Uniformly ^{14}C-labelled plant cell walls: production, analysis and behaviour in rat gastrointestinal tract

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Uniformly ^{14}C-labelled primary cell walls (^{14}C-PCW) were purified from suspension-cultured cells of spinach (*Spinacia oleracea* L.) grown in a medium containing D-[U-^{14}C]glucose. The approximate polymer composition of the ^{14}C-PCW preparation (% total ^{14}C) was homogalacturonan 30, rhamnogalacturonan 23, xyloglucan 10, other hemicelluloses 3, cellulose 21, lignin 0, ^{14}C-labelled protein < 3 and [^{14}C]starch < 2. The degree of methyl esterification of the pectic polysaccharides was about 25%. The ^{14}C-PCW contained about 4% *O*-acetyl and 3% non-volatile ester-linked residues. When tracer levels of these ^{14}C-PCW were fed to rats, only about 18% of the ^{14}C appeared in the faeces; negligible levels of [14]C (0·07%) remained in the gut contents 4 d after feeding. Some ^{14}C was present in the carcass. The results show that U-^{14}C-labelled primary cell walls can be purified and radiochemically analysed by the methods developed here, and that primary cell walls are extensively fermented by the gut microflora of the rat.

Dietary fibre: Plant cell walls: Fermentation: Rat

The plant cell wall (PCW) is becoming increasingly widely recognized as an important part of a healthy diet as its fate in the gastrointestinal (GI) tract becomes apparent (Prynne & Southgate, 1979; Høverstad *et al.* 1982; Eastwood, 1987; Stephen *et al.* 1987; Trowell & Burkitt, 1987; Hosoya *et al.* 1988). PCW material is the major source of dietary fibre (DF) in our diet. PCW are composed largely of three classes of polysaccharide: cellulose, pectins and hemicelluloses. Cellulose (β-(1 → 4)-D-glucan) constitutes the 'skeletal' microfibrils of the PCW; pectins and hemicelluloses are classed as matrix polysaccharides. The major pectins are homogalacturonans and rhamnogalacturonans, and the major hemicelluloses are xylans, xyloglucans, mannans, β-(1 → 3)-glucans and β-(1 → 3),(1 → 4)-glucans (McNeil *et al.* 1984; Fry, 1988). The polysaccharides often also contain *O*-acetyl and methyl ester groups, and phenolic residues e.g. *O*-feruloyl esters (Deuel & Stutz, 1958; Fry, 1982; York *et al.* 1988). The presence of these ester-linked groups may influence the susceptibility of the polysaccharides to enzymic digestion. In certain mature plant tissues, the phenolic polymer lignin is also a major component of the cell wall (Northcote, 1972).

All the matrix polysaccharides are essentially insoluble when they are integrated within the intact PCW, although most of them can be rendered water-soluble by treatment with hot water (e.g. pH 3–8) or cold aqueous alkali, or both (Fry, 1986). Many studies of the role of DF have followed the fate of individual matrix polysaccharides solubilized from PCW (Eastwood *et al.* 1986; Englyst *et al.* 1987; Bonhomme-Florentin, 1988). Studies have also been undertaken of the fate of purified α-[^{14}C]cellulose, obtained after solubilization of all

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matrix polysaccharides from PCW (Carryer *et al.* 1982; Kelleher *et al.* 1984; Walters *et al.* 1989). However, the mode of action, and fate, of DF may be influenced by the interaction of different polymeric components within the coherent structure of the PCW. Solubilization will cause these interactions to be lost; heating will reduce the relative molecular mass (M_r) of the pectic polysaccharides, and alkali will remove all ester-linked substituents (Fry, 1988). Thus, solubilization of cell wall polymers will undoubtedly affect their fate in the GI tract. A more meaningful picture is likely to emerge from a study of the fate of polysaccharides which are still part of an intact PCW.

In the present paper we describe the production, purification and analysis of ^{14}C-labelled primary cell walls of a dicotyledonous plant and an initial study of their use in feeding trials in laboratory rats. Plant cells were grown in a simple, fully-defined culture medium containing D-[U-^{14}C]glucose (Glc) as the only C source. This resulted in the uniform ^{14}C-labelling of the PCW to yield a product which retained the natural inter-polymeric associations and whose fate in the GI tract could be followed whether the ^{14}C-PCW were fed to the animal in a high- or low-fibre diet.

<div align="center">METHODS</div>

Production of U-^{14}C-labelled plant cell walls (^{14}C-PCW)

Cell suspension cultures of spinach (*Spinacia oleracea* L. cv Monstrous Viroflay) were grown as described by Fry & Street (1980) except that 5 g D-[U-^{14}C]Glc (3·3 GBq/mol)/l was the only C source. After 7 d, when about 80% of the ^{14}C had been removed from the medium, the cells were harvested by filtration through nylon mesh (64 μm pores), washed with distilled water and frozen in 10 g portions. Each portion was thawed, suspended in 100 ml of a solution containing 20 g sodium dodecyl sulphate/l, 20 mM-HEPES and 10 mM-ascorbate, pH 7·4 and sonicated in an MSE 'Soniprep'. Completion of cell disruption was checked by microscopy. Each suspension was filtered through nylon mesh, and the PCW-enriched residue was washed in the HEPES buffer and transferred into 100 ml phenol–acetic acid–water (2:1:1, w/v/v, PAW) at 20°.

