Symposium 3

The use of ^{14}C-labelled substrates to study plant cell wall breakdown in the gastrointestinal tract

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In both herbivores and omnivores (e.g. man) plant material is an important component of the diet. Much of the dietary plant material (e.g. starch, proteins, lipids) is digested in the upper gut but a significant proportion resists digestion and reaches the caecum and colon. The bulk of non-digestible plant material is cell wall (CW). The mammalian small intestine does not produce any enzymes capable of degrading CW polysaccharides (Miller *et al.* 1995). However, on reaching the caecum and colon, CW polysaccharides are extensively degraded to simple sugars and uronic acids by caecal and colonic bacteria. Bacteria generate energy by the fermentation of sugars and uronic acids and generate endproducts such as volatile fatty acids (VFA), other carboxylic acids and gases (Kelleher *et al.* 1984; Bonhomme-Florentin, 1988; Cummings & Macfarlane, 1991). These may be a significant source of endogenous short-chain fatty acids such as acetate and propionate in mammals (Scheppach *et al.* 1991). Fermentation endproducts may subsequently be absorbed by the gut mucosa and transported to the liver via the portal blood system. These fermentation endproducts are presumed to be metabolized by the liver and their products exported to the other body tissues (Cummings & Macfarlane, 1991) and may represent a significant salvage of C especially in starved or actively-growing animals (Bugaut & Bentéjac, 1993).

CELL WALL STRUCTURE

Parenchymatous cells are the most prevalent cell type in dietary plant material such as fruit and vegetables (McDougall *et al.* 1996). We have used cultured spinach (*Spinacia oleracea* L., cv. Monstrous Viroflay) cells in our studies as they can be grown heterotrophically in a simple, fully-defined medium and their CW have a well characterized chemical composition. Cultured spinach cells are rapidly-growing undifferentiated cells and possess only primary CW, whose composition is typical of that of the parenchymatous CW of many plants (Gray *et al.* 1993*a*).

Primary CW are composites of three major classes of polysaccharides: cellulose, hemicelluloses and pectins. Primary CW also contain structural glycoproteins and small amounts of phenolic material. Pectins are a group of heterogeneous polysaccharides of high molecular weight (MW) that are present in the CW and middle lamellae and constitute 30–50% of the total CW polysaccharide (Fry, 1988). Pectins are rich in D-galacturonic acid (GalA) units linked through α-(1→4) glycosidic bonds. Within the pectin molecule there

are areas of homogalacturonan composed mainly of unbranched GalA residues with occasional rhamnose units. There are also areas of pectinase (*EC* 3.2.1.15)-resistant rhamnogalacturonans which are composed of GalA plus other sugar residues such as rhamnose, galactose (Gal) and arabinose (Ara; O'Neill *et al.* 1990). Some of the sugar residues in CW hemicelluloses and pectins may be esterified to molecules such as methanol, acetate or phenolic acids. For example, acetate is esterified to the Gal residues of xyloglucans or the mannose residues of glucomannans (York *et al.* 1988). In pectins GalA residues may bear acetyl groups esterified to the -OH groups and methyl groups esterified to the -COOH groups. In addition to methanol and acetate the polysaccharides of plant CW may carry esters of phenolic acids such as ferulic acid (FA) and *p*-coumaric acid (PCA; Wallace & Fry, 1994). Of the saponifiable FA in spinach primary CW 60% is attached to pectins, esterified to Gal or Ara residues in rhamnogalacturonans (Fry, 1982, 1983). Most of the remainder is present in an enzyme-resistant fraction. Fry (1984) proposed a role for peroxidase (*EC* 1.11.1.7)-mediated phenolic coupling in strengthening CW by cross-linking polymers via phenol groups. A highly-cross-linked CW would also impede the penetration of hydrolytic enzymes into the CW matrix and, therefore, limit degradation.

