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Validation of the design of feeding experiments involving [^{14}C]substrates used to monitor metabolic flux in higher plants

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ABSTRACT

The aim of this study was to examine whether flux through the pathways of carbohydrate oxidation is accurately reflected in the pattern of $^{14}CO_2$ release from positionally labelled [^{14}C]substrates in conventional radiolabel feeding studies. Heterotrophic cell suspension cultures of *Arabidopsis thaliana* were used for this work. The presence of an alkaline trap to capture metabolically generated $^{14}CO_2$ had no significant effect on the ratio of $^{14}CO_2$ release from specifically labelled [^{14}C]substrates, or on the metabolism of [U-^{14}C]glucose by the cells. Although the amount of $^{14}CO_2$ captured in a conventional time-course study was only about half of that released from a sample acidified at an equivalent time point, the ratios of $^{14}CO_2$ released from different positionally labelled [^{14}C]glucose and [1-^{14}C]gluconate were the same in untreated and acidified samples. Less than 5% of radioactivity supplied to the growth medium as [^{14}C]bicarbonate was incorporated into acid-stable compounds, and there was no evidence for appreciable reassimilation of $^{14}CO_2$ generated intracellularly during oxidation of [1-^{14}C]gluconate by the cells. It is concluded that the ratio of label captured from specifically labelled [^{14}C]glucose is a valid and convenient measure of the relative rates of oxidation of the different positional carbon atoms within the supplied respiratory substrate. However, it is argued that failure to compensate for the incomplete absorption of $^{14}CO_2$ by an alkaline trap may distort estimates of respiration that rely on an absolute measure of the amount of $^{14}CO_2$ generated by metabolism.

1. Introduction

Feeding experiments involving radio-isotopically labelled substrates have underpinned the study of plant metabolism for more than half a century and have been important in elucidating the major metabolic pathways of carbon metabolism (Axelrod and Beevers, 1956; Bassham and Calvin, 1957; Beevers, 1961). Such studies typically entail incubating tissue with a ^{14}C-labelled compound and monitoring the appearance of the radioactivity in metabolic products generated by the cells. One common application of this approach involves determining the relative rates of $^{14}CO_2$ release from specific carbon positions within ^{14}C-labelled substrates and this provides a convenient approach for estimating flux through the diverse pathways of carbohydrate oxidation in non-photosynthesising plant cells (ap Rees, 1980). Usually, in such studies, plant tissue is incubated in a sealed flask with a positionally labelled [^{14}C]substrate and the radioactivity released as $^{14}CO_2$ is quantified following capture by an alkaline trap (Gibbs and Beevers, 1955; Stitt and ap Rees, 1978; Averill et al., 1998; Garlick et al., 2002; Nunes-Nesi et al., 2005; Malone et al. 2006; Kruger et al., 2007; Studart-Guimarães et al., 2007; Centeno et al., 2008; Morgan et al., 2008; Sienkiewicz-Porzucek et al., 2008). Recently, there has been renewed interest in exploiting the potential of respirometric CO_2 labelling from positionally labelled substrates as an approach for quantifying flux through metabolic networks (Yang et al., 2005, 2006).

Despite the widespread use and importance of approaches based on respiratory $^{14}CO_2$ release, theoretical consideration of the underlying processes suggests that the design of many radiolabel feeding experiments may be flawed. Problems arise from the assumption that $^{14}CO_2$ released by the cells is quantitatively captured by an alkaline trap. This creates a conflict between, on one side, the need to collect effectively all of the CO_2 released by the cells in a given sampling period and, on the other, the metabolic requirement of the cells for CO_2. Even in non-photosynthesising tissues, carboxylation reactions occur in several metabolic processes (Yang et al., 2005) and these may be restricted by depletion of available CO_2. For example, phosphophenolpyruvate carboxylase is responsible for replenishing intermediates of the tricarboxylic acid cycle through the anapleurotic production of oxaloacetate/malate, and any limitation in the activity of this enzyme is likely to constrain the withdrawal of intermediates for biosynthesis. This problem is likely to be particularly marked in plant cells, in which flux through the reaction catalysed by phosphoenolpyruvate carboxylase is appreciable (ap Rees et al., 1981; Bryce and ap Rees,

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1985; Edwards et al., 1998). Thus, if an alkaline trap depletes CO_2 concentrations sufficiently to perturb metabolism, then ^{14}C-feeding experiments will systematically under-estimate flux through anapleurotic and biosynthetic processes that require CO_2. Alternatively, if the alkaline trap does not effectively absorb the available CO_2, then these studies will under-estimate the extent to which each of the individual carbon atoms of respiratory substrate has been oxidised to CO_2. In either instance, the data obtained will fail to reflect the metabolic activity of the tissue under normal conditions. A further potential complication is introduced if CO_2 is not quantitatively captured by the alkaline trap. Under such circumstances, $^{14}CO_2$ released during oxidation of positionally labelled substrates may be reassimilated through carboxylation reactions. This could lead to labelling of intermediates in carbon positions unrelated to those in the original [^{14}C]substrate thereby confounding interpretation of flux through the metabolic network.

The aim of this study is to assess the extent to which the problems considered above are likely to distort the pattern of metabolism or its interpretation. A heterotrophic cell suspension culture of *Arabidopsis thaliana* is used to address two points: first, the extent to which the presence of an alkaline trap perturbs metabolism under conditions typical of those used in ^{14}C-feeding experiments; and secondly, the degree to which respired $^{14}CO_2$ is reincorporated into metabolic intermediates during such studies.