The pooled PAW suspensions were stirred for 16 h at 20° to extract proteins (other than those covalently associated with the PCW; Fry, 1991) and filtered through muslin. The PAW treatment of the PCW residue was repeated several times until the ammonium formate–acetone-precipitation test for proteins (Fry, 1988) proved negative. The PCW residue was washed four to five times in distilled water to remove PAW and starch was extracted with 900 ml dimethylsulphoxide (DMSO)/l (Selvendran *et al.* 1985). The suspension was stirred for 16 h at 20°, filtered on nylon mesh, washed four to five times in distilled water, dialysed against distilled water for 16 h to remove final traces of the DMSO, and freeze-dried. Fig. 1 shows a representative sample of the PCW preparation.

Analysis of ^{14}C-PCW

Ester-linked fraction (ELF). ^{14}C-PCW (1 mg) were hydrolysed in 0·1 ml 0·1 M-NaOH in a nitrogen-filled tube at 25° for 16 h to release ester-linked groups (e.g. as [^{14}C]ferulate, [^{14}C]acetate and [^{14}C]methanol) and the hydrolysate was brought to approximately pH 4·7 with 0·1 ml 0·2 M-acetic acid. Solubilized polymers were precipitated with 1 ml ethanol and pelleted at 2500 g for 10 min; the pellet was rinsed with 0·5 ml ethanol (830 ml/l) and the pooled supernatant fractions (ELF) were divided into three 0·5 ml portions, which were treated as follows: portion 1 was added to 0·5 ml water and assayed for total ^{14}C; portion 2 was added to 0·1 ml 2 M-NaOH to convert ^{14}C-labelled acids to their non-volatile sodium salts and dried under vacuum to remove [^{14}C]methanol, and the residue was re-dissolved in 1·0 ml ethanol (415 ml/l) and assayed for ^{14}C; portion 3 was added to 0·1 ml glacial
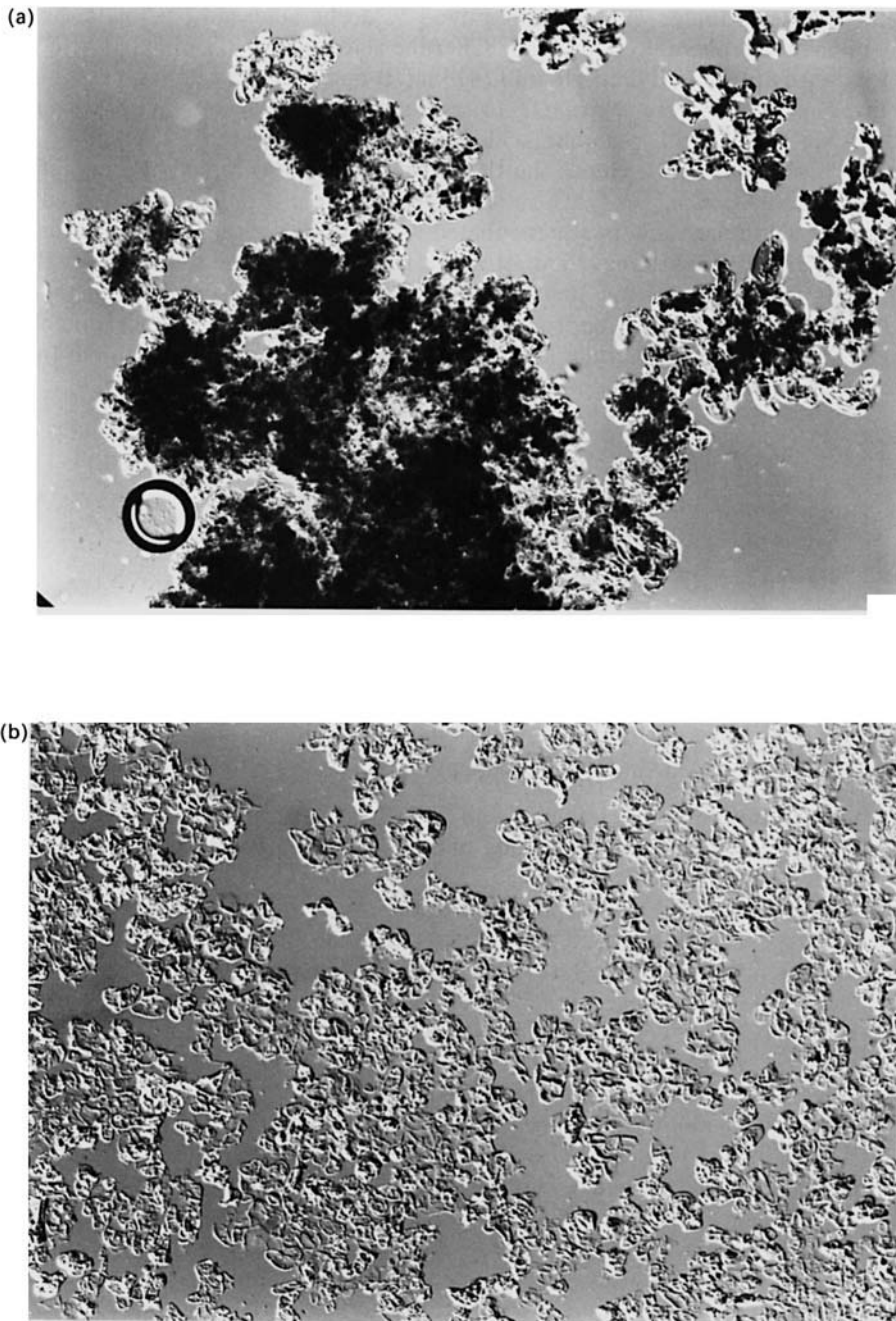
Fig. 1. (a) Freeze-dried cultured spinach (*Spinacia oleracea* L.) cells, and (b) cell walls isolated from the cells shown in (a). Both preparations were stained with Evans' Blue to reveal protein and photographed under DC optics. The cells (a) are strongly aggregated and highly cytoplasmic compared with the isolated walls (b). Magnification: × 110.