USE OF ^{14}C-LABELLED SUBSTRATES IN NUTRITION

Studies of colonic carbohydrate metabolism in mammals have used polymers extracted from CW (Carryer *et al.* 1982; Kelleher *et al.* 1984; Eastwood *et al.* 1986; Bonhomme-Florentin, 1988; Walters *et al.* 1989) or radio-labelled, poorly-absorbable, monosaccharides (Brydon *et al.* 1987). The extraction processes may alter the chemical and physical properties of the polysaccharide; thus, the metabolism of CW polysaccharides may differ between extracted polymers and polymers *in situ* in the cell wall. Even where chemical modification of polymers during extraction is minimized, it is still likely that a soluble polysaccharide would differ from an otherwise identical wall-bound polysaccharide in its susceptibility to enzymic digestion. Studies using extracted polymers involve the feeding of large amounts of the test substance which also may interfere with the normal bowel functioning. The availability of a chemically-defined radioactively-labelled plant CW (U-^{14}C-labelled CW) preparation has facilitated direct investigation of the fate of CW sugar residues as they pass along the rat gastrointestinal tract (Gray *et al.* 1993*a*,*b*). It also enables the subsequent redistribution of the ^{14}C in the host tissues, excretion products and expired gases to be studied. Recently, uniformly-labelled plant CW have been used to study the degradation of plant CW in fowl and, in particular, the effects of pre-processing on CW degradation (Savory, 1992*a*,*b*).

By using radioactive U-^{14}C-labelled CW, an investigation of all CW-derived organic metabolites is possible irrespective of their chemical nature and destination in the body tissues, urine, faeces, or expired gas. The high specific activity of the ^{14}C-labelled CW has advantages in that only small amounts (typically 5–10 mg) need to be fed to the rats. Therefore, there is minimal interference with the host diet or metabolism. Feeding of intact labelled CW more accurately reflects the fate of these residues in the gut than would be possible using purified polysaccharide preparations.

Whole CW of cultured spinach cells were labelled with ^{14}C in specific C atoms to provide substrates with which to investigate the fermentation of the pectic components (in

Table 1. *Distribution of ^{14}C within labelled spinach cell-wall preparations* (From Fry, 1984; Gray *et al.* 1993*a*; Miller *et al.* 1994)

Source of cell-wall radioactivity . . . Component	Percentage of total radioactivity				
	[U-^{14}C] Glucose	[6-^{14}C] Glucuronate	[*methyl*-^{14}C] Methionine	[1-^{14}C] Acetate	*trans*-[U-^{14}C] Cinnamic acid
Glucose*	22·5	0	<1	0	0
Galactose	5·0	0	<1	0	0
Arabinose	8·5	0	<1	0	0
Xylose	1·7	0	<1	0	0
Galacturonic acid	31·3	85·5	<1	0	0
XG2	6·7	0	<1	0	0
Methyl ester	1·3	0	90·0	0	0
Acetyl ester	4·0	0	0	39	0
N-acetyl amide	<1	0	0	25	0
Phenolic esters	<2·9	0	0	0	60
R_f zero†	4·3	12·7	7·5	ND	ND

XG2, Xylosyl-α-(1→6)glucose; ND, not determined.

* Other than included in XG2.

† Chromatographically immobile (e.g. paper chromatography in butan-1-ol–acetic acid–water; 12:3:5 by vol.) after Driselase digestion.

intact CW) in the rat gastrointestinal tract. Although there are differences between cultured cells and intact plant material (e.g. the presence of secondary wall material in the latter), feeding of intact ^{14}C-pectin-labelled CW more accurately reflects the fate of dietary pectic residues in the gut than would be possible using solubilized and purified polysaccharide preparations.

