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Density-labelling of cell wall polysaccharides in cultured rose cells: comparison of incorporation of ^{2}H and ^{13}C from exogenous glucose

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Abstract

Labelling with stable isotopes has under-exploited potential for studies of polysaccharide endotransglycosylation in vivo. Ideally, the labelled polysaccharides should have the highest possible buoyant density. Although [$^{13}C_6$]glucose has previously been used as a precursor, it was unclear whether ^{2}H would be efficiently incorporated from [^{2}H]glucose or lost as D_2O. Rose (*Rosa* sp.) cell-suspension cultures efficiently incorporated ^{13}C from D-[$^{13}C_6$,$^{2}H_7$]glucose into wall polysaccharides with negligible dilution from atmospheric $^{12}CO_2$. Also, ~70% of the ^{2}H atoms in D-[$^{13}C_6$,$^{2}H_7$]glucose were retained during polysaccharide biosynthesis. This shows that relatively few cycles of intermediary metabolism leading to the release of D_2O occurred before sugar residues were incorporated into wall polysaccharides. In agreement with these observations, isopycnic centrifugation in caesium trifluoroacetate gradients showed that the hydrated buoyant density of xyloglucan synthesised by rose cells growing on [$^{13}C_6$,$^{2}H_7$]glucose and [$^{13}C_6$]glucose was 3.7 and 2.6% higher, respectively, than in isotopically non-labelled cultures. Thus, [^{13}C,^{2}H]glucose-feeding enabled a 42% better resolution of 'heavy' from 'light' xyloglucan than [^{13}C]glucose-feeding.

Keywords: Xyloglucan; Biosynthesis; Isopycnic centrifugation; Caesium trifluoroacetate (CsTFA™); Transglycosylation; Density-labelling

1. Introduction

Plant cell-wall polysaccharides undergo several covalent and non-covalent modifications in vivo, including enzymic hydrolysis and transglycosylation,[1] the making and breaking of hydrogen bonds,[2] cross-linking[3,4] and possibly oxidative scission.[5] These processes are relevant to the mechanism and control of cell wall loosening, thought to be necessary for cell expansion, fruit softening and leaf abscission.[6] The interpolymeric transglycosylation of xyloglucan, the major hemicellulose (alkali-extractable, non-pectic polysaccharide) in the primary cell walls of dicotyledonous plants, is of particular interest. This reaction is catalysed by xyloglucan endotransglycosylase (XET) and results in the 'cutting and pasting' of portions of xyloglucan chains.[7–11] Such transglycosylation could provide a mechanism by which lengths of newly secreted xyloglucan become integrated into the existing wall fabric,[12,13] and the breaking and re-joining of the existing framework could loosen the wall and enable cell expansion without permanent weakening of the wall.[8]

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Investigation of interpolymeric transglycosylation in vivo is not straightforward because the substrates may be chemically identical with the reaction products. We recently used dual isotopic labelling to detect such reactions in vivo.[12] The two labels used were (a) a stable isotope (the cells were fed substrate concentrations of D-[$^{13}C_6$]glucose), which introduced into the cell walls a population of 'heavy' xyloglucan molecules that could be partially resolved from the normal ('light') population by isopycnic centrifugation; and (b) a radioisotope (the cells were fed a trace of L-[1-^{3}H]arabinose), which introduced an easily detectable second label into the pentose residues of the xyloglucan.[14] We detected the transfer of ^{3}H between the 'heavy' and 'light' populations, thus providing the first experimental demonstration of interpolymeric transglycosylation using endogenous substrates in vivo.[12]

This method of observing interpolymeric transglycosylation in vivo would be improved if the resolution of the 'heavy' and 'light' populations could be enhanced. Two approaches to increasing the buoyant density of the 'heavy' population can be considered: (a) minimising contamination with ^{12}C during ^{13}C incorporation; and (b) supplying a second stable isotope such as ^{2}H or ^{18}O in addition to the ^{13}C.