acetic acid and dried under vacuum to remove [^{14}C]acetic acid and [^{14}C]methanol whilst retaining ester-linked phenolic acids e.g. [^{14}C]ferulic acid and p-[^{14}C]coumaric acid and the residue was re-dissolved in 1·0 ml ethanol (415 ml/l) and assayed for ^{14}C.

The three portions were expected to contain the following major ester-linked components, respectively: 1, phenolic acids + acetic acid + methanol; 2, phenolic acids + acetic acid; 3, phenolic acids. Hence the ^{14}C in each of these components was estimated.

Driselase-soluble fraction (DSF). The ethanol-insoluble pellet remaining after removal of the ELF was dried under vacuum and incubated with 1 ml Driselase (Sigma Chemical Co., Poole, Dorset; 10 g/l) in pyridine (10 ml/l), acetic acid (10 ml/l) and sodium azide (0·5 g/l), approximately pH 4·5 at 37° for 16 h. Driselase, a mixture of enzymes from the fungus *Irpex lacteus*, can break down approximately 98% of generally-tritiated spinach PCW material to mono- and disaccharides (Fry, 1988; S. C. Fry, unpublished results). Its action on various substrates may be summarized as:

xyloglucan	→ isoprimeverose (α-D-xylopyranosyl-(1 → 6)-D-Glc; XG2), Glc, D-galactose (Gal), L-fucose (Fuc), L-arabinose (Ara)^{tr},
xylans	→ D-xylose (Xyl), Ara, xylobiose^{tr}, D-glucuronic acid (GlcA)-containing oligosaccharides)^{tr},
homogalacturonan	→ D-galacturonic acid (GalA),
rhamnogalacturonan-I	→ GalA, L-rhamnose (Rha), Ara, Gal, Fuc^{tr},
mannans	→ D-mannose (Man), Glc, Gal,
cellulose	→ Glc,
starch	→ medium-M_r oligosaccharides, maltose^{tr}, Glc^{tr},
proteins	→ medium-M_r oligopeptides,

where tr is trace amounts (S. Aldington and S. C. Fry, unpublished results). Before use, Driselase was freed of non-proteinaceous and insoluble material, as described by Fry (1982).

After hydrolysis, the DSF was separated from the pellet. One portion of the DSF was assayed directly for ^{14}C and a second portion was paper chromatographed in butanol–acetic acid–water (12:3:5, by vol.)–ethyl acetate–pyridine–water (8:2:1, by vol.) (BAW–EPW). Strips of the paper were assayed for ^{14}C and those strips comprising the incompletely-resolved GalA–XG2–Gal–Glc zone were recovered from the scintillation fluid, washed in toluene, dried and eluted with water. The pooled eluate was re-chromatographed for 48 h in EPW, which gave almost baseline-resolution of the four sugars mentioned.

Acid-hydrolysed fraction (AHF). The small, Driselase-insoluble residue was hydrolysed in 6 M-HCl at 120° for 16 h, cooled and bench-centrifuged; both the clear supernatant fraction and the (very slight) pellet were assayed for ^{14}C.

[^{14}C]*Starch content.* ^{14}C-PCW (10 mg) were added to 4·5 ml 0·2 M-acetate (Na$^+$, pH 4·5), heated at 100° for 4 h to gelatinize starch, and cooled to 50°. Amyloglucosidase (*EC* 3.2.1.3) solution (1 ml, containing 1000 Units; BDH) was added and incubated at 50° for 16 h. A control sample lacked amyloglucosidase. Solubilized material was assayed for ^{14}C (it contained about 30% of the total ^{14}C, whether or not amylglucosidase had been used, largely representing the solubilization of pectic polysaccharides at 100°). A portion of the solution was paper chromatographed in BAW–EPW and [^{14}C]Glc (which was absent in the enzyme-free control) was assayed.