PREPARATION AND ANALYSIS OF LABELLED CELL WALLS

Radioactively-labelled plant CW are prepared by dosing cell cultures with a suitable radioactive metabolic precursor. Once the label has been incorporated, the cells are harvested and the CW isolated. In this way uniformly-labelled CW (U-^{14}C-labelled CW) have been produced and characterized in detail (Gray *et al.* 1993*b*) and their composition is shown (along with that of other CW preparations) in summary in Table 1. Since D-[U-^{14}C]glucose was the only C source for the cultures it follows that all organic components of the CW will be labelled to approximately the same atomic specific radioactivity. U-^{14}C-labelled CW provide a useful substrate for determining the total extent of CW breakdown in the gut before studies of specific polysaccharide residues.

The polygalacturonate backbone of the pectin molecules was labelled by dosing the cell cultures with D-[6-^{14}C]GalA. After harvesting, the CW were analysed by enzymic degradation and the nature of the labelled sugar residues was determined by chromatography. ^{14}C from exogenous D-[6-^{14}C]glucuronic acid becomes incorporated into UDP-[^{14}C]uronic acids. These are not reduced to UDP-[^{14}C]glucose or UDP-[^{14}C]Gal, but may be decarboxylated to UDP-xylose whereupon the ^{14}C is lost as $^{14}CO_2$. Of the ^{14}C in the CW 85% had been incorporated into GalA of pectins. There was no evidence for the

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incorporation of radioactivity into neutral sugars. The remaining radioactivity may include uronic acid-containing polysaccharides that are resistant to enzymic hydrolysis (e.g. rhamnogalacturonan-II) and/or tightly associated with phenolic material.

Pectins possess methyl ester groups which were labelled by feeding cell cultures L-[*methyl*-^{14}C]methionine. The position of the label was determined by hydrolysis, (enzymically or with acid or alkali), which releases ^{14}C-labelled methyl esters as [^{14}C]methanol. Of the ^{14}C 90% was volatile (presumably as $^{14}CH_3OH$) after hydrolysis suggesting that the ^{14}C in these CW was located in the methyl ester groups. We partially characterized the remaining 10% of the ^{14}C that was not ^{14}C-labelled methyl ester. This fraction included methyl etherified sugars such as 2-*O*-methylxylose and 2-*O*-methylfucose, in addition to ^{14}C-labelled methoxylated phenolics such as FA and its degradation products.

Polysaccharides such as pectins and hemicelluloses may contain sugar residues which are esterified with acetate. To label these groups we fed cell suspension cultures with [1-^{14}C]acetate. Analysis of the labelled CW using acid- or alkaline-hydrolysis indicated that 39% of the ^{14}C was present as *O*-acetyl esters and a further 26% as *N*-acetyl amides. The remaining ^{14}C was distributed among a range of unidentified CW components (each comprising less than 5% of the remaining ^{14}C), including a lipid-like component, although not among any of the major CW sugars (Miller *et al.* 1994).

Phenolic residues in spinach CW were ^{14}C-labelled by supplying cultured spinach cells with *trans*-[U-^{14}C]cinnamic acid. Cinnamic acid is rapidly taken up by plant cells and may be converted to several hydroxycinnamic acids (e.g. PCA, caffeic, FA, or sinapic acids). CW labelled in this manner have been characterized and found to contain ^{14}C-labelled feruloyl (Fer) and *p*-coumaroyl residues (Fry, 1984). Of the Fer residues in spinach CW 60% are esterified to Gal or Ara residues of polysaccharides, and are released on Driselase digestion as the Fer disaccharides, Fer-Ara_2 and Fer-Gal_2. Most of the coumaroyl groups appear to be linked in a similar manner. A significant proportion of the ^{14}C was incorporated into Driselase-resistant material that on saponification yielded chromatographically-immobile material presumed to include oxidatively-coupled phenols (Fry, 1984).