2. Results and discussion

2.1. Influence of an alkaline CO_2-trap on metabolism of specifically labelled [^{14}C]substrates by Arabidopsis cells

Arabidopsis cell suspension cultures showed the anticipated pattern of metabolism of positionally labelled respiratory substrate in a typical time-course study in which the alkaline CO_2-trap was replaced at intervals throughout a 24-h incubation period (Fig. 1, see also graphical abstract for scale expansion of $^{14}CO_2$ release

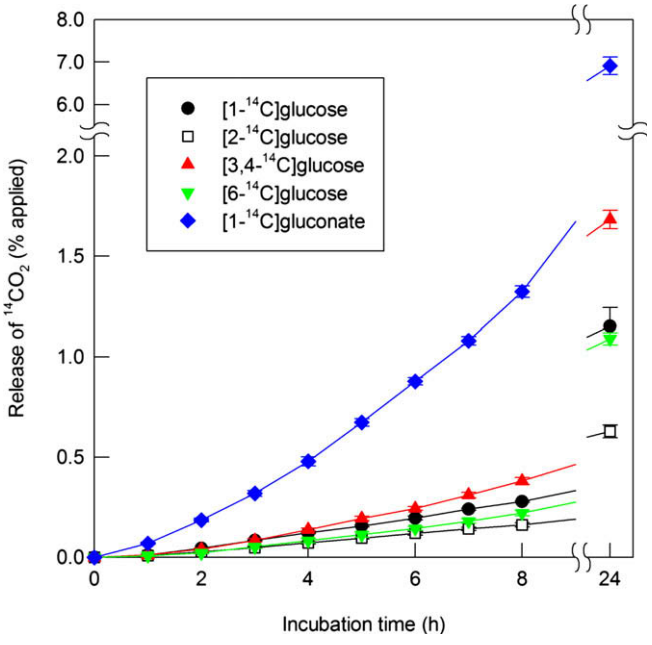

Fig. 1. Oxidation of specifically labelled [^{14}C]substrates by a cell suspension culture of *Arabidopsis*. Samples from a 7-day-old culture were incubated in [1-^{14}C]glucose (●), [2-^{14}C]glucose (□), [3,4-^{14}C]glucose (▲), [6-^{14}C]glucose (▼), or [1-^{14}C]gluconate (♦) for 24 h. Released $^{14}CO_2$ was collected in alkaline traps that were replaced at the time intervals indicated. Cumulative $^{14}CO_2$ release is presented as the percentage of applied label. Each value is the mean ± SE from four replicate samples.

from [^{14}C]glucose over the first 8 h). Following a lag of 2–4 h, the release of $^{14}CO_2$ from [1-^{14}C]- and [2-^{14}C]-glucose, was approximately linear while that from [3,4-^{14}C]- and [6-^{14}C]-glucose, and [^{14}C]gluconate continued to increase progressively over the remainder of the time-course. As expected, $^{14}CO_2$ was released in the order C3,4 > C1 > C6 > C2 from specific positions in glucose. The ratios of $^{14}CO_2$ release from different carbon positions within [^{14}C]glucose and [1-^{14}C]gluconate (Fig. 2) are consistent with the likely activities of glycolysis, the oxidative pentose phosphate pathway, the tricarboxylic acid cycle, recycling of triose phosphates to hexose phosphates, and pentan synthesis (Davies et al., 1964; ap Rees, 1980; Malone et al., 2006; Kruger et al., 2007). The changes that occurred in these ratios during the initial phase of the time-course may be explained by differences in the time taken to isotopically label the pools of metabolites that are the immediate precursors for decarboxylation of C-groups derived from specific positions within the original [^{14}C]substrate, and suggest that most metabolic intermediates had achieved isotopic equilibrium within about 4 h.

To examine the influence of the presence of the alkaline trap on metabolism, cells were incubated with [1-^{14}C]-, [2-^{14}C]-, [3,4-^{14}C]- and [6-^{14}C]-glucose, and [1-^{14}C]gluconate in sealed flasks in either the absence or presence of a vial containing KOH. The incubation was terminated after 4 h by acidifying the medium, and the released $^{14}CO_2$ was determined after adding KOH to the vials of those samples from which it was absent during the incubation. For each [^{14}C]substrate, the amounts of $^{14}CO_2$ recovered in the alkaline trap were equivalent under the two conditions, but were about twice those obtained during the equivalent period of the time-course study undertaken at the same time (Table 1). Comparison of $^{14}CO_2$ release by MANOVA revealed no significant difference between incubations conducted in sealed flasks in the presence or absence of the alkaline trap, whereas $^{14}CO_2$ capture in the equivalent samples from the time-course study was significantly lower than that obtained in sealed flasks for each of the labelled substrates ($V = 1.483$, $F = 3.44$, $df = 10,12$, $P = 0.023$; $\Lambda = 0.004$, $F = 14.03$, $df = 10,10$, $P < 0.001$). However, despite the marked differences in yield of $^{14}CO_2$ between samples incubated in different ways, statistical comparison of the ratios of $^{14}CO_2$ release from specific carbon positions within [^{14}C]glucose and [1-^{14}C]gluconate by MANOVA showed no significant difference between cells incubated in the presence or absence of an alkaline trap and those 4 h into the time-course study (Table 2). The ratios compared in this analysis are those used in previous studies to report directly on relative flux through different sections of the central pathways of carbohydrate oxidation (Davies et al., 1964; ap Rees, 1980; Garlick et al., 2002; Malone et al., 2006). The results show that the presence of an alkaline CO_2 trap has no appreciable impact on metabolism. Moreover, they indicate that even though the sampling strategy used in conventional time-course studies does not quantitatively capture CO_2, it provides an accurate reflection of the ratio of $^{14}CO_2$ released from different positional ^{14}C-labelled substrates.