Susceptibility to pancreatin. ^{14}C-PCW (1 mg) were shaken gently at 37° for 16 h in 1 ml porcine pancreatin (1 g/l) in 50 mM-phosphate (Na$^+$, pH 7·5), containing 0·5 g NaN$_3$/l. ^{14}C-Labelled material thereby solubilized was paper chromatographed in BAW–EPW.

Paper chromatography and electrophoresis

Chromatography and electrophoresis were performed on Whatman no. 1 paper (for details, see Fry, 1988). Marker sugars were stained with aniline hydrogen phthalate.

Chromatography was by the descending method for 16 h unless otherwise stated. Solvents used were BAW–EPW, the two solvents (BAW and EPW) being used sequentially (16 h development in each), in the same dimension, with thorough drying of the paper after the first (BAW) run.

Electrophoresis was performed in pH 3·5 buffer (acetic acid–pyridine–water (10:1:189, by vol.) at 2 kV for 3 h in a water-cooled flat-bed apparatus, under which conditions GlcA migrated considerably faster than GalA.

Assay of ^{14}C

Aqueous solutions (1 ml) were mixed with 10 ml scintillant A (PPO (3·3 g/l) and POPOP (0·3 g/l) in toluene–Triton X-100 (2:1, v/v)) and assayed at an efficiency determined by use of a quench curve. Strips of chromatography paper were placed directly into 2 ml scintillant B (PPO (5 g/l) and POPOP (0·5 g/l) in toluene). For samples analysed by paper chromatography or electrophoresis, a small portion of the sample solution was assayed directly in scintillant A to obtain an absolute measure of the total ^{14}C present, and then a further portion was chromatographed and the relative distribution of ^{14}C along the chromatogram was assessed by scintillation-counting of strips in scintillant B. For assay of ^{14}C in animal tissues, faeces etc., weighed portions (less than 0·2 g) were oxidized in a stream of O_2 using a Packard 306 sample oxidizer. The $^{14}CO_2$ produced was collected in Carbosorb–Permafluor (4:5, v/v) and assayed by liquid-scintillation counting.

Animal feeding experiment

Male albino Wistar rats were housed in solid-bottomed cages with wood shavings for bedding. The room was maintained at 20° with 12 h light/d and noise was kept to a minimum. The stock diet CRM(X) (Special Diet Services Ltd, Witham, Essex), which contains (/kg dry matter): total non-starch polysaccharides (NSP) 133 g, insoluble NSP 102 g, cellulose 26 g and soluble NSP 31 g (kindly analysed by Dr H. Englyst, Cambridge)) in pelleted form and water were supplied *ad lib*. The ^{14}C-PCW (40 kBq; about 4 mg, i.e. about 0·1 % of a rat's daily intake of dry matter) were suspended in water and administered orally (volume given < 3 ml). The syringe barrels were washed out and assayed for ^{14}C to permit determination of the exact dose supplied. The dosed animals were housed in metabolism cages with a broad-spaced gridded floor to minimize coprophagy. Faeces and urine were collected separately into vials containing 0·1 M-NaOH to halt further fermentation. This concentration of NaOH would not cause significant oxidation of polysaccharides and would not hydrolyse glycosidic linkages, although any ester-linked groups (methyl, acetyl, feruloyl etc.) remaining in the faeces would be hydrolysed from the polysaccharides. The samples were removed at 24 h intervals and freeze-dried (this would result in the loss of any saponified [^{14}C]methyl groups as $^{14}CH_3OH$, but retention of ^{14}C-labelled polysaccharides and any [^{14}C]acetate, [^{14}C]ferulate etc.). After 4 d, the animals were killed by cervical dislocation and dissected immediately. The post mortem and dissection were performed by experienced animal house technicians. The organs were identified by blunt dissection. Adipose tissue was identified and removed by blunt dissection. The pelt was removed *in toto* from the animal and the adipose tissue was removed by scraping. Small intestines, caecum and colon were isolated and removed and their contents were freeze-dried. The intestinal tissues were washed in water and frozen.

Table 1. *Distribution of radioactivity in a* ^{14}C*-labelled plant cell wall* (*PCW*) *preparation from cultured spinach* (Spinacia oleracea L.) *cells*

(Values are means with their standard errors for six measurements from separate analyses of the same ^{14}C-PCW preparation)