DIGESTION OF U-^{14}C-LABELLED CELL WALLS IN THE GUT

Studies using uniformly-labelled primary plant CW have shown that they are extensively degraded in the rat gut. Radioactivity disappeared from the gut contents and was subsequently detected in the urine, CO_2 and body tissues (Table 2) after several hours (Gray *et al.* 1993*a*,*b*). Unabsorbed ^{14}C in the gut contents would include undegraded CW material, bacterial cells and metabolites. The liver and pelt were highly labelled and, since they are the largest body organs, contained the greatest accumulation of ^{14}C in any of the tissues (Gray, 1993*b*; Buchanan *et al.* 1994*a*). Liver tissue was partitioned into a chloroform-soluble phase (mainly lipid), an aqueous methanol (500 ml/l)-soluble phase (sugars, gangliosides, free amino acids and other water-soluble material) and an insoluble fraction (mainly protein and glycogen). The chloroform fraction contained 61% of the ^{14}C, 19% was present in the aqueous phase and 20% was insoluble. The bulk of the radioactivity in the lipid-containing fraction was phospholipids with a minor amount present as triacylglycerols. Radioactivity in the pelt was present as chloroform-soluble material

Table 2. *Distribution of ^{14}C in rat tissues and excreta after dosage with ^{14}C-labelled primary plant cell walls* (From Gray *et al.* 1993*a*,*b*; Buchanan *et al.* 1994*a*,*b*, 1995*a*,*b*, 1996)

Position of labelling	Fraction	Percentage of dosage at time-intervals (h) after administration			
		2	4	6	18
Uniform	Gut contents and faeces	ND	ND	75	11
	Body tissues	ND	ND	18	30
	Urine	ND	ND	ND	2·3
	CO_2	2	3·5	8	25
Uronate (C-6, pectins)	Gut contents and faeces	81	82	43	8·9
	Body tissues	3·6	9·8	27	21
	Urine	0·04	0·24	0·26	2·2
	CO_2	0·00	0·38	3·9	37
Methyl esters (pectins)	Gut contents and faeces	110	72	66	5·0
	Body tissues	5·0	11	21	60
	Urine	0·12	0·21	0·56	1·9
	CO_2	0·11	0·53	3·6	18
Acetyl esters (pectins and hemicelluloses)	Gut contents and faeces	90	85	68·5	ND
	Body tissues	15	26	19	ND
	Urine	0·18	0·23	0·71	ND
	CO_2	0·05	4·1	4·0	ND
Phenolics (pectins and hemicelluloses)	Gut contents and faeces	74	67	70	24
	Body tissues	26	33	42	34
	Urine	0·9	1·7	2·6	20
	CO_2	0·0	0·0	0·0	0·3

ND, not determined.

(55%), which was characterized as the fatty acid moiety of steryl esters. The bulk of the remaining ^{14}C in the pelt was insoluble, and acid-hydrolysis released this mainly as amino acids, principally alanine and glutamic acid. Analysis of radioactivity remaining in the faeces indicated that most of the major CW sugars had been digested, including half the glucose residues attributable to cellulose (Gray *et al.* 1993*a*).

DIGESTION OF *URONATE*-6-^{14}C-LABELLED CELL WALLS IN THE GUT

Under simulated conditions of the stomach and small intestine some solubilization of labelled uronate residues from spinach CW was observed, although the solubilized material was polymeric and there was no evidence of degradation to oligogalacturonides or monosaccharides (Miller *et al.* 1995). In agreement with this observation, only small amounts of ^{14}C were solubilized during passage through the small intestine of the rat, but

on reaching the caecum the ^{14}C became rapidly soluble. The soluble material contained labelled VFA, mainly propionate and higher VFA, with a small amount of formate. The remaining water-soluble radioactivity was non-volatile and included some high MW material which may be soluble bacterial protein or pectins (or oligogalacturonides) released from the plant CW. Also present was some low MW acidic material which may include free GalA or carboxylic acids produced by bacterial fermentation (Buchanan *et al.* 1995*b*).

