation that the very-poorly-digested faeces-derived cell walls float on our gradients, whereas the highly digestible mesophyll cell walls sink, emphasizes this connection. It is also noteworthy that the ratio of glucose to xylose shows a regular trend from 1.4 in the cell walls isolated

from the faeces of grass-fed sheep (Morris & Bacon, 1977), through the values shown in Tables 1 and 2, to 3.2 or more in mesophyll cell walls (Gordon *et al.*, 1977).

The use of different solvents may introduce complications into the relationship between different cell-wall types. We have not determined, for example, how much different wall types swell in the presence of alcohols, and whether any minor constituents of the walls such as waxes and cutins are extracted by the mixed solvent systems. It may be significant that the differences in Morrison lignin content between light and heavy fractions were not as large when butanol was used to form the density gradient, as with ethanol or light petroleum. That the cell-wall material as a whole has different densities in different solvent systems is indicated by the ranges of density (g/cm³ at 20°C) within which fractionation was obtained. These were 1.370–1.416 for methanol, 1.393–1.449 for ethanol, 1.445–1.468 for *n*-butanol and 1.450–1.496 for light petroleum. There were indications that the densities of wall materials differed somewhat from batch to batch, but insufficient evidence has so far been accumulated to relate this to the species difference, or to conditions of cultivation.

It is difficult to compare our results with the conjectures about cell-wall composition made previously by staining techniques. Meier & Wilkie (1959) analysed the cell walls of the tracheids of *Pinus silvestris* (Scots pine) in which secondary thickening was in progress. Although direct methods of chemical analysis were used, the compositions of successive layers were calculated by difference, and the errors of these calculations increased as thickening progressed. No comparable investigation has been made of cell walls of monocotyledonous plants.

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