Variation in oxygen isotope fractionation during cellulose synthesis: intramolecular and biosynthetic effects

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ABSTRACT

The oxygen isotopic composition of plant cellulose is commonly used for the interpretations of climate, ecophysiology and dendrochronology in both modern and palaeoenvironments. Further applications of this analytical tool depends on our in-depth knowledge of the isotopic fractionations associated with the biochemical pathways leading to cellulose. Here, we test two important assumptions regarding isotopic effects resulting from the location of oxygen in the carbohydrate moiety and the biosynthetic pathway towards cellulose synthesis. We show that the oxygen isotopic fractionation of the oxygen attached to carbon 2 of the glucose moieties differs from the average fractionation of the oxygen attached to carbons 3–6 from cellulose by at least 9%, for cellulose synthesized within seedlings of Triticum aestivum L. and Ricinus communis L.). The fractionation for a given oxygen in cellulose synthesized by the Triticum seedlings, which have starch as their primary carbon source, is different than the corresponding fractionation in Ricinus seedlings, within which lipids are the primary carbon source. This observation shows that the biosynthetic pathway towards cellulose affects oxygen isotope partitioning, a fact heretofore undemonstrated. Our findings may explain the species-dependent variability in the overall oxygen isotope fractionation during cellulose synthesis, and may provide much-needed insight for palaeoclimate reconstruction using fossil cellulose.

Key-words: oxygen-18; oxygen isotope ratios; palaeoclimate.

INTRODUCTION

Oxygen isotope ratios of plant cellulose are commonly invoked as a quantitative indicator of several abiotic and biotic factors integrated during cellulose synthesis. Initial isotopic studies of aquatic plant cellulose revealed that δ18O values (see Eqn 2 for a definition of δ18O) of cellulose (δ18Ocel) were ~27‰ greater than the δ18O value of water (δw) at the site of synthesis (Epstein, Thompson & Yapp 1977; DeNiro & Epstein 1981). This has led to the conclusion that this enrichment factor results from a 2/3 contribution of ambient CO2 (which has equilibrated with ambient water and has a δ18O value ~41‰ greater than the δ18O value of ambient water) and a 1/3 contribution from unfrac-tioned environmental water (Epstein et al. 1977). Hence,

\[
\delta^{18}O_{\text{cel}} = \frac{1}{3} \delta_w + \frac{2}{3} \delta_{w+41} = \delta_w + 27.3\%.
\] (1)

If Eqn 1 is valid, there should be a temperature effect on the isotopic composition of cellulose, because the isotopic equilibration between CO2 and water is temperature sensitive (Vogel, Groates & Mark 1970). However, no such temperature effect has been observed, indicating that some additional processes have contributed to the oxygen isotope signature of cellulose (DeNiro & Epstein 1981). Further evidence against a simplistic adoption of Eqn 1 is provided by tissue cultures, seed germination and other experiments within which cellulose synthesized heterotrophically showed post-photosynthetic isotopic exchange between the oxygen in intermediate carbohydrates and water (Sternberg, DeNiro & Savidge 1986; Yakir & DeNiro 1990; Luo & Sternberg 1992). Studies show that, depending on the substrate available for cellulose synthesis, 29–77% of the oxygen isotope content of heterotrophically synthesized cellulose is determined by the oxygen isotope content of water. Furthermore, cellulose synthesized from sucrose, the most common carbohydrate transported within plants, acquires about 40% of its oxygen from water (Sternberg et al. 1986; Yakir & DeNiro 1990; Roden & Ehleringer 1999). Current mechanistic models meant to explain δ18O values in plant cellulose implicitly assume that the bulk of the isotopic signature acquired during cellulose synthesis occurs via the carbonyl hydration reaction. This assumption is supported by the observation that the fractionation factor for the carbonyl oxygen in an in vitro acetone–water system is 27‰ and is not subject to temperature effects (Sternberg & DeNiro 1983). The second implicit assumption is that oxygen isotope fractionation during heterotrophic cellulose synthesis relative to the in situ water remains constant regardless of the location of the oxygen in the carbohydrate moiety or the metabolic pathway leading to cellulose synthesis. Although widely used, these assumptions have not
been tested, and several pathways other than the carbonyl hydration reaction by which the oxygen in the glucose moieties could acquire their particular isotopic signature during cellulose synthesis exists (Schmidt, Werner & Rossmann 2001). Furthermore, carbonyl hydration reactions may not achieve complete equilibrium (Schmidt et al. 2001). Some studies have indicated that the named oxygen isotopic fractionations vary and could be substantially below 27%, as exemplified by the case of cellulose synthesized directly from sucrose and from starch (Sternberg et al. 1986; Luo & Sternberg 1992). These studies reported oxygen isotopic fractionation ranging from 16.3 to 22.0‰, as calculated for cellulose relative to the 18O content of in situ water surrounding cellulose synthesis (Sternberg et al. 1986; Luo & Sternberg 1992). Although this evidence suggests that the second implicit assumption previously listed may not be valid, no study has yet been performed to explicitly test the true applicability of the assumption. Here, we report on experiments designed to specifically test the following assumptions: (1) the fractionation factors for oxygen atoms in a carbohydrate moiety are all similar to 27%, regardless of the position of the oxygen within the carbohydrate molecule destined for cellulose synthesis, and (2) the metabolic pathway leading to cellulose synthesis is unimportant isotopically. We improved a previously developed technique (Sternberg, Anderson & Morrison 2003) so that we could determine the oxygen isotope ratios of the oxygen atoms specifically attached to the second carbon (cf. carbons 3–6) within the cellulose glucose moieties to test the first assumption. To test the second assumption, we compared the isotopic signature of cellulose that had been synthesized heterotrophically via two different biosynthetic pathways: using starch or lipid as the primary substrate. Using these approaches, we shed new light on the molecular and biosynthetic aspect of plant oxygen isotope fractionation.