Although only small amounts of ^{14}C had reached the caecum 2 h after oral administration, many body tissues contained some label; later most body tissues accumulated label in increasing quantities (Table 2). The distribution of label in the body tissues was similar to that observed with uniformly-labelled CW. The liver and pelt are the largest organs and accumulated most label. The radioactivity in the liver (1·4% of intake) was shown to be mainly due to lipid-soluble material (62% of label in liver). However, a significant amount of label (32% of label in liver) was water soluble but was not identified. This water-soluble material may contain free amino acids or Krebs' cycle intermediates. In lipid extracted from the liver, the label was present mainly as phospholipid with a minor amount attributable to cholesteryl esters. The remaining radioactivity in the liver was present as insoluble material. The pelt was highly labelled (12–15% of intake), and the radioactivity was shown to be entirely associated with the insoluble fraction. Alkaline-hydrolysis of the rat pelt chloroform-insoluble material revealed that the label was predominantly present as non-essential amino acids such as aspartic acid, asparagine, glutamic acid and alanine (Buchanan *et al.* 1994*a*).

DIGESTION OF CELL-WALL METHYL AND ACETYL ESTER GROUPS IN THE GUT

Artificial pancreatic juice caused the slow, non-enzymic removal of methyl ester groups (half-life 25 h) from intact CW containing [*methyl*-^{14}C]pectin (Miller *et al.* 1995). However, since the CW are only present in the upper gut for 2–3 h, non-enzymic hydrolysis can only account for the release of a small proportion of the methyl ester residues in the upper gut. *In vivo* only small amounts of ^{14}C were solubilized from *methyl*-^{14}C-labelled CW during passage through the small intestine, although ^{14}C was rapidly solubilized when it reached the caecum. Samples of the soluble radioactivity contained volatile ^{14}C which was present as both [^{14}C]methanol (35–52% of the soluble ^{14}C) and ^{14}C-labelled VFA (35–58% of the soluble ^{14}C). The VFA fraction contained labelled acetate plus higher VFA (Buchanan *et al.* 1995*b*). *In vitro* experiments using fresh rat caecal contents suggest that initially [^{14}C]methanol is released from *methyl*-^{14}C-labelled CW and is then converted to VFA, presumably by the activities of intestinal methylotrophic bacteria (C. J. Buchanan, S. C. Fry and M. A. Eastwood, unpublished results). The production of both acetate (Englyst *et al.* 1987) and formate (Werch *et al.* 1942) has been demonstrated during the fermentation of soluble pectin in mammals and the release of methanol from soluble pectins by human colonic bacteria has been demonstrated *in vitro* (Siragusa *et al.* 1988).

All the body tissues were labelled, although the liver and pelt accumulated most radioactivity. The label present in the liver (3% of intake) was mainly lipophilic (92% of the label in the liver). In liver-lipid extracts the ^{14}C was predominantly associated with phospholipid with some minor label in triacylglycerols. The pelt was highly labelled (6% of intake) and two-thirds of the label in the pelt was present as the fatty acid moiety of

steryl esters while the remaining label had been incorporated into non-essential amino acids such as alanine (Buchanan *et al.* 1994*b*).

Some [^{14}C]acetate (approximately 10%) was released from *acetyl*-^{14}C-labelled CW during passage through the small intestine (Buchanan *et al.* 1995*a*). The pancreatic secretions of mammals contain esterases which are involved in the digestion of dietary lipids. However, such esterases are not active on acetyl esters in dietary CW (Miller *et al.* 1995). The alkaline conditions of the small intestine may contribute to the partial de-esterification (ester bonds are alkali-labile). However, *in vitro* studies have suggested that little of the loss of acetyl groups is due to the endogenous secretions of the gastrointestinal tract, as acetyl ester groups are more stable than methyl ester groups in this environment (Miller *et al.* 1995). The most likely explanation for the observed release of acetate from CW is the initiation of degradation of CW by indigenous micro-organisms inhabiting the small intestine (Macy *et al.* 1982) of the rat. On reaching the caecum the remaining labelled acetyl groups were rapidly released from the CW as [^{14}C]acetate (Buchanan *et al.* 1995*a*).