PCW analysis products	Radioactivity $(10^{-4} \times \text{dpm per mg PCW})$		% total ^{14}C in PCW material	Molar ratio¶
	Mean	SE		
ELF	5·31	0·58	8·2	
[^{14}C]methyl-ester*	0·84	0·07	1·3	1·3
O-[^{14}C]acetyl†	2·60	0·06	4·0	2·0
Non-volatile‡	1·87		2·9	0·3
DSF	58·0		90·0	
R_F zero§	2·74	0·02	4·3	0·7
Galacturonic acid	20·2	0·18	31·3	5·2
XG2	4·29	0·06	6·7	0·6
Galactose	3·25	0·08	5·0	0·8
Glucose	14·5	0·02	22·5	3·7
Mannose	0·33	0·02	0·5	0·1
Arabinose	5·46	0·03	8·5	1·7
Xylose	1·10	0·02	1·7	0·3
Fucose	0·77	0·03	1·2	0·2
Rhamnose	1·88	0·04	2·9	0·5
Remainder‖	3·34		5·2	0·9
AHF‖	1·15		1·8	0·3
Hydrochloric acid-insoluble	undetectable		0·0	0·0
Total	64·5		100·0	—

ELF, ester-linked fraction; DSF, Driselase-solubilized fraction; AHF, acid-hydrolysed fraction; R_F, relative band speed; XG2, isoprimeverose (α-D-xylose p-(1 → 6)-D-glucose); dpm, disintegrations/min.

  * Volatile from acidic and alkaline solutions.

  † Volatile from acidic but not alkaline solutions.

  ‡ Includes ester-linked phenolic residues e.g. O-feruloyl (molar ratio has been calculated for a C_{10} compound).

  § Chromatographically-immobile material: includes products from [^{14}C]starch and [^{14}C]protein (molar ratio has been estimated assuming C_6 residues).

  ‖ Molar ratio has been calculated assuming that this material is composed of C_6 residues.

  ¶ % ^{14}C in component : no. of C atoms in component.

Adipose tissue, liver and skin were also frozen immediately after dissection. Each sample was weighed and a portion thereof was assayed for ^{14}C.

RESULTS AND DISCUSSION

Analysis of ^{14}C-PCW samples

Two batches of ^{14}C-PCW were produced and analysed independently with similar results (Gray, 1989). The batch used in the present experiment showed a total activity of about 10·7 kBq/mg dry PCW material (sum of ELF + DSF + AHF), which is close to the specific activity (18·5 kBq/mg) of the D-[U-^{14}C]Glc supplied to the spinach cells.

ELF. Significant radioactivity (about 8 % of the total ^{14}C) was associated with presumptive ester-linked groups i.e. material that became ethanol-soluble after alkaline hydrolysis (Table 1). The most abundant ester was volatile at low but not high pH. Although our method does not distinguish [^{14}C]acetic acid from other volatile acids, acetate
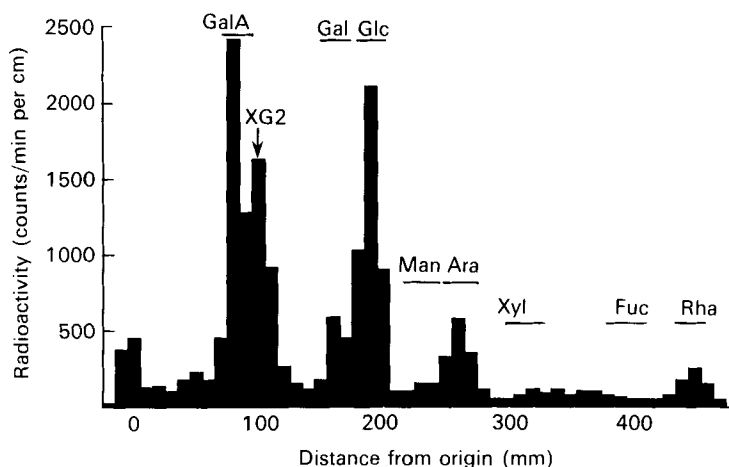
Fig. 2. Paper chromatography in butanol–acetic acid–water (12:3:5, by vol.)–ethyl acetate–pyridine–water (8:2:1, by vol.) of the Driselase-solubilized fraction of ^{14}C-labelled cell walls from cultured spinach (*Spinacia oleracea* L.) cells. The positions of markers are indicated: Ara, L-arabinose; Fuc, L-fucose; Gal, D-galactose; GalA, D-galacturonic acid; Glc, D-glucose; Man, D-mannose; Rha, L-rhamnose; Xyl, D-xylose; XG2, isoprimeverose (α-D-xylopyranosyl-(1 → 6)-D-Glc). For details of procedures, see pp. 180–181.

is the most likely identity of this material because it is the only volatile acid commonly esterified to PCW polysaccharides. Acetate is linked to xyloglucans (York *et al.* 1988), xylans (Northcote, 1972) and pectins (Deuel & Stutz, 1958; O'Neill *et al.* 1990). Appreciable amounts of labelled material that was volatile at high pH were also present and this is presumed to be [^{14}C]methanol derived from the methyl-esterified carboxy (—COOCH$_3$) groups of pectic polysaccharides. Also present in the ELF was some labelled material that was non-volatile at both high and low pH, and this would have included ferulic and *p*-coumaric acids, which are esterified to Ara and Gal residues of pectic polysaccharides in spinach (Fry, 1982, 1983).

