Fermentation and subsequent disposition of ^{14}C-labelled plant cell wall material in the rat

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A ^{14}C-labelled plant cell wall preparation (^{14}C-PCW) produced from spinach (*Spinacia oleracea* L.) cell culture exhibits uniform labelling of the major polysaccharide groups (%): pectins 53, hemicellulose 13, cellulose 21, starch 3. This ^{14}C-PCW preparation has been used in rat studies as a marker for plant cell wall metabolism. Metabolism of the ^{14}C-PCW occurred largely over the first 24 h. This was due to fermentation in the caecum. The pectic fraction of the plant cell walls was degraded completely in the rat gastrointestinal tract, but some [^{14}C]cellulose was still detected after 24 h in the colon. Of the ^{14}C, 22% was recovered in the host liver, adipose tissue and skin, 26% excreted as $^{14}CO_2$ and up to 18% was excreted in the faeces. There was no urinary excretion of ^{14}C. In vitro fermentation using a caecal inoculum showed reduced $^{14}CO_2$ production, 12% compared with 26% in the intact rat. ^{14}C-PCW is a useful marker to investigate the fate of plant cell wall materials in the gastrointestinal tract. These studies show both bacterial fermentation of the ^{14}C-PCW and host metabolism of the ^{14}C-labelled fermentation products.

Dietary fibre: Plant cell walls: Fermentation: Rat

Plant cell wall material (PCW; dietary fibre; Trowell *et al.* 1976) is an important contributor to dietary complex carbohydrates. PCW is composed of three major classes of polysaccharides, pectin, hemicellulose and cellulose (Selvendran, 1985). Within these three polysaccharide classes there are many individual distinct structures, functions and properties. It is the diversity of these polysaccharide types and their varying abundance in PCW that has led to the difficulties in study design and interpretation (Eastwood & Passmore, 1983). Many studies have been conducted on individual PCW and other extracted materials giving important, but of necessity, indirect evidence of their action and metabolism in the colon by bacteria (Eastwood *et al.* 1986).

The availability of a chemically-defined, radioactivity-labelled (^{14}C) plant cell wall (^{14}C-PCW) facilitates direct investigation of the fate of individual sugars within the PCW, as they pass along the rat gastrointestinal tract. This enables the subsequent redistribution of the radioactive labelling in the host tissues, excretion products and expired gases to be studied. The details of the production and analysis of the ^{14}C-PCW have been reported elsewhere (Gray *et al.* 1993). The use of radioactivity in dietary fibre feeding trials is not new and includes studies of cellulose (Carryer *et al.* 1982; Kellehar *et al.* 1984; Walters

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Table 1. *Deduced ^{14}C distribution in polysaccharide groups in spinach* (Spinacia oleracea *L.*) *cell wall*

Polysaccharide	Residue	Molar ratio§	^{14}C (%)‖
Pectins	Arabinose	0·42	7·5
	Galactose	0·21	4·4
	Galacturonic acid	1·47	31·3
	Rhamnose	0·14	2·9
	Methyl ester	0·37	1·3
	O-Acetyl ester	0·42	3·0
	O-Feruloyl etc.	0·08	2·9
	Total		53·3
Hemicelluloses	Arabinose	0·06	1·0
	Fucose	0·03	0·6
	Galactose	0·03	0·6
	Glucose*	0·06	1·2
	Mannose	0·02	0·5
	XG2†	0·17	6·7
	Xylose*‡	0·10	1·7
	O-Acetyl ester	0·14	1·0
	Total		13·3
Cellulose	Glucose	1·00	21·3
Starch	Glucose	0·12	2·6

* Other than that included in XG2.
† Xylosyl-α-(1→6)-glucose, derived from xyloglucan.
‡ Derived from xylans.
§ Mol ^{14}C-labelled residue per mol cellulosic [^{14}C]glucose residues.
‖ Percent of total ^{14}C in plant cell wall material.

et al. 1989). This is the first report to utilize intact ^{14}C-PCW in which all three major polysaccharide groups are labelled.