METHODS AND MATERIALS

Heterotrophic cellulose synthesis and extraction

Cellulose was synthesized heterotrophically during the germination and growth of seedlings in complete darkness by two species: *Triticum aestivum* L. (within which starch comprises the major substrate for heterotrophic cellulose synthesis) and *Ricinus communis* L. (within which lipids comprise the major substrate for heterotrophic cellulose synthesis). We define heterotrophic cellulose synthesis as the synthesis of cellulose occurring in a sink tissue (e.g. stems, roots or seedlings grown in the dark) using carbohydrates transported from a source tissue (e.g. leaves or seed endosperm/cotyledons). Germination and growth of seedlings using water with variable oxygen isotopic ratios followed previous procedures (Sternberg et al. 1986; Luo & Sternberg 1992; Sternberg et al. 2003). After germination and stem/foliage growth, the fibrous stems, leaves and roots were separated, dried and ground to a fine powder. Cellulose extraction was performed with the classical methods described within Sternberg (1989a).

**Cellulose hydrolysis and derivation**

Extracted cellulose was hydrolyzed either with cellulase [enzyme class (EC 3.2.1.4)] or trifluoracetic acid (TFA). For the enzymatic (cellulase) hydrolysis, we used a lower and more purified quantity of enzyme (Sigma-C-9422, 7.8 units mg⁻¹ of solid, EC 3.2.1.4; Sigma-Aldrich, St. Louis, MO, USA) than previously reported (Sternberg et al. 2003). Although this product contains a high proportion of cellulose, it also contains cellobiase (EC 3.2.1.21) as per its activity specification (1 unit will liberate 1.0 μmol of glucose from cellulose in 1 h at pH 5.0 and 37 °C). One hundred milligrams of cellulose was added to 0.5 g of cellulose in 25 mL of a sodium acetate buffer (pH 5.0) and incubated at 40 °C for a period of 96 h. After incubation, the enzyme was precipitated by bringing the hydrolysis solution to a concentration of 75% MetOH, centrifuging and rotoevaporating the supernatant to recover the glucose. Acid hydrolysis was performed according to the method of Fengel & Wegener (1979) using 99% TFA, but with a smaller starting quantity of cellulose (0.3 g). Purity of the cellulose hydrolysate was confirmed by comparing its high-performance liquid chromatography (HPLC) chromatogram with that of a standard glucose solution via an Econosil-NH₄ 10 μ column (Alltech, Deerfield, IL, USA) with a 3:1 acetonitrile : water solution as the mobile phase. Sigma α-cellulose (C-8002, lot 11k0246; Sigma-Aldrich) was hydrolyzed by TFA using water of variable oxygen isotope signatures to determine whether the δ18O of the hydrolysis solution affects the δ18O value of the final phenylglucosazone derivative. Glucose from the cellulose hydrolysate was derived to phenyl glucosazone using the method described within Oikawa et al. (1998).

**Isotopic analysis of cellulose, phenyl glucosazone and water**

Oxygen isotope analyses of cellulose and phenyl glucosazone were carried out as previously mentioned (Sternberg et al. 2003). Oxygen isotope ratios of the water available during seed germination and synthesis of heterotrophic cellulose were determined by equilibration of the water with CO₂ over a period of 12 h at 40 °C in a multiflow system connected to an Isoprime isotope ratio mass spectrometer (IRMS; GV Instruments, Manchester, UK). All oxygen isotope ratios reported here are in δ (%) units, relative to Vienna Standard Mean Ocean Water (vSMOW) and are given by

\[
\delta^{18}O = \left[ \frac{R_{\text{sample}}}{R_{\text{vsmow}}} - 1 \right] \cdot 1000. \tag{2}
\]

*R_{\text{sample}}* and *R_{\text{vsmow}}* represent the molar ratio of 18O/16O of the sample and Vienna-based standard mean ocean water. The precision of analysis was ± 0.36‰ for the organic oxygen isotope determination (Sternberg et al. 2003) and ± 0.1‰ for the water samples.
Data analysis

Calculation of the $\delta^{18}O$ value of the oxygen attached to carbon 2 of the glucose moieties of cellulose followed the procedure of Sternberg et al. (2003) and is described by the following equation:

$$\delta^{18}O_2 = (5 \times \delta^{18}O_{\text{cell}}) - (4 \times \delta^{18}O_{\text{p–g}}).$$  \hspace{1cm} (3)

The terms of the previous equation: $\delta^{18}O_2$, $\delta^{18}O_{\text{cell}}$ and $\delta^{18}O_{\text{p–g}}$ represent the $\delta^{18}O$ values of the oxygen attached to the second carbon of the glucose moiety of cellulose, of cellulose and of phenylglucosazone, respectively. Regression values of slopes and intercepts for the linear relationship between $\delta^{18}O_2$, $\delta^{18}O_{\text{cell}}$ and $\delta^{18}O_{\text{p–g}}$ versus the $\delta^{18}O$ value of water available during seed germination and development were compared using the statistical methods of Armitage (1980). If the slopes were significantly different, then no comparison between the intercepts was performed. Error propagation for the calculations of the oxygen isotopic fractionation factor for each of the exchange reactions during cellulose synthesis was performed using the quadrature method (Taylor 1997).

RESULTS

Cellulose hydrolysis

A chromatogram of the TFA-generated cellulose hydrolysate was similar to that of the standard glucose solution, but with an additional minor peak appearing about 30 min after injection (Fig. 1). Oxygen isotope ratios of phenylglucosazone synthesized from the TFA-generated hydrolysates of Sigma $\alpha$-cellulose averaged 23.4 ± 2.2‰, even though the hydrolysis solutions used spanned a range of $\delta^{18}O$ values over 250‰ (Fig. 2).

Oxygen isotope ratios of Triticum generated cellulose and phenylglucosazone

Oxygen isotope ratios of heterotrophically synthesized cellulose ($\delta^{18}O_{\text{cell}}$), phenylglucosazone ($\delta^{18}O_{\text{p–g}}$) and the oxygen attached to the second carbon of the glucose moiety ($\delta^{18}O_2$) were all linearly related to the oxygen isotope ratios of the water ($\delta^{18}O$) available during seed germination and growth (Fig. 3, Table 1). There were, however, significant differences in the slopes of these linear relationships within the Triticum experiment. The linear relationship between $\delta^{18}O_2$ value versus the $\delta^{18}O$ value of water exhibited the highest slope (0.652 and 0.700 for the enzymatic and TFA hydrolysis, respectively) followed by that of cellulose (0.402) and finally, by that of phenylglucosazone (0.339 and 0.336 for the enzymatic and TFA hydrolysis, respectively). Although there were clear differences in the isotopic ratios of the $y$-intercept for the previously mentioned linear relationships (Table 1), they could not be compared statistically because of the extent to which the slope of the regressions differed.

A lower proportion of oxygen associated with carbon 2 exchanged with water during cellulose synthesis by the Triticum seedlings compared with a previous report (Sternberg et al. 2003). This discrepancy might be caused by the current use of a purer enzyme preparation, or by hydrolysis with TFA, which does not leave any residues. In Sternberg et al. (2003), a large quantity of low-purity enzyme (0.5 g) was used for hydrolysis and glucose, often present in some cellulase preparations, might have contaminated the hydrolysate. For these reasons, we prefer the values of isotopic exchange generated by this study as an improvement over Sternberg et al. (2003).

Oxygen isotope ratios of Ricinus generated cellulose and phenylglucosazone

No significant differences were found between the slopes for the linear relationships between $\delta^{18}O_2$, $\delta^{18}O_{\text{cell}}$ and $\delta^{18}O_{\text{p–g}}$ values versus the $\delta^{18}O$ value of the water available for the Ricinus generated cellulose (Fig. 3). A statistical comparison of the $y$-intercepts indicated significant differences: the $\delta^{18}O_2$ versus the $\delta^{18}O_{\text{cell}}$ linear relationship had the highest $y$-intercept (51.05 ± 5.0‰), followed by the intercept of the $\delta^{18}O_{\text{cell}}$ versus $\delta^{18}O_{\text{p–g}}$ relationship (28.4 ± 0.42‰) and the
Figure 3. Oxygen isotope ratios of cellulose (a & b), phenylglucosazone (c & d) and the oxygen attached to carbon 2 of the glucose moieties (e & f) versus the $\delta^{18}O$ value of the water present during heterotrophic cellulose synthesis in both Triticum and Ricinus seedlings. Triangles (c & e) represent the case where cellulose was hydrolyzed enzymatically for the Triticum experiment; circles represent products from cellulose hydrolyzed by trifluoracetic acid (TFA) for both experiments.

Table 1. The slope ($F_i$), intercept ($I_i$) and the correlation coefficient ($R^2$) for the relationship between the $\delta^{18}O$ value of cellulose, phenylglucosazone and oxygen attached to carbon 2 of the glucose moieties within heterotrophically synthesized cellulose versus that of the water within which the cellulose was synthesized for two species (Triticum and Ricinus)

<table>
<thead>
<tr>
<th>Seed type</th>
<th>Oxygens in</th>
<th>$F_i$</th>
<th>$I_i$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triticum</td>
<td>Cellulose</td>
<td>0.402 ± 0.007$^a$</td>
<td>28.6 ± 0.6$^a$</td>
<td>0.995</td>
</tr>
<tr>
<td></td>
<td>Phenylglucosazone (enzyme)</td>
<td>0.339 ± 0.003$^b$</td>
<td>30.5 ± 0.2$^a$</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td>Phenylglucosazone (TFA)</td>
<td>0.336 ± 0.003$^b$</td>
<td>30.8 ± 0.2$^a$</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td>O in carbon 2 (enzyme)</td>
<td>0.652 ± 0.030$^a$</td>
<td>21.3 ± 3.2$^a$</td>
<td>0.944</td>
</tr>
<tr>
<td></td>
<td>O in carbon 2 (TFA)</td>
<td>0.700 ± 0.030$^a$</td>
<td>19.6 ± 3.4$^a$</td>
<td>0.958</td>
</tr>
<tr>
<td>Ricinus</td>
<td>Cellulose</td>
<td>0.905 ± 0.005$^a$</td>
<td>28.4 ± 0.4$^a$</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>Phenylglucosazone (TFA)</td>
<td>0.924 ± 0.012$^a$</td>
<td>22.5 ± 1.0$^a$</td>
<td>0.997</td>
</tr>
<tr>
<td></td>
<td>O in carbon 2 (TFA)</td>
<td>0.858 ± 0.064$^a$</td>
<td>51.0 ± 5.0$^a$</td>
<td>0.948</td>
</tr>
</tbody>
</table>

In the case of Triticum, the cellulose was hydrolyzed either by enzymatic hydrolysis or by TFA (see Methods and Materials). The slope $F_i$ represents the proportion of oxygen that was either exchanged or added by water during cellulose synthesis. Slope and/or intercept values superscripted for each species indicate a significant difference between slopes and/or intercept. In Triticum, the slopes were significantly different and therefore, the difference between intercepts could not be tested.

TFA, trifluoracetic acid.
lowest intercept for the phenylglucosazone derivative (22.5 ± 1.0‰).

DISCUSSION

Cellulose hydrolyses by enzyme and TFA

Cellulose synthesized from Triticum seeds were hydrolyzed in two ways: with an enzyme preparation or with TFA. We observed no significant differences between the oxygen isotope ratios of phenylglucosazone (δ\(^{18}O_{p-g}\)) and the oxygen attached to carbon 2 (δ\(^{18}O_2\)) for cellulose hydrolyzed by either method. Therefore, even though the enzyme incompletely hydrolyzed the cellulose, there are no fractionation effects from the incomplete hydrolysis.

The cellulose hydrolysis by TFA yielded a hydrolysate highly concentrated in glucose with a minor component (probably mannose) eluting approximately 5 min after the glucose eluted (Fig. 1). Cellulose hydrolysis by TFA is a much faster method of hydrolysis compared with the enzymatic method: cellulose can be completely hydrolyzed in 2 h with TFA, whereas enzymatic hydrolysis requires an incubation period of nearly 4 d. In addition, the TFA method yields a much greater quantity of hydrolysate than the enzymatic method and requires less starting material, which can be a critical consideration when analyzing fossil samples (Jahren & Sternberg 2003). Lastly, cellulose hydrolysis by TFA ensures against contamination of the hydrolysate, because TFA is completely removed during rotoevaporation (Fengel & Wegener 1979).

Isotopic effects during cellulose hydrolysis

It is important to determine any isotopic signal originating from the hydrolysis of cellulose when considering the phenylglucosazone derivative. If such a signal exists, it could complicate the use of the isotopic ratios of phenylglucosazone to interpret isotopic values resulting from cellulose synthesis within plants. Cellulose hydrolysis involves the insertion of one water molecule between two consecutive glucose moieties (Fig. 4). A hydroxyl group, which contains one oxygen atom, could be added either to carbon 1 or 4 of the glucose moieties (Fig. 4). Hydrolysis of cellulose with \(^{18}O\)-enriched water, followed by the derivation and isotopic analysis of phenylglucosazone, can determine which carbon of the glucose moiety receives an oxygen atom from water. If the \(^{18}O\) from the hydrolysis solution is added to carbon 1, no isotopic enrichment will be apparent because of the fact that both oxygens attached to carbon 1 and 2 are eliminated during derivation of phenylglucosazone (Fig. 4). In contrast, if the oxygen atom from water is being added to the carbon 4 of the glucose moiety, phenylglucosazone will have a greater δ\(^{18}O\) value than that produced from hydrolysis of cellulose in \(^{18}O\)-depleted water (Fig. 4). No significant difference between the δ\(^{18}O\) values of phenylglucosazone derived from cellulose hydrolysates in the

![Figure 4](image_url). Comparison of possible outcomes from the addition of a hydroxyl group to two different positions (carbons 1 or 4) of the glucose moieties during the hydrolysis of cellulose.
presence of water having oxygen isotope ratios spanning 250‰ was observed (Fig. 2). From this, we conclude that the hydroxyl group from the hydrolysis solution is added to carbon 1 of the glucose moiety and was then eliminated when phenylglucosazone was derived. This means that the phenylglucosazone isotopic signal results exclusively from in situ biochemical reactions that occur during cellulose synthesis in plants and not from other processes that take place during hydrolysis.

**Relationship between δ18O values of cellulose and intramolecular oxygen with those of germination water**

The linear regression of δ18Ocell, δ18Op–g and δ18O2 values against the δ18O value of water present during germination and seedling growth (δw) can be understood as the following (Sternberg et al. 1986):

\[
\delta^{18}O_{\text{cell}} = [F_{\text{cell}} \cdot \delta_w] + [(F_{\text{cell}} \cdot \Delta_{\text{cell}}) + (1 - F_{\text{cell}}) \cdot \delta_{w}^{\text{ne}}] 
\]

(4)

\[
\delta^{18}O_{p-g} = [F_{p-g} \cdot \delta_w] + [(F_{p-g} \cdot \Delta_{p-g}) + (1 - F_{p-g}) \cdot \delta_{p-g}^{\text{ne}}] 
\]

(5)

\[
\delta^{18}O^2 = [F_2 \cdot \delta_w] + [(F_2 \cdot \Delta_2) + (1 - F_2) \cdot \delta_{w}^{\text{ne}}]. 
\]

(6)

The slope of the linear relationship (F) is equivalent to the proportion of oxygen in the substrate (i.e. starch or lipid) that either exchanged with or were added by water during cellulose synthesis. These values are specific to the oxygen atoms in the cellulose molecule (attached to carbons 2–6 for Fcell, carbons 3–6 for Fp–g and carbon 2 for F2). The overall isotopic fractionation for the oxygen that exchanged with or was added by water during cellulose synthesis (Δcell) is composed of a 4/5 contribution from the overall fractionation of the oxygen associated with carbons 3–6 (Δp–g) and a 1/5 contribution from the fractionation in the oxygen associated with carbon 2 (Δ2). Likewise, the isotopic value of the oxygen atoms in the substrate that does not exchange with or are not added by water is designated as δwne for the respective oxygen atoms in the glucose moieties. The y-intercept of the previous linear relationships (designated here as I) is equivalent to the isotopic fraction of the oxygen that did not exchange with, or was not added on, by water during cellulose synthesis [(1 – F2)·δwne], as well as to a fractionation factor component (Fcell·Δcell).

**Proportion of intramolecular oxygen exchanging with water during heterotrophic cellulose synthesis**

We observed a greater proportion of isotopic exchange between the oxygen attached to carbon 2 and water compared with the proportional exchange of oxygen attached to carbons 3–6 during cellulose synthesis by *Triticum*. The isotopic exchange during cellulose synthesis is expected to be greater in the oxygen associated with carbon 2 than with those attached to carbons 3–6, because glucose molecules from starch would have to first be converted to fructose-bis-phosphate, thus exposing the oxygen attached to carbon 2 to exchange, before it is converted to a triose phosphate and exposing the oxygen attached to carbons 3–6 to exchange.

Our results imply that complete isotopic exchange occurred between the oxygen in the *Ricinus* (i.e. oxygen-poor lipid substrate) synthesized cellulose and water. The slope of the linear relationship of cellulose and derivatives against the in situ water ranged from 0.86 to 0.92. Previous experiments using *Ricinus* seeds resulted in a lower slope of the linear relationship, indicating that only about 70% of the cellulose oxygen molecules had been isotopically labeled by water during its synthesis (Sternberg et al. 1986; Luo & Sternberg 1992). Oxygen isotope analyses in both of these previous experiments were performed using isoquinoline (Sternberg 1989a), which carries an oxygen isotopic ‘memory effect’, thus dampening isotopic labelling and lowering the slope of the relationship relative to that presented here. There are no reports describing a 1:1 relationship between the δ18O value of autotrophically synthesized cellulose and that of water. Autotrophically synthesized cellulose should have its oxygen isotope ratios completely determined by the oxygen isotope ratios of the *in situ* water. Previous studies of autotrophically synthesized cellulose usually measured slopes between 0.48 and 0.88 (Cooper & DeNiro 1989; Yakir & DeNiro 1990; Sauer, Miller & Overpeck 2001). Previous explanations proposed that a change in the isotopic composition of the *in situ* water available during the growth of the plant material caused the slope to be less than expected (Yakir & DeNiro 1990). However, a different autotrophic cellulose experiment showed that the changes in the δ18O value of water during plant growth were too small to account for a slope of less than 1 (Sauer et al. 2001). Our results show that all the oxygen atoms in the heterotrophically synthesized cellulose in the *Ricinus* experiment were derived from the *in situ* water, because the slopes we obtained were similar to, or higher than, those observed for experiments that generated cellulose autotrophically.

**Intramolecular and biosynthetic effects in the oxygen isotope fractionation during heterotrophic cellulose synthesis**

We tested the assumption that the oxygen isotopic fractionation between oxygen in the carbohydrate intermediates and oxygen within the *in situ* water is approximately 27‰ regardless of (1) the oxygen position in the carbohydrate molecule and (2) the metabolic pathway leading to cellulose. If the first assumption was correct, then the y-intercepts of Eqns 5 and 6 can be written as

\[
I_{p-g} = (F_{p-g} \cdot \Delta) + (1 - F_{p-g}) \cdot \delta_{p-g}^{\text{ne}} \quad \text{and} 
\]

(7)

\[
I_2 = (F_2 \cdot \Delta) + (1 - F_2) \cdot \delta_{w}^{\text{ne}}, \quad \text{respectively.} 
\]

(8)

For Eqns 7 and 8 above, ∆ represents a single fractionation factor regardless of the oxygen position in the carbohydrate leading to cellulose synthesis (Case 1). In the same vein, assuming that the non-exchangeable oxygen attached to
carbon 2 and those attached to carbons 3–6 are similar to the respective oxygen of the original substrate (e.g. starch), it follows that

$$\delta_{starch} = (0.20 \cdot \Delta_2) + (0.80 \cdot \Delta_{p-g}) \quad (9)$$

We measured the $\delta^{18}O$ value of starch from the *Triticum* seedlings ($\delta_{starch}$). Therefore, Eqs 7–9 only have three unknowns ($\Delta_2$, $\Delta_{p-g}$, and $\Delta_{cell}$) and can be solved simultaneously for $\Delta$. Calculation of $\Delta$ under these assumptions showed a mean fractionation factor of 23.5‰ (Table 2), which is low compared to the 27‰ observed during autotrophic cellulose synthesis (Epstein et al. 1977; DeNiro & Epstein 1981). A small error is associated with Eqn 9, because about 70% of starch is composed of amyllopectin with branch lengths of about 20 glucose moieties (Blennow et al. 2000). This shorter branch length would be associated with several oxygen atoms attached to the first terminal of the carbon glucose moieties. As a result, the factors of 0.20 and 0.80 previously mentioned would be an overestimate by a small amount, and the oxygen isotopic signature of the oxygen attached to carbon 1 would be unknown. However, a sensitivity analysis, assuming that the $\delta^{18}O$ value of the oxygen atoms in carbon 1 of the glucose moieties deviated from the average of starch by 100‰, gave an error of only $\sim \pm 0.09$‰.

The assumption that the fractionation factors between each oxygen in the glucose moieties of cellulose are similar, regardless of the oxygen position, leads to a contradiction of previous well-established observations and is therefore shown to be false. Further evidence that the fractionation factor for the oxygen associated with carbon 2 is different than the average for the oxygen associated with carbons 3–6 is provided when these two fractionation factors are calculated independently (Case 2). The only required condition for this calculation is that the relative contribution of each fractionation factor will add up to the reported average oxygen isotopic fractionation of autotrophically synthesized cellulose: 27‰ (DeNiro & Epstein 1981; Sternberg 1989b). Hence,

$$\Delta_{cell} = 27‰ = (0.20 \cdot \Delta_2) + (0.80 \cdot \Delta_{p-g}) \quad (10)$$

and the y-intercept in equations 5 and 6 become

$$I_2 = (F_2 \cdot \Delta_2) + (1 - F_2) \cdot \delta_{p-g} \quad \text{and}$$

$$I_{p-g} = (F_{p-g} \cdot \Delta_{p-g}) + (1 - F_{p-g}) \cdot \delta_{p-g} \quad (12)$$

Equations 9–12 have four unknowns: the fractionation factors $\Delta_2$ and $\Delta_{p-g}$, and the non-exchangeable oxygen isotope ratios of the substrate oxygen associated with carbon 2 ($\delta^{18}O$) and 3–6 ($\delta_{p-g}$). These four equations can be solved simultaneously for the unknowns, including $\Delta_2$ and $\Delta_{p-g}$. The resulting fractionation factor for the oxygen associated with carbon 2 (averaging 19.6‰) is different than the fractionation for the oxygen associated with carbons 3–6 (averaging 28.8‰, Table 2), therefore negating the first assumption.

The results of the *Ricinus* experiment, where lipids are the major substrate for cellulose synthesis, provide evidence that fractionation factors not only differ within the cellulose molecule, but also depend on the metabolic pathway by which cellulose is synthesized. This possibility was first mentioned by Schmidt et al. (2001); here we quantify this effect. The oxygen isotope fractionation factors resulting from the *Ricinus* experiment show large differences between oxygen associated with carbon 2 compared with the oxygen associated with carbons 3–6 of the glucose moieties (Table 2). The metabolic pathway from lipid to cellulose in *Ricinus* is obviously different than the pathway transforming starch into cellulose in *Triticum*. The observation that the fractionation for the oxygen associated with carbon 2 in the *Ricinus* experiment ($\Delta_2 = 51$‰) differed greatly from that observed for the *Triticum* experiment ($\Delta_2 = 19.6$‰) leads us to the conclusion that the particular cellulose biosynthetic pathway exerts an influence over the fractionation effects of individual oxygen atoms, therefore negating the second assumption previously detailed. The differences between cellulose synthesized from starch versus that synthesized from lipids in terms of oxygen addition or exchange lies in several reactions occurring during

<table>
<thead>
<tr>
<th>Seed type and hydrolysis method</th>
<th>$\Delta_2$</th>
<th>$\Delta_{p-g}$</th>
<th>$\Delta_{cell}$</th>
<th>Starch $\delta_2$</th>
<th>Starch $\delta_{p-g}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Triticum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case 1</td>
<td></td>
<td></td>
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<tr>
<td>Enzyme hydrolysis</td>
<td>24.0 ± 2.9</td>
<td>24.0 ± 2.9</td>
<td>24.0 ± 2.9</td>
<td>16.4</td>
<td>33.7</td>
</tr>
<tr>
<td>TFA hydrolysis</td>
<td>22.9 ± 3.1</td>
<td>22.9 ± 3.1</td>
<td>22.9 ± 3.1</td>
<td>12.0</td>
<td>34.8</td>
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<tr>
<td>Enzyme hydrolysis</td>
<td>18.9 ± 2.6</td>
<td>29.0 ± 3.9</td>
<td>27.0</td>
<td>26.5</td>
<td>31.2</td>
</tr>
<tr>
<td>TFA hydrolysis</td>
<td>20.2 ± 2.7</td>
<td>28.7 ± 3.9</td>
<td>27.0</td>
<td>23.8</td>
<td>31.9</td>
</tr>
<tr>
<td><strong>Ricinus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TFA hydrolysis</td>
<td>51.0 ± 5.0</td>
<td>22.5 ± 1.0</td>
<td>28.2 ± 0.4</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

For the *Triticum* experiment, fractionation factors were calculated for two cases: case 1 assumed that all fractionation factors are the same regardless of the position in the glucose moiety, and case 2 made no assumption on the fractionation factors other than they must average to 27‰. For the *Ricinus* experiment, we assumed complete addition/exchange between the oxygen of the glucose moieties and water during cellulose synthesis, that is, $F = 1$.

n.a., not applicable; TFA, trifluoracetic acid.

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glucose moieties generated from starch hydrolysis have all the oxygen atoms found in the final cellulose product, and their final isotopic identity is mostly acquired during carboxyl hydration and the aldolase catalyzed reaction occurring during the futile cycle from glucose-6-phosphate to the triose phosphates (Schmidt et al. 2001). Carboxyl hydration reactions may be facilitated and may reach equilibrium through enzyme catalysis (Schmidt et al. 2001). Glucose moieties generated from lipids, however, will have all the oxygen atoms derived from water through reactions catalyzed by enolhydratase, fumarase and enolase, and the final isotopic identity of the oxygen atoms in the cellulose molecule may be determined by reactions during which the newly added oxygen is incompletely exchanged with water (Schmidt et al. 2001). It has been suggested, for example, that the oxygen added during gluconeogenesis in position 6 of the glucose moiety does not exchange with water, because it is protected by a phosphate group (Schmidt et al. 2001).

The source of variation in oxygen isotope fractionation during cellulose synthesis

The commonly used average 27‰ enrichment factor for the oxygen isotope ratios of cellulose relative to the source water is based on several measurements of the oxygen isotope ratios of cellulose and the source water of submerged aquatic plants (DeNiro & Epstein 1981; Sternberg, DeNiro & Keeley 1984; Sternberg 1989b; Sauer et al. 2001). This value is, in fact, a mean of the previous measurements having a range of about ±4‰ (DeNiro & Epstein 1981; Sternberg 1989b). The reasons for this variation is as yet unknown, but careful monitoring of source water indicates that this variation is not due to isotopic drift of the source water during the growth of the aquatic plant (Sauer et al. 2001). Furthermore, different species grown within the same aquarium will have different cellulose oxygen isotope ratios (Sternberg et al. 1984). We propose that the intramolecular and the biosynthetic pathway isotopic effects demonstrated here may in part be responsible for the previously observed variation. The differences in the fractionations associated with particular oxygen positions in the glucose moiety can affect the final average cellulose oxygen isotope ratio relative to that of the source water. There are likely differences in the extent of the (hexose-triose) futile cycle (Hill et al. 1995) occurring in the cellulose synthesizing tissue, depending on species and even environmental conditions (Sauer, Aellen & Siegwolf 1997). This difference would not only affect the proportion of oxygen atoms in the glucose moiety that interact with the water in the cellulose synthesizing cell, but it would also affect which oxygen atom interacts with the cell water. The net outcome will be a difference in the average isotopic fractionation during cellulose synthesis, because the isotopic fractionations for different oxygen positions in the glucose differ. Biosynthetic pathways are also important in determining the final oxygen isotope ratios of cellulose even if the immediate precursor for cellulose synthesis is a carbohydrate (e.g. sucrose). We note that, although the ultimate carbon source for cellulose synthesis in the Ricinus seedlings is lipids, the pathway towards cellulose synthesis probably involves gluconeogenesis from lipids stored in the cotyledons, conversion to sucrose, translocation to sink tissue and finally, cellulose synthesis. Evidently, the isotopic labelling occurring during gluconeogenesis is sufficiently robust to persist through the entire cellulose synthesis process, including the sucrose intermediate, and to affect the oxygen isotope ratios of cellulose: the final biosynthetic product.

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REFERENCES


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