DSF. [^{14}C]GalA and [^{14}C]Glc were the major monosaccharides released after Driselase hydrolysis (Figs. 2 and 3; Table 1). This suggests that the ^{14}C-PCW contained large amounts of [^{14}C]pectin and [^{14}C]cellulose. The [^{14}C]uronic acid spot was found by paper electrophoresis to be almost entirely galacturonic acid. Smaller amounts of XG2, Gal, Man, Ara, Xyl, Fuc and Rha were also detected. Chromatographically-immobile components of the DSF would have included the partial digestion products of [^{14}C]starch.

AHF. The ^{14}C-labelled material that was insoluble in Driselase but solubilized in hot HCl (which would include [^{14}C]protein) accounted for less than 2% of the total activity of the ^{14}C-PCW (Table 1). The final HCl-insoluble residue, which would have included lignin if present, was not radioactive. These cultured spinach cells do not show histochemically stainable lignin, although some other primary cell walls do contain lignin.

Starch content. Amyloglucosidase liberated small amounts of [^{14}C]Glc, from which the [^{14}C]starch content was estimated to be about 2·6% of the total ^{14}C-PCW.

Pancreatin digestibility. To assess possible interference by [^{14}C]starch and ^{14}C-labelled protein in our animal experiments, we treated samples of the ^{14}C-PCW with pancreatin, which contains amylase and proteases. DF is, by definition, not digested by the secretions of the pancreas. Pancreatin did not solubilize more material from ^{14}C-PCW than did buffer alone. The pancreatin-solubilized ^{14}C-labelled products were immobile in BAW–EPW (Gray, 1989); distance (mm) moved from the origin in this chromatographic system are: Glc 150, maltose 60, maltotriose 30, maltotetraose 10 and all common amino and imino acids more than 40 (Fry, 1988). Thus, although the work with amyloglucosidase indicated
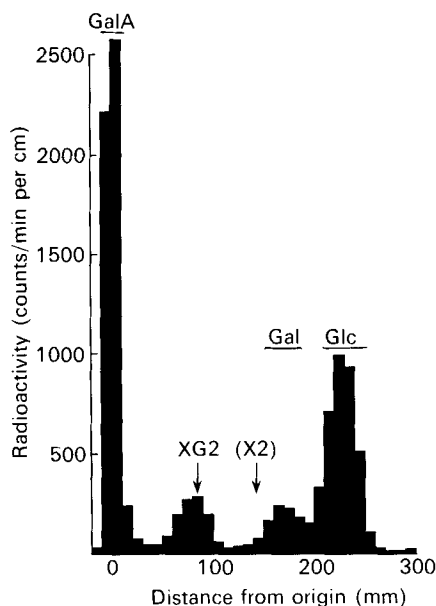
Fig. 3. Re-chromatography for 48 h in ethyl acetate–pyridine–water (8:2:1, by vol.) of the zone containing D-galacturonic acid, isoprimeverose (α-D-xylopyranosol-(1 → 6)-D-glucose), D-galactose and D-glucose after elution from the appropriate strips of the paper chromatogram in butanol–acetic acid–water (12:3:5, by vol.)–ethyl acetate–pyridine–water (8:2:1, by vol.) of the Driselase-solubilized fraction of ^{14}C-labelled cell walls from cultured spinach (*Spinacia oleracea* L.) cells. The positions of markers are indicated: Gal, D-galactose; GalA, D-galacturonic acid; Glc, D-glucose; XG2, isoprimeverose (α-D-xylopyranosyl-(1 ⟩ 6)-D-Glc); X2, xylobiose (β-D-xylpyranosyl-(1 ⟩ 4)-D-Xyl). For details of procedures, see pp. 180–181.

the presence of [14C]starch, this was not readily digestible to malto-oligosaccharides without previous gelatinization at 100°. Similarly, the ^{14}C-labelled protein was not readily digested by pancreatin.

Deduction of polymeric distribution of [14]C

It is possible to make some deductions about the ratios of the ^{14}C-labelled polymers present in the ^{14}C-PCW (summarized in Table 2), based on knowledge of the chemistry of typical PCW polysaccharides (for review, see Fry, 1988). Although these deductions are not precise analyses of polymer composition, we believe that they nevertheless provide a helpful picture of the nature of the labelled DF used in the animal experiments.

Consider first the pectic material. All GalA is assumed to be associated with pectic polysaccharides and all Rha with rhamnogalacturonan. If rhamnogalacturonan is assumed to have a GalA:Rha value of 1:1 (mol/mol) (as in the more abundant of the known rhamnogalacturonans, rhamnogalacturonan-I; O'Neill *et al.* 1990), then the GalA is distributed between homogalacturonan and rhamnogalacturonan in the ratio approximately 9:1 ((5·2–0·5)/0·5). Assuming that most of the non-pectic Ara and Gal residues are associated with xylans and xyloglucans respectively, then from the information on these hemicelluloses (see p. 185) the amount of pectic Ara and Gal can be estimated. Most of the [14C]methyl ester groups occur on GalA residues, which are thus estimated to possess a degree of methyl esterification of 25 % (1·3/5·2). *O*-Acetyl groups are found in both pectins and hemicelluloses and the distribution between these two polysaccharides is not known. At most, 38 % (2·0/5·2) of the GalA residues could have been *O*-acetylated. (It has been shown (p. 186) that most of the acetate groups must be associated with pectins; as a rough