The label released from the CW was absorbed from the gut and incorporated into the body tissues (Table 2). Radioactivity was present in all the body tissues but predominantly in the liver (3·7–4·4% of intake) and pelt (5·6–7·5% of intake). In the liver, most label was incorporated into amino acids. Marty & Vernay (1984) demonstrated that [1-^{14}C]acetate introduced directly into intestinal loops (caecum) of rabbits is rapidly absorbed and transported to the liver; much of the label was then available to other tissues. In excised liver, [1-^{14}C]acetate was rapidly incorporated into free amino acids, other carboxylic acids and sugars (Marty & Vernay, 1984).

DIGESTION OF PRIMARY-CELL-WALL PHENOLICS

The rate of degradation of CW may be influenced by the presence of wall-bound phenolic groups. In ruminants, CW-bound phenolic acids limit the degradability of forage material (Hartley & Jones, 1977) and a similar inhibition may occur in single-stomached animals. *In vitro* the rate of degradation of CW polysaccharides by rumen micro-organisms is depressed by free phenolic acids (Jung, 1985; Jung & Sahlu, 1986), which inhibit the growth and enzyme activity of bacteria (Chesson *et al.* 1982; Martin & Akin, 1988).

After rats were dosed with *phenolic*-^{14}C-labelled CW, the presence of radioactivity in the body tissues and excreta of the rat was determined as an assessment of the degree to which the phenolic residues of the CW were taken up and metabolized by the host (Table 2). At 2 h after oral administration most label was present in the stomach and small intestine with only a minor proportion reaching the lower gut. However, by 2 h a significant proportion of the label was present in the body tissues or had been excreted in urine, indicating that some label had been released from the CW and absorbed by the host animal. In both the stomach and small intestine ^{14}C was associated mainly with the insoluble fraction. Phenolic acids which had been released from CW may have been rapidly absorbed by the gastric or intestinal mucosa and, therefore, were not evident in the gut contents (Buchanan *et al.* 1996). Phenolic-sugar esters are hydrolysed by porcine pancreatic esterases (Kato & Nevins, 1985), although the removal of phenolic ester groups in the small intestine may be limited by the inability of enzymes to penetrate the intact CW. Fer esters are considerably more alkali-stable (Fry, 1982) than methyl and acetyl esters and, thus, would be essentially resistant to non-enzymic hydrolysis in the gut. The removal of

phenolic groups from CW in the small intestine is likely to be due partly to bacterial activity.

Most of the labelled phenolic groups in the CW survived passage through the upper gut and reached the caecum. On reaching the caecum, labelled phenolic acids (PCA and FA) and soluble, chromatographically-immobile phenolic material, derived from *phenolic*-^{14}C-labelled CW, and likely to contain oxidatively-cross-linked phenolics, were released. There was evidence for the removal of free phenolic acids from the gut (presumably by absorption into the body tissues) and for the accumulation in the gut contents of chromatographically-immobile material, much of which survived passage through the gut to be excreted in faeces. Phase-partitioning of the soluble material in the gut contents suggested that hydrophilic (sugar-associated) ^{14}C-labelled phenolics are released from the CW and subsequently become less hydrophilic, presumably as sugar groups are removed (Buchanan *et al.* 1996).

CONCLUSION

Intra-caecal and/or colonic metabolism of plant CW polysaccharides by gut bacteria may have considerable benefit to the host animal in terms of recovery of dietary C and may provide valuable contributions to both energy and supply of precursors of structural compounds. Most of the observed labelled compounds (lipids and amino acids) in the body tissues could be derived from Krebs' cycle intermediates and it is likely that bacterial fermentation endproducts are sequestered to Krebs' cycle on entering the liver. The presence of simple phenolic esters did not limit the extent of degradation of CW in the rat intestine as evidenced by the almost complete removal of such groups by the caecal and colonic bacteria. However, the presence of oxidatively-coupled phenols (chromatographically-immobile fraction) may be a significant factor in limiting CW digestion since these compounds were the major ^{14}C-labelled phenolic fraction to survive passage through the gut into the faeces.

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