2.2. Influence of an alkaline CO_2-trap on metabolism of [U-^{14}C]glucose by Arabidopsis cells

The influence of an alkaline trap on carbohydrate metabolism in cell cultures was further examined by comparing the redistribution of label following incubation with [U-^{14}C]glucose in the absence or presence of a vial containing KOH. These incubations were conducted in sealed flasks under conditions equivalent to those used to monitor oxidation of positionally labelled [^{14}C]substrates as described in the previous section, and the studies were extended to include two treatments likely to alter flux through the central pathways of carbon metabolism. In one, the cells were incubated with nitrite which is taken up and converted to ammonia thereby

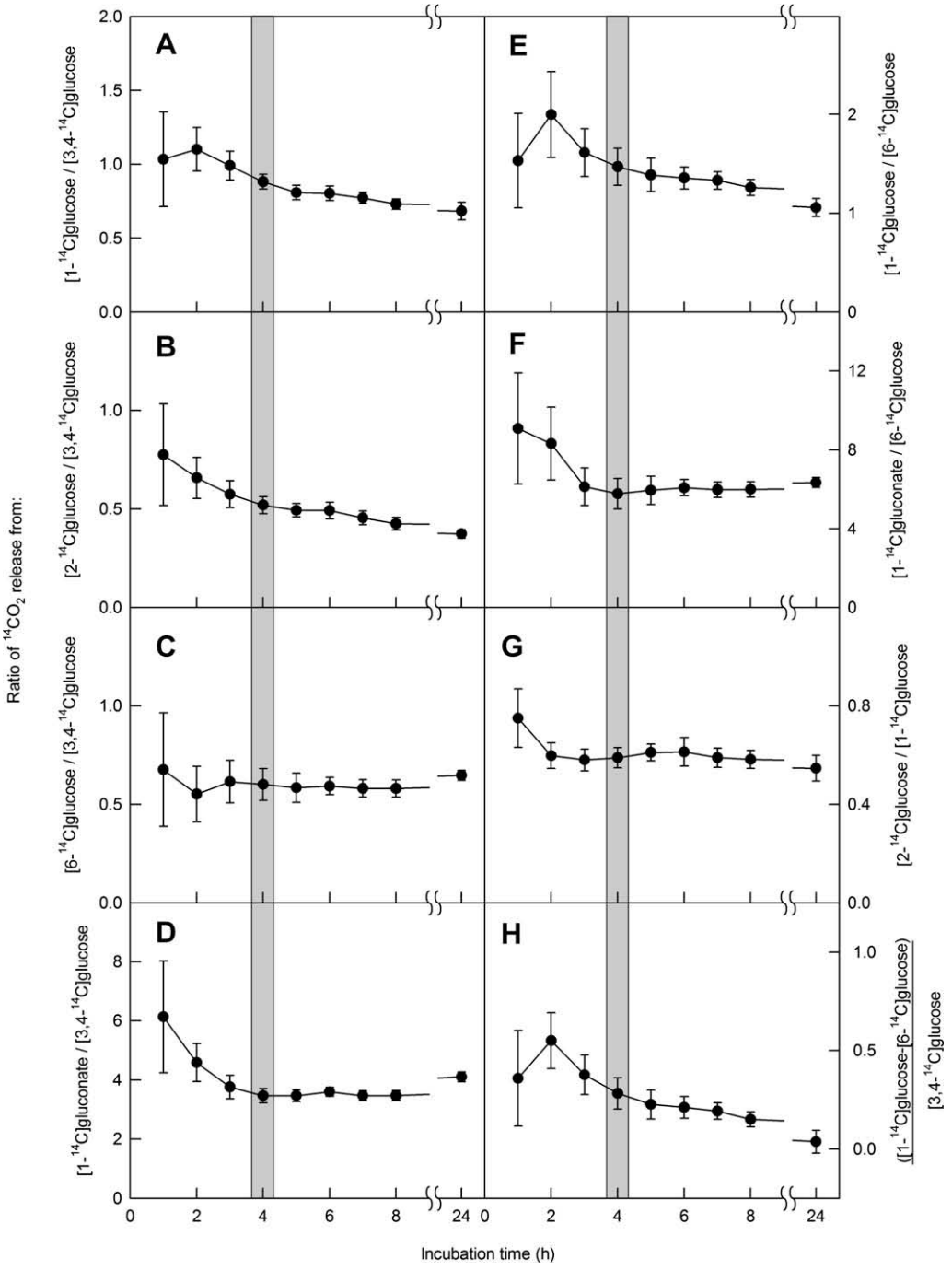

Fig. 2. Comparison of relative rates of oxidation of specific positions within labelled substrates by a cell suspension culture of *Arabidopsis*. Ratios of $^{14}CO_2$ release from different combinations of positionally labelled substrates were calculated for cumulative $^{14}CO_2$ release during defined time intervals following metabolism of ^{14}C-labelled substrate by a cell suspension culture using data presented in Fig. 1. Shown are the ratios of $^{14}CO_2$ release from: A, [1-^{14}C]glucose:[3,4-^{14}C]glucose; B, [2-^{14}C]glucose:[3,4-^{14}C]glucose; C, [6-^{14}C]glucose:[3,4-^{14}C]glucose; D, [1-^{14}C]gluconate:[3,4-^{14}C]glucose; E, [1-^{14}C]glucose:[6-^{14}C]glucose; F, [1-^{14}C]gluconate:[6-^{14}C]glucose; G, [2-^{14}C]glucose:[1-^{14}C]glucose; H, ([1-^{14}C]glucose – [6-^{14}C]glucose):[3,4-^{14}C]glucose. Each value is the mean ± SE for ratios determined from four separate samples of cell culture. Shading indicates the ratios after 4 h used for comparison in Table 2.

increasing demand for NADPH and stimulating flux through the oxidative pentose phosphate pathway (Butt and Beevers, 1961; Averill et al., 1998). In the other, the cells were exposed to α-cyano-4-hydroxycinnamic acid which inhibits mitochondrial pyruvate transport (Halestrap, 1975; Dry and Wiskich, 1985), and may divert carbon through an alternative route involving phosphoenolpyruvate carboxylase (ap Rees, 1980). In none of the conditions studied was there any significant effect of the presence of an alkaline trap on either the amount of [U-^{14}C]glucose metabolised or the redistribution of label during the 4 h incubation as assessed by Student's *t*-test and MANOVA, respectively (Tables 3 and 4).