Table 2. *Deduced ^{14}C distribution between major polymeric groups in ^{14}C-labelled cell walls from cultured spinach* (Spinacia oleracea *L.*) *cells*

Polymer	Residue	Molar ratio	% of [14]C	
Pectins				
Homogalacturonan	Galacturonic acid	4·7	28·3	
	Methyl ester	1·3	1·3	28–33*
	O-Acetyl ester	1·5(?)	3·0	
Rhamnogalacturonan	Galacturonic acid	0·5	3·0	
	Rhamnose	0·5	2·9	21–25*
	Arabinose†	1·5	7·5	
	Galactose†	0·7	4·4	
	Ferulate etc.	0·3	2·9	
Hemicelluloses				
Xyloglucan	XG2	0·6	6·7	
	Fucose	0·1	0·6	
	Galactose	0·1	0·6	9–10*
	Glucose	0·2	1·2	
	O-Acetyl ester	0·5(?)	1·0	
Xylans	Xylose	0·3	1·7	about 3*
	Arabinose	0·2	1·0	
Mannans	Mannose	0·1		0·5
Cellulose	Glucose	3·5		21·3
Non-wall components				
Starch	Glucose	0·4		2·6
Protein		0·3		1·8
Lignin	(HCl-insoluble)	0·0		0·0

XG2, isoprimeverose (α-D-xylopyranosyl-(1 → 6)-D-glucose).

* The range quoted arises because the distribution of methyl and O-acetyl esters between the various polysaccharides is unknown.

† Arabinose and galactose are arbitrarily included under rhamnogalacturonan, but may include some arabinan, galactan and arabinogalactan not directly associated with galacturonic acid-rich polysaccharides.

approximation, it is suggested that approximately 30% of the GalA residues are O-acetylated.) The composition (mol/mol) of the pectic polysaccharides can, thus, be summarized (Table 2).

The hemicelluloses of these cell walls are mainly xyloglucans and xylans (Fry, 1988). Assuming a xyloglucan composition typical of other dicotyledons (XG2:Glc:Gal:Fuc approximately 6:2:1:1 mol/mol; Fry, 1989), then, since all XG2 in the DSF arises from xyloglucan, the [14C]XG2 content allows an estimate to be made of the proportion of the Gal and Glc that is derived from xyloglucan. The remainder of the Gal (i.e. the majority) is assumed to be pectic and the remainder of the Glc (also the majority) is assumed to be cellulosic. The observed XG2:Fuc ratio (6:1 mol/mol) is compatible with essentially all the fucose residues in the spinach cell walls occurring in xyloglucan. Similarly, assuming a xylan composition typical of other dicotyledons (Xyl:Ara approximately 3:2, with traces of GlcA), then, since all xylose arises from xylan, the [14C]Xyl content of the ^{14}C-PCW allows an estimate to be made of the proportion of the Ara that is derived from xylan. The remainder (the majority) is assumed to be of pectic origin. Traces of mannose were detected, indicating the presence of traces of mannans; the other sugars possibly associated with these putative mannans would be negligible. The hemicelluloses will have O-acetyl groups attached to the Gal residues of xyloglucan and the Xyl residues of xylan. In the ^{14}C-PCW there is about a 4-fold molar excess of [14C]acetate over what would be needed to

provide one *O*-acetyl group per Xyl residue in xylan and per Gal residue in xyloglucan. Thus, although the degree of acetylation of the hemicellulose is unknown, it is likely that most of the [^{14}C]acetate was of pectin origin (Table 2).

Two remaining polysaccharides are cellulose and starch. [^{14}C]Glc in the DSF will be mainly cellulosic in origin, minus the amount formed from the non-xylosylated Glc residues of xyloglucan (Table 2). Starch is poorly digested by Driselase (S. Aldington & S. C. Fry, unpublished results) and will contribute mainly to the chromatographically-immobile material in the DSF. It has been shown in the present study that [^{14}C]starch accounts for only 2·6% of the total ^{14}C, and the failure of pancreatin to yield any [^{14}C]Glc or [^{14}C]maltose suggests that the trace of starch present was a relatively enzyme-resistant form consistent with its inextractability by DMSO (900 ml/l).

Proteins are also partially degraded by Driselase and will contribute water-soluble products that are immobile in BAW–EPW. The 1·8% of the total ^{14}C that was solubilized by HCl may also have been derived from protein. Again, the resistance of the ^{14}C-PCW material to conversion to chromatographically-mobile products by pancreatin argues against protein making an appreciable contribution to our results.