METHODS

PCW were prepared according to the previous description of Gray *et al.* (1993) and the deduced ^{14}C distribution in the polysaccharide groups is shown in Table 1 (values from Gray *et al.* 1993). The molar ratio values were calculated by fixing the value of cellulosic [^{14}C]glucose residues at 1·00. Of the residue 9·5% could not be accounted for. The PCW were isolated from cell suspension cultures of spinach (*Spinacia oleracea* L.) grown with D-[U-^{14}C]glucose (5 g/l) as the only energy source. This ensures that every C atom will have an equivalent atomic specific radioactivity and assumes that the cells do not discriminate between ^{14}C and ^{12}C.

An initial study in the rat has shown that by 24 h much of the ^{14}C-PCW had disappeared from the gastrointestinal tract of the rat and the ^{14}C was found elsewhere (Gray *et al.* 1993). During the first 24 h 16% of the orally administered PCW was excreted in faecal material, whereas in the second 24 h only 1·6% of the administered dose was excreted in faeces. By 96 h the faecal excretion of ^{14}C-PCW was negligible at 0·6% of the administered dose. The experiments described in the present paper refer to the first 24 h following administration of the dose.

Three male Albino Wistar rats were studied for each time-interval, i.e. 3, 6, 12 and 24 h after administration of the dose. They had previously been fed on a stock diet (CRM (X); Special Diet Services Ltd in pellet form); the analysis of the dietary constituents is shown in Table 2.

Table 2. *Detailed analysis of CRM(X) diet (g/kg)**

Crude oil	30
Crude protein	183
Non-starch polysaccharide (NSP)†	133
Insoluble NSP	102
Soluble NSP	31
Cellulose	26
Total carbohydrate content	569
Vitamin	10
Mineral	45

* Values supplied by Special Diet Services Ltd.
† Englyst *et al.* (1982).

The animals were dosed with a known amount of approximately 40 kBq (weighing approximately 4 mg, which is 0·1 % dry weight of the daily diet) U-^{14}C-PCW suspended in less than 3 ml water, by oral administration. This took place at 10.00 hours in each experiment to reduce the effects of diurnal variation. The syringe barrels were washed out and the residual activity determined by sample oxidation, providing an accurate measure of the ^{14}C dosage.

After dosing by oral administration the animals were housed individually in metabolism cages with a broad-spaced-grid floor to minimize coprophagy. Faeces and urine were collected separately into vials containing 0·1 M-NaOH to halt any further fermentation. The samples were removed at regular intervals during the day and freeze-dried. The animals were killed in groups of three by cervical dislocation and dissected immediately at 3, 6, 12 and 24 h after administration of ^{14}C-PCW. The small intestine, caecum and colon were identified, isolated and removed and their contents freeze-dried. The intestinal tissues were washed in water and frozen. Adipose tissue, liver and skin were also frozen immediately after dissection. A portion of each sample was assayed for ^{14}C.

Weighed portions of each sample ($< 0{\cdot}2$ g) were oxidized in a stream of O_2 using a Packard 306 sample oxidizer. The CO_2 produced was collected in carbosorb–permafluor (4:5, v/v) and ^{14}C content determined by liquid-scintillation spectroscopy.

To determine the ^{14}C lost in expired air by the rats, $^{14}CO_2$ production in the rats during the first 24 h was studied. Two rats were housed separately in metabolism respiration cages after oral administration of ^{14}C-PCW. The air was drawn through the metabolism cage with a pump, into a silica-gel drying column before passing through two CO_2 traps containing carbosorb. Portions (1 ml) of carbosorb were removed from the CO_2 trap and to this was added 7 ml fresh carbosorb and 10 ml permafluor for scintillation counting.

In order to detect the individual plant polysaccharides in the gastrointestinal contents, 400 kBq ^{14}C-PCW was administered to two rats at varying time-intervals to enable the ^{14}C profile to be investigated, e.g. 3 h stomach, 24 h faeces. The freeze-dried enteric contents were obtained in the same way as outlined previously, and exposed to mild alkaline hydrolysis, followed by Driselase (Sigma Chemical Co., Poole, Dorset) digestion as described by Gray *et al.* (1993). The soluble fraction was loaded onto chromatography paper and developed in butan-1-ol–acetic acid–water (12:3:5, by vol.; BAW) followed in the same dimension by ethyl acetate–pyridine–water (8:2:1, by vol.: EPW), for 16 h in each run (Gray *et al.* 1993; Fry, 1988). The profile of ^{14}C was determined by scintillation counting.

In order to investigate the fermentation of the ^{14}C-PCW by rat caecal bacteria, fermentation was conducted in 100 ml Quickfit wide-mouthed flasks containing 40 ml buffered medium (Goering & Van Soest, 1979) in a shaking water-bath at 37°. The flasks

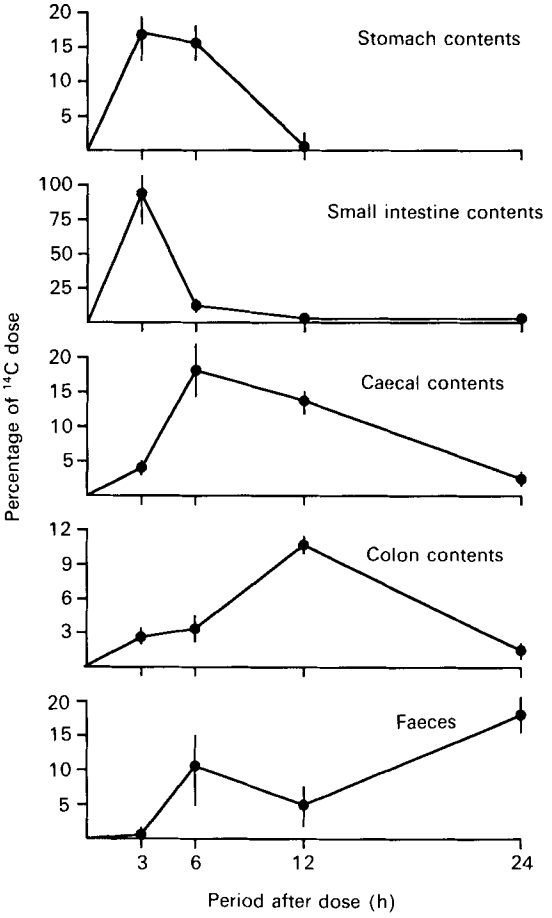

Fig. 1. Distribution of ^{14}C-labelled plant cell walls from spinach (*Spinacia oleracea* L.) in the gastrointestinal tract of rats over 24 h after oral administration of ^{14}C-labelled plant cell walls. Values are means with their standard errors represented by vertical bars. For details of procedures, see pp. 190–192.

were fitted with air-tight stoppers and the gas inlet–outlet connected to a CO_2 trap and a culture sample tube. Medium was added to the flask containing 4 g unlabelled spinach PCW and a known amount of ^{14}C-PCW. The inlet tube was connected to a gas cylinder containing CO_2–N_2 (5:95, v/v). Reducing solution, 2 ml (g/l; cysteine hydrochloride 6·25, NaOH 1·6, $Na_2S.9H_2O$ 6·25) was added after 40 min to the culture and CO_2 was admitted until reazurin added to the medium became colourless, indicating anaerobic conditions. Rat caecal contents were added as soon as the medium was seen to be reduced by loss of pink colouration. The flasks were incubated for 24 h and culture samples removed at 3, 6, 12, and 24 h. The production of $^{14}CO_2$ was monitored in the same way as described in the animal experiment.

RESULTS

The results (Figs. 1 and 2) show the presence of ^{14}C-PCW in the stomach and small intestine during the first 3–6 h. The apparent recovery of ^{14}C at 3 h in excess of 100% is probably due to the administration technique being relatively imprecise; this does not affect the overall results. After 6 h there was a progressive movement of ^{14}C along the intestine and

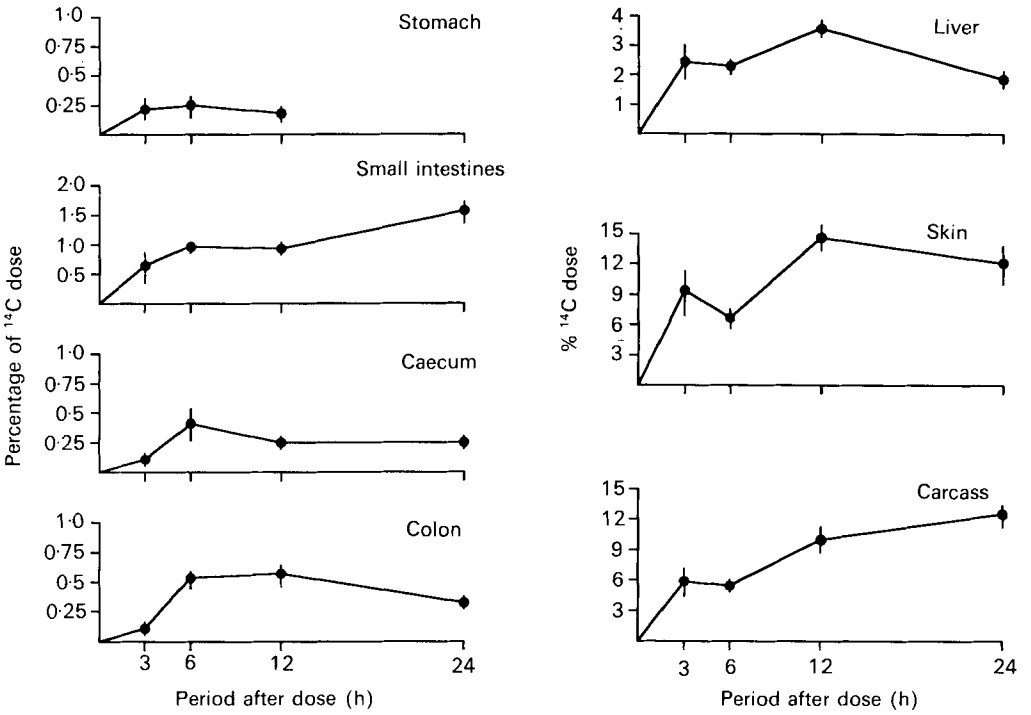

Fig. 2. Distribution of ^{14}C in the rat tissues over 24 h after oral administration of ^{14}C-labelled plant cell walls from spinach (*Spinacia oleracea* L.). Values are means with their standard errors represented by vertical bars. For details of procedures, see pp. 190–192.

colon with the progressive appearance of ^{14}C in the body tissues and faeces. After 12 h most of the ^{14}C-PCW was found in the caecal and colonic contents, faeces and liver, skin and carcass tissues. There was an increase in ^{14}C in the caecal and colon tissues at 6 h and the colon tissue at 12 h.

Fig. 3 shows the radioactive profiles of the chromatograms obtained from the ^{14}C-PCW preparation. Driselase digestion of the purified ^{14}C-PCW was about 90% complete, the products being mainly mono- and disaccharides (Fig. 3(*a*). Good chromatographic separations were also obtained with Driselase digests of ^{14}C-PCW material in the presence of gut contents (Fig. 3(*b–e*)). Paper chromatographic separations in the solvent system used were not compromised by the presence of quite large amounts of contaminating salts, proteins etc. (Fry, 1988). The fates of the cellulose (glucose) and pectin (galacturonic acid) fractions were the easiest to follow. There was a persistent peak at the origin which may have been due to glycoprotein which had not been degraded, or residual polysaccharide. The nature of this finding will form part of a further study. In the PCW, the galacturonic acid peak was considerably larger than the glucose peak. After 12 h in the caecum the glucose peak exceeded the galacturonic acid peak. These were relative values, and the main conclusion was that pectin had been fermented more quickly than cellulose. The administered ^{14}C-PCW contained approximately 21% [^{14}C]cellulose. At 12 h, 14% of the ^{14}C was to be found in caecal contents and approximately 45% of this was cellulose (i.e. 6·3% of the dose); 10% was to be found in the colon, of which 36% was cellulose (3·6% of the dose), and 5% was found in the faeces, of which approximately 50% was cellulose (2·5% of the dose). This implied that 12% of the ^{14}C-PCW was still cellulose, and overall this meant that about 41% of the [^{14}C]cellulose had been digested after 12 h.

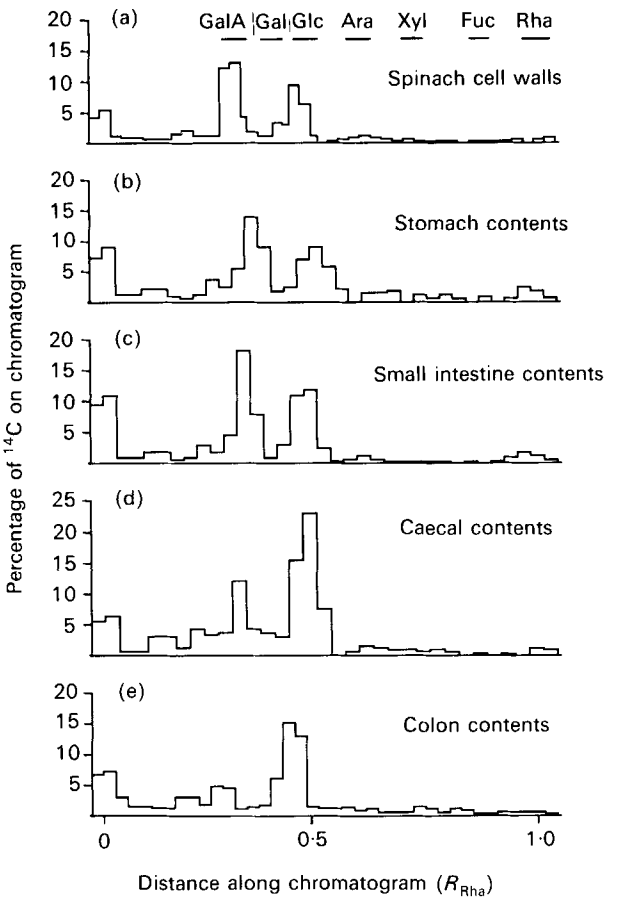

Fig. 3. Paper chromatography profiles of Driselase-digested ^{14}C-labelled plant cell walls from spinach (*Spinacia oleracea* L.) before ingestion (a) compared with contents of the gastrointestinal tract (b–e) of two rats fed on the same material. The difference between chromatograms has been corrected using the mobility of rhamnose (R_{Rha}) GalA, galacturonic acid; Gal, galactose; Glc, glucose; Ara, arabinose; Xyl, xylose; Fuc, fucose; Rha, rhamnose. For details of procedures, see pp. 190–192.

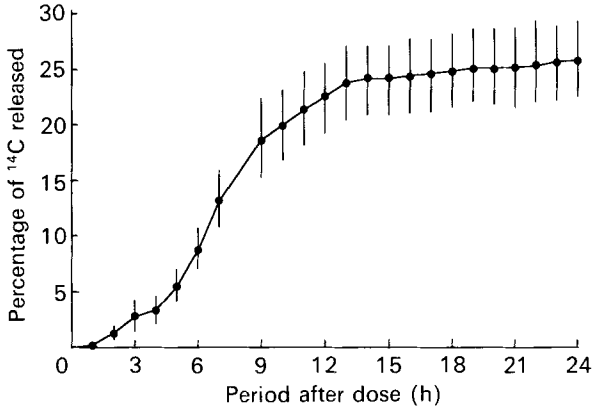

Fig. 4. Production of $^{14}CO_2$ from rats after oral administration of ^{14}C-labelled plant cell walls from spinach (*Spinacia oleracea* L.) Values are means with their standard errors represented by vertical bars. For details of procedures, see pp. 190–192.

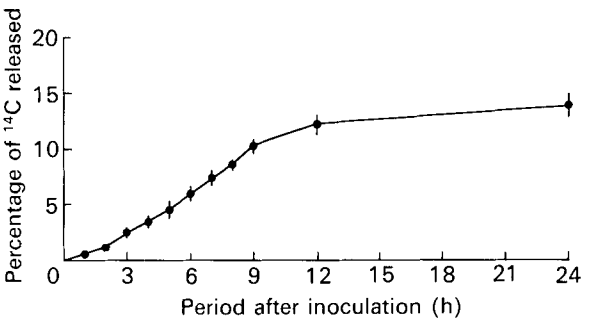

Fig. 5. Production of $^{14}CO_2$ from in vitro fermentation containing ^{14}C-labelled plant cell walls from spinach (*Spinacia oleracea* L.) and a caecal inoculum after 24 h. Values are means with their standard errors represented by vertical bars. For details of procedures, see pp. 190–192.

Fig. 4 shows the exhalation of $^{14}CO_2$ during the 24 h of study. There was an initial delay in the appearance of $^{14}CO_2$ in the expired gases, respiratory and rectal. The production reached its maximum at between 4 and 9 h; although there was a decline after 13 h, there was still a gradual production over the entire 24 h.

Fig. 5 shows the production of $^{14}CO_2$ from the *in vitro* fermentation process. Values are expressed as a percentage of the original ^{14}C dose. The bacteria appear to adapt rapidly to their new environment and produce $^{14}CO_2$ rapidly. The production (15%) is less than that *in vivo* using the rat (25%).

DISCUSSION

These studies examine the utilization of intact labelled PCW as an indicator of the fate of dietary fibre in the gastrointestinal tract. Understanding this fate has been limited by the methodology available, so these experiments represent the development of a new approach. It is reasonable to regard the action of fibre in the upper intestine as being a consequence of the whole conglomerate of fibre acting as a hydrated mass so that the fate of individual sugar residues is irrelevent. In the colon the fate of individual sugar residues following bacterial fermentation is of interest. The metabolic products of the bacterial fermentation of these sugar residues include the short-chain fatty acids acetate, butyrate and propionate and CO_2 (Cummings & Englyst, 1987). These fatty acids can either be metabolized (butyrate) in the colonic mucosa or absorbed and pass to the liver and systemic circulation (acetate, propionate) and peripheral tissue. However, the precise relationship between the original individual sugar residues in the plant cell wall and the metabolic products disposed throughout the body is not at all clear.

The spinach cultures used were rapidly-growing undifferentiated cells and the wall structure was relatively uniform in composition. The PCW residues were uniformly labelled because the cells were grown on [U-^{14}C]glucose as the only C source. The high specific activity of the ^{14}C-PCW has advantages. This eliminates any interference with the host diet during the experiment. The radioactive marker makes a measurement of all PCW-derived metabolites possible irrespective of their destination in the body tissues, urine, faeces or expired gas.

The PCW used in these experiments contained (g/kg) pectin 530, hemicellulose 130, cellulose 210, with a small starch content.

A problem with the method was the use of oral administration. The particular nature of the PCW does not allow easy administration, particularly using such a small volume (less than 3 ml). This was indicated in the 3 h experiments where greater than 100% recovery of ^{14}C was obtained and this was due to high counts in one animal. The overall picture is no way distorted by this value.

It is clear, however, that there is rapid movement of dietary fibre along the gastrointestinal tract and metabolism in the caecum. By 6–12 h most of the ^{14}C was found in the caecum. It is of interest that the proportion of radioactivity passing out in the faeces was small and did not exceed 18% in a 24 h period. Also of interest is that there is an accumulation of ^{14}C in the liver, skin and carcass, indicating that the metabolites of the PCW are incorporated into these tissues. This suggests that the fermented PCW generate metabolic products, e.g. short-chain fatty acids, which act as metabolic sources throughout the body. It is of interest that little or none of the ^{14}C appears in the urine.

About 25% of the C from ^{14}C-PCW was expired as $^{14}CO_2$. It is difficult to separate the evolution of CO_2 from bacterial and host tissues. The delay in the onset of the evolution of $^{14}CO_2$ gives only a partial clue. This is because there may be a slow onset of fermentation of PCW or a rapid metabolism of these fermentation products by the host animal. The small amounts found in the liver, however, raise the possibility that there is rapid metabolism of the acetate, and possibly propionate, by the liver with a consequent evolution of CO_2. From the chromatographic profies of the intestinal contents it was clear that the bacteria degrade the pectic fraction (galacturonic acid residues) in preference to the cellulose fraction (glucose residues). There may well be a hierarchy of polysaccharide utilization in the large gut which could result from the chemical and physical properties of the individual polysaccharides. It is reasonable to suppose that the pectins would be more fermentable by the caecal bacteria than the celluloses. It is unlikely that the ^{14}C in the faeces after 24 h (18% of the total ^{14}C dose) was due entirely to undegraded cellulose. Some of the ^{14}C would be incorporated into the bacterial cells. Hosoya *et al.* (1988) have suggested that 10% of the ^{14}C from ^{14}C-labelled fructo-oligosaccharides incubated with faecal inoculum will become incorporated into bacterial cells. If a similar value is applied to the 82% of the ^{14}C-PCW material that did not appear in the faeces, then approximately 8% of the dose would be predicted to be excreted in stool as ^{14}C-labelled bacterial cells. Thus, of the ^{14}C (18% of dose) that actually appeared in the faeces, approximately 8 out of 18 would have been ^{14}C-labelled cells, the remainder (10% of ^{14}C dose) being undigested ^{14}C-PCW material, probably mainly [^{14}C]cellulose. Since the dose was approximately 21% [^{14}C]cellulose it is, therefore, likely that well over half of the cellulose was fermented by the gut flora.

In the ^{14}C-PCW fermentation study using rat caecal contents under anaerobic conditions the bacteria adapted quickly to the new environment and fermented the ^{14}C-PCW immediately. There was a constant high rate of production of $^{14}CO_2$ between 3 and 9 h. Total $^{14}CO_2$ production in vitro, approximately 15% of the ^{14}C dose, was considerably less than the level produced in the in vivo experiments (approximately 25% of the ^{14}C dose). Whilst the majority of the expired $^{14}CO_2$ may well be of bacterial origin it is not unreasonable to suppose that there was metabolism of the evolved short-chain fatty acids by the rat resulting in expired $^{14}CO_2$ of mammalian origin.

A further stage of the present work will be to identify the endproducts of the metabolites to be found in the carcass and skin.

The present paper shows the use of a chemically-defined ^{14}C-labelled PCW in studying the fate of PCW after ingestion by the rat. It shows the subsequent distribution of the radioactive label, wherein 25% of the PCW is expired principally in breath but also in flatus as $^{14}CO_2$. There is significant metabolism in the caecum and the appearance of ^{14}C in the liver, adipose tissue and skin. Less than 20% of the ^{14}C appears in the faeces. The use of such labelled PCW offers a new development in our methodology for the study of PCW fermentation in the rat. An extension of this work will be to label the PCW with different labelled sugar residues and follow their subsequent fate.

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