Further comparisons were made after combining data from incubations conducted in the presence and absence of an alkaline trap. Neither nitrite nor α-cyano-4-hydroxycinnamic acid had a significant effect on the amount of [U-^{14}C]glucose metabolised by the cell cultures (Tables 3 and 4). However, MANOVA revealed significant differences in the redistribution of label, expressed as a percent ^{14}C metabolised, between cells incubated in the presence

Table 1
Influence of an alkaline trap on release of $^{14}CO_2$ from specifically labelled substrates by a cell suspension culture of *Arabidopsis*.

Substrate	$^{14}CO_2$ released (% applied)		
	KOH present	KOH absent	During time-course (4 h)
[1-^{14}C]glucose	0.272 ± 0.007	0.271 ± 0.024	0.122 ± 0.003
[2-^{14}C]glucose	0.139 ± 0.008	0.132 ± 0.007	0.072 ± 0.005
[3,4-^{14}C]glucose	0.279 ± 0.006	0.307 ± 0.003	0.138 ± 0.007
[6-^{14}C]glucose	0.168 ± 0.007	0.183 ± 0.009	0.083 ± 0.010
[1-^{14}C]gluconate	0.911 ± 0.026	1.070 ± 0.028	0.479 ± 0.023

Samples from a 7-day-old culture were incubated in 5 ml growth medium supplemented with specifically labelled substrate in the dark in sealed flasks in the presence or absence of a vial containing KOH. Cells were killed after 4 h by acidification of the sample, and $^{14}CO_2$ released through metabolism was captured by KOH already present, or added to the vial following acidification. Data for cumulative $^{14}CO_2$ release from the equivalent period of the time-course described in Fig. 1 are presented for comparison. Each value is the mean ± SE of measurements from four separate samples.

Table 2
Relative rates of oxidation of specific positions within labelled substrates by a cell suspension culture of *Arabidopsis* in the presence and absence of an alkaline trap.

Position of label	Ratio of $^{14}CO_2$ release from specifically labelled substrates		
	KOH present	KOH absent	During time-course (4 h)
C1/C3,4	0.977 ± 0.034	0.885 ± 0.078	0.883 ± 0.050
C2/C3,4	0.498 ± 0.033	0.431 ± 0.023	0.519 ± 0.043
C6/C3,4	0.602 ± 0.027	0.596 ± 0.028	0.600 ± 0.080
C1-GlcA/C3,4	3.268 ± 0.118	3.490 ± 0.096	3.466 ± 0.240
C1/C6	1.623 ± 0.077	1.486 ± 0.147	1.471 ± 0.187
C1-GlcA/C6	5.433 ± 0.265	5.862 ± 0.312	5.776 ± 0.767
C2/C1	0.510 ± 0.033	0.487 ± 0.050	0.588 ± 0.042
(C1–C6)/C3,4	0.375 ± 0.036	0.289 ± 0.082	0.283 ± 0.080

Ratios of $^{14}CO_2$ release from different combinations of positionally labelled substrates were calculated using data presented in Table 1. The ratios are based on release from the indicated C positions of glucose except for C1-GlcA which refers to release from C1 of gluconate. Each value is the mean ± SE for ratios determined from four separate samples of cell culture.

and absence of nitrite ($V = 0.96$, $F = 12.5$, $df = 10,5$, $P = 0.006$; $\Lambda = 0.039$, $F = 12.5$, $df = 10,5$, $P = 0.006$) although the differences were confined to minor changes in labelling of the acidic, basic and residual components of the EtOH-insoluble fraction. Similar analyses showed that α-cyano-4-hydroxycinnamic acid had a significant effect on the redistribution of metabolised ^{14}C ($V = 0.96$, $F = 12.7$, $df = 10,5$, $P = 0.006$; $\Lambda = 0.038$, $F = 12.7$, $df = 10,5$, $P = 0.006$) with statistically large effects on the proportion of label accumulating in sucrose ($r_{pb} = 0.87$), the basic fraction of EtOH-soluble compounds ($r_{pb} = 0.72$), the neutral, acidic and basic fractions of EtOH-insoluble compounds ($r_{pb} = 0.54$, 0.56 and 0.65, respectively) and carbon dioxide ($r_{pb} = 0.68$). The increase in labelling of sucrose and decrease in labelling of the other fractions in cells treated with α-cyano-4-hydroxycinnamic acid is consistent with an inhibition of mitochondrial pyruvate uptake and a restriction of entry of carbon into the tricarboxylic acid cycle. Overall, these results demonstrate that even under conditions in which fluxes through the major pathways of carbohydrate oxidation are varied, inclusion of an alkaline CO_2 trap does not itself perturb metabolism.

2.3. Assimilation of $^{14}CO_2$ by heterotrophic Arabidopsis cells

A cell culture was incubated in a medium supplemented with NaH[^{14}C]O_3 in either the presence or absence of an alkaline trap to assess the likely extent of reassimilation of $^{14}CO_2$ produced during the metabolism of labelled respiratory substrates. Over a 4-h incubation about 5% of the radiolabel was incorporated into acid-stable compounds, and the presence of KOH had no significant effect on the assimilation of $^{14}CO_2$ as assessed by Student's *t*-test (Table 5). This result has two implications. The first is that the presence of an alkaline trap does not deplete available CO_2 in the medium to the extent that it impairs the activity of phosphoenolpyruvate carboxylase and other carboxylating enzymes. This is supported by analysis of control samples lacking the cell culture in which only about 20% of the label added to the medium as NaH[^{14}C]O_3 was captured in the alkaline CO_2-trap during a 4-h incubation (data not shown). The second implication is that the extent of dark fixation of CO_2 by heterotrophic cell cultures is relatively low. However, these measurements may underestimate the degree of reassimilation of metabolically derived $^{14}CO_2$ because they rely on the uptake of inorganic carbon from the medium and thus may not reflect the actual rate of intracellular CO_2 fixation. This consideration was assessed by examining the redistribution of label by a cell culture incubated in [1-^{14}C]gluconate. This substrate is taken up by plant cells, phosphorylated to 6-phosphogluconate and thereafter metabolised via the oxidative pentose phosphate pathway in which radiolabel present at C-1 is converted exclusively to $^{14}CO_2$ (Garlick et al., 2002). Thus, labelling of other compounds provides a direct indication of reassimilation of this metabolically generated $^{14}CO_2$. Accordingly, cells were incubated in [1-^{14}C]gluconate for 4 h under conditions equivalent to those used to monitor metabolism of [^{14}C]substrates as described in the previous section before being extracted and subjected to metabolic fractionation. There was no significant difference in the redistribution of label between cells incubated in the presence and absence of an alkali trap (Table 6). Under the fractionation conditions used, the vast majority of label in the EtOH-soluble material was recovered in the acidic fraction that bound to Dowex 1 anion exchange resin and eluted with 80 mM potassium succinate (pH 5.5), although about 20% of the label was recovered in the neutral fraction and less than 2% in the basic components. Labelling of the EtOH-insoluble material was negligible. Excluding the release of $^{14}CO_2$, the distribution of label in the cell extracts was identical to that in [1-^{14}C]gluconate fed to the cell culture (Table 6). These results suggest that essentially all of the radiolabel in the cell extracts is confined to gluconate and its immediate product 6-phosphogluconate, and are consistent with there being very limited reassimilation of metabolically generated $^{14}CO_2$ by these cells.

3. Concluding remarks

This study presents two lines of evidence demonstrating that metabolism in cell cultures is not perturbed by the presence of an alkali trap as employed in conventional ^{14}C-feeding experiments. First, the ratios of $^{14}CO_2$ release from different combinations of positionally labelled substrates are not significantly altered by the presence of a vial containing KOH (Table 2). Secondly, the redistribution of label following metabolism of [U-^{14}C]glucose is the same in the presence and absence of an alkali CO_2 trap, and this equivalence is maintained under a range of conditions likely to alter relative flux through the major pathways of carbohydrate oxidation (Tables 3 and 4). These results indicate concerns that metabolism will be perturbed by the presence of an alkali trap are unfounded.

The likely reason why an alkali trap has no measurable impact on metabolism is that it does not, by itself, facilitate rapid and complete removal of CO_2 from the medium. This is suggested by the observation that the amount of $^{14}CO_2$ captured in a conventional time-course study is typically only about half of that released following acidification of the medium at the equivalent time point (Table 1), and is supported by the demonstration that only about 20% of label added to the incubation medium as H[^{14}C]O_3^- is captured in an alkaline trap within the 1–8 h timeframe of a typical metabolic study. This implies that, despite the presence

Table 3
Influence of nitrite on metabolism of [U-^{14}C]glucose by a cell suspension culture of *Arabidopsis* incubated in the presence or absence of an alkaline trap.

Fraction	Radioactivity in specified fraction			
	Control		Nitrite	
	KOH present	KOH absent	KOH present	KOH absent
Ethanol soluble	2,712,468 ± 129,463	2,728,356 ± 40,806	2,792,110 ± 9595	2,637,458 ± 136,972
Neutral	2,679,988 ± 128,966	2,697,656 ± 40,054	2,764,178 ± 8867	2,605,114 ± 141,025
Glucose	2653901 ± 123354	2,628,502 ± 41,245	2,757,801 ± 45354	2,576,944 ± 166,638
Sucrose	22181 ± 1098	19,008 ± 2028	195,723 ± 1064	22,664 ± 2878
	(27.8 ± 1.5)	(26.2 ± 1.8)	(28.1 ± 1.1)	(30.2 ± 0.8)
Fructose	4714 ± 260	4337 ± 413	4149 ± 179	5058 ± 767
	(5.9 ± 0.4)	(6.0 ± 0.4)	(6.0 ± 0.2)	(6.7 ± 0.2)
Acidic	24002 ± 1685	22,151 ± 2087	19,943 ± 1133	24,604 ± 5161
	(30.0 ± 1.5)	(30.7 ± 2.5)	(28.7 ± 1.3)	(31.3 ± 3.4)
Basic	8478 ± 548	6547 ± 1973	7990 ± 535	7740 ± 440
	(10.8 ± 1.3)	(8.8 ± 2.6)	(11.5 ± 0.8)	(11.2 ± 2.5)
Ethanol insoluble	9273 ± 2633	7373 ± 445	6751 ± 818	6098 ± 1217
	(11.3 ± 2.8)	(10.4 ± 1.1)	(9.7 ± 1.0)	(8.0 ± 1.2)
Neutral	2718 ± 1605	1429 ± 204	2828 ± 646	3037 ± 945
	(3.3 ± 1.8)	(2.0 ± 0.4)	(4.0 ± 0.8)	(3.8 ± 1.1)
Acidic	735 ± 150	687 ± 40	576 ± 44	364 ± 69
	(0.9 ± 0.2)	(1.0 ± 0.1)	(0.8 ± 0.1)	(0.5 ± 0.1)
Basic	2689 ± 487	2654 ± 29	2076 ± 90	1441 ± 155
	(3.3 ± 0.5)	(3.7 ± 0.2)	(3.0 ± 0.1)	(1.9 ± 0.1)
Residue	3132 ± 539	2602 ± 315	1270 ± 141	1255 ± 190
	(3.9 ± 0.6)	(3.7 ± 0.6)	(1.8 ± 0.2)	(1.8 ± 0.4)
Carbon dioxide	11,571 ± 1932	12,616 ± 2483	11,125 ± 1297	9309 ± 1289
	(14.2 ± 1.7)	(17.9 ± 4.0)	(16.1 ± 2.0)	(12.6 ± 1.5)
Total ^{14}C metabolised (dpm)	80,220 ± 4623	72,032 ± 3044	69,531 ± 1908	75,472 ± 9853
Total recovery of ^{14}C (%)	100.1 ± 2.1	99.1 ± 1.4	101.3 ± 1.7	100.4 ± 1.1

Samples from a 7-day-old culture were incubated with 37 kBq [U-^{14}C]glucose for 4 h in either the absence or presence of 0.5 mM sodium nitrite prior to extraction in HCO_2H/EtOH and subsequent metabolic fractionation. The amount of radioactivity in each fraction is presented as dpm, and is also expressed in parentheses as a percentage of total ^{14}C metabolised (the sum of radioactivity in carbon dioxide, the ethanol-insoluble fraction, and the ethanol-soluble fraction excluding that present in glucose in each cell culture). Each value is the mean ± SE from four separate culture samples. Total recovery is expressed as a percentage of ^{14}C applied to the flask.

Table 4
Influence α-cyano-4-hydroxycinnamic acid on metabolism of [U-^{14}C]glucose by a cell suspension culture of *Arabidopsis* the presence or absence of an alkaline trap.

Fraction	Radioactivity in specified fraction			
	Ethanol		α-Cyano-4-hydroxycinnamic acid	
	KOH present	KOH absent	KOH present	KOH absent
Ethanol soluble	2,766,523 ± 32,039	2,742,016 ± 25,495	2,790,097 ± 101,331	2929610 ± 63995
Neutral	2,742,774 ± 29,282	2,719,116 ± 26,963	2,765,175 ± 101,181	2904167 ± 68115
Glucose	2,757,198 ± 56958	2,677,236 ± 45,837	2,646,716 ± 1,000,812	2869893 ± 53232.2
Sucrose	15,511 ± 2385	15,662 ± 1519	21,070 ± 3274	23666 ± 5476
	(26.6 ± 0.8)	(28.2 ± 1.2)	(34.6 ± 1.4)	(37.4 ± 1.3)
Fructose	3679 ± 574	3455 ± 326	3700 ± 540	3867 ± 703
	(6.3 ± 0.2)	(6.2 ± 0.2)	(6.1 ± 0.2)	(6.3 ± 0.1)
Acidic	18,218 ± 2942	17,783 ± 1557	21,084 ± 3378	20983 ± 4712
	(31.2 ± 1.3)	(32.0 ± 0.9)	(34.5 ± 1.4)	(33.3 ± 1.6)
Basic	5530 ± 389	5118 ± 270	3838 ± 244	4460 ± 449
	(9.8 ± 0.6)	(9.3 ± 0.5)	(6.6 ± 0.7)	(7.5 ± 0.6)
Ethanol insoluble	5682 ± 1146	3585 ± 225	3311 ± 438	3212 ± 247
	(9.6 ± 1.3)	(6.5 ± 0.4)	(5.6 ± 0.8)	(5.6 ± 0.8)
Neutral	2284 ± 694	1156 ± 165	986 ± 130	872 ± 83
	(3.7 ± 0.8)	(2.1 ± 0.3)	(1.8 ± 0.4)	(1.5 ± 0.2)
Acidic	496 ± 41	397 ± 59	348 ± 24	295 ± 36
	(0.9 ± 0.1)	(0.7 ± 0.1)	(0.6 ± 0.1)	(0.5 ± 0.1)
Basic	1130 ± 207	828 ± 12	663 ± 37	654 ± 73
	(1.9 ± 0.3)	(1.5 ± 0.1)	(1.1 ± 0.1)	(1.1 ± 0.2)
Residue	1772 ± 253	1204 ± 93	1314 ± 372	1391 ± 140
	(3.1 ± 0.4)	(2.2 ± 0.1)	(2.1 ± 0.5)	(2.4 ± 0.5)
Carbon dioxide	9192 ± 215	9692 ± 258	7280 ± 734	6041 ± 1139
	(16.5 ± 1.6)	(17.7 ± 1.2)	(12.5 ± 1.8)	(10.0 ± 1.8)
Total ^{14}C metabolised (dpm)	57,812 ± 7218	55,294 ± 3628	60,284 ± 7861	62229 ± 11995
Total recovery of ^{14}C (%)	101.8 ± 1.9	99.6 ± 0.9	97.0 ± 1.3	100.1 ± 1.3

Samples from a 7-day-old culture were incubated with 37 kBq [U-^{14}C]glucose for 4 h in either 1.0 mM sodium α-cyano-4-hydroxycinnamic acid (supplied as a concentrated stock solution dissolved in 50 μl EtOH) or an equivalent volume of EtOH prior to extraction in HCO_2H/EtOH and subsequent metabolic fractionation. The amount of radioactivity in each fraction is presented as dpm, and is also expressed in parentheses as a percentage of total ^{14}C metabolised (calculated as described in Table 3). Each value is the mean ± SE from four separate culture samples. Total recovery is expressed as a percentage of ^{14}C applied to the flask.

of an alkaline trap, an appreciable proportion of the inorganic carbon remains dissolved in the incubation medium and cells, and is therefore still available as a substrate for carboxylation reactions, such as that catalysed by phosphoenolpyruvate carboxylase.

Table 5
Non-photosynthetic assimilation of $^{14}CO_2$ by a cell suspension culture of *Arabidopsis*.

Incubation conditions	^{14}C incorporated into acid-stable compounds	
	dpm	% applied[a]
KOH present	122,004 ± 2293	5.62 ± 0.11
KOH absent	118,964 ± 1362	5.48 ± 0.06

Samples from a 7-day-old culture were incubated in 5 ml growth medium supplemented with 50 mM $NaH^{14}CO_3$ (specific activity 148 MBq mol^{-1}) for 4 h in the dark in either the presence or absence of an alkaline CO_2 trap. Metabolism was stopped by acidification using HCO_2H and the incorporation of ^{14}C into acid-stable compounds was determined. Each value is the mean ± SE of measurements on four separate samples.

[a] Total activity applied was 2,171,912 ± 10,027 dpm.

Table 6
Metabolism of [1-^{14}C]gluconate by a cell suspension culture of *Arabidopsis*.

Fraction	Radioactivity in specified fraction		
	Cell extract		[1-^{14}C]gluconate (supplied to cells)
	KOH present	KOH absent	
Ethanol soluble			
Neutral	612.9 ± 28.1 (19.9 ± 1.1)	613.1 ± 29.1 (19.7 ± 1.2)	3482 ± 146 (19.8 ± 1.1)
Acidic – succinate eluate	2283.3 ± 79.1 (74.0 ± 1.7)	2312.5 ± 80.1 (74.1 ± 1.9)	13049 ± 532 (74.0 ± 1.8)
Acidic – formate eluate	147.3 ± 24.9 (4.8 ± 0.9)	153.3 ± 22.5 (4.9 ± 0.8)	868 ± 114 (5.0 ± 0.7)
Basic	42.1 ± 30.1 (1.3 ± 0.9)	40.2 ± 30.7 (1.3 ± 0.9)	222 ± 169 (1.2 ± 0.9)
Ethanol insoluble	n.s.	n.s.	–
Carbon dioxide	2122.4 ± 57.7	2275.4 ± 62.2	–

Samples from a 7-day-old culture were incubated in 5 ml growth medium supplemented with 0.3 mM [1-^{14}C]gluconate in the dark in sealed flasks in the presence or absence of a vial containing KOH. After 4 h, cells were harvested and washed prior to extraction in HCO_2H/EtOH and subsequent metabolic fractionation. Acidic compounds retained by Dowex 1 anion exchange resin were eluted with 80 mM K-succinate (succinate eluate) followed by 4 M HCO_2H (formate eluate). The amount of radioactivity in each fraction is presented as dpm, and in parentheses as a percentage of the label in the ethanol-soluble fraction. Each value is the mean ± SE of measurements from four separate samples. The distribution of radioactivity in samples of the [1-^{14}C]gluconate supplied to the cells is presented for comparison. n.s. indicates values not significantly above background.

Even though CO_2 is not quantitatively removed by the alkaline trap, there is no evidence to suggest that reassimilation of metabolically generated $^{14}CO_2$ leads to a significant distortion in the pattern of label release from different carbon positions in the supplied glucose. Estimates based on the assimilation of label added to the incubation medium as $H[^{14}C]O_3^-$ suggest that less than 5% of metabolically generated $^{14}CO_2$ is reincorporated into metabolic intermediates (Table 5). Furthermore, there is no indication of appreciable reassimilation of intracellular $^{14}CO_2$ generated during the metabolism of [1-^{14}C]gluconate (Table 6). However, care must be exercised in extending these conclusions to other plant systems with more pronounced biosynthetic activity, and in particular developing oilseeds in which CO_2 recapture by Rubisco seems to be an important process (Schwender et al., 2004; Goffman et al., 2005). For such material it may be necessary to use an open system in which the incubation vessel is continuously flushed with air and the exhaust gas bubbled through an alkaline trap to permit adequate recovery of respiratory $^{14}CO_2$ and limit its reassimilation by the tissue (Schwender et al., 2004).

Despite the inability of an alkaline trap to capture all of the $^{14}CO_2$ generated in a conventional time-course study, it would appear that a representative proportion of CO_2 produced by metabolism is absorbed, since the ratios of $^{14}CO_2$ released from particular combinations of positionally labelled [^{14}C]substrates measured in the standard time-course procedure are identical to those obtained following acidification of the medium at the equivalent time (Table 2). These results establish that the ratio of label captured in alkaline CO_2 traps from specifically labelled [^{14}C]glucose remains a valid and convenient measure of the relative rates of oxidation of the different positional carbon atoms within the supplied substrate.

However, the demonstration that $^{14}CO_2$ is not quantitatively removed from the incubation medium by a conventional alkaline trap has potentially important implications for flux determination based on the metabolism of [U-^{14}C]glucose or equivalent [^{14}C]substrates. In this approach the relative respiratory flux is calculated by integrating the proportion of label evolved as CO_2 with that recovered in organic acids, amino acids and protein (Bindon and Botha, 2002; Geigenberger et al., 2005; Davies et al., 2005; Malone et al., 2006; Oliver et al., 2008). Typically, such studies rely on the passive diffusion of $^{14}CO_2$ from the tissue and medium, and are thus likely to under-estimate the amount of $^{14}CO_2$ generated by metabolism and, in turn, under-estimate the rate of respiration. The degree to which published estimates of carbohydrate metabolism are distorted by this effect will depend on both the respiratory activity of the tissue (specifically, the proportion of substrate that is oxidised to CO_2) and on the effectiveness of alkali trapping of $^{14}CO_2$. The latter is likely to vary between studies and will be influenced by the solubility of CO_2 in the incubation medium and by equilibration between the medium and the air space in the incubation vessel, both of which will depend on, *inter alia*, pH and temperature. These considerations suggest that considerable caution must be exercised in evaluating the absolute amount of $^{14}CO_2$ generated in published feeding studies, and that in future work it will be necessary to assess the efficiency of $^{14}CO_2$ capture by an alkaline trap particularly when using procedures that do not permit acidification of the sample and medium at the end of the incubation period.

4. Experimental

4.1. Materials

Cell suspension cultures of *Arabidopsis thaliana* (L.) Heynh. ecotype Landsberg *erecta*, derived from an original stock established by May and Leaver (1993), were grown in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), supplemented with 160 mM glucose, 0.5 μg.ml^{-1} naphtheleneacetic acid and 0.05 μg ml^{-1} kinetin at 22 °C in the light in a controlled environment and aerated on a rotary shaker at 100 rpm. The suspension was subcultured every 7 d, by adding 10 ml of culture to 90 ml of fresh MS medium in a 250 ml conical flask.

Sodium [^{14}C]bicarbonate (specific activity 1.92 GBq $mmol^{-1}$), [1-^{14}C]glucose (specific activity 2.00 GBq $mmol^{-1}$), [6-^{14}C]glucose (specific activity 2.07 GBq $mmol^{-1}$), and [U-^{14}C]glucose (specific activity 0.14 GBq $mmol^{-1}$) were purchased from GE Healthcare Life Sciences (http://www1.gelifesciences.com/). [1-^{14}C]gluconate (specific activity 2.04 GBq $mmol^{-1}$) and [3,4-^{14}C]glucose (specific activity 1.11 GBq $mmol^{-1}$) were obtained from American Radiolabeled Chemicals Inc. (http://www.arc-inc.com/). [2-^{14}C]glucose (specific activity 1.67 GBq $mmol^{-1}$) was from PerkinElmer NEN (http://las.perkinelmer.com/). All enzymes were from Roche Diagnostics Ltd. (http://www.roche.com/). General chemicals and chromatography resins were purchased from Sigma–Aldrich (http://www.sigmaaldrich.com/) or Merck (http://www.merckbiosciences.co.uk/).

4.2. Monitoring release of $^{14}CO_2$ by Arabidopsis cell culture metabolising positional [^{14}C]-labelled substrates

A 1 ml aliquot of a 7-day-old *Arabidopsis* cell suspension was incubated in the dark at 22 °C in a 100 ml conical flask in a total

volume of 5 ml MS medium containing 3.7 kBq specifically labelled [^{14}C]glucose or [1-^{14}C]gluconate. Each flask was then sealed with a rubber bung and aerated on an orbital shaker at 100 rpm. For incubations involving [1-^{14}C]gluconate, the concentration of gluconate in the incubation medium was adjusted to 0.3 mM. Released $^{14}CO_2$ was collected in 0.5 ml of 10% (w/v) KOH in a vial suspended in the flask. The KOH solution was replaced every hour for 8 h and again 24 h after the beginning of the incubation. This protocol is similar, or identical, to that used in previous studies (Gibbs and Beevers, 1955; Stitt and ap Rees, 1978; Garlick et al., 2002; Malone et al., 2006).

4.3. Metabolism of [^{14}C]substrates by Arabidopsis cell culture

Cell cultures were incubated with 3.7 kBq specifically labelled [^{14}C]glucose, 3.7 kBq [1-^{14}C]gluconate or 37 kBq [U-^{14}C]glucose in conditions identical to those described under "*4.2 Monitoring time-course of release of $^{14}CO_2$ by Arabidopsis cell culture metabolising positional [^{14}C]-labelled substrates*" except that each flask was sealed with a suba-seal bung from which was suspended a 1.5 ml vial to hold the alkaline CO_2-trap. After 4 h the incubation was terminated by injecting 1 ml 25 M HCO_2H into the incubation medium through the bung. $^{14}CO_2$ released from the cells and acidified medium was captured by 0.5 ml 10% KOH that was either present throughout the incubation in the vial suspended in the flask or added to the vial following acidification of the sample. The flask was incubated for a further 1 h with shaking at 22 °C and then at 4 °C for 8–16 h before extraction of the incubation mixture.

4.4. Ethanol extraction of Arabidopsis cell culture

The acidified incubation mixture was quantitatively transferred to a 15 ml tube using a total of 6 ml EtOH. The tube was sealed and incubated at 80 °C for 60 min. After cooling, the extract was centrifuged at 3500g for 5 min and the supernatant was removed. The pellet was washed with 5 ml EtOH thrice and the washings added to the supernatant. The combined soluble fraction derived from each sample was reduced to dryness by rotary evaporation at 40 °C and redissolved in 4 ml 50 mM Na-OAc buffer (pH 5.5). The insoluble residue following EtOH extraction was resuspended in water to a final volume of 1 ml. All fractions were stored at −80 °C prior to fractionation.

4.5. Fractionation of ^{14}C-labelled extracts

The soluble fraction obtained by extraction of the cell culture in EtOH was separated into acidic, basic and neutral components by ion-exchange chromatography through Dowex 50Wx8-200 (H^+ form) and Dowex 1x8-200 (OAc^- form) using a vacuum manifold as described by Kruger et al. (2007). The neutral component of the soluble fraction was evaporated to dryness, resuspended in water, and further fractionated by TLC as described by Scott and Kruger (1995). The EtOH-insoluble residue remaining following extraction of the cell culture was degraded and fractionated as described by Kruger et al. (2007).

4.6. $^{14}CO_2$ metabolism by Arabidopsis cell culture

Cell cultures were incubated in MS medium supplemented with 50 mM $NaH^{14}CO_3$ (specific activity 148 MBq mol^{-1}) in conditions identical to those described under "*4.3 Metabolism of [^{14}C]substrates by Arabidopsis cell culture*". The incubation was terminated after 4 h by acidification of the cell culture, and the alkali CO_2-trap (whether present throughout the incubation or added following acidification) was removed. To determine incorporation of ^{14}C into acid-stable metabolic products, the acidified incubation mixture was evaporated to dryness on a heating block at 60 °C, resuspended in 1 ml 25 M HCO_2H, dried down again, and then resuspended in 1 ml H_2O. An aliquot of the extract was combined with an equal volume of Scintran tissue solubiliser (Merck) and incubated for 16 h at 60 °C prior to determination of ^{14}C (Runquist and Kruger, 1999).

4.7. Determination of radioactivity

The amount of ^{14}C in aqueous samples was determined by liquid scintillation counting after addition of four volumes of Optiphase 'HiSafe' 3 (Wallac, http://las.perkinelmer.co.uk/). The efficiency of counting was typically greater than 90%.

4.8. Statistical analysis

The mean ± standard error (SE) of ratios of independent unpaired samples were determined as described by Motulsky (1995). Student's *t*-test (two-tailed, unequal variance) and MANOVA (based on Type III sum-of-squares) were conducted using SPSS 12.0 for Windows (SPSS, http://www.spss.com/). The arcsine [$\arcsin(y/100)^{0.5}$] of percentage values and the logarithm of ratios were used in these analyses (Wardlaw, 1985). Homogeneity of variance of the dependent variable(s) was confirmed by Levene's test. MANOVA outputs were assessed by Pillai's trace (V) and Wilks' Lamda (Λ). Only statistical comparisons for which $P < 0.05$ are considered significant, and large effects are defined as those with a point-biserial (Pearson) correlation coefficient (r_{pb}) > 0.5 (Cohen, 1992).

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