Study of the long-term fate of ^{14}C-PCW in the rat

An experiment was conducted to determine the fate of the ^{14}C-PCW characterized in the present paper when fed as DF to rats. The ^{14}C-PCW were supplied as a pulse, at 'tracer' levels, to rats raised on a diet containing 133 g NSP (as defined by the Englyst *et al.* (1982) technique)/kg dry weight. It was, therefore, expected that the dose of ^{14}C-PCW would not of itself evoke any appreciable change in the nature or behaviour of the gut microflora. The trial period lasted 4 d in order to maximize the output of ^{14}C. The aim of the experiment was to investigate the degradation of ^{14}C-PCW when fed to rats. In the present work, only the non-gaseous ^{14}C-labelled products were investigated and no attempt has been made to identify the chemical nature of the labelled products; a complete description of the fate of this labelled DF will require measurement of ^{14}CO$_2$ output and characterization of the ^{14}C-labelled polysaccharides, microbial biomass and short-chain fatty acids in the gut contents and faeces, as well as of the mammalian metabolites in the carcass.

Table 3 shows the distribution of ^{14}C in the faeces and urine of the rats after 1, 2 and 3 + 4 d, and in the caecal contents and selected tissues of the carcass after 4 d. Only about 20% of the ^{14}C was excreted in faeces and urine over the 4 d period, the majority in the first 24 h, and this will be an overestimate of the residual ^{14}C-PCW as there will have been some incorporation of ^{14}C into bacterial cells. This means that a very high proportion of this ^{14}C-PCW preparation must have been fermented during transit through the GI tract. Cellulose is probably the least readily fermented component of the ^{14}C-PCW; however, since they contained about 21% [^{14}C]cellulose, at least some of the cellulose must have been fermented. The great majority of the pectic and hemicellulosic polysaccharides must also have been fermented.

The low level of ^{14}C in the caecal contents indicates that almost all the C derived from the spinach PCW had passed out of the GI tract by day 4. Although small quantities of ^{14}C were recovered in the urine, there were traces of particulate material in the urine samples suggesting some faecal contamination; it seems clear that little ^{14}C was excreted in the urine.

The ^{14}C in faeces, urine and caecal contents together accounted for about 20% of the total ^{14}C dose (Table 3). Very little additional ^{14}C was recovered in the caecal tissue (which might be expected to take up products of bacterial fermentation) and only about 1% in the liver. Adipose tissue (1 g) contained 0·25% of the dosed ^{14}C, and the mean body-weight of the rats was 185 g; this might indicate that a significant proportion of the ^{14}C can be

Table 3. *Distribution of* ^{14}C *from* ^{14}C-*labelled cell walls from cultured spinach* (Spinacia oleracea L.) *cells fed to rats**

(Mean values with their standard errors for three rats)

Sample	Percentage of dose ^{14}C recovered†			Mean	SE
	Rat A	Rat B	Rat C		
Faeces					
Day 1	10·9	16·4	20·3	15·9	1·6
Day 2	2·51	1·17	1·20	1·63	0·26
Days 3 + 4	0·47	0·64	0·70	0·60	0·04
Urine					
Day 1	0·70	3·06	0·81	1·52	0·44
Day 2	0·21	0·31	0·31	0·28	0·02
Days 3 + 4	0·21	0·17	0·23	0·20	0·01
Caecal contents					
Day 4	0·07	0·06	0·07	0·07	;0·01
Tissues (day 4)					
Liver	1·06	1·25	1·42	1·24	0·06
Caecum	0·14	0·09	0·23	0·15	0·02
Adipose‡	0·21	0·32	0·23	0·25	0·02
Skin‡	0·15	0·11	0·12	0·13	0·01
Body wt (g)	190	162	204		

* For details of procedures, see pp. 181–182.
† Means of two measurements from each sample.
‡ Values for adipose and skin are given as % (of dosed ^{14}C) recovered/g tissue.

attributed to the remaining carcass, although probably not as much as 46 % (0·25 × 185) since the value in the adipose tissue may be misleadingly high (cf. skin values in Table 3).

Substantial amounts of the ^{14}C were, thus, not recovered, and are presumed to have been lost as ^{14}CO$_2$, directly by the respiratory activity of the gut microflora or by respiration (in rat tissues) of compounds such as short-chain fatty acids generated by micro-organisms, or both. Some of the ^{14}C may also have been lost as ^{14}CH$_4$.

In conclusion, the ^{14}C-labelled primary cell walls of spinach, although being resistant to digestion by pancreatin, were extensively (> 80 %) fermented in the rat GI tract. The wall material contained (approximate %) pectic polysaccharides 53, hemicelluloses 13 and cellulose 21; the fermentation was, therefore, so extensive that not only the matrix polysaccharides but also the microfibrillar cellulose must have been at least partially fermented. The wall material contained about 4 % acetyl ester groups and up to about 3 % saponifiable phenolic residues (probably mainly attached to pectic polysaccharides); these substituents, although able to impede the enzymic digestion of polysaccharides in vitro, were clearly not able to prevent the extensive fermentation of spinach cell walls in the large intestine of the rat. The radiochemical analytical methods developed here will enable a more detailed monitoring of the sequence in which the wall polysaccharides are degraded as they pass along the GI tract. However, the present results confirm that DF in the form of primary cell walls can be extensively digested and can, therefore, make a contribution to the animal's C and energy requirements.

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