Concerning the minimisation of dilution with ^{12}C, the plant cells used were heterotrophic and grew using glucose as their sole carbon source. However, all heterotrophic cells contain a range of carboxylases, e.g., phospho-enol-pyruvate carboxylase, which could cause the incorporation of appreciable amounts of ^{12}C from atmospheric CO_2 into central metabolic pathways. To test this, we have now studied the proportion of carbon atoms in wall polysaccharides that were derived from exogenous [^{13}C]glucose.

Concerning the introduction of a second stable isotope, the Aldrich Chemical Company agreed to prepare [$^{13}C_6$,$^{2}H_7$]glucose, but it was not certain what proportion of the ^{2}H would be lost during the intermediary metabolism that occurs before a given sugar residue becomes incorporated into a wall polysaccharide. Glucose 6-phosphate, formed from exogenous glucose, may undergo various reversible reactions and 'futile cycles',[15] during which ^{2}H would be lost as D_2O, before its carbon skeleton enters UDP-glucose and thence polysaccharides. For example, the readily reversible phosphohexose isomerase reaction results in the loss of ^{2}H from C-2, and the triose phosphate isomerase reaction also releases ^{2}H from C-2 of glyceraldehyde 3-phosphate (formerly C-5 of glucose). Similarly, the conversion of glucose 6-phosphate to ribulose 5-phosphate (in the first three steps of the oxidative pentose phosphate pathway) results in the loss of ^{2}H from C-1 and C-3 of the glucose, even though some or all of the carbon atoms of the ribulose may subsequently re-enter hexoses. The incorporation of ^{2}H from exogenous glucose into wall polysaccharides thus depends on the extent of these and other metabolic interconversions occurring before a given hexose is channelled into UDP-glucose. We have therefore now tested how efficiently ^{2}H from exogenous glucose is incorporated into wall polysaccharides in plant cell-suspension cultures.

2. Experimental

Materials.—'Heavy' glucose was D-[U-^{13}C; 1,2,3,4,5,6,6-^{2}H]glucose (Aldrich Chemical Co., Poole, Dorset, UK), in which 99% of the C atoms were ^{13}C and ~50% of the non-exchangeable H atoms were ^{2}H. L-[1-^{3}H]Arabinose (148 GBq/mmol) was from Amersham International. Caesium trifluoroacetate (CsTFA™) was from Pharmacia Biotech. Driselase (Sigma, Poole, Dorset, UK) was partially purified by $(NH_4)_2SO_4$ precipitation followed by de-salting.[16]

Culture conditions.—Cell-suspension cultures of rose (*Rosa* sp., 'Paul's Scarlet') were routinely sub-cultured once per fortnight by dilution into 10 vols of a medium[17] containing 2% D-glucose as the sole carbon source. Cultures were incubated under constant dim illumination on an orbital shaker at 25 °C.

Preparation of (^{13}C,^{2}H)-labelled cell walls for mass spectrometry.—An aliquot of standard 14 day old rose cell culture (2 mL) was transferred aseptically to a sterile 15 mL plas-

tic centrifuge tube. The cells were allowed to sediment and the supernatant was removed. The cells were then resuspended in 10 mL of glucose-free medium and the sedimentation was repeated three times to remove extracellular [^{12}C,^{1}H]glucose. The cells were resuspended in 2 mL of glucose-free rose medium and a 1.7-mL portion of this suspension was added to a 100 mL conical flask containing 17 mL of medium with 1.1% [^{13}C,^{2}H]glucose as the sole carbon source. This culture thus received the normal 11-fold dilution upon sub-culture; it was incubated under normal conditions for 14 days.

After 14 days, a 13-mL aliquot of the culture was transferred into a 500-mL conical flask containing 130 mL of medium with 0.55% [^{13}C,^{2}H]glucose as the sole carbon source. This culture was incubated under standard conditions; 10-μL aliquots of medium were removed at intervals and assayed for remaining glucose by the *p*-hydroxybenzoic acid hydrazide (PAHBAH) assay.[18]

When the concentration of extracellular glucose had dropped from 0.5 to 0.05% w/v (9 days after sub-culturing; referred to below as time '*T*'), 5.4 mL of the 'heavy' culture was transferred into a Petri dish, to which was added 0.6 mL of medium containing 5% w/v [^{13}C,^{2}H]glucose. This culture was incubated for a further 5 days, by which time the cells had been growing in [^{13}C,^{2}H]glucose for a total of 28 days.

Mass spectrometry (MS).—Cells from the 28-day (^{13}C,^{2}H)-labelled culture were washed with glucose-free medium and 70% EtOH to yield an alcohol-insoluble residue, which was dried and digested (48 h, 37 °C) with partially purified 'Driselase' (1% w/v in 1:1:98 v/v/v py–AcOH–water, pH 4.7, containing 0.5% chlorobutanol[16]). The major products of the action of Driselase on xyloglucan are isoprimeverose [α-D-Xyl*p*-(1→6)-D-Glc], glucose and galactose. Driselase also releases glucose from cellulose (but little from starch), and galactose from pectic polysaccharides, but isoprimeverose is a unique indicator of xyloglucan. The enzyme was then inactivated by the addition of formic acid to 15% (w/v) and the digestion products were separated by preparative paper chromatography in 12:3:5 v/v/v, butanol–AcOH–water (20 h). Appropriate zones (isoprimeverose and glucose + galactose) were eluted[19] and re-run by preparative paper chromatography in 8:2:1 v/v/v, EtOAc–py–water (EPW; 16 h). The isoprimeverose, glucose and galactose zones were eluted and further purified by passage through a 100-mg bed of BondElut C_{18} in water: the colourless material that failed to bind to the BondElut was collected. Portions of the three sugar samples were tested for purity by TLC in 9:6:3:1 butan-1-ol–AcOH–Et_2O–water and by analytical paper chromatography in EPW for 48 h. Duplicate TLC plates were either sprayed with 10% H_2SO_4 in MeOH and then charred at 120 °C to reveal general organic compounds, or dipped through aniline hydrogenphthalate and heated at 105 °C for 5 min to reveal reducing sugars.[16] Paper chromatograms were stained by the latter method.

Additional portions of each of the three sugars were dissolved at 10 μg/mL in 50% MeOH and analysed by single-quadrupole electrospray MS on a Platform II mass spectrometer (Micromass Ltd., Manchester, UK) in positive ionisation mode. The samples were infused at 5 μL/min and the ions were produced in an atmospheric pressure ionisation–ESI ion source. Source temperature was 65 °C and the drying and nebulising gas flow rates were 300 and 20 L/h, respectively. A potential of 3.0–3.5 kV was applied to the probe tip and the effect on sample signal intensity of varying the cone voltage (30–100 V) was determined. The peaks reported on in the present paper all persisted when the cone voltage was increased, compatible with their being due to metal ion adducts. The quadrupole mass analyser was scanned at 100 AMU/s following appropriate external calibration. The mass accuracy of measurements was within 0.1–0.2 Da on the m/z scale. The MS analyses were kindly performed by Dr A.M. Gouldsworthy, Chemistry Department, The University of Edinburgh, UK.

Preparation of (^{13}C,^{2}H,^{3}H)-labelled xyloglucan for isopycnic centrifugation.—A further 18-mL portion of the 'heavy' culture at time '*T*' (see above) was dispensed into a Petri dish. [^{13}C,^{2}H]Glucose (20% w/v in 2 mL medium) was added and the culture was incu-

bated for 30 min before the addition of [^{3}H]arabinose (5 MBq in 0.1 mL glucose-free medium). Samples (1.5 mL) of the culture were removed at intervals (0–7 days), filtered, washed and frozen (-80 °C) for later analysis. The [^{3}H]xyloglucan did not change in buoyant density between 0 and 7 days; therefore data for all time points have been pooled.

A control culture was maintained under identical conditions but with 'light' medium (containing [^{12}C,^{1}H]glucose) throughout. Radio-labelling with tracer quantities of [^{3}H]arabinose was as above.

Extraction and buoyant density measurements of xyloglucan.—The ^{3}H-labelled rose cells (pre-grown in either [^{13}C,^{2}H]- or [^{12}C,^{1}H]glucose) in each 1.5 mL sample were filtered, washed with sugar-free medium and stored at -80 °C. The frozen cells were thawed into 5 mL of 6 M NaOH containing 1.0% $NaBH_4$ and shaken at 37 °C for 24 h. The solubilised hemicellulose was neutralised, dialysed against water, freeze-dried, and redissolved in 0.5 mL of 8 mM PyOAc (pH 4.7). Xyloglucan was precipitated from the total hemicellulose fraction by addition of EtOH to 50% (v/v) and incubation at 25 °C for 18 h; the xyloglucan-enriched pellet was dried, redissolved in 1 mL of 8 mM PyOAc containing 8 M urea (pH 5.3) (urea–PyOAc) and applied to Q-Sepharose FastFlow (2 mL bed, pre-equilibrated with urea–PyOAc). Neutral xyloglucan was eluted with a further 2 mL of urea–PyOAc. The eluted product contained 70% of the total cellular xyloglucan, at 95% radiochemical purity.[20] A portion (2.5 mL) of the neutral xyloglucan solution was mixed with 9 mL of CsTFA™ solution (density 2.0 g/mL) and 2.75 mL of 500 mM PyOAc (pH 4.7, containing 1.7 kBq of O-[^{14}C]acetylated tamarind xyloglucan [very low degree of acetylation] as an internal marker[12]) to give a solution of density 1.6 g/mL. The solution was centrifuged in a vertical rotor (Sorvall Step-Saver 65V13 at 255,000*g* for 40 h at 18 °C with slow deceleration) to produce an approximately linear density gradient (1.45–1.75 g/mL). Gradients were collected in 250 μL fractions by downward displacement with white mineral oil at 0.5 mL/min. The density of each fraction was estimated from the weight of a 200 μL portion, which was then assayed for ^{3}H and ^{14}C by scintillation counting. Curves were fitted and mean buoyant densities estimated as described.[12]

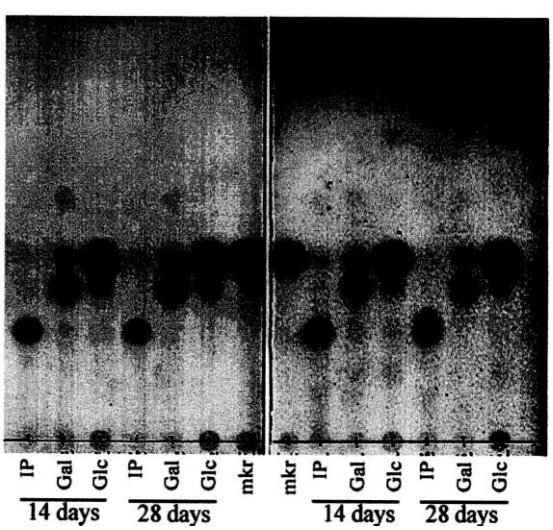

Fig. 1. TLC of sugar samples used for MS. Rose cells were cultured for 14 or 28 days with [^{13}C,^{2}H]glucose as sole carbon source, then the wall polysaccharides were digested with Driselase and three major products [isoprimeverose (IP), galactose and glucose] were partially purified by preparative paper chromatography. Portions of each preparation were analysed by TLC and stained with aniline hydrogen-phthalate (a) or H_2SO_4–MeOH (b); mkr = marker glucose.

3. Results and discussion

Mass spectrometry.—Isoprimeverose, glucose and galactose were isolated from the wall polysaccharides of a rose cell culture that had been incubated with [^{13}C,^{2}H]glucose as the sole carbon source for 28 days. TLC demonstrated an acceptably high degree of purity and only slight cross-contamination between galactose and glucose (Fig. 1).

To determine ^{13}C and ^{2}H incorporation, we analysed these three sugars as metal ion adducts by MS. The isoprimeverose, glucose and galactose each showed a small peak (1.9, 3.4 and 3.0% of the total respective Na^+ adducts) corresponding to the Na^+ adduct of the unlabelled sugar ($m/z = 335$, 203 and 203; Fig. 2). These completely unlabelled (^{12}C,^{1}H)-molecules must represent sugar residues that had been present in the cell wall for >28 days.

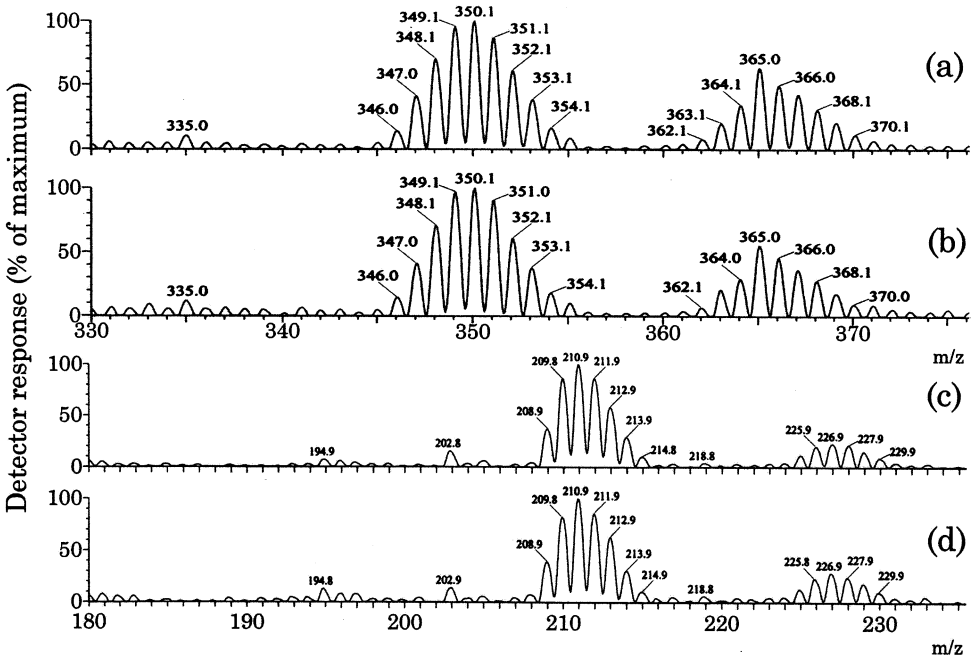

Fig. 2. Representative positive-mode mass spectra of sugars isolated from the polysaccharides of density-labelled rose cells. MS of the isoprimeverose (a,b) and glucose (c,d) purified from (^{13}C,^{2}H)-labelled cells as described in Fig. 1. In (a) and (c) the cone voltage was 100 V; in (b) and (d) it was 80 V. Results for galactose were similar to those for glucose but additional small peaks due to an unidentified contaminant were present at m/z 187.9 and 203.8.

The remaining 96.6–98.1% of the Na^+ adducts were present as a series of peaks corresponding to the fully ^{13}C-labelled Na^+ adducts with zero to several ^{2}H atoms (Fig. 2). Each series of Na^+ adducts was accompanied by a similar pattern of smaller peaks with m/z values 16 units higher, attributable to K^+ adducts. Omitting the 1.9–3.4% of the Na^+ adducts that were unlabelled, the remainder had mean m/z values of 350.1 (isoprimeverose·Na^+), 211.4 (glucose·Na^+) and 211.6 (galactose·Na^+), indicating mean values of about 4.1, 2.4 and 2.6 ^{2}H atoms per molecule, respectively (Table 1). These values correspond to ^{2}H enrichments of 32, 34 and 37%, respectively, of the total non-exchangeable (i.e., C-bonded) H atoms.

^{13}C and ^{2}H enrichments of 96.6–98.1 and 32–37%, respectively, compare with enrichments of approximately 99% ^{13}C and 50% ^{2}H in the 'heavy' glucose supplied to the cells. Thus, the vast majority of the C atoms of the cell wall polysaccharides were derived from the last 28 days' supply of exogenous glucose. The only alternative — incorporation of atmospheric CO_2 — was negligible, indicating that little heterotrophic carboxylation occurred.

In contrast to the C atoms, the non-exchangeable H atoms of the wall polysaccharides were expected to be derived only partly from the exogenous glucose and partly from the water of the culture medium. H atoms would be introduced from water into sugars during metabolic steps such as those involved in the glycolysis–gluconeogenesis and the pentose-phosphate-pathway–gluconeogenesis 'futile cycles'.[15] The MS data show that 64–74% of the non-exchangeable H atoms were derived from exogenous glucose, the remainder presumably originating from water. Among other possible interpretations, a 71% ($=5/7$) retention would correspond to loss of the ^{2}H atoms from only C-2 and C-5 of the glucose owing to the glycolysis–gluconeogenesis futile cycle proceeding as far as the triose phosphates. Thus the use of [^{13}C,^{2}H]glucose was a significant improvement over [^{13}C]glucose for the practical goal of density-labelling wall polysaccharides.

Theoretical density of unhydrated, labelled xyloglucans.—The measured isotopic enrichments imply that the typical repeat-unit (Glc_8·Xyl_6·Gal·Fuc = XXXGXXFG in the abbreviated nomenclature of Ref. 21) of newly

synthesised xyloglucan[22,23] would have a mean M_r in the (^{13}C,^{2}H)-labelled cells of ~2501 (assuming 99% ^{13}C and the same ^{2}H enrichment for fucose as for glucose) instead of the normal 2379 (assuming ~1% natural ^{13}C and no ^{2}H). This would give an unhydrated density for the dual-labelled xyloglucan 5.1% higher than normal. With [U-^{13}C]glucose (lacking ^{2}H) as the precursor, the density of unhydrated xyloglucan would be predicted to be only 3.7% higher than normal. Therefore, the use of [^{13}C,^{2}H]glucose instead of [^{13}C]glucose is predicted to boost the maximum attainable difference in density between 'heavy' and 'light' xyloglucan by about 38%.

Buoyant density measurements.—Isopycnic centrifugation provides estimates of the densities of hydrated, not dry, polysaccharides. (^{13}C,^{2}H)-Labelled xyloglucan will thus have a measured density less than 5.1% higher than unlabelled. When rose cells were grown in [^{13}C,^{2}H]glucose medium for 23 days and then

Table 1
Mass spectral data of wall carbohydrates isolated from rose cells pre-cultured on [^{13}C,^{2}H]glucose [a]

Species	(m/z)	No. of ^{13}C atoms [b]	No. of ^{2}H atoms [b]	Proportion of total ^{13}C-labelled Na^+ adducts (%) [c]
Isoprimeverose·Na^+		(11) [d]	(13) [d]	
	335	0	0	(1.9) [e]
	346	11	0	2.5
	347	11	1	7.8
	348	11	2	13.2
	349	11	3	17.8
	350	11	4	18.7
	351	11	5	16.6
	352	11	6	11.3
	353	11	7	7.1
	354	11	8	3.2
	355	11	9	1.9
Glucose·Na^+		(6) [d]	(7) [d]	
	203	0	0	(3.4) [e]
	209	6	0	9.1
	210	6	1	21.0
	211	6	2	24.5
	212	6	3	21.0
	213	6	4	14.7
	214	6	5	7.2
	215	6	6	2.6
Galactose·Na^+		(6) [d]	(7) [d]	
	203	0	0	(3.0) [e]
	209	6	0	7.4
	210	6	1	21.8
	211	6	2	23.1
	212	6	3	20.0
	213	6	4	15.0
	214	6	5	8.7
	215	6	6	3.9

[a] Cell walls from the density-labelled cells were digested with Driselase and the resulting isoprimeverose, glucose and galactose were purified chromatographically and analysed by MS. Only data for the Na^+ adducts are presented; data for the K^+ adducts are in agreement.

[b] Assuming all-or-none ^{13}C-labelling.

[c] Excluding unlabelled (^{12}C,^{1}H) molecules. Based on peak heights (mean of two determinations with cone voltage 80 and 100 V, respectively).

[d] In parentheses: theoretical maximum.

[e] Not included in total.

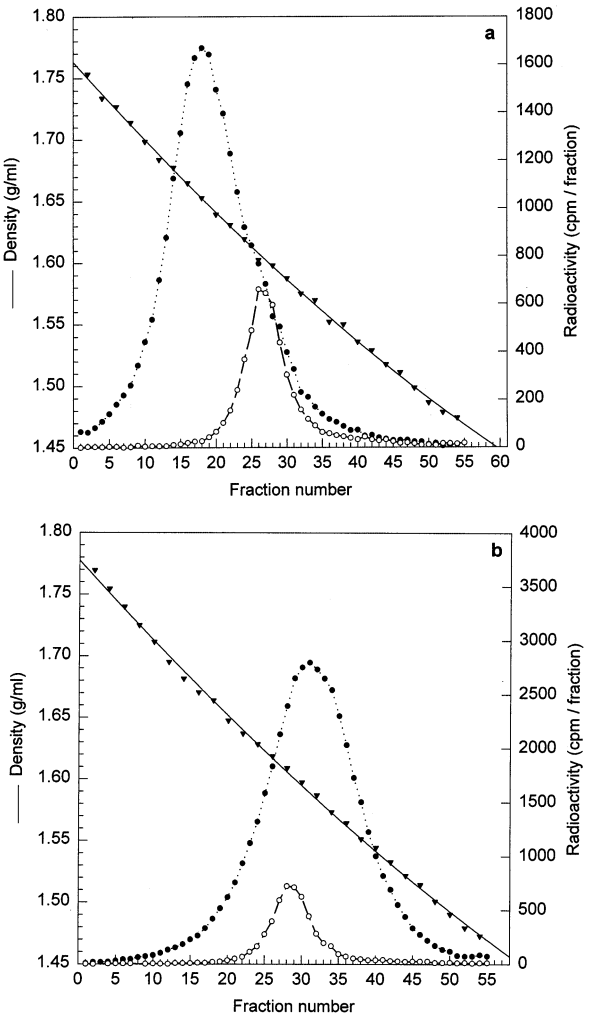

Fig. 3. Representative buoyant density profiles of xyloglucan produced in (^{13}C,^{2}H)-labelled rose cells. Rose cells were cultured for 23 days with (a) [^{13}C,^{2}H]glucose or (b) [^{12}C,^{1}H]glucose as sole carbon source, then pulse-labelled with [^{3}H]arabinose. The neutral xyloglucan was extracted and purified, then fractionated by isopycnic centrifugation in a CsTFA gradient. ●·····●, profile of radiolabelled rose xyloglucan; ○– – –○, profile of [^{14}C]acetylated tamarind xyloglucan added as internal marker during each centrifuge run; ▼——▼, density of CsTFA solution.

pulse-labelled with a trace of [^{3}H]arabinose, isopycnic centrifugation of the extracted radioactive xyloglucan revealed a mean buoyant density of 1.6432 ± 0.0010 g/mL (e.g., Fig. 3(a)), compared with 1.5846 ± 0.0006 g/mL for radioactive xyloglucan produced in a control culture raised on [^{12}C,^{1}H]glucose throughout (e.g., Fig. 3(b)). Thus the (^{13}C,^{2}H)-labelled, hydrated xyloglucan was 3.7% denser than normal. In contrast, the xyloglucan produced in rose cultures that had been raised on [^{13}C]glucose (without ^{2}H) had a hydrated density only 2.6% higher than normal (e.g., figure 6(b) of Ref. 12). Therefore, the use of [^{13}C,^{2}H]glucose instead of [^{13}C]glucose was observed to boost the maximum attainable difference in buoyant density between 'heavy' and 'light' xyloglucan by about 42%, in good agreement with the 38% predicted from MS.

4. Conclusions

We conclude that heterotrophic rose cells utilise the carbon of the supplied glucose for biosynthesis of polysaccharides and do not detectably dilute this carbon with that derived from atmospheric CO_2. In addition, we have shown that $\sim 70\%$ of the ^{2}H atoms present in supplied [$^{2}H_7$]glucose are retained during incorporation of sugars into wall polysaccharides. Therefore, rose cells growing on [$^{13}C_6$,$^{2}H_7$]glucose synthesise 'heavy' polysaccharides with an appreciably higher buoyant density than those on [$^{13}C_6$]glucose. This finding is valuable for the design of experiments that exploit isopycnic centrifugation and radiolabelling techniques[12] to monitor the post-synthetic modifications, especially endo-transglycosylation, of hemicelluloses and possibly also pectins in the walls of living plant